UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of putative lateral habenula and entopeduncular nucleus neuronal responses to noxious stimuli in the anesthesized mouse

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2013

The thesis of Li Yin Lan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013

DEDICATION

For my family.
For my friends.

EPIGRAPH

Men love to wonder,
and that is the seed of science.
-Ralph Waldo Emerson

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Professor Roberto Malinow for his patience and support as chair of my committee. The opportunity to work and learn at the forefront of neuroscience in his lab as an undergraduate and then as a graduate student, has helped me to develop my critical thinking skills and to become a better scientist.

I would also like to thank Dr. Steven Shabel for his never ending patience, guidance, and mentorship. Additionally, I would like to express gratitude to Professor Takaki Komiyama for providing advice, as well as physical space to work on this project. Finally, thanks to all the current and past members of the Malinow lab for their help and support.

ABSTRACT OF THE THESIS

Characterization of putative lateral habenula and entopeduncular nucleus neuronal responses to noxious stimuli in the anesthesized mouse

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Master of Science in Biology

University of California, San Diego, 2013

Professor Roberto Malinow, Chair

Recently, there have been studies on the projection from the entopeduncular nucleus (EP) to the lateral habenula (LHb) and its relationship to the reward and disappointment systems. Specifically, the projection from the EP to the LHb have been found to be excitatory and behaviorally aversive in rats. However, the response to pain in the projection from the EP to the LHb has not yet been studied or identified. Here, we find that many neurons are initially excited, then inhibited in both the EP and LHb, but only the EP has a large proportion of cells that are only inhibited after the shock. We also

found that the majority of inhibited EP cells had high baseline firing rates, whereas the majority of LHb neurons with high baseline firing rates were excited by tailshock. These results suggest that there at least two populations of cells in the EP that project to the LHb and are activated by shock. One population sends excitatory projections to the LHb and is excited initially, then inhibited by shock. The other population sends inhibitory projections to the LHb and is only inhibited by shock.

Introduction:

Part of the larger habenular structure and located in the epithalamus, the lateral habenula (LHb) is a highly conserved brain structure amongst most vertebrates, and has been known to be involved in depression (Hikosaka, 2010). In patients with depression, the neurons in the LHb are hyperactive and the disorder can be treated with deep brain stimulation in the LHb, which is thought to inhibit the LHb (Sartorius and Henn, 2007). Researchers have also studied the cellular basis of depression in the LHb using rodent models of depression. Through these studies, it has been observed that excitatory synapses of neurons in the LHb are hyperactive in depressed animals as compared to wildtype animals (Li et al., 2011).

The LHb has also been implicated in negative event processing. In rhesus monkeys, LHb neurons behaved in an opposite manner to midbrain dopaminergic neurons behavior to reward and no-reward predictors. Specifically, LHb neurons were excited and inhibited by no-reward-predicting and reward-predicting targets, respectively, while dopaminergic neurons were excited and inhibited by reward-predicting and no-reward-predicting targets, respectively (Matsumoto and Hikosaka, 2007). These results suggest that the LHb has an important role in negative event processing and together with the depression literature are consistent with the hypothesis that a hyperactive habenula contribute to depression by enhancing processing of aversive events.

In addition, various studies have implicated the role of the habenula in pain processing, a form of negative event. Rats injected with thiopental, an anesthesiant, were exposed to a noxious stimulus resulting in increased activity in the habenula (Archer et al., 1995). In addition, the response of the lateral habenula to noxious pain has been

examined in the rat. Two populations of cells were found--one exhibiting an excitatory response and one exhibiting an inhibitory response following high intensity tail shock in the anesthesized rat (Benabid and Jeaugey, 1989). These results strongly implicate the LHb's role in pain processing.

Although the LHb has been well studied, the roles of upstream projections from the LHb have not yet been fully identified, specifically the entopeduncular nucleus (EP), located within the basal ganglia. The projection from the EP to the LHb had been previously identified through cellular labeling (Parent et al., 2001). However, its function has not been studied in depth until recently. In one study, Hong and Hikosaka saw that the cells in the borders of the globus pallidus internal segment (GPi), the primate homolog of the EP, were excited by non-reward-predictors and inhibited by reward-predictors, similar to those observed in LHb neurons. The timing of excitation in the GPi was slightly earlier than those in the LHb, suggesting that the GPi is upstream of the LHb (Hong and Hikosaka, 2008). These results suggest that the GPi is important for sending negative stimulus information to the LHb.

However, these are observed responses in the primate and not in rodents. In an alternate rodent study regarding this specific pathway, the input from the EP to the LHb was found to be excitatory with predominately glutamatergic cells, behaviorally aversive, and its activity suppressed by serotonin in rats (Shabel et al., 2012). The rodent EP and its response to pain has not yet been studied specifically. Therefore, the motivation for this study is to develop a system in which we can use to investigate the neural circuitry of negative event processing. Once more evidence of how the EP processes pain is

collected, further experiments may be performed to examine how the EP contributes to LHb processing of noxious stimuli.

We used a noxious stimulus (tail shock) as an environmental cue of a negative outcome and investigated the independent responses in EP and LHb neurons to such stimuli. We found that a majority of neurons in the EP and LHb responded to noxious stimuli. More EP and LHb cells than expected responded with excitation followed by inhibition after tail shock, more cells than expected in only the EP responded with inhibition followed by inhibition after tail shock, and on average the activities of high firing rate EP cells (>5Hz) were inhibited by shock, while high firing rate LHb cells (>3Hz) were excited by shock. These results suggest that there exist at least two populations of cells in the EP that project to the LHb and are activated by the tail shock. One population sends excitatory projections to the LHb and is excited initially, then inhibited. The other population sends inhibitory projections to the LHb and is only inhibited by tail shock.

