Published in final edited form as:

Brain Struct Funct. 2014 July ; 219(4): 1231–1237. doi:10.1007/s00429-013-0560-4.

GABAergic neurons in the medial septal-diagonal band (MSDB) are important for acquisition of the classically conditioned eyeblink response

J.J. Roland^{1,*}, K.L. Janke², R.J. Servatius^{1,2,3}, and K.C.H. Pang^{1,2,3}

¹Stress and Motivated Behavior Institute, NJMS-UMDNJ, Newark, NJ 07103

²Graduate School of Biomedical Sciences, NJMS-UMDNJ, Newark, NJ 07103

³Neurobehavioral Research Laboratory (129), DVA Medical Center, NJHCS, East Orange, NJ 07018

Abstract

The medial septum and diagonal band of Broca (MSDB) influence hippocampal function through cholinergic, GABAergic and glutamatergic septohippocampal neurons. Nonselective damage of the MSDB or intraseptal scopolamine impairs classical conditioning of the eyeblink response (CCER). Scopolamine preferentially inhibits GABAergic MSDB neurons suggesting that these neurons may be an important modulator of delay CCER, a form of CCER not dependent on the hippocampus. The current study directly examined the importance of GABAergic MSDB neurons in acquisition of delay CCER. Adult male Sprague-Dawley rats received either a sham (PBS) or GABAergic MSDB lesion using GAT1-saporin (SAP). Rats were given two consecutive days of delay eyeblink conditioning with 100 conditioned stimulus (CS)-unconditioned stimulus (US) paired trials. Intraseptal GAT1-SAP impaired acquisition of CCER. The impairment was observed on the first day with sham and lesion groups reaching similar performance by the end of the second day. Our results provide evidence that GABAergic MSDB neurons are an important modulator of delay CCER. The pathways by which MSDB neurons influence the neural circuits necessary for delay CCER are discussed.

Keywords

hippocampus; septohippocampal; delay eyeblink; microdialysis; acetylcholine; GAT1-saporin

Introduction

The medial septum and vertical limb of the diagonal band of Broca (MSDB) are important for learning and memory; lesions of the MSDB impair performance on a variety of learning and memory tasks (Kesner et al. 1986). Inactivation of the MSDB neurons has effects similar to lesions (Givens and Olton 1990; Mizumori et al. 1990). Given the strong connections between the MSDB and hippocampus, damage of the hippocampus generally

*Corresponding Author: Neurobehavioral Laboratory, VA Medical Center, 385 Tremont Avenue, Mailstop 129, 11-160, East Orange, NJ 07018, jessica.roland80@gmail.com.

produces similar effects to MSDB dysfunction (Gray and McNaughton 1983). However, damage of the two structures can lead to divergent results in some tasks, and delay eyeblink conditioning is an example. Understanding the reasons for the differential effects result can provide important information regarding the relationship between MSDB and its target regions, both hippocampal and non-hippocampal.

Damage to the MSDB, but not the hippocampus, impairs the delay paradigm of classical conditioning of the eyeblink response (CCER). CCER is a simple form of associative learning where a neutral stimulus (conditioned stimulus, CS) is paired with a reflex-eliciting stimulus to the periorbital muscles (unconditioned stimulus, US). In the delay paradigm of CCER, CS and US co-terminate, and the neural circuit that is necessary and sufficient involves brainstem and cerebellar nuclei (Thompson 1986; Lavond et al. 1993; Raymond et al. 1996). Damage of the hippocampus either has no effect or facilitates acquisition of delay CCER (Schmaltz and Theios 1972; Solomon and Moore 1975; Akase et al. 1989; Shohamy et al. 2000; Beylin et al. 2001; Lee and Kim 2004). In contrast, damage of the MSDB slows the acquisition of delay CCER (Moore et al. 1976; Berry and Thompson 1979; Solomon and Gottfried 1981; Solomon et al. 1983; Allen et al. 2002; Salvatierra and Berry 1989; Lockhart and Moore 1975).

The septohippocampal pathway is composed of axons from a heterogeneous population of neurons. The MSDB contains cholinergic, GABAergic, glutamatergic and peptidergic neurons (Amaral and Kurz 1985; Sotty et al. 2003; Freund and Antal 1988; Gulyas et al. 1990). Cholinergic MSDB neurons are projection cells that send axons to both the principle cells and interneurons within the hippocampus (Frotscher and Leranth 1985). In fact, cholinergic MSDB neurons are the predominant source of acetylcholine (ACh) in the hippocampus (Mesulam et al. 1983). The MSDB GABAergic population is composed of distinct subtypes. GABAergic projection cells send terminals to the hippocampus and contain the calcium binding protein parvalbumin (PV) (Freund 1989). Additionally, GABAergic interneurons make synaptic contacts with other MSDB neurons, including cholinergic neurons (Freund and Antal 1988). Less is known about the local circuitry of glutamatergic MSDB neurons; it has been suggested that they are both projection and interneurons (Gritti et al. 2006; Manseau et al. 2005). With regard to learning and memory, cholinergic neurons have been studied for many years (see Parent and Baxter 2004 for a review), but investigation of the function of GABAergic and glutamatergic MSDB is in its infancy.

