Functional contributions of multiple cancellation signals in suppressing predictable electrosensory noise

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Abstract

The principal electrosensory neurons (AENs) in the medulla of little skates extract relevant signals from a noisy electrosensory background generated by the animal's own behaviors (reafference). Research supports the existence of an adaptive filter in the cerebellar-like electrosensory nuclei of skates. Internal reference signals (e.g. motor commands and sensory feedback) in the molecular layer carry information pertaining to ongoing behaviors and supply AENs with cancellation signals (i.e. inverse predictions of electrosensory input), which when added to the actual electrosensory input, negate reafference. Thus the removal of predictable electrosensory input and preservation of novel signals in AENs are achieved.

In this study, predictable electrosensory input was experimentally created by repetitive pairings of a peripheral excitatory electrosensory stimulus with either ventilatory motor commands or passive fin movements, both of which are known to be represented among the internal reference signals of the molecular layer. In both cases, the development of a cancellation signal that suppresses predictable electrosensory input was apparent in AEN responses: the response to the electrosensory stimulus declined during repeated pairings, and removal of the electrosensory stimulus revealed suppressed activity time-locked to the period of the suddenly absent electrosensory stimulus. Using this coupling paradigm, the functional properties of multiple cancellation signals within a single AEN were examined. First, multiple cancellation signals
associated with distinct internal reference signals in the molecular layer are shown to exist within a single AEN and are modified independently, suggesting that separate cancellation signals for distinct behaviors may also exist, and can be stored and modified without affecting one another. Second, overlapping cancellation signals associated with distinct internal reference signals are additive in a subset of AENs, although not all AENs had combined cancellation signals that were significant or stronger than singular cancellation signals. These results support the idea that a single behavior may have several component cancellation signals that are associated with different aspects of the behavior. Together, the component cancellation signals may work in conjunction to more effectively suppress reafference associated with that behavior. We propose that a functional role for multiple cancellation signals, shown in this study to be independent and additive, may be to provide sensory predictions that suppress dynamic, complex patterns of reafference that result from continuously, intermittently or concurrently performed behaviors.
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Chapter 1:

A review of electro sensory noise suppression in elasmobranchs

Function of electro sensory systems

The acute sensitivity of most sensory systems becomes problematic when irrelevant noise overloads and interferes with sensing of weak signals. Electroreceptive fish in marine environments demonstrate sensitivity to electric fields as weak as 1 to 5 nV cm\(^{-1}\) (Wilkens and Hoffman 2005). In skates, *Raja erinacea*, the animal’s own behaviors, such as breathing and swimming, cause continuous, undesirable self-stimulation (or reafference) of the peripheral electroreceptors and subsequent primary afferent neurons (Figure 1A). Biologically relevant signals from the external environment are thus difficult to extract from a background of self-generated noise. Despite receiving input directly from primary afferent neurons, the principal neurons of the electro sensory nucleus in the skate medulla seem unaffected by reafference and show a marked improvement in their signal:noise ratios (Figure 1B; Bodznick and Montgomery 1993; Bodznick, Montgomery and Carey 1999). Principle electro sensory neurons of mormyrid and gymnotid electro sensory lateral line (ELL) are similarly unaffected by reafference (Bell 1981; Bell, Bodznick, Montgomery and Bastian 1997). In all three systems, removal of redundant reafferent signals is essential to facilitate extraction of pertinent environmental signals. Useful extrinsic signals include bioelectric fields generated by other...
Figure 1. Reafference (self-stimulation) in the skate’s electrosensory system.

A. *Left:* The activity of primary afferent neurons (Aff) is tonic in paralyzed fish, but becomes strongly modulated by breathing (ventilation, V) and swimming (fin movements, FM) behaviors. *Right:* Responses to excitatory electrosensory stimuli (E) are masked by reafference (self-stimulation).

B. *Left:* Secondary AENs receive direct input from primary afferent neurons, yet reafference is suppressed. Spontaneous activity in AENs is low and not strongly affected by behaviors. *Right:* The AENs maintain sensitivity to weak electrosensory stimuli.

For both A and B, electrosensory stimuli onset (E) are indicated by upward deflections, intensity 5 μV. Numbers below Aff and AEN traces indicate spike rate in Hz; vertical scale bars, 0.5mV.

C. A diagram depicting the electrosensory pathway of skates: external electrosensory stimuli are detected by electroreceptors on the peripheral body and transmitted to secondary AENs in the medulla via primary afferent neurons (Adapted from Bodznick, Montgomery and Carey 1999). *Inset:* Distribution of electroreceptors on ventral (V) and dorsal (D) surface of the skate (Adapted from Bodznick, Montgomery and Tricas 2003).
A  Primary afferent

Paralyzed Fish  |  Paralyzed + E
Breathing Fish  |  Breathing + E
Swimming Fish  |  Swimming + E

B  Secondary AEN

1  |  2

C  Electrosensory pathway

Molecular Layer

Secondary Neuron (AEN)

Recording Electrode

Hindbrain

Body

Electroreceptor

Electrosensory Stimulus
living organisms, such as prey, predators and conspecifics. Skates, which are bottom-feeding animals, use their electrosense to uncover and capture buried prey (Kalmijn 1997). In locating conspecifics, female stingrays move to position themselves nearby a buried dipole emitting simulated bioelectric fields comparable to those produced by other females. On the other hand, male stingrays, searching for reproductively active females, perform abrupt turns upon detection of these fields and attempt to excavate the dipole. The males would then normally begin courtship behaviors afterward (Tricas, Scott and Sisnersos 1995). Although use of electroreception for predator avoidance has not explicitly been tested, small sharks prefer attacking smaller metal objects in favor of large metal objects. The interpretation is that higher intensity electric fields emanate from larger animals, which may pose a predatory threat (Johnson, Scronce and McManus 1984). Additionally, the electrosense may be used for detecting non-bioelectric fields, such as in navigation. Electroreceptive fish are sensitive to magnetic fields at geological magnitudes, as well as the direction and polarity of electric field voltage gradients. In experimental settings, stingrays are able to use magnetic fields to orient and perform learned behaviors (Kalmijn 1978).

*Self-modulation of electroreceptors*

Electroreceptors, which are distributed widely around the body (Figure 1C) measure the difference in the strength of electric fields between an external
pore and the internal reference (Hofmann, Chagnaud and Wilkens 2005). Recordings of transdermal potential difference, which also compare the internal and external milieu of the animal, show fluctuations that correspond to ventilatory inhalation and exhalation phases in skates (Bodznick, Montgomery and Bradley 1992). The driving force that creates the transdermal potential difference is thought to be created by active osmoregulatory ion-pumping and passive ionic currents across permeable epithelial layers in contact with seawater such as the gills, spiracles and mouth. In naturally behaving skates, opening and closing of the mouth associated with breathing alters the transdermal potential difference. Experimental hypo- or hypertonic disruption of the osmoregulatory balance brought on by transferring the skate to dilute seawater or salt-loading the skate with saline injection resulted in predictable enhancements or reductions of the transdermal ventilatory potential (Bodznick et al. 1992). Altering the electrical conductivity using weak depolarizing or hyperpolarizing current injections into the animal’s body via a salt-bridge electrode also predictably shifted the transdermal potential (Bodznick et al. 1992). The activity of primary afferent neurons closely reflects fluctuations in transdermal potential, thus ventilation—a behavior that modulates transdermal potential—generates electrosensory reafference that must be eliminated via sensory processing.

Swimming behaviors also weakly stimulate the electroreceptors (Figure 1B). Fin movements cause mechanical perturbations of the electroreceptor
organ, and activation or suppression of primary afferent neurons (Figure 1B; Conley 1995). Moreover, deflections of the fin change the spatial positions of electroreceptors on the fin, as well as partially screen electroreceptors on the body from external electric fields (Conley and Bodznick 1994). Thus although an external electric field may be constant, detection of electric fields at various electroreceptor pores on the body or fin will be transformed to varying amounts by fin movements during swimming.

Clearly peripheral sensory input fluctuates during performance of different behaviors, hence there is a necessity for accurate integration of proprioceptive and electrosensory information during sensory processing in order to determine appropriate behavioral outcomes.

*Circuitry of electrosensory system in elasmobranchs*

Primary afferent neurons carry electrosensory signals from peripheral electroreceptors to the first order electrosensory processing nucleus in the skate medulla, called dorsal octavolateralis nucleus (DON). Within the DON are principal neurons (ascending efferent neurons, AENs), which have large multipolar cell bodies (20-40 µm length, 10-15 µm width) situated beneath a superficial molecular layer. The spiny apical dendrites of AENs extend above into the molecular layer and receive many thousands of synapses from excitatory parallel fibers and inhibitory stellate cells. The AENs also have smooth basal dendrites that protrude into the central zone and receive comparatively fewer,
yet very strong excitatory input from 10 to 20 primary afferent neurons (Figure 2; Bodznick and Schmidt 1984).

*Molecular layer inputs*

The molecular layer comprises beams of unmyelinated parallel fibers, which are the axons of granule cells. Granule cell bodies are situated in the dorsal granular ridge (DGR), superficial to the DON (Figure 2). Afferent inputs to DGR carry internal reference signals pertaining to ongoing behaviors, including copies of motor commands, proprioceptive and electroreceptive signals (Hjelmstad, Parks and Bodznick 1996; Conley and Bodznick 1994).

Afferent cells to DGR carry copies of motor commands associated with different phases of ventilation (e.g. inhalation, exhalation, ventilatory coughs). Simultaneous recordings of an efferent cranial nerve carrying ventilatory motor commands showed that there is synchronous bursting activity in DGR afferents that were temporally coincident with ventilatory movements (Hjelmstad et al. 1996).