Methods:

Animals:

C57/BL6 male wild-type mice were housed individually and kept on a 12/12 hour light dark cycle. The mice underwent head bar implantation, at approximately 60 days of age. Anesthetized with isoflurane, the mice had the locations of the EP (AP: -1.2 mm from bregma; ML: 1.85 mm) and LHb (AP: -1.8 mm from bregma; ML: 0.5 mm) marked with a permanent marker directly on the skull, and the head bar secured with super glue and dental cement. The mice were then used to record neural activity typically within 15 days following head bar implantation.

Pipettes and internal solution:

Pipettes were made from borosilicate (OD: 1.5 mm; ID: 0.86 mm) and pulled with the Sutter Instrument P-2000 (laser based micropipette puller) to achieve a tapered shank length of 0.75 cm. The tips were then broken to a size of 1-2 µm, resulting in a final resistance of 5-15 MOhms in the brain. A solution of 2% Neurobiotin and 3% NaCl or 2% Pontamine Sky Blue (PSB) was used to fill the pipettes.

Recording setup:

Mice were anesthesized with urethane (1.3g/kg) prior to recording. Once sufficiently anesthesized, the animal was placed in a head restraint, its temperature regulated at 37 degrees Celsius via heating pad and rectal probe, and two 30 gauge hypodermic needles inserted lightly 0.75 cm and 2 cm from the tip of the tail. The locations of the EP and LHb on the skull were then drilled open with a dental drill, taking

care to keep the hole small, about the size of the drill bit. Dura mater was delicately removed, exposing the brain underneath. Pipettes were then stereotactically lowered into the brain to the EP (3800-5000 μm) and LHb (2500-3500 μm) for extracellular recording of cells. The exposed brain was kept moist with repeated applications of ACSF in the drilled hole.

Cells were located with an Axopatch 1D amplifier with a 5kHz sampling frequency and the filter set at a -3dB cutoff frequency of 5kHz. Under current clamp mode, pipettes were advanced in 2 μm steps until a 1.0 to 1.5 mV neural signal was recorded from the cell. The tail shock protocol utilized an external grass stimulator that evoked a 2 mA high frequency of shocks every millisecond for 10 milliseconds into the mouse tail via the hypodermic needles.

Perfusion and Tissue Processing/Histology:

Multiple methods were utilized to try to achieve marking pipette positions in the EP and the LHb. Unfortunately, none of the following techniques were successful for cell labeling.

1. Juxtacellular recording with neurobiotin was attempted (Joshi and Hawken, 2006).
2. Extracellular recording with neurobiotin (positive current supplied).
3. Extracellular recording with neurobiotin (positive pressure).
4. Extracellular recording with 2% PSB internal solution (negative current).
5. Extracellular recording with 2% PSB internal solution (positive current).
6. Extracellular recording with fluorescent beads (positive pressure).

7. Pipettes with varying tip sizes and lengths were tried.

The following protocol was utilized for any kind of recording with neurobiotin. After recording, the animals were given an overdose of urethane and then were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). The brain was then extracted, allowed to sit in PFA for at least 12 hours, and sliced into sections of 75 µm. The slices were stained for Neurobiotin with the Vectastain ABC Standard Kit followed by Vectastain DAB Peroxidase Substrate Kit. The ABC Standard Kit was prepared according to the suggested protocol, along with 0.2% Triton X to allow for permeabilization of the cell membranes. The slices were incubated for one hour with the ABC Standard Kit. Lastly, the slices were stained for 2 minutes with the DAB peroxidase substrate kit, also prepared according to the suggested protocol. The slices were then visualized underneath a light microscope.

The following protocol was utilized for any kind of recording with PSB and fluorescent beads. After recording, the animals were given an overdose of urethane and sacrificed. The brain was extracted, allowed to sit in PFA for at least 12 hours and sliced into sections of 75 µm. The slices were then visualized underneath a light microscope.

Data Analysis:

Neuronal spikes were sorted with the MiniAnalysis program and plotted with Matlab for frequency of spikes against time for each recorded cell. Averages were obtained two seconds prior to the shock stimulus and for the 0 to 1 second timeframe and the 1 to 2 second timeframe following the shock stimulus (Figure 2).

Statistical Analysis:

Statistical significance was determined after performing a χ^2 test in the following manner: Expected values were calculated from baseline periods (-5 to 0 seconds). Baseline periods for all cells in the EP and the LHb were broken up into 4 consecutive second timeframes. From within the 4 second timeframes, baseline periods were further divided into the first 2 second "baseline" followed by the 2 remaining second "response" sections. "Response" sections were divided into the first second and the second second and its average response categorized after comparing its activities to the averaged "baseline" section. Following this, the "baseline" and "response" sections swapped roles ("baseline" now treated like "response" section and "response" section treated like "baseline" section) and their responses also quantified. Following quantification of various responses, the number of responses obtained were averaged to calculate the percent population expected. χ^2 test was then performed on the expected and observed values. Lastly, responses that were more common than expected were considered significant if the p value of the response was greater than 0.05.

Z score was utilized for normalization of responses with the following equation:

$$z\ score = \frac{x - \mu}{\sigma}$$

where x is the observed value, μ is the mean and σ is the standard deviation.

Results:

We obtained responses from 27 out of 35 cells in the EP and 29 out of 30 cells in the LHb. On average, four to five cells were recorded from a single animal. Trials were evoked every 15 seconds and lasted 14.336 seconds. For a given cell, average firing rates relative to the shock (time = 0) were calculated using 100 ms bins.

Excitation, inhibition response is more common than expected in the EP and the LHb.

To better understand the responses of cells to tail shock, the responses of cells in the EP and the LHb were categorized into five categories based on percent change from the baseline average in the 0-1 second time period following the shock and the 1-2 second time period following the shock (Figure 2). The five categories were excitation, excitation; excitation, inhibition; inhibition, inhibition; inhibition, excitation; and no response. The pattern of responses in the EP and LHb were more different than that predicted by chance, with 23% of EP cells and 43% of LHb cells responding with excitation then inhibition following tail shock (Figure 3). Figure 4 shows that the excitation, inhibition response occurred twice as frequently than expected in the EP and occurred 4 times as frequently than expected in the LHb.

Since the excitation, inhibition responses were more common than expected in both the EP and the LHb, we were curious to see whether there were any time course differences between EP and LHb cells following tail shock. EP and LHb cells that exhibited the excitation, inhibition responses were normalized and plotted as illustrated by figure 6. EP and LHb normalized responses were similar, with about a three-fold

increase in activity in the 0 to 1 second timeframe and a 50% decrease in activity in the 1 to 2 second timeframe.

All EP cells and LHb cells were normalized and plotted, revealing that the populations as a whole respond similarly with a strong excitation followed by weak inhibition (Figure 5).

Inhibition, inhibition response is more common than expected in the EP, but not in the LHb.

In addition to seeing more cells than expected for the excitation, inhibition response in both the EP and the LHb, it was observed that the inhibition, inhibition response was more common than expected in the EP, but not the LHb. As indicated in figure 3, 31.43% of EP cells and only 13.33% of LHb cells responded with inhibition, inhibition following tail shock. The inhibition, inhibition response in the EP occurred less than twice as frequently than expected while the inhibition, inhibition response occurred half as many times as expected in the LHb (Figure 4). With these data, it is evident that the inhibition, inhibition response is more common than expected in the EP, but not in the LHb.

EP high firing rate cells on average are inhibited by shock. LHb high firing rate cells on average are excited by shock.

Since the baseline firing rates varied from cell to cell, we examined the relationship between baseline firing rates and response to tail shock. EP cells with a frequency greater than 5 Hz and LHb cells with a frequency greater than 3 Hz were

averaged and plotted (Figure 7 and 8). A different pattern emerged when analyzing these cells; high firing rate EP cells, on average, were inhibited following shock while high firing rate LHb cells, on average, were excited following shock. There is a delayed response in these cells when compared to the faster responding excitation, inhibition cells (Figure 6). In addition, high firing EP cells are correlated with inhibition while high firing LHb cells are correlated with excitation (Figure 9).

Discussion:

The results shed light on the processing of noxious stimuli through the EP to LHb pathway. The simplest model to explain our results is that there exists two different populations of EP cells that project to the LHb in response to noxious stimuli: one population is excited and then inhibited by shock, sending excitatory projections to the LHb, while the other is inhibited by shock and sends inhibitory projections to the LHb.

The common excitation, inhibition response in both the EP and the LHb suggests that these cells in the EP project to the LHb with excitatory connections. The presence of prevalent inhibition, inhibition responses in the EP and excitation, excitation responses in the LHb are consistent with the hypothesis that EP neurons whose activity is suppressed by tailshock send inhibitory projections to the LHb (Figure 3 and 4). Lastly, the activities of high firing rate cells in the EP and the LHb were different. High firing cells in the EP on average were inhibited by shock, while high firing cells in the LHb on average were excited by shock. This result suggests that these high firing cells in the EP inhibit LHb high firing cells; thus aversive stimuli that inhibit EP cells would excite LHb cells. Taken together, these results suggest both excitatory and inhibitory projections from the EP to the LHb are active in response to noxious stimuli.

Previous studies that investigated tail shock responses in the LHb revealed two populations: one population was inhibited while another was excited by tail shock (Benabid and Jeaugey, 1989). Interestingly in spite of these previously published results, we did not observe any cells displaying pure inhibition, but instead observed cells that were excited. A possible reason that no cells in the LHb displaying inhibition were that

the intensity tail shock that was utilized in this study was considerably lower (2-5mA vs. 30-50mA).

In line with our experiment, the GPi was found to send reward-related signals to the LHb, identifying two distinct populations of cells in the GPi, the reward negative type and the reward positive type (Hong and Hikosaka, 2008). The reward negative type exhibited a fast response to reward or no reward and is typically excited by no reward. These cells are analogous to EP cells that responded in an excitatory, inhibitory manner. The reward positive type exhibited a slower response to reward and no reward and is typically inhibited by no reward. These cells are analogous to EP cells that responded in an inhibitory, inhibitory manner to tail shock.

However, further studies are necessary to verify these putative results. Unfortunately, the exact locations of responding cells in both the EP and LHb were never marked despite months of troubleshooting with various pipette tip sizes and shapes, as well as various internal solutions and marking reagents. The histology revealed only pipette tracts in or near the site of the EP as indicated in figure 1. The tracts of pipettes targeted to the LHb could not be identified. Hence, determining the exact location of these cells will prove advantageous towards future studies regarding this projection. In addition, it is unclear whether responding EP cells project to the LHb. It is possible that some of these cells actually project to the motor thalamus or brainstem, an alternate projection that has been studied in detail (Goldberg and Bergman, 2011).