GABAergic MSDB neurons may be an important modulator of delay CCER. Intraseptal scopolamine, a muscarinic cholinergic antagonist, impairs delay CCER similar to MSDB lesions (Solomon and Gottfried 1981). Moreover, scopolamine preferentially inhibits GABAergic more than cholinergic MSDB neurons (Wu et al. 2000; Alreja et al. 2000). Therefore, the indirect evidence suggests that loss of GABAergic MSDB neurons would impair acquisition of delay CCER.

We have recently used a novel GABAergic immunotoxin to examine the function of GABAergic MSDB neurons; GAT1-saporin (GAT1-SAP) combines a rabbit polyclonal antibody to the GABA-transporter-1 with the ribosomal toxin saporin. When administered

into the MSDB, GAT1-SAP reduces the number of PV-immunoreactive (ir) GABAergic neurons, while sparing cholinergic neurons (Pang et al. 2011; Koppen et al. 2012). In the present study, we used intraseptal GAT1-SAP to directly test the importance of GABAergic MSDB neurons in delay CCER.

Methods

Subjects

Male Sprague-Dawley rats were obtained from Charles River (Wilmington, DE) and housed individually with free access to food and water. They were maintained on a 12 h photoperiod with the onset of light at 0700 h. Rats were kept under these conditions for 3 weeks before the study began, and weighed an average of 375 g at the start of the study.

Surgery

Rats were weighed and randomly assigned to receive either phosphate-buffered saline (PBS) or GAT1-SAP into the MSDB. Rats were anesthetized with a Ketamine (80mg/kg)/xylazine (10mg/kg) mixture (i.p., supplemented as necessary). With a level skull, PBS (0.1M) or GAT1-SAP (Advanced Targeting Systems, 0.325µg/µl in PBS) was administered into the MS (0.6mm posterior to bregma, 1.5mm lateral to midline, 6.6 mm ventral to brain surface, needle angled 15° toward midline; 0.5µl) and each DB (0.6mm posterior to bregma, +/-0.5mm lateral to midline, 7.8mm ventral to brain surface; 0.4 µl). PBS and GAT1-SAP were injected at a rate of 0.1 µl/min. After each injection, GAT1-SAP or PBS was allowed to diffuse 5 min before removal of the syringe needle. Rats were allowed at least 2 weeks recovery before eyeblink electrode implantation.

All rats were prepared for recording eyelid electromyography (EMG) signals and delivery of electrical stimulation to the periorbital muscles (Servatius 2000). Rats were anesthetized with a Ketamine (80mg/kg)/xylazine (10mg/kg) mixture (i.p., supplemented as necessary) and then fitted with a head-stage containing four Teflon-coated stainless steel wires (A-M Systems, 75 µm diameter). The wires were threaded subcutaneously and emerged through the eyelid. Two wires were used to record eyelid EMG and the other two wires delivered electrical stimulation. Animals were allowed at least 72h to recover before eyelid conditioning commenced.

Apparatus

Eyelid conditioning was conducted in a 27cm × 29cm × 43cm sound-attenuating test chamber (Med Associates, St. Albans, VT). The EMG electrodes were connected to a differential AC amplifier equipped with a 300–500Hz band pass filter (A-M Systems Model 1700, Everett, WA). Signals were amplified 10,000X, digitized at a 1000Hz-sampling rate (National Instruments, Austin, TX) and recorded on a computer. A program written in LabVIEW (National Instruments) controlled the timing of the EMG recordings and stimulus presentations. The conditioned stimulus (CS) was a 500ms, 82-dB white noise pulse with a rise/fall of 10ms. The unconditioned stimulus (US) was a 10ms, 10-V square-wave stimulus (Bioelectric Stimulus Isolator, Coulbourn Instruments, Whitehall, PA) delivered to the periorbital muscle.

Eyeblink Conditioning

Rats are conditioned in 4 identically constructed chambers at the same time, two rats from each treatment group (PBS and GAT1-SAP). Initially, rats were acclimated to the experimental apparatus for thirty minutes and their EMG signals evaluated to determine signal quality. All rats were trained on a delay CCER paradigm for two consecutive sessions. The delay CCER training consisted of 100 trials of paired CS-US presentations with CS and US co-terminating. The intertrial interval (ITI) was 25–35s with an average of 30s.

