Medial auditory thalamus is necessary for acquisition and retention of eyeblink conditioning to cochlear nucleus stimulation

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Associative learning tasks commonly involve an auditory stimulus, which must be projected through the auditory system to the sites of memory induction for learning to occur. The cochlear nucleus (CN) projection to the pontine nuclei has been posited as the necessary auditory pathway for cerebellar learning, including eyeblink conditioning. However, the medial auditory thalamic nuclei (MATN), consisting of the medial division of the medial geniculate, suprageniculate, and posterior interlaminar nucleus have also been implicated as a critical auditory relay to the pontine nuclei for cerebellum-dependent motor learning. The MATN also conveys auditory information to the amygdala necessary for avoidance and fear conditioning. The current study used CN stimulation to increase activity in the pontine nuclei, relative to a tone stimulus, and possibly provide sufficient input to the cerebellum for acquisition or retention of eyeblink conditioning during MATN inactivation. Primary and secondary effects of CN stimulation and MATN inactivation were examined using 2-deoxy-glucose autoradiography. Stimulation of CN increased activity in the pontine nuclei, however, this increase was not sufficient for cerebellar learning during MATN inactivation. Results of the current experiment provide additional evidence indicating the MATN may be the critical auditory relay for many associative learning tasks.

Pavlovian eyeblink conditioning has been widely used to examine the behavioral and neural correlates of associative learning and memory (Gormezano et al. 1983; Christian and Thompson 2003; Freeman and Steinmetz 2011). This task is established by pairing a conditioned stimulus (CS), typically a tone, with an unconditioned stimulus (US), mild periorbital shock, that evokes an eyeblink reflex. After repeated CS–US presentations, an adaptive eyeblink conditioned response (CR) emerges prior to US onset. Converging lines of evidence indicate that the cerebellum, specifically the interpositus nucleus and cerebellar cortex, are essential sites of memory formation and storage for this task (McCormick et al. 1982; McCormick and Thompson 1984a,b; Yeo et al. 1985; Freeman et al. 1995, 2005; Perrett and Mauk 1995; Krupa and Thompson 1997; Garcia and Mauk 1998; Nicholson and Freeman 2002; Jirenhed et al. 2007; Halverson et al. 2010). However, the specific anatomical pathway by which critical auditory CS inputs from cochlear nucleus (CN) reach this cerebellar circuit during learning are currently unclear.

Mossy fiber projections from the pontine nuclei to the cerebellum relay stimulus information from various sensory modalities, including auditory (Steinmetz et al. 1986, 1987; Lewis et al. 1987; Knowlton and Thompson 1988; Tracy et al. 1998; Hesslow et al. 1999; Bao et al. 2000; Halverson and Freeman 2010a,b). The monosynaptic auditory pathway from the CN to the lateral pontine nuclei (LPN) has been suggested as a possible pathway relaying auditory information to the cerebellum (Steinmetz et al. 1987), but has not been manipulated experimentally. Projections from CN to LPN and other auditory structures are primarily contralateral, the CN also has polysynaptic paths to additional nuclei within the auditory system which then target LPN (Campolattaro et al. 2007; Halverson et al. 2010). These connections are through auditory structures such as the medial auditory thalamic nuclei (MATN), including the medial division of the medial geniculate

(MGm), suprageniculate (SG), and posterior interlaminar nucleus (PIN) (Malmierca et al. 2002; Halverson et al. 2010). The unilateral projection from MATN to the LPN is a necessary and sufficient cerebellar input for acquisition and expression of auditory eyeblink conditioning (Halverson and Freeman 2006, 2010a; Campolattaro et al. 2007; Halverson et al. 2008).

In addition to cerebellar learning, the MATN also plays an important role in relaying auditory information to the amygdala during fear conditioning (LeDoux et al. 1984, 1990; McCabe et al. 1993; Campeau and Davis 1995; Poremba and Gabriel 1997). Tetanic stimulation of MATN is sufficient for enhancement of the amygdala-dependent acoustic startle reflex (Huang et al. 2005). Stimulation of the CN and MATN are also each sufficient for rapid acquisition and expression of eyeblink CRs (Nowak et al. 1999; Campolattaro et al. 2007; Freeman and Duffel 2008; Halverson and Freeman 2010a).

The current experiment used CN stimulation ipsilateral to the trained eye (left) to increase activity in the contralateral (right) pontine nuclei, relative to a tone stimulus from a previous study (Halverson et al. 2008), in an attempt to provide sufficient input to the cerebellum for eyeblink conditioning during contralateral (right) MATN inactivation. In addition, 2-deoxy-glucose (2-DG) autoradiography was used to examine the metabolic consequences of CN stimulation combined with MATN inactivation. Increasing the input from CN to the pontine nuclei, in the absence of MATN input, should support at least partial acquisition or expression of eyeblink conditioning if this source of auditory mossy fiber information is involved with cerebellar learning.

Results

Cannula and electrode placements

Cannula placements in the MATN were verified by examining serial coronal sections. All placements ($n = 18$) were in or within 0.5 mm of the right MGm. Muscimol infusions through cannula placements within this range of the MGm block acquisition and retention of learned eyeblink responses to a tone (Halverson et al. 2008). Figure 1A shows a representative cannula placement in the MATN. All cannula placements were similar to the example shown in Figure 1 with respect to location within the MGm and rostral/caudal extent of the entire MATN.

Electrode placements in the CN were also verified by examining serial coronal sections. Placements were in the left ventral ($n = 13$) and dorsal ($n = 5$) divisions of the CN. No differences were observed in the rate of learning between rats with dorsal or ventral electrode placements. Figure 1B shows a representative electrode placement in the CN.

Eyeblink conditioning

Acquisition of eyeblink conditioning was tested with left CN stimulation combined with muscimol inactivation of the right MATN (phase 1). Conditioned eyeblink response expression with MATN inactivation was also tested with both stimulation and a tone to identify any differences in response performance between stimulation and a peripheral tone while the MATN was inactivated. If the short-latency (CN to LPN) pathway is involved in sending auditory information to the pontine nuclei sufficient for eyeblink conditioning, maximizing input from the CN with stimulation during MATN inactivation should support an increase in CR percentage during acquisition, and support at least partial expression of responses during a muscimol retention test by overriding the loss of modulatory input to the LPN from the MATN. However, if direct input from the CN to LPN is not sufficient for cerebellar

learning, the muscimol group should fail to learn during infusion sessions, and show a significant decrease in CR percentage during the muscimol retention test despite the facilitation of the CN to LPN pathway provided by stimulation.

To examine the effects of MATN inactivation during phase 1 on savings in phase 2, a group of control rats were given three sessions of US alone trials (phase 1) followed by three sessions of paired training with cochlear stimulation to compare their subsequent acquisition rate with the muscimol group during post-MATN infusion acquisition training (phase 2). The US exposure group controlled for potential sources of behavioral inhibition that could impair CR acquisition rate in the muscimol group during phase 2 as a consequence of blocking auditory information sufficient for learning from reaching the cerebellum with MATN inactivation. If auditory information important for learning is projected to the cerebellum with CN stimulation during MATN inactivation, there should be some evidence of associative learning either during the initial training sessions or during post-infusion training. Alternatively, if auditory information sufficient for learning does not reach the cerebellum in this case, the muscimol and US exposure groups will show identical learning rates during phases 1 and 2.

Muscimol infusions into the MATN during phase 1 blocked acquisition of conditioned eyeblink responses to CN stimulation (sessions 1–3; Fig. 2A). The muscimol group showed an identical acquisition rate in phase 2 of training (sessions 4–6) relative to the US exposure control group that received US-alone presentations during phase 1 (Fig. 2A,B). The CR percentage data for the muscimol, saline, and US exposure groups for the three acquisition phases (sessions 1–6 and sessions 9–11) (Fig. 2A,B) were examined with a repeated-measures ANOVA, which revealed an interaction of the group, session, and phase factors, $F_{(8,60)} = 17.50$, $P < 0.001$. Tukey's honestly significant difference (HSD) post hoc tests indicated that the saline group produced more CRs than the muscimol and US exposure groups on sessions 1–4 (all comparisons, $P < 0.05$). The percentage of learned responses did not differ between the muscimol and US exposure groups during the first two phases of training ($P < 0.05$), indicating no associative learning in the muscimol group during phase 1. The muscimol and US exposure groups also showed significantly more CRs in the second and third sessions of phase 2 (sessions 5–6) and all sessions of phase 3 (sessions 9–11) compared with all sessions of phase 1 (sessions 1–3) ($P < 0.05$ for each case). This result indicated that conditioning rates during inactivation of MATN while using CN stimulation as a CS were not significantly different from conditioning while receiving presentations of the US alone.

Muscimol infusions into the MATN blocked CRs to both CN stimulation and tone stimuli during the muscimol retention tests (sessions 7 and 12) (Fig. 2A). Repeated-measures ANOVA for both the muscimol retention tests (sessions 6–8, 11–13) revealed a main effect of the session factor for both stimulation, $F_{(2,22)} = 552.07$, $P < 0.0001$, and tone retention tests, $F_{(2,22)} = 892.26$, $P < 0.0001$ (Fig. 2A). Post hoc tests (HSD) indicated that all rats emitted fewer CRs on the CN stimulation–muscimol retention test (session 7) than on sessions 6 and 8 ($P < 0.05$ for each case). All rats also emitted fewer CRs on the tone muscimol retention test (session 12) than on sessions 11 and 13 ($P < 0.05$). These results indicated that inactivation of the MATN with muscimol produced similar impairments in CR frequency with either CN stimulation or a peripheral tone.

More intense or salient stimuli produce an increase in the rate of acquisition in eyeblink conditioning (Scavio and Gormezano 1974). To examine the saliency of CN stimulation relative to a peripheral tone, the acquisition rates during phases 1 and 2 were compared with acquisition rates of a previous study (Halverson et al. 2008) in which rats received the same training sequence
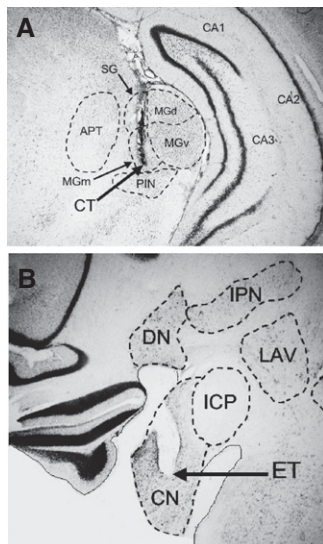
Figure 1. (*A*) Coronal section of the MATN showing a representative cannula placement in the MGm. (APT) anterior pretectal nucleus, (SG) suprageniculate, (MGm) medial division of the medial geniculate, (MGd) dorsal division of the medial geniculate, (MGv) ventral division of the medial geniculate, (CT) cannula tip. (*B*) Coronal section of the cerebellum and brainstem showing a representative electrode placement in the CN. (DN) dentate nucleus, (IPN) interpositus nucleus, (LAV) lateral vestibular nucleus, (ICP) inferior cerebellar peduncle, (CN) cochlear nucleus, (ET) electrode tip. Magnification, 2.5×.
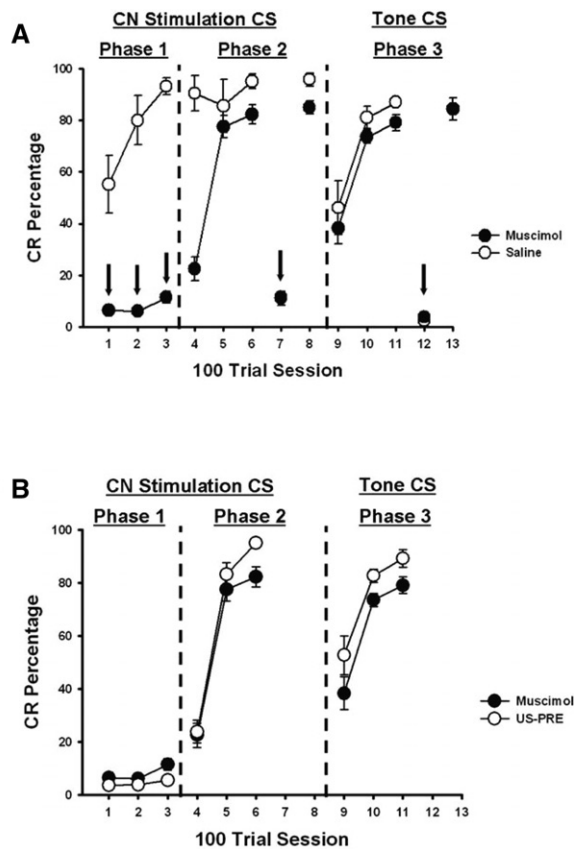
Figure 2. (*A*) Mean ± SE conditioned response (CR) percentage for rats given muscimol (black arrows) or saline during acquisition training (sessions 1–3). All rats received CN stimulation on sessions 1–8, and a tone stimulus on sessions 9–13. All rats received muscimol (black arrows) on sessions 7 and 12. Rats given muscimol during acquisition showed no evidence of learning and all rats showed a severe reduction in CRs on the muscimol retention tests (sessions 7 and 12). (*B*) Mean ± SE CR percentage for rats given muscimol or US exposure during acquisition training (sessions 1–3). Rats in both groups showed identical acquisition rates on subsequent paired training (sessions 4–6) with CN stimulation and similar amounts of savings when switched to the tone (sessions 9–11).

(saline or muscimol, followed by no infusions) with the same tone CS used in phase 3 of the current experiment. Repeated-measures ANOVA on the CR percentage data for phases 1 and 2 with CN or tone stimuli revealed a three-way interaction of the phase, session and group factors, $F_{(6,50)} = 45.58$, $P < 0.0001$. Tukey's HSD post hoc tests on the data for the saline groups from both experiments indicated that the animals receiving CN stimulation (current experiment) emitted more CRs on sessions 1 (55.2% versus 16.9%) and 2 (79.9% versus 55.9%) than those receiving the tone (previous experiment) ($P < 0.05$ in each case). Post hoc tests on the data for muscimol groups indicated that the animals receiving CN stimulation showed more CRs on session 5 (77.4% versus 36.2%) than those receiving the tone from the previous study ($P < 0.05$). These results indicated that the CN stimulation CS was more salient and supported faster acquisition than the tone CS for both saline and muscimol groups.

Measures of response performance were taken from CS-alone trials to avoid contamination from the cerebellum-independent reflexive response to the US. Response measures were reported on sessions without infusions since the number of CRs during infusion sessions was insufficient for performing statistical analyses. Rats in the muscimol group showed later onset and peak latencies relative to the US exposure and saline groups during the first stimulation session without infusions. Results were examined with repeated-measures ANOVA on the onset and peak latency data for phase 2 which revealed an interaction of the group and session factors for onset, $F_{(4,30)} = 6.86$, $P < 0.0001$, and peak latency, $F_{(4,30)} = 3.872$; $P < 0.012$. Tukey's HSD post hoc tests indicated that rats in the muscimol group showed later onset and peak latencies than both US exposure and saline groups on session 4 ($P < 0.05$). Rats in the US exposure group also showed later CR onsets on session 4 relative to the saline group ($P < 0.05$). This was an expected result since later onset and peak latency observed in the muscimol and US exposure groups is indicative of earlier stages of learning (Gormezano 1972). All three groups showed onset and peak latencies during the first session of tone conditioning (phase 3, session 9) that indicated new learning to the tone. Results were examined with a repeated-measures ANOVA on the onset and peak latency data, which revealed a main effect of the session factor onset, $F_{(2,30)} = 7.00$, $P < 0.003$, and peak latency, $F_{(2,30)} = 5.18$, $P < 0.012$. Tukey's HSD post hoc tests revealed that rats in all three groups showed later onset and peak latencies on session 9 than all other tone sessions without infusions ($P < 0.05$).

As animals acquire eyeblink conditioning the amplitude of the response increases in parallel with an increase in CR percentage, which makes changes in amplitude an additional index to investigate learning (Gormezano 1972). Repeated-measures ANOVA on the amplitude data revealed an interaction of the group and session factors for phase 2, $F_{(4,30)} = 4.27$, $P < 0.007$, and a main effect of the session factor for phase 3, $F_{(2,30)} = 13.18$, $P < 0.0001$. Post hoc tests (HSD) indicated that the muscimol and US exposure groups showed lower amplitude responses on session 4 relative to the saline group, and the muscimol and US exposure groups showed an increase in response amplitude from session 4 to session 5 ($P < 0.05$). Post hoc tests also indicated that all three groups showed lower response amplitudes on session 9 than all other tone sessions without infusions during which amplitude was not different between groups ($P < 0.05$ in all cases). The delayed onset and peak latency data combined with the lower response amplitude data serves as evidence that new learning was occurring during the first session when rats were transferred from a CN stimulation CS to the tone.

The startle or α response to CS onset has been classified in rats and mice and is associated with more intense auditory stimulation (Skelton 1988; Boele et al. 2010). This response is triggered by stimulus onset and, unlike the CR, the α response is cerebellar independent (Skelton 1988; Boele et al. 2010). One rat in the saline group consistently showed α responses to very low-intensity CN stimulation (5 μA) even during the muscimol retention test (Fig. 3). Muscimol inactivation of the MATN blocked CRs in this rat but not α responses. All other rats typically showed infrequent small amplitude α responses to CN stimulation that were attenuated by muscimol infusions into MATN (Fig. 3A,C). Besides the effect on CRs muscimol infusions into MATN or CN stimulation did not produce any overt behavioral changes or ataxia. This result indicates that while low-intensity CN stimulation can be a highly salient stimulus capable of evoking robust behavioral α responses and rapid CR acquisition, this stimulation is insufficient for the learned cerebellar-dependent response without input from the MATN.

2-DG autoradiography

Examining CR acquisition rate along with the metabolic effects of both CN stimulation and tone stimuli in the pontine nuclei under MATN inactivation provided evidence of the increase in stimulus saliency produced by CN stimulation. During presentations of left CN stimulation metabolic activity in the right MATN was severely
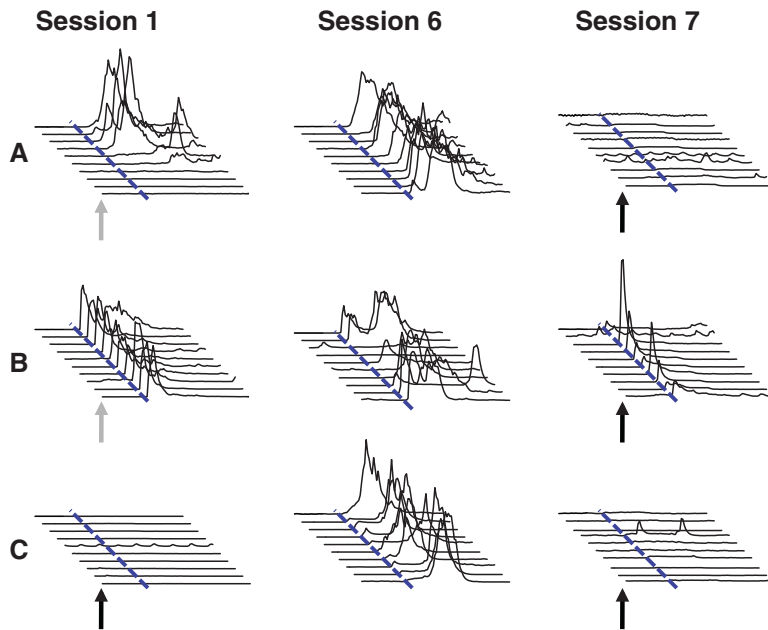
Figure 3. Integrated eyelid EMG traces from CN stimulation-alone trials demonstrating muscimol effects on acquisition (session 1) and expression (sessions 6,7) of conditioned responses. Gray arrows represent saline infusion before training, black arrows represent muscimol infusion before training. Eyelid traces are stacked in order of the session with the first trial in front and the last trial in back of each set. Each trace represents 1 sec. (*A*) Data from a rat in the saline group showing rapid CR acquisition during session 1, CR expression during session 6 followed by CR abolition during MATN muscimol inactivation in session 7. (*B*) Data from a second rat in the saline group showing a similar pattern of responses as seen in *A*. CN stimulation (5 μA) evoked α responses after stimulus onset (dotted blue line) that were not affected by MATN muscimol inactivation. (*C*) Data from a rat in the muscimol group showing blocked CR acquisition (session 1) and expression (session 7) during MATN inactivation.

the group and side factors for the pontine nuclei, $F_{(1,7)} = 76.98$, $P < 0.001$ (Figs. 4, 5). Tukey's HSD post hoc tests indicated that the saline group showed more 2-DG uptake in the right and left pontine nuclei than the right side of pons in the muscimol group ($P < 0.05$). Uptake for the left side of the pons in the muscimol group was not significantly different from either side of the saline group ($P > 0.05$; Figs. 4, 5).

The goal of the current experiment was to increase activity in the pontine nuclei by using CN stimulation in an attempt to overcome the decrease in pons activity under MATN inactivation observed in a previous study using a tone (Halverson et al. 2008). The 2-DG methods and infusion procedures were the same between the Halverson et al. (2008) study and the current study which allows for direct comparisons to be made between the metabolic activity for rats receiving either CN stimulation or a tone as the training stimulus. Repeated measures ANOVA on the normalized region-of-interest 2-DG readings for the pontine nuclei revealed an interaction of the group and side factors, $F_{(3,12)} = 26.20$, $P < 0.0001$. HSD post hoc tests indicated that rats receiving CN stimulation had more 2-DG uptake on both sides of the pontine nuclei than rats receiving the tone in both groups, saline (left = 167.8 nCi/g versus 134.1, right = 174.3 nCi/g versus 134.7) and muscimol (left = 166.9 nCi/g versus 118.5. right = 115.5 nCi/g versus 67.4) ($P < 0.05$). This result indicates that CN stimulation effectively increased the metabolic activity in the pontine nuclei, even during MATN inactivation, above the level observed with a tone CS.

Discussion

Acquisition and expression of cerebellar-dependent eyeblink CRs with CN stimulation ipsilateral to the trained eye (left) were severely impaired by muscimol inactivation of the contralateral (right) MATN. Retention of CRs with a tone CS was also severely impaired by muscimol inactivation of the MATN, as seen in a previous study (Halverson et al. 2008). Rats in the muscimol group showed no evidence of associative learning during initial acquisition in phase 1 (Fig. 2A, sessions 1–3) and a significantly impaired acquisition rate during post-infusion training in phase 2 (Fig. 2A, sessions 4–6) that was very similar to the US exposure control group (Fig. 2B). A muscimol retention test (Fig. 2A, sessions 7 and 12) blocked CRs in all rats with both CN stimulation and tone stimuli, which confirmed similar cannula placements in the MATN between the saline and muscimol groups. Metabolic activity in the MATN was severely reduced in the muscimol group compared with animals that received saline infusions (Fig. 4). Metabolic activity in the right pontine nuclei was also significantly reduced relative to the left side of pons in the muscimol group and in relation to the metabolic activity observed in the saline group (Figs. 4, 5).

The rapid CR acquisition rate observed with CN stimulation relative to a previous study using a tone CS (Halverson et al. 2008)

reduced in the muscimol group compared with the saline group (Fig. 4). Qualitative assessment of MATN inactivation was done due to the increased 2-DG uptake by gliosis from the cannula track that would confound quantitative readings (Halverson et al. 2008). Metabolic activity in the right auditory cortex was also decreased in the muscimol group relative to the saline group (Fig. 4) most likely due to inactivation of neighboring ventral division of the medial geniculate nucleus (MGv) (Halverson et al. 2008). Bilateral metabolic activity was observed in the CN in both muscimol and saline groups. The bilateral activity in the CN was likely reflective of other auditory stimuli that were present during 2-DG uptake including sound from the chamber fan, the rat moving inside the conditioning chamber, and ambient sounds from the laboratory. Bilateral 2-DG uptake was also observed in the superior olive, inferior colliculus, and lateral lemniscus (Fig. 4). Cerebellar activity in the muscimol and saline groups, was similar to the observations with MATN inactivation and presentations of a tone stimulus from a previous study (Halverson et al. 2008) which found no differences between groups. These results replicated the findings of the Halverson et al. (2008) study which used a tone stimulus and inactivation of the right MATN, the focus of this study was on possible differences at the level of the pontine nuclei due to stimulation of the left CN. Region-of-interest measurements were taken from the pontine nuclei to investigate secondary inactivation of the right pontine nuclei relative to the left between the muscimol and saline groups. The right pontine nuclei are important as they are the primary target of both left CN and right MATN and also the source of auditory mossy fibers necessary for the left cerebellum to learn and express eyeblink CRs. Repeated-measures ANOVA on the normalized region-of-interest 2-DG measurements revealed an interaction of
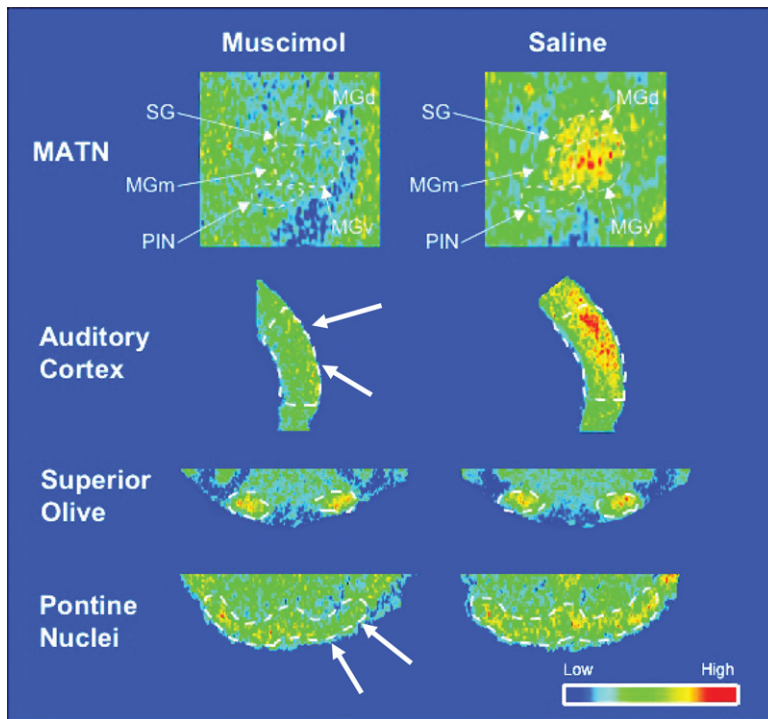
Figure 4. Autoradiographs showing uptake of 2-DG radiolabeled with [14]C (red indicates strongest 2-DG uptake). Dotted lines: Boundaries of the medial auditory thalamic nuclei (MATN), auditory cortex, superior olive, and pontine nuclei. Boundaries were drawn based on the same thionin-stained sections. *Upper* row: Representative MATN sections showing activity in the auditory thalamus in the muscimol and saline groups. Second row: auditory cortex; third row: superior olive; *lower* row: pontine sections show secondary inactivation (white arrows) after ipsilateral muscimol infusion and saline infusions into MATN.

suggests that CN stimulation serves as a very salient stimulus. Stimulation of the CN could possibly increase activity in the pontine nuclei (relative to a tone CS) to a level sufficient to overcome MATN inactivation and support learning. The use of left CN stimulation produced a differential unilateral increase in metabolic activity in the right pontine nuclei during inactivation of the right MATN compared with the metabolic activity observed in the same side of pons while using the tone stimulus from the Halverson et al. (2008) study. This increase provided by CN stimulation in the direct input from CN to the contralateral pontine nuclei would have likely provided adequate input to the cerebellum for at least partial acquisition or expression of eyeblink conditioning during MATN inactivation. Inactivation of the right MATN produced a decrease in metabolic activity in the pons in this study, this decrease in activity was likely due to decreased input from both MATN and auditory cortex (Fig. 4), since both areas showed decreases in 2-DG uptake during MATN inactivation (Halverson et al. 2008).

Rats were unable to acquire or express conditioned eyeblink responses with ipsilateral CN stimulation under contralateral MATN inactivation. This result provides compelling evidence that information in the auditory system must be relayed through the MATN to the pontine nuclei for both acquisition and expression of cerebellar-dependent eyeblink CRs. It is still possible that the direct CN to LPN projection is somehow involved with sending auditory information to the cerebellum for learning. This projection will need to be silenced during eyeblink conditioning using a technique like optogenetics to rule out any involvement with cerebellar learning. Future studies may also extend this auditory-evoked engagement of MATN to associative learning in

general. Muscimol inactivation during the initial acquisition sessions (sessions 1–3) also slowed acquisition rates in the subsequent post-muscimol training (session 4) relative to the first session in phase 1 of the saline group. A control group that initially received three US-alone sessions displayed a similar delay in CR acquisition rate on the first paired session with cochlear stimulation (Fig. 3, session 4). This result provides evidence that blocking auditory stimulation at the level of the MATN during paired training is similar to training with presentations of the US alone at the level of the cerebellum. The increased input from the CN to the pontine nuclei and cerebellum was not sufficient to support acquisition or expression of CRs in the absence of input from the MATN. Unlike the learned cerebellar-dependent CRs, startle responses elicited by CN stimulation in one rat were not blocked by MATN inactivation (Fig. 3B), smaller startle responses in all other rats were typically attenuated during MATN inactivation. Thus, MATN inactivation selectively blocked the auditory information sufficient for CR acquisition and expression from being relayed to the pontine nuclei and into the cerebellum.

The results of this study provide additional evidence that convergent auditory inputs to the MATN contralateral to the trained eye and the corresponding ipsilateral MATN projection to the LPN comprise the necessary auditory pathway for acquisition and expression of cerebellar-dependent eyeblink CRs to auditory stimuli (Fig. 6; Halverson and Freeman 2006, 2010a; Campolattaro et al. 2007; Freeman et al. 2007; Halverson et al. 2008). The laterality of CN stimulation and MATN inactivation in the current experiment was selected based on the connectivity within the auditory system and how the auditory system interfaces with the eyeblink
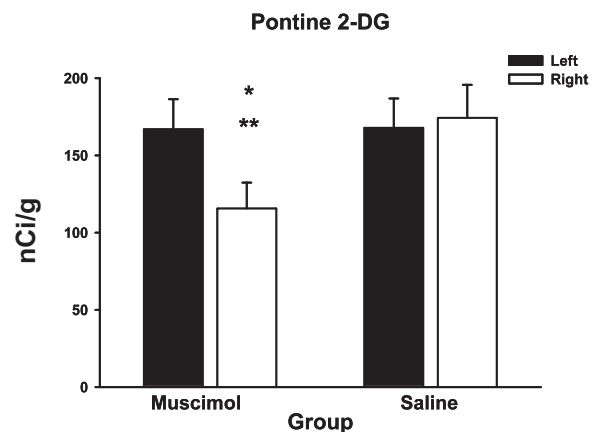

Figure 5. Mean ± SE nCi/g of 2-DG uptake in the *left* and *right* side of the basal pontine nuclei in the muscimol and saline groups. (*) Significant difference ($P < 0.05$) between the muscimol and saline groups; (**) significant difference ($P < 0.05$) between *left* and *right* side within groups.

Figure 6. The auditory pathway for associative fear and cerebellar-dependent motor learning. Parallel inputs into the MATN from contralateral cochlear nucleus (CN), and primarily unilateral inputs from superior olive (SO), lateral lemniscus (LL), and inferior colliculus (IC). Direct unilateral projection from MATN to the LPN. LPN mossy fiber projection into the contralateral cerebellum (cortex [CTX] and interpositus nucleus [IPN]), and IPN output to red nucleus (RN) for CR expression, IPN also has feedback to MATN. The MATN also has a reciprocal connection with the amygdala (AMYG), which is necessary for fear and avoidance learning. The AMYG also projects to LPN and could modulate cerebellar-dependent motor learning via this pathway. Blue boxes indicate sites of memory formation and storage. Dotted lines indicate modulatory inputs.

et al. 2002; Fanselow and Poulos 2005; Boele et al. 2010). Appetitive conditioning with an auditory stimulus also leads to learning-related potentiation in auditory thalamic firing (Disterhoft and Olds 1972; Nienhuis and Olds 1978), but has not been investigated to the same extent as thalamic involvement in eyeblink or fear conditioning. Acquisition of eyeblink or fear conditioning can enhance conditioning in the other paradigm while using the same auditory stimulus. This may be due to learning-related changes in the MATN that are established during the initial phase of learning that then subsequently facilitates MATN input important for later learning in the other paradigm (Neufeld and Mintz 2001; Lindquist et al. 2010). Similar to eyeblink conditioning, if the US used in fear conditioning is unilateral (e.g., eye shock) the MATN and amygdala engaged in learning and expressing conditioned fear responses are also unilateral (Fig. 6; LeDoux et al. 1991; Blair et al. 2005). Amygdala modulation of cerebellar-dependent motor learning could therefore be influenced from either the direct amygdala projection to LPN or from the reciprocal connection between the amygdala and MATN (Prince and Amaral 1981; Maren et al. 2001; Poremba and Gabriel 2001; Taub and Mintz 2010; Ng and Freeman 2013).

Ipsilateral (to the trained eye) CN stimulation was used in an attempt to increase the activity in the CN projection to the contralateral pons during eyeblink conditioning and possibly overcome the effect of MATN inactivation on CR acquisition and/or expression. Although increased activity in the pontine nuclei was observed with CN stimulation (relative to a tone), rats did not acquire or express CRs under MATN inactivation. The MATN is the necessary auditory input to the LPN for cerebellar-dependent eyeblink conditioning and is also engaged in many other associative learning paradigms including appetitive, avoidance, and fear learning. The MATN may, therefore, be a centerpiece of auditory associative learning regardless of the reinforcing events or responses measured.

conditioning circuitry (LPN). Stimulation of CN bilaterally or even contralateral to the trained eye was not used in the current experiment due to the connections in the auditory system and how auditory structures project onto the known eyeblink conditioning circuitry highlighted above and illustrated in Figure 6. Contralateral (right) CN stimulation would likely be a sufficient CS for eyeblink conditioning, however, because CN has primarily contralateral projections to higher auditory structures and to LPN, stimulating the right CN would not engage the eyeblink conditioning circuitry as directly as the stimulation used in the current experiment.

Learning-dependent cerebellar feedback to MATN may enhance thalamic output to the LPN related to the stimulus used in training and facilitate further plasticity in the cerebellum (Halverson et al. 2010). The MATN, along with auditory cortex, is also part of the auditory pathway necessary for amygdala-dependent fear conditioning (Romanski and LeDoux 1992). Stimulation of the auditory cortex is a sufficient CS for eyeblink conditioning (Knowlton and Thompson 1992; Nowak et al. 1999). However, lesions of the entire cerebral cortex (including auditory cortex) do not prevent acquisition or retention of eyeblink conditioning to an auditory stimulus (Oakley and Russell 1972, 1977). In addition to the MATN being important for the relay of auditory information to the cerebellum and amygdala, both fear and eyeblink conditioning produce short-latency plasticity (>50 msec) in MATN within the first few learning trials (Gabriel et al. 1975; Quirk et al. 1995; Maren et al. 2001; Poremba and Gabriel 2001; Halverson et al. 2010). The many similarities between cerebellar-dependent motor and amygdala-dependent fear conditioning have been reviewed in detail while identifying different auditory pathways for both types of learning (Medina

Materials and Methods

Subjects
The subjects were 18 male Long-Evans rats (250–400 g). The rats were housed in the animal colony in Spence Laboratories of Psychology at the University of Iowa (Iowa City, IA). All rats were maintained on a 12 h light–dark cycle and given ad libitum access to food and water. All procedures involving animals were approved by the University of Iowa Institutional Animal Care and Use Committee.

Surgery
One week before training, rats were removed from their home cages and anesthetized with isoflurane. After anesthesia onset, rats were fitted with differential electromyograph (EMG) electrodes (stainless steel) implanted into the upper left orbicularis oculi muscle. The reference electrode was a silver wire attached to a stainless steel skull screw. The EMG electrode leads terminated in

gold pins in a plastic connecter. A bipolar stimulating electrode (Plastics One) for shock US delivery was implanted subdermally, caudal to the left eye. A 23-gauge guide cannula was implanted 2.0 mm dorsal to the right MGm. A 30-gauge stylet was inserted into the guide cannula and extended 1.0 mm from the end of the guide. Stereotaxic coordinates for the guide cannula were 5.5 mm posterior to bregma, 3.1 mm lateral to midline, and 6.3 mm ventral to skull surface. A bipolar stimulating electrode was implanted into the left dorsal or ventral CN (10.9 mm posterior to bregma, 3.8 mm lateral to midline, and 8.4 mm ventral to skull surface). The plastic connector housing the EMG electrode leads, both bipolar stimulating electrodes, the guide cannula, and three skull screws were secured to the skull with Osteobond copolymer bone cement (Zimmer). Animals were maintained on 0.006% Sulfatrim (Hi-Tech Pharmacal Co.) in water for 4 d after surgery.

Muscimol infusion procedure

The γ-aminobutyric acid (GABA) receptor agonist muscimol was used to create a temporary localized lesion by hyperpolarizing cells around the infusion site. Cells that are hyperpolarized by muscimol are less likely to fire action potentials and remain in that inactivated state until the drug is no longer effective (Majchrzak and Di Scala 2000). Muscimol binds with the GABA$_A$ receptor, which allows chloride ions to enter the cell, effectively decreasing neural activity beginning 30 min after infusion with the effect lasting at least 2 h post-infusion (Martin 1991).

Before the muscimol infusions, the stylet was removed from the guide cannula and replaced with a 30-gauge infusion cannula that extended 2.0 mm beyond the guide cannula. The infusion cannula was connected to polyethylene tubing (PE 10; 110–120 cm), which was connected to a 10 µL gas tight syringe (Hamilton). The syringe was placed in an infusion pump (Harvard Apparatus), and 1.0 µL of muscimol (10 mM, pH = 7.4) or saline was infused over 2 min at a rate of 30 µL/h. The large infusion volume (1.0 µL) was used to target all the nuclei of the MATN (MGm, PIN, and SG) with a single infusion. After the infusion, the tubing connected to the infusion cannula was cut and sealed with candle wax. The infusion cannula remained in place for the duration of the experimental session and was replaced by the stylet after each session.

Apparatus

The conditioning apparatus consisted of two small sound-attenuating chambers (BRS/LVE). Within each sound-attenuating chamber was a small-animal operant chamber (BRS/LVE) in which the rats were kept during conditioning. One wall of the operant chamber was fitted with two speakers that independently produced tones of up to 120 dB with a ~1000–9000 Hz frequency range. A wall-mounted exhaust fan provided a 65 dB masking noise. The CN stimulation used in training was a 300 msec train of 0.1-msec biphasic square wave pulses (5–50 µA) at 50 Hz. Stimulation level was independently set for each rat by starting at 50 µA and determining the intensity that produced a behavioral response before training, then reducing the current in 5 µA increments until no behavioral response was observed. This individual stimulation level for each rat was then used throughout the experiment. Typical behavioral responses to CN stimulation were head turns, ear/whisker movement, and rearing. One rat displayed α or startle responses to CN stimulation at 5 µA and was trained at this low-intensity level to investigate the effect of MATN inactivation on the stimulation elicited α responses. The tone stimulus used in training was a 2000 Hz pure tone (85 dB; range in conditioning chamber, 83–87 dB). The electrode leads from the rat's headstage were connected to peripheral equipment by lightweight cables that allowed the rat to move freely during conditioning. A desktop computer was connected to the peripheral equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs). The periorbital shock stimulus was delivered through a stimulus isolator (model 365A; World Precision Instruments). Eyelid EMG activity was amplified differentially (gain, 2000; sampling rate,

250 Hz), filtered (500–5000 Hz), and integrated (time constant, 20 msec). The intensity of the shock US was set at two times the threshold for eliciting a discrete eyeblink (typical range of final current intensity, 2–4 mA; 60 Hz; constant current). Current intensity of the US was determined during the first few training trails of the first day of training and held at that level throughout training for each rat.

Conditioning procedure

Rats adapted to the training environment for 5 min before each training session. During phase 1, saline ($n = 6$) or muscimol ($n = 7$) was infused into the MATN 30 min prior to each of three delay eyeblink conditioning sessions with each session consisting of 100 trials per day. Trials were presented with a pseudorandom distribution of intertrial intervals between 18 and 42 sec (30 sec average), in which 300 msec CN stimulation coterminated with a 25-msec shock US, yielding an interstimulus interval of 275 msec. Each of the first three sessions was separated by 3 d to prevent tolerance to muscimol. Following the three infusion sessions all rats were given three daily sessions without infusions in order to examine the amount of savings from the initial training (phase 2). Behavioral savings observed in the muscimol group during the post-infusion sessions would indicate acquisition of associative learning during the initial infusion sessions even though performance was impaired. All rats then received a final infusion of muscimol followed by a recovery session. Following training with CN stimulation, all rats received three sessions of delay conditioning with the tone (300 msec, 2 kHz) and shock US (phase 3). Following these three sessions, all rats received an additional infusion of muscimol followed by a recovery session. A control group ($n = 5$) was given three sessions of US alone presentations (phase 1) followed by three sessions of paired training with CN stimulation (phase 2), and finally three sessions with the tone (phase 3). The US exposure group was used to determine whether muscimol inactivation of the MATN was behaviorally equivalent to presenting no conditioned stimulus during training. This is particularly important for assessing performance during phase 2 because a difference in savings between the saline and muscimol groups could be affected by US exposure, context exposure, or context conditioning in addition to differences in learning during phase 1. Daily training sessions consisted of 10 blocks of nine paired CN stimulation or tone-US presentations followed by a CN stimulation or tone alone trial. The values relayed to the computer software from the EMG integrator were voltage values of integrated EMG activity. The CR threshold was set to 0.4 V above the amplified and integrated EMG activity at baseline. The EMG baseline was typically zero. Integrated EMG responses that exceeded the threshold value during the first 80 msec of stimulus onset were considered startle or α responses; responses that exceeded the threshold value during the last 195 msec of the CS were considered CRs; responses that crossed the threshold after US onset were defined as unconditioned responses.

Histology

After training, the rats were killed with a lethal injection of sodium pentobarbital (150 mg/kg) and transcardially perfused with 100 mL of physiological saline followed by 300 mL of 10% neutral buffered formalin (Surgipath). After perfusion, the brains were post-fixed in the same fixative for a minimum of 24 h, cryoprotected in a 30% sucrose in formalin solution, and subsequently sectioned at 50 µm with a sliding microtome. Sections were stained with thionin. The locations of the cannula and electrode placements were verified using a light microscope (Leica DMLS, Wetzlar, Germany) and stereotaxic brain atlas (Paxinos and Watson 1998).

2-DG procedure and analysis

After training, some of the rats were given saline ($n = 5$) or muscimol ($n = 7$) infusions into the MATN 30 min before exposure to the CN stimulation used in training. Twenty minutes after the

infusion, each rat received an intraperitoneal injection of a glucose analog, 2-DG radiolabeled with [14]C (20 µCi/100 g of body weight; American Radiolabeled Chemicals) in 0.3 mL of sterile saline. After the injection the rats were put into the training chamber and presented with 60 CN stimulation-alone trials during the 45 min of 2-DG uptake. Rats were immediately decapitated, brains were quickly removed, frozen at −37°C, and stored at −80°C. Brains were sectioned on a cryostat at 40 µm. Serial sections were quick dried on glass coverslips, glued on poster board, and exposed to ^{14}C phosphor imaging screens for 36 h along with ^{14}C standards (Amersham Biosciences). The screens were read and digitized by a phosphor imaging system (Cyclone Storage Phosphor System; PerkinElmer Life Sciences). Individual calibration curves were calculated based on absolute gray levels of the ^{14}C standards on each film. Subsequent densitometric measures were then automatically converted to units of nanoCuries per gram (nCi/g). Region-of-interest measurements (ImageJ, NIH) were taken from the pontine nuclei to examine the secondary effects of MATN inactivation during CN stimulation. Measurements were taken from the calibrated film by tracing around each side of the pontine nuclei throughout its extent. The 2-DG techniques were similar to previous studies that examined metabolic activity of the cerebellum and MATN after inactivation with muscimol (Freeman et al. 2005; Halverson et al. 2008). This mapping technique has previously been used to identify possible metabolic differences in the cerebellum during trace and delay eyeblink conditioning, and also to explore metabolic differences in various neural structures including thalamus and auditory cortex during conditioned bradycardia (Gonzalez-Lima and Scheich 1986a,b; Plakke et al. 2007).

Acknowledgments

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