Despite these shortcomings, more can be learned about the activities of these cells by labeling for various neurotransmitters. In these experiments, we would expect to see excitatory cells labeling for glutamate and inhibitory cells label for GABA. The observed

responses of these cells can possibly be explained with the results of these proposed experiments.

Figures:

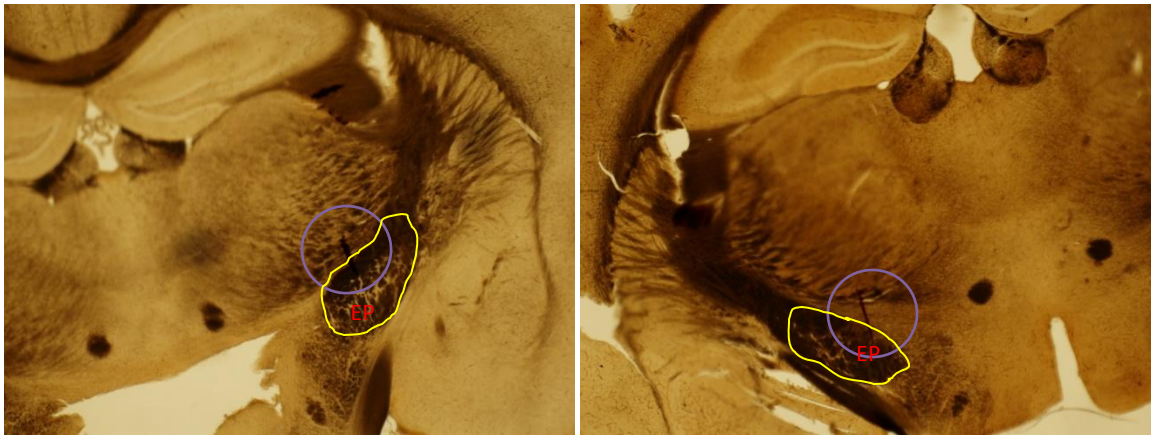

Figure 1: Two examples of pipette tracts in the EP. The location of the EP is indicated by the yellow outline. Pipettes tracts are circled in lavender. Exact locations of the recorded cells were never marked. However, the trail left by the pipette into the EP indicates that most pipette tips were near or within the EP. No pipette tracts were seen in the LHb.

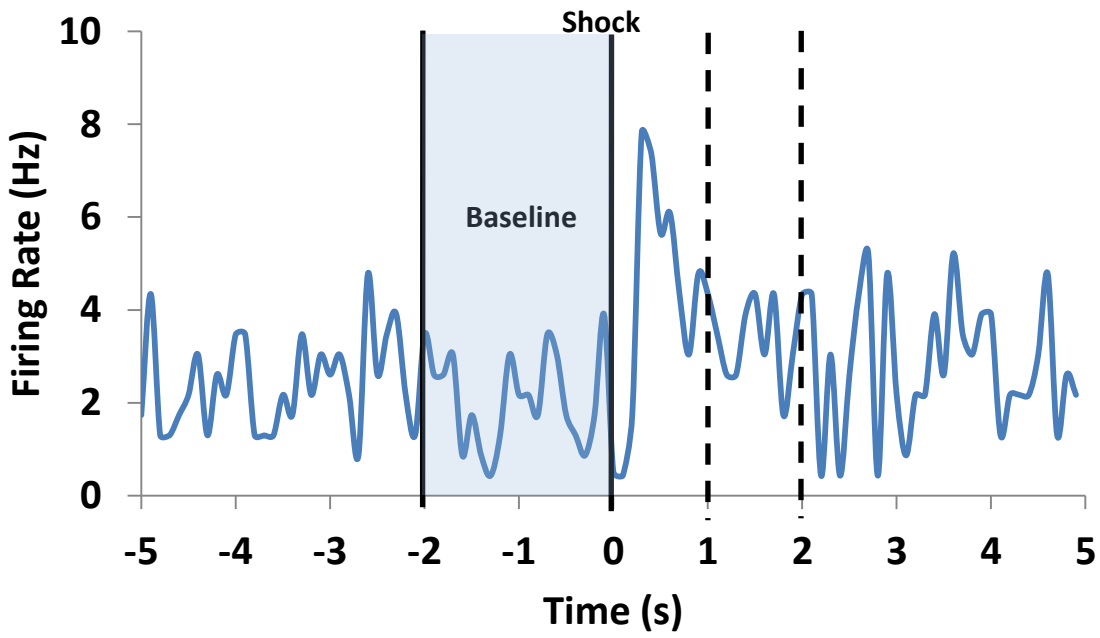

Figure 2: Example of a single cell response to tail shock. The administration of the tail shock began at time 0, with 10 shocks every 1 millisecond, resulting in a 10 ms shock length. Baseline averages were taken from -2 to 0 seconds and compared to 0 to 1 second and 1 to 2 second sections respectively to allow for categorization into five categories: excitation, excitation; excitation, inhibition; inhibition, inhibition; inhibition, excitation; and no response. Cells must exhibit a change greater than 15% to allow for classification into any category.