Data processing and statistics

Only animals with accurate placement of GABAergic MSDB lesions were included in the final data analysis. All EMG data was analyzed on a trial-by-trial basis and only animals with reliable recordings across both days of training were included in the final data analysis. The EMG data were analyzed as described previously (Servatius 2000). The 250ms period prior to the CS was treated as a baseline. A confidence threshold was established for each trial as the maximum baseline EMG was added to four times the standard deviation of the baseline; EMG activity in the response windows that exceeded this value was an otherwise rare event and indicative of an eyeblink response. An eyeblink was recorded when EMG activity between CS and US onsets exceeded the threshold (Servatius 2000). Responses during the first 30ms of the CS onset were treated as orienting responses (ORs) and were not reported as CRs (eyeblinks elicited during the remainder of the CS period). ORs rarely occurred in our preparation because of the gradual rise/fall of the acoustic CS. Performance was assessed as probability of a CR being elicited for a block of 20 trials.

All statistical analyses were performed using SPSS for Windows (version 12.0.1, SPSS, Chicago, IL) with $\alpha = 0.05$. Mixed design analyses of variance (ANOVA) were used to analyze the data. F-tests for simple effects were used for post-hoc analyses. Planned comparisons were made to assess performance during each acquisition session. All data are expressed as mean $\pm$ standard error of the mean.

Histology and immunocytochemistry

At the end of behavioral testing, rats were perfused intracardially with saline followed by 10% formalin. Brains were extracted, immersed overnight in formalin followed by 30% sucrose. Brains were sectioned (50 μ m) through the MSDB. Sections were incubated in antibodies to choline acetyltransferase (ChAT; 1:500 dilution, AB144P, Chemicon International, Temecula, CA) and parvalbumin (PV; 1:1,000 dilution, P3088, Sigma Immunochemicals, St. Louis, MO) for 16 hours at 25°C, then in the appropriate biotinylated secondary antibodies (1:200 dilution, Jackson Immuno-Research Laboratory) for 2 hours at 25°C. Visualization was performed using the avidin–biotin method (Standard Vectastain ABC Kit, Vector Laboratory, Burlingame, CA) with nickel-enhanced diaminobenzidine (Horikawa and Armstrong 1988).

Unbiased stereology

The number of ChAT-ir and PV-ir MSDB neurons was estimated using standard stereology procedures (West 1999). Every third section of the entire MSDB was counted from the

anterior pole of the MS to the crossing of the anterior commissure and included the MS, vertical limb of the DB, and portions of the horizontal limb of the DB. Stereology was performed by a person blind to the treatment of the rats, using the optical fractionator method (Stereo Investigator v.7.0, MicroBrightField, Colchester, VT) on a microscope with an x-, y-, and z-axis motorized stage (Bio Point 30, Ludl Electronic Products, Hawthorne, NY). Leading edges of ChAT-ir or PV-ir somas were counted using a 40x objective lens (Carl Zeiss, NeoFluar, 0.75 NA). Cells in the uppermost focal plane (2 μ m) were not counted. The counting frame and grid size were 75 μ m $\times$ 100 μ m and 150 μ m $\times$ 200 μ m, respectively. Numbers of cells in the PBS and GAT1-SAP groups were compared using separate independent sample t-tests for each neuronal type and all data are expressed as mean $\pm$ standard error of the mean.

Results

Histology

Six rats in each treatment condition were randomly selected for stereological analysis. Representative PV-ir and ChAT-ir sections from both groups can be seen in Fig 1a. Intraseptal GAT1-SAP reduced PV-ir GABAergic but not ChAT-ir neurons in the MSDB (Fig 1b). GAT1-SAP reduced the number of PV-ir MSDB neurons by 75% (PBS: 4650 $\pm$ 540; GAT1-SAP: 1129 $\pm$ 234; t [10] = 5.98, p < 0.0001). In contrast, the reduction of ChAT-ir cells was not significant (PBS: 6290 $\pm$ 784; GAT1-SAP: 6093 $\pm$ 682; t [10] = .19). Thus, GAT1-SAP when infused into the MSDB selectively damaged PV-ir GABAergic MSDB neurons and spared cholinergic MSDB neurons.