Proprioceptive afferents to DGR show sensitivity to fin position, movement and direction. Information from stretch receptors in the fin muscles reaches DGR via the spinal cord (Schmidt and Bodznick 1987). DGR also receives incoming cranial projections from the paralemniscal nucleus, nuclei F and K. Nucleus F has been implicated as a vestibular nucleus, while the paralemniscal
Figure 2. Dorsal octavolateralis nucleus in the skate medulla. A. Drawing of a skate brain. Horizontal line indicates transverse section through the hindbrain of the slice shown in B. B. Cresyl-stain of a slice through the hindbrain showing the electrosensory nucleus (called dorsal octavolateralis nucleus, DON) with neurons (not drawn to scale) of the electrosensory circuit overlaid. The cell bodies of AENs are situated in the peripheral zone (pz). The basal dendrites of AENs extend into the central zone (cz) and receive peripheral electrosensory input from primary afferent neurons. The apical dendrites of AENs extend into the molecular layer (ml) and receive inputs from dorsal granular ridge (DGR) granule cells, which provide information about ongoing behaviors. Both apical and basal dendrites also receive inhibitory input via inhibitory interneurons (IN). MB, midbrain; HB, hindbrain; CB, cerebellum; ALLN, anterior lateral line nerve (Adapted from Bodznick, Montgomery and Tricas 2003).
nucleus is a relay nucleus for descending electrosensory feedback from the lateral mesencephalic electrosensory nucleus (Schmidt and Bodznick 1987).

Electroreceptive afferents to DGR have broad receptive fields that encompass many smaller receptive fields of adjacent AENs, which suggest inputs converge from higher order electrosensory areas (Schmidt and Bodznick 1987).

The dense parallel fiber and stellate cell system of the molecular layer provides the AENs with massive amounts of information (e.g. motor commands and sensory feedback) that pertain to ongoing behaviors and that serve as internal reference signals. Montgomery and Bodznick (1994) propose that a mechanism (termed the adaptive filter model) exists in the electrosensory DON of skates that suppresses reaference and preserves novel signals from the environment in the AEN. Specifically the adaptive filter model posits the internal reference signals delivered to AENs via the molecular layer are used to generate inverse sensory predictions (or cancellation signals), which when added to the peripherally detected electrosensory input arriving on basal dendrites of AENs, results in the negation of predictable reaference.

*Coupling experiments provide evidence for the adaptive filter model*

Montgomery and Bodznick (1994) developed coupling experiments to test the adaptive filter (Figure 3A). During the pre-coupling phase, baseline activity in the AEN was recorded. Next in the peri-coupling phase, electrosensory stimuli were paired to a specific phase of the skate’s ventilatory motor command
cycles. Initially, responses were robust, but a 44% decline over ten minutes of coupling was seen in 36/57 (63%) AENs (Montgomery and Bodznick 1994). The adaptive filter model suggests that the attenuation in response is an indication of a developing cancellation signal (i.e. molecular layer inputs generate the inverse of the expected electrosensory input). Cancellation signals can also be detected in the post-coupling phase. At stimulus offset, a period of reduced activity (or negative image) exists where electrosensory stimuli were previously present. As behavior continues without the electrosensory stimuli, the negative image disappears and the AEN resumes the original baseline activity (Montgomery and Bodznick 1994).

Synaptic plasticity at molecular layer-AEN synapses

The development of cancellation signals is achieved through anti-Hebbian plasticity at molecular layer-AEN synapses (Montgomery and Bodznick 1994). The adaptive filter model proposes that during coupling, repetitive co-activation of the AEN and molecular layer inputs results in a relative decrease in excitatory parallel fiber-AEN synaptic strengths, and/or increase in inhibitory stellate cell-AEN synaptic strengths (Figure 3B: peri-coupling). This may explain how continued pairings of electrosensory stimuli to a specific internal reference signal leads to attenuation of the AEN's initially robust response (i.e. electrosensory input becomes predictable with repeated pairings, and through anti-Hebbian synaptic plasticity described above, the response to the
electrosensory input is suppressed). The relative decrease in excitation and/or increase in inhibition is also discernible as a negative image at stimulus offset. The adaptive filter model posits that during post-coupling, as molecular layer inputs are active when the AEN is not, excitatory parallel fiber-AEN synapses strengthen and/or inhibitory stellate cell-AEN synapses weaken (Figure 3B: post-coupling). The adjustment in synaptic weightings is such that the cancellation signal is modified to reflect the absence of electrosensory input arriving via the AEN's basal dendrites. Lastly, the adaptive filter model predicts that when molecular layer inputs are inactive, synapses are stable and do not change (Figure 3B: note the inactive input). A memory trace for cancellation signals may be stored in the relative weightings of synapses, and furthermore may be ready to use when activity in the molecular layer inputs (in conjunction with their associated behaviors) resume.

The adaptive filter provides an efficacious mechanism to counter fluctuating, complex patterns of reaference generated by continuously, intermittently, or concurrently performed behaviors. Only those molecular layer inputs that correlate with AEN activity are selectively modified, thus each AEN develops a unique set of relative synaptic weightings that are dynamically adjusted, and that serve to suppress a very specific pattern of reaference it receives based on where the AEN's receptive field is on the body. Signals that are impossible to predict (i.e. those not generated by the animal's own behaviors, or external) are passed onto higher order electrosensory processing areas.
Figure 3. A coupling experiment provides evidence for an adaptive filter mechanism. **A.** Stimulus time histograms show distribution of AEN activity throughout ventilatory cycles. *Baseline (V):* The activity of the AEN is dispersed evenly throughout the entire ventilatory cycle. *Peri-coupling (V+E):* Presentation of the electrosensory stimuli at a specific phase in the ventilatory cycle leads to a vigorous response in the AEN, but after repeated electrosensory stimuli pairings to ventilation, a cancellation signal develops. Note the decline in response during peri-coupling. *Post-coupling (V):* The cancellation signal is also apparent at stimulus offset. Note the period of suppressed activity (negative image) where the electrosensory stimuli were previously present. The onset of electrosensory stimuli are indicated by solid lines beneath histograms during peri-coupling, and offset by a dotted line during post-coupling. Ex, exhalation; In, inhalation; V, ventilation; E, electrosensory stimulus. **B.** Schematic diagrams depicting changes in AEN inputs and outputs as predicted by the adaptive filter model. The net apical input from the molecular layer (ML) is summed with the net basal input from primary afferent neurons (Aff) to generate a net AEN output (net inputs and outputs shown in red). *Pre-coupling:* During baseline activity, some molecular layer inputs are active, while others are not. Total net input and output of AENs is neutral. *Early peri-coupling:* The onset of electrosensory stimuli generates a net Aff input, which is reflected in the net AEN output. *Late peri-coupling:* In the molecular layer, synapses of excitatory parallel fibers (in white) that are repeatedly co-active with the AEN weaken, while repeatedly co-
active inhibitory stellate cells (in black) strengthen. The cancellation signal delivered via the molecular layer inputs begins to resemble the inverse of the expected net Aff input. Summation of ML and Aff inputs decreases the net AEN output. Coincident activity between pre- and post-synaptic neurons is a requirement for changes in synaptic plasticity, thus changes occur in a specific set of molecular layer-AEN synapses that are relevant to suppressing the electrosensory input. Early post-coupling: At stimulus offset, the net Aff input becomes silent. The cancellation signal provided by the molecular layer generates a negative image in the net AEN output, and has the shape of the now absent electrosensory stimulus. Late post-coupling: With continued absence of electrosensory stimuli, excitatory parallel fiber synapses strengthen and inhibitory stellate cell synapses weaken, restoring AEN activity to baseline (diagram not shown). Note the ML synapse, whether excitatory or inhibitory, does not change if inactive.
Several lines of evidence indicate that the site for plasticity resides at molecular layer-AEN synapses. Bodznick, Montgomery and Carey (1999) substituted electrosensory stimuli with depolarizing and hyperpolarizing current injections as a means to activate or inhibit AENs. In addition, direct stimulation of the parallel fibers replaced molecular layer inputs normally driven by behavior (e.g. breathing, swimming). During the experiment, compensatory changes in the AEN’s response indicated that synaptic plasticity relies on coincident activity between presynaptic molecular layer inputs and postsynaptic AEN. A decline in response was observed during coupling, as was a negative image at stimulus offset. Disengaging molecular layer inputs by DGR lesions eliminated the typical response seen in AENs during coupling (Bodznick et al. 1999), and led to a massive upsurge of AEN spontaneous activity and indiscriminate sensitivity to external electrosensory stimuli (Conley 1995).

Repetitive coincident activity between presynaptic and postsynaptic neurons is associated with changes in synaptic strength (Hebb 1949). Zhang (2008) provided evidence that NMDA receptors are involved as coincident detectors at molecular layer-AEN synapses. Blockade of NMDA receptors using competitive antagonist AP5 reversibly prevented the development of cancellation signals.

The postsynaptic potentials measured in AENs appear to reflect changes in molecular layer-AEN synaptic strengths, as predicted by the adaptive filter model. Bertetto (2007) showed that the magnitude of excitatory postsynaptic
potentials in the AENs declined in amplitude during coupling and increased during recovery, indicating that the strengths of activated synapses are modified during the coupling experiment according to the predictions of the adaptive filter model. Depression and potentiation of EPSPs occurred on different time scales, which suggest that different mechanisms mediate the augmentation and diminution of synaptic strength.