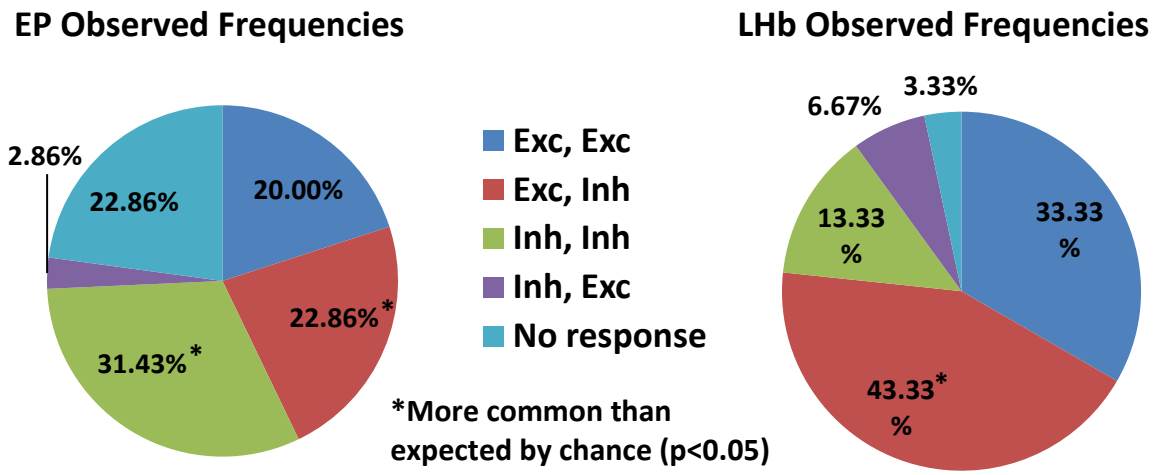

Figure 3: Categorical quantification of the responses of EP cells and LHb cells to tail shock. In the EP, 22.86% of cells responded with excitation (>15% change from baseline) followed by inhibition (>15% change from baseline) in the 0-1 second and 1-2 second time periods, respectively, following shock. In the LHb, 43.33% of cells responded with excitation followed by inhibition in the 0-1 second and 1-2 second time periods, respectively, following shock. The excitation, inhibition responding cells in both the EP and the LHb were of a higher percentage than is expected by chance. In addition, 31.43% of EP cells responded with inhibition, inhibition, more than is expected by chance. Note that the EP has many cells that respond with excitatory, inhibitory and inhibitory, inhibitory responses. Also, the LHb has many cells that respond with excitation, inhibition and excitation, excitation. As a whole, the EP and LHb population responses were more different than expected by chance in both the EP and the LHb (EP: n = 35 cells total, χ^2 population = 14.58, p = 0.0057; LHb: n = 30 cells total, χ^2 population = 32.1, p = 0.000002).

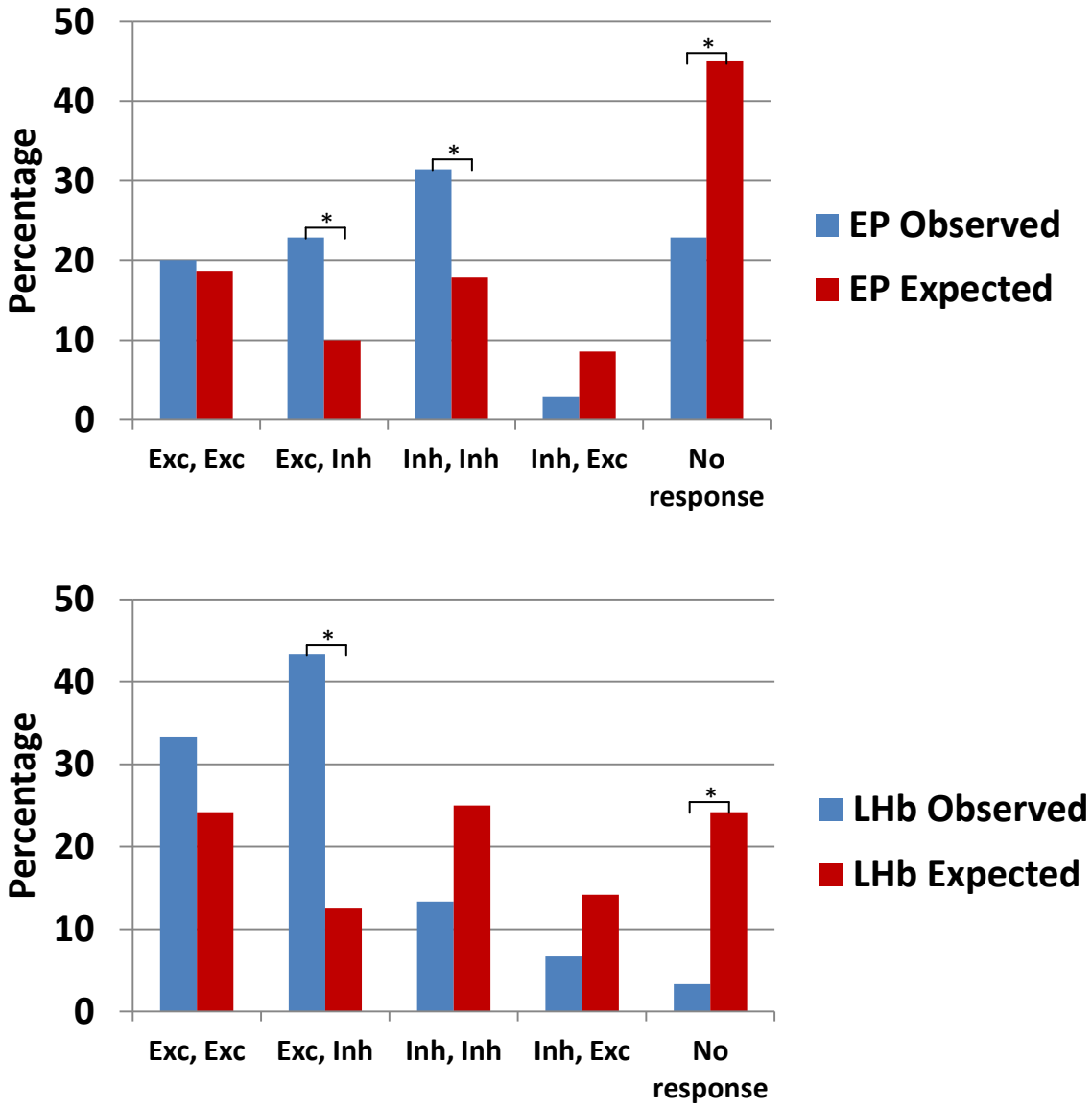

Figure 4: Percentage of EP and LHb cells observed and expected for each category of response. In the EP, cells that responded with excitation then inhibition in the 0-1 second and 1-2 second time frames, respectively, were more common than expected (8 of 35 cells, $\chi^2 = 6.4286$, $p = 0.0112$). Cells that responded with inhibition followed by inhibition in the EP were also more common than expected (11 out of 35 cells, $\chi^2 = 4.3948$, $p = 0.036$). Cells that had no response in the EP were less common than expected (8 out of 35 cells, $\chi^2 = 6.9336$, $p = 0.0085$). In the LHb, cells that responded with excitation then inhibition in the 0-1 second and 1-2 second time frames, respectively, were more common than expected (13 out of 30 cells, $\chi^2 = 26.0762$, $p < 0.0001$). Cells that had no response in the LHb were less common than expected (1 out of 30 cells, $\chi^2 = 7.105$, $p = 0.0077$).

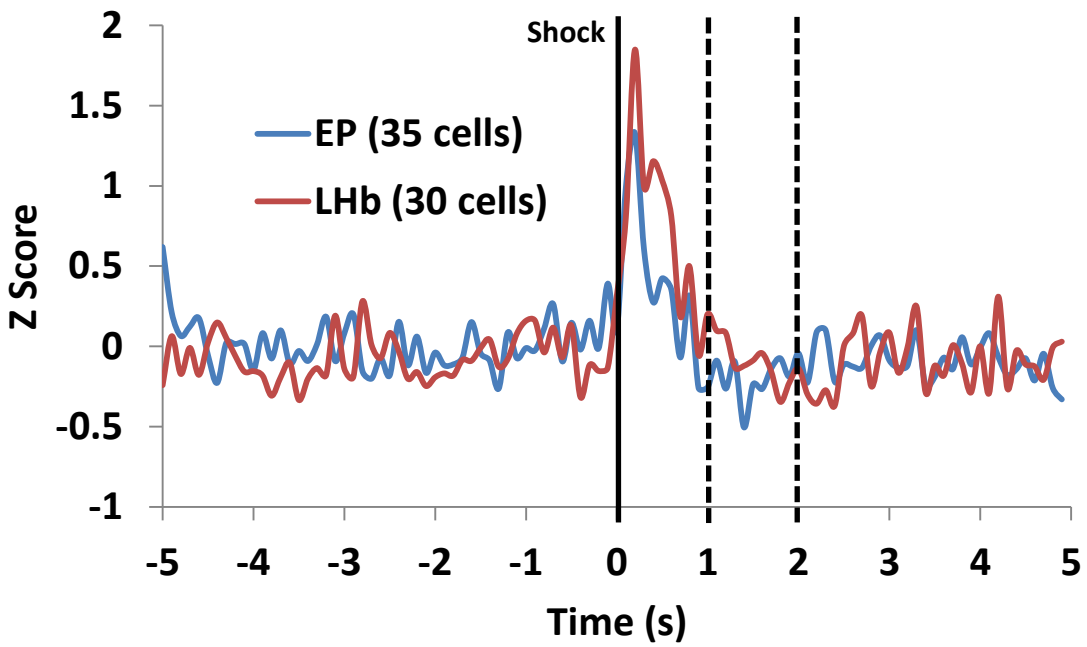

Figure 5: Normalized EP and LHb population responses to tail shock. All cells in the EP and the LHb were normalized utilizing Z score, averaged, and average values plotted. As a population, EP and LHb cells respond similarly--excitation after shock in the first second, followed by a slight inhibition, and then a return to baseline values.

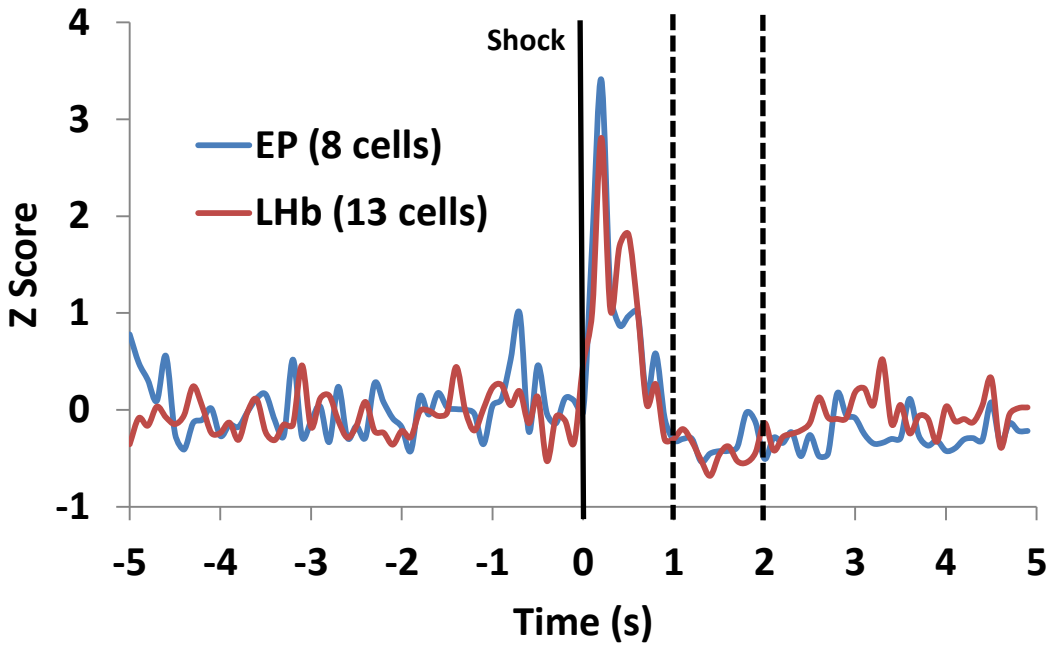

Figure 6: Normalized EP and LHb excitation, inhibition responses. EP and LHb cells categorized with the excitation, inhibition response were normalized, averaged, and averages plotted. Cells in both the EP and the LHb follow the same time course--excitation after shock in the 0-1 second timeframe, followed by inhibition in the 1-2 second timeframe.

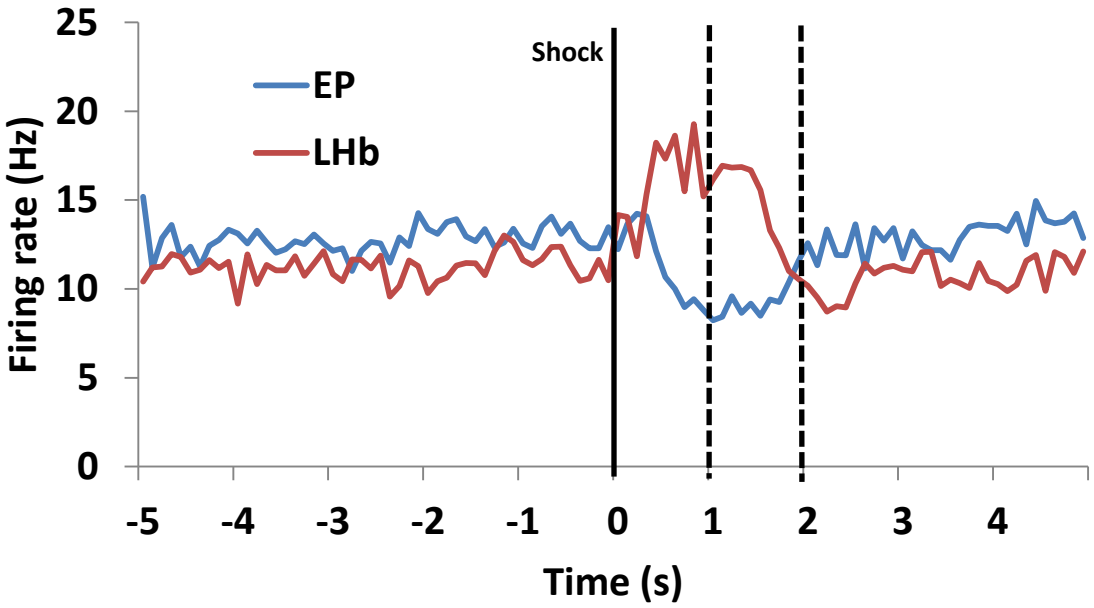

Figure 7: High firing rate cells in the EP (>5 Hz) and the LHb (>3Hz) averaged and plotted. EP cells with high firing rates (n=6) have delayed inhibition following shock relative to faster responding excitation, inhibition cells. LHb cells with high firing rates (n=6) have delayed excitation following shock relative to faster responding excitation, inhibition cells.

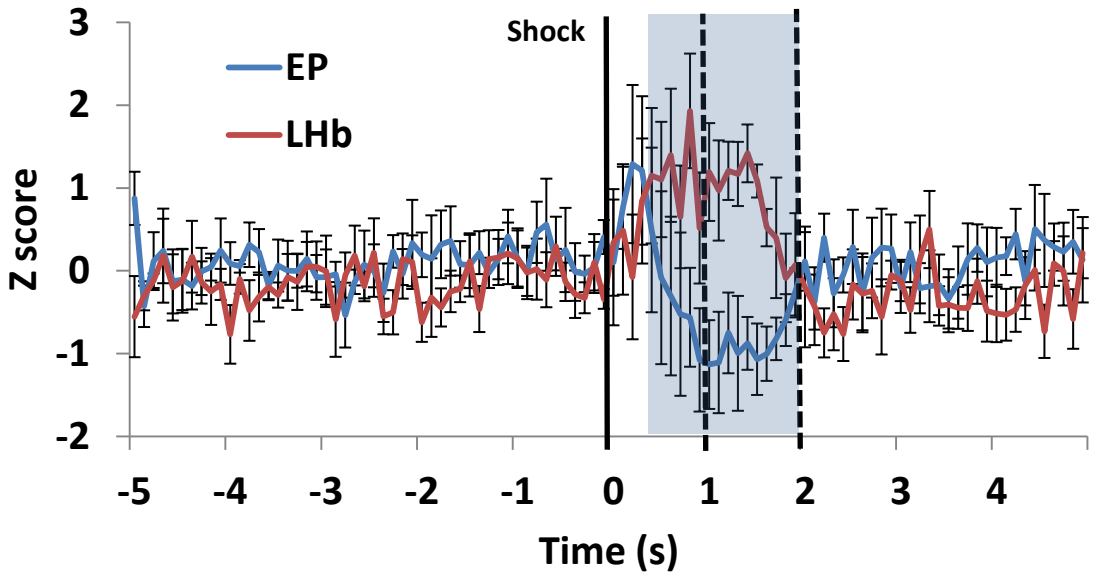

Figure 8: High firing cells in the EP (>5Hz) and the LHb (>3Hz) were normalized, averaged, and averages plotted. Similar to the non-normalized high firing rate cells, cells in the EP (n=6) on average are inhibited following shock while cells in the LHb (n=6) on average are excited following shock. There is a delayed reaction as indicated in the shaded portion.

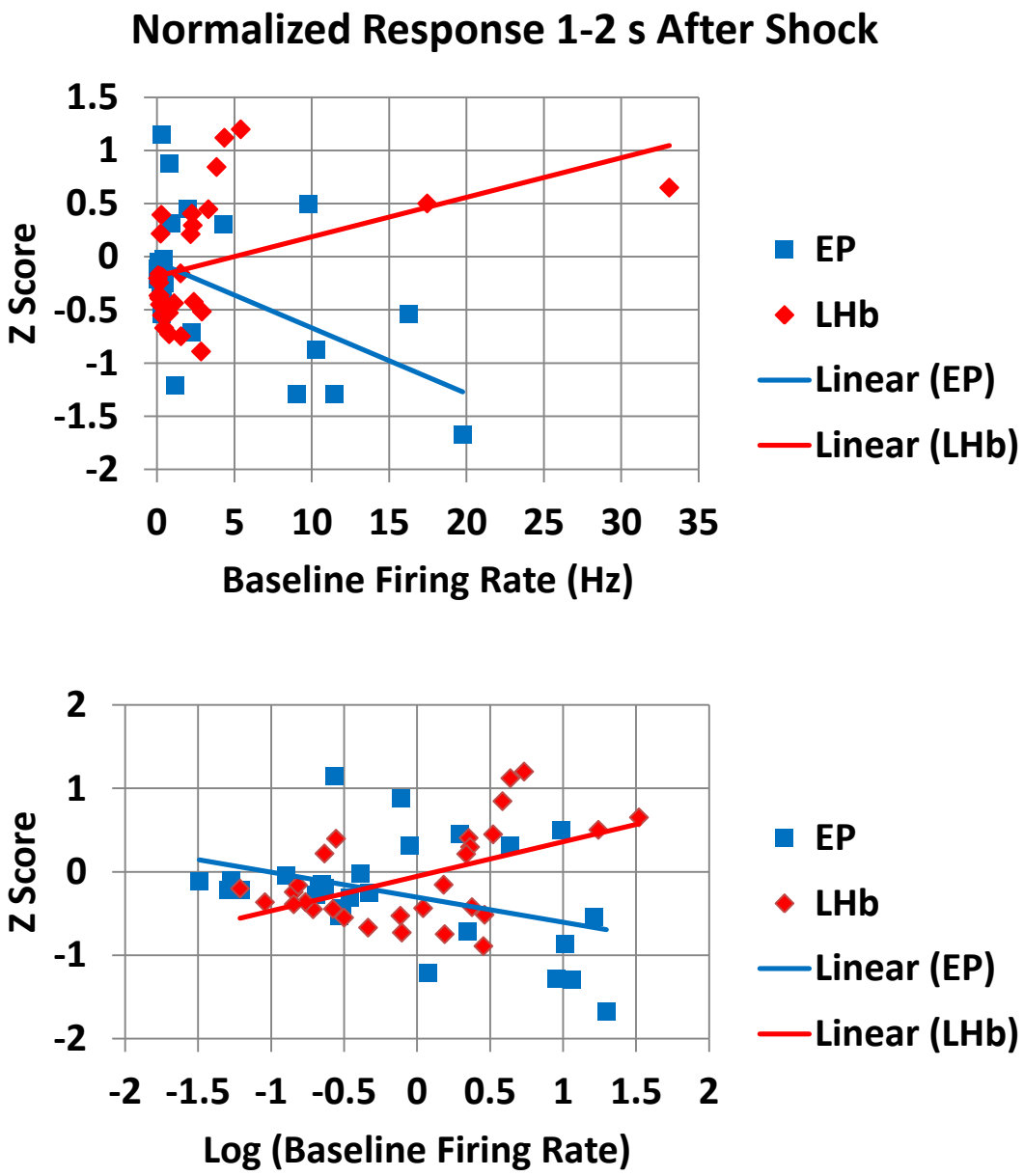

Figure 9: EP and LHb normalized response 1 to 2 seconds following shock are plotted and trend lines included. Only cells with categorical responses are included. A: In the EP, there is now more of a negative change in firing rate following shock (correlation coefficient = - 0.52443, p = 0.005). In the LHb, there is again greater change in firing rate following shock (correlation coefficient = 0.431806, p = 0.019). B: Cells exhibit similar trends after taking the log value of baseline firing rates (EP: correlation coefficient = -0.38577, p = 0.0469, LHb: correlation coefficient = 0.504146, p = 0.0053). Cells with higher baseline firing rates are correlated with inhibition in the EP or excitation in the LHb.

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