Delay eyeblink conditioning

Intraseptal GAT1-SAP retarded acquisition of delay CCER (PBS: N = 13; GAT1-SAP: N = 14; Fig 2). The percentage of conditioned responses (CR) was analyzed in a 2 $\times$ 2 $\times$ 5 (treatment $\times$ session $\times$ trial block) ANOVA. Overall, rats acquired CCER as demonstrated by main effects of session (F [1,25] = 42.53; p < .0001) and trial block (F [4,100] = 23.09; p < .0001). The interaction of session $\times$ trial block (F [4,100] = 2.34; p = .06) trended toward significance. Intraseptal GAT1-SAP impaired acquisition of CCER, as the main effect of treatment was significant (F [1,25] = 5.01; p < .05). Moreover, the treatment $\times$ session $\times$ trial block interaction also trended toward significance (F [4,100] = 2.15; p = .08). The treatment $\times$ session (F [1,25] = 2.08), and treatment $\times$ trial block interactions (F [4,100] = .09) were not significant.

Planned comparisons were performed for each acquisition session. During the first session, significant main effects were found for treatment (F [1,25] = 6.08; p < .05) and trial block (F [4,100] = 18.14; p < .0001), but treatment and trial block did not interact (F [4,100] = 1.22). During the second session, the main effect of trial block was significant (F [4,100] = 6.52; p < .0001), but neither the main effect of treatment (F [1,25] = 1.83) nor the treatment $\times$ trial block interaction (F [4,100] = 1.02) was significant. Thus, intraseptal GAT1-SAP significantly impaired acquisition of delay CCER during the first but not second session.

Discussion

Intraseptal GAT1-SAP administration selectively reduced GABAergic septohippocampal neurons (PV-ir), while not significantly altering the number of cholinergic neurons. These results are similar to the selective GABAergic damage observed previously in the MSDB (Pang et al. 2011; Koppen et al. 2012) and BNST (Radley et al. 2009). GAT1-SAP at the same dose used in the current study reduced PV-ir and GAD67-ir MSDB neurons to similar degrees (Pang et al. 2011), evidence that GAT1-SAP does not selectively target the hippocampally projecting PV-ir GABAergic neurons.

Intraseptal GAT1-SAP treatment slowed the acquisition of delay CCER. While the overall analysis suggested a general impairment of delay CCER acquisition, visualization of the results suggests that the effects of intraseptal GAT1-SAP were predominantly during the first session with both treatment groups reaching similar performance by the end of the second acquisition session. This observation was supported by the trend of the treatment x session x trials interaction toward significance and the planned comparison analysis. This result provides the first direct evidence that MSDB GABAergic neurons importantly modulate delay CCER, a task that is not dependent on the hippocampus (Schmaltz and Theios 1972; Solomon and Gottfried 1981). Moreover, these neurons may be essential to the effects of scopolamine on delay CCER (Solomon et al. 1983), as scopolamine preferentially inhibits MSDB GABAergic compared to cholinergic neurons (Alreja et al. 2000; Wu et al. 2000).

While it is possible that intraseptal GAT1-SAP impairs delay CCER via cholinergic MSDB neurons, we think this mechanism is unlikely. GAT1-SAP did not alter the number of ChAT-ir neurons in the MSDB. Damage to GABAergic MSDB neurons with GAT1-SAP did not alter basal or exploration evoked hippocampal ACh release (unpublished observations). Moreover, the muscarinic antagonist scopolamine did not impair delay CCER when administered into the hippocampus, the target of cholinergic MSDB neurons (Solomon and Gottfried 1981). Thus, cholinergic MSDB neurons are unlikely to be involved in the effects of intraseptal GAT1-SAP on acquisition of delay CCER.

Intraseptal GAT1-SAP could interfere with acquisition of delay CCER by disrupting hippocampal activity and enhancing “noise” within the hippocampus. Systemic scopolamine impairs delay CCER and hippocampal lesions abolish this of scopolamine (Solomon et al. 1983). Manipulations that interfere with normal hippocampal activity also impair CCER (Salafia et al. 1977; Berger et al. 1980). These results suggest that abnormal activity is more detrimental to learning CCER than not having a hippocampus (Solomon and Gottfried 1981; Solomon et al. 1983).

One way that damage of GABAergic MSDB neurons alters hippocampal activity is to attenuate hippocampal theta rhythm. The presence of hippocampal theta rhythm facilitates the acquisition of delay CCER. In rabbits, hippocampal theta is predictive of faster delay CCER acquisition (Berry and Thompson 1978). Furthermore, pairing CS and US when hippocampal theta rhythm is present facilitates acquisition compared to CS-US pairing when theta rhythm is not present (Asaka et al. 2002; Seager et al. 2002; Nokia et al. 2008; Asaka

et al. 2005). Preferential damage of MSDB GABAergic neurons with intraseptal kainic acid eliminates type II theta rhythm and attenuates type I theta rhythm (Yoder and Pang 2005), and intraseptal GAT1-SAP likely does the same (unpublished observations), as GABAergic septohippocampal neurons are the pacemaker for hippocampal theta rhythm (Borhegyi et al. 2004; Simon et al. 2006; Brazhnik and Fox 1999).

However, if hippocampal theta rhythm were important for acquisition of delay CCER, it is unclear why hippocampal lesions do not show a similar retardation of acquisition as CS-US pairings during non-theta periods. It is possible that hippocampal theta rhythm is not the critical influence on CCER acquisition but that activity or oscillatory rhythm in another brain region that may be correlated with hippocampal theta rhythm is the important factor. Theta rhythm in the cerebellum can synchronize with hippocampal theta rhythm (Hoffmann and Berry 2009; Wikgren et al. 2010); CS-US pairings during cerebellar theta facilitates acquisition of CCER similar to that seen for hippocampal theta rhythm (Berry and Hoffmann 2011; Nokia et al. 2008; Green and Arenos 2007). At present, the influence of MSDB neurons on cerebellar theta rhythm is unknown, nor is the pathway by which this modulation might occur.

The MSDB could also modulate acquisition of delay CCER via non-hippocampal targets. Neurons of the medial septum make direct connections to the central nucleus of the amygdala (Volz et al. 1990; Russchen 1982), and the central nucleus of the amygdala projects to brain stem areas essential for the CCER (Hopkins and Holstege 1978; Whalen and Kapp 1991; Harvey et al. 1984). Amygdala lesions significantly slow the rate of CR acquisition depending on the conditioning parameters (Weisz et al. 1992). However, damage of the amygdala reduces the asymptotic level of learning (Blankenship et al. 2005; Sakamoto and Endo 2010), as it likely reduces the appreciation of the US (Christian and Thompson 2003; Whalen and Kapp 1991). This is a different pattern than obtained in the present study where intraseptal GAT1-SAP eventually reached the same level of performance, as sham-lesioned animals, during the second session. Thus, GABAergic MSDB lesions are unlikely to modify the acquisition of delay CCER through modulation of the amygdala.

The medial portion of the horizontal diagonal band of Broca projects to the medial prefrontal cortex (mPFC) (Gaykema et al. 1990), and lesions of the mPFC impair acquisition of delay CCER (Wu et al. 2012; Powell et al. 2005). The pattern of impairment following mPFC lesions is similar to those observed in the present study, a slowed acquisition that eventually reaches the same performance of sham lesions. However, the region of the diagonal band of Broca that projects to the mPFC is more posterior than our injection sites. Still, GAT1-SAP may impair axons projecting anteriorly to the mPFC.

In summary, intraseptal GAT1-SAP preferentially destroyed GABAergic MSDB neurons while leaving cholinergic MSDB neurons intact. Damage of GABAergic MSDB neurons impaired the initial acquisition of the conditioned eyeblink response, but did not prevent the acquisition of CCER as treated animals were able to acquire the delay eyeblink conditioned response to the same level as PBS treated sham animals with further training. Thus, GABAergic MSDB neurons are important for the initial stages of classical conditioning. The

exact targets by which GABAergic MSDB neurons modulate classical conditioning still need to be determined.

References

- Akase E, Alkon DL, Disterhoft JF. Hippocampal lesions impair memory of short-delay conditioned eye blink in rabbits. *Behav Neurosci.* 1989; 103 (5):935–943. [PubMed: 2803560]
- Allen MT, Padilla Y, Gluck MA. Ibotenic acid lesions of the medial septum retard delay eyeblink conditioning in rabbits (*Oryctolagus cuniculus*). *Behav Neurosci.* 2002; 116 (4):733–738. [PubMed: 12148941]
- Alreja M, Wu M, Liu W, Atkins JB, Leranath C, Shanabrough M. Muscarinic tone sustains impulse flow in the septohippocampal GABA but not cholinergic pathway: implications for learning and memory. *J Neurosci.* 2000; 20 (21):8103–8110. [PubMed: 11050132]
- Amaral DG, Kurz J. An analysis of the origins of the cholinergic and noncholinergic septal projections to the hippocampal formation of the rat. *J Comp Neurol.* 1985; 240:37–59. [PubMed: 4056104]
- Asaka Y, Griffin AL, Berry SD. Reversible septal inactivation disrupts hippocampal slow-wave and unit activity and impairs trace conditioning in rabbits (*Oryctolagus cuniculus*). *Behav Neurosci.* 2002; 116 (3):434–442. [PubMed: 12049324]
- Asaka Y, Mauldin KN, Griffin AL, Seager MA, Shurell E, Berry SD. Nonpharmacological amelioration of age-related learning deficits: the impact of hippocampal theta-triggered training. *Proc Natl Acad Sci U S A.* 2005; 102 (37):13284–13288. [PubMed: 16150707]
- Berger TW, Clark GA, Thompson RF. Learning-dependent neuronal responses recorded from limbic system brain structures during classical-conditioning. *Physiological Psychology.* 1980; 8 (2):155–167.
- Berry SD, Hoffmann LC. Hippocampal theta-dependent eyeblink classical conditioning: coordination of a distributed learning system. *Neurobiol Learn Mem.* 2011; 95 (2):185–189. [PubMed: 21129492]
- Berry SD, Thompson RF. Prediction of learning rate from the hippocampal electroencephalogram. *Science.* 1978; 200:1298–1300. [PubMed: 663612]
- Berry SD, Thompson RF. Medial septal lesions retard classical conditioning of the nictitating membrane response in rabbits. *Science.* 1979; 205:209–211. [PubMed: 451592]
- Beylin AV, Gandhi CC, Wood GE, Talk AC, Matzel LD, Shors TJ. The role of the hippocampus in trace conditioning: temporal discontinuity or task difficulty? *Neurobiol Learn Mem.* 2001; 76:447–461. [PubMed: 11726247]
- Blankenship MR, Huckfeldt R, Steinmetz JJ, Steinmetz JE. The effects of amygdala lesions on hippocampal activity and classical eyeblink conditioning in rats. *Brain Res.* 2005; 1035 (2):120–130. [PubMed: 15722052]
- Borhegyi Z, Varga V, Szilagyi N, Fabo D, Freund TF. Phase segregation of medial septal GABAergic neurons during hippocampal theta activity. *J Neurosci.* 2004; 24 (39):8470–8479. [PubMed: 15456820]
- Brazhnik ES, Fox SE. Action potentials and relations to the theta rhythm of medial septal neurons in vivo. *Exp Brain Res.* 1999; 127:244–258. [PubMed: 10452212]
- Christian KM, Thompson RF. Neural substrates of eyeblink conditioning: acquisition and retention. *Learn Mem.* 2003; 10:427–455. [PubMed: 14657256]
- Freund TF. GABAergic septohippocampal neurons contain parvalbumin. *Brain Res.* 1989; 478:375–381. [PubMed: 2924136]
- Freund TF, Antal M. GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Letters to Nature.* 1988; 336:170–173.
- Frotscher M, Leranath C. Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscopic study. *J Comp Neurol.* 1985; 239:237–246. [PubMed: 4044938]
- Gaykema RPA, Luiten PGM, Nyakas C, Traber J. Cortical projection patterns of the medial septum-diagonal band complex. *J Comp Neurol.* 1990; 293:103–124. [PubMed: 2312788]

- Givens BS, Olton DS. Cholinergic and GABAergic modulation of medial septal Area: effect on working memory. *Behav Neurosci*. 1990; 104 (6):849–855. [PubMed: 2178347]
- Gray JA, McNaughton N. Comparison between the behavioural effects of septal and hippocampal lesions: a review. *Neurosci Biobehav Rev*. 1983; 7:119–188. [PubMed: 6348604]
- Green JT, Arenos JD. Hippocampal and cerebellar single-unit activity during delay and trace eyeblink conditioning in the rat. *Neurobiol Learn Mem*. 2007; 87:269–284. [PubMed: 17046292]
- Gritti I, Henny P, Galloni F, Mainville L, Mariotti M, Jones BE. Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. *Neuroscience*. 2006; 143:1051–1064. [PubMed: 17084984]
- Gulyas AI, Gorcs TJ, Freund TF. Innervation of different peptide-containing neurons in the hippocampus by GABAergic septal afferents. *Neuroscience*. 1990; 37 (1):31–44. [PubMed: 1978740]
- Harvey JA, Land T, McMaster E. Anatomical study of the rabbit's corneal-VIth nerve reflex: connections between cornea, trigeminal sensory complex, and the abducens and accessory abducens nuclei. *Brain Res*. 1984; 301:307–321. [PubMed: 6733495]
- Hoffmann LC, Berry SD. Cerebellar theta oscillations are synchronized during hippocampal theta-contingent trace conditioning. *Proc Natl Acad Sci U S A*. 2009; 106 (50):21371–21376. [PubMed: 19940240]
- Hopkins DA, Holstege G. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. *Exp Brain Res*. 1978; 32:529–547. [PubMed: 689127]
- Horikawa K, Armstrong WE. A versatile means of intracellular labeling: injection of biocytin and its detection with avidin conjugates. *J Neurosci Methods*. 1988; 25:1–11. [PubMed: 3146670]
- Kesner RP, Crutcher KA, Measom MO. Medial septal and nucleus basalis magnocellularis lesions produce order memory deficit in rats which mimic symptomatology of Alzheimer's disease. *Neurobiol Aging*. 1986; 7:287–295. [PubMed: 3528890]
- Koppen JR, Winter SS, Stuebing SL, Cheatwood JL, Wallace DG. Infusion of GAT1-saporin into the medial septum/vertical limb of the diagonal band disrupts self-movement cue processing and spares mnemonic function. *Brain Structure and Function*. 2012 in press.
- Lavond DG, Kim JJ, Thompson RF. Mammalian substrates of aversive classical conditioning. *Annual Review of Psychology*. 1993; 44:317–342.
- Lee T, Kim JJ. Differential effects of cerebellar, amygdalar, and hippocampal lesions on classical eyeblink conditioning in rats. *J Neurosci*. 2004; 24 (13):3242–3250. [PubMed: 15056703]
- Lockhart M, Moore JW. Classical differential and operant conditioning in rabbits (*Oryctolagus cuniculus*) with septal lesions. *Journal of Comparative and Physiological Psychology*. 1975; 88:147–154. [PubMed: 1120791]
- Manseau F, Danik M, Williams S. A functional glutamatergic neurone network in the medial septum and diagonal band area. *J Physiol*. 2005; 566:865–884. [PubMed: 15919710]
- Mesulam MM, Mufson EJ, Wainer BH, Levey AI. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). *Neuroscience*. 1983; 10 (4):1185–1201. [PubMed: 6320048]
- Mizumori SJY, Perez GM, Alvarado MC, Barnes CA, McNaughton BL. Reversible inactivation of the medial septum differentially affects two forms of learning in rats. *Brain Res*. 1990; 528:12–20. [PubMed: 2245328]
- Moore JW, Goodell N, Solomon PA. Central cholinergic blockade by scopolamine and habituation, classical conditioning, and latent inhibition of the rabbit's nictitating membrane response. *Physiological Psychology*. 1976; 4:395–399.
- Nokia MS, Penttonen M, Korhonen T, Wikgren J. Hippocampal theta (3–8 Hz) activity during classical eyeblink conditioning in rabbits. *Neurobiol Learn Mem*. 2008; 90:62–70. [PubMed: 18294872]
- Pang KCH, Jiao X, Sinha S, Beck KD, Servatius RJ. Damage of GABAergic neurons in the medial septum impairs spatial working memory and extinction of active avoidance: effects on proactive interference. *Hippocampus*. 2011; 21 (8):835–846. [PubMed: 20865731]

- Parent MB, Baxter MG. Septohippocampal acetylcholine: involved in but not necessary for learning and memory? *Learn Memory*. 2004; 11:9–20.
- Powell DA, Churchwell J, Burriss L. Medial prefrontal lesions and Pavlovian eyeblink and heart rate conditioning: effects of partial reinforcement on delay and trace conditioning in rabbits (*Oryctolagus cuniculus*). *Behav Neurosci*. 2005; 119 (1):180–189. [PubMed: 15727523]
- Radley JJ, Gosselink KL, Sawchenko PE. A discrete GABAergic relay mediates medial prefrontal cortical inhibition of the neuroendocrine stress response. *J Neurosci*. 2009; 29 (22):7330–7340. [PubMed: 19494154]
- Raymond JL, Lisberger SG, Mauk MD. The cerebellum: a neuronal learning machine? *Science*. 1996; 272:1126–1131. [PubMed: 8638157]
- Russchen FT. Amygdalopetal projections in the cat: II. subcortical afferent connections. A study with retrograde tracing techniques. *J Comp Neurol*. 1982; 207:157–176. [PubMed: 7096644]
- Sakamoto T, Endo S. Amygdala, deep cerebellar nuclei and red nucleus contribute to delay eyeblink conditioning in C57BL/6 mice. *Behav Neurosci*. 2010; 32:1537–1551.
- Salafia WR, Romano AG, Tynan T, Host KC. Disruption of rabbit (*Oryctolagus cuniculus*) nictitating membrane conditioning by posttrial electrical stimulation of hippocampus. *Physiol Behav*. 1977; 18 (2):207–212. [PubMed: 866457]
- Salvatierra A, Berry S. Scopolamine disruption of septohippocampal activity and classical conditioning. *Behav Neurosci*. 1989; 103:715–721. [PubMed: 2765176]
- Schmaltz LW, Theios J. Acquisition and extinction of a classically conditioned response in hippocampectomized rabbits (*Oryctolagus cuniculus*). *Journal of Comparative and Physiological Psychology*. 1972; 95:322–330.
- Seager MA, Johnson LD, Chabot ES, Asaka Y, Berry SD. Oscillatory brain states and learning: impact of hippocampal theta-contingent training. *Proc Natl Acad Sci U S A*. 2002; 99:1616–1620. [PubMed: 11818559]
- Servatius RJ. Eyeblink conditioning in the freely moving rat: square-wave stimulation as the unconditioned stimulus. *J Neurosci Methods*. 2000; 102:35–42. [PubMed: 11000409]
- Shohamy D, Allen MT, Gluck MA. Dissociating entorhinal and hippocampal involvement in latent inhibition. *Behav Neurosci*. 2000; 114 (5):867–874. [PubMed: 11085600]
- Simon AP, Poindessous-Jazat F, Dutar P, Epelbaum J, Bassant MH. Firing properties of anatomically identified neurons in the medial septum of anesthetized and unanesthetized restrained rats. *J Neurosci*. 2006; 26:9038–9046. [PubMed: 16943562]
- Solomon PR, Gottfried KE. The septohippocampal cholinergic system and classical conditioning of the rabbit's nictitating membrane response. *Journal of Comparative and Physiological Psychology*. 1981; 95 (2):322–330. [PubMed: 7229163]
- Solomon PR, Moore J. Latent inhibition and stimulus generalization of the classically conditioned nictitating membrane response in rabbits (*Oryctolagus cuniculus*) following dorsal hippocampal ablation. *Journal of Comparative and Physiological Psychology*. 1975; 89:1192–1203. [PubMed: 1202093]
- Solomon PR, Solomon SD, VanderSchaaf E, Perry HE. Altered activity in the hippocampus is more detrimental to classical conditioning than removing the structure. *Science*. 1983; 220 (4594):329–331. [PubMed: 6836277]
- Sotty F, Danik M, Manseau F, Laplante F, Quirion R, Williams S. Distinct electrophysiological properties of glutamatergic, cholinergic and GABAergic rat septohippocampal neurons: novel implications for hippocampal rhythmicity. *J Physiol*. 2003; 551:927–943. [PubMed: 12865506]
- Thompson RF. The neurobiology of learning and memory. *Science*. 1986; 233:941–947. [PubMed: 3738519]
- Volz HP, Rehbein G, Triepel J, Knuepfer MM, Stumpf H, Stock G. Afferent connections of the nucleus centralis amygdalae. *Anatomy and Embryology*. 1990; 181:177–194. [PubMed: 2327598]
- Weisz DJ, Harden DG, Xiang Z. Effects of amygdala lesions on reflex facilitation and conditioned response acquisition during nictitating membrane response conditioning in rabbit. *Behav Neurosci*. 1992; 106:262–273. [PubMed: 1317182]
- West MJ. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *Trends in Neuroscience*. 1999; 22:51–61.

- Whalen PJ, Kapp BS. Contributions of the amygdaloid central nucleus to the modulation of the nictitating membrane reflex in the rabbit. *Behav Neurosci.* 1991; 105:141–153. [PubMed: 2025386]
- Wikgren J, Nokia MS, Penttonen M. Hippocampal-cerebellar theta band phase synchrony in rabbits. *Neuroscience.* 2010; 165 (4):1538–1545. [PubMed: 19945512]
- Wu G, Yao J, Zhang L, Li X, Fan Z, Yang Y, Sui J. Reevaluating the role of the medial prefrontal cortex in delay eyeblink conditioning. *Neurobiol Learn Mem.* 2012; 97:277–288. [PubMed: 22387661]
- Wu M, Shanabrough M, Leranth C, Alreja M. Cholinergic excitation of septohippocampal GABA but not cholinergic neurons: implications for learning and memory. *J Neurosci.* 2000; 20 (10):3900–3908. [PubMed: 10804229]
- Yoder RM, Pang KCH. Involvement of GABAergic and cholinergic medial septal neurons in hippocampal theta rhythm. *Hippocampus.* 2005; 15 (3):381–392. [PubMed: 15630696]

Fig 1.

(a) Photomicrographs of the medial septum and diagonal band of Broca in both MSDB sham (PBS) and GAT1-SAP treated animals. Immunoreactivity for Parvalbumin and choline acetyltransferase was used to visualize GABAergic and cholinergic MSDB neurons, respectively. Scale bar (right lower panel) = 500µm. (b) Intraseptal GAT1-SAP treatment significantly reduced the number of parvalbumin-ir neurons by 75%. In contrast, there was a non-significant (24%) reduction in cholinergic-ir neurons.

Fig 2.

Probability of a CR over time (20 trials per block; mean $\pm$ SEM) from delay eyeblink acquisition (Sham = 13; GAT1-SAP = 14) in both MSDB sham (PBS) and GAT1-SAP treated animals. Intraseptal GAT1-SAP treatment significantly impaired the acquisition of the delay eyeblink conditioned response on day 1 of testing. However, on day 2 of acquisition training GAT1-SAP treated animals reached similar performance as sham (PBS) treated animals.