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14. ABSTRACT

In this project, we have been developing a novel mouse model of MS with a focus on cognitive dysfunction and defective myelin using homologous recombination in embryonic stem cells. We obtained homologous recombinants at high frequency; however, difficulties in deriving mice from the ES cells delayed our progress. We repeated the recombination experiments and again obtained high level recombination from our screens of embryonic stem cell clones but could not derive animals from these cells. We have discontinued business with the company used for the embryonic stem cell work and we are currently testing another facility to determine if we can continue our project. Future experiments will be funded from internal funding sources to ensure that we obtain the mouse model proposed in the current project. In addition to generation of our novel model, we developed neurophysiological techniques to assess cognitive deficits in our novel mouse model. These experiments involve using the auditory pathway to test neural processing in the superior olivary complex We have published one of these studies.

15. SUBJECT TERMS
mouse model, multiple sclerosis, myelin, auditory pathway, cognition, homologous recombination, conditional knockout

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INTRODUCTION

The pathophysiology of demyelinating plaques in MS have been characterized in great detail and many of the underlying mechanisms causing physical disability in the relapsing/remitting and progressive phases of the disease have been elucidated. However, more subtle aspects of the disease which affect the day-to-day quality of life of patients have received much less attention, including cognitive and learning deficits, memory loss and difficulties with vision and hearing. This issues are of importance because they affect more than 50% of MS patients at all stages of the disease. Published clinical and case studies suggest that these symptoms stem from demyelinating/remyelinating lesions in white and gray matter regions, resulting in slower conduction velocity or intermittent conduction block through the lesions. If so, then cognitive and sensory deficits likely arise from structural abnormalities such as thin myelin, which has lower electrical resistance than normal and does not support rapid conduction velocities in small diameter axons. In this project, we will develop a knockout mouse with abnormalities in CNS myelin sheaths that mimic the electrical properties of myelin typically found in demyelinating/remyelinating MS plaques. The contributions of this dysfunctional myelin to hearing abnormalities and cognitive deficits will be determined using several electrophysiological tests.

BODY

Task 1 - Generate the targeting construct for homologous recombination

1a) Clone a LoxP site upstream of exon 1 in a 6kb fragment of the mouse Claudin 11 gene
Completed

1b) Clone a floxed PGKneo cassette into a unique restriction site downstream of exon
Completed

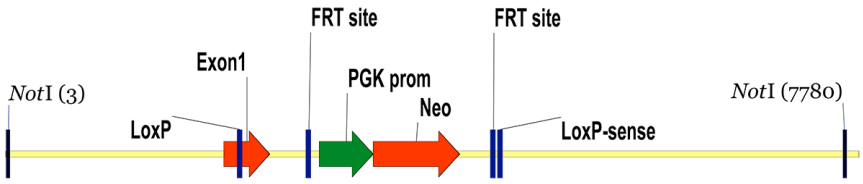

Fig. 1 – schematic of Claudin 11 knockout construct generated for homologous recombination

Task 2 - Electroporate mouse ES cells and harvest 600 neomycin-resistant clones

2a) Electroporate ES cells with the DNA construct
Completed by the Nationwide transgenic facility at University of Ohio.

2b) Select for neomycin-resistant cells and allow to form colonies in G418 selection medium
Completed by the Nationwide transgenic facility at University of Ohio.

2c) Pick 600 resistant colonies and grow in 96-well plates for PCR screening
Completed by the Nationwide transgenic facility at University of Ohio for 400 clones.

Task 3 - Genotype 600 clones by long-range PCR using TaKaRa polymerase

3a) Purify DNA from clones and perform PCR screens
Purified genomic DNA from 400 ES cell clones delivered to us by the Nationwide transgenic facility at University of Ohio. Completed TaKaRa genotyping for the 400 clones.

# ES clones harvested	# DNA clones yielding DNA	# ES cell DNAs genotyped	# ES clones correct	# ES clones expanded for blastocyst injection
400	400	400	240 (60%)	12

Task 4 - Grow all ES cell recombinants identified by PCR for Southern blotting and injections
4a) Grow ES cell recombinants in 6 well dishes sufficient for DNA testing and injections
Completed by the Nationwide transgenic facility at University of Ohio.
4b) Purify DNA from ES cell clones and run agarose gels for Southern blotting
Completed.
4c) Probe Southern blots to ensure homologous recombinants have recombined correctly
Completed.

Task 5 - Set up breeders to expand our existing CNP-Cre:FLPeR colony to generate females
5a) Set up 10 breeding pairs of CNP-Cre:FLPeR mice (C57Bl/6J) from our existing colonies
Completed.
5b) Mice are born and females are genotyped to identify the CNP-Cre transgene
Completed.

Task 6 - Inject ES cells into blastocysts to obtain highly penetrant mouse chimeras
6a) Inject the 2 best clones into blastocysts and implant into pseudopregnant dams
Completed by the Nationwide transgenic facility at University of Ohio.
6b) Mouse chimeras are born and reared to breeding age
Completed by the Nationwide transgenic facility at University of Ohio.

Task 7 - Breed chimeras to generate Cldn11+/-:CNP-Cre:FLPeR mice
7a) Set up chimera x CNP-Cre:FLPeR breeding pairs
We were not able to identify any mouse pups harboring both the *FLPeR* transgene and the homologous recombinant allele from 3 litters and 19 mouse progeny. We tested other breeding strategies but could not move beyond this point. This problem was not associated with linkage of the *FLPeR* transgene and the homologous recombination locus or toxicity associated with recombination in the *Cldn11* gene but we were unable to ascertain the problem.

Tasks 2 – 4b were repeated with new embryonic stem cells. However, rather than inject the embryonic stems cells into blastocysts, this time we performed an additional round of electroporation of 2 selected homologous recombinant clones to introduce a *Flp* recombinase plasmid into the cells. This procedure is a little faster than breeding recombinant mice with the *FLPeR* strain of mice but carries slightly higher risk of damaging the ES cell recombinants so that they do not generate germline transmission of the homologously recombined gene. We obtained 400 clones from this procedure, none of which carried the *Flp*-recombined allele. The most obvious interpretation of this result is that the company performing the work for us did not culture the cells under appropriate "non-selective" conditions; however, we could not prove this. Accordingly, we ceased doing business with the company. We are currently testing another company for the work and will continue with the project with funding from internal sources.

7b) We could not progress to this task because of the failure of Task 7a.

Task 8 - Establish and maintain breeding colony to generate Cldn11-/-:CNP-Cre mice
8a) Could not be completed due to failure of Task 7
8b) Could not be completed due to failure of Task 7

Task 9 - Perform ABR and PPI on Cldn11-/-:CNP-Cre mice and controls
To move forward with ABR and behavioral testing, we first used wild type mice to develop a novel anesthesia regimen which extends anesthesia time from 30 min to 60 min. This was necessary to accommodate the extended ABR tests we needed to perform. The major advantage of our regimen is that it does not significantly impact ABR characteristics, unlike commonly used anesthetics such as a Ketamine/Xylazine cocktail. We completed this study, which has been recently published in the Journal of Neuroscience Methods (Maheras and Gow, 2013). We did not perform PPI tests on the wild type mice because we have already established protocols for this procedure.

Second, we used our novel anesthesia cocktail to develop interaural hearing experiments and measure

binaural interactions using a relatively non-invasive procedure that allows us to test animals at multiple ages. We used wild type mice to establish this procedure and are now testing a *Cldn11* knockout compound transgenic mouse that we developed in a different project (Wu et al., 2012). Unlike the original *Cldn11* knockout mouse which is deaf (Gow et al., 2004), hearing in these compound transgenic mice has been rescued by a *Cldn11* genomic transgene (unpublished data). These mice are a poor substitute for the conditional knockout mice we have been attempting to generate in the current project but they have enabled us to perform limited neurophysiology and neurochemistry experiments [one phenotype not rescued by the genomic transgene is hind limb weakness (Gow et al., 1999), which precludes PPI experiments]. The data from these studies suggest that neural processing is perturbed in the absence of claudin 11 in myelin (Fig. 2).

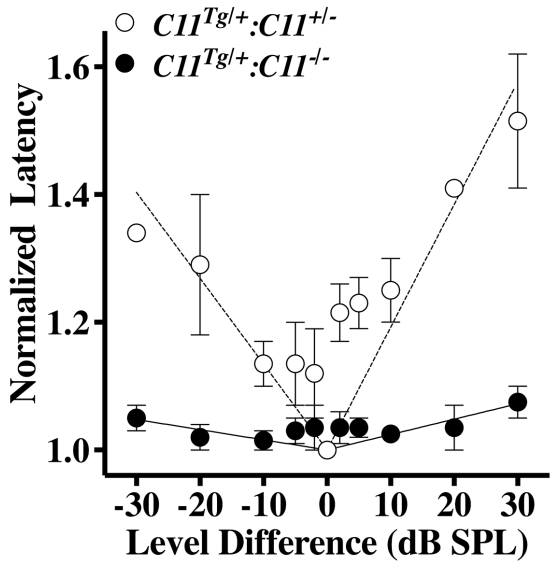

Fig. 2 – Binaural interaction component (BIC) is perturbed in the absence of claudin 11
Plot of superior olive BIC latencies from control (white circles; $C11^{Tg/+}:C11^{+/-}$) and *Cldn11* null (black circles; $C11^{Tg/+}:C11^{-/-}$) mice at six weeks of age as a function of sound stimulation level differences between the left and right ears. When left and right ears are stimulated with sound of equal intensity (i.e. $X = 0$ dB SPL) the normalized latency of the output from the superior olive is short (i.e. $Y = 1$). As sound intensity levels to each ear increasingly differ ($X < > 0$), the latencies increase symmetrically. This latency change reflects the ability to localize sound in space. For the mutant mice, the plot is essentially horizontal, indicating that these mutants cannot localize sounds in space.

A well-known characteristic of signal flow through the auditory pathway is that the latency of the signal is inversely proportional to amplitude. Thus, large amplitudes have short latencies. We can interpret the data in Fig. 1 for the control mice as revealing the strong dependence between sound intensity balance and superior olive output (i.e. short latency associated with a large amplitude), where large imbalances (i.e. -30 or +30 dB SPL) cause increases in output latency because superior olive signal output is small. In this light, the independence of the mutant mouse latencies on the sound intensity balance indicates that superior olive signal output is always maximal. These data suggest that there should be increased neural activity in the superior olive compared to controls.

Third, we performed an initial experiment by magnetic resonance spectroscopy (MRS) using an 11 Tesla magnet (500 MHz) to determine if we can observe changes in neurotransmitters and a number of other major metabolites (summarized in Table 1). Significantly increased levels ($p < 0.05$) of glutamine (+31%), glutamate (+15%), glutamate/creatine (+10%), glutamine/creatine (+28%), glutamine/glutamate (+20%), Glx (+19%), Glx/creatine (+15%), and succinate (+33%). Significantly decreased levels ($p < 0.05$) of GABA/glutamate (-19%) were found in the mutants compared to the controls.

The region-specific change in excitatory neurotransmitter signifies increased neural activity in the superior olive from the mutants. In view of the data in Fig. 2, the neurochemical changes in the mutants may be a consequence of perturbed neural processing of auditory signals and is consistent with the notion that abnormal myelin can have significant consequences for neurons and neurotransmitter systems. After these initial promising findings, we have continued to collect tissue samples for MRI neurochemistry analysis. However, the

magnet is currently under repair and progress on these experiments has stalled. Repairs are expected by the end of summer 2013.

Table 1 – neurotransmitter levels in the superior olivary complex of Claudin 11 null mice and controls

Neurotransmitter
Membrane Turnover
Energy Status
2nd Messenger
Osmolarity

Neurochemical Profile of the SOC for *Cldn11*$^{-/-}$ Mice

3°	2°	1°		Superior Olivary Complex
			Glycine	
			GABA	
			GABA/CRE	
			Asparate	
			Glutamine	
			Glutamate	
			GLU/CRE	
			GLN/CRE	
			GLN/GLU	
			GABA/GLU	
			GLX/CRE	
			GLX	
			N-AcAsp-Glu	
			Taurine	
			Betaine	
			Choline	
			Gly-P-Cho	
			GPC/CRE	
			P-Et-Amine	
			P-Choline	
			Cholines	
			CHOs/CRE	
			Creatine	
			Creatine2	
			Lactate	
			LAC/CRE	
			Succinate	
			N-Ac-Asp	
			NAA/CRE	
			NAAx	
			NAAx/CRE	
			Inositol	
			INS/CRE	
			INS/GLY	

Significant **Increase** $p < .05$

Significant **Decrease** $p < .05$

Abbreviation	Chemical
CRE	Creatine
GABA	gamma Amino Butyric Acid
GLY	Glycine
GLN	Glutamine
GLU	Glutamate
GSH	Glutathione
INS	Myo-Inositol
LAC	Lactate
NAA	N-Acetyl Aspartate
SUC	Succinate
TAU	Taurine
CHO	Choline
PCh	Phosphocholine
GPC	GlyceroPhosphocholine
CHOLINES	CHO + PCh + GPC

KEY RESEARCH ACCOMPLISHMENTS

1. Development of a novel anesthesia cocktail of 375 mg/kg avertin – 200 mg/kg chloral hydrate, which provides an anesthesia time of 60 min. Publication of this protocol showing that the anesthetic cocktail does not significantly depress ABRs in mice, in contrast to commonly used drug cocktails (Maheras and Gow, 2013).
2. Development of a neurophysiological protocol to assess neural processing of binaural auditory signals in the superior olivary complex of the mouse (Fig. 2). Preliminary findings indicate that the absence of claudin 11 in myelin of the auditory pathway perturbs bilateral auditory information processing in the superior olive and likely impairs the localization of sound in space. In humans, localization of sound in space is important for understanding complex sounds such as speech in noisy environments. Indeed, demyelinating lesions in the central auditory pathway underlies one of the common symptoms of multiple sclerosis known as the “cocktail party effect”. Accordingly, our findings lend support to the notion that myelin integrity is an important component of neural processing. These data can be extended to provide a molecular basis for anecdotal evidence in the clinical literature that abnormal myelin is commonly observed in patients with behavioral abnormalities.

3. Measurement of abnormal excitatory neurotransmitter levels in the superior olivary complex of *Cldn11* knockout mice (Table 1). Increases in the levels of glutamate and glutamine in the superior olivary complex as a result of myelin dysfunction suggest that the neurons in this region may be hyperactive. This is consistent with our neurophysiologic data showing that auditory pathway signals always maximally stimulate the superior olive.

REPORTABLE OUTCOMES

Publications:

1. Maheras KJ, Gow A (2013) Increased anesthesia time using 2,2,2-tribromoethanol-chloral hydrate with low impact on mouse psychoacoustics J Neurosci Methods, doi:10.1016/j.jneumeth.2013.07.004.

Abstracts:

1. Maheras K, Vengalil, M, Gow A (2011) Cognitive deficits associated with CNS myelin dysfunction. Great Lakes Glia, Traverse City, MI, Sept 24-26.
2. Maheras K, Gow A (2011) Cognitive deficits associated with CNS myelin dysfunction. Society for Neuroscience, Washington DC, Nov 12-16.
3. Denninger AR, Maheras K, Kirschner DA, Gow A (2011) Claudin 11 regulates the permeability of the CNS intramyelinic compartment. Society for Neuroscience, Washington DC, Nov 12-16.
4. Maheras K, Gow A (2011) Cognitive deficits associated with CNS myelin dysfunction. Am. Society for Cell Biol., Denver, CO, Dec 3-7.
5. Maheras K, Galloway M, Douglas MS, Ghoddoussi F, Gow A (2012) Cognitive Deficits Associated with Myelin Dysfunction and Neuronal Dyssynchrony. Society for Neuroscience, New Orleans, LA, Oct 13-17.
6. Maheras KJ, Douglas M, Vengalil M, Ghoddoussi F, Galloway M, Commissaris R, Perrine SA, Gow A (2013) Cognitive deficits associated with myelin dysfunction and neuronal dyssynchrony. Dept Psychiatry and Behavioral Neurosciences Alumni Symposium and Research Day, WSU, MI, Mar 1.
7. Maheras KJ, Galloway M, Ghoddoussi F, Gow A (2013) The potential relevance of myelin dysfunction on behavior. Society for Neuroscience, San Diego, CA, Nov 8 - 14.

Informatics:

1. Inducible knockout of the *Cldn11* gene in mice

Grant applications:

1. 1 F31 MH097469-01, NIH, NINDS (PI: K. Maheras) 4/1/12–3/31/14.
   Title: The role of Claudin 11 on neural processing and behavioral abnormalities
   Requested: $74,118.
   Not funded.

2. 1 F31 MH097469-01A1, NIH, NINDS (PI: K. Maheras) 9/1/12–8/31/14.
   Title: The role of Claudin 11 on neural processing and behavioral abnormalities
   Requested: $84,464.
   Not funded.

3. P.I., Research Grant, Childrens Research Center of Michigan 1/1/13–12/31/14.
   Title: Exploring the etiology of auditory processing disorder
   Requested: $100,000.
   Not Funded.

4. P.I., Research Grant, Carls Foundation of Michigan 1/1/13–6/30/14.
   Title: Exploring the etiology of auditory processing disorder
   Awarded: $50,000.
   Funded. No overlap with the current DOD project.

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1.	K. Maheras, PhD graduate student, Wayne State University	6/31/11.
2.	M. Vengalil, freshman year Princeton University, New Jersey Summer Undergraduate Research Program, Wayne State University	6/30/11–8/15/11.
3.	K. Maheras, advance to PhD candidacy status, Wayne State University	1/31/12.

Personnel receiving pay from this award:
Alexander Gow
Kathleen Maheras

CONCLUSION

Although the specific goals of our research to generate a conditional knockout of the *Cldn11* gene in mice and assess neural processing in the auditory pathway could not be completed, we have made substantial progress and completion for developing the necessary protocols to measure changes in behavior and cognition from the perspectives of neurophysiology and neurochemistry. We anticipate applying these techniques to the conditional knockout mice when we complete the task of generating them. In addition, we have been very active in collaborating with other groups to develop and analyze these mice in terms of published abstracts, student training and grant submissions.

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Gow, A., Davies, C., Southwood, C.M., Frolenkov, G., Chrustowski, M., Ng, L., Yamauchi, D., Marcus, D.M., and Kachar, B. (2004). Deafness in *Claudin 11*-null mice reveals the critical contribution of basal cell tight junctions to stria vascularis function. J Neurosci *24*, 7051-7062.

Gow, A., Southwood, C.M., Li, J.S., Pariali, M., Riordan, G.P., Brodie, S.E., Danias, J., Bronstein, J.M., Kachar, B., and Lazzarini, R.A. (1999). CNS myelin and Sertoli cell tight junction strands are absent In *Osp/Claudin 11*-null mice. Cell *99*, 649-659.

Maheras, K.J., and Gow, A. (2013). Increased anesthesia time using 2,2,2-tribromoethanol-chloral hydrate with low impact on mouse psychoacoustics. Journal of neuroscience methods *219*, 61-69.

Wu, X., Peppi, M., Vengalil, M.J., Maheras, K.J., Southwood, C.M., Bradley, M., and Gow, A. (2012). Transgene-Mediated Rescue of Spermatogenesis in Cldn11-Null Mice. Biol Reprod *86*, 131-139.

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Basic Neuroscience

Increased anesthesia time using 2,2,2-tribromoethanol–chloral hydrate with low impact on mouse psychoacoustics

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[b] Carman and Ann Adams Department of Pediatrics, Wayne State University School of Medicine, Detroit, MI 48201, USA
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HIGHLIGHTS

- Combination avertin–chloral hydrate increases anesthesia in mice to 1 h.
- Avertin–chloral hydrate anesthesia slightly suppresses ABR Wave I, II and V amplitudes.
- Avertin–chloral hydrate has minimal effects on ABR Wave I, III and V latencies.
- Avertin–chloral hydrate causes mild intestinal vasodilation.
- Avertin–chloral hydrate has low impact on mouse health in the post-anesthetic period.

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ABSTRACT

Background: To examine psychoacoustics in mice, we have used 2,2,2-tribromoethanol anesthesia in multiple studies. We find this drug is fast-acting and yields consistent results, providing 25–30 min of anesthesia. Our recent studies in binaural hearing prompted development of a regimen to anesthesia time to 1 h. We tested a novel cocktail using 2,2,2-tribromoethanol coupled with low dose chloral hydrate to extend the effective anesthesia time.
New method: We have established an intraperitoneal dosing regimen for 2,2,2-tribromoethanol–chloral hydrate anesthesia. To measure efficacy of the drug cocktail, we measured auditory brainstem responses (ABRs) at 10 min intervals to determine the effects on hearing thresholds and wave amplitudes and latencies.
Results: This novel drug combination increases effective anesthesia to 1 h. ABR Wave I amplitudes, but not latencies, are marginally suppressed. Additionally, amplitudes of the centrally derived Waves III and V show significant inter-animal variability that is independent of stimulus intensity. These data argue against the systematic suppression of ABRs by the drug cocktail.
Comparison with existing methods: Using 2,2,2-tribromoethanol–chloral hydrate combination in psychoacoustic studies has several advantages over other drug cocktails, the most important being preservation of latencies from centrally- and peripherally-derived ABR waves. In addition, hearing thresholds are unchanged and wave amplitudes are not systematically suppressed, although they exhibit greater variability.
Conclusions: We demonstrate that 375 mg/kg 2,2,2-tribromoethanol followed after 5 min by 200 mg/kg chloral hydrate provides an anesthesia time of 60 min, has negligible effects on ABR wave latencies and thresholds and non-systematic effects on amplitudes.

1. Introduction

The use of rodent models for psychoacoustic studies has increased markedly in recent decades. In contrast to clinical and research studies of audition in humans using surface electrodes, many simple animal experiments are performed using subdermal electrodes under anesthesia. The least invasive method for examining the auditory pathway is to administer a temporary anesthetic, which avoids more complex surgical procedures involving

Abbreviations: ABR, auditory brainstem response; ANOVA, analysis of variance; CH, chloral hydrate; dB SPL, decibels sound pressure level; EEG, electroencephalogram; i.p., intraperitoneal; kHz, kilohertz; PBS, phosphate buffered saline; SD, standard deviation; SOC, superior olivary complex; TBE, 2,2,2-tribromoethanol.

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the insertion of intracranial electrodes, and the subsequent complications associated with recovery and postoperative infection. Injectable anesthetics are typically administered via venous, peritoneal, muscular or subcutaneous routes depending on the differing properties of the drugs and their absorption rates (Anesthesia and Analgesia in Laboratory Animals, 2008; Flecknell, 1996). The most commonly recommended anesthetics for use in rodents include barbiturates, steroid cocktails, dissociative agents and neuroleptanalgesics.

Anesthetic drugs most commonly used for auditory studies in rodents have been limited to a combination of a dissociative anesthetic with a powerful sedative/analgesic such as ketamine/xylazine, which confers effective anesthesia over a period of 1–2 h. Alternatively, more mild fast acting drugs such as 2,2,2-tribromoethanol (Forrest et al., 1996; Gow et al., 2004; Zheng et al., 1999) have also been employed. However, when compared with psychoacoustic measures in awake mice, ketamine/xylazine anesthesia has been shown to alter waveform structure (McGee et al., 1983), increase wave latencies by as much as 1 ms, and decrease wave amplitudes up to 20% (van Looij et al., 2004). Additionally, if injected at high doses, it causes significant respiratory depression in rodents and requires prolonged post-procedure recovery (Anesthesia and Analgesia in Laboratory Animals, 2008). Similarly, volatile inhaled anesthetics such as isoflurane have been also reported to alter ABR waveform morphology, decrease wave amplitudes and increase wave latencies and hearing thresholds in rodents (Santarelli et al., 2003; Stronks et al., 2010). The centrally derived waveforms are predominantly affected in a dose dependent manner.

In contrast to such anesthetics, 2,2,2-tribromoethanol is an effective anesthesia with rapid onset and recovery times. A major advantage of this drug is its safety in rodents, with therapeutic doses far below the lethal dose. Furthermore, it neither impairs early and middle latency auditory evoked responses (Gow et al., 2004) nor significantly suppresses respiration. However, 2,2,2-tribromoethanol is a relatively short-acting drug at doses useful for psychoacoustics, which confines auditory experiments to 25–30 min in duration thereby limiting experimental complexity and the extent of data collection.

The goal of the current study has been to develop an anesthesia regimen that increases the effective anesthesia time in mice, compared to a single dose of 2,2,2-tribromoethanol, without significantly compromising the quality of ABRs acquired using subdermal electrodes. Accordingly, we compared our standard regimen of 375 mg/kg 2,2,2-tribromoethanol with a drug combination comprising 375 mg/kg 2,2,2-tribromoethanol followed after 5 min by 200 mg/kg chloral hydrate. Our data show that we can increase effective anesthesia almost 2-fold without compromising ABR quality.

2. Materials and methods

2.1. Mice

The Department of Laboratory and Animal Resources at Wayne State University maintained all mice in this study. All experiments on mice were performed in accordance with an Institutional Animal Care and Use Committee protocol approved by the Wayne State University Animal Investigation Committee. The mice used in this study were between 6 weeks and 4 months of age and were on a randomly mixed genetic background comprised of C57BL/6J and 129 Sv/Ev strains. We observed only mild age-dependent ABR threshold increases, which were consistent with previous studies on this mixed background (Gow et al., 1999, 2004).

2.2. Chemicals

A 40× stock solution of 2,2,2-tribromoethanol (Sigma, St. Louis, MO) (100% weight/volume) was prepared by dissolving 10 g of 2,2,2-tribromoethanol in 6.2 ml of 2-methylbutan-2-ol (Sigma, St. Louis, MO) and adjusting to 10 ml. The stock was aliquoted at 250 μl in 1.5 ml plastic tubes (Thermo Fisher Scientific, Waltham, MA) and stored at −20 °C. Working solutions of 2.5% 2,2,2-tribromoethanol were prepared from a fresh aliquot of stock dissolved by vortexing in 9.75 ml sterile PBS, pH 7.4 (Cellgro, Mannassas, VA). Working solutions were stored away from light at 4 °C up to 2 weeks.

Chloral hydrate (Sigma, St. Louis, MO) was prepared as a 2% working solution by dissolving 0.5 g in 25 ml of sterile PBS. Working solutions were stored sterile and away from light at 25 °C. The PBS was filter-sterilized using a 10 ml disposable syringe (Luer-Lok tip, BD Falcon, Franklin Lakes NJ) with a 0.22 μm filter attachment (Millipore, Carrighwahill, Ireland).

2.3. Anesthesia

Mice were anesthetized by i.p. injection with fresh 2,2,2-tribromoethanol in sterile PBS at a dose of 375 mg/kg (2,2,2-tribromoethanol cohort) as previously described (Gow et al., 2004), and placed on a 39 °C heating pad for experimentation. The mice were monitored for the absence of tail, foot, and ear reflexes, as well as reduced respiratory rate, which are all indications of effective anesthesia. Alternatively, mice received a combination of fresh 375 mg/kg, i.p., 2,2,2-tribromoethanol in sterile PBS followed by 200 mg/kg, i.p., chloral hydrate in sterile PBS (2,2,2-tribromoethanol–chloral hydrate cohort) 5 min after 2,2,2-tribromoethanol anesthesia was confirmed. After the completion of experiments, the mice were allowed to recover in a separate cage on a 39 °C heating pad until they regained normal activity and could be placed back in their home cage. The mice were monitored periodically in their home cage for 24 h to ensure they remained active and healthy.

2.4. Auditory brainstem response measurements

After confirming anesthesia, the fur along the top of the head and middle of the back of each mouse was sterilized/moistened with alcohol prep pads (Triad Group, Inc., Hartland, WI). Exposing the eyes to the alcohol pad was avoided. Subdermal platinum electrodes (Grass Technologies, West Warwick, RI) were then placed at the right and left mastoids (Negative), vertex (Reference) and hindquarters along the midline (Ground). Ten minutes post 2,2,2-tribromoethanol injection, and at subsequent 10 min intervals, mice were presented with 1024 pure-tone stimuli through bilateral sound tubes in 10 dB SPL decrements from 80 to 20 dB SPL at 32 kHz (102 μs duration; Blackman envelope, which is a commonly used stimulus and comprises constant rise and fall phases of the stimulus intensity of similar shape to a Gaussian curve) and a stimulus rate of 29.1/s. Both right and left ears were tested successively and ipsilateral EEGs were pre-amplified (100,000×), band pass filtered (0.3–3 kHz) and recorded for 12.5 ms (Sepwin software version 5.1, Intelligent Hearing Systems, Miami, FL).

Amplitudes of the ABR waves were measured from the first derivative of the wave peak to that of the following trough and latencies were measured from the stimulus onset to the first derivative of each wave peak. As expected, plots of ABR wave amplitude versus increasing stimulus intensity exhibited positive slopes for all mice while the slopes of latency versus stimulus intensity plots were negative. Hearing thresholds were approximated as the lowest stimulus intensity that could evoke an ABR Wave I response.

To examine the amplitudes of Waves I, III and V as functions of anesthesia time, we initially plotted absolute amplitudes. Although

we observed low inter-animal variability for the Wave I data, there was significant stochastic variability for Waves III and V that was not correlated with either anesthesia time or stimulus intensity. Alternate plots of sensation level (defined as $SL = A_{stim} - A_{thres}$, where A_{stim} is the stimulus amplitude and A_{thres} is the threshold amplitude) as a function of anesthesia time did not resolve this variability (data not shown).

2.5. Peritoneal cavity examination

Mice received two i.p. injections, 1 day apart, of sterile saline, 375 mg/kg 2,2,2-tribromoethanol in sterile saline or 2,2,2-tribromoethanol followed by 200 mg/kg chloral hydrate in sterile saline. The anesthetized mice were allowed to fully recover on a 39 °C heating pad then placed back in their home cages and monitored for 24 h. Three days after the second injection, the mice were sacrificed and their peritoneal cavities were immediately exposed and photographed (Coolpix 950, Nikon, Melville, NY) followed by a detailed analysis of the abdominal organs for signs of persistent anesthesia-induced pathology. Two independent observers blinded to the injected agent were asked to evaluate the organs using several criteria: general appearance, organ color and size relative to one another, signs of inflammation, vasodilation and the presence or absence of ascites. Eight mice were examined including two sterile PBS injected controls, three 2,2,2-tribromoethanol and three 2,2,2-tribromoethanol–chloral hydrate injected mice.

2.6. Statistics

Statistical analyses were performed using GraphPad Prism (version 5, La Jolla, CA). The latency/intensity data comparing 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate in Fig. 2 were fit with a one-phase exponential decay model. Regression coefficients, R^2, were also calculated. The data for each 10 min interval was analyzed using a one-way ANOVA with Bonferroni post hoc testing to compare matching intensities between the anesthetic treatments. In Fig. 3, normalized (to the 50 min data point) latencies and amplitudes for Waves I from each animal, and the average values from the cohort, were fit using linear regression constrained at (50,1). *F*-tests were used to identify significant deviances of the regression line slopes from zero. Similarly, in Fig. 4, normalized (to the 50 min data point) latencies and amplitudes for Waves V from each animal, and the average values from the cohort, were fit using linear regression constrained at (50,1). *F*-tests were used to identify significant deviances of the regression line slopes from zero. In Fig. 5, normalized (to the Day 0 data point) average weights were fit using linear regression constrained at (0,1). An *F*-test was used to determine if the slope of the regression line differs from zero.

3. Results

3.1. Minimal differences in ABR thresholds and Wave I latencies for 2,2,2-tribromoethanol versus 2,2,2-tribromoethanol–chloral hydrate anesthesia

To ascertain the impact of a 2,2,2-tribromoethanol–chloral hydrate anesthetic cocktail on mouse psychoacoustics, we examined several characteristic features of the ABRs (Fig. 1A). First, we show that the five characteristic peaks and troughs of the ABR arising from generators in the eighth cranial nerve, cochlear nucleus, SOC, lateral lemniscus and inferior colliculus are evoked during either anesthetic. Second, the latency of each of these waves occurs at characteristic intervals. The first wave (Wave I) arises approximately 1.5 ms post-stimulus, followed by Waves II–V at roughly 1 ms intervals thereafter. Third, we find that the latency

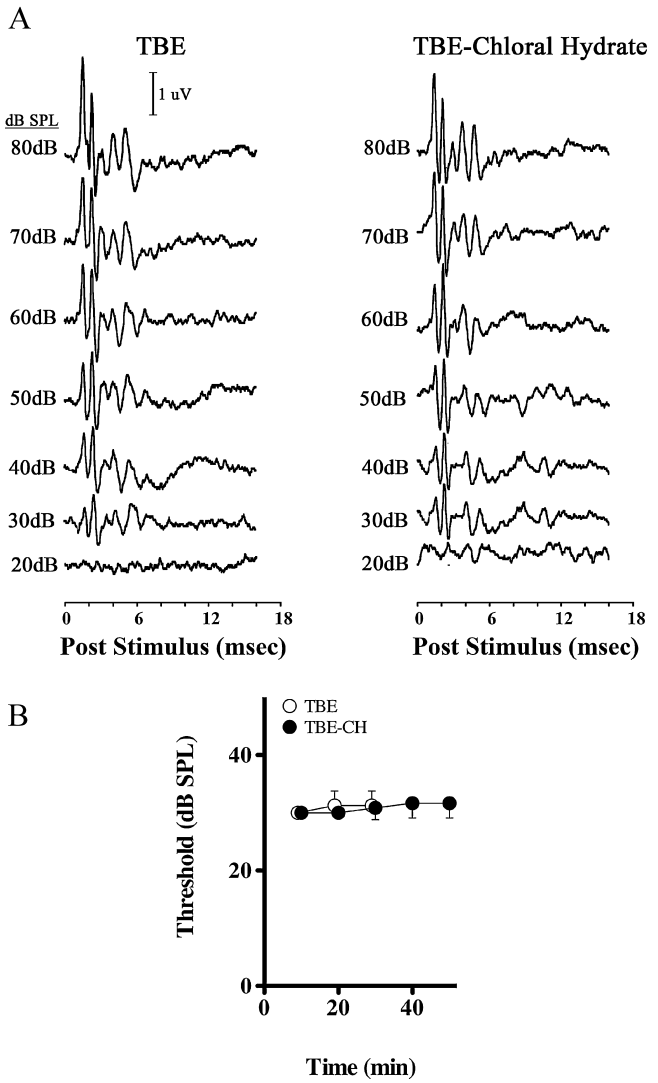

Fig. 1. Latency-intensity series of Wave I from 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate anesthetized mice. (A) Representative ABR intensity series from 2,2,2-tribromoethanol (TBE) or 2,2,2-tribromoethanol–chloral hydrate (TBE-CH) anesthetized mice measured at 10 min intervals post-injection anesthesia. (B) ABR hearing thresholds from 2,2,2-tribromoethanol (TBE) and 2,2,2-tribromoethanol–chloral hydrate (TBE-CH) anesthetized mice measured at 10 min intervals post-anesthesia.

of each wave increases slightly as the stimulus intensity is progressively decreased (i.e. ABRs are right-shifted as stimulus intensity decreases) (Table 1).

Fourth, the lowest stimulus intensity that elicits an identifiable ABR Wave I approximates the hearing threshold of the mice (Fig. 1B), the time course of which is not statistically different between mice anesthetized with 2,2,2-tribromoethanol (ANOVA; $F(2,10)$, $P = 0.5471$) or 2,2,2-tribromoethanol–chloral

Table 1
F and *P* values for one-way ANOVAs comparing Wave I latencies between the 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate cohorts (Fig. 2). No statistically significant differences are observed between corresponding intensities.

Intensity (dB)	$F(11,48)$	P
80	1.524	>0.05
70	1.560	>0.05
60	1.923	>0.05
50	2.431	>0.05
40	1.742	>0.05
30	2.468	>0.05

Table 2
Interpeak latencies of 2,2,2-tribromoethanol (TBE) and 2,2,2-tribromoethanol–chloral hydrate (TBE–CH) anesthetized mice at 10 min intervals.

Anesthesia time (min)	ABR interpeak latencies (ms)					
	V–I		III–I		V–III	
	TBE	TBE-CH	TBE	TBE-CH	TBE	TBE-CH
10	3.75 ± 0.13	3.60 ± 0.14	1.64 ± 0.09	1.64 ± 0.10	2.11 ± 0.14	1.95 ± 0.19
20	3.70 ± 0.19	3.59 ± 0.20	1.67 ± 0.07	1.64 ± 0.11	2.03 ± 0.21	1.96 ± 0.22
30	3.68 ± 0.22	3.60 ± 0.16	1.64 ± 0.05	1.69 ± 0.07	2.04 ± 0.23	1.91 ± 0.07
40		3.63 ± 0.13		1.74 ± 0.10		1.89 ± 0.11
50		3.56 ± 0.14		1.65 ± 0.09		1.91 ± 0.16

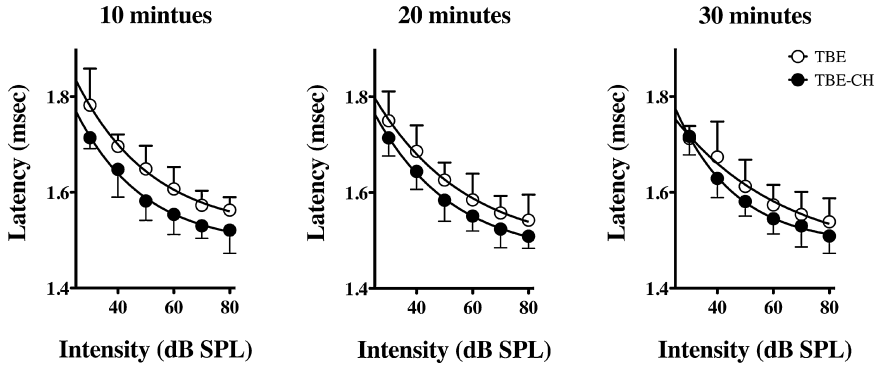

Fig. 2. ABR Wave I thresholds from 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate anesthetized cohorts. Quantified latency-intensity series of ABR Waves I under 2,2,2-tribromoethanol (TBE) or 2,2,2-tribromoethanol–chloral hydrate (TBE-CH) anesthesia at 10 min post-injection intervals. Data are plotted as mean ± SD for five mice per treatment.

hydrate (ANOVA; $F(4,25)$, $P = 0.3393$). Regression lines for these threshold series have positive slopes ($P < 0.05$); however, the average threshold change is negligible at <7%. Finally, we find no statistical differences in the Wave I latencies between the anesthesia groups at any stimulus intensity (Table 2), indicating that both drug regimens have similar initial effects on the ABRs. Moreover, we find no statistical differences from an ANOVA (Table 2) between the cohorts for interpeak latencies of the total ABR (Waves V–I; $F(5,26)$, $P = 0.91$), the peripherally derived ABR components (Waves III–I; $F(5,25)$, $P = 0.55$), or centrally derived components (Waves V–III; $F(5,25)$, $P = 0.81$).

We also compared Wave I latencies as a function of stimulus intensity at 10 min intervals between 10 and 30 min for the 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate cohorts (Fig. 2), after which the 2,2,2-tribromoethanol group regain consciousness. The average latencies of Wave I for all intensities (80–30 dB SPL) are adequately described by a one-phase exponential decay model using non-linear regression ($0.66 < R^2 < 0.83$). The regression lines for each group converge with time, but never differ by more than 4% on the ordinate axis at any stimulus intensity or time point, and the ordinate intercepts are not statistically significant at any time point ($P > 0.37$). Moreover, the Wave I latencies between the 10 and 30 min time points differ by no more than 4% within each cohort. Together, these data indicate that there are negligible differences in ABR waveforms, latencies or hearing thresholds between the treatment groups over the time course of the experiment.

3.2. ABR amplitudes and latencies are modestly altered by 2,2,2-tribromoethanol–chloral hydrate

To determine the effects of 2,2,2-tribromoethanol–chloral hydrate on Wave I amplitudes with time, we measured ABRs in anesthetized mice at 10 min intervals for 50 min at 32 kHz. Our previous studies have shown this to be the optimal stimulus frequency for hearing experiments, providing greatest amplitudes and lowest thresholds (Gow et al., 2004). Amplitudes of the waves were obtained from 80 dB SPL to approximately 30 dB SPL, depending on the hearing threshold of individual mice. At a stimulus intensity of 80 dB SPL (Fig. 3A), we observe consistent and statistically significant reductions in Wave I amplitudes ($\alpha = 0.05$; Table 3) at early time points during anesthesia, as indicated by the positive slopes of the regression lines for all mice. Regression lines at other stimulus intensities between 70 and 50 dB SPL also have positive slopes for most of the mice (Fig. 3B–D). Furthermore, the regression lines for the average amplitudes differ statistically from zero ($\alpha = 0.05$; Table 3).

Despite these non-zero slopes, the physiological effect of 2,2,2-tribromoethanol–chloral hydrate on Wave I amplitudes is relatively small. Thus, the average amplitude we observe at 80 dB SPL after 10 min of anesthesia is reduced by 19%, compared to the 50 min time point when the mice are beginning to regain consciousness. At lower stimulus intensities, average reductions in amplitudes are 12% at 70 dB SPL (Fig. 3B) and at most 6% for 60–40 dB SPL stimuli. Around the hearing threshold of 30 dB SPL, the anesthesia has little affect on Wave I amplitudes.

Similar to Wave I amplitudes, the regression lines for normalized Wave I latencies (Fig. 3F–H) are statistically non-zero for most

Table 3
Tabulated P values of normalized Wave I amplitudes from 2,2,2-tribromoethanol–chloral hydrate anesthetized mice (Fig. 2A–D). F-tests were used to identify statistically significant differences between the null hypothesis ($m = 0$), that regression line slopes do not differ from zero, for several mice (bold, italicized).

Stimulus (dB SPL)	P values, [$F(1,3)$]					
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Group
80	***0.006***	***0.004***	***0.001***	***0.001***	***<0.001***	***<0.001***
70	***0.030***	***0.005***	0.054	***0.006***	***0.009***	***0.003***
60	0.542	***0.005***	0.732	0.112	***<0.001***	0.051
50	0.060	0.051	0.139	0.588	0.137	***0.022***
40	***0.004***	0.977	***0.006***	***0.006***	***0.030***	***<0.001***
30	0.127	***0.009***	0.090	1.000	***0.010***	0.239

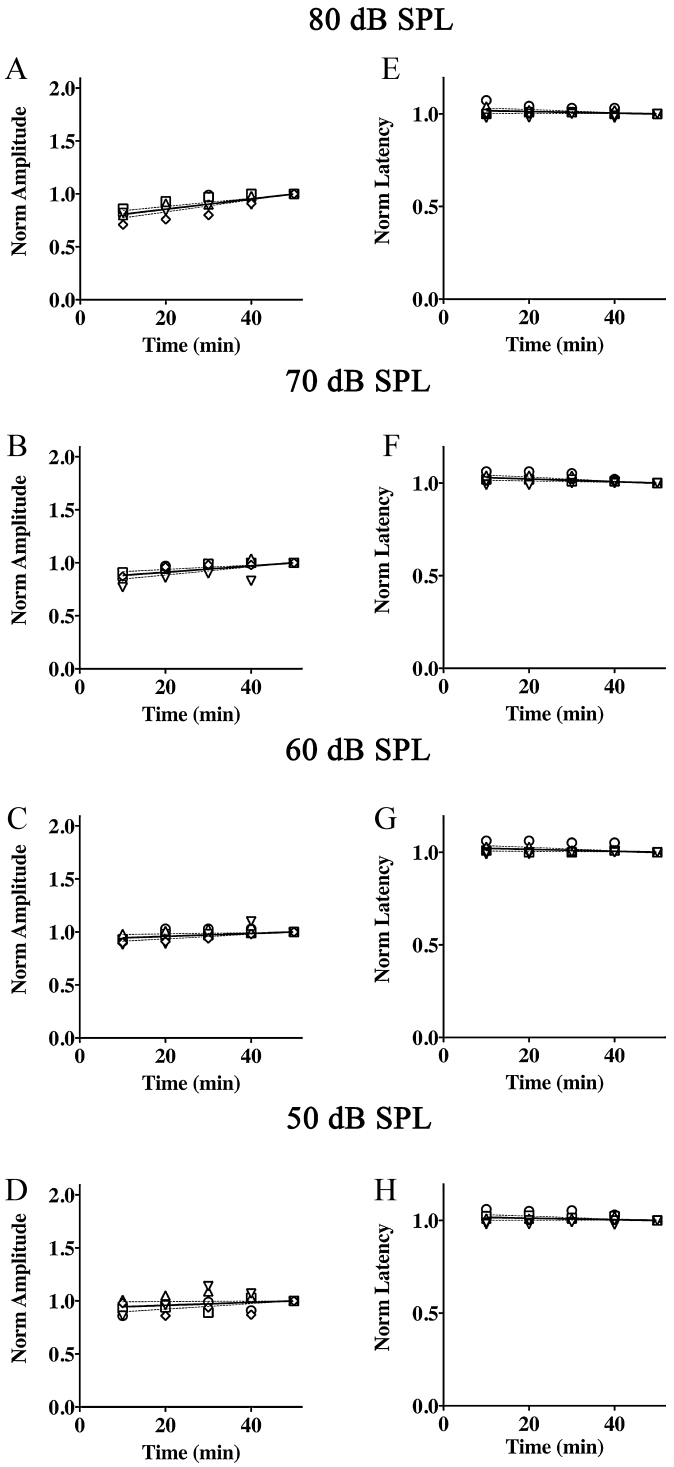

Fig. 3. Normalized amplitudes and latencies of Wave I from 2,2,2-tribromoethanol–chloral hydrate anesthetized mice. ABR threshold series were obtained at 10 min intervals during anesthesia for 50 min. Normalized Wave I amplitudes are plotted as a function of time for stimulus intensities of 80 dB SPL (A), 70 dB SPL (B), 60 dB SPL (C), and 50 dB SPL (D). The data for each mouse are normalized to amplitude at 50 min and the average linear regression line constrained at (50,1) is shown (solid line) ±95% confidence interval (dashed lines). Normalized Wave I latencies with the average regression line constrained at (50,1) is also shown (solid line) ±95% confidence interval (dashed lines) for intensities of 80 dB SPL (E), 70 dB SPL (F), 60 dB SPL (G), and 50 dB SPL (H).

Table 4
Tabulated *P* values for normalized Wave I latencies from 2,2,2-tribromoethanol–chloral hydrate anesthetized mice (Fig. 2E–H). *F*-tests were used to identify statistically significant differences between the null hypothesis ($m = 0$), that regression line slopes do not differ from zero, for several mice (bold, italicized).

Stimulus (dB SPL)	*P* values, [*F*(1,3)]					
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Group
80	***0.001***	0.166	***0.004***	0.236	0.166	***<0.001***
70	***0.001***	***0.001***	***0.002***	***0.670***	0.689	***0.002***
60	***0.005***	***0.166***	***0.001***	0.261	0.099	***0.004***
50	***0.001***	***0.010***	0.112	0.193	***0.001***	***0.005***
40	***0.046***	***0.004***	***0.003***	0.052	***0.014***	0.148
30	***0.004***	***0.031***	***0.002***	0.501	0.166	***<0.001***

individual mice ($\alpha = 0.05$; Table 4), and all of the averaged data down to the hearing threshold have negative slopes. Nevertheless the absolute changes in the latencies, which are increased at early time points, are minimal. Thus, for all stimulus intensities between 80 and 30 dB SPL, we observe an increase of 3% or less compared to the 50 min time point. Together, these data demonstrate that the 2,2,2-tribromoethanol–chloral hydrate anesthesia causes minor reductions in Wave I ABR amplitudes and negligible changes in latencies.

The size and stability of Waves I provide the greatest sensitivity for measuring changes in ABRs, but anesthetics typically have less impact on compared to subsequent waves, particularly those derived from central signal generators. Thus, to determine the effect of 2,2,2-tribromoethanol–chloral hydrate anesthesia on the major centrally derived components, we examined normalized amplitudes and latencies of ABR Waves III (data not shown) and Waves V (Fig. 4).

The Wave III amplitudes (not shown) are qualitatively similar to the Wave I data in that the slopes of the regression lines are positive; however the rates of change with time are much greater. Thus, maximal average amplitudes at 10 min are 1.5–3-fold smaller than those at 50 min, which suggests that Wave III may be strongly affected by 2,2,2-tribromoethanol–chloral hydrate. Unexpectedly, the magnitudes of the amplitude data are independent of stimulus intensity, which suggests a stochastic effect of the combination anesthesia. In contrast to the amplitude changes, the normalized average Wave III latencies are invariant with stimulus intensity or anesthesia time and the slopes of the regression lines are zero ($\alpha = 0.05$). Together, these data suggest that the Wave III amplitudes vary in a stochastic rather than systematic manner, which may argue against a strong suppression and more toward increased amplitude variability during anesthesia. Indeed, this variability is reflected in the large 95% confidence intervals in the 80–60 dB SPL data.

Fig. 4A–D shows normalized amplitudes of Waves V at 10 min intervals up to 50 min under combination 2,2,2-tribromoethanol–chloral hydrate anesthesia, and normalized amplitudes are shown from 80 to 50 dB SPL. The regression lines of the averaged amplitude data between 80 and 60 dB SPL have non-zero slopes ($\alpha = 0.05$), with maximum amplitudes of 10–21% greater than the 50 min time point. However, in contrast to the Wave I and Wave III data (Fig. 3A–D), slopes of the regression lines in Fig. 4A–D are negative, which indicates that Wave V amplitudes are supernormal at early anesthesia time points and decrease as the mice approach consciousness. In addition, the magnitudes of these changes are independent of stimulus intensity. These data suggest that 2,2,2-tribromoethanol–chloral hydrate does not depress Wave V amplitudes but rather enhances them, which differs from other common anesthetic combinations used in the hearing field such as ketamine/xylazine. However, we cannot

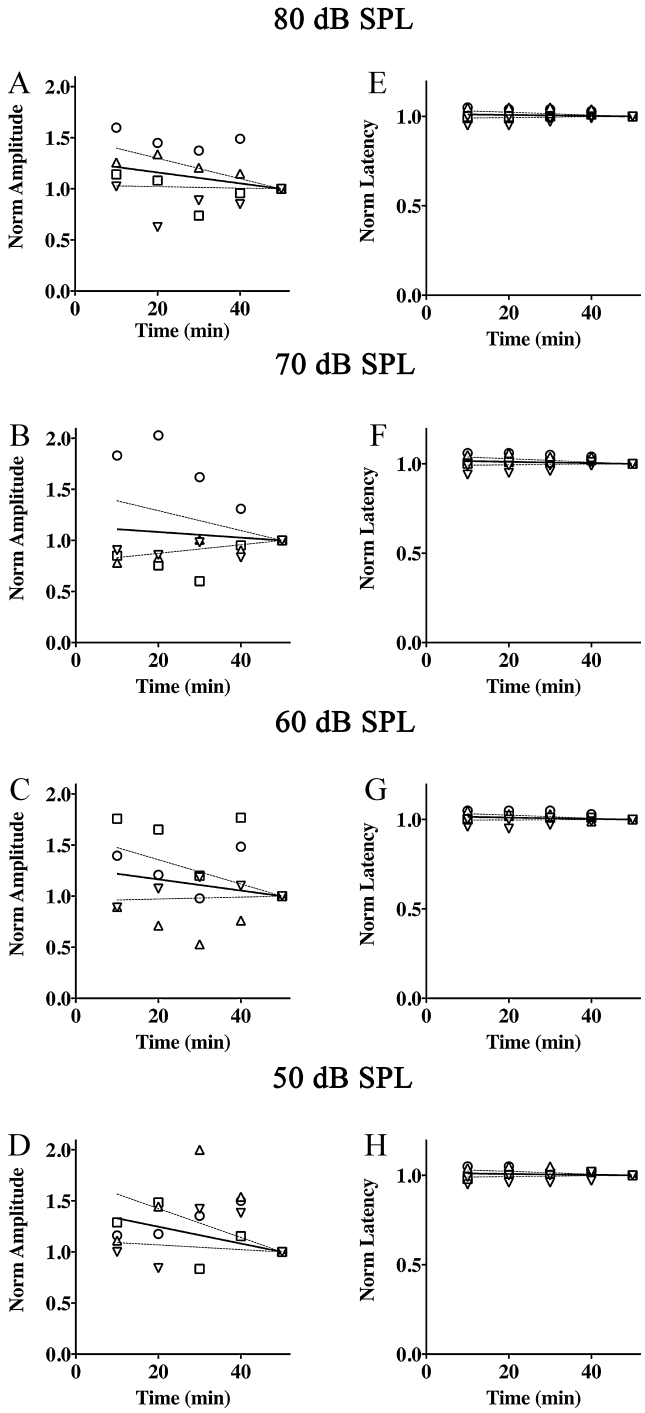

Fig. 4. Normalized amplitudes and latencies of Wave V from 2,2,2-tribromoethanol–chloral hydrate anesthetized mice. ABR threshold series were obtained at 10 min intervals during anesthesia for 50 min. Normalized Wave V amplitudes are plotted as a function of time for stimulus intensities of 80 dB SPL (A), 70 dB SPL (B), 60 dB SPL (C), and 50 dB SPL (D). The data for each mouse are normalized to amplitude at 50 min and the average linear regression line constrained at (50,1) is shown (solid line) ±95% confidence interval (dashed lines). Normalized Wave V latencies with the average regression lines constrained at (50,1) is also shown (solid line) ±95% confidence interval (dashed lines) for intensities of 80 dB SPL (E), 70 dB SPL (F), 60 dB SPL (G), and 50 dB SPL (H).

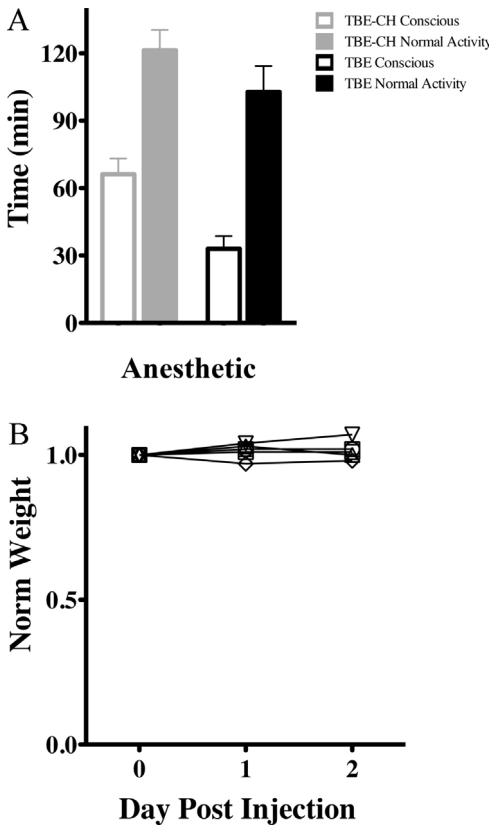

Fig. 5. Post anesthesia effect on recovery time and weight. (A) Average times for mice to become conscious from anesthesia and resume normal activity. Averages are for six mice (mean ± SD) for awake and fully recovered states. 2,2,2-Tribromoethanol–chloral hydrate (TBE–CH) anesthetic combination persists for 60 min with full recovery observed by 2 h, where as 2,2,2-tribromoethanol alone persists for 30 min with full recovery by an hour and a half. (B) The weights of six mice are normalized to their weight on the day of anesthesia. There is no statistically significant change in weight for this cohort over 3 days following anesthesia (ANOVA; $P = 0.272$).

rule out the possibility of stochastic anesthesia effects, similar to the Wave III amplitudes, because of the large variability in the amplitudes between mice.

Wave V latencies for 80–50 dB SPL are shown in Fig. 4E–H. The regression lines for the 80–60 dB SPL latencies have zero slope ($\alpha = 0.05$) while the latencies at 50 dB SPL are 3% greater at 10 min than at 50 min. These data demonstrate that there is little effect of 2,2,2-tribromoethanol–chloral hydrate on Wave V latencies even though the drug combination generally increases the amplitudes of these waves in our study cohort.

3.3. Rapid recovery of mice from 2,2,2-tribromoethanol–chloral hydrate anesthesia and normal weight maintenance

In addition to determining the effect of 2,2,2-tribromoethanol–chloral hydrate anesthesia on ABRs, we examined post-anesthesia recovery times and the safety of the anesthesia in terms of mouse weight over the subsequent 2 days. Consciousness was judged to coincide with several coincident behavioral characteristics: the reappearance of tail and ear pinch reflexes, an increase in respiratory rate and attempts to ambulate. Normal activity coincided with the reacquisition of persistent exploring, foraging, eating and drinking behaviors.

Fig. 5A shows average times taken for six mice to regain consciousness and resume normal activity in the cage following 2,2,2-tribromoethanol or 2,2,2-tribromoethanol–chloral hydrate anesthesia. Although the time to consciousness doubles from 30 min for 2,2,2-tribromoethanol to 60 min for the combination anesthetic, there is a modest 20% increase in the time to regain normal activity for the combination cocktail. Thus, the addition of

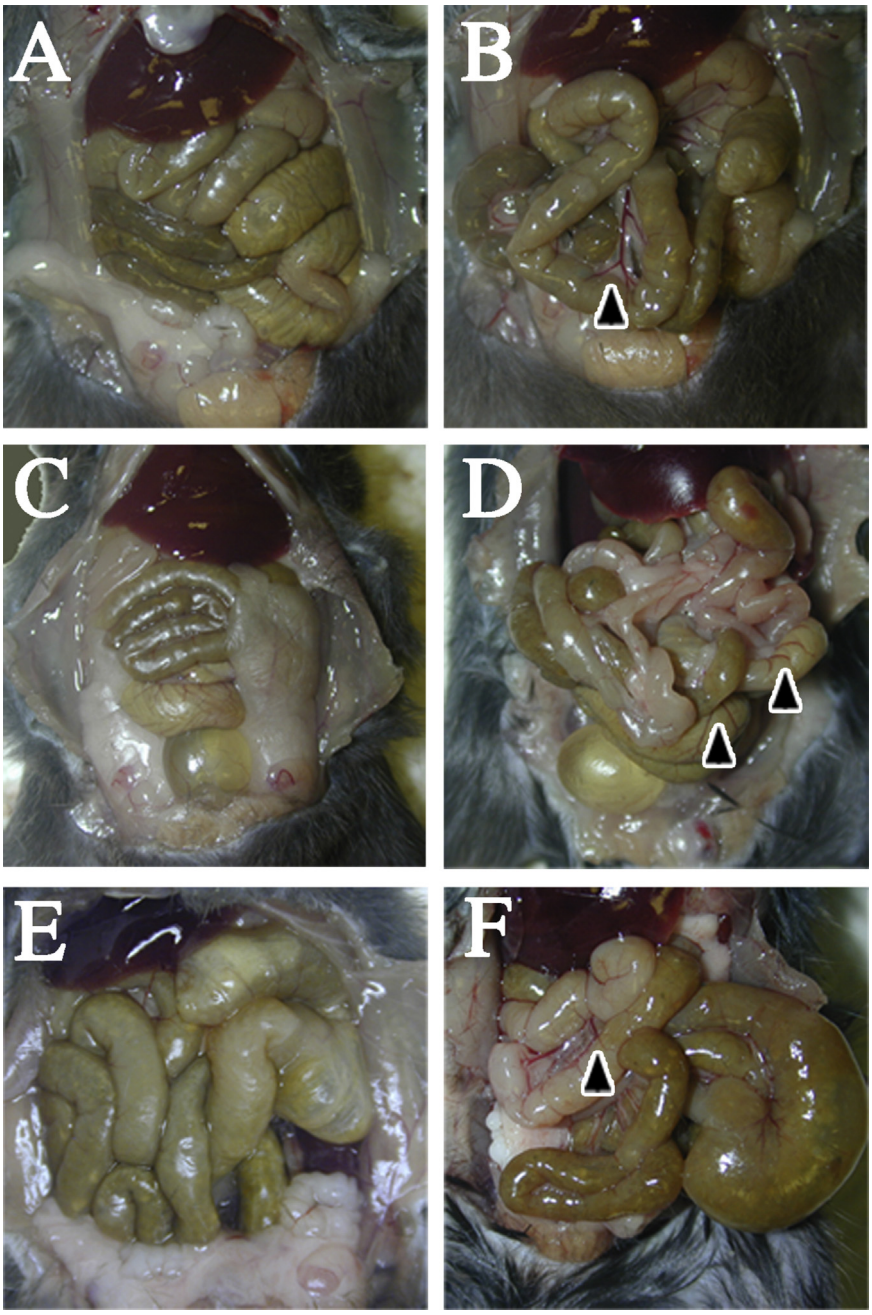

Fig. 6. Mild acute vasodilation in 2,2,2-tribromoethanol–chloral hydrate injected abdomens. Saline injected (A and B), 2,2,2-tribromoethanol injected (C and D) and 2,2,2-tribromoethanol–chloral hydrate injected (E and F) abdominal cavities post sacrifice indicate normal coloration, morphology and shape of abdominal structures. Control (B), 2,2,2-tribromoethanol injected (E), and 2,2,2-tribromoethanol–chloral hydrate injected (F) intestines upon further examination highlight differences in vasodilation along intestines and septum. Black arrowheads indicate increased vasodilation in the peritoneal cavity.

low dose choral hydrate extends anesthesia but does not markedly increase recovery time from consciousness to normal activity.

A useful indication of the short and medium term effects of anesthesia on mouse health is obtained by monitoring changes in weight during the post anesthesia period. Fig. 5B shows the weights of six animals anesthetized with 2,2,2-tribromoethanol–chloral hydrate normalized to the weight of the mice just prior to injection (Day 0). An average weight gain of 5% was observed for most mice after 2 days, and none of them lost more than 1.2% of their pre-anesthesia body weight in the post-anesthesia period. Accordingly, we conclude that the acute after effects of the 2,2,2-tribromoethanol–chloral hydrate combination is not detrimental to the normal behavior of the mice.

3.4. Mild peritoneal vasodilation following 2,2,2-tribromoethanol–chloral hydrate anesthesia

Finally, we examined the comparative medium term effects of two doses of anesthetic, 1 day apart, on peritoneal cavity appearance in mice injected with PBS, 2,2,2-tribromoethanol, or 2,2,2-tribromoethanol–chloral hydrate. Fig. 6 shows the peritoneal cavities of representative mice before (Fig. 6A, C, and E) and following (Fig. 6B, D, and F) detailed examination of the abdominal organs. Both observers were in agreement that the superficial appearance of the abdominal organs from the 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate cohorts are indistinguishable from the controls at 3 days post-injection and gut content and

color indicates that all three cohorts were actively feeding. Further, ascites is absent in all three cohorts, which suggests there is little or no inflammation, and the organs have normal size, shape and color.

The observers identified several areas of apparent vasodilation in the intestinal vasculature for both 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate cohorts compared to the controls, but the anesthesia cohorts were indistinguishable from each other. Accordingly, we observe persistent damage to the peritoneal cavity of mice following a single dose of 2,2,2-tribromoethanol or the 2,2,2-tribromoethanol–chloral hydrate cocktail, although the pathology is mild and non-inflammatory. This vasodilation likely stems from mild irritation caused by 2,2,2-tribromoethanol and/or 2-methylbutan-2-ol because of the similarity of the changes in both anesthetized cohorts.

4. Discussion

We have used 2,2,2-tribromoethanol anesthesia to examine the psychoacoustics of mice in multiple studies (Gow et al., 1999, 2004; Southwood et al., 2004) and find that this drug is fast-acting, safe and yields consistent results. Nevertheless, a single dose provides only 25–30 min of anesthesia, which limits the scope of experiments that can be performed. Our interest in characterizing binaural hearing in mice prompted a search for anesthetics that increase anesthesia time to 1 h. Rather than a major change to different drugs, which would yield results that may be difficult to compare with our previous studies, we opted to test a novel cocktail using 2,2,2-tribromoethanol as a base anesthetic overlaid with low dose chloral hydrate to extend effective anesthesia time.

Our overall goal in the current study has been to determine if the combined use of 2,2,2-tribromoethanol and chloral hydrate in mice increases the duration of effective anesthesia while minimally changing the fundamental characteristics of the centrally derived ABR waveforms. 2,2,2-Tribromoethanol at 375 mg/kg is the routine dose that we use for psychoacoustic studies (Gow et al., 2004). We have observed previously that increasing this dose significantly, for example to 500 mg/kg as used in another study (Zheng et al., 1999), increases anesthesia time but at the cost of significant reductions in the ABR amplitudes.

In contrast to combination anesthesia, we find that repeated i.p. injections of 375 mg/kg within the same testing session are impractical for several reasons. First, the difficulty in accounting for residual drug from the initial injection may increase the variability of anesthesia as well as the risk of overdosing. Second, repeated dosing may displace the electrodes and/or sound tubes, which could alter the ABR wave amplitudes. Finally, we do not observe adverse effects from single 2,2,2-tribronoethanol doses given 2 days apart for up to 1 week (over a wide age range of the mice from 4 weeks to 18 months); multiple doses per session would increase the risk of abdominal pathology and discomfort for the mice.

Herein, we demonstrate that 375 mg/kg 2,2,2-tribromoethanol followed after 5 min by 200 mg/kg chloral hydrate is safe for mice, roughly doubles the anesthesia time to around 60 min, and has non-systematic effects on the ABR amplitudes. A more reliable measure of these waves is to examine latencies, as shown in Fig. 4A–D, the regression lines for the averaged latencies have slightly negative or zero slope. Because of the tight relationship between ABR wave amplitudes and latencies, it seems reasonable to conclude that the variable amplitudes we observe stem from inter-subject effects rather than systematic suppression of the ABRs by the anesthetic combination.

Accordingly, 2,2,2-tribromoethanol–chloral hydrate anesthesia is superior to other commonly used anesthetics in psychoacoustic studies. Amplitudes and latencies of ABRs measured from awake mice are similar to the values we observed under 2,2,2-tribromoethanol anesthesia alone (van Looij et al., 2004). Other anesthetics, such as isoflurane and ketamine/xylazine, have been shown to suppress ABR amplitudes by 20% and increase wave latencies by 1 ms in rodents when compared to awake recordings (McGee et al., 1983; Santarelli et al., 2003; Stronks et al., 2010; van Looij et al., 2004). Using the combination 2,2,2-tribromoethanol–chloral hydrate anesthesia, we observe negligible increases in wave latencies and non-systematic changes in amplitude, which is a preferable and more consistent method for collecting and analyzing psychoacoustics.

We find that the timing for the administration of 2,2,2-tribromoethanol and chloral hydrate to mice is important. Initial experiments involved injecting these anesthetics in quick succession, first 2,2,2-tribromoethanol then chloral hydrate. However, this protocol leads to acute gasping and body twitching in some mice and occasionally a failure to induce effective anesthesia. However, administration of 2,2,2-tribromoethanol to rapidly induce unconsciousness at a level where reflexes are absent, followed by chloral hydrate, yields no adverse effects. Typically, we use a time delay between 2,2,2-tribromoethanol and chloral hydrate injections of approximately 5 min.

The most common side effects from i.p. injected anesthetics include transient weight loss and peritoneal irritation or inflammation (Gaertner et al., 2008). In Fig. 5B, we demonstrate using ANOVA that there are no statistically significant changes in weight for 2,2,2-tribromoethanol–chloral hydrate injected mice over the 3 days following anesthesia ($P = 0.272$). Fig. 6 shows that the peritoneal cavities of these mice sacrificed 3 days post injection with 2,2,2-tribromoethanol–chloral hydrate (Fig. 6E and F), 2,2,2-tribromoethanol alone (Fig. 6C and D) and even saline injected controls (Fig. 6A and B) exhibit mild signs of vasodilation around the intestines and normal abdominal organ color and morphology. The similar extent of vasodilation between the 2,2,2-tribromoethanol and 2,2,2-tribromoethanol–chloral hydrate cohorts suggests that chloral hydrate contributes minimally to peritoneal irritation. Finally, there is no evidence of ascites in the peritoneal cavity of the anesthetized mice, which indicates that inflammatory responses are minimal.

For decades, many studies in rodents have employed 2,2,2-tribromoethanol anesthesia because of the ease of preparation and use (Gaertner et al., 2008; Papaioannou and Fox, 1993; Weiss and Zimmermann, 1999): 2,2,2-tribromoethanol is not a controlled substance, is easily prepared, and is a safe and rapid-onset anesthetic that can be used over a wide dosage range. Anesthesia with this drug causes loss of righting reflexes, absence of responses to tail and ear pinching, muscle relaxation and decreased skeletal respiratory activity within 1–2 min (Gaertner et al., 2008; Norris and Turner, 1983; Papaioannou and Fox, 1993). However, incorrect preparation or storage of 2,2,2-tribromoethanol stocks increases the risk of mortality (Buetow et al., 1999; Gaertner et al., 2008; Weiss and Zimmermann, 1999). The decomposition products, hydrobromic acid and dibromoacetaldehyde, decrease the pH of the solution below 5 and irritate the peritoneal cavity (Buetow et al., 1999; Nicol et al., 1965; Norris and Turner, 1983; Papaioannou and Fox, 1993). To minimize complications, 2,2,2-tribromoethanol is dissolved in 2-methylbutan-2-ol and stored at 4 °C and away from light for no longer than 2 weeks (Weiss and Zimmermann, 1999).

Chloral hydrate has been used as a reliable sedative for more than a century (Gaertner et al., 2008; Hetzler and Dyer, 1984). It is often considered to be a suboptimal anesthetic for rodents because it is a poor analgesic and, indeed, most contemporary surgical procedures involve anesthetics with strong analgesic properties (Flecknell, 1996). Typical doses of chloral hydrate for rodents range from 300 to 400 mg/kg, which produces effective anesthesia for 1–2 h. A major advantage of chloral hydrate is the relatively shallow level of anesthesia conferred, as well as the relatively prolonged

effective anesthesia time achieved (Field et al., 1993; Sisson and Siegel, 1989). At doses around 300 mg/kg, chloral hydrate has been shown to preserve the EEG waveforms in rats (Sisson and Siegel, 1989) which is an important consideration for psychoacoustic studies.

5. Conclusions

Herein, we demonstrate that 375 mg/kg 2,2,2-tribromoethanol followed after 5 min by 200 mg/kg chloral hydrate roughly doubles anesthesia time in mice to around 60 min. Psychoacoustics of early latency peripheral auditory components exhibit normal latencies and attenuation of wave amplitudes by only a few percent at high stimulus intensities. The latencies of centrally derived components are unchanged, and we observe variability in the amplitudes although group averages are normal. Because of the interdependence of wave latency and amplitude, it is reasonable to conclude that the amplitude variability stems from inter-subject differences and is not a systematic effect of the anesthetic combination.

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