Learning-related neuronal activity in the ventral lateral geniculate nucleus during associative cerebellar learning

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Submitted 12 March 2013; accepted in final form 8 August 2014

Kashef A, Campolattaro MM, Freeman JH. Learning-related neuronal activity in the ventral lateral geniculate nucleus during delay eyeblink conditioning. J Neurophysiol 112: 2234–2250, 2014. First published August 13, 2014; doi:10.1152/jn.00185.2013.—During delay eyeblink conditioning, rats learn to produce an eyelid-closure conditioned response (CR) to a conditioned stimulus (CS), such as a light, which precedes and coterminates with an unconditioned stimulus (US). The inferior olive provides input to the cerebellum, where the memory underlying eyeblink conditioning is formed and stored (Freeman and Steinmetz 2011; Mauk and Thompson 2009). The inferior olive may play a role in visual eyeblink conditioning by supplying visual sensory input to the pontine nuclei (PN) and also receiving feedback from the cerebellum. No prior study has investigated LGNv neuronal activity during eyeblink conditioning. The present study used multiple tetrodes to monitor single-unit activity in the rat LGNv during pre-exposure (CS only), unpaired CS/US, and paired CS-US training conditions. This behavioral-training sequence was used to investigate nonassociative- and associative-driven neuronal activity in the LGNv during training. LGNv neuronal activity habituated during unpaired training and then recovered from habituation during subsequent paired training, which may indicate that the LGNv plays a role in attention to the CS. The amplitude of LGNv neuronal activity correlated with CR production during paired but not unpaired CS/US training. Cerebellar feedback to the LGNv may play a role in modulating LGNv activity and attention to the CS during paired training. Based on the present findings, we hypothesize that the role of LGNv in visual eyeblink conditioning goes beyond simply routing visual CS information to the PN and involves modulation of attention.

associative learning; eyeblink conditioning; lateral geniculate; cerebellum

EYEBLINK CONDITIONING PROCEDURES have been used extensively to investigate the neural mechanisms underlying associative learning (Freeman and Steinmetz 2011). It is well established that the cerebellum plays a central role in acquisition and retention of eyeblink conditioning and other types of motor learning (Barmack and Simpson 1980; Freeman and Steinmetz 2011; Gilbert and Thach 1977; Graf et al. 1988). Convergence of conditioned stimulus (CS) and unconditioned stimulus (US) information occurs within the cerebellar cortex and interpositus nucleus, where the memory underlying eyeblink conditioning is formed and stored (Freeman and Steinmetz 2011; Mauk and Donegan 1997; Steinmetz et al. 1989; Thompson and Steinmetz 2009). The inferior olive provides input to the cerebellum about the US (Mauk et al. 1986; Steinmetz et al. 1989), and the pontine nuclei (PN) provide input to the cerebellum about the CS (Halverson and Freeman 2010a, b; Steinmetz et al. 1986, 1989).

Multiple cortical and subcortical visual-processing areas project to the PN, such as lateral geniculate nucleus, superior colliculus, visual cortex, and pretectal nuclei (Graybiel 1974; Holstege and Collewijn 1982; Moore et al. 2000; Ribak and Peters 1975; Wells et al. 1989). Among these areas, the ventral lateral geniculate nucleus (LGNv) may play a critical role in eyeblink conditioning with a visual CS, considering that inactivation of the LGNv impairs retention of visual eyeblink conditioning (Steinmetz et al. 2013), and direct stimulation of the LGNv is sufficient to establish eyeblink conditioning (Halverson et al. 2009). Moreover, inactivation of the medial PN, which receives input from the LGNv, significantly impairs eyeblink conditioning with a visual CS (Halverson and Freeman 2010b; Koutalidis et al. 1988). The cerebellum projects to the LGNv (Zimny et al. 1986), which further strengthens the possibility that the LGNv may play a role in eyeblink conditioning with a visual CS.

To our knowledge, no prior study has directly investigated LGNv neuronal activity during associative learning in anesthetized animals (Davidowa and Albrecht 1992; Miller et al. 2008). Multiple studies, however, have shown that learning-related neuronal activity occurs in specific auditory thalamic nuclei in awake animals during associative conditioning (Disterhoft and Olds 1972; Gabriel et al. 1975; O’Connor et al. 1997; Ryugo and Weinberger 1978). For example, Halverson et al. (2010) found that neuronal plasticity occurs in the medial division of medial geniculate (MGM) and suprageniculate nucleus during eyeblink conditioning with an auditory CS. Neuronal plasticity has also been found in the MGM during fear conditioning and discriminative-avoidance conditioning (Bordi and LeDoux 1994; Gabriel et al. 1975). Currently, there is no consensus regarding the origin of this neural plasticity or its functional contribution to associative learning (Maren et al. 2001; Parsons et al. 2006; Poremba and Gabriel 2001; Weinberger 2011). It is therefore of interest to determine whether the occurrence of learning-related neuronal changes during associative learning is also a feature of visual thalamic neurophysiology. The LGNv may simply relay visual input to the PN, or it could play an active role in the learning process, similar to the MGM (Poremba and Gabriel 1997).

The present study used multiple tetrode recordings to monitor and investigate learning-related changes in LGNv neuronal activity during delay eyeblink conditioning in rats. Single-unit activity was recorded in the LGNv during pre-exposure (CS only), unpaired CS/US, and paired CS-US training to differentiate changes due to associative learning from changes due to
nonassociative factors (e.g., sensitization) and conditioned response (CR) production.

MATERIALS AND METHODS

Subjects

Subjects were 10 male Long-Evan rats (200–250 g). The rats were housed in the Spence Laboratories of Psychology at the University of Iowa on a 12-h light-dark cycle with ad libitum access to food and water. All procedures were approved by the University of Iowa Institutional Animal Care and Use Committee and the National Institutes of Health.

Surgery

Stereotaxic surgery was performed ~2 wk before the start of behavioral training. After the rat was anesthetized with isoflurane (1.5–3.0%, 0.7 l/min), a mid sagittal scalp incision was made to expose the underlying skull. Stainless-steel screws were threaded into the skull (two into each bone) until they reached just above the skull’s inner surface. A burr hole (~2 mm) was drilled over the right LGNv (anteroposterior, ~4.5; mediolateral, ~4.0), using a small trephine drill bit. Next, the dura mater, exposed by this hole, was removed carefully, leaving minimal damage to the cortex. A custom-made hyperdrive, consisting of six recording tetrodes and one reference tetrode, was implanted, placing its outlet just above the exposed surface of cortex. The portion of the skull opening not covered by the drive outlet was sealed with low-viscosity silicon (Kwik-Sil; World Precision Instruments, Sarasota, FL). The hyperdrive assembly was grounded to a silver wire connected to a stainless-steel screw, secured in the right frontal bone. Two electromyography (EMG) stainless-steel microwires were implanted into the upper-left orbicularis oculi muscle, and a silver wire grounded the EMG to a stainless-steel screw. Each EMG microwire terminated in a gold pin, held in a plastic connector. A bipolar-stimulating electrode, for delivering the US, was implanted subdermally, immediately caudal to the left eye. The tetrode hyperdrive assembly, EMG, and bipolar electrode were fixed to the skull and skull-screw anchors with bone cement (Zimmer, Warsaw, IN).

Tetrode Hyperdrive

The hyperdrive assembly consisted of seven custom-made microdrivers and a 27-channel electronic interface board (EIB-27; Neuralynx, Bozeman, MT). Each of the individually moveable microdrivers was loaded with a single tetrode. Each tetrode was made by twisting four insulated nichrome microwires (12 μm diameter; Kanthal Palm Coast, Palm Coast, FL) that were fused together by partially melting their insulation with heat. Each microwire in a tetrode was secured to an individual recording channel on the EIB-27 with a small gold pin, except for the tetrode, which was designated as a reference that had all four microwires connected to the reference channel. Each tetrode was gold plated (Sifco Process) to adjust its impedance to ~350–500 kΩ (IMP-1 impedance tester; Bak Electronics, Germantown, MD) by passing a small amount of current (~0.1 μA) through its tip. Tetrodes were lowered into the brain [dorsoventral (DV), −2.0] immediately after surgery and were left there during the recovery week. One week after surgery, tetrodes were lowered gradually over a period of 5 days toward their targeted location (DV, −5.5). The reference tetrode was lowered into or near the corpus callosum, where there was minimal neuronal activity. Three days before the start of training, tetrodes were lowered up to 100 μm/day until they reached their targeted location. Once training began, tetrodes were lowered to increase the quality of recording as necessary but no more than 100 μm on any single day. A 2-h waiting period was allotted to the animal after tetrodes were lowered and before the start of a training session.

Conditioning Apparatus

The conditioning apparatus consisted of a rat operant chamber, housed within a shielded sound-attenuation chamber (BRS/LVE, Laurel, MD). A light bulb (1.65 W, 100 lx), used to deliver the CS, was located on the back wall of the sound-attenuation chamber, directly behind the operant chamber. A stimulus isolator (model number 365A; World Precision Instruments) was used to generate a periorbital shock US [1.0–2.0 mA direct current (DC)]. The electrode leads from the rat’s headstage were connected to a motorized commutator above the sound-attenuation chamber and connected to peripheral equipment and a desktop computer. Computer software controlled the delivery of stimuli and recorded eyelid EMG activity (JSA Designs, Raleigh, NC). EMG activity was recorded differentially, band-pass filtered (500–5,000 Hz), amplified, rectified, and integrated (JSA Designs). Before rectifying the EMG signal, the amplified output was also sent to a Neuralynx Lynx amplifier, which was further filtered (1–9,000), digitized (1,894 Hz), and recorded (Neuralynx).

Conditioning Procedures

Training started ~2 wk after the day of the surgery. A summary of the entire experimental design is shown in Fig. 1. The duration of the light CS was 475 ms on all trials. On paired CS-US trials, the CS was terminated with the 25-ms periorbital shock (US), producing a 450-ms interstimulus interval (ISI). On day 1, rats received 80 CS-alone trials with an intertrial interval (ITI) that averaged 30 s (range: 20–40 s). This “Pre-exposure” session was used to investigate how neurons in the LGNv respond to the light CS before a rat was exposed to the US. The pre-exposure session was followed immediately by 80 unpaired presentations of the CS and US (“Pseudoconditioning-1”) with an ITI that averaged 15 s (range: 10–20 s). The training sequence on day 1 was used to determine if nonassociative factors, due to the US, produce changes in CS-elicited LGNv neuronal activity after the US was added to the training context but not explicitly paired with the CS. On day 2, rats received a second unpaired CS/US training session of 80 trials (“Pseudoconditioning-2”), followed immediately by 100 paired CS-US trials (”Acquisition-1”). Paired trials had an average ITI of 30 s (range: 15–45 s), and every 10th trial of paired training was a CS-alone probe trial (i.e., 10 probe trials/100-trial session). On days 3–9, rats received paired CS-US training with 100 trials each day (“Acquisition-2” to “Acquisition-8”) to establish significantly high percentages of eyeblink CRs to the light CS. Based on previous studies, this amount of training is sufficient for rats to reach an asymptotic level of CR production. On day 10, rats received 80 paired CS-US presentations (“Overtraining”), followed by 80 trials of extinction training with unpaired presentations of the CS and US (“Extinction-1”). On day 11, rats were given additional trials of extinction training (“Extinction-2”), followed immediately by 100 paired trials (“Reacquisition-1”), and on day 12, rats received 80 paired trials (“Reacquisition-2”), followed by extinction training with 80 unpaired CS-US presentations (“Re-extinction”). In summary, training consisted of day 1: pre-exposure → pseudoconditioning-1; day 2: pseudoconditioning-2 → acquisition-1; days 3–9: acquisition-2 to acquisition-8; day 10: overtraining → extinction-1; day 11: extinction-2 → reacquisition-1; and day 12: reacquisition-2 → re-extinction.

The number of trials presented during different sessions was chosen based on different factors. The minimum possible number of trials was used to minimize the number of times that a rat was exposed to the US. At the same time, we ensured enough numbers of trials to induce significant learning and CR production to generate enough numbers of trials for analysis. The number of unpaired trials was generally chosen to be lower compared with paired trials. This decision took into account the CS-alone probe trials, which were given during the CS-US paired training. Furthermore, the presentation of a large number of unpaired CS-US trials can risk producing...
significant learned irrelevance to a CS, which slows down the rate of learning during subsequent paired training with that CS (Overmier 2002; Overmier and Seligman 1967). Pilot results also found that it took more paired trials for rats to develop CRs and learning-related modifications of LGNv neural activity than unpaired trials to develop neuronal habituation and extinguishment of CRs.

The training design of the present experiment allowed us to correlate changes in the LGNv neuronal activity with behavior during different stages of eyelid conditioning (i.e., learning-related, neuronal-activity changes). These learning-related changes in neuronal activity can have different components. Changes in neuronal activity may occur: due to 1) the general effects of US exposure (such as sensitization), 2) acquired paired and unpaired CS/US associations, and/or 3) CR production (CR related). The comparison of LGNv neuronal activity during pre-exposure (CS only), unpaired CS/US, paired CS-US, and CR/no-CR trials allowed us to differentiate further learning-related neuronal activity into its different components. This design also allowed us to record the same neurons under different training conditions, which strengthened the power of our analyses.

The acquisition-1 session was important for investigating the development of learning-related neuronal activity before the appearance of CRs. With the start of the acquisition-2 session, some rats showed a significant percentage of CRs. Rats learn eyelid conditioning at different rates; therefore, on any single acquisition day, they might be at different levels of learning and CR performance. To correlate the neuronal data and CR production, this heterogeneity of speed and stage of learning should be taken into account. Behaviorally defined criteria were used to select appropriate sessions to be able to correlate the development of learning-related neuronal activity to the CR production. The first 25 CR trials on the first session that rats made at least 25 CRs, plus the first 25 no-CR trials on that same session, were placed in a group named “Acquisition-early” sessions. This group was used to investigate the relationship between LGNv neuronal activity and CR production when rats reached the asymptotic level of performance.

The analysis of the extinction, reacquisition, and re-extinction trials had the advantage of examining the LGNv neuronal activity during both paired CS-US and unpaired CS/US conditions, while rats produced CRs under both conditions. The transition from paired to unpaired sessions (and vice versa) in well-trained rats also provides the opportunity to observe changes in learning-related neuronal activity over a relatively short period of time. Unlike naïve rats that require many training sessions for initial memory formation, well-trained rats rapidly retrieve previously acquired memories.

To simplify the analyses, reacquisition sessions and extinction and re-extinction sessions were placed in two groups named “Reacquisition-avg” and “Extinction-avg” sessions, respectively.

Neuronal Recording and Single-Unit Sorting

Signals from each recording channel were passed through a headstage unity-gain preamplifier. The preamplifier output was then fed into programmable amplifiers, passing through a motorized commutator. This signal was band-pass filtered (600–6,000 Hz) and amplified at a gain of 10,000–20,000. The outputs of the amplifiers were digitized (32 kHz) and stored in a computer-controlled acquisition system when they passed the threshold using tetrode configuration (Neuralynx). An automatic spike-sorting software program (KlustaKwik; http://klustakwik.sourceforge.net) was used to isolate single units from multunit recordings. This software program identifies clusters of spikes based on the similarity of their waveform properties. Clusters were then inspected and further refined manually by an interactive software program (MClust; http://redshlab.neuroscience.unm.edu/MClust/MClust.html). It is assumed that spikes with similar properties originate from the same neuron. Neuronal data were analyzed relative to the onsets of the CS, US, and CR (NeuroExplorer; Nex Technologies, Madison, AL).

Eyelid EMG and Neuronal Recording Analyses

The EMG signal, recorded through Neuralynx, was rectified and divided into 2.5-ms bins. This signal was used to detect the CR-onset latency. To do that, the running average of the EMG activity in the CS time window was compared with the baseline average for each
individual trial. If the difference was at least four SDs higher than the baseline average, then it was marked as the start of eyelid movement (i.e., CR onset). Any eyelid movement that occurred during the first 80 ms of the CS duration was considered an alpha (startle) response and was disregarded from the analysis. Eyelid EMG activity was also visually inspected for each trial to verify the accuracy of CR onset and was manually determined if the automatic algorithm failed to detect the CR onset accurately (e.g., due to noise).