Concluding remarks

Multiple studies have provided evidence that the adaptive filter model serves as an effective mechanism for suppressing reafference in AENs without loss of sensitivity to weak external electric fields. Research on the elasmobranch DON has established a well-defined neural architecture and function. The elasmobranch DON provides an ideal system for studying broader principles of physiology, such as long term potentiation/depression and experience-dependent synaptic plasticity. On a cellular and molecular level, it may also provide insight on receptor properties and chemical signaling pathways. The elasmobranch DON is a cerebellar-like structure, thus research may also have implications for understanding cerebellar function, including reversible learning, memory storage, and use of internal sensory predictions to guide motor behaviors. In this study, multiple cancellation signals were developed within an AEN to examine their putative independent and additive functional properties in suppressing electrosensory reafference.
Chapter 2:

Functional contributions of multiple cancellation signals in suppressing predictable electrosensory noise

Introduction

In any sensory system, relevant signals must be extracted from a noisy background. In skates, the animal’s own behaviors, such as breathing and swimming, stimulate electroreceptors (reafference). The animal must discern pertinent, external signals from those that are self-generated and hence redundant. The primary afferents deliver complex electrosensory patterns of activation to the dorsal octavolateralis nucleus in the skate medulla. The lack of self-modulation seen in many of the principal electrosensory neurons (called ascending efferent neuron, or AEN) suggests that a robust mechanism exists to suppress the modulation caused by the behaving animal (Figure 1).

An adaptive filter may serve as a mechanism to remove reaferrence in AENs (Montgomery and Bodznick 1994). Peripheral electrosensory input detected by electroreceptors on the body is transmitted to basal dendrites of AENs via primary afferent neurons. Internal reference signals, which are known to exist in the molecular layer and carry information pertaining to ongoing behaviors, arrive on the apical dendrites of AENs (Hjelmstad et al. 1996). In coupling experiments, AENs showed attenuating responses to external electrosensory stimuli paired repeatedly with an internal reference signal over
the course of minutes, as well as the appearance of an apparent negative image when the electrosensory stimulus was removed. A negative image is defined as a period of significantly lower (or higher) activity found time-locked to the period of the previously presented excitatory (or inhibitory) electrosensory stimulus (Montgomery and Bodznick 1994; Duman and Bodznick 1996). These findings were interpreted as the development of a cancellation signal, which is the inverse of the expected electrosensory input and that adds to the actual electrosensory input to suppress AENs’ responses to it (Montgomery and Bodznick 1994). Experiments in recent years have outlined intriguing properties of cancellation signals, and suggest that multiple cancellation signals are capable of working in concert to eliminate patterns of self-stimulation associated with various behaviors.

Properties of cancellation signals

One of the earliest experiments on the adaptive filter showed that cancellation signals are AEN-specific (Bodznick 1993). Only AENs whose receptive fields were activated by electrosensory stimuli during coupling experiments developed cancellation signals. Later recordings in the same animal from different AENs that had not undergone the coupling experiment showed baseline levels of activity, yet were capable of developing cancellation signals when tested.
Cancellation signals show temporal specificity. When electrosensory stimuli were paired at different time delays during ventilatory motor command cycles, AEN responses and negative images were formed at matching time delays (Figure 4A; Bodznick et al. 1999). Internally generated ventilatory motor commands were accessed by recording the neuronal multi-unit discharge from an efferent cranial nerve carrying ventilatory motor commands. Hjelmstad, Parks and Bodznick (1996) offer a reasonable explanation for temporal precision: each behavior generates a variety of internal reference signals in the molecular layer, and any of these may serve as cancellation signals. For example, separate motor commands exist to direct movements of the mouth, spiracle and branchial musculature, and each of these may have different temporal epochs during the ventilatory cycle (e.g. inhalation or exhalation phases, ventilatory coughs). Multiple motor commands provide a neural substrate that enables temporally precise cancellation signals to form at any point in time during the ventilatory cycle.

A variety of internal reference signals, including motor commands and sensory feedback signals, exist in the molecular layer, and each one is capable of producing a cancellation signal. Bodznick, Montgomery and Carey (1999) showed that pairing electrosensory stimuli to any one of ventilatory motor commands, proprioceptive or electrosensory signals was sufficient to produce a decline in the AEN response and create a negative image at stimulus offset (Figure 4A and 4B). Proprioceptive signals were induced experimentally
Figure 4. Properties of cancellation signals. **A.** The formation of cancellation signals is temporally precise. The response to electrosensory stimuli during coupling, as well as the appearance of negative images after coupling match the time delay of electrosensory stimuli (E) presentations after the onset (black arrows) of ventilatory motor command (VMC) cycles (Bodznick, Montgomery and Carey 1999). **B.** Cancellation signals can be generated using a variety of internal reference signals, including propriosensory or electrosensory signals that are paired repeatedly with electrosensory stimuli. Propriosensory signals were generated by fin movements (FM), and electrosensory signals by whole-body electrosensory stimulation through the gut (G) (Bodznick et al. 1999). **C.** Development of cancellation signals need not occur in a single continuous coupling session, but can accumulate incrementally through multiple, short coupling sessions separated by rest periods (Zhang and Bodznick 2008).
by passive movements of the ipsilateral pectoral fin, and electro sensory signals by stimulation of the whole body using a gut electrode. The presence of a variety of motor commands and sensory feedback signals suggests that each behavior may have a diverse set of cancellation signals that operate in conjunction with one another to form a composite cancellation signal, which perhaps may be more efficacious than any of its individual components in suppressing behavior-specific reafference.

Cancellation signals develop incrementally. Zhang and Bodznick (2008) show that a series of brief fin movement coupling sessions interspersed with sporadic rest periods (i.e. pauses in fin movement) are capable of developing cancellation signals incrementally over time (Figure 4C). This suggests that cancellation signals persist even as fin movements are paused (presumably, proprio sensory signals in the molecular layer become temporarily inactive; consequently the adaptive filter model predicts their synapses are stable). Furthermore, cancellation signals can be strengthened in an additive manner through subsequent coupling sessions.

*Functional contributions of multiple cancellation signals*

Since each behavior in the skate’s repertoire generates different patterns of reafference (Figure 1), and some behaviors are intermittent (e.g. swimming), while others are continuous (e.g. ventilation), an obvious assumption is that the reafference generated in naturally behaving animals may be complex and
dynamically changing. To effectively suppress electrosensory reaference, the adaptive filter model assumes that AENs have available a variety of predictive internal reference signals in the molecular layer, and that each AEN selects, through coincident activity and synaptic plasticity at molecular layer-AEN synapses, those signals that are relevant for removing the specific pattern of reaference it receives (Bodznick and Montgomery 1994). In the present study, two separate experiments were performed with the intent of developing multiple cancellation signals within a single AEN in order to examine their independent and additive functional contributions, and furthermore, to test the assumptions of the adaptive filter model.

In the first experiment, we hypothesize that within a single AEN, separate and distinct cancellation signals exist simultaneously for different behaviors. Cancellation signals associated with one particular behavior should form independently and ought to be modifiable without affecting cancellation signals associated with another behavior. The adaptive filter model proposes that the neuronal engram for this is that cancellation signals associated with different behaviors may arrive through separate, non-overlapping molecular layer inputs and that activity across different sets of molecular layer fibers does not affect one another (i.e. the plasticity is homosynaptic). If this is true, then multiple cancellation signals for distinct behaviors can be stored simultaneously in relative synaptic weights. The experiment was designed to first develop a cancellation signal for fin movements (mimicking swimming, FM); secondly, to
develop a cancellation signal for ventilatory motor commands (mimicking breathing, VMC); and finally, to check if the cancellation signal for fin movements persist given that the fin movements were paused during development of the VMC cancellation signal. Additionally, the design of this experiment tests the assumption of the adaptive filter model that if molecular layer inputs are paused (assuming that ceasing fin movements will suspend activity in those molecular layer inputs that carry proprioceptive signals related to fin movements), then cancellation signals ought to be maintained until fin movements are resumed, even though there may be activity in other sets of molecular layer inputs.

In the second experiment, we test the assumption that each behavior has associated with it a number of component internal reference signals, of which any can be used to form a composite cancellation signal. Overlapping contributions of multiple cancellation signals may summate to enhance the elimination of behavior-specific reaference in AENs. An example of a composite cancellation signal for breathing has been suggested to include component internal references signals associated with multiple ventilatory motor commands that direct the mouth, spiracle, and branchial muscles; proprioceptive signals that carry information about movements of the mouth and spiracles; and electrosensory information that feeds back from higher order electrosensory processing areas (Hjelmstad et al. 1996). Hence, the hypothesis is that a composite cancellation signal (i.e. a joint VMC and FM cancellation signal) should be stronger than any one of its component cancellation signal alone (i.e.}
either singular VMC or FM cancellation signal). Additionally, as this experiment utilizes concurrent, yet temporally unrelated presentations of two internal reference signals, the specificity of cancellation signals to a specific internal reference signal with which electrosensory stimuli are paired can also be tested.
Methods

Animals

Little skates (*Raja erinacea*) were collected from Long Island Sound, Connecticut and Vineyard Sound, Massachusetts and maintained in tanks of artificial seawater with salinity 30 parts per thousand, pH 8.2, and temperature 10-13°C. Skates were anesthetized by immersion in 0.04% benzocaine in seawater and placed on ice for surgery. The cranium roof was opened and bilateral incisions of the brain at the optic chiasm decerebrated the animals. During experiments, skates were submerged in circulating seawater, 10°C, with the exposed brain stabilized above the seawater level by a head clamp. A flow of oxygenated seawater, 0.4 L min⁻¹, directed through the mouth provided artificial respiration.

During experiments, animals were immobilized by intravenous paralyzing drug injections of 2.0 mg mL⁻¹ pancuronium bromide in skate ringer. Paralysis of animals was preferable to spinalization in order to preserve the spinal propriosensory inputs to the brain, however, paralysis eliminated breathing movements. Thus fictive ventilation was monitored by recording from an efferent cranial nerve carrying ventilatory motor commands (VMC). VMC signals have been shown to be an effective predictor for developing cancellation signals (Bodznick et al. 1999).
Stimulation

Local electric fields were generated by a dipole electrode, which could be moved to locate and provide excitatory stimulation of electrosensory neurons’ receptive fields. The dipole electrode consisted of a pair of 2 mm hollow glass tubes separated by 1 cm at the tip and filled with 1.5% agar in seawater. The dipole electrode was connected to a stimulator via a constant current isolator and calibrated to deliver 2-5 μV voltage steps when presented 1 cm from the electroreceptor pore opening.

Propriosensory signals were induced by manual deflections of the ipsilateral pectoral fin. A non-conducting rubber clamp attached to the fin was driven by a sinusoidal wave output (0.6 Hz, 0.5-1.0 cm fin movement) from a function generator and moving mechanical arm. Passive fin movements generate proprioceptive responses in stretch receptors of the fin muscles (Conley and Bodznick 1994) and are delivered via the spinal cord to the dorsal granular ridge and further projected onto the apical dendrites of AENs (Schmidt and Bodznick 1987).

Recordings

Ventilatory motor commands (VMC) are carried to the respiratory musculature via the hyomandibular seventh cranial nerve, and central copies of these motor commands also reach the apical dendrites of AENs (Hjelmstad et al. 1996). The hyomandibular seventh cranial nerve was exposed from behind the
spiracle and a suction electrode was used to record the multi-unit discharge. The VMC signals were preamplified, filtered 1-3 kHz, and acquired on a computer at 2 kHz sampling rate. A window discriminator converted the signals into event pulses, which were used to trigger stimulus time histograms for VMC cycles in offline data analysis.

Primary afferent neurons were recorded extracellularly from the ipsilateral anterior lateral line nerve by glass micropipettes (2-5 μm tip, 20-30 MΩ resistance, filled with 4 mol L⁻¹ NaCl). Electroreceptive units were identified by their high tonic spike rates (> 15 Hz) in paralyzed animals and by their responsiveness to 2 μV dipole fields.

Secondary AENs were recorded extracellularly from the ipsilateral dorsal octavolateralis nucleus (i.e. the first order electrosensoric nucleus) in the medulla using platinum-tipped indium-filled glass capillary electrodes (2-5 μm ball tip, 2-8 MΩ resistance). AENs have characteristically low spontaneous activity of approximately 2-5 Hz, but respond robustly to weak dipole fields of 2 μV. Units were verified as AENs by their antidromic spikes when stimulated from their projection site in the lateral mesencephalic nucleus of the contralateral midbrain. The stimulating electrode (a concentric bipolar electrode, 2-8 V stimulus intensity) was positioned to give an optimized evoked antidromic field potential in the DON. Signals from electrosensoric neurons were preamplified, filtered 0.1-5 kHz, and acquired at 6 kHz sampling rate. Spikes were also fed through a window discriminator, converted into event pulses, and
recorded as spike events at 100 Hz sampling rate to create stimulus time histograms.
Data analysis

Using offline analysis, the AENs were analyzed to examine changes in their response profile during a series of coupling sessions. Units that were lost midway through the experiment, or that had interference from a second unit were discarded. Occasionally, units failed to show any significant change in spiking from the coupling experiment (i.e. non-plastic AENs) and were excluded from further analysis.

Normalized spike count

A normalized spike count was calculated for each trial (i.e. each cycle of ventilatory motor command or fin movement), and used as a measure to compare changes in AEN firing throughout the coupling experiment. The normalized spike count gives an indication of the spike count during the stimulus interval while accounting for background firing outside the stimulus interval. Specifically, it counts the number of spikes that occur within the stimulus interval (‘IN’), and subtracts from it the scaled number of spikes that occur outside the stimulus interval (‘OUT’). Scaling adjusts the relative weight of spikes occurring outside the stimulus interval to compensate for the longer time duration of the outside stimulus interval relative to the time duration of the inside stimulus interval.

\[
\text{Normalized Spike Count} = (\text{ Spike Count}_{\text{IN}}) - \left( \text{ Spike Count}_{\text{OUT}} \times \frac{\text{Time}_{\text{IN}}}{\text{Time}_{\text{OUT}}} \right)
\]
The development of cancellation signals were assessed using two indicators: first, a decline in normalized spike count during peri-coupling, and second, significantly reduced normalized spike count at stimulus offset compared to baseline (i.e. indicative of a negative image). The first indicator compares the first and last 40 cycles of normalized spike counts during peri-coupling in order to assess the statistical significance of attenuation in the AEN's response. The second indicator compares the last 40 cycles before coupling (i.e. baseline) to the first 40 cycles after coupling (i.e. immediately at stimulus offset) to assess the statistical significance of the negative image. Student's t-tests were performed on the two sample sets of 40 cycles to test that the difference in the means of the two normally distributed populations was statistically significant (p<0.05).

Subtracted spike count

The subtracted spike count was used in the second additive experiment to determine which cancellation signal (either singular or combined) was stronger. The subtracted spike count quantifies the magnitude of the negative image for each cancellation signal. First, the last 40 cycles before coupling was averaged (i.e. mean of baseline). Next, the normalized spike count of the first 40 cycles after coupling (i.e. immediately at stimulus offset) was subtracted from the mean of baseline for each of the cycles to generate a set of 40 subtracted
spike counts. The mean of these 40 subtracted spike counts was assigned as the magnitude of the negative image. The negative image with the higher mean subtracted spike count was considered the stronger cancellation signal.

Mean subtracted spike count = (Mean of baseline) – (Mean of early post-coupling)

Student’s t-test compared the set of 40 subtracted spike counts of one negative image with the 40 subtracted spike counts of another negative image to determine whether the difference in the means of the two populations was statistically significant (p<0.05).

*Stimulus time histograms*

Stimulus time histograms were used to visualize the rate and timing of AEN spikes relative to cycles of internal reference signals. Comparisons of stimulus time histograms generated for pre-coupling, peri-coupling and post-coupling phases give an indication for changes in the AEN response profile during the course of the experiment. Often during the experiment, two internal reference signals were presented (e.g. ventilatory motor commands and fin movements), thus two separate stimulus time histograms (one set for each internal reference signal) were composed from the same AEN in order to represent the distribution of spiking according to its own internal reference signal.
Figure 5. Creating separate sets of stimulus time histograms for each internal reference signal. Each stimulus time histogram is triggered by the onset of a new cycle (e.g. one VMC cycle includes one inhalation and one exhalation; one FM cycle is one sinusoidal wave). The x-axis is equivalent to one cycle of an internal reference signal binned into 0.034 s segments, and the y-axis represents the cumulative number of spikes. For each phase (i.e. pre-, peri- or post-coupling), the number of spikes falling into each bin is counted per cycle and summed for 40 cycles. Stimulus time histograms triggered by different internal reference signals will have different response profiles in order to represent the distribution of spiking according to its own internal reference signal, even though data is from the same AEN recording. Stimulus time histograms triggered by VMC cycles are shown in green, and FM cycles in orange.
**Experimental procedures**

*Multiple cancellation signals have independent contributions*

The first experiment tests whether multiple cancellation signals that are associated with separate internal reference signals can develop within a single AEN, and if they independently form, co-exist, and are modified without affecting one another. Two internal reference signals of the molecular layer were used: proprioceptive signals driven by fin movements (FM) and ventilatory motor commands (VMC). Fin movements consisted of 0.5-1.0 cm sinusoidal fin deflections at 0.6 Hz (1.7 s cycles), whereas internally generated ventilatory motor commands were recorded from the hyomandibular seventh cranial nerve (approximately 2 s cycles). Using the coupling paradigm (Montgomery and Bodznick 1994), a cancellation signal was first developed for fin movement (FM) by pairing electrosensory stimuli repeatedly to the same point in time of the FM cycle for 5 minutes. The electrosensory stimulus (E) was turned off briefly for 1 minute and a negative image was observed to confirm the development of the cancellation signal (C.S.FM). After 5 minutes of FM coupling, if AENs did not show a cancellation signal, they were considered non-plastic and abandoned. For those that did show a cancellation signal in the form of a negative image, it was necessary to subsequently reinforce the C.S.FM further with a second 5-minute FM coupling session since observing the negative image weakens C.S.FM. Following reinforcement, both FM and E were simultaneously turned off, and then a separate cancellation signal was developed for ventilatory motor
commands by 5 minutes of VMC coupling. At stimulus offset, C.S.\textsubscript{VMC} was noted by the presence of a negative image. Then over the course of 5 minutes, AEN activity was allowed to resume near baseline levels (i.e. degradation of C.S.\textsubscript{VMC}). Afterward, fin movements were resumed without the electrosensory stimuli to check for the persistence of C.S.\textsubscript{FM} delay.

*Multiple cancellation signals have additive contributions*

The second experiment tests whether two cancellation signals associated with separate internal reference signals can combine to suppress reaference associated with one particular behavior. Each AEN underwent three coupling sessions in series: FM, VMC, and VMC+FM. Since FM and VMC cycles have different time durations and presentation frequencies, the number of cycles was set at 150 cycles during the peri-coupling phase, and at least 200 cycles during post-coupling phase. The first session developed a singular cancellation signal for ventilatory motor commands (C.S.\textsubscript{VMC}), and the second session developed a singular cancellation signal for fin movements (C.S.\textsubscript{FM}). In both of these sessions, VMC and FM were presented concurrently, yet were temporally unrelated (i.e. they had different presentation rates that were not multiples of each other); and only one internal reference signal received pairing with the electrosensory stimulus at a time. In the third coupling session, VMC triggers were used to initiate FM cycles, thus both VMC+FM cycles were presented synchronously. The mean subtracted spike counts were calculated for each cancellation signal to
assess if the combined cancellation signal, C.S.\textsubscript{VMC+FM}, was stronger than either singular cancellation signal, C.S.\textsubscript{VMC} or C.S.\textsubscript{FM} alone. Controlled variables were kept constant across all three coupling sessions for each AEN recording (e.g. 150 cycles during peri-coupling; electrosensory stimulus intensity, duration, and delay; and frequency, amplitude and offset of fin movements).

Additionally, the first two coupling sessions were used to test for input specificity of internal reference signals. In each coupling session, the electrosensory stimulus was paired with only one internal reference signal, thus a cancellation signal should develop specific to the paired internal reference signal. There should be no discernable pattern of response in the raster plots or stimulus time histograms of the unpaired internal reference signal.
Results

*Plastic and non-plastic AENs*

All cells identified as AENs were analyzed to obtain their normalized spike counts (i.e. a measurement of spiking within the electrosensory stimulus interval that accounts for background firing outside the stimulus interval). There was variability in the attenuation of AEN responses to the electrosensory stimulus during coupling; not all AENs demonstrated a significant decline. Additionally, not all AENs demonstrated a statistically significant negative image (i.e. period of reduced firing found during the stimulus interval at stimulus offset, p<0.05 when compared to baseline). Those that exhibited significant negative images were classified as ‘plastic,’ whereas those that did not have clear negative images were labeled ‘non-plastic.’ Considering the differences in response profiles, it is reasonable to suspect that AENs comprise a heterogeneous population (Bastian, Chacron and Maler 2004). As this study is concerned with examining cancellation signals, plastic AENs were of interest, and thus non-plastic AENs were excluded from further analysis. Of the total 38 AENs reported in this study, five (13%) were deemed non-plastic (p>0.05). The true proportion of non-plastic AENs is largely underreported in this study since numerous AENs were encountered that did not develop cancellation signals upon initial coupling tests, and were abandoned in favor of AENs that demonstrate greater plasticity. The possible functions and significance of non-plastic AENs are discussed later.
Multiple cancellation signals have independent contributions

The first experiment tests whether an existing cancellation signal associated with one internal reference signal (e.g. proprioceptive signals driven by fin movement, FM) is stable and maintained in spite of the development of another cancellation signal associated with a separate internal reference signal (i.e. ventilatory motor commands, VMC).

A series of coupling sessions were performed with the same AEN. An example of the changing response profile of a plastic AEN is shown in the raster plots and stimulus histograms of Figure 6. Separate raster plots triggered by VMC or FM cycles from the same AEN recording illustrate the different distribution in spiking patterns that depend on which internal reference signal is used as the trigger (Figure 6A). After baseline recording of fin movements (precoupling; normalized spike count = 0.2±0.9 spks), electrosensory stimuli were paired to fin movements. The AEN responded robustly initially (early peri-coupling; normalized spike count = 2.8±1.8 spks), but declined 30% (p<0.019) over the course of 5 minutes (late peri-coupling; normalized spike count = 1.9±1.8 spks). Attenuation in response during coupling is one indication of a developing cancellation signal. A second indication is a negative image at stimulus offset, which is a period of suppressed activity found time-locked to the period of the absent electrosensory stimulus. Upon visual confirmation of C.S.FM, the cancellation signal was reinforced and fin movements were suspended. With cessation of fin movements, the activity of the molecular layer inputs associated
with proprioceptive signals are presumably halted, and the adaptive filter model proposes that synaptic weightings remain unchanged if molecular layer inputs are inactive (Bodznick et al. 1994). The experiment then proceeded with a session of VMC coupling. Pairing of electrosensory stimuli to ventilatory motor commands elicited a similar response profile in the AEN (pre-coupling; normalized spike count = 0.4±2.0 spks). A robust response was observed (early peri-coupling; normalized spike count = 3.8±2.0 spks), which declined by 46% (p<0.0001) over 5 minutes (late peri-coupling; normalized spike count = 2.0±1.7 spks). The presence of a negative image was also observed (early post-coupling; normalized spike count = -0.4±0.8 spks, p<0.020 compared to baseline). With continued cycles of ventilatory motor commands in the absence of peripheral electrosensory input, AEN activity recovered to near baseline levels over the course of 5 minutes (late post-coupling; normalized spike count = 0.1±0.8 spks). Lastly, fin movements were resumed to verify whether C.S.FM delay persisted in spite of the development of a separate cancellation signal for C.S.VMC. In this AEN, the negative image for C.S.FM delay was found to have persisted (early post-coupling; normalized spike count = -0.5±0.7 spks, p<0.0001 compared to baseline). C.S.FM delay then faded (late post-coupling; normalized spike count = 0.1±0.8 spks) with continued fin movement in the absence of electrosensory stimulation. Stimulus time histograms illustrate that both the heightened spiking during peri-coupling and the negative image during post-coupling are
Figure 6. Multiple cancellation signals may form and are modified independently within a single AEN. A. Raster plots show distribution of AEN firing triggered by fin movement (FM, left) and ventilatory motor command (VMC, right) cycles. Data is from one cell. Baseline activity was recorded for both VMC and FM (pre-coupling). FM coupling session: Electrosensory stimuli (E) were paired to fin movements, and a cancellation signal developed, was observed, and then reinforced (peri-coupling: FM+E, post-coupling: FM, reinforce: FM+E respectively), after which fin movements were subsequently shut off. VMC coupling session: Pairings of electrosensory stimuli with VMC cycles developed a cancellation signal for VMC, as seen by a significant reduction in AEN firing during the period where electrosensory stimuli were presented (peri-coupling: VMC+E), as well as the presence of a negative image at stimulus offset (post-coupling: VMC). FM session resumed: Resuming fin movements (post-coupling: FM) demonstrated that the negative image for fin movements had persisted. Solid black bars (E) above the raster plots indicate the phase in the cycle where the electrosensory stimuli was paired to. Vertical green and orange bars between raster plots indicate the 40 cycles from which stimulus time histograms were drawn. B. Stimulus time histograms. Pre-coupling: AEN activity is distributed throughout both VMC and FM cycles during baseline. Peri-coupling: AEN responded robustly initially, but declined with continued paired presentations of electrosensory stimuli to the internal reference signal. Post-coupling: A negative image was visible at stimulus offset (early post coupling),
indicating the development of cancellation signals for both FM and VMC. Notably the cancellation signal for FM was still present when fin movements were resumed, which then faded with continued fin movements in the absence of a time-locked electrosensory stimulus. The period between dotted lines denote the electrosensory stimulus interval.
temporally specific: both are found within the electrosensory stimulus interval (denoted by dotted lines in Figure 6B) in FM and VMC stimulus histograms.

Of the 23 AENs analyzed for the independent coupling paradigm, three were determined to be non-plastic and excluded (i.e. did not show C.S.\text{FM} after the first 5 minutes of FM coupling, p>0.05 compared to baseline). Of the 20 remaining AENs deemed plastic, 11 cells (55%) demonstrated the existence of two distinct C.S.\text{FM} and C.S.\text{VMC}, providing evidence that multiple cancellation signals associated with separate internal reference signals can exist simultaneously within a single AEN. When fin movements were resumed after the VMC coupling session, 17 of 20 AENs (85%) demonstrated a persistent C.S.\text{FM} delay. Finally among the 20 plastic AENs, eight cells (35%) demonstrated triple cancellation signals for C.S.\text{FM}, C.S.\text{VMC}, and C.S.\text{FM} delay. This last result provides the clearest indication that multiple cancellation signals can develop within an AEN, and that they are updated independently without affecting one another. Figure 7 provides a summary of these findings.
Figure 7. Pie chart showing number of plastic AENs exhibiting independent cancellation signals. Only AENs that developed a cancellation signal for fin movements, C.S.\textsubscript{FM}, after the first 5 minutes of FM coupling were considered plastic (n=20). Of the 20 plastic AENs, 11 further developed a cancellation signal for ventilatory motor commands, C.S.\textsubscript{VMC}, although only 8 exhibited a persistent C.S.\textsubscript{FM} delay when fin movements were resumed. In total, 17 of 20 AENs exhibited persistent C.S.\textsubscript{FM} delay, although not all demonstrated the intermediary C.S.\textsubscript{VMC}.
Plastic AENs that all developed C.S.FM during the first 5 minutes of FM coupling (n=20)

- n=9: 45% (C.S.FM and C.S.VMC)
- n=3: 15% (C.S.FM and C.S.FM delay)
- n=8: 40% (C.S.FM, C.S.VMC and C.S.FM delay)
The 8 plastic cells that demonstrated all three cancellation signals were analyzed to obtain population data for this subgroup of AENs. The negative images for C.S.\textsubscript{VMC} (average p<0.011) and C.S.\textsubscript{FM delay} (average p<0.005) were both statistically significant (n=8). Of the eight cells, five AENs also demonstrated a significant response decline during VMC coupling (mean decline = 27±12\%, p<0.0001) and FM coupling (mean decline = 21±9\%, p=0.009). A comparison of the changing response profiles during each phase of coupling to VMC or FM cycles for the eight AENS is shown in Figure 8.
Figure 8. Bar chart showing changes in AEN activity during each phase of the coupling session. There were 8 AENs that demonstrated significant negative images for C.S.\textsubscript{FM}, C.S.\textsubscript{VMC} and C.S.\textsubscript{FM%delay} (by comparing normalized spike counts of baseline to early post-coupling phase). Of the eight, five AENs also demonstrated a significant decline in response during coupling (by comparing early to late peri-coupling phase). Error bars shown are standard errors; asterisks indicate the difference between the mean normalized spike counts of two phases is statistically significant (p<0.001). The mean normalized spike count is a measure of the number of spikes occurring within the electro sensory stimulus interval while accounting for background firing outside the stimulus interval, averaged for 40 cycles per phase.
Baseline
Early peri-coupling
Late peri-coupling
Early post-coupling
Late post-coupling

Mean normalized spike count

* n=8
* n=5

VMC
FM
Multiple cancellation signals have additive contributions

The adaptive filter suggests that learning of cancellation signals is mediated by plasticity of individual synapses, and that incremental synaptic effects may be temporally and spatially summated in the AEN (Zhang and Bodznick 2008). The gradual reduction in response during the peri-coupling phase, as well as gradual restoration to baseline during post-coupling supports the notion that changes in response occur in incremental, cumulative fashion. The second experiment tests the hypothesis that when two cancellation signals using distinct internal reference signals are developed synchronously and are overlapping, the effect of the cancellation signals may be added and should appear stronger than either one cancellation signal developed alone. If indeed component cancellation signals can combine to provide a composite cancellation signal, the summated inverse sensory prediction may more efficaciously suppress the reafference associated with the particular behavior.

In this experiment, three coupling sessions were performed on a single AEN sequentially: first, VMC coupling; second, FM coupling; and third, VMC+FM coupling. In the first two sessions, VMC and FM molecular layer inputs were ongoing, but had no temporal relationship with each other (i.e. presented at different rates that were not multiples of one another). During each session, electrosensory stimuli were paired to only one internal reference signal at a time. Figure 9 depicts separate raster plots and stimulus time histograms triggered by VMC or FM cycles of an example AEN. This AEN did not show a
significant decline in response to electrosensory stimuli during the VMC coupling session (p=0.49). On the other hand, the appearance of a statistically significant negative image at stimulus offset was observed (normalized spike count = −0.4±0.7 spks, p=0.033 compared to baseline). In the second FM coupling session, the AEN demonstrated both a significant response attenuation during coupling (response declined 25% from 4.2±1.9 to 3.2±2.0 spks, p=0.008), and a negative image at stimulus offset (normalized spike count = −0.6±0.7 spks, p=0.007). In the third session, the onset of ventilatory motor commands was used to initiate fin movements, thus VMC and FM molecular layer inputs occurred in synchrony. In the same AEN, a response decline was observed during coupling (4.7±2.2 to 2.6±1.8 spks). The decline in response was greatest in VMC+FM coupling (43%), compared to a non-significant decline in VMC coupling and a 25% decline in FM coupling. The clearest negative image (normalized spike count = −0.6±0.6 spks) was also detected at stimulus offset for VMC+FM coupling compared to VMC or FM coupling, as assessed by the smaller p value obtained from Student t-tests (p=0.033 for VMC, p=0.007 for FM, and p<0.0001 for VMC+FM).
Figure 9. Multiple cancellation signals may add to strengthen one another within a single AEN. **A.** Raster plots display changes in AEN spiking profile during a series of coupling sessions that developed a singular cancellation signal for VMC (C.S.\textsubscript{VMC}, green), a singular cancellation signal for FM (C.S.\textsubscript{FM}, orange), and a combined cancellation signal for VMC+FM (C.S.\textsubscript{VMC+FM}, red). **Pre-coupling:** In each session, baseline activity was recorded. **Peri-coupling:** AENs responded robustly to electro sensory stimuli paired repeatedly to one internal reference signal: either VMC, FM, or VMC+FM. **Post-coupling:** The appearance of a negative image at stimulus offset was used as an indicator for the development of a cancellation signal. **Left,** raster plot triggered by VMC; **right,** raster plot triggered by FM from the same AEN recording. In the third VMC+FM coupling session, fin movements were synchronized to ventilatory motor commands, hence their identical raster plots. Solid black bar indicates the phase in the cycle where electrosensory stimuli (E) were paired to. Vertical green, orange and red bars between raster plots indicate the 40 cycles used to draw stimulus time histograms. **B.** Stimulus time histograms demonstrate that changes in AEN responses were time-locked to the electrosensory stimulus interval (denoted between dotted lines). Negative images were evident at stimulus offset. In this AEN, the combined cancellation signal C.S.\textsubscript{VMC+FM} was stronger than C.S.\textsubscript{VMC} or C.S.\textsubscript{FM} alone (see early post-coupling histograms).
A

Pre-coupling: VMC, (FM)
Peri-coupling: VMC+E, (FM)
Post-coupling: VMC, (FM)

Pre-coupling: FM, (VMC)
Peri-coupling: FM+E, (VMC)
Post-coupling: FM, (VMC)

Pre-coupling: VMC+FM
Peri-coupling: VMC+FM+E
Post-coupling: VMC+FM

B

VMC coupling (FM) ➔ FM coupling (VMC) ➔ VMC + FM coupling

Baseline
Early Coupling
Late Coupling
Early Post-coupling
Late Post-coupling
In order to compare the strength of singular C.S.\textsubscript{FM} and C.S.\textsubscript{VMC} against combined C.S.\textsubscript{VMC+FM}, it was necessary to quantify the magnitude of cancellation signals. The mean subtracted spike count (i.e. the mean baseline – mean early post-coupling normalized spike counts) was calculated for all three cancellation signals (C.S.\textsubscript{FM}, C.S.\textsubscript{VMC} and C.S.\textsubscript{VMC+FM}) and used as the quantitative measure for the strength of their negative images (see ‘Data Analysis’).

Of the 15 AENs tested using the additive coupling paradigm, two AENs did not develop a singular cancellation signal (p>0.05) and were deemed non-plastic and excluded. A separate four AENs had developed significant negative images for either singular C.S.\textsubscript{VMC} or singular C.S.\textsubscript{FM}, but surprisingly, not combined C.S.\textsubscript{VMC+FM}. These four AENs counter the adaptive filter model’s hypothesis: they clearly demonstrate plasticity and are able to develop singular cancellation signals, but fail to repeat the development of a cancellation signal when the same two internal reference signals are presented synchronously in the third coupling session. For reasons not yet known, the combined cancellation signal C.S.\textsubscript{VMC+FM} failed to reach statistical significance, but possible explanations are addressed in the discussion. In any case, the majority of plastic AENs (9/13 AENs, 69%) did develop significant combined cancellation signals, C.S.\textsubscript{VMC+FM} (Figure 10 summarizes findings).
Figure 10. Pie chart showing numbers of plastic AENs exhibiting additive cancellation signals. Of the 13 AENs deemed plastic (i.e. developed either one of the singular cancellation signals), four did not develop a significant combined cancellation signals, C.S.\textsubscript{VMC+FM} (i.e. negative image did not reach statistical significance when early post-coupling was compared to baseline). Of the remaining nine that had statistically significant negative images for combined C.S.\textsubscript{VMC+FM}, two AENs had weaker combined C.S.\textsubscript{VMC+FM} than either singular C.S.\textsubscript{VMC} or C.S.\textsubscript{FM}. In seven AENs, the combined C.S.\textsubscript{VMC+FM} was stronger than singular cancellation signals, although only two reached statistical significance for strength.
Plastic AENs that showed at least one of the singular cancellation signal (n=13)

- n=2 (15%) no VMC+FM
- n=4 (31%) VMC+FM weaker
- n=5 (39%) VMC+FM not significantly stronger
- n=2 (15%) VMC+FM significantly stronger
The singular cancellation signals were compared against the combined cancellation signal by the strength of their negative images using mean subtracted spike counts. In two of nine plastic AENs, the combined cancellation signals was found weaker than one of the singular cancellation signals (Figure 11C; mean subtracted spike count for first cell: 1.50 vs. 0.90 spks for singular C.S.\textsubscript{VMC} vs. combined C.S.\textsubscript{VMC+FM} respectively; second cell: 0.60 vs. 0.35 spks for singular C.S.\textsubscript{VMC} vs. combined C.S.\textsubscript{VMC+FM} respectively). Nevertheless, seven of nine AENs appeared to have stronger combined C.S.\textsubscript{VMC+FM} (i.e. higher mean subtracted spike count values for combined C.S.\textsubscript{VMC+FM} compared to singular C.S.\textsubscript{VMC} or C.S.\textsubscript{FM}). By using student’s t-test to compare sets of 40 subtracted spike counts, two of these seven AENs reached statistical significance in having stronger combined C.S.\textsubscript{VMC+FM} than both C.S.\textsubscript{VMC} and C.S.\textsubscript{FM} (first cell: p<0.01 for VMC vs. VMC+FM comparison and p<0.001 for FM vs. VMC+FM comparison; second cell: p<0.001 for VMC vs. VMC+FM comparison and p<0.001 for FM vs. VMC+FM comparison). In three of seven AENs, C.S.\textsubscript{VMC+FM} was only significantly stronger than C.S.\textsubscript{VMC}; and in another AEN, C.S.\textsubscript{VMC+FM} was only significantly stronger than C.S.\textsubscript{FM}. Finally for one of the seven AENs, C.S.\textsubscript{VMC+FM} had the highest mean subtracted spike count, but was not significantly stronger than either C.S.\textsubscript{VMC} or C.S.\textsubscript{FM} (Figure 11A). Possible explanations for the observed weakness in the combined cancellation signal are discussed later.
Figure 11. Mean subtracted spike count used to compare the strength of negative images from different cancellation signals. Each line represents a separate AEN recording. The mean subtracted spike count calculates the difference in normalized spike count between baseline and early post-coupling, averaged for 40 cycles.  

A. In seven AENs, combined C.S.VMC+FM had the highest mean subtracted spike count. In two of the seven AENs (purple and dark blue), combined C.S_{VMC+FM} exhibited a statistically significant stronger cancellation signal than both singular C.S_{VMC} and C.S_{FM} (p<0.05). In five AENs, combined C.S_{VMC+FM} were significantly stronger than only one of the singular cancellation signals, either C.S_{VMC} or C.S_{FM}, but not both. In one AEN, combined C.S_{VMC+FM} had a higher mean subtracted spike count than both singular cancellation signals, but the difference in strength was not statistically significant. Closed circles indicate combined C.S_{VMC+FM} was significantly stronger than singular C.S_{VMC} or C.S_{FM} (p<0.05); open circles indicate C.S_{VMC+FM} had a higher mean subtracted spike score than singular C.S_{VMC} or C.S_{FM}, but did not reach statistical significance (p>0.05).  

B. In two AENs, combined C.S_{VMC+FM} was weaker (i.e. lower mean subtracted spike score) than one of the singular cancellation signals.
A. Combined C.S.\textsubscript{VMC+FM} is stronger than singular C.S.\textsubscript{VMC} or C.S.\textsubscript{FM} (n=7)

B. Combined C.S.\textsubscript{VMC+FM} is weaker than singular C.S.\textsubscript{VMC} or C.S.\textsubscript{FM} (n=2)
The design of the additive experiment allowed us to test whether the response profiles of AENs are specific to the internal reference signal that electrosensory stimuli were paired to. Using the first VMC coupling session, two sets of stimulus time histograms were drawn: one set was triggered by VMC cycles and another by FM cycles (Figure 12A). The stimulus time histograms demonstrate that although two internal reference signals were active, only the signal that was consistently paired with electrosensory stimuli showed a robust response during coupling and a negative image during recovery.

