Mechanisms of Synchronization in the Vocal Central Pattern Generator of *Xenopus laevis*

A Thesis
Presented to
The Division of Mathematics and Natural Sciences
Reed College

In Partial Fulfillment
of the Requirements for the Degree
Bachelor of Arts

Joseph Perry
May 2013

Approved for the Division
(Biology)

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Erik Zornik

Acknowledgments

AMBER BANG
bam! right in the n.IX-X!

BEN PULIAFITO
it's definitely all about alternative splicing

MICA PEACOCK
thanks for humoring my wild ramblings and speculations

TERRA VLEESHOUWER-NEUMANN
i totally won that O-week bet

ENYO SHERMAN
i think i lost that O-week bet

TESS MYERS
for being a badass

ZINA JENNY
:)

I feel lucky to be part of the first group of thesis students to have come through Erik's lab. I think that electrophysiology is some of the coolest, most hands-on biology around, and it was gratifying to gradually become proficient in these technically and manually complicated procedures. Getting your first singing brain is such a rush – there's this suspense in the application of 5-HT, stepping back and watching the trace on the monitor for any sign of CAPs, and listening to the crackle of static from the attached speaker. After so many failures, would this finally be the one that works? My first singing brain came during a surgery that I thought I had messed up during an early step but went through the motions anyway with significantly more speed and less care than normal. It felt like a life lesson. Learning electrophysiology for me became more of a mind game (how do I have to *think* in order to make this work?) than just blindly learning a skill.

I've actively tried to hold on to my initial unease at killing *Xenopus*. Despite an initial visceral reaction to the dissection (we must have a hardwired propensity to react as such somewhere in the brain, something like with spiders and snakes. Man, the thing about working with CPGs is that I've started to understand how much of our experiences really are just consequences of physical mechanisms), I stopped reacting physiologically after the first couple of surgeries. This was a little worrying to me – it brought to mind the summer that I worked clipping and counting salmon fry and the high-school students would throw fish down each other's shirts to amuse themselves. They had a total disregard for the animals we exercised absolute power over. I didn't want that, so I always put on my serious face and try to have some respect for the frogs while I'm sacrificing them.

I predict that we'll have figured out the entire Xenopus vocal CPG within the next four years – seven years tops. We have the theory, we just need to confirm it. How complicated could it be?

Finally, I humbly apologize for my gratuitous use of footnotes, semicolons, commas, parenthetical statements, and the words “however”, “via”, and “thus” in this document.[1]

1 However; I regret nothing (thus, via this, I demonstrate all of the above).

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Abstract

I examined the neural circuitry responsible for advertisement calls in the African clawed frog *Xenopus laevis* by dissecting out the brain and keeping it alive in oxygenated saline. In this preparation, bath-applied serotonin evokes activity in the laryngeal nerve that matches *in vivo* recordings during vocalizations. This "fictive calling" is produced by a central pattern generator consisting of two reciprocally-connected nuclei: the motor nucleus IX-X (n.IX-X) and the dorsal tegmental area of the medulla (DTAM). There is a population of neurons in DTAM that seem to control the "fast trill" phase of calls, dubbed fast trill neurons (FTNs).

I recorded from FTNs after transecting the brain between n.IX-X and DTAM to reveal the role of the reciprocal connections in FTN firing patterns. I found that FTNs in the transected brain [1] still produce synchronized long-lasting depolarizations [2] continue to receive excitatory input during trills [3] have an increased variability in spike rate. Taken as a whole, my results suggest that FTNs synchronize the onset and offset of their long-lasting depolarizations via local excitation in DTAM which is normally synchronized by inhibitory connections from n.IX-X.

For Steve Arch
the best damn teacher I've had.
He showed me how to not be scared of the science,
how to make it my own,
and how to weave
criticism with
compassion.

Introduction

Neurons control behavior; movement essential for an animal's survival is the result of muscles contracting and relaxing, which do so at the whim of the nervous system. Staving off death is thus a simple matter of determining the correct order to fire one's neurons. While there's usually considerable leeway in obtaining non-fatal outcomes[2]*, many situations require a specific and/or repetitive movement in order to interact with the environment. These activities range from the slow, rhythmic pulsing of a jellyfish to the rapid, precise, and flexible song of a Lyre bird. Behaviors that involve the coordinated activity of many muscles require a specific set of neurons to be able to produce the appropriate pattern of activity at the appropriate times.*

Understandably, attempts to map the systems which evolution has produced to perform the staggering diversity of behaviors we see in the world have met with considerable difficulties. Compared to the brain, no other organ has been studied with so much enthusiasm and yet surrendered so few of its secrets. The historically popular solution to this problem is to simplify it by studying simple behaviors in invertebrates. They have drastically fewer neurons than vertebrates, and their circuits are remarkably consistent. Every *C. elegans*, for example, has precisely 302 neurons and their synapses are consistent enough to be mapped (White et al., 1986). Studying these simpler systems has allowed neuroscientists to identify circuits that generate stereotyped patterns of activity and send output to muscles. These circuits are called "central pattern generators" (CPGs), and control virtually all rhythmic behavior in both invertebrates (e.g., swimming (Pearce and Friesen, 1984), eating (Benjamin et al., 2000), and flight (Wendler, 1974)) and in vertebrates (Duysens and Van de Crommert, 1998).

2 At least, not immediately fatal. There has not yet been an observed non-fatal final outcome for any organism.

The mating call of the African clawed frog *Xenopus laevis* consists of a series of clicks produced by rapid muscle contractions in the larynx. Clicking comes in ~300 ms bursts of ~60 Hz "fast trill" followed by ~700 ms trains of ~30 Hz "slow trill". The central pattern generator responsible for this activity can be studied *in vitro* by dissecting out the brain and keeping it alive in oxygenated saline. In this preparation, bath-applied serotonin evokes activity in the laryngeal nerve that matches in vivo recordings. This "fictive calling" is produced by a circuit consisting of two reciprocally-connected nuclei: the motor nucleus IX-X (n.IX-X) and the dorsal tegmental area of the medulla (DTAM). There is a population of neurons in DTAM called fast trill neurons (FTNs) which produce long-lasting depolarizations (LLDs) that correlate each fast trill and action potentials that correlate with each fictive click – thought to dictate fast trill duration and click rhythm, respectively.

FTNs can be observed individually with a whole-cell clamp or *en masse* with local field potential (LFP) recordings. LFP waves correlate with both fictive trills and LLDs, and have phasic "spikes" that roughly correlate with each click. It's possible to transect the brain between n.IX-X and DTAM and continue to observe LFP waves – though their phasic trill-like activity is lost. I hypothesized that this was due to a desynchronization of FTN action potentials but that they were maintaining the synchronization of their LLDs.

In this thesis, I recorded from FTNs in transected brains in order to directly observe any changes in their firing pattern. I found that they still produce synchronized LLDs, continue to receive excitatory input during trills, and have an increased variability in spike rate. Taken as a whole, my results suggest that FTNs are able to synchronize the onset and offset of their LLDs despite a desynchronization of their excitatory connections in the transected brain (due to a loss of inhibitory input from n.IX-X).

NEURONS

OVERVIEW

First, it will be useful to understand how neurons work.

Neuronal signaling is generally one-way[3]: a neuron that has been sufficiently excited will send an action potential (a wave of excitation) down the length of its axon. Neurotransmitter is released from the end of the axon onto waiting dendrites from other neurons. A dendrite can potentially receive thousands of simultaneous inputs from passing axons, all of which the neuron sums up at a single point: the axon hillock. When the hillock is sufficiently excited, it triggers a new action potential and the cycle begins anew.

CELLULAR MECHANICS

Membrane Potential

The 'excitation' of a cell refers to the voltage between the inside and the outside of the cell's membrane. The membrane voltage (or potential) is determined in large part by ion concentration gradients. Since there are fewer sodium ions (Na^+) and a lot more negatively-charged molecules inside the cell than outside the cell, there is a negative electrical potential (a voltage) across the membrane. Classically[4], a neuron's resting potential is around -70 mV. Since both the concentration gradient and voltage across the membrane are in favor of sodium moving inward (there's more Na^+ outside and the positive ion likes to move towards the negative interior), when sodium channels open Na^+ will quickly flow into the cell. This continues until the voltage of the interior of the cell becomes positive enough that it resists additional sodium entry. For sodium, this "reversal potential"[5] is typically about +40 mV. Thus, a neuron at rest can rapidly change its

3 Except for "retrograde" action potentials (see footnote 9).

4 There is a *lot* of variation in these numbers across cells and species. Throughout this section I'm going to give oversimplified textbook-type voltages in order to illustrate their general relationships.

5 It's called a reversal potential because if a cell's voltage goes past it, sodium will start trying to flow *out* of the cell instead.

membrane potential by opening sodium channels.

Action Potentials & Ionic Currents

A neuron uses this ability to rapidly transmit information down its axon in the form of a positive-feedback loop we call an action potential (sometime abbreviated to AP). Axons express voltage-gated sodium channels (VGSCs) that open when the membrane potential is made more positive ("depolarized", since the cell is normally polarized to its resting potential) past a threshold around -55 mV. The inrush of Na^+ through these newly-opened VGSCs depolarize the local potential even further, which triggers the opening of additional nearby VGSCs.

My favorite metaphor for this process is a trail of gunpowder: a spark at one end (typically at the axon hillock[6]) sets off a chain-reaction that travels along some predetermined path until it reaches the end. However, a gunpowder trail can only go off once. This is not the case in neurons; they're able to fire an AP every couple of milliseconds. But the VGSC chain-reaction only explains how a neuron is able to become depolarized. After they open, the neuron would be stuck at sodium's reversal potential because opening more sodium channels wouldn't do anything.

How, then, do neurons get back to their resting potential after becoming depolarized during an action potential? VGSCs contain a mechanism that causes them to deactivate after a short time (an attached clump of protein swings up into the open channel and clogs it), but that's closing the barn door after the horse is out; there's already enough Na^+ in the cell to keep it depolarized. They could pump out the sodium (and are actually continuously doing so) but they can't do it fast enough to cause the quick repolarization that we observe.

Here we remember that sodium is not the only ion in the universe. Potassium ions (K^+) are found in high concentrations inside the cell and low concentrations outside. Neurons also express voltage-gated potassium channels (K_v channels) which will open when the cell becomes depolarized. Therefore, following the VGSC-caused

6 The axon hillock is the closest location to the dendrite that expresses VGSCs. Thus, changes in potential throughout the dendrite will be felt at the axon hillock the strongest, and accordingly VGSCs at the hillock will be the first ones to open in response to dendritic excitation.

depolarization, K_v channels open and K^+ ions will flow outwards, causing the cell to become more negative (since positive charge is leaving) until it hits potassium's reversal potential of about -90 mV. Since this happens as rapidly as the depolarizing Na^+ current, neurons use large-conductance calcium- and voltage-gated potassium channels (BK_{Ca}[7]) that open during the VGSC-caused depolarization to repolarize the cell and bring the voltage back down to resting potential.[8]

SIGNALING

Dendrites & Synapses

Unlike axons, dendrites do not express VGSCs or experience action potentials.[9] During development, dendrites grow many small spines that come very close to touching axons from other neurons.[10] These spaces are called synapses. Presynaptic (ie, on the axon) voltage-dependent calcium channels selectively allow Ca^{2+} ions to enter the cell during an action potential (Ca^{2+} ions, like Na^+, have a higher concentration outside the cell than inside). Once inside the cell, Ca^{2+} interacts with membrane-bound protein complexes which snare neurotransmitter-containing vesicles and cause them to release their contents into the synapse. Postsynaptic (i.e., on the dendrite) membrane-bound receptors then bind the released transmitter and, depending on the receptor, open ion channels or activate other intracellular secondary signals.

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7 So named for their big konductance (of K^+ ions).

8 The fact that the reversal potential of K^+ is about the same as a neuron's resting potential is not a coincidence. At rest, the membrane isn't very permeable to any ion, but it is the *most* permeable to potassium due to “leakage” channels. You can think of the membrane potential as a weighted average of all the ion reversal potentials, with the weights for each set by the permeability of the membrane to that ion. Thus, the final voltage will be closest to the reversal potential of the ion to which the membrane is the most permeable.

9 Mostly. Some voltage-sensitive Na+ and Ca2+ channels have been found on the dendrite itself (Kim, 1993), but only seem to open when VGSCs on the axon have also opened and massively depolarized the cell. Such "retrograde action potentials" are thought to have some role in altering the strength of synapses as a function of the neuron's activity, perhaps via NMDA receptors.

10 Not all connections are the standard axon → dendritic spine. Some axons synapse onto main branch of the dendrite, the soma, and even other axons! In a strictly modulatory sense, of course.

Excitatory Ionotropic Receptors

Glutamate is the ubiquitous excitatory neurotransmitter in vertebrates and many invertebrates. Its primary effect as a neurotransmitter is via binding to the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. An AMPA receptor is a membrane ion channel that opens when bound to glutamate. They are primarily permeable to cations and usually have a net equilibrium potential of about 0 mV. Therefore, when an axon releases glutamate into a synapse, the glutamate will bind to waiting dendritic AMPA receptors and depolarize the dendrite for as long as it remains bound (this event is called an excitatory postsynaptic potential, or EPSP).

If a dendrite is sufficiently depolarized by EPSPs, the depolarization is seen by the VGSCs at the axon hillock who open and trigger an action potential. However, not all EPSPs are created equal. Some will have greater or lesser influence on the hillock's decision to fire.

- The size of the EPSP is the most straightforward factor; its magnitude can vary as a function of the amount of transmitter released and the number of receptors available to bind and open.
- Since voltage falls off as a function of distance across the membrane, the axon hillock will see a greater fraction of an EPSP on a spine closer to the base of the dendrite than one more distal.
- AMPA receptor currents decay over the course of a few milliseconds (Colquhoun et al., 1992; Clements et al., 1998) due to glutamate unbinding and receptor desensitization (Trussell et al., 1993). Since the hillock needs to be pushed over a threshold and it is unlikely a single EPSP will sufficiently depolarize it, two EPSPs which arrive synchronously will be more likely to cause an AP than two equivalent EPSPs which arrive separately.

N-Methyl-D-aspartate (NMDA) receptors differ from AMPA receptors in a few very important ways. While NMDA receptors are also glutamate-binding cation channels

with a reversal potential of about 0 mV, they also require bound glycine[11], allow calcium into the cell (important for triggering a multitude of secondary effects), and are blocked by a magnesium ion while the membrane is at resting potential. Even while glutamate is opening adjacent AMPA receptors, no ions will flow through NMDA receptors until the membrane becomes depolarized enough to (briefly) displace the magnesium ion. These properties mean that NMDA receptors are effectively ligand- *and* voltage-gated, which allows them to function as "coincidence detectors"; calcium entry into the cell during synaptic input becomes a function of whether the neuron is currently firing an action potential (thus being massively depolarized) in response to input. Especially since intracellular calcium levels can regulate the strength of a synapse, NMDA receptors have been extensively studied as possible mechanism for Hebbian learning ("Neurons that fire together, wire together.")

Inhibitory Ionotropic Receptors

Not all input is excitatory. Gamma-aminobutyric acid (GABA) receptors are selectively permeable to Cl^-, which are found in higher concentrations outside the cell than inside and have a reversal potential of about -65 mV. When GABA binds to a GABA receptor, negatively-charged chloride ions flow into the cell and cause an inhibitory postsynaptic potential (IPSP). Neurons receiving IPSPs need a greater amount of excitation to exceed their VGSC threshold, so their firing is consequently suppressed.

Inhibitory input is an essential component of many neuronal circuits – reciprocal inhibition can cause rhythmic alternation in the activity of two neurons, prolonged periods of inhibition can trigger depolarizing post-inhibitory rebounds, and pulses of inhibition can reset the phase of intrinsic oscillations.

11 Why is glycine needed, functionally? What's it doing? I have never found a satisfactory answer. NMDA receptors are *everywhere*, but everybody just ignores glycine.

Metabotropic Receptors

Not all receptors are ion channels. More complicated, longer-term effects are mediated via metabotropic receptors. The largest class of these are the G protein-coupled receptors (GPCRs), a class of membrane-bound receptors which release an activated G protein into the cytoplasm upon transmitter binding. The G protein goes on to activate second messenger systems, which can alter gene expression, regulate the localization of receptors to the membrane, and change the sensitivity or permeability of other channels via phosphorylation (figure 1).

Electrical Signaling

Some neurons are directly connected to other neurons by small pores called gap junctions, which allow small molecules (such as ions) to flow from one cell to the other. Neurons connected by gap junctions can therefore quickly pass electrical signals to one another without the need for a chemical synapse. Systems that require quick and accurate message transduction – such as the control of a heartbeat – can use gap junctions to efficiently synchronize events. A standard test for whether a cell expresses gap junctions is to inject it with a non-membrane-permeable dye whose molecules are small enough to fit through the gap and see if it spreads to adjacent cells.

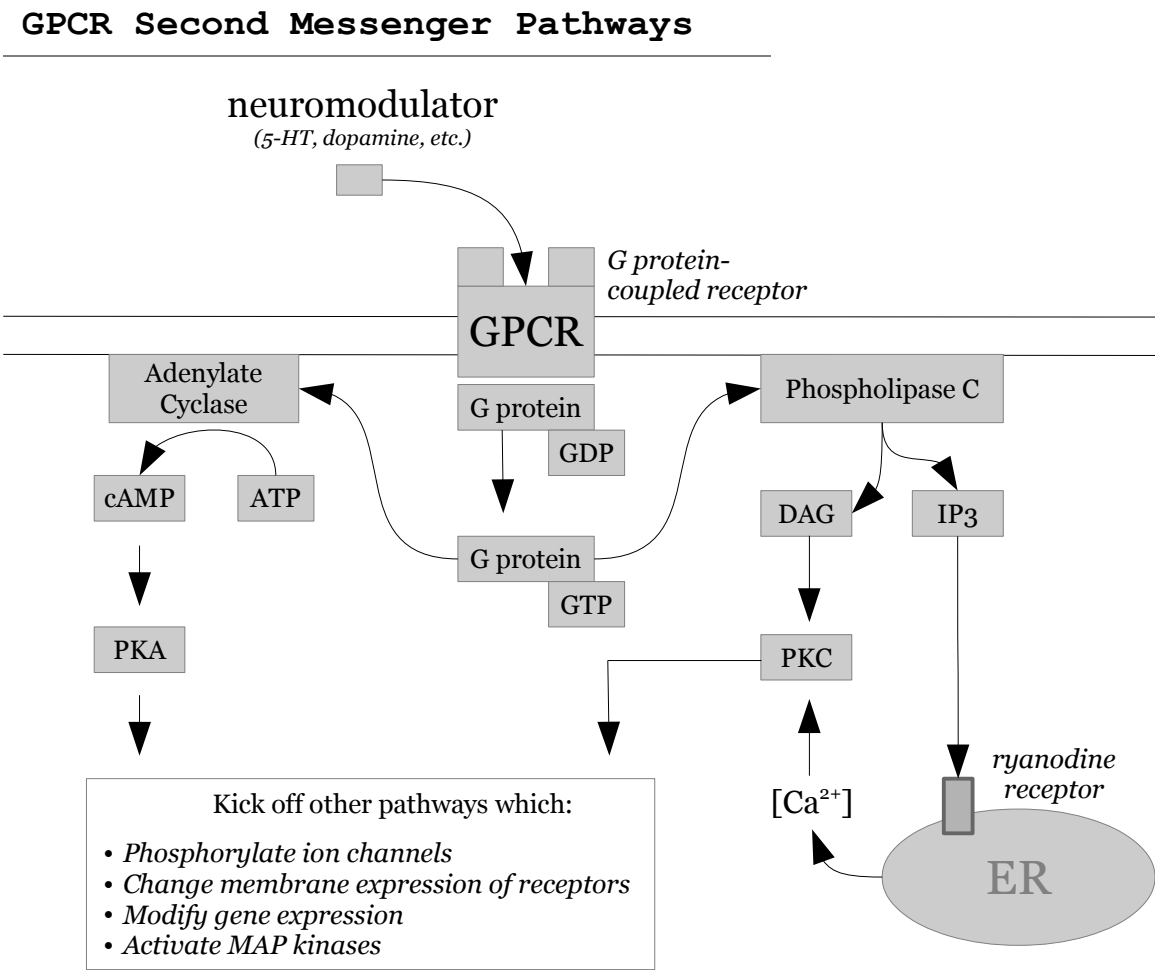


Figure 1. G protein-coupled receptor second messenger pathways. Once a ligand binds to G protein coupled receptor (GPCR) the attached G protein is activated by trading its GDP for GTP. From that point, there are two primary pathways mediating the effects of metabotropic receptors:

PKA pathway: the activated G protein phosphorylates (or dephosphorylates, in the case of inhibitory G proteins) adenylate cyclase, which starts converting adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Protein kinase A (PKA) requires bound cAMP in order to phosphorylate its many possible downstream targets.

PKC pathway: the activated G protein activates phospholipase C, which cleaves a molecule from the phospholipid bilayer into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 acts on ryanodine receptors on the endoplasmic reticulum, which allows calcium to be released from their intracellular stores. DAG and the increased $[Ca^{2+}]$ both bind to and activate protein kinase C.

The targets of phosphorylation by PKA and PKC vary significantly from cell-to-cell, but they have been found to be involved in changing the sensitivities of ion channels, altering the cycle of receptor endo- and exocytosis leading to a shift in their surface expression, and triggering changes in gene expression.

The signal ends when the G protein inactivates itself by cleaving its bound GTP back to GDP and rebinds to an empty GPCR.

CENTRAL PATTERN GENERATORS

OVERVIEW

Many CPGs seem to share similar components in terms of generating rhythms. Some mechanisms are intrinsic to individual neurons, while others emerge via the communication of multiple neurons. The rhythm of a CPG is produced by a combination of these intrinsic- and network-based components. Feedback to a CPG can be transmitted by sensory neurons[12] or neuromodulators (such as serotonin and dopamine), which can make both short- and long-term modifications to a CPG's output. An animal's final behavior may depend upon multiple linked CPGs, each controlling a separate aspect of a unified movement.

INTRINSIC PROPERTIES

Pacemaker Cells & Intrinsic Oscillations

Some neurons display fluctuations in membrane potential in the absence of synaptic input (figure 2A). These cells express ion channels that are tuned to open and close at some frequency, which can result in spikeless sub-threshold oscillations (STOs) or can depolarize the cell past threshold and trigger regular action potentials (or bursts of action potentials). Either way, intrinsic oscillations can help set a cell's frequency of spiking at a rate independent of synaptic input. While the ion channels responsible for oscillations vary from system-to-system, the currents generally look a bit like a slow action potential: one or more depolarizing inward currents (sodium or calcium) trigger a hyperpolarizing outward current (potassium). The oscillations can be modified as a function of membrane voltage. Many only appear within a narrow range of potentials (usually below or at the threshold for APs) (McCormick and Pape, 1990) while others experience voltage-dependent frequency modulation (Desmaisons et al., 1999).

12 Blurring the line between a modular, independent central pattern generator and a simple reflex loop.

Mechanisms of CPGs

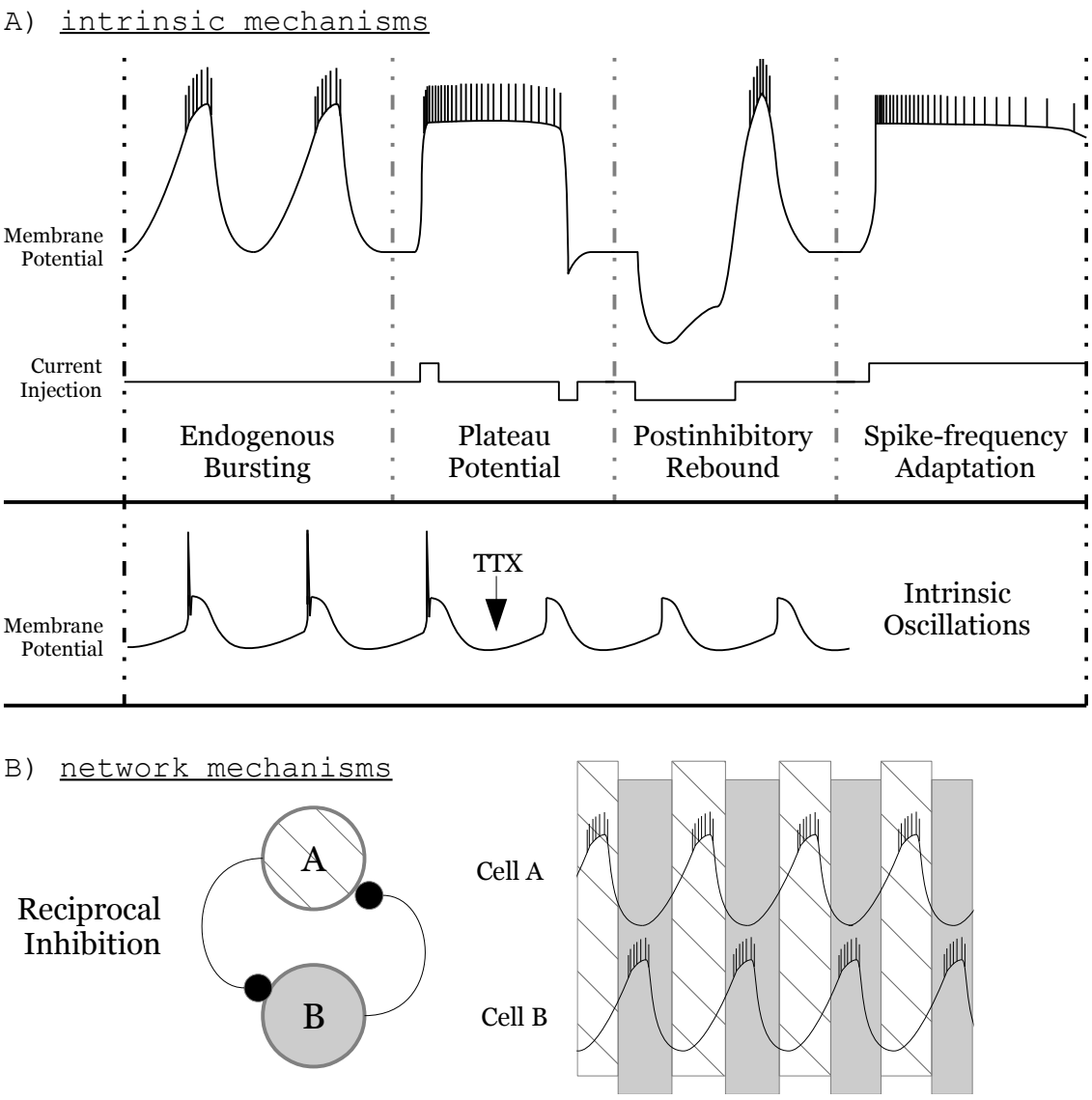


Figure 2. Common mechanisms underlying pattern generation. Rhythms are produced by a combination of intrinsic cellular- and collective network-based mechanisms. Modified from (Marder, 2001) and (McCormick, 1990)

A – Intrinsic Mechanisms: Some neurons display distinct behaviors when exposed to pulses of excitatory or inhibitory current. Endogenously bursting neurons can produce rhythmic bursts of action potentials without any synaptic input. A brief excitatory pulse will trigger bistable neurons to maintain a depolarized "plateau potential", which can be ended with a brief inhibitory pulse. Neurons with postinhibitory rebounds will transiently depolarize following a period of inhibition. Spike-frequency adaptation describes a decrease in the frequency of firing during an extended depolarization. Intrinsic oscillations are often TTX-insensitive and can set the frequency of action potential generation.

B – Network Mechanisms: Two spontaneously active cells that inhibit each other (represented here by black circles) produce alternating bursts of action potentials, especially if they exhibit spike-frequency adaptation (ending the current burst) and postinhibitory rebounds (kick-starting the next burst).

Sodium currents can be part of slow (2-5 Hz) (Chapman and Lacaille, 1999) or fast (10-50 Hz) (Desmaisons et al., 1999; Rubin and Cleland, 2006)) oscillations. These currents are abolished by tetrodotoxin[13] (TTX) and are frequently voltage-sensitive (i.e., they are only active within some range of membrane potentials). Of particular note is the **queer current** (I_h)[14], a slow hyperpolarization-activated sodium current which is a component for a number of behaviors (figure 3).

Calcium currents involved in intrinsic oscillations are mediated by voltage-sensitive quickly-inactivating **T-type calcium channels**[15] or slowly-inactivating **L-type calcium channels**[16] (Perez-Reyes, 2003). Both types of channels will open above a certain membrane potential, but will close and inactivate after a delay. Inactivated channels will stay closed regardless of the level of depolarization. Once the membrane potential returns to a polarized state, the channels become "de-inactivated" and once again can open upon depolarization. T-type calcium channels can cause rhythmic calcium spikes as they undergo activation → inactivation → de-inactivation (figure 3A).

Potassium currents are not an essential component of STOs like they are for action potentials (Desmaisons et al., 1999), but they can play a role. Multiple K^+ channels have been found to be involved in the polarized phase of some STOs and individually blocking them can produce a markedly different effects (Charles and Hales, 1995). The final oscillation can be produced by an interaction of multiple channel types – for example, a tidy STO can be produced by [Ca^{2+}]-dependent K^+ channels alternating with a depolarizing calcium current.

13 TTX is a neurotoxin that binds to and blocks the voltage-gated sodium channels responsible for action potentials. It's nasty stuff – used as a natural toxin by fugu pufferfish, predatory moon snails, blue-ringed octopuses, and rough-skinned newts. This is the reason we wear close-toed shoes in lab.

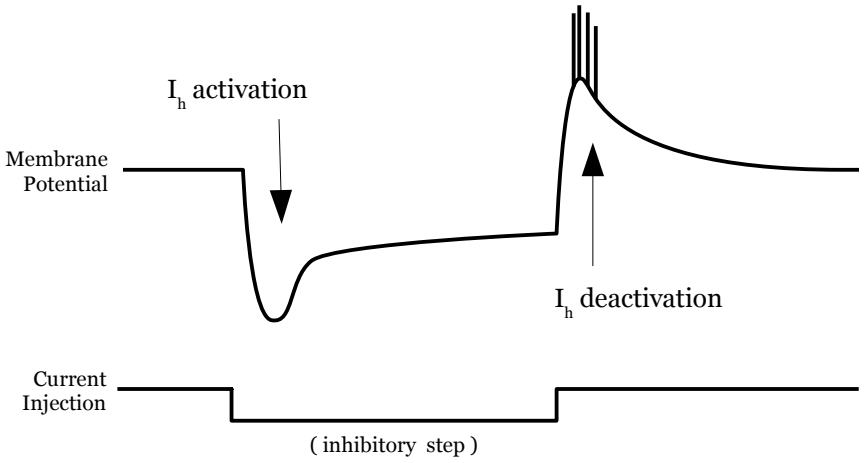
14 Also called the hyperpolarization-activated current (I_h), the sag current, the funny current (I_f), the anomalous rectifying current (I_{AR}), and the inward rectifying current (I_{IR}) (not to be confused with the K^+ inward rectifier). I_h is the preferred terminology in the literature (Pape, 1996), but I am going to refer to it as the queer current because that one is my favorite.

15 So named for the transient nature of the current they produce (lasting ~15-30 ms) (Fishman, 1981).

16 So named for the long-lasting nature of the current they produce (lasting more than 2000 ms). There is also a N-type, which somewhat mysteriously refers to channels that fall into neither category.

Queer Current; I_h

A) postinhibitory rebound



B) intrinsic oscillations

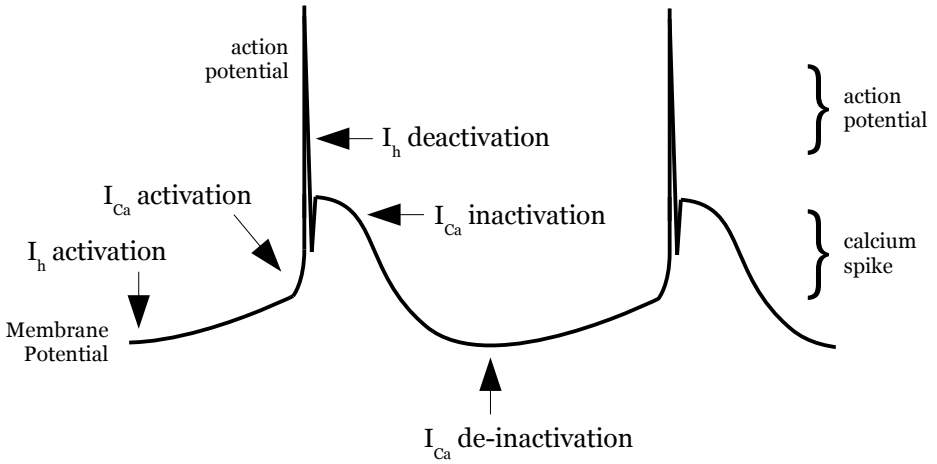


Figure 3. Roles of the queer current. The queer current (I_h) is a slow hyperpolarization-activated sodium current. Modified from (Pape, 1996), (McCormick, 1990), and (Perez-Reyes, 2003).

A – Postinhibitory Rebound: The queer current slowly activates and starts depolarizing the neuron during an inhibitory step. Since it is also slow to deactivate, once inhibition is released it will continue to depolarize the neuron, resulting in a postinhibitory rebound.

B – Intrinsic Oscillations: In conjunction with T-type voltage-sensitive inactivating calcium current (I_{Ca}) (and if the cell is at an appropriate membrane potential) the queer current can help to produce regular calcium spikes. I_h slowly depolarizes the cell until I_{Ca} is triggered and pushes the cell over the threshold for an action potential. The large depolarization ends I_h and triggers the inactivation of I_{Ca}. Once the cell returns to its resting potential, I_{Ca} can de-inactivate and I_h can begin to restart the cycle.

Desmaisons et al. (1999) notes that while STOs can set a neuron's rate of firing (figure 4B), this mechanism only works if the EPSP kinetics are such that they depolarize the cell long enough for the STO to reach its depolarized phase. If the hyperpolarized phase of the oscillation is too long, an EPSP could completely decay before it has a chance to trigger an action potential. An STO with a sufficiently long wavelength could theoretically thus "gate" EPSPs and discard those that are not received in-phase, but this mechanism does not seem to have yet been observed in nature.

Some neurons require exposure to neuromodulators to produce intrinsic oscillations, and different neuromodulators can produce different patterns of activity. For example, the anterior burster neuron in the lobster Panulirus interruptus displays at least three distinct modes of bursting in response to 5-HT, dopamine, and octopamine (Harriswarrick and Flamm, 1987), each of which require distinct sets of sodium, calcium, and potassium channels.[17] Since all three modes of bursting can be exhibited by a single cell, an overall "natural" STO may be a result of multiple oscillations which are simultaneously active.

Spike-frequency Adaptation

Some neurons produce trains of actions potentials whose firing frequency gradually decreases as the train progresses (figure 2A). Spike-frequency adaptation is likely useful in capping the length of a burst and protects a neuron from endless spike trains. Several ion channels have been found whose activity can result in spike-frequency adaptation:

- **SK channels**[18] are calcium-dependent low-conductance potassium channels that can open during a plateau due to an increase in $[Ca^{2+}]$ (via either a calcium channel or release from intracellular stores in the ER) (Madison and Nicoll, 1984; Xia, 1998) . As $[Ca^{2+}]$ increases, potassium current via SK

17 E.g., bursts produced during 5-HT and octopamine application are abolished with TTX, but dopamine-evoked rhythmic long-lasting depolarizations will continue (sans action potentials, as a result of the TTX). They do share some properties, such as requiring calcium entry for burst termination, but there are also other differences between the amines, including the levels of calcium-dependence for burst termination and polarizing currents in between bursts.

18 So named for their small konductance (of K+ ions).

channels slowly builds up and begins inhibiting spike formation.

- **M-channels**[19] are voltage-dependent non-inactivating potassium channels (Madison and Nicoll, 1984) whose conductances are a function of membrane potential; they're slightly open at rest but have increased conductance during depolarization. The current can cumulatively increase with each action potential until the progressive hyperpolarization starts to limit the rate of firing (Sekirnjak and du Lac, 2002).
- **VGSCs** (the voltage-sensitive sodium channels responsible responsible for action potentials) can undergo a slow inactivation over the course of several seconds (Fleidervish et al., 1996). Though the molecular basis is unclear, there is a progressive removal of VGSCs from the available pool over the course of a sustained depolarization, leading to a decreased rate of firing.

Postinhibitory Rebound

Some neurons will immediately fire a burst of action potentials following a period of hyperpolarization (figure 2A). Three mechanisms for postinhibitory rebound are cited in (Angstadt et al., 2005):

> "(1) activation of I_h , (2) deinactivation of an inward current, and (3) deactivation of a slow outward current."

- (1) **The queer current** (I_h, see footnote 14) is a result of hyperpolarization-activated sodium channels (Straub, 2001; Ascoli et al., 2010). It is typically identified by the presence of a slow partial depolarization during an extended period of hyperpolarization, and is blocked by addition of Cs^+ (Pape, 1996) or ZD7288 (Harris, 1995). Since the queer current is slow to inactivate, a sudden release of inhibition causes the cell to overshoot its resting potential and briefly depolarize (figure 3A).
- (2) **Deinactivation of an inward current** during hyperpolarization in leech motoneurons is described by Angstadt et al. (Angstadt et al., 2005). They

19 So named for the G protein-coupled muscarinic acetylcholine receptor which, when ligand-bound, causes a deactivation of M-channels (Brown, 1980).

found voltage-sensitive calcium channels that are open above a relatively polarized voltage (around -60 mV) but deactivate once the cell reaches a certain level of depolarization. However, this deactivation is released during hyperpolarization. Once the inhibition is released and the cell returns to -60 mV, these channels open and depolarize the cell, resulting in a postinhibitory rebound (until they automatically deactivate again).

- (3) **Deactivation of a slow outward current** is described in (Sekirnjak and du Lac, 2002) and is similar to the M-channels responsible for spike-frequency adaptation. They describe a slow voltage-sensitive potassium current that builds during normal firing and is responsible for maintaining the resting potential. Once the cell is hyperpolarized by inhibitory input, this current will deactivate, resulting in a brief depolarization as the current slowly builds back up.

Plateau Potentials

Some neurons produce extended depolarizations in response to brief depolarizing stimuli. Though in some neurons these "plateau potentials" can end spontaneously, they can also be ended by a brief hyperpolarizing stimulus (figure 2A). Neurons displaying plateau potentials can thus serve as switches; they can be toggled between an active depolarized state and an inactive resting state.[20]

Plateau potentials can be maintained by a calcium current through either slowly- or non-inactivating L-type calcium channels (Kiehn and Eken, 1998).[21] Like VGSCs, these channels are voltage-sensitive and will open if the membrane potential goes above a threshold. Unlike VGSCs, however, they will stay open until some other factor (e.g., an IPSP, artificially injected hyperpolarizing current, or a change in the conductance of other ion channels) hyperpolarizes the cell back below the L-type threshold. Until then, they permit a calcium current that maintains the depolarization.

It's not uncommon that a neuron only displays bistable behavior in the presence

20 Some neurons may fire spontaneously even during their "inactive" state.
21 Though sodium current-maintained plateaux have been reported (Llinás, 1979).

of a neuromodulator.[22] In (Hounsgaard and Kiehn, 1989), they describe turtle motoneurons which exhibit 5-HT-dependent plateau potentials. Hounsgaard et al. postulate that in this system, plateau potentials are normally prevented by the action of calcium-dependent potassium channels, though other factors may also contribute to the removal of plateaux (they include Ca^{2+} buffering systems and the conductance of the calcium channels responsible for the plateaux as other possible variables that could effect the cell's bistability). Whatever the mechanism, application of 5-HT can eliminate this tonic repression of bistability and allow L-type calcium channels to produce plateau potentials.

MULTI-CELL BEHAVIORS

Reciprocal Inhibition

When two cells are tonically active and inhibit each other, they will naturally begin fire alternating bursts of action potentials (figure 2B). Reciprocal inhibition is one of the best-studied methods that neuronal circuits use to produce rhythmic behavior. The rhythm produced can be further fine-tuned by a neuron's intrinsic properties. The degree to which postinhibitory rebounds are displayed will alter the speed of transition between the neurons (Satterlie, 1985). Plateau potentials can help maintain the burst and provide a mechanism for expeditious cessation of the burst. Spike-frequency adaptation can limit the duration of a burst, and thus controls the rate of alternation between the two neurons.

The leech heart[23] is the textbook[24] example of a circuit that uses reciprocal inhibition. Constrictions are controlled by a series of reciprocally-connected inhibitory interneurons which alternatively burst with a cycle period of about 10 sec (Angstadt and Calabrese, 1991). These interneurons display queer currents which allow them to escape inhibition and voltage-dependent calcium plateau currents which maintain their own inhibitory burst (Arbas and Calabrese, 1987a; Arbas and Calabrese, 1987b).

22 Though in some neurons, serotonin can instead quench plateaux (Perrier, 2008).

23 The "heart" of a leech is actually just a pair of rhythmically contracting tubes.

24 To be precise, *Neurons and Networks: An Introduction to Neuroscience* by John E. Dowling. I got my copy off Amazon.com for a penny + $3.99 shipping & handling. It has a calligraphic monkey in a tree on the cover. It's great.

Pacemaker-driven Networks

The rhythm produced by the stomatogastric ganglion of lobsters and crabs is driven primarily by a single cell; the anterior burster (AB) neuron. It spontaneously bursts and is electrically coupled to several other motoneurons in the ganglion (which accordingly fire in phase with the AB neuron) while projecting inhibitory connections to others (causing them to fire out of phase). There is some reciprocal inhibition in the system – out of phase motoneurons reciprocally inhibit each other to produce sub-alternating rhythms and inhibit motoneurons that are electrically coupled to the AB neuron (Dowling, 1992) – but the speed of the network's output is primarily a function of the AB neuron's activity.[25]

Synchronization Via Inhibitory Input

Inhibitory input can reset the phase of a neuron's STO (Desmaisons et al., 1999). This means that an IPSP simultaneously delivered to two intrinsically oscillating cells can cause them to become synchronized (figure 4A). Since STOs can modify the frequency of action potentials triggered by EPSPs (figure 4B), simultaneous inhibitory input can cause a group of neurons displaying STOs to start firing their action potentials in synch.

An example of a system that appears to use this method of synchronization is described by (Desmaisons et al., 1999) while studying inhibitory granule cells that synapse onto intrinsically oscillating mitral cells in the rat olfactory bulb:

> "A single granule cell which may be connected to several mitral cells could therefore effectively synchronize their intrinsic oscillations. ... This possibility is supported by both physiological observations (Isaacson and Strowbridge, 1998), synaptic organization (Price and Powell, 1970), and the estimated ratio of granule cells to mitral cells of 100:1 (for review, see Mori, 1987; Shepherd and Greer, 1998). Furthermore, because granule cells are electrotonic-coupled via gap junctions (Reyher et al., 1991), granule cell activity may be synchronized, thus permitting synchronization of GABA release onto clusters of mitral cells."

He also proposes that, given the correct resting potential and a sufficiently large IPSP, an inhibitory input could trigger a post-inhibitory rebound and yield immediate synchronized spikes in target neurons.

25 Though the activity of the AB neuron can be significantly affected by the activity of its electrically-coupled neurons (Kepler, 1990).

Sub-threshold Oscillations

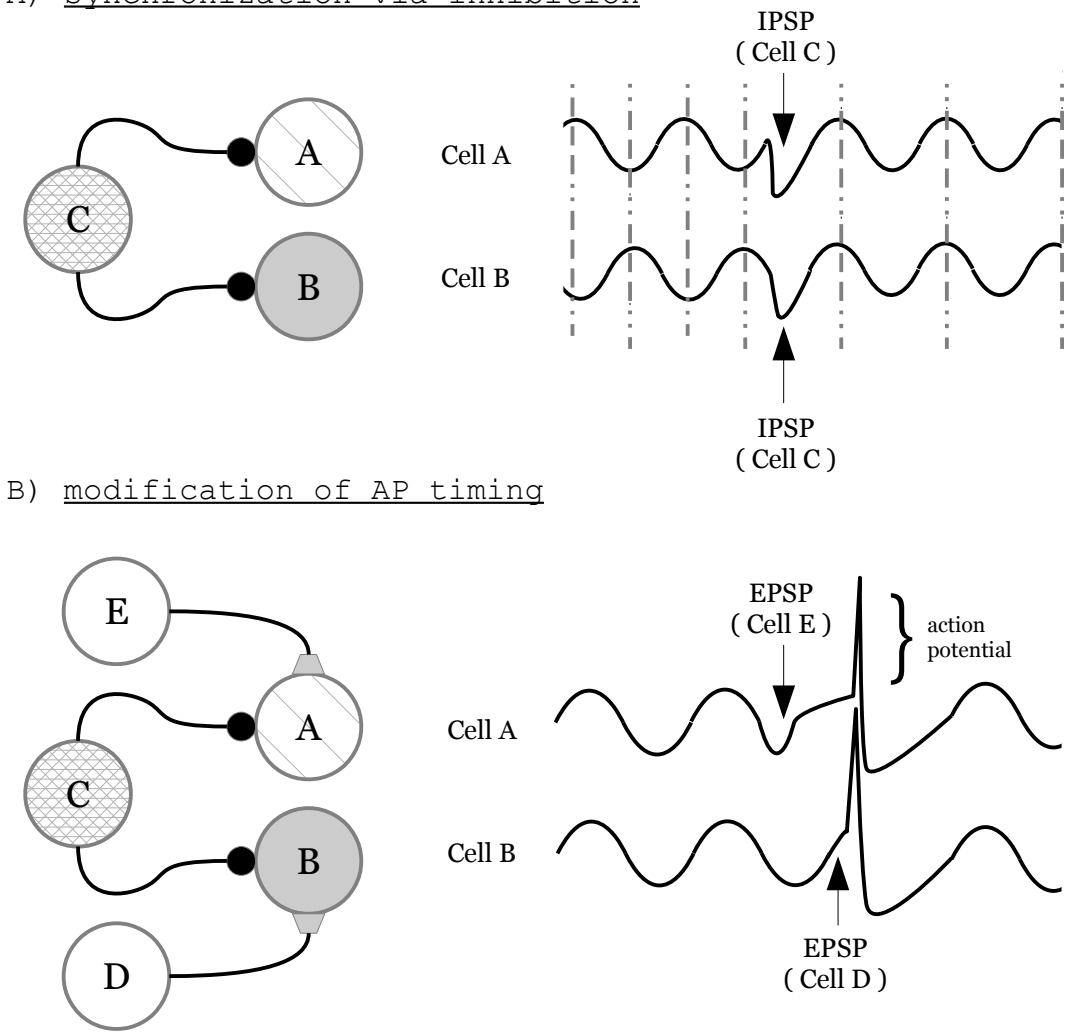


Figure 4. Synchronization of action potentials by sub-threshold oscillations. Modified from (Desmaisons, 1999).

A – Synchronization via Inhibition: Cells with sub-threshold oscillations (STOs) can have their phases reset by an inhibitory input. Hence, when cells with similar STOs can be synchronized by simultaneous inhibitory post-synaptic potentials (IPSPs). If the cells display postinhibitory rebounds, such an inhibitory input could produce immediate synchronized action potentials (not pictured).

B – Modification of AP timing: STOs are able to modify the timing of the conversion of an excitatory post-synaptic potential (EPSP) into an action potential (AP). If the neuron receives an EPSP during the polarized phase of its STO, it won't fire until it reaches threshold during the depolarized phase. If two cells have synchronized STOs, they are more likely to fire synchronized APs even when receiving desynchronous EPSPs.

MODULATION OF GENERATORS

Neuromodulators

CPGs, whether they operate continuously or episodically, need to be able to update their patterns in response to the organism's changing circumstances. Sensory feedback can provide immediate modification of a CPG (e.g., the feeling a rock in a shoe altering your gait), but many intermediate and long-term changes to a network are mediated by neuromodulators (such as 5-HT, dopamine, and acetylcholine).

Neuromodulators can alter a neuron's activity by tweaking the properties of its ion channels. Application of substance P, a neuropeptide usually associated with sensory pathways involving pain, increases the frequency of bursting ~300% by neurons in the lamprey locomotor CPG (Parker and Grillner, 1998). Parker et al. found two synaptic mechanism's of substance P's effect: 1) it activates PKC, which phosphorylates NMDA receptors and increases the amount of current they permit, increasing the size of EPSPs, and 2) it increases the frequency of spontaneous miniature EPSPs in a calcium-independent manner (implying that the base probability for transmitter release has gone up, which likely results in a greater amount of transmitter being released after each action potential). Svensson (Svensson et al., 2001) goes on to further characterize substance P's effect on the locomotor CPG in relation to other neurotransmitters (figure 5).

Bath-applied serotonin has been found in a number of systems to activate a CPG *in vitro*. Straub et al. (Straub and Benjamin, 2001) found that exposing neurons in the *Lymnaea* (an aquatic snail) feeding CPG to 5-HT causes them to depolarize, begin bursting, and display queer current-mediated postinhibitory rebounds (as in figure 3A). 5-HT thus both gates the fictive behavior (it can't take place without the 5-HT signal) and controls its rhythm in a dose-dependent manner (the greater the amplitude of the rebounds, the greater the frequency of bursting).

In vivo, starved leeches have higher circulating levels of 5-HT than satiated ones. As in *Lymnaea*, Angstadt et al. (Angstadt et al., 2005) found that 5-HT causes neurons in the locomotor CPG to begin displaying larger and shorter postinhibitory

rebounds – however, since it persists in the presence of Cs^+, the underlying current is not queer. They hypothesize that 5-HT is either increasing the calcium conductances of the responsible ion channels or is inhibiting some limiting potassium current.

Memory

It is critical that organisms are able to draw from previous experience in reacting to novel stimuli. Long-term memory can be represented by modification of connections between neurons (the most intensively studied of which is long-term potentiation, or LTP) or intrinsic changes within a single cell (Sangha et al., 2004). While the body of research on LTP is staggering (for an excellent review, read Malenka and Bear, 2004), much of it examines cells from brain slices that are isolated from sensation or behavior.

However, the cellular basis of memory has been studied in the fictive breathing behavior of *Lymnaea*. Aerial breathing in *Lymnaea* is controlled by a CPG consisting of only three interneurons (Syed and Winlow, 1991) which can survive dissection and continue to function in vitro (Syed et al., 1990). It is thus possible to condition snails to cease aerial respiration[26] *in vivo* and then see how their respiratory CPG behaves *in vitro* compared to a yoked control.

Unfortunately, it turns out that there's not a simple answer to memory formation even in such a simple system. Following such a procedure, Lukowiak and Syed (1999) found that each snail would undergo a different type of change in their respiratory CPG that resulted in the cessation of activity. For example: spontaneous firing of the right pedal dorsal 1 (RPeD1) neuron is crucial in controlling the activity of the respiratory CPG. In a large number of conditioned snails, RPeD1 had been silenced – thus eliminating fictive respiration – but some snails had been conditioned without a silenced RPeD1 and instead had other changes throughout the CPG that eliminated the fictive behavior. How conditioning affects these sufficient-but-not-necessary changes (and which changes they do affect) is not well understood.

26 It's OK, Lymnaea can just breath through their skin instead.

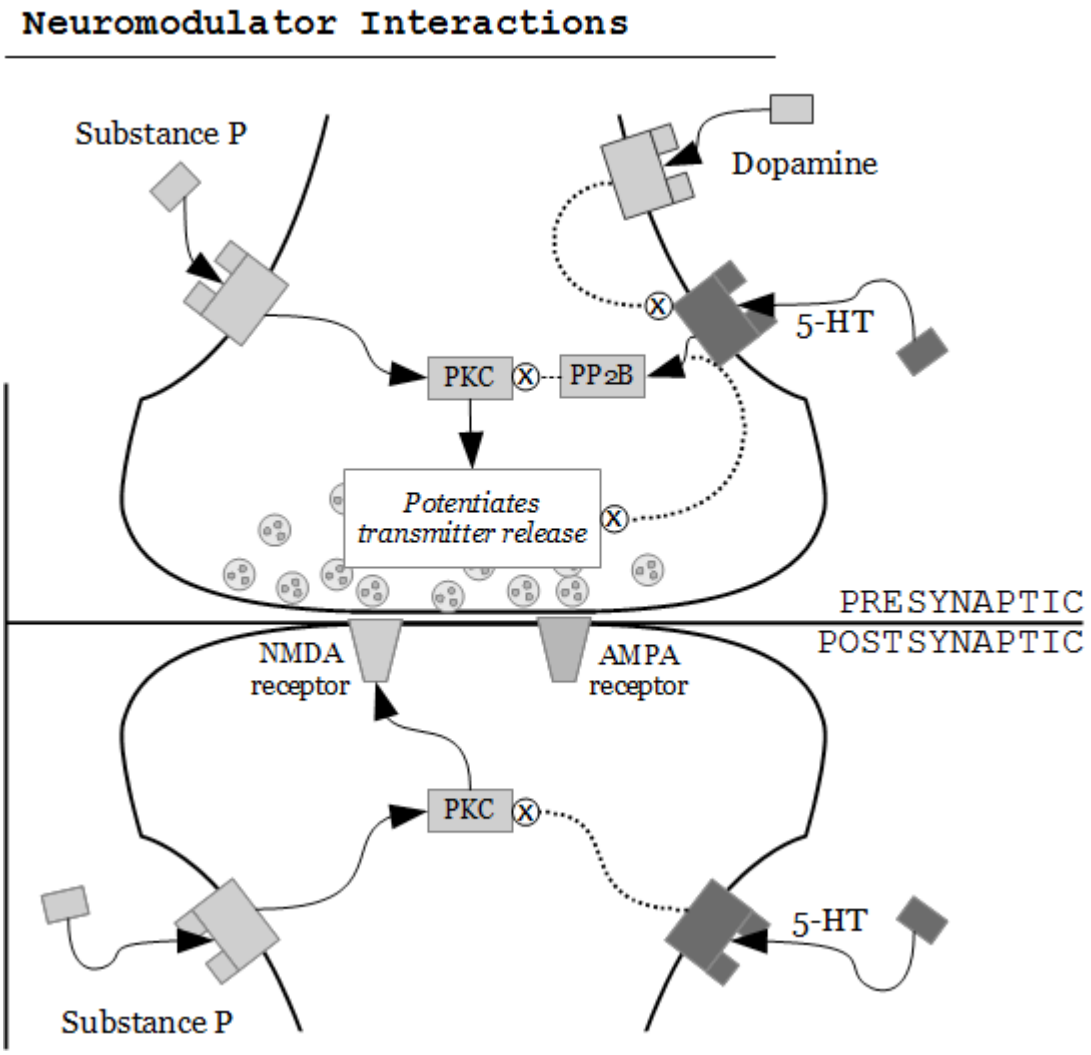


Figure 5. Neuromodulator interactions in the lamprey locomotor CPG. Substance P increases the excitability of the synapse, 5-HT blocks that increase, and dopamine partially blocks that blockage. This system of neuromodulators allows the lamprey to: 1) perform long-term potentiation of its motor network (possibly for migration to upstream spawning grounds), 2) prevent that modification until the appropriate time, and 3) obtain short-term potentiation of the network without permanent effects. Modified from (Svensson, 2001).

Substance P: Application of substance P alone evokes a long-term dramatic increase in the frequency of bursting via concurrent presynaptic and postsynaptic effects. Presynaptically, it binds a GPCR which causes the activation of PKC, which goes on to increase the release probability of excitatory transmitter. It activates PKC postsynaptically in a similar fashion, where PKC goes on to potentiate NMDA receptors.

Serotonin: 5-HT blocks the presynaptic effects of substance P via protein phosphatase 2B (PP2B), which dephosphorylates PKC. Postsynaptically, a serotonergic GPCR blocks the effects of PKC via an unknown pathway. Thus, 5-HT may be preventing the long-term effects of substance P until the appropriate time.

Dopamine: Serotonin's presynaptic blockage of substance P's potentiation of excitability is blocked by a dopamine GPCR. Thus, with the application of all three transmitters, there is a nonpermanent increase in the frequency of bursting.

Interestingly, to form new long-term memories, the *Lymnaea* respiratory CPG seems to require the soma of the RPeD1; animals with RPeD1's soma ablated but neurites left intact are still able to perform aerial respiration and form short-term memories, but do not retain conditioning for more than a few hours (Scheibenstock et al., 2002). However, previously conditioned snails that undergo the RPeD1 soma-ablation surgery are able to retain their conditioning but can't form new long-term memories. It seems that some alteration in gene expression (or some other nuclear-specific process) is required in RPeD1 to make enduring changes in the behavioral output of the entire CPG. Similar to many forms of LTP, this process is NDMA receptor-, PKC-, and MAPK-depedent (Rosenegger and Lukowiak, 2010).

Systems Of Inter-CPG Coordination

For the escape reflex in the mollusc *Tritonia*, which move with simple alternating contractions between two muscle groups, a single CPG that can be turned on and off is sufficient to encode the behavior. For added flexibility, the rhythm of even that single CPG can be modified by neurotransmitters. If, however, an animal needs to coordinate additional muscle groups, more elaborate CPGs and networks of CPGs are required. For the undulating swimming movement in lampreys, waves of unilateral alternating contractions need to move up their bodies. Each section of the lamprey contains a CPG functionally similar to the one in *Tritonia*, but they keep their phases offset in order to produce the correct order of contractions (Pearce, 1984).

Many terrestrial animals face an even more difficult challenge: walking. Locomotion in the stick insect *Cuniculina*, for example, requires 1) controlling each joint of a leg, 2) coordinating between joints to produce a type of step, and 3) alternating activity between legs to produce a gait. Separate CPGs are responsible for each aspect of a step, and need to be informed by direct sensory afferents (reflex pathways), coordinate their activity between themselves, and act on their motor nuclei (Bassler, 1993). Complex movements require specific sets of CPGs to be activated, and *in vitro* fictive behavior can sometimes resemble a muddled mix of many patterns (Bassler and Wegner, 1983).

THE XENOPUS PARADIGM

THE *XENOPUS LAEVIS* FICTIVE CALLING PREPARATION

The Behavior In Question

Xenopus males produce a clicking advertisement call that alternates between 60 Hz “fast trills” and 30 Hz “slow trills”. Clicks are produced by two discs in the larynx being snapped apart by contracting muscles. Laryngeal muscle contraction is controlled by the hindbrain via the descending cranial nerve bundle IX-X (N.IX-X). It is possible to record from N.IX-X in free-swimming animals (Yamaguchi and Kelley, 2000) and see compound action potentials (CAPs) in N.IX-X that precede larynx-produced clicks with a 1:1 correspondence.

Fictive Calling

Rhodes et al. (Rhodes et al., 2007) showed that it was possible to surgically isolate the brain and, by bath-applying 5-HT, elicit a pattern of CAPs in N.IX-X (figure 6) that matched the in-vivo recording. Central pattern generators in vertebrates are usually less-easily studied than those in invertebrates due to a harder-to-access nervous system, increased complexity of behavior, and a drastic increase in the number of neurons involved. This in-vitro *Xenopus* fictive calling preparation is one of the few that confers easy access to a vertebrate central pattern generator, allowing a simple fictive behavior to be observed real-time in response to manipulations.

Anatomy Of The CPG

Tract-tracing experiments (Zornik and Kelley, 2007; Brahic and Kelley, 2003; Wetzel et al., 1985) reveal at the base of N.IX-X the primary motor nucleus that controls laryngeal muscle contraction: nucleus IX-X (n.IX-X). Interneurons in n.IX-X are reciprocally connected to a more-anterior nucleus called DTAM[27]. Bath-applied 5-HT is thought to activate this vocal CPG via 5-HT_{2C}-like receptors (Yu and Yamaguchi, 2010) in the dorsal raphe nucleus, which is also located in the brainstem and contains serotonergic neurons projecting to both n.IX-X and DTAM[28]. It is possible to bisect the

27 The "pretrigeminal nucleus of the dorsal tegmental area of the medulla" also abbreviated to PbN.

28 Endogenous 5-HT pathways (activated by application of a serotonin-selective reuptake inhibitor (Yu, 2009) and direct electrical stimulation to DTAM (Rhodes, 2007) can also induce fictive vocalization.

brain just anterior to DTAM and still get fictive calling[29] (Rhodes et al., 2007), indicating that the nuclei in this area are all that are necessary to produce fictive vocal activity.

DTAM Control Of Trills

The duration and rate of fast trills are temperature-dependent; cooling a brain will result in a longer and slower trill, and heating the brain gives a shorter and faster trill. It is also possible to change the water temperature in the tanks of live animals and see similar results *in vivo*. However, selectively cooling DTAM results in a longer fast trill, but cooling n.IX-X has no effect on fictive call duration (Zornik et al., 2010). A process localized to DTAM appears to control the length of fast trills (Zornik and Kelley, 2008).

DTAM appears to directly excite n.IX-X motoneurons. Local field potential recordings (LFP, they show the overall activity of all neurons near the tip of an inserted wire) from DTAM during fictive calls pick up depolarization waves which co-occur with N.IX-X CAPs. Additionally, antidromatic stimulation of n.IX-X produces a signal in DTAM within several milliseconds, indicating that at least some of the axons projecting to n.IX-X are directly connected to cells in DTAM.

Fast Trill Neurons

Individual cells in DTAM have been characterized in the intact brain (Zornik and Yamaguchi, 2012). Whole-cell recordings have revealed a population of "fast trill neurons" (FTNs), which are active exclusively preceding and/or during a fast trill. FTNs produce bursts of 60 Hz action potentials on top of long-lasting depolarizations (LLDs), closely matching the pattern seen in LFP recordings. FTNs thus collectively seem to be responsible for controlling the CAP rate and wave duration, respectively, seen in LFP recordings.

29 Fast trills were roughly twice as long in brains with transected telencephalons. However, when bilateral connections between DTAM nuclei were severed, FT duration returned to its normal length. Weird.

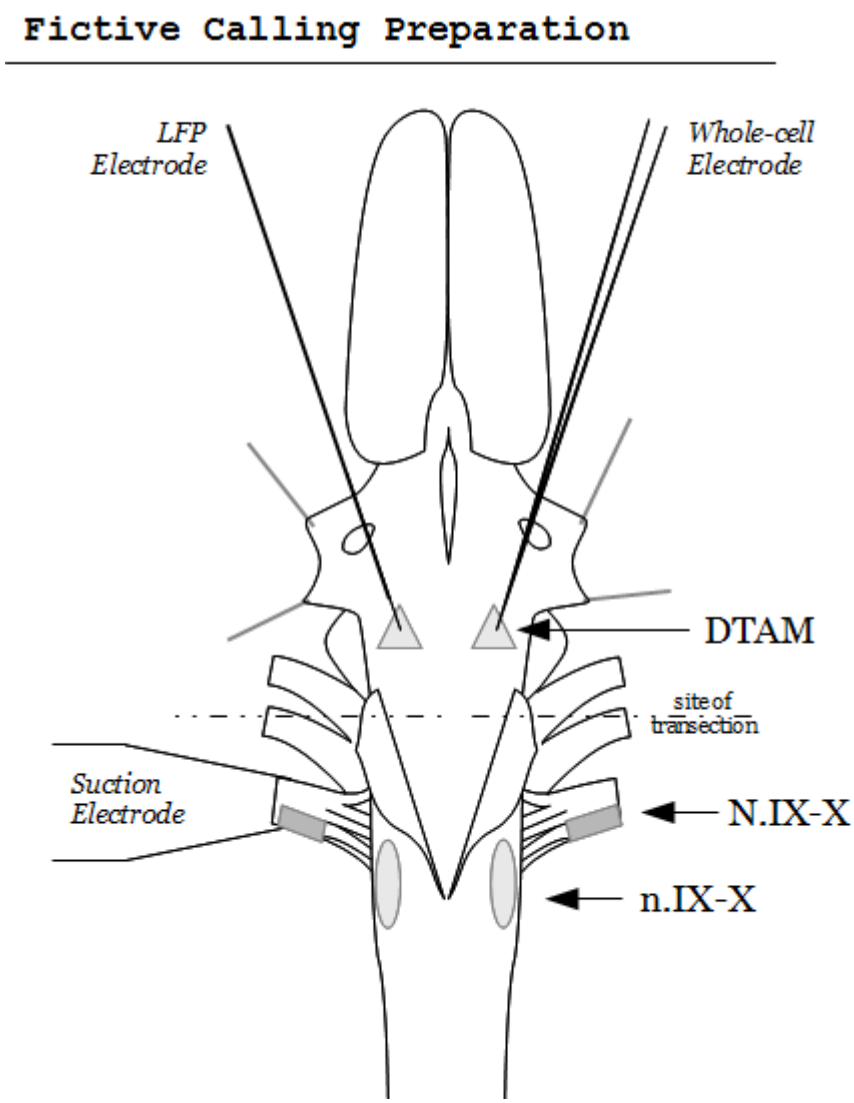


Figure 6. Fictive calling preparation. An extracted *Xenopus* brain that has been bisected between the forth and fifth ventricles. Pins are keeping the optic tectum open for easy access to the dorsal tegmental area of the medulla (DTAM). During a trial, a local field potential (LFP) electrode may unilaterally record activity in DTAM while a glass pipette containing a whole-cell (WC) electrode may record from the opposite nucleus. The cranial nerve bundle IX-X (N.IX-X) has all but the most posterior rootlets cut (not shown) and a suction electrode is used to record its activity. In transection experiments the brain would be cut at the indicated site, severing the reciprocal connections between DTAM and the motor nucleus IX-X (n.IX-X).

Additional properties of FTNs:

- FTNs display voltage-gated 60 Hz sub-threshold oscillations, which can be seen during the brief depolarized period preceding a spike train and during artificial depolarizing current steps. These STOs are only seen during periods (natural or artificial) of depolarization. A different rate of oscillation have not been observed, even under varying levels of injected current.
- Voltage-clamp experiments show that FTNs receive a concurrent inhibitory and excitatory input following each CAP.
- Like the LFP wave, the LLDs are NMDAR-dependent.
- Each FTN begins their LLD at different points during trills. When they start is conserved from trill-to-trill; some are early-bursters which begin spiking even before a wave begins while others are late-bursters and don't begin spiking until two-thirds of the way through.
- They seem to monosynaptically synapse into n.IX-X; stimulation in n.IX-X will evoke an antidromatic spike within a couple milliseconds in a recorded FTN (Zornik and Yamaguchi, 2012).
- Most FTNs display a queer current.

In pyramidal neurons from the prefrontal cortex, 5-$HT_{2A/C}$ receptor agonists increase NMDAR-dependent bursting via PKC activation (Zhong et al., 2008). Especially since we suspect that 5-HT_{2C} receptors are critical for fictive fast trill, it's possible that 5-HT is acting through a similar pathway to enable FTN LLDs.

CAP SYNCHRONIZATION

One would be forgiven for wondering what role n.IX-X even plays! Does DTAM just project to n.IX-X motoneurons and tell them when to fire? Not, perhaps, the most interesting circuit. However, it is possible to transect the brain above n.IX-X (thereby cutting it off from the rest of the CPG) and continue to record the LFP in DTAM. While the waves still persist, their 60 Hz CAPs are lost and are replaced by high frequency noise (Zornik et al., 2010).

Since we know that FTNs intrinsically want to spike at 60 Hz and have not observed a way that this rate is modified (apart from temperature manipulations), it may be that they require input from n.IX-X in order to synchronize firing, and in the transected brain they lack that input. Since FTNs receive a concurrent EPSP and IPSP following each action potential in a burst, we can't say if the synchronizing input from n.IX-X is inhibitory or excitatory. However, since FTNs display STOs, it's possible that the synchronization of STOs via inhibitory input (see the according section in the introduction) is the mechanism that causes synchronization of CAPs. In either case, the noisy wave we see in transected-brain LFP recordings could possibly be a result of desynchronous 60 Hz bursts by FTNs.

Since I will be recording from FTNs in a transected brain, I will be able to see the difference (or lack thereof) in the firing pattern of FTNs in the transected brain versus those from intact brains. I will also attempt voltage-clamp experiments to see what inputs are lost after transection.

LLD SYNCHRONIZATION

The fact that LFP waves persist in a transected brain implies that FTNs are able to synchronize their LLDs without input from n.IX-X. They do not appear to be electrically coupled; filling an FTN with dye does not spread to adjacent neurons. What's more, it is possible to evoke LLDs by application of NMDA and 5-HT in a bath containing TTX, so most FTNs must be able to produce LLDs without any synaptic input. We construct our models accordingly; intra-DTAM connections must be able to control and synchronize LLDs, but are not required for them to be produced.

Wave Initiation

In the whole brain, EPSPs appear to build up and eventually trigger the intrinsic mechanism that sustains an LLD (likely NMDA channels, since the wave is blocked by NMDAR antagonists). However, remember that different FTNs start their LLDs at different times. We can construct two possible (non-exclusive) models of connectivity for the initiation and continuation of a trill:

- FTNs which begin their LLD late in a trill may be primarily receiving input from FTNs that begin bursting slightly sooner, and so forth. Some population of early-bursters thus can start a “rolling recruitment” where over the course of a trill there is a chain reaction of FTNs exciting and triggering LLDs in neurons “downstream” of themselves.
- The point in a trill that a FTN begins to spike is determined by some threshold of excitatory input that it requires in order to begin its LLD (which would be persistent trill-to-trill, and dependent on NMDAR expression, number of outward current channels, etc.). There's still a rolling recruitment, but no special pattern of connectivity is required. The early-bursters are merely FTNs with a low enough threshold that they don't require special excitement to start their own LLDs.

Wave Termination

We have no direct evidence for how an LLD is terminated. However, since we know that LLDs are NMDAR-dependent, a buildup of calcium may play a role. The termination of LLDs is not perfectly synchronous; early-bursters tend to end their LLD slightly before the end of the LFP wave, while late-bursters tend to end their LLD slightly after.

The ion channels responsible for spike-frequency adaptation are attractive mechanisms for the auto-termination of a trill. Since SK channels are sensitive to the buildup of calcium, calcium entry via open NMDA channels during a trill may serve as a trigger for these polarizing channels to open and destabilize the depolarization (though mere depolarization-sensitive M-channels could also be sufficient). However, there are a couple of problems with these channels as the only mechanisms. First, spike-frequency adaptation is generally a gradual process; one sees a slow reduction in the frequency of spikes as the polarizing current builds up rather than the sudden and controlled end of an LLD. Second, a way that intra-DTAM signals could control the timing of the channels finally overcoming the depolarized plateau and ending the LLD does not immediately present itself.

Perhaps once the trill has been maintained for a time and SK channels (or another depolarization-activated channel) are open but are not yet producing a large enough polarizing current to end the LLD, FTNs become dependent on incoming excitatory synaptic input to oppose polarization and maintain the LLD. If a number of early-bursting FTNs eventually auto-terminate and stop exciting other FTNs, those FTNs may be forced to end their own LLDs. The more FTNs whose LLDs end, the less excitation the remaining FTNs would receive and thus be more likely to end themselves. LLDs from late-bursting FTNs are the last ones to end because they would have been active for the shortest time and thus would have the smallest polarizing currents and require the least amount of excitation to continue, and vice-versa for LLDs from early-bursters.

Materials & Methods

Frogs were anesthetized with a subcutaneous injection of ˜0.6 ml 1.3% tricaine methanesulfonate (MS-222). When they became unresponsive, they were weighed, measured, and then buried in ice on the dissecting tray for ten minutes. Before dissection began, a toe pinch was applied to confirm the absence of a reflex response (and thus, the absence of functioning neurons). The spine was cut and the skull plus surrounding tissue was extracted and briefly washed in oxygenated saline before being placed underneath a dissecting scope in the dissecting dish (a large petri dish lined with a silicone elastomer [Sylgard, Dow]) filled with oxygenated saline (in mm : 96 NaCl, 20 $NaHCO_3$, 2 $CaCl_2$, 2 KCl, 0.5 $MgCl_2$, 10 HEPES, and 11 glucose, pH 7.8).

Excess fat and muscle tissue were cut away to expose the skull and spine. A sceleral punch was then used to peel back the skull, chip away the spine, and collapse the otic capsules in order to expose the brain and cranial nerves. The spinal cord and nerves were cut using a pair of iridectomy scissors and the isolated brain was pinned (two pins for each olfactary nerve and one pin at the back of the cord) in a dissecting dish with fresh oxygenated saline. The meninges were removed, all rootlets entering N.IX-X apart from the most caudal (forth) were cut, and the dorsal midbrain was bisected between the forth and fifth ventricles. The brain was then allowed to rest in oxygenated saline for at least an hour in order to ensure that the MS-222 had worn off.

The brain was transferred and re-pinned into a smaller recording dish, with additional pins opening the ventricle cut to allow access to DTAM. Fresh, oxygenated saline was perfused into the dish at ˜200 ml/hr. A suction electrode was used to attach N.IX-X to allow recording of laryngeal motor neuron activity. This signal was run through a differential AC amplifier (A-M systems, Model 1700) and digitized (Molecular Devices, Digidata 1440A) before being sent to the computer and recorded with Clampex and MultiClamp 700b Commander before being analyzed in Clampfit (Molecular Devices). Local field potentials (LFPs) were recorded by shallowly inserting a tungsten electrode into DTAM and placing a ground in the recording dish.

To confirm functional fictive calling, saline perfusion was halted and 5 µl of 60mm 5-HT was applied via micropipette into the ˜6ml bath, giving a final concentration of about 4 µm. If fictive calling was weak, perfusion was reinitiated and the brain was rested for at least and hour (as was the case between every 5-HT application) before another identical 5-HT application. Once healthy fictive calling was confirmed, a glass micropipette (see Appendix A1) was filled with intracellular solution (in mm : 115 KCl, 2 $MgCl_2$, 2 EGTA, 10 HEPES, 2 MgATP, and 0.2 NaGTP, pH 7.8) and placed into a micro manipulator. A neuron in DTAM was then whole-cell patch-clamped using the blind technique (the state of the electrode is monitored solely via insertion depth and resistance, see Appendix A2).

Results

Whole-Cell Recordings of FTNs in the Transected Brain

Four fast trill neurons were successfully recorded: one in an intact brain and three in transected brains (figure 7). At first glance, it seems that FTNs recorded in transected brains do not have drastically altered activity patterns; they still produce a LLD and burst of action potentials correlated with the LFP wave. All recorded neurons displayed a robust queer current.

The first FTN to be recorded was in the whole-brain (figure 7A). It seemed to be a relatively hyperpolarized neuron: it did not produce inter-trill action potentials, had large LLDs[30], and frequently displayed truncated bursts of action potentials during the LLD. There were prominent 50 Hz STOs during all of its LLDs. It received bumps in membrane potential that looked like EPSPs between bouts of song, and was hyperpolarized during breathing. Transection of the brain was attempted during this clamp, but the procedure moved the brain enough that access to the cell was lost.

The second FTN was found in the same brain following transection (figure 7B). It was the most anomalous of all recorded FTNs; while it did undergo a period of depolarization during some LFP waves, it never fired any action potentials. This may indicate that it was damaged from the clamp, hyperpolarized due to the transection, or our access was just too limited or distal to detect action potentials. The rapid EPSP-like events during its LLD are further analyzed in figure 9. Depolarizing current steps larger than 200 pA resulted in a rapid hyperpolarizing current (see Appendix A3).

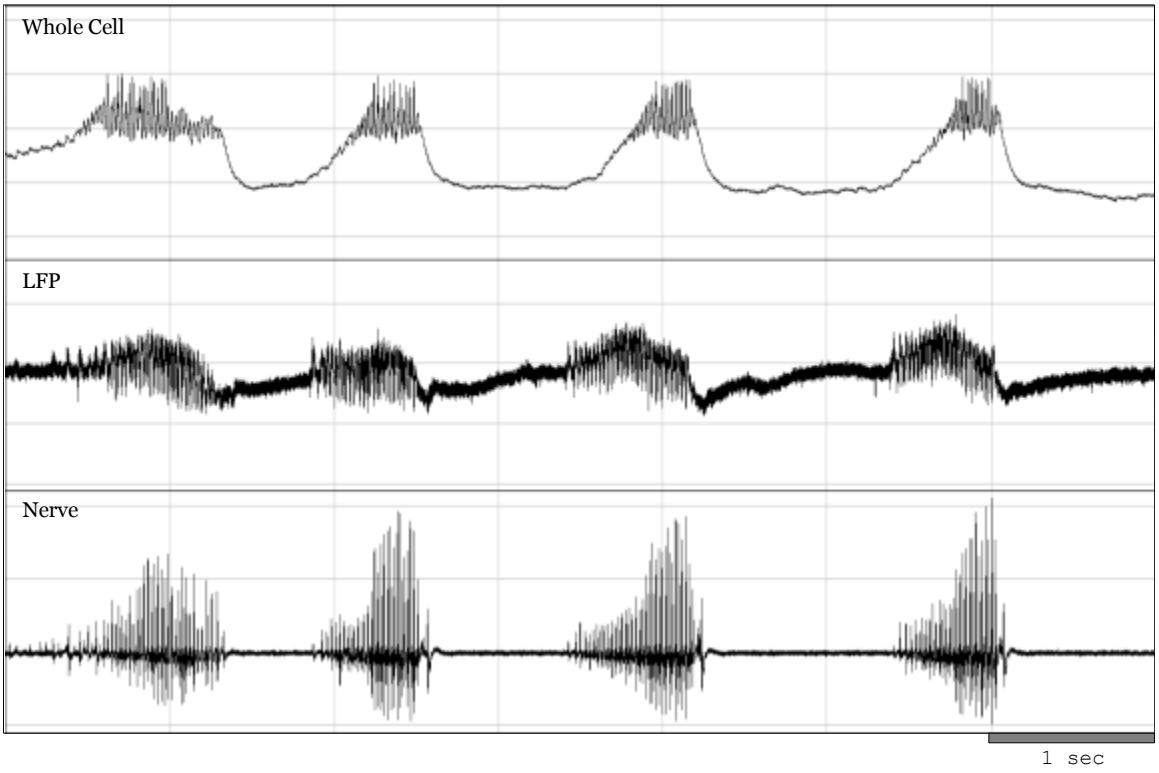
The third FTN was found in the same brain as the first two cells. It seemed to be more depolarized than the cell found in the whole-brain; it displayed more robust firing during and between trills, and possessed relatively shallow LLDs.

The fourth FTN was found in a second transected brain. There was consistent inter-trill firing which increased in rate on application of 5-HT. LLDs were almost invisible due to – presumably – a relatively depolarized baseline.

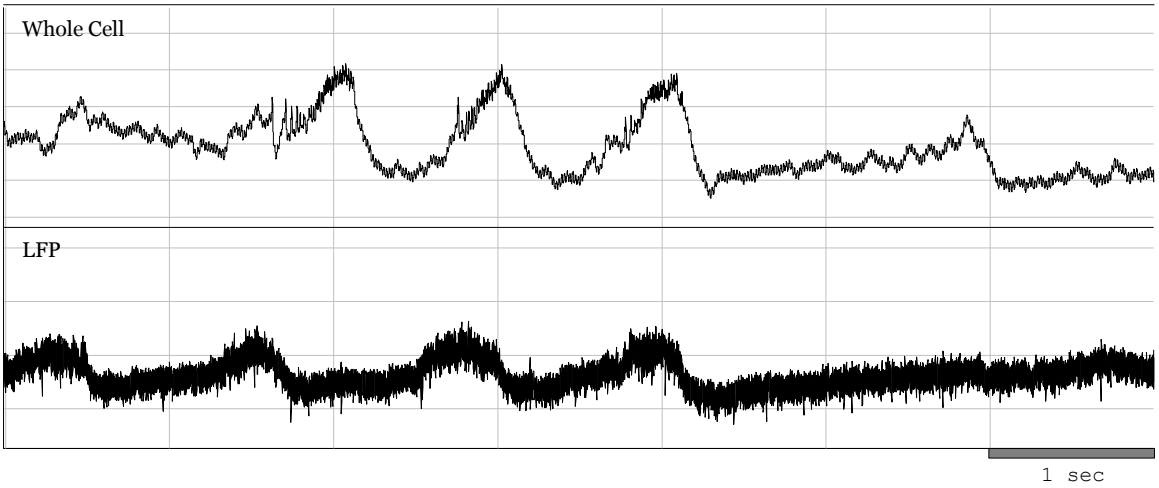
30 A neuron with a hyperpolarized resting potential can produce larger LLDs than a neuron with a relatively depolarized resting potential because the cation reversal potential is further removed; it has more distance to fall.

Example FTN Traces

A) FTN 1 – Brain #1 – Intact

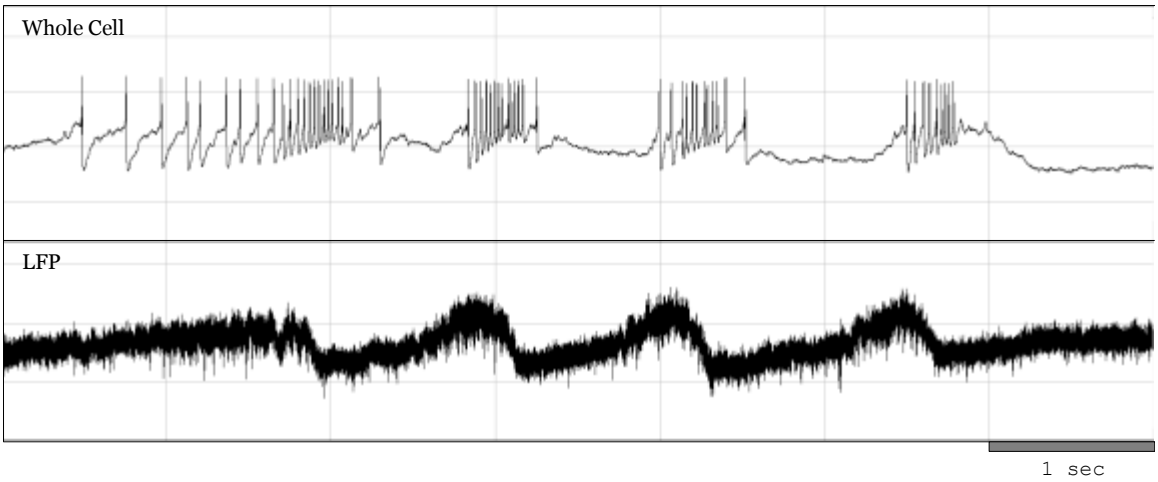


B) FTN 2 – Brain #1 – Transected



Example FTN Traces

C) FTN 3 – Brain #1 – Transected



D) FTN 4 – Brain #2 – Transected

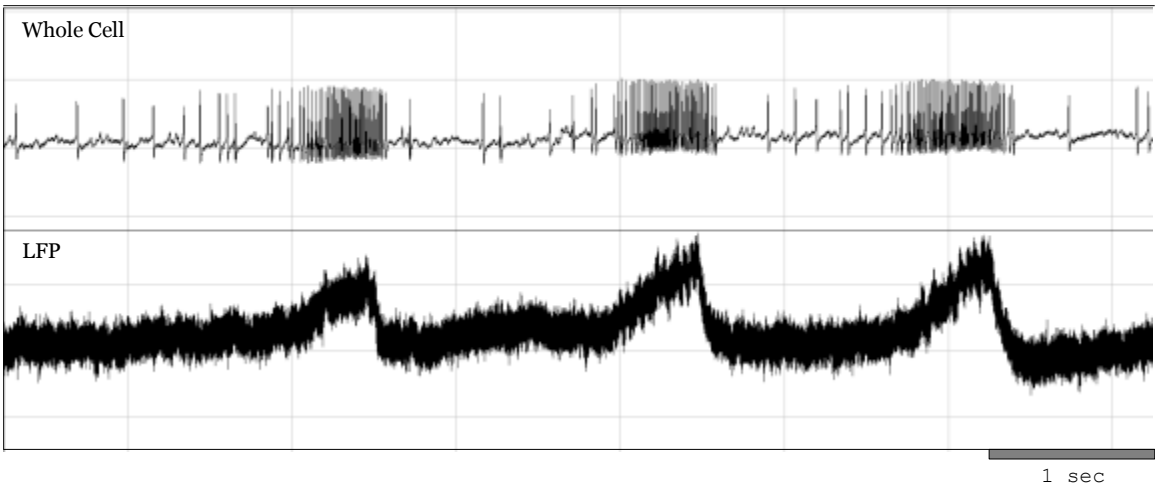


Figure 7. Examples of three different FTNs during 5-HT application. Due to incorrect program settings, a vertical scale bar is not available.

A – FTN 1: This cell is the only one recorded as part of the intact circuit. It produced large LLDs and fired exclusively during fictive fast trill. Prominent STOs are also present; in many cases the cell failed to fire an action potential (e.g., halfway through the first trill) but still produces a significant depolarization.

B – FTN 2: This cell never spiked. However, we were able to detect small and rapid EPSP-like depolarizations during LLDs, possibly implying a loss of the synchronized excitatory input seen in whole brain preparations.

C – FTN 3: This cell's LLDs and action potentials were roughly comparable to those seen in the whole brain. The shallow LLDs are probably due to a relatively depolarized resting potential.

D – FTN 4: This cell was the most depolarized of all clamped cells; it produced rapid trains of APs, consistently fired in between bouts of trill, and its LLDs were almost nonexistent (see footnote 30).

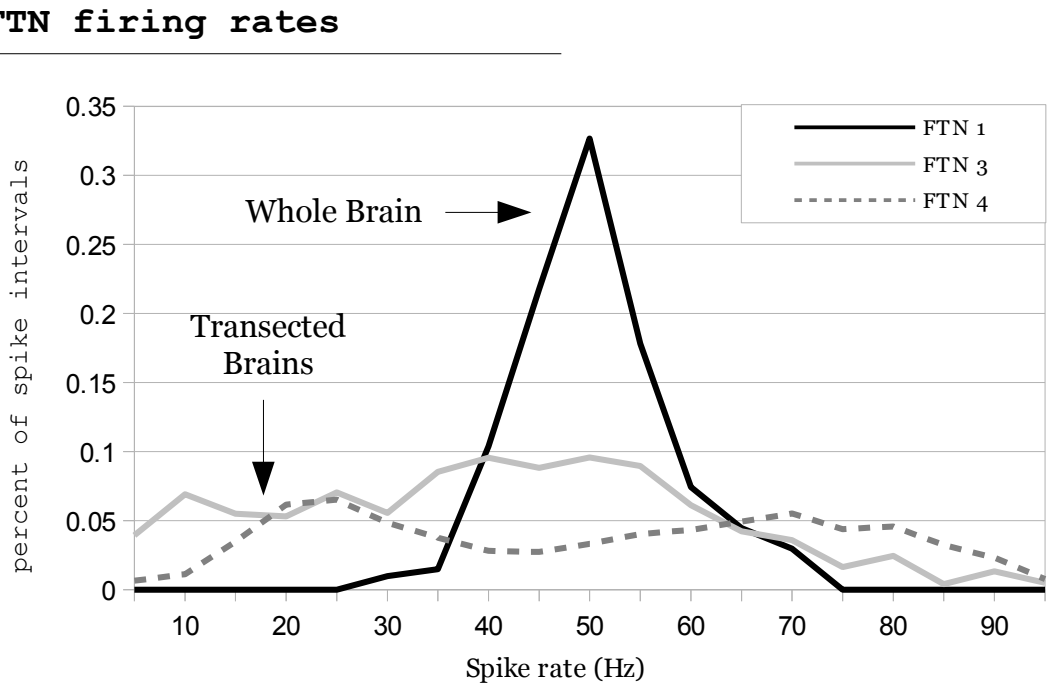


Figure 8. Histogram of FTN firing rates during fictive calling in whole and transected brains (bin width = 5 Hz). Spike rates were analyzed over the course of several bouts in each neuron. Interestingly, the two FTNs from separate transected brains have different average rates of fast trill spiking.

FTN 1 (n=202 spikes) is from an intact brain shows a significant spike at 50 Hz (these data are a mixture of action potentials and STOs).

FTN 3 (n=867 spikes) was found in the same brain as FTN 1 after it had been transected, and shares peaks at ~ 40–50 Hz. It also displays lower-frequency peaks at 10 and 25 Hz, representing inter-trill spiking.

FTN 4 (n=2155 spikes) fired faster on average that either of the other FTNs with a peak at 70 Hz. It also displays a peak at 25 Hz, also representing inter-trill spikes.

Frequency Of Action Potentials

Action potentials during LFP waves were peak-picked and sorted into a normalized frequency histogram (figure 8). The FTN recorded in the whole-brain experiment shows a prominent narrow peak at 50 Hz. Peaks from neurons recorded in transected brains are much wider due to a greater variability in spike rate. The transected-brain FTNs also had different rates of firing during fast trill.

Peaks at 25 Hz in the third and forth FTN represent, for the most part, inter-trill and introductory spikes. The histogram from the whole-brain FTN likely does not display this peak because it did not produce intro phase trill spikes (figure 7A), while the other two neurons displayed substantial slow spiking between trills (figure 7C,D)

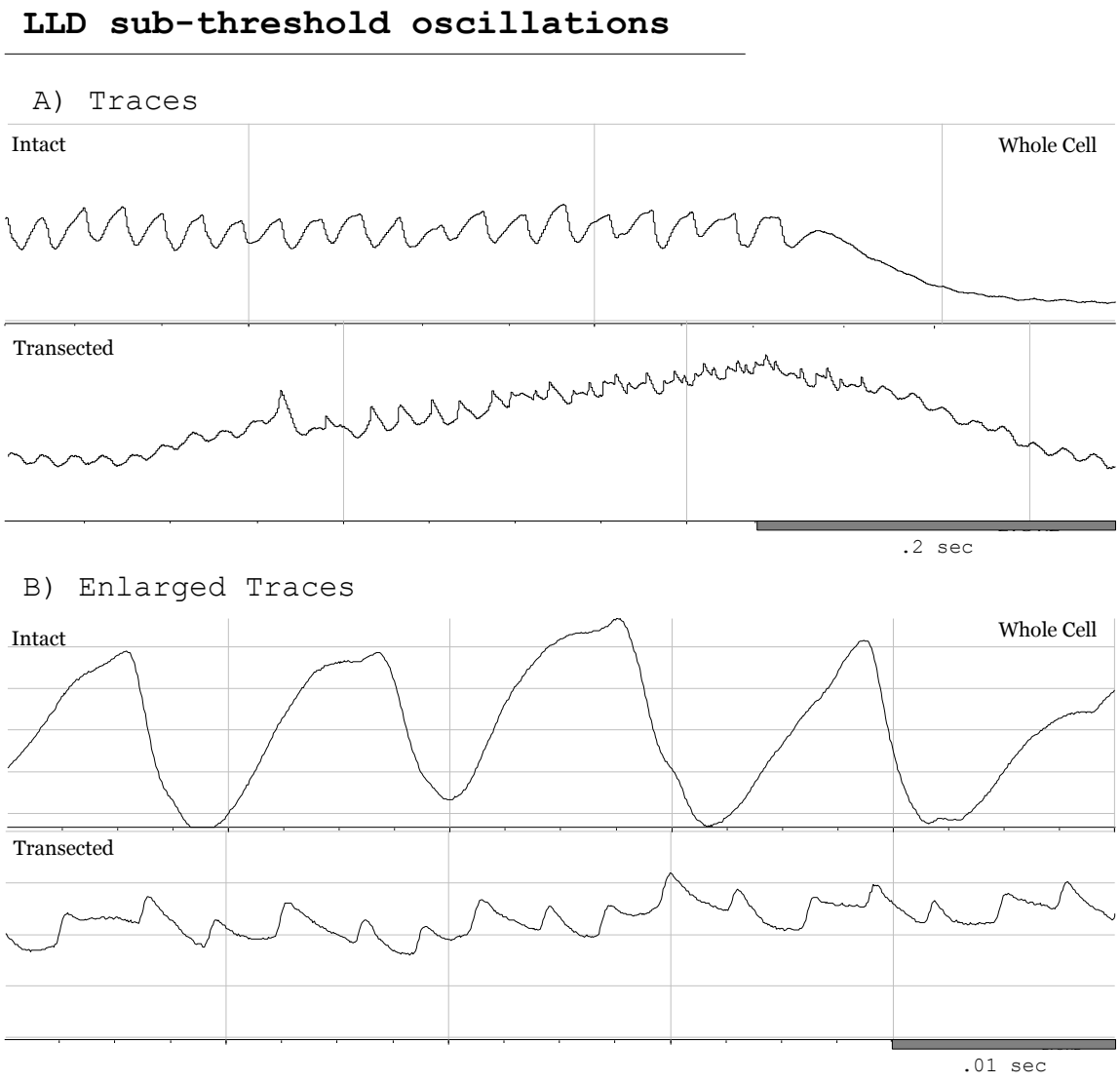


Figure 9. Sub-threshold activity during LLDs produced by neurons from intact and transected brains. Both y-axes are on the same scale of arbitrary units.

The trace from the **intact brain** show a section of an LLD where the FTN failed to produce action potentials, revealing the large 60 Hz phase-locked oscillations underlying normal trills. The decay slopes seen in **B** are not exponential; the cell is probably being polarized by a combination of intrinsic currents and the IPSPs that follow every CAP.

STOs from the **transected brain** are significantly faster and smaller than those in the intact brain. They have an exponential decay characteristic of EPSPs (seen more clearly in **B**). The transected trace also contains baseline 60 Hz noise, seen preceding and following the LLD in **A**.

Sub-threshold Activity

A single FTN from a transected brain produced LLDs but no action potentials (figure 7B). Compared to a spikeless section of a cell from an intact brain, the sub-threshold events in the transected brain are much smaller and are a different shape than those in the intact brain (figure 9B). Their decay slopes look exponential – a characteristic of EPSPs rather than intrinsic STOs. The steeper decay slopes from the intact brain are likely due at least in part to phase-locked IPSPs, which have been previously observed in voltage-clamped FTNs from intact brains. The absence of IPSP-like activity and the continued presence of EPSPs suggests that connections in DTAM may be primarily excitatory while input from n.IX-X are inhibitory in nature.

Trains of these spikes were not found during all waves; the neuron would sometimes exhibit an LLD-like depolarization during a wave without visibly distinct STOs or EPSPs (see Appendix A3). One reasonable interpretation of this is that there may be EPSPs too faint or distal from the clamp point to distinguish, and the sudden appearance of EPSPs is a result of a new FTN (or group of FTNs) that suddenly become active during the trill.[31]

LFP Decay Slopes

If FTNs lose CAP synchrony in the transected brain, it's possible that LFP wave synchrony could also be affected. Since waves are still produced, they obviously do not completely lose coherence. However, slightly desynchronized LLDs could result in wider and shorter LFP waves. There is not a consistent way to identify the start of a LFP wave, so they were characterized by applying a 5 Hz low-pass filter and then calculating the slope between the wave's peak and the first minimum following the wave (figure 10).

Though both brains showed a significant shift in their normalized decay slopes following transection, they were in opposite directions. It's possible that a trend would emerge with a greater number of trials, but at present we can't claim that there is a consistent shift in the decay slope of LFP waves following transection.

31 The implication being that there is a population of FTNs which are occasionally not recruited to spike during transected fictive vocalizations.

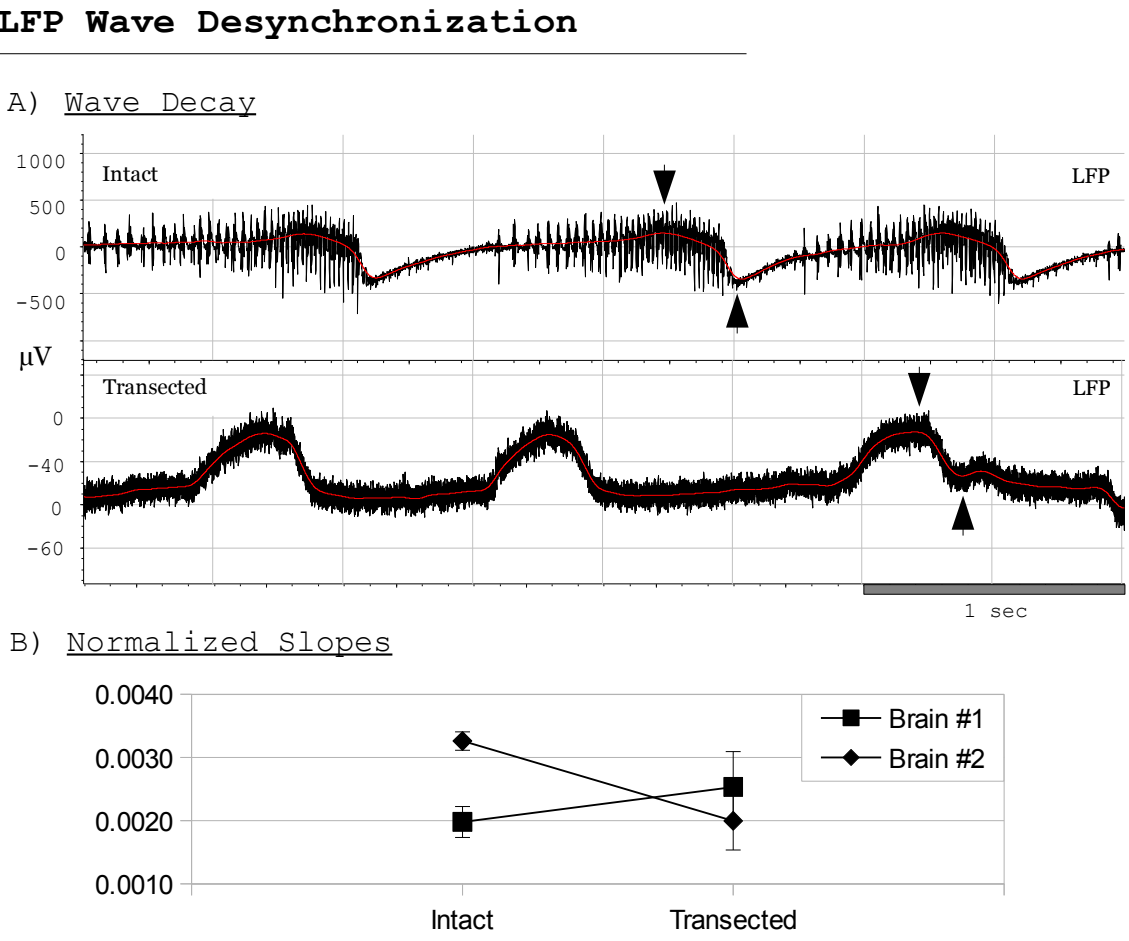


Figure 10. Normalized wave decay slopes.

A – Wave Decay: Waves were characterized by running a 5 Hz low-pass filter (shown as a grey line inside of both traces) and then peak-picking its minima and maxima during waves (arrows). Slopes were calculated as the change in amplitude divided by the time interval between the two points.

B – Normalized Slopes: Due to the variability in scale between LFP recordings, slopes were normalized to the largest change in amplitude during their bout (e.g., the slope of the largest wave became 1 / interval). No clear trend for the change of the average slope was found following transection; though each shift was individually significant, they were in opposite directions.

Discussion

Review

Bath-applied 5-HT will cause extracted *Xenopus* brains to produce patterns of laryngeal nerve activity resembling that of an animal producing advertisement calls. Fast trill neurons in DTAM seem to be the primary drivers of the laryngeal motoneurons during fictive calling. They undergo a long-lasting depolarization for the duration of each fast trill during 5-HT-evoked fictive calling. During these LLDs, they produce ~60 Hz subthreshold oscillations and action potentials, and receive simultaneous EPSPs and IPSPs following each spike. Local field potential recordings pick up the net activity of FTNs during fast trill, and show corresponding waves (due to the overall depolarization of the area due to LLDs) with phasic activity (presumably due to the synchronized firing of action potentials).

LFP waves are still produced in brains that have been transected between n.IX-X and DTAM, but they lose their phasic activity. I hypothesized that the loss of phasic activity in transected-brain LFP waves was due to a loss of synchronization between the action potentials of fast trill neurons. Recordings of FTNs in the transected brain support the model that the connection to n.IX-X normally synchronizes FTN action potentials, but connections within DTAM are sufficient to synchronize LLDs.

Fast Trill Neurons in the Transected Brain

In the absence of the ability to simultaneously record from two FTNs to directly observe any change in synchronization following transection, evidence of transected-brain AP desynchronization has to come from single-FTN and LFP recordings. As previously reported, I found that phasic LFP spikes are lost in transected brains. I have shown that spike rates in transected-brain FTNs on average remain roughly the same as in the whole brain[32] but show a marked increase in the variation of spike rates (figure 8), implying a loss of some regulatory influence – presumably coming from n.IX-X.

The two spiking FTNs I found in the transected brain had different peaks in

32 Maybe a little bit faster – which makes sense if they're losing an inhibitory signal.

spike frequency (figure 8). The first neuron roughly matched the whole-cell neuron found in the same brain at 40 and 50 Hz, while the second spiked considerably faster at an average of 70 Hz. This difference in speed may reflect a difference in the natural spiking frequency of FTNs once released from n.IX-X regulation, a difference in the health of the clamped neuron, or a difference in the health between brains.

Additionally, I found a single FTN that did not produce APs during its LLD, revealing the underlying subthreshold activity present during trills. The ~60 Hz STOs seen in whole-brain preparations were absent, replaced by smaller and more rapid spikes (figure 9). These spikes had the characteristic shape of EPSPs; a sharp increase followed by and exponential decay. The lack of any IPSP-like events suggest that transecting the brain eliminates the normal phase-locked inhibitory input to FTNs. If this is the case, it would support the model that n.IX-X → DTAM connections are inhibitory.

The rate of these (presumably) EPSPs had a range from 20–220 Hz (see Appendix A3). Assuming this neuron was not damaged in some exotic way, it appears that it could be receiving desynchronized excitatory inputs (separate ~60 Hz trains of spikes). Given this interpretation, this FTN could only share excitatory synapses with a handful of other neurons (many desynchronous inputs would result in even higher rates of EPSPs). Until more neurons of this type are found or more DTAM connectivity data are collected, it's difficult to make further assertions.

Given the loss of synchronization of action potentials in the transected brain, I asked if the synchronization of LLDs into LFP waves were also affected. A desynchronous wave would be lower-amplitude and wider, so I analyzed LFP decay slopes (figure 10) in order to see if the ability of FTNs to form coherent waves is reduced by transection. In both brains, I saw a significant shift in the decay slope, but in opposite directions (i.e., one brain's waves began having a sharper termination post-transection and the other's became more shallow). It's possible that a trend would emerge given additional data, but at the moment it does not appear that transection causes a drastic shift in the overall shape of an LLD. This indicates that the circuit synchronizing the onset and offset of LLDs is contained entirely within DTAM.

The Model, as it Stands

Taken as a whole, these data support a model where n.IX-X synchronizes the action potentials of fast trill neurons via inhibitory input[33], and that fast trill neurons in transected brains are able to synchronize the onset and offset of their long-lasting depolarizations[34] despite the desynchronization[35] of their excitatory[36] connections.

The model of the fast trill circuit currently stands as such:

- 5-HT acts directly on FTNs (and likely elsewhere in the circuit) via 5-HT_{2C} receptors to depolarize them and enable LLDs.
- FTNs project excitatory connections to: 1) FTNs 2) n.IX-X motoneurons and 3) n.IX-X interneurons.
- While n.IX-X motoneurons project their axons down N.IX-X, interneurons project inhibitory connections back to DTAM. Acting as an efferent motor copy, this inhibition synchronizes the STOs of fast trill neurons, causing the synchronization of FTN action potentials to become a positive-feedback loop: the more synchronized input n.IX-X receives from DTAM, the more effectively it would synchronize FTNs.
- Excitatory connections between FTNs within DTAM are sufficient to produce a rolling recruitment of FTNs producing synchronous NMDAR-dependent TTX-insensitive LLDs.
- LLDs trigger depolarization-activated ~60 Hz STOs which set the correct spike timing and rate of action potentials during the trill.
- LLDs synchronously end by an unknown mechanism – possibly due to a buildup a calcium during the trill causing an increasing need for excitation to maintain the trill, setting up a positive-feedback loop for trill termination.

33 Given the lack of IPSPs seen in the first transected-brain FTN.
34 Given the lack of a drastic change in the shape of LFP decay slopes.
35 Given the increased variation in firing rates.
36 Given the presence of EPSPs seen at the start of each LLD and the pattern of EPSPs seen in the first transected-brain FTN.

Appendix

A1. Pipette Manufacture

Micropipettes used for whole-cell recordings were made from borosilicate glass (with filament) capillary tubes with diameters 1.5 mm outside and .86mm inside. They were pulled with a Sutter Instrument Flaming / Brown Model P-1000 pipette puller under the program:

Heat 319 | Pull 0 | Velocity 50 | Time 150 | Pressure 500

Bath resistances usually ranged from 7 – 10 MΩ.

A2. Blind Patch-Clamp Technique

A glass micropipette was filled with intracellular solution (see materials and methods) and placed into an electrode micro-manipulator. A disposable syringe was used to apply 0.04 ml positive pressure. The pipette was lowered into the dish and positioned over DTAM in voltage clamp mode with holding at 0 mV with steps of -10 mV.

The pipette was advanced until its resistance showed a sharp increase, indicating contact with the tissue. A positionometer's baseline was reset to track the depth of penetration. The pipette was quickly pulsed back and forth, staying approximately within 80 µm of the surface. Once the pipette was advanced to ˜80 µm and resistance had popped back down to near-bath levels, I would begin searching for cells.

To find a cell, I would steadily advance the pipette at ˜1 µm/s until there was a small but rapid increase in resistance (usually about up to 20 MΩ). Upon contact, I retracted the pipette 10 µm before advancing it again to see if the contact was repeatable. Once I found a consistent presence of a cell, I would release the positive pressure and mouth-apply a slight suction to the back of the syringe. At this point, either the resistance would jump to somewhere between 300 to several thousand MΩ, or would become clogged and I would start over.

Upon obtaining a good seal, I would switch the holding potential to -60 mV and apply a sharp-but-brief inward suction to the syringe (making a "smeck") noise to break into the cell. As I was learning the technique, it seldom worked on the first try. A successful break-in would drop the resistance back down to the tens of MΩ, and I would confirm the cell clamp by running a series of increasing current injections until I saw a queer current and action potentials.

A3. FTN #2

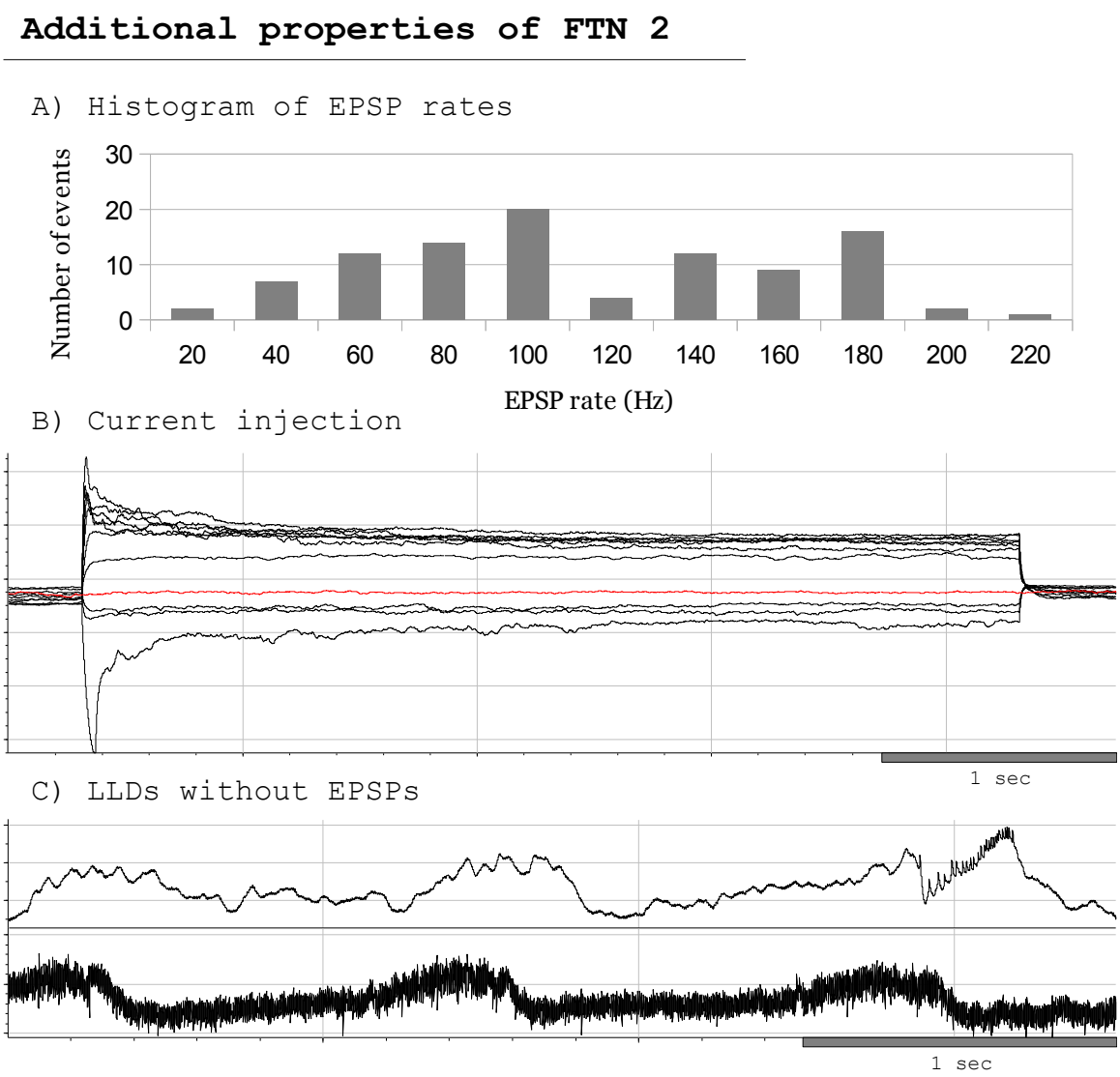


Figure 11. Additional properties of FTN 2.

A – Histogram of EPSP rates: EPSP rates were collected from three separate trills and are aggregated here. There seems to be a gap at 120 Hz – possibly indicating some unknown mechanism preventing FTNs from firing their ~60 Hz APs exactly out of phase.

B – Current injection: Current injections starting at -300 pA and increasing by 100 pA per sweep were applied to the cell. Queer current and a depolarization-activated hyperpolarizing current are displayed.

C – LLDs without EPSPs: Not all long-lasting depolarization events in this cell also received EPSPs. It's possible that the cell is being excited in a manner we can't directly see (e.g., distal EPSPs) and only during particular bouts do excitatory synapses we can detect become active.

A4. Summary: Ten-Hundred Most Common Words

I study the brain of a jumping animal that lives in the water and makes noise. I want to find out how it makes that noise. So I take out its brain and put the brain in a cup of sweet water that has air in it (so can eat and breathe). If I put in a kind of brain water and then listen very hard with a computer I can hear it trying to make the noise!

That is really cool, but I still don't know how it's making the noise. Other people have done things that help me understand. One person put dark water in the noise-making part of the animal to see where in the brain the pretend noise is coming from. This dark water went inside the brain cells that made the noise making part go. They put in more dark water, and saw that part they found liked to talk with a different part, which liked to talk to the first part at the same time.

(The brain is a very confusing place! Many parts talk to other parts and it's hard to keep track of what they all are saying. For making pretend noise, it only needs these two parts. That makes studying this a lot easier!)

Cells in the second part talk and get quiet fast all at the same time (about as fast as air-lights go on and off). They do this in bursts, and are also able to all stop at the end of the burst at the same time. It sounds just like the pretend noise! But if we cut the brain between the two parts, it still tries to talk in bursts but it doesn't sound like anything anymore. This tells us that to talk and get quiet at the same time, the second part needs to be able to hear the first part. But the second part doesn't need to hear anything else to talk in bursts!

(I think it has gotten confused and the cells can't talk at the same time because they don't have the first part telling them when it's fine to say stuff. The first part might be like one of those red and green lights that tell cars to stop and go, only it gets them all to go at the same time.)

The problem is, since cells in the second part also talk to each other, we don't know who is telling them the things we see them hear. I am going to try to cut the brain in half while watching what cells in the second part are hearing. The stuff they stop hearing after I cut the brain in half will probably be the stuff the first part was telling them, and the stuff they keep hearing will be the stuff they're telling themselves. If it works, it will answer both how they get quiet and loud really fast all at the same time AND how they all know when to end their burst!

The biggest problem is that I am running out of time to learn how to listen to the very small cells and write my final big paper for college, and I keep not doing these things because the computer has simple word boxes which are much more fun to write in than the big word boxes.

(Also, I have to kill the jumping animals in order to find out these things, and that makes me sad.)

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