Peristimulus time histograms (PSTHs) of LGNv single-unit activity were generated relative to the onset of the CS. Each PSTH had a 112.5-ms pre-CS baseline and 450-ms CS time period (i.e., the 450-ms ISI for paired CS-US trials). The durations of the pre-CS and CS time periods were divided into 11.25-ms bins, producing 10 pre-CS bins and 40 CS bins. The PSTH for each single unit shows that unit’s firing rate (spikes/s) during each bin (i.e., bin value). These pre-CS and CS bins were each grouped into 10-bin intervals, creating one pre-CS interval and four CS intervals. For each single unit, the bin values for each of the four CS time intervals were compared separately with the bin values for the pre-CS time interval using Wilcoxon signed-rank tests (Campolattaro et al. 2011; Freeman and Nicholson 1999; Halverson et al. 2010; Kubota et al. 1996), and significant differences were determined at \( P < 0.05 \). Based on the Wilcoxon signed-rank test results, the activity of each of the four CS intervals was characterized as higher (excited), lower (inhibited), or the same as the baseline recording (unresponsive). Single units that had higher activity relative to its baseline, in at least one of the CS time intervals, were categorized as excited neurons. If activity in at least one CS time interval was lower than its baseline, then the single unit was categorized as inhibited neurons. Single units were categorized as unresponsive neurons if none of the four CS time intervals was different than baseline. Very few single units showed a mixture of intervals with higher and lower activity than baseline. These single units were not included in the analyses.

Additionally, CS-elicited activity for each single unit was normalized to compare response magnitude across LGNv neurons, irrespective of their individual level of baseline activity. Normalized response values were calculated by subtracting the average of pre-CS baseline bin values from each CS bin value and dividing that difference by the SD of the pre-CS baseline bin values (Freeman and Nicholson 1999). To simplify the analyses, CS bins were grouped into eight blocks of five bins. Each block value was equal to the sum of the normalized bin values for that block.

Learning-related neuronal activity modifications can be in the form of the following: 1) changes in the recruitment of neurons due to training and/or 2) changes in the response magnitude of these recruited neurons. To detect the first type of neuronal changes, the number of neurons that showed excited activity at each interval (independent of the activity of these neurons during other intervals) was divided by the total number of recorded neurons (regardless of their activity type) during that interval. This calculation resulted in the percentages of neurons that showed excited activity at each CS interval (one to four) for different stages of training. Similar analyses were also performed for the neurons that showed inhibited activity. To detect the second type of neuronal activity changes (i.e., changes in the magnitude of neuronal responses), analysis was performed on the averaged, normalized PSTHs for different categories of neurons (i.e., excited and inhibited) during different stages of training.

Furthermore, periresponse time histograms (PRTHs) were generated to determine how LGNv neuronal activity relates to CR production. These PRTHs included single-unit activity during the 200-ms time periods before and after CR onset, which was normalized to the unit’s pre-CS baseline period of its corresponding PSTHs. Normalization was performed using the same bin and block size values described above for PSTHs.

**Statistical Analysis**

\[ \chi^2 \] Tests were performed to compare the percentages of neurons with increased activity (i.e., excited neurons) across the different sessions and training conditions (paired vs. unpaired) with trials separated by response type (CR vs. no-CR). These \( \chi^2 \) tests were done separately for each of the four CS time intervals (one to four). The same analyses were performed for the percentages of neurons with decreased activity (i.e., inhibited neurons).

Linear mixed models (LMMs) with repeated measures were used to analyze behavioral and normalized neural (PSTHs and PRTHs) data (SAS 9.2; SAS Institute, Cary, NC). Analyses included different factors, such as session (levels: pre-exposure, pseudoconditioning-1, pseudoconditioning-2, acquisition-1, acquisition-early, acquisition-criterion, overtraining, extinction-1, extinction-2, reacquisition-1, reacquisition-2, re-extinction, extinction-avg, reacquisition-avg); CR (levels: CR, no-CR), pairing (levels: paired, unpaired), and block (levels: one through eight). For the statistical analysis of the neuronal data, “subjects” were individual neurons. If the spike form of a single unit remained stable during the sessions on the same day, then that single unit was considered as the same neuron and was treated as one subject. Therefore, sessions on the same day were considered as different levels of the within-subjects factor of session. Neurons recorded during different days were treated as different subjects; therefore, sessions on different days were considered as different levels of the between-subjects factor of session. The Tukey-Kramer procedure and step-down Bonferroni correction were used, where appropriate, to correct for the family-wise error rate. A linear trend was tested for some of the analyses. This statistical procedure tests whether a line with a nonzero slope is able to be fitted on the values of consecutive data points. An increasing linear trend means the slope of the fitted line is positive, and a decreasing linear trend means the slope of the fitted line is negative.

**Tetrode Placement**

At the end of training, each rat was placed under anesthesia, and tetrode-ending positions were marked with small lesions made by passing 10 \( \mu \)A of DC for 10 s through one of the channels in each tetrode. The rat was deeply anesthetized the next day with an overdose of sodium pentobarbital (90 mg/kg) and transectarily perfused with 0.9% PBS, followed by 4% buffered formalin. The brain was removed from the skull and postfixed in 30% sucrose-formalin and subsequently, sectioned at 50 \( \mu \)m on a sliding microtome (American Optical, Buffalo, NY). All sections were mounted on slides, stained with thionin, and examined for tetrode-tips placement. Only recordings in the LGNv and intergeniculate leaflet were included in the analyses.

**RESULTS**

**Behavioral Results**

Behavioral data were analyzed for pre-exposure, pseudoconditioning-1, pseudoconditioning-2, acquisition-1 to acquisition-8, acquisition-criterion, overtraining, extinction-1, extinction-2, reacquisition-1, reacquisition-2, re-extinction, extinction-avg, and reacquisition-avg sessions. Percentage of CRs for all sessions is shown in Fig. 2. Statistical analysis using session as a within-subjects factor was significant \([F_{(19, 99)}] = 13.33, P < 0.001\). Rats showed low levels of blinking in response to the CS during the pre-exposure, pseudoconditioning-1, pseudoconditioning-2, and acquisition-1 sessions. CR% increased during paired training and was significantly higher during the acquisition-3 through the acquisition-8 sessions compared with the pseudoconditioning-2 session (\( P < 0.020 \)).
Behavioral Results

Rats approached an asymptotic level of CR performance during the overtraining session (~90%). CR% dropped when rats received extinction training. Nevertheless, CR percentages for the extinction sessions were significantly higher than the pseudoconditioning-2 session (P < 0.026), except for the re-extinction session, which was close to significantly higher than the pseudoconditioning-2 session (P < 0.072). CR% increased when the rats resumed paired training during the reacquisition sessions and again approached asymptote during the reacquisition-2 session. There was no significant difference in CR% between the reacquisition-2 and overtraining sessions. The reacquisition-2 and overtraining sessions showed a significantly higher CR% than the reacquisition-1 and extinction sessions (P < 0.052). There was no significant difference in CR% between the extinction sessions or among the extinction and reacquisition-1 sessions.

Table 1. Number of excited neurons and percentages of neurons that showed excited responses during different CS intervals in each session, separated for CR, no-CR trials (PSTHs)

<table>
<thead>
<tr>
<th>Session</th>
<th>n</th>
<th>Interval 1, %</th>
<th>Interval 2, %</th>
<th>Interval 3, %</th>
<th>Interval 4, %</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exposure</td>
<td>22</td>
<td>15.9</td>
<td>36.4</td>
<td>25.0</td>
<td>27.3</td>
<td>44</td>
</tr>
<tr>
<td>Pseudoconditioning-1</td>
<td>21</td>
<td>22.7</td>
<td>40.9</td>
<td>40.9</td>
<td>27.3</td>
<td>44</td>
</tr>
<tr>
<td>Pseudoconditioning-2</td>
<td>31</td>
<td>13.2</td>
<td>26.0</td>
<td>35.7</td>
<td>35.7</td>
<td>84</td>
</tr>
<tr>
<td>Acquisition-1</td>
<td>27</td>
<td>11.5</td>
<td>21.3</td>
<td>18.9</td>
<td>37.6</td>
<td>61</td>
</tr>
<tr>
<td>Acquisition-early/CR</td>
<td>34</td>
<td>18.9</td>
<td>34.0</td>
<td>35.7</td>
<td>37.6</td>
<td>80</td>
</tr>
<tr>
<td>Acquisition-early/no-CR</td>
<td>34</td>
<td>18.9</td>
<td>34.0</td>
<td>35.7</td>
<td>37.6</td>
<td>80</td>
</tr>
<tr>
<td>Acquisition-criterion/CR</td>
<td>43</td>
<td>16.7</td>
<td>39.3</td>
<td>35.7</td>
<td>35.7</td>
<td>84</td>
</tr>
<tr>
<td>Acquisition-criterion/no-CR</td>
<td>42</td>
<td>13.1</td>
<td>29.8</td>
<td>21.4</td>
<td>20.2</td>
<td>84</td>
</tr>
<tr>
<td>Overtraining</td>
<td>26</td>
<td>10.3</td>
<td>38.5</td>
<td>35.9</td>
<td>17.9</td>
<td>39</td>
</tr>
<tr>
<td>Extinction-avg/CR</td>
<td>46</td>
<td>9.9</td>
<td>17.8</td>
<td>23.8</td>
<td>18.8</td>
<td>101</td>
</tr>
<tr>
<td>Extinction-avg/no-CR</td>
<td>47</td>
<td>12.2</td>
<td>28.0</td>
<td>20.7</td>
<td>12.2</td>
<td>82</td>
</tr>
<tr>
<td>Reacquisition-avg/CR</td>
<td>40</td>
<td>16.4</td>
<td>47.3</td>
<td>49.1</td>
<td>30.9</td>
<td>55</td>
</tr>
<tr>
<td>Reacquisition-avg/no-CR</td>
<td>17</td>
<td>10.1</td>
<td>22.0</td>
<td>20.3</td>
<td>22.0</td>
<td>59</td>
</tr>
</tbody>
</table>

CS, conditioned stimulus; CR, conditioned response; PSTHs, peristimulus time histograms; n, number of excited neurons recorded during each session; N, total number of neurons (regardless of category of response) recorded during each session.

Neuronal Results

Neural data were analyzed for pre-exposure, pseudoconditioning-1, pseudoconditioning-2, acquisition-1, acquisition-early, acquisition-criterion, overtraining, extinction-1, extinction-2, reacquisition-2, reacquisition-1, extinction-avg, reacquisition-avg, and extinction-avg sessions. Rats reached an asymptotic CR percentage during the overtraining session, leaving too few no-CR trials for a complete analysis of those trials. Therefore, only CR trials were analyzed for this session. Preliminary analyses of neuronal data revealed no major differences between reacquisition sessions or among extinction and re-extinction sessions. The data for reacquisition and extinction/re-extinction sessions were therefore grouped into the reacquisition-avg and extinction-avg sessions, respectively. Additional behaviorally defined sessions were created for the acquisition sessions (i.e., acquisition-early and acquisition-criterion sessions; see MATERIALS AND METHODS for details). Neuronal data are reported for these sessions.

Activity from 322 LGNv neurons was analyzed in this experiment; 200 (62%) showed excitation, 51 (16%) showed inhibition, and 71 (22%) were unresponsive to the CS. Similar patterns of LGNv neuronal activity have been detected in other studies [e.g., Sumitomo et al. (1979)]. The number of excited and inhibited neurons recorded during the different training sessions is shown in Tables 1 and 2. Figure 3 shows the

Fig. 2: A: mean (±SE) eyeblink conditioned response (CR) percentage for different training sessions. B: mean (±SE) CR percentages calculated for different session types. "Acquisition-criterion": average CR percentage obtained on the 1st session of paired training when rats produced CRs on at least 50% of trials; "Extinction-avg": average CR percentage during extinction-1, extinction-2, and re-extinction sessions; "Reacquisition-avg": average CR percentage during reacquisition-1 and reacquisition-2 sessions.
position of tetrode tips in the LGNv, where neural activity was recorded. The average baseline firing rates for excited, inhibited, and unresponsive neurons over all of the sessions were 12.3, 11.7, and 7.8 spikes/s, respectively. Figure 4 shows examples of excited, inhibited, and unresponsive neurons. LGNv activity was examined at different stages of training, separated into CR and no-CR trial types. Complete neuronal analyses were performed on data obtained only from excited, single-unit recordings.

Table 2. Number of inhibited neurons and percentages of neurons that showed inhibited responses during different CS intervals grouped in paired (acquisition-criterion, overtraining, and reacquisition-avg sessions) and unpaired (extinction-avg) sessions, separated for CR, no-CR trials (PSTHs)

<table>
<thead>
<tr>
<th>Session</th>
<th>n</th>
<th>Interval 1, %</th>
<th>Interval 2, %</th>
<th>Interval 3, %</th>
<th>Interval 4, %</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired/CR</td>
<td>27</td>
<td>1.7</td>
<td>6.4</td>
<td>4.1</td>
<td>4.7</td>
<td>172</td>
</tr>
<tr>
<td>Paired/no-CR</td>
<td>16</td>
<td>1.8</td>
<td>5.4</td>
<td>5.4</td>
<td>3.6</td>
<td>111</td>
</tr>
<tr>
<td>Unpaired/CR</td>
<td>15</td>
<td>2.4</td>
<td>3.6</td>
<td>7.2</td>
<td>3.6</td>
<td>83</td>
</tr>
<tr>
<td>Unpaired/no-CR</td>
<td>21</td>
<td>4.5</td>
<td>9.0</td>
<td>4.5</td>
<td>4.5</td>
<td>67</td>
</tr>
</tbody>
</table>

n, number of inhibited neurons recorded during each session type; N, same as Table 1.

Percentage of neurons that showed stimulus-related, excited responses during the pre-exposure, pseudoconditioning-1, pseudoconditioning-2, acquisition-1, and acquisition-criterion sessions. These analyses were performed to compare the percentages of neurons that showed CS-elicited, excited responses during CS-only, unpaired CS/US, and paired CS-US training. These comparisons allowed us to detect how LGNv neuronal responses modified as training started, and the CS-US relationship changed across the different stages of training. The per-

Fig. 3. Left: Nissl-stained coronal sections of the thalamus showing examples of tetrode placements in the LGNv. Arrows point to the location of tetrode tips. Right: coronal views showing where in the LGNv tetrode recordings obtained activity for units categorized as excited (green circles), inhibited (red circles), and unresponsive (black circles). LGNd, dorsal lateral geniculate nucleus; IGL, intergeniculate leaflet.
Percentage of excited neurons decreased during unpaired training (i.e., neural habituation) and subsequently increased during paired training, primarily when CRs were produced (i.e., recovery from habituation).

There was no significant difference in the percentage of excited neurons between the pre-exposure (CS only) and pseudoconditioning-1 (unpaired CS/US) sessions, indicating that the introduction of the US to the training context had no significant effect on CS-elicited neuronal responses in the LGNv (Fig. 5A). Fewer excited neurons were found during the pseudoconditioning-2 session than the pseudoconditioning-1 session ($P < 0.050$; Fig. 5A). This finding shows that the LGNv neuronal response to the CS habituated as unpaired CS/US training continued. LGNv neuronal activity recovered from this habituation when paired CS-US training started, and rats reached significant levels of CR performance. This conclusion was based on the finding that more excited neurons were found during the acquisition-criterion/CR trials compared with the pseudoconditioning-2 session ($P < 0.021$; Fig. 5A vs. B). Some recovery from habituation was also found during the acquisition-1 session and the acquisition-criterion/no-CR trials. These conclusions were based on the observation that more
excited neurons were found only for the third time interval of the pseudoconditioning-1 session compared with the acquisition-1 session (41% vs. 14.8%; \( P < 0.002 \), and \( P < 0.019 \); Fig. 5A) and acquisition-criterion/no-CR trials (41% vs. 21%; \( P < 0.019 \)); Fig. 5, A vs. B), whereas the pseudoconditioning-1 session showed a higher number of excited neurons during both the second (41% vs. 21%) and third (41% vs. 13%) intervals (\( P < 0.050 \); Fig. 5A) compared with the pseudoconditioning-2 session. However, this level of recovery from habituation was not large enough for the acquisition-1 session and acquisition-criterion/no-CR trials to become significantly different from the pseudoconditioning-2 session (Table 1). The largest recovery from habituation was observed during the acquisition-criterion/CR trials. There was no significant difference in LGNv responses between the pseudoconditioning-1 session and acquisition-criterion/CR trials. These findings suggest that the main effect of paired CS-US training was recovery from habituation that developed during continued unpaired training (i.e., the pseudoconditioning-2 session) and not an increase in the neuronal activity compared with the baseline unpaired CS/US training (pseudoconditioning-1 session) before neuronal habituation developed. The comparison of acquisition-criterion/CR with acquisition-criterion/no-CR trials is discussed next.

Percentage of neurons that showed stimulus-related, excited responses during the acquisition-criterion, overtraining, extinction-avg, and reacquisition-avg sessions. These analyses investigated the percentages of neurons that showed CS-elicited, excited responses during different stages of training (paired vs. unpaired) when rats produced frequent CRs. The goals of these analyses were to compare neuronal activity during the paired and unpaired training and CR and no-CR trials. The factors of pairing, session, and CR production were used in these analyses. Only CR trials were examined for the overtraining session, due to the low number of no-CR trials. When combined, paired (CS-US) sessions (i.e., the acquisition-criterion, overtraining, and reacquisition-avg sessions) had a higher percentage of excited neurons compared with unpaired (CS/US) sessions (i.e., the extinction-avg) when a CR was produced (\( P < 0.035 \)), but no significant difference was observed when a CR was not produced (Fig. 5, B and C). Moreover, these analyses showed a higher percentage of excited neurons during acquisition-criterion CR trials compared with no-CR trials (\( P < 0.040 \); Fig. 5B). These results showed

![Graph A](http://jn.physiology.org/)

**A**

Pre-exposure (N: 44)

Pseudoconditioning-1 (N: 44)

Pseudoconditioning-2 (N: 61)

Acquisition-1 (N: 61)

![Graph B](http://jn.physiology.org/)

Acquisition-early/CR (N: 53)

Acquisition-early/no-CR (N: 53)

Acquisition-criterion/CR (N: 84)

Acquisition-criterion/no-CR (N: 84)

![Graph C](http://jn.physiology.org/)

Overtraining/CR (N: 39)

Extinction-avg/CR (N: 101)

Extinction-avg/no-CR (N: 82)

Reacquisition-avg/CR (N: 55)

Reacquisition-avg/no-CR (N: 59)

Fig. 5. Percentage of neurons that exhibited significant excited responses compared with the baseline during CS intervals for different stages of training. **A**: habituation developed when unpaired training continued (pseudoconditioning-1 > pseudoconditioning-2). Some recovery from this habituation was observed when paired CS-US training started (acquisition-1). **B**: the percentages of neurons showing excited activity increased to the prehabituation level (pseudoconditioning-1) during paired trials when rats produced frequent CRs (acquisition-criterion). **C**: the LGNv activity habituated again and then recovered from habituation when rats received extinction and reacquisition training, respectively. Notice that overtraining activity was significantly higher than the habituated level (pseudoconditioning-2). However, normalized neuronal responses of excited neurons during overtraining were not significantly different than the pseudoconditioning-2 (see Fig. 6, A and C), which shows a dissociation between the measure of the percentage of excited neurons and the measure of the magnitude of normalized response of excited neurons during overtraining. In all of the other sessions, these 2 different measures of neuronal activity changed in the same direction. "Acquisition-early" sessions: data combined for the 1st 25 CR and 25 no-CR trials from the 1st session of paired training when rats made at least 25 CRs. White symbols: sessions when rats received either CS-only or unpaired CS/US training; black symbols: sessions when rats received paired CS-US training; red lines (B and C): CR trials; black lines (B and C): no-CR trials. N, total number of neurons (regardless of category of response) recorded during each session.
that learning-related changes developed in LGNv neuronal activity. CS-elicited LGNv neuronal activity increased during paired trials when rats produced a CR in anticipation of a US. This neuronal response decreased when the CS no longer predicted the US (i.e., during unpaired extinction training), even though rats continued to produce CRs.

Higher percentages of excited neurons were found during reacquisition-avg/CR trials compared with extinction-avg/CR trials ($P < 0.001$; Fig. 5B). Higher percentages of excited neurons were also found during reacquisition-avg/CR trials compared with reacquisition-avg/no-CR trials ($P < 0.005$). There were no significant differences in neural responding between reacquisition-avg/no-CR trials and extinction-avg/no-CR trials. There was also no significant difference between extinction-avg/CR trials and extinction-avg/no-CR trials. Therefore, higher percentages of excited neurons were observed on CR trials only during paired training.

Percentage of excited neurons for overtraining/CR trials and extinction-avg/CR trials was compared with the pseudoconditioning-2 session to determine if any learning-related neuronal activity occurred during these sessions. Higher percentages of excited neurons were found during overtraining/CR trials compared with pseudoconditioning-2 trials. However, the level of LGNv neuronal responding did not differ between the extinction trials and unpaired pretraining trials when the LGNv neuronal activity was normalized (i.e., pseudoconditioning-2 session; Fig. 5A and C).

Normalized, stimulus-related activity of excited neurons during the pre-exposure, pseudoconditioning-1, pseudoconditioning-2, acquisition-1, and acquisition-criterion sessions. These analyses compared the magnitude of CS-elicited LGNv neuronal activity obtained from excited neurons during CS-only, unpaired CS/US, and paired CS/US training. The comparison of neuronal responses, obtained during pre-exposure, unpaired, and paired CS/US training, allowed us to differentiate CS-elicited LGNv activity, due to nonassociative processes (e.g., sensitization induced by US exposure), from CS-elicited LGNv activity, due to associative learning (i.e., acquiring the CS-US-associative relationship). Furthermore, trials during the acquisition-criterion sessions were divided into CR and no-CR trial types (the acquisition-criterion/CR and acquisition-criterion/no-CR trials, respectively) to determine if learning-related changes in the LGNv neuronal activity, due to paired training, would occur exclusively when rats produced CRs. Neuronal habituation was observed during the second session of unpaired CS/US training (i.e., the pseudoconditioning-2 session). LGNv neuronal activity recovered from this habituation after paired CS/US training began. Moreover, this learning-related increase in neuronal activity was mainly observed during paired CR trials (Fig. 6A and B).

These conclusions were based on the following analyses that tested specific individual hypotheses. For performing these analyses, LMMs, with the factors of session and block, were used. Session was used as a within-subjects factor for analyzing sessions given on the same day and as a between-subjects factor for analyzing sessions given on different days. It was assumed that the same neurons were recorded during sessions on the same day, and different neurons were recorded during sessions on different days. Block was always used as a within-subjects factor. Moreover, acquisition-criterion trials were analyzed separately based on CR production.

To test the effect of introducing unpaired US on the LGNv neuronal response to the CS, pre-exposure and pseudoconditioning-1 sessions were compared. This analysis did not show any significant differences between these two sessions. The pseudoconditioning-2 session showed lower neuronal activity compared with the pseudoconditioning-1 session over the average of all blocks [$F_{(4, 50)} = 3.89, P < 0.055$; Fig. 6A], indicating that LGNv activity habituated as unpaired training continued. The interaction of session and block was not significant. The testing of the effect of block at different levels of session revealed that a close to significantly larger neuronal response occurred during the fifth block of the pseudoconditioning-1 session ($P < 0.058$). Analyses did not reveal any significant differences in neuronal activity between the acquisition-1 and pseudoconditioning-1 sessions or between the acquisition-1 and pseudoconditioning-2 sessions. These findings indicate that the initial paired training resulted in some modest recovery from habituation but not enough to make a significant difference when the acquisition-1 session was compared with the pseudoconditioning-2 session. Neuronal activity during acquisition-criterion/CR trials was not significantly different from the neuronal activity during the pseudoconditioning-1 session, indicating that paired CS-US training only resulted in recovery from habituation and not an increase in the magnitude of the LGNv neuronal response compared with the baseline unpaired CS/US training before habituation developed (i.e., the pseudoconditioning-1 session). Neuronal activity during acquisition-criterion/CR trials was significantly greater than the pseudoconditioning-2 session over the average of all blocks [$F_{(4, 75.9)} = 6.49, P < 0.013$]. The session and block interaction was also significant [$F_{(7, 145)} = 2.25, P < 0.034$]. The examination of activity during acquisition-criterion/CR trials vs. the pseudoconditioning-2 session at different levels of block revealed that activity during acquisition-criterion/CR trials was significantly greater than during the pseudoconditioning-2 session in blocks 5–8 ($P < 0.030$). Acquisition-criterion/CR trial activity was not significantly different from the acquisition-1 session over the average of all blocks, but the session and block interaction was significant [$F_{(7, 141)} = 2.18, P < 0.040$]. Tests of the interaction revealed that the acquisition-criterion/CR session had greater neuronal activity during the seventh block relative to the acquisition-1 session ($P < 0.012$). Acquisition-criterion/no-CR trial activity was not significantly different from the pseudoconditioning-1 or pseudoconditioning-2 sessions, indicating that the main effect of paired training manifested during the trials in which a CR was produced, although these results show some recovery from the habituation that occurred during no-CR trials. These findings suggest that habituation of the LGNv neuronal response developed during continued unpaired training. Subsequently, LGNv recovered from this habituation as paired training began and when CRs were produced. In support of these findings, individual single-unit recordings were detected, which showed rapid habituation and subsequent recovery when the state of training changed. This recovery was persistent and resulted in higher neuronal responding when the rats transitioned from unpaired to paired training (Fig. 7).
Normalized, stimulus-related activity of excited neurons during the acquisition-early sessions: comparison of CR and no-CR trials. To investigate further the development of learning-related neuronal changes as paired CS-US training sessions progressed, the first 25 trials on the first session when rats made at least 25 CRs (acquisition-early/CR) were compared with the first 25 no-CR trials on the same session (acquisition-early/no-CR). There were no significant differences in the magnitude of the LGNv activity between these CR and no-CR trial types (Fig. 6B); however, CR-related neuronal changes in LGNv activity developed during subsequent paired training sessions and when rats produced high percentages of CRs (described next).

Normalized, stimulus-related activity of excited neurons during the acquisition-criterion sessions: comparison of CR and no-CR trials. This analysis investigated the relationship between CR production and LGNv neuronal-response magnitude during the acquisition-criterion sessions. The acquisition-criterion group consisted of the sessions when rats first produced CRs on at least 50% of trials. Neuronal activity on CR trials was compared with no-CR trials during the acquisition-criterion sessions, using a within-subjects design. Results showed that neuronal activity during CR trials was significantly greater than no-CR trials, especially during the second half of the CS time window, which shows that these learning-related neuronal changes were mostly related to CR production (i.e., CR related), rather than the result of changes in the early CS-evoked sensory response.

The above-mentioned conclusions were based on the LMM analysis with within-subjects factors of CR and block. The main factor of CR was significant in this analysis \([F(1, 43.1) = 7.21, P < 0.011]\). The interaction of CR and block was not significant \([F(7, 201) = 0.96, P < 0.465]\). However, when the effect of CR was examined at different blocks, while \(P\) values adjusted by Bonferroni’s method, significantly more neuronal responding was shown during CR trials compared with no-CR trials in blocks during the second half of the CS (i.e., blocks 5–8; \(P < 0.024\); Fig. 6B). This finding supports the conclusion that in spite of a nonsignificant CR and block interaction, the effect of CR was larger during the second half of the CS window. Therefore, it can be concluded that the learning-related neuronal activity habituated with continued unpaired training when rats were expressing frequent CRs (i.e., acquisition-criterion/CR > pseudoconditioning-2). Habituation was observed again when rats reached asymptotic CR performance, as overtraining/CR was not significantly different than pseudoconditioning-2. This habituation trend was again substantially reversed when rats received reacquisition training (see Fig. 9). Symbols and line colors are as in Fig. 5. n, number of excited neurons recorded during each session.

Fig. 6. Averaged, normalized CS time histograms (±SE) for excited neurons during pre-exposure, pseudoconditioning, acquisition, and overtraining. A: CS-elicited neuronal activity during the pseudoconditioning-1 session, when averaged over all blocks, was significantly greater than the pseudoconditioning-2 session. This finding shows that continued unpaired training resulted in habituation of LGNv neurons. Some recovery from habituation was observed when paired training started during the acquisition-1 session. B: magnitude of the neuronal activity between acquisition-early/CR and no-CR trials did not differ; however, neuronal activity on CR trials was significantly greater than no-CR trials once rats reached learning criteria (acquisition-criterion). This learning-related neuronal activity was greatest during blocks 5–8, which are the same time periods when CRs are typically produced (i.e., CR related). C: summary of the main findings and overtraining. The magnitude of the LGNv neuronal activity habituated with continued unpaired training (i.e., pseudoconditioning-1 > pseudoconditioning-2). LGNv single units recovered from habituation during subsequent paired training when rats were expressing frequent CRs (i.e., acquisition-criterion/CR > pseudoconditioning-2). Habituation was observed again when rats reached asymptotic CR performance, as overtraining/CR was not significantly different than pseudoconditioning-2. This habituation trend was again substantially reversed when rats received reacquisition training (see Fig. 9). Symbols and line colors are as in Fig. 5. n, number of excited neurons recorded during each session.
related activity, which was observed during the CR trials, compared with the no-CR trials, was CR related rather than related to the CS-evoked sensory response. Figure 8 shows examples of neurons with greater neuronal response during CR than no-CR trials.

Normalized, stimulus-related activity of excited neurons during the acquisition-criterion/CR, overtraining/CR, and reacquisition-avg/CR. These analyses investigated changes in CS-elicited LGNv neuronal responses on CR trials during the acquisition-criterion, overtraining, and reacquisition-avg sessions. Rats received only paired training in these sessions, and only CR trials were analyzed; therefore, any difference in the LGNv neuronal response to the CS was related to the amount of training. Results showed that the amplitude of the neuronal activity was larger on acquisition-criterion/CR and reacquisition-avg/CR trials compared with overtraining/CR trials. These findings indicate that the magnitude of the LGNv neuronal response gradually developed habituation as paired training

Fig. 7. Examples of PSTHs (spikes/s) and raster plots of LGNv activity on days 1 and 2 of training. PSTHs were generated using activity recorded during the 1st and 2nd half of each session. The 2 top rows (neurons A and B) are examples of neurons that exhibited rapid habituation between the 1st and 2nd halves of pre-exposure (CS-alone training) and then recovered from habituation during the 1st half of the following pseudoconditioning-1 session (unpaired CS/US training). Some habituation recurred between the 1st and 2nd halves of pseudoconditioning-1. The 2 bottom rows are examples of neurons (C and D) that showed some recovery from habituation acquired during pseudoconditioning-2 (i.e., the 2nd unpaired CS/US session) when rats completed acquisition-1 (i.e., the 1st session of paired CS-US training). Arrows indicate that transitions between different sessions occurred on the same day. The vertical lines at 0 s mark the CS onset.
continued, and rats approached asymptotic levels of CR performance. This habituation trend reversed when rats received extinction training, followed by additional paired training (Figs. 6, B and C, and 9).

LMM analysis was performed using session as between- and block as within-subjects factors. With the comparison of the acquisition-criterion and overtraining sessions, the factor of session was not significant \( F(1, 74.4) = 2.91, P = 0.092 \). However, the interaction of session by block was significant \( F(1, 135) = 2.16, P = 0.042 \). The testing of the effect of session at different blocks showed that the neuronal activity during the end of the CS was significantly larger in the acquisition-criterion sessions compared with the overtraining session \( (P = 0.004; \text{Fig. 6C}) \). There were no significant differences when acquisition-criterion and reacquisition-avg sessions were compared. Reacquisition-avg sessions showed a larger magnitude neuronal response compared with the overtraining session \( [F(1, 79) = 4.14, P = 0.045] \). The interaction of session and block factors was not significant.

To investigate the degree of neuronal habituation during the overtraining session, the magnitude of neuronal response during this session was compared with the pseudoconditioning-2 session. The interaction of session and block was significant \( [F(7, 98.2) = 2.19, P = 0.042] \), but individual comparisons did not reveal any significant differences between these two sessions (Fig. 6, A and C).

Normalized, stimulus-related activity of excited neurons during the extinction-avg and reacquisition-avg sessions. This analysis investigated changes in the magnitude of learning-related neuronal activity in well-trained rats, as they transitioned from paired to unpaired training and vice versa. A larger neuronal response occurred on CR trials compared with no-CR trials only for paired trials. Analysis was also performed to compare the amplitude of the LGNv neuronal response on CR trials between paired and unpaired sessions. Results showed that on average, the amplitude of the neuronal activity was larger on paired CR trials compared with unpaired CR trials only for reacquisition-avg sessions compared with the extinction-avg sessions. The interaction of session and block was significant \( [F(7, 98.2) = 2.19, P < 0.042] \), but individual comparisons did not reveal any significant differences between these two sessions (Fig. 6, A and C).

Fig. 8. PSTHs (spikes/s) with raster plots for 2 different excited neurons showing greater LGNv activity during CR trials (left) compared with their activity on no-CR trials (right) during acquisition sessions. Black triangles: CR onset for each trial in the raster plots. The vertical lines at 0 s mark the CS onset.

Fig. 9. Averaged, normalized CS time histograms (±SE) for excited neurons during extinction-avg and reacquisition-avg sessions. Neuronal activity during the reacquisition-avg trials, in which a CR was produced, on average, was greater than no-CR trials. There was no difference between neuronal activity during CR and no-CR trials for the extinction-avg sessions. Neuronal activity during trials in which CR was produced was, on average, greater for reacquisition-avg sessions than during the extinction-avg sessions. Findings indicate that learning-related neuronal activity developed during the paired CS-US trials when CR was produced. White symbols: sessions when rats received unpaired CS/US training; black symbols: sessions when rats received paired CS-US training; red lines: CR trials; black lines: no-CR trials. n, number of excited neurons recorded during each session.
LMM analysis was performed between extinction-avg and reacquisition-avg sessions using session, CR, and block as within-subjects factors. The factor of CR was significant \(F_{1, 112} = 13.16, P < 0.001\); the interaction of session and CR was also significant \(F_{3, 119} = 3.90, P < 0.051\), but the interaction of session, CR, and block factors was not significant. On average, there was significantly greater neuronal activity on CR trials compared with no-CR trials during the reacquisition-avg sessions \(P < 0.003\). However, there were no significant differences between CR and no-CR trials during the extinction-avg sessions. When the effect of CR was tested at different levels of block for the reacquisition-avg sessions, the second block was significantly larger during CR trials compared with no-CR trials \(P < 0.002\). Moreover, the amplitude of neuronal activity during the CR trials in reacquisition-avg sessions was, on average, larger than CR trials during extinction-avg sessions \(P < 0.051\). When the effect of CR was tested at different levels of block between reacquisition-avg and extinction-avg sessions, the neuronal activity during the reacquisition-avg session was larger than the extinction-avg session in the second block when CRs were produced \(P < 0.002\). Analysis of neuronal data did not show any significant differences between no-CR trials during the reacquisition-avg and extinction-avg sessions (Fig. 9). To investigate if any learning-related neuronal activity was evident during the extinction-avg/CR trials, the response magnitude during these trials was compared with the pseudoconditioning-2 session, which showed the highest neuronal habituation. No significant differences were found between these sessions (Figs. 6A and 9).

Normalized, response-related activity of excited and inhibited neurons during the acquisition-criterion, overtraining, extinction-avg, and reacquisition-avg sessions. To investigate further the relationship between the LGNv neuronal response and CR production, PRTHs were examined for peri-CR linear trends of LGNv neuronal activity. Pre-CR ramping of neuronal activity for the excited neurons was found in all sessions (Fig. 10A). Also, there was a post-CR-decreasing trend in the neuronal activity of excited neurons. That is, the magnitude of the LGNv neuronal activity increased before the CR onset and then decreased after the CR started. Inhibited neurons showed a pre-CR-decreasing trend in the neuronal activity that reached a post-CR plateau (Fig. 10B). The presence of peri-CR linear trends suggests that interactions exist between the LGNv and the neuronal circuitry responsible for CR production.

LMM analysis was performed on the normalized PRTHs of excited neurons, with session as between- and block as within-subjects factors. The factor of block was significant \(F_{1, 112} = 10.86, P < 0.001\). Peri-CR neuronal activity was evaluated for the presence of linear trends. The testing of the linear trend for the pre-CR neuronal activity for different sessions showed a significant increasing trend for all studied sessions \(P < 0.009\). A pre-CR-increasing linear trend indicates that neurons showed a positive ramping of activity before CR onset, which suggests a possible relationship with CR production. The linear trend for the post-CR neuronal activity showed a significant negative slope in all studied sessions \(P < 0.024\). That is, the magnitude of the LGNv neuronal activity decreased following CR onset. The linear trend for the complete peri-CR neuronal activity was not significant in any of the sessions.

Peri-CR linear trends were also tested for the inhibited neurons. There were few inhibited neurons; therefore, the paired and unpaired sessions were combined together, and LMM analysis was performed with pairing as a between- and block as a within-subjects factor. No significant differences were found between the two groups, which were therefore combined, and peri-CR trends were tested. A negative pre-CR linear trend was found \(P < 0.009\), which shows that the activity of LGNv-inhibited neurons decreased continuously before the CR onset. A post-CR trend was not detected. Also,
Comparison of changes in the percentages of neurons that showed excited responses during different CS intervals (neuronal recruitment) with changes in the averaged, normalized activity of excited neurons (magnitude of neuronal response). The range of changes was overall larger for the percentages of neurons that showed excited responses compared with the averaged, normalized responses of excited neurons (Figs. 5, 6, and 9). However, these measures of learning-related neuronal changes generally showed similar trends. That is, the magnitude of LGNv activity, revealed by averaged, normalized responses, tended to increase or decrease as the percentages of excited neurons increased or decreased, respectively. One exception was the overtraining session, where no difference was found in the magnitude of the averaged, normalized neuronal responses compared with the pseudoconditioning-2 session, even though the percentages of excited neurons were greater in this session compared with the pseudoconditioning-2 session (Figs. 5, A and C, and 6, A and C). The overtraining/CR and extinction-avg/CR trials were compared with test for similar dissociation between these two measures, and CRs were also expressed during unpaired training condition. There was, again, no difference in the magnitude of the normalized neuronal responses between these two sessions, but the percentages of excited neurons were significantly larger for the overtraining/CR trials ($P < 0.010$; Figs. 5C, 6C, and 9). Another unique property of the overtraining session was that the level of baseline neuronal activity during this session was significantly lower than the average level of baseline activity when all of the other sessions combined together ($P < 0.023$). These results indicate that there were a few differences in neuronal recruitment that were not reflected in changes in the average magnitude of neuronal activity. Thus the analyses of the percentage of neurons showing excitation and the normalized activity were both necessary to characterize fully learning-related changes in LGNv activity.

**DISCUSSION**

Learning-related modifications in LGNv neuronal activity were observed in rats given delay eyeblink conditioning with a visual CS. Neuronal activity in the LGNv habituated during unpaired CS/US pretraining and recovered from habituation after rats began paired CS-US training. This recovery from habituation was most prominent during trials when rats produced CRs (Fig. 6C). Our findings suggest that the LGNv may play a role in attention by maintaining a robust neuronal response to stimuli that predicts the US and attenuating the neuronal response to nonpredictive or otherwise irrelevant stimuli.

Brain connectivity data have not identified direct anatomical convergence of the CS and US sensory pathways in the LGNv; it is therefore not likely that the LGNv is capable of developing learning-related neuronal modifications through direct association of stimuli. However, there are indirect routes by which US information and information about the CS-US association can reach the LGNv. The LGNv receives inputs from brain stem-ascending neuromodulatory systems, which are activated by the US, including the ascending dorsal cholinergic bundle, locus coeruleus, and dorsal raphe nucleus (Brauer et al. 1984; Cosenza and Moore 1984; Fuxe and Jonsson 1974; Graybiel 1974; Luth and Brauer 1983; Papadopoulos and Parnavelas 1990). The combination of CS and neuromodulatory inputs to the LGNv might be sufficient to induce synaptic plasticity or increased excitability.

The cerebellum may also play a role in facilitating LGNv neuronal activity during eyeblink conditioning with a visual CS. The present experiment found that overall greater neuronal activity occurred in the LGNv during paired training trials with CRs relative to trials without CRs (Fig. 6B). This enhanced neuronal activity is likely CR related, because it was detected mostly during the second half of the CS time period, when CRs are produced. It needs to be emphasized that by “CR-related activity,” we are referring to the neuronal activity that is related to CR production and starts before CR performance. Furthermore, CR-related neuronal activity in the LGNv did not appear immediately when paired training first began; rather, it emerged later in training when rats were frequently expressing CRs. Therefore, LGNv CR-related neuronal activity appeared after learning-related plasticity had already developed in the cerebellum. A direct anatomical connection has been shown from the cerebellum to the LGNv (Zimny et al. 1986). The cerebellar output, which drives motoneurons to produce the eyeblink CR, may therefore provide feedback directly to the LGNv and be the origin of the CR-related activity in the LGNv. CR-related neuronal activity has been observed in various brain nuclei that have both afferent and efferent connections with the cerebellum. This CR-related neuronal response typically “ramps up” before the CR is expressed, which is the characteristic response profile of cerebellar interpositus neurons that drives the eyeblink CR (Bao et al. 2000; Campolattaro et al. 2011; Clark et al. 1997; Halverson et al. 2010). Clark et al. (1997) showed that CR-related neuronal activity in the PN could be blocked by cooling the interpositus nucleus, further strengthening the hypothesis that the CR-related activity observed in these brain structures during the eyeblink conditioning originates in the cerebellum. The present study also found ramping up of the LGNv pre-CR activity, as activity for excited LGNv neurons increased, and activity for inhibited LGNv neurons decreased, before CR onset (Fig. 10, A and B).

Cerebellar feedback to the LGNv may help direct selective attention to the visual CS during associative learning (Clark et al. 1997). Tracy et al. (2013) showed that a lower-intensity electrical-stimulation CS was needed to induce eyeblink conditioning when applied to PN regions where CR-related activity occurred compared with stimulation in PN regions that did not show CR-related activity. This finding suggests that PN CR-related activity, which is likely driven by cerebellar feedback, might enhance attention to the CS (Clark et al. 1997).

CR-related activity in other brain structures that receive cerebellar feedback (such as LGNv) might play a similar functional role in enhancing attention to the predictive CS. This simple model (Fig. 11) is consistent with the bidirectional relationship of attention and associative learning. For the animal to learn a CS-US association, it needs to pay attention to the CS. In turn, the amount of attention the animal directs toward the CS is related to how reliably the CS has predicted the US in the past (i.e., the previously learned CS-US association) (Bouton 2007; Bushnell 1998; Mackintosh 1975; Pearce and Hall 1980). The larger magnitude of LGNv neuronal response during acquisition-criterion and reacquisition-avg sessions and the reduced
LGNv neuronal response during overtraining and extinction-avg sessions indicates that the LGNv neuronal response was not simply a function of automatic attention to the CS-US association or CR performance. Modifications in LGNv activity were rather related to the state of association between the CS and US and the surprisingness of this association (i.e., controlled attention). This controlled attention may be used to enable the eyeblink neural circuitry to learn the CS-US association when it is still new and not fully learned (Bushnell 1998; Pearce and Hall 1980).

With the analysis of the LGNv neuronal response during visual eyeblink conditioning, the confounding effect of CR expression must be taken into consideration. During visual eyeblink conditioning, in contrast to auditory eyeblink conditioning, the eyelid closure CR attenuates the CS (i.e., physically blocks the light CS from reaching the retina). This CS attenuation effect of the CR could be the source of LGNv CR-related changes in activity observed during the present experiment. However, we found that CR-related increases in LGNv neuronal activity did not occur during overtraining and extinction sessions, even though rats were producing CRs at a high rate (Figs. 6C and 9). Also, excited neurons showed an increasing trend in activity before CR onset and a decreasing trend after CR onset (Fig. 10A). These findings show that CR onset and the possible blocking of retinal stimulation by the eyelid closure produce a general decrease in LGNv neuronal activity rather than an increase. The possibility that activity in some LGNv neurons changes when the visual CS is physically blocked by eyelid closure cannot be dismissed, but the present findings do not support the assertion that those changes are the source of CR-related LGNv activity observed in the present experiment. Furthermore, some of the learning-related neuronal modifications that we observed in the LGNv were unrelated to CR production. LGNv neuronal activity developed habituation during continued unpaired pretraining and showed some recovery from this habituation as paired CS-US training started (acquisition-avg) and before CRs appeared. Also, the amplitude of neuronal activity during the no-CR trials showed some increases compared with the habituated LGNv neuronal activity during unpaired training. Moreover, during the reacquisition training in the already well-trained rats (reacquisition-avg sessions), the LGNv showed a general increase in the neuronal activity during the CS time window when a CR was produced compared with the unpaired extinction training (extinction-avg sessions; Fig. 9). This general increase in the neuronal activity was specifically most prominent during the early CS-evoked neuronal response. The mentioned changes in the neuronal activity were not specifically CR related, which, in general, supports the hypothesis that the LGNv neuronal response is capable of modification during learning.

The present findings are consistent with results reported in prior studies that recorded neuronal activity in thalamic nuclei other than the LGNv during associative learning. For example, Gibbs et al. (1986) found that learning-related changes in the dorsal lateral geniculate occurred as a “lack of [neuronal] habituation,” when anesthetized pigeons were trained with a light CS, paired with a foot-shock US. Two other studies observed a neuronal habituation trend in the MGm in animals trained with unpaired presentations of a tone CS and a foot-shock US (Maren et al. 2001; Poremba and Gabriel 2001). Interestingly, the Poremba and Gabriel (2001) study also showed that amygdalar inactivation at the onset of training blocked this trend of habituation in MGm neuronal response to unpaired CS/US presentations [also, Talk et al. (2004)]. In another study that simultaneously recorded amygdalar and MGm neurons during heart-rate conditioning in rabbits, neu-
neuronal habituation was observed in both structures after rabbits received a CS-only pretraining session. Both structures recovered from this habituation when paired CS-US training started (McEchron et al. 1995). The results of these studies and the current study suggest the possibility that neuronal habituation to a nonpredictive stimulus might be a common feature among some thalamic nuclei involved in learning and possibly influenced by the amygdala.

To our knowledge, only one study [i.e., Davidowa and Albrecht (1992)] has previously recorded LGNv neuronal activity during associative learning. That study conditioned anesthetized rats with a visual CS (light), paired with a shock US, applied to the rat’s tail. Davidowa and Albrecht (1992) observed that during forward-paired CS-US training, the number of neurons showing facilitation canceled out the number of neurons showing suppression, resulting in no net change in LGNv activity. As the present study also showed, forward-paired training did not produce overall greater LGNv neuronal responses compared with the pretraining baseline, but the main effect of paired training was to overcome the habituation that occurred with continued unpaired training. However, Davidowa and Albrecht (1992) found that LGNv activity showed overall facilitation when the rats were given backward pairings of CS and US (US-CS training). Davidowa and Albrecht (1992) concluded that the modulation of LGNv neuronal activity might be due to nonassociative facilitation produced by the arousing effect of the US. Our findings argue against that conclusion, as unpaired presentations of the CS and US in the present experiment eventually resulted in habituation of a LGNv neuronal response. Interestingly, Davidowa and Albrecht (1992) showed that local infusion of atropine, an ACh antagonist, into the LGNv, abolished both facilitation and suppression of LGNv neurons in both forward and backward training. This finding highlights the potential importance of ACh in modulating LGNv neuronal activity during associative learning. Some studies have shown that the cholinergic system can have increasing or decreasing effects on the LGNv neuronal response, depending on the level of activation (Guo et al. 2005, 2010; McCormick and Prince 1987). ACh, therefore, may influence neuronal facilitation or suppression in the LGNv, which explains why different effects occur with paired and unpaired CS-US training. Also, it needs to be taken into consideration that Davidowa and Albrecht (1992) used anesthetized rats in their experiments, because an animal’s state of arousal (e.g., animal is awake or in slow-wave sleep) affects how ACh influences the dorsal lateral geniculate nucleus (Marks and Roffwarg 1989).

In summary, neuronal activity in the LGNv changed, depending on whether the CS and US were paired or unpaired, the amount of training, and CR production. The neuronal changes observed in the LGNv were mostly in the form of habituation during unpaired sessions and recovery from this habituation during paired sessions. CR-related neuronal activity was also evident as enhanced neuronal activity during the paired trials, in which rats produced CRs. These findings suggest an important role for LGNv in attention to the predictive CS during visual eyeblink conditioning. It is possible that changes in LGNv activity are influenced by brain stem-ascending neuromodulatory systems (e.g., ACh) and feedback from the cerebellum. Future studies that manipulate the function of these brain structures and their inputs to the LGNv may help provide a more detailed understanding of the mechanisms underlying modifications of neuronal activity in the LGNvs during associative learning.

ACKNOWLEDGMENTS

We thank Inah Lee, Jadin Jackson, and Ka Ng for advice and technical assistance.

GRANTS

Support for this work was provided by the National Institute of Mental Health (Grant MH080005 to J. H. Freeman).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.K. and J.H.F. conception and design of research; A.K. performed experiments; A.K. and M.M.C. analyzed data; A.K. and J.H.F. interpreted results of experiments; A.K. prepared figures; A.K. drafted manuscript; A.K., M.M.C., and J.H.F. edited and revised manuscript; A.K., M.M.C., and J.H.F. approved final version of manuscript.

REFERENCES


LATERAL GENICULATE ACTIVITY DURING ASSOCIATIVE LEARNING


Halverson HE, Freeman JH. Ventral lateral geniculate input to the medial pons is necessary for visual eyelid conditioning in rats. Learn Mem 17: 80–85, 2010b.


Weinberger NM. The medial geniculate, not the amygdala, as the root of auditory fear conditioning. Hear Res 274: 61–74, 2011.