The converse test was performed in the same AEN using data from the second FM coupling session (Figure 12B). During coupling, stimulus time histograms triggered by FM showed that responses to the electrosensory stimuli were time-locked to a particular point in the FM cycle. As VMC inputs were not paired, VMC-triggered stimulus time histograms have dispersed spiking throughout the VMC cycle. At stimulus offset, FM-triggered stimulus time histograms displayed a clear negative image, whereas no discernable pattern was observed in VMC-triggered histograms. Thus development of cancellation signals was restricted to the internal reference signal that was reliably active with the AEN. Input-specificity could be clearly observed by visual inspection in all 15 AENs tested (see raster plots in Figures 6A, 9A, 12A and 12B).
Figure 12. Input-specificity of AEN response profiles. A. During a ventilatory motor command (VMC) coupling experiment, fin movements (FM) were ongoing but not time-locked to electrosensory stimuli (E) presentations. Data from one cell. The raster plots, upper, and stimulus time histograms, lower, shown on the left are triggered by VMC: responses were time-locked during coupling, and a negative image was also found time-locked after coupling. In contrast, raster plots and stimulus time histograms shown on the right are triggered by ongoing, out-of-phase FM cycles: there was no distinguishable pattern of response. Note the black bar found only above the VMC raster plot indicates the time-locked nature of electrosensory stimuli presentation, but is missing above the FM raster plot as presentations of electrosensory stimuli were constantly shifting (i.e. temporally unrelated). B. Conversely, FM coupling indicates clear coupling responses and cancellation signals for FM, right, whereas AEN spiking was widely dispersed throughout ongoing, out-of-phase VMC cycles, left. Stimulus histograms were drawn using 40 cycles.
A  C.S.\textsuperscript{VMC-specific}

Pre-coupling: VMC, (FM)
Peri-coupling: VMC+E, (FM)
Post-coupling: VMC, (FM)

B  C.S.\textsuperscript{FM-specific}

Pre-coupling: FM, (VMC)
Peri-coupling: FM+E, (VMC)
Post-coupling: FM, (VMC)
Additional observation

The proposed purpose of the adaptive filter is to eliminate reafference. However the AENs used in these coupling experiments rarely reach complete response suppression to the electrosensory stimulus. The kinetics of response suppression vary across AENs and even within the same AEN. Bertetto (2007) showed from recordings of post-synaptic potentials that potentiation and depression of synapses occur on different time scales in AENs. Are some AENs slow to develop cancellation signals? Here I present one example of a prolonged AEN recording in a FM coupling experiment (Figure 13).

Electrosensory stimuli (5 µV) were paired with a specific point in fin movement cycles for 2 hours. A gradual yet significant decline in response was seen in the first hour (43% decline from 7.2±1.4 to 4.1±1.5 spks, p<0.0001). In the second hour of coupling, response decreased at a similar rate (44% decline from 4.1±1.5 to 2.3±1.3 spks, p<0.0001). It seemed that the response to the electrosensory stimulus would decline further with continued coupling, but at this point, the electrosensory stimulus was turned off and a strong negative image was observed (normalized spike count = −1.8±0.8 spks, p<0.0001 compared to baseline). The fin movement remained off for an extended period of 10 hours to examine the persistence of C.S.FM delay. Regular probes using brief fin movements indicated that C.S.FM delay persisted throughout the course of the fin movement pause (normalized spike count at 6 hour delay = −1.0±0.7 spks, p<0.0001 compared to baseline). After 10 hours, fin movements were resumed
for two hours, and the negative image began to fade (post-coupling normalized spike count at 0 hour = –0.7±0.5 spks; at 1 hour = –0.4±0.5 spks; at 2 hour = – 0.4±0.7 spks). After two hours of continuous fin movements, AEN activity had recovered significantly (p=0.013, compared to early post-coupling), but had not reached pre-coupling baseline levels, which is in agreement with Bertetto’s (2007) finding that potentiation after coupling occurs more slowly than depression during coupling.
Figure 13. A slow developing cancellation signal in one AEN. In a long recording, electrosensory stimuli (E) were paired with fin movements (FM) for 2 hours. A slow, but significant decline in response was observed (87%, p<0.0001). At stimulus offset (i.e. initial recovery), a strong negative image was observed, then fin movements were paused for 10 hours. A probe using brief fin movements at 6 hours indicated the negative image was still persistent. When fin movements were resumed continuously for 2 hours, the negative image faded slowly.

Stimulus time histograms separated by 1 hour intervals were drawn for the peri-coupling and post-coupling phase. Each stimulus time histogram is composed of 3 minutes of cycles denoted between the horizontal dotted lines on the raster plots. Vertical dotted lines denote the electrosensory stimulus interval.

Horizontal scale bar, 1sec; vertical scale bar, 30mins.
Discussion

The goal of this study was to examine the properties of multiple cancellation signals that may work jointly to suppress patterns of reaference associated with different behaviors. Suppression of reaference is important in sensory systems, such as the skate’s dorsal octavolateralis nucleus, as it enables the principal neurons (ascending efferent neurons, AENs) to attend to external relevant signals, while selectively ignoring signals that are self-generated and redundant. In this study, an external electrosensory input was paired to ventilatory motor commands or fin movements, both of which are known to be represented as internal reference signals in the molecular layer. Apparent cancellation signals developed, which were capable of suppressing predictable electrosensory input. Evidence for the development of a cancellation signal was measured by two indicators: first, a time-locked decline in the AEN’s response to electrosensory stimuli, and second, a negative image found time-locked to the stimulus interval at electrosensory stimulus offset.

Multiple cancellation signals have independent contributions

There were three main findings from the first experiment that support the adaptive filter model and past research findings (Montgomery and Bodznick 1994; Zhang and Bodznick 2008). First, this experiment provided the first evidence that multiple cancellation signals associated with distinct internal reference signals can exist simultaneously within a single AEN. Secondly,
development and degradation of a cancellation signal for ventilatory motor commands did not affect the persistence of an existing cancellation signal for fin movements. Lastly, the cancellation signal for fin movements was stable throughout the period that fin movements were paused, and re-activated when the fin movements resumed.

Previous studies have demonstrated that a variety of internal reference signals are each capable of producing a cancellation signal within an AEN, including ventilatory motor commands, propriosensory and electrosensory signals (Bodznick et al. 1999). Although the studies developed separate cancellation signals in separate AENs, the expectation is that each AEN has access to a multitude of internal reference signals and selects those it needs; therefore it is not surprising that multiple cancellation signals developed within a single AEN, as demonstrated here.

How are distinct FM and VMC cancellation signals achieved, and what may the neural correlates be? One possibility is that each parallel fiber of the molecular layer carries a distinct—in other words, only one type of—internal reference signal. This hypothesis is suggested by research showing that there are several, separate afferent inputs to the dorsal granular ridge (where granule cell bodies are situated) and each afferent input carries distinct internal reference signals (Hjelmstad et al. 1996); also that the outputs of dorsal granular ridge project homotopically to the DON (Conley and Bodznick 1994). An alternative hypothesis is that convergence occurs in the dorsal granular ridge, thus some
parallel fibers carry multiple types of internal reference signals. This second hypothesis seems less likely because if two types of internal reference signals were delivered via one subset of molecular layer input, then modification of one cancellation signal (either strengthening or degrading) should be reflected in the modification of the other cancellation signal. This study found 11 candidate cells that developed distinct C.S._FM and C.S._VMC. The majority (8/11 cells, 73%) showed that C.S._FM delay was persistent, despite the development and degradation of C.S._VMC, thus suggesting the majority of internal reference signals arrive via discrete molecular layer inputs. However, in 3 of 11 (27%) AENs, C.S._FM delay failed to persist (p=0.245, 0.298, 0.366; n=3). Possible reasons for this may be that a small subset of molecular layer inputs carries multiple types of internal reference signals, or alternatively that the first C.S._FM was not strong enough initially to persist. Recordings from granule cells in dorsal granular ridge may help elucidate reasons for failure of C.S._FM delay to persist.

As the majority of cells show cancellation signals are independent, it argues in favor of homosynaptic plasticity of molecular-layer AEN synapses (i.e. activity in one subset of molecular layer inputs does not seem to spread and affect activity in another subset, or if so, the effect is small). It was found that 17 of 20 plastic AENs developed C.S._FM and furthermore maintained C.S._FM delay during a pause. Thus cancellation signals (i.e. C.S._FM and C.S._FM delay) were generally well preserved and unaffected by other internal reference signals, which probably arrive through separate molecular layer inputs. Additionally,
input-specificity is evidenced by visual examination of raster plots and stimulus time histograms. Cancellation signals did not spread indiscriminately to other internal reference signals that were not paired to the electrosensory stimulus. These findings are in agreement with previous studies (Bertetto 2007; Zhang 2008) in that coincident activity is a requirement for changes in molecular layer-AEN synaptic strength.

The first experiment supports the hypothesis of the adaptive filter model that distinct cancellation signals arrive through separate, non-overlapping sets of parallel fibers and stellate cells, and that these sets of molecular inputs do not affect one another.

*Multiple cancellation signals have additive contributions*

The second experiment attempts to verify an additive property for cancellation signals within a single AEN: whether multiple cancellation signals can summate to create a stronger composite cancellation signal compared to its component parts. This experiment found three different outcomes that confound the answer. In 13 plastic AENs, four cells did not develop combined cancellation signals, although they did develop singular cancellation signals. In two cells, the combined cancellation signal was weaker than one of the singular cancellation signals. Finally, seven cells met the prediction of the adaptive filter model in that the combined cancellation signal was stronger than each of the singular cancellation signals (although only two of seven reached statistical significance
for being stronger than both singular cancellation signals, and five of seven reached statistical significance for being stronger than one singular cancellation signal).

The four cells that developed singular cancellation signals but not combined cancellation signals is a puzzling result. They show development of a singular cancellation signal in one condition (i.e. two ongoing internal reference signals with electrosensory stimuli paired to only one of these signals), but not in another similar condition (i.e. same two internal reference signals, now occurring in synchrony with the electrosensory stimulus paired to both). If we put aside the question of additivity momentarily, the expectation is that if the AEN demonstrated the development of a cancellation signal for a singular internal reference signal, at the very least it should be repeated when two internal reference signals are presented simultaneously given that they are independent (as shown by the first experiment). One possible reason as to why a combined cancellation signal did not develop a significant negative image in the second condition may be due to the high variability in AEN responses during coupling experiments. During experiments, multiple runs of the coupling experiment were performed on the same AEN. It was found that a cancellation signal sometimes developed in one run, but not in a second run (data not shown). I believe this finding is symptomatic of the recording and analytical techniques used, rather than the AEN failing to develop a cancellation signal. In this study, two indicators were used to ascertain the development of a
cancellation signal, however not all AENs showed a significant decline during coupling, nor did all show significant negative images during stimulus offset. The issue is confounded by generally low rates of firing in AENs during baseline (2-5Hz), making it difficult for a negative image to be discerned. The measure of negative images relies heavily on the absence of spiking during the stimulus interval as well as the subtraction of spikes that occur outside of the stimulus interval. The analytical parameters used in this study attempt to extract a reduced period of activity from a neuron that is already firing at low rates (p-values for the four cells that did not develop combined cancellation signals: p=0.068, 0.063, 0.141, 0.342). The four cells that did not develop combined cancellation signals were not tested with a repeat run, thus it is possible that variability in AEN responses may account for the lack of a combined cancellation signal. An alternative explanation is that these four cells represent a population of AENs that do not show an additive property, however this explanation seems unlikely since the majority of cells (7/13 AENs, 54%) do show a slight additive component, although not always statistically significant.

Another unusual outcome of this experiment was that two cells had stronger singular cancellation signals than combined cancellation signals. It should again be noted that a cancellation signal was determined to be stronger or not using statistical analyses that measure negative images, which have the limitations discussed above. An additional limitation is that many singular cancellation signals produced initially strong negative images (p<0.001), which
makes detecting a stronger effect in the combined condition difficult (p would need to be << 0.001). It is plausible that the strength of the negative image is not adequately captured by the analytical techniques used in this. To avoid a floor effect produced by initially strong singular cancellation signals, future experiments should seek to develop weaker cancellation signals in order to allow the strength of combined C.S.\textsubscript{VMC+FM} to be revealed. This may be achieved using extracellular recording techniques and spike count analyses (such as those used in this study), but employing shorter coupling periods, weaker electrosensory stimuli, smaller deflections of the fin, etc.; or else, using patch-clamp techniques to examine changes in the magnitude of summated postsynaptic potentials in AENs from multiple molecular layer inputs.

Alternatively, different analytical measures for cancellation signals may be sought. Candidate techniques include time latency for spikes to recover within the stimulus interval during post-coupling, measuring the increases in spike rate outside the stimulus interval, and measuring the negative images of increased (as opposed to decreased) activity at stimulus offset when AENs are presented with inhibitory (as opposed to excitatory) electrosensory stimuli.

\textit{Non-plastic and plastic AENs}

The results from this study are consistent with other studies on the adaptive filter in skate dorsal octavolateralis nuclei (Montgomery and Bodznick 1994) in that a sizable and, in this study, underreported population of AENs did
not develop cancellation signals (i.e. non-plastic). In some AENs, despite repeated runs of coupling experiments, and changing experimental parameters such as increasing the coupling period, intensity of the electrosensory stimulus, and magnitude of the internal reference signal in the case of fin movements, AENs still did not develop significant cancellation signals. While a more sensitive measure for cancellation signals might reveal a greater proportion of seemingly non-plastic, but actually plastic AENs reaching statistical significance, I am inclined to agree with the experimental results produced here and in previous studies (Montgomery and Bodznick 1994; Bodznick et al. 1999) that some AENs are not plastic, and comprise a separate population from those AENs that do show plasticity.

Studies of principal cells in gymnotid electro sensory lateral line (ELL), which have similar characteristics to skate DON due to their cerebellar-like nature of the electrosensory nuclei, suggest a functional role for non-plastic principal cells (Bastian, Chacron and Maler 2004). Analogous to skates, the principal cells of gymnotid ELL receive inputs on their apical dendrites from an extensive excitatory parallel fiber network as well as indirect inhibition from interneurons. An anti-Hebbian mechanism at the apical synapses of AENs allows the generation of inverse sensory predictions necessary for electrosensory processing (in the case of gymnotids, to eliminate reaference generated by tail bends and the associated electric organ; Bell et al. 1997). Bastian et al. (2004) found that non-plastic cells have a functional role in the gymnotid ELL. Rather
than eliminating predictable electrosensory input entirely, it may be encoded in a separate channel (i.e. non-plastic principal cells), which enables the animal to preserve global information (e.g. whole body stimulation) that may be used in other calculations needed for electrosensory processing.

Additional observation

The experimental observation that at least one AEN developed a steadily increasing cancellation signal slowly over a two hour period adds another layer of complexity to electrosensory reaference suppression. It seems likely that different mechanisms mediate the plasticity in AENs with slow developing cancellation signals (e.g. at least 2 hours as shown here) versus AENs with rapidly developing cancellation signals (e.g. five 1-minute coupling sessions separated by rest periods as shown in Zhang and Bodznick 2008). A closer examination is needed to determine whether those characterized as non-plastic AENs are actually slow learners, or if there are three distinct populations of AENs (non-plastic, slow plastic, or quick plastic). For that, it would be necessary to characterize morphological, physiological and immunohistochemical properties in order to develop an appreciation for the diversity of neuronal populations in the dorsal octavolateralis nucleus and what putative functions each might have.
Implications of cerebellar-like structures for cerebellar function

Cerebellum and cerebellar-like structures have similar anatomical structures and neural circuitry (Devor 2000), and they have been suggested to share common patterns of gene expression, and perhaps have similar developmental and evolutionary origins (Bell 2002). The elasmobranch DON, gymnotid ELL, mormyrid ELL, and mammalian dorsal cochlear nucleus are examples of cerebellar-like structures (Bell et al. 1997). The predominant feature in cerebellar-like structures is the presence of a layer of large principal cells (similar to the Purkinje cell in the cerebellum) with extensively branching apical dendrites, which receive a rich variety of information from the parallel fibers and inhibitory interneurons in the molecular layer. The principal cells in cerebellar-like structures receive tonic peripheral sensory input via primary afferents terminating on their basal dendrites, whereas the Purkinje cells in cerebellum receive infrequent, powerful spikes from a single climbing fiber on its apical dendrite from the inferior olive (Sawtell and Bell 2008). Plasticity has been demonstrated in both structures to occur in analogous sites. In cerebellar-like structures, plasticity occurs between molecular layer and principal cell synapses, driven by correlations between peripheral sensory input and predictive signals. On the other hand in cerebellum, a single complex spike delivered by the climbing fiber is sufficient to alter the Purkinje cell’s output through plasticity at molecular layer-Purkinje cell synapses (Devor 2000). Additionally, there is a greater diversity of cells in the granular layer of
cerebellum, including unipolar brush cells and Golgi cells (Sawtell and Bell 2008).

Despite anatomical and input differences, studying cerebellar-like structures may provide insight into the function of cerebellum, particularly in the context of learning and sensory predictions. In the skate DON, where research has revealed an understanding for both structure (i.e. connectivity of neurons, distal inputs from other areas of the brain) and function (i.e. generation of sensory predictions to suppress behavioral reafference), cerebellar-like structures may provide a useful model to study parallels in cerebellar function (e.g. learning a motor skill using sensory feedback to modify new movements, vestibulo-ocular reflex to stabilize a visual image on the retina during head movements) (Brashers-Krug, Shadmehr and Bizzi 1996; Boyden, Katoh and Raymond 2004).

**Future directions**

All animals must deal with the extraction of meaningful information from redundant reafference. One mechanism proposed, which has similar features across phylogenetically separate electroreceptive fish, is that an internally generated inverse sensory prediction is compared with the actual electrosensory input, which when added together, removes reafference in a temporally specific manner. There are a number of different avenues that need to be investigated, including an exploration of how these inverse sensory predictions are generated
and the specific cellular and molecular mechanisms that achieve it (e.g. receptor trafficking, molecular pathways). The relative contribution of inhibition in these structures has not received as much attention as the effects of excitation. In cerebellum, the sites of plasticity for enhanced inhibition onto principal neurons were found at inputs to interneurons that feedforward onto Purkinje cells (Smith and Otis 2005). Whether this is also true of cerebellar-like structures, or if plasticity occurs elsewhere (e.g. interneuron-principal cell synapse) remains to be seen. It would be important to determine if the inputs of interneurons are convergent or if their outputs diverge, and specifically whether interneuron-principal synapse change strength, as the adaptive filter model suggests.

**Summary**

In this study, findings show that multiple cancellation signals associated with different internal reference signals can exist within the same AEN. Different internal reference signals seem to arrive through separate, non-overlapping molecular layer inputs, allowing for the independent nature of cancellation signals. Cancellation signals are additive in a subset of AENs, although not all developed combined cancellation signals that were significant or stronger than singular cancellation signals. Finally, some AENs are slow to develop cancellation signals. I propose that the functional relevance of multiple cancellation signals, which may be independent and additive, is to provide
sensory predictions that suppress dynamic, complex patterns of reaference that result from continuously, intermittently or concurrently performed behaviors.
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