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THE COGNITIVE EFFECTS OF NATURALLY OCCURRING DOMOIC ACID TOXICOSIS IN WILD CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*)

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List of Abbreviations

CSF: Cerebrospinal Fluid

CSL: California sea lion

DA: Domoic Acid

LML: Long Marine Lab

MRI: Magnetic Resonance Imaging

TMMC: The Marine Mammal Center

UCSC: University of California Santa Cruz

General Abstract

Peter Cook

THE COGNITIVE EFFECTS OF NATURALLY OCCURRING DOMOIC ACID TOXICOSIS IN WILD CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*)

This dissertation comprises three separate studies making use of a unique model for conducting cognitive neuroscience in a wild population: California sea lions (CSLs) (*Zalophus californianus*) undergoing rehabilitation for toxic exposure to algal metabolite domoic acid (DA). The approach described here presents some notable advantages over typical research with laboratory animals, featuring as it does large samples of big-brained, socially complex animals with broad genetic diversity and variable life histories. The first study assessed the feasibility of using an auditory habituation measure as a behavioral diagnostic assay for identifying CSLs with DA toxicosis in the rehabilitation setting—it was found that initial responsivity to repeated auditory tones was a strong predictor of post-hoc veterinary diagnoses. The second study replicated the behavioral findings of the first, but also added structural brain imaging, allowing quantitative correlation of test behavior to volumetric measures of hippocampal and parahippocampal regions. Regional brain volumes did not predict responsivity in this study, suggesting that the behavioral results were driven by some other neurological feature of DA toxicosis, likely epilepsy. The third study compared quantitative measurements of regional temporal lobe damage to performance in a delayed alternation task and a once-daily foraging task, the latter allowing both a measure of spatial working memory and long-term allocentric spatial memory. Right hippocampal formation

volume predicted performance in all three measures. This suggests that DA toxicosis causes substantial impairments in hippocampal-dependent function, including working and spatial memory, and, further, that these cognitive mechanisms are at least partially lateralized to the right hippocampal formation. Results from these studies are used to argue for the value, both applied and theoretical, of naturalistic models for studying brain and behavior.

Dedication

To my family

Acknowledgments

The research described in this dissertation was conducted entirely between 2008 and 2012, during my time as a doctoral student at University of California Santa Cruz (UCSC). Data were collected both at The Marine Mammal Center (TMMC), in Sausalito, CA, and at UCSC's Long Marine Lab (LML). The text of this dissertation (specifically Chapter 1) includes a reprint of the following previously published material: Cook, P., Reichmuth, C., & Gulland, F. (2011). Rapid Behavioral Diagnosis of Domoic Acid Toxicosis in California Sea Lions. *Biology Letters, 7,* 536–538. One of the co-authors listed in this publication (Colleen Reichmuth) directed and supervised the research which forms the basis for the dissertation. The other co-author listed (Frances Gulland) has granted approval for this material to be used. I took the primary role on all aspects of this publication. I devised the experiment, collected and analyzed all the data, and wrote the paper.

Chapters 2 and 3 are each made up of manuscripts specifically prepared for submission to particular target journals (*PLoS One* in the case of Chapter 2, and *The Journal of Cognitive Neuroscience* in the case of Chapter 3). As such, the structure and formatting of each chapter has been necessarily constrained, and the overall dissertation does not have the stylistic cohesion it would had each chapter been written subordinate to a whole. However, given that, at the time of its completion, all parts of my dissertation have been, or will soon be ready to be, submitted to peer-reviewed journals, featuring actual manuscripts represents a pragmatic choice that was made in consultation with my committee. Brief introductions to each chapter discuss the audience for which the chapter's manuscript has

been targeted. Using concise manuscript submissions as the chapters of my dissertation necessitated some redundancy in introductory sections on the topics of DA and its particular effect on CSLs. Due to this, I've kept background discussion of DA in the following general introductory section to a minimum, choosing instead to focus on broader theoretical concerns linking my three chapters that would not be appropriate in detail in the individual manuscripts.

As must nearly always be the case with a contemporary dissertation in the sciences, the research collected here was highly collaborative in nature. It will be evident from the subsequent, and far from comprehensive, list of acknowledgements, that a great many animals, both human and non-, were integral to my work. That said, I took the primary role in designing, conducting, analyzing, and writing all research laid out in this dissertation.

Among those most worthy of note who played a role in the research collected here: My advisors, Margaret Wilson and Colleen Reichmuth, both of whom were extremely supportive of my growth as a scientist, and of my pursuing alternative and, at times, decidedly non-conventional avenues in my work; Charan Ranganath, who served on both my qualifying and dissertation committees, and who connected me to a broader world of cognitive neuroscience research that is currently under-represented at UCSC; Andrew Rouse, who first began work with me in 2009 as an undergraduate intern, and who, in 2013, has become one of my most trusted collaborators; Frances Gulland, whose unique dedication to furthering both veterinary medicine and marine mammal science paved the way for most of this work with TMMC; Bill Van Bonn, who facilitated many a CSL MRI, and

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I also gratefully acknowledge the National Science Foundation, which funded me with a graduate fellowship for three years, and the Packard Endowment for Ocean Sciences at UCSC, the Friends of Long Marine Lab, and the Earl and Ethel Meyers Oceanographic Trust, which each provided targeted funding in support of these projects. A special thanks to Ron

Schusterman, who was integral in my joining the Pinniped Lab in the first place, and who provided customarily sage feedback (and a necessary kick in the tailbone) during the instigation of data collection for what became Chapter 1. And last, but certainly not least, the many fine CSLs, both with and without DA toxicosis, who were my surprisingly willing, curious, bright, and sometimes frustrating (but nearly always endearing) research subjects for the work described here-in. If any of this research promotes CSL welfare in any small way, it will have been well worth doing.

General Introduction

This dissertation contains three chapters, each exploring the relationship between brain structure and function through a mix of imaging and behavioral approaches. My subjects in these studies were wild CSLs temporarily in captivity undergoing rehabilitation. Many of these animals were exposed in the wild to DA, a naturally occurring neurotoxin produced by algae of the genus pseudo-nitzschia, common off the California coast. DA is a glutamate agonist with high affinity for AMPA and kainate receptors. In some cases, exposure leads to DA toxicosis, which presents acutely as seizures and related neurological symptoms, and, when the initial exposure is not fatal, can progress into a chronic condition characterized by persistent epilepsy and progressive hippocampal damage (Goldstein *et al*., 2008).

These studies are linked by the research model used. They also share a broader concern: exploring an alternative approach to comparative cognitive neuroscience research. Typical laboratory animal models make use of genetically constrained subjects with highly impoverished rearing conditions. By opportunistically using wild CSLs with naturally occurring brain damage, I was able to draw large sample sizes from a genetically diverse wild population with variable life histories. This also allowed me to extend prior findings in cognitive neuroscience into an understudied species. In addition to the potential benefits of a wild model, CSLs are long-lived, big-brained, inquisitive animals with complex social and foraging behavior, and have been shown to be very amenable to behavioral study.

Summary of empirical research

Chapters 1 and 2 discuss applied attempts to develop rapid behavioral diagnostic assays for use in identifying CSLs with DA toxicosis. Such methods would be of substantial practical value given the immense effort expended in treating stranded CSLs and the prior difficulty of developing a rapid, effective, reliable assay for toxic exposure to DA. The first two chapters also provide insight into the relationship between behavioral habituation, hippocampal damage, and epilepsy. Chapter 3 discusses findings linking quantitative assessments of brain damage with fine-scale behavioral measures of memory obtained with relatively rapid remote training methods. The research in Chapter 3 was conducted with dual purpose—first, to provide quantitative assessments of impairment from DA toxicosis, and second, to explore a novel, humane model for studying the cognitive effects of hippocampal damage.

The behavioral diagnostic measures described in Chapters 1 and 2 involved habituation and dishabituation of an orienting response (*i.e.,* "responsivity") to repeated auditory cues. Orienting behavior is easy to elicit in untrained animals, easy to measure with simple tools and analyses, and linked via prior research to the hippocampus (Stoppel *et al*., 2009; Honey *et al*., 2007; Yamaguchi *et al.,* 2004; Sokolov, 1990). In the manuscript making up Chapter 1, I reported that *post-hoc* veterinary diagnoses (established following each subject's rehabilitation and final disposition) were strongly predicted by slowed habituation of an orienting response (*i.e.*, increased or extended responsivity) to a repeated auditory tone. In the work reported in Chapter 2, I replicated the initial finding in Chapter 1, but also added hippocampal and parahippocampal volumes (as measured by volumetric tracing from MRI)

as independent measures. In contrast to prior work with this population of animals using solely qualitative assessments of brain damage (*e.g.*, Thomas, Harvey, Goldstein, Barakos, & Gulland, 2009), the volumetric measurements of the hippocampus and parahippocampus used in the present studies allowed for a nuanced comparison of behavioral measures to damage in relevant brain regions. Neither hippocampal volume nor parahippocampal volume reliably predicted the habituation rates reported in Chapter 2. The absence of strong correlations suggests that some other factor affecting *post-hoc* diagnoses, but not directly dependent on extent of brain lesions, may explain the strong relationship between habituation rates and diagnoses. Possibilities are discussed in Chapter 2. Of note, these two studies (comprising Chapters 1 and 2) strongly indicate that a simple and efficient behavioral metric based on observed habituation of an orienting response to repeated auditory cues could be of real use for aiding diagnosis of DA toxicosis in a veterinary setting.

In Chapter 3, the behavioral measures employed were more complex and required laboratory testing over a period of weeks. The tasks were delayed alternation in a two-choice maze, which has been shown to be highly sensitive to hippocampal damage (Ainge, van der Weer, Langstrom & Wood, 2007; Bannerman *et al*., 2001; Maruki, Izaki, Hori, Nomura, & Yamauchi, 2001; Aggleton, Neave, Nagle, & Hunt, 1995; Olton, 1986), and a once-daily foraging task similar to certain applications of two commonly used rodent mazes, the Morris water maze (Morris, 1984) and radial arm maze (Olton, 1997). The two behavioral tasks used here were selected because they are reliant on hippocampal mechanisms that have been localized to the CA3/CA1 axis (the most reliable location of damage with DA toxicosis (Goldstein *et al*., 2008)). Delayed alternation is likely dependent

on an animal's ability to accurately represent recent events and their sequence, which should be hippocampal-dependent (Eichenbaum, Sauvage, Fortin, Komorowski, & Lipton, 2012), while across-session latency in the foraging task, in which subjects must attempt to recall a previously rewarded location on successive days, is likely dependent in part on allocentric spatial memory (Paul, Magda & Abel, 2009; Squire, 1992). A separate measure in the foraging task—the number of incorrect revisits to non-baited locations within a testing session (that is, within-session errors)—should be dependent on spatial working memory (Wilkerson & Levin, 1999). The research described in Chapter 3 compared behavioral results to hippocampal formation volumes determined from MRI, as in Chapter 2. The results showed that right hippocampal volume was a significant predictor of performance in the delayed alternation task, and across-session latency and within-session errors on the once-daily foraging task, while left hippocampal volume was not.

Although I believe each chapter of my dissertation stands on its own as a valuable empirical contribution, and they collectively contribute to understanding of the impact of DA on CSLs and other marine life, the work discussed herein is also unified by a far-reaching theoretical concern with research methodology, a concern that served as one of my primary motivators in embarking on this series of experiments. By studying large, wild animals with naturally occurring brain damage, I was able to make use of a unique model for cognitive neuroscience research. The ongoing, apparently unavoidable environmental exposure of CSLs to a potent neurotoxin allowed me to establish an alternative to more traditional approaches using small, shorter-lived laboratory animals. I'll argue here that there is inherent value in exploring such alternative models.

Common Laboratory Models for Behavioral Neuroscience Research

While cognitive neuroscience is, as a field, concerned with broadly applicable explanations for how the brain acts across different levels of organization to produce behavior in diverse groups of human and non-human animals, the vast bulk of the non-human experimental literature in cognitive neuroscience is solely dependent on artificially constrained models (predominately rodent). In this, cognitive neuroscience follows in the larger footsteps of biomedical research more broadly, in which the traditional approach relies on strains of animals that have been inbred over generations to produce behaviorally tractable animals with limited genetic variability (Wahlsten, 1972). The most commonly used strain of research animal is the "Black 6" mouse, which has been bred for laboratory use over the past century, and was the first non-human species to have its genome fully sequenced (Battey, Jordan, Cox, & Dove, 1999). More recently, heavily funded international initiatives (such as the International Mouse Phenotyping Consortium (IMPC) supported in part by NIH) have enabled researchers to access "bespoke" mice, with particular genes disabled *in utero*, or "knocked out." Traditionally, experimental psychology studies have relied more heavily on rats (and to a lesser extent monkeys) than mice, but these models are similarly constrained (Bodkin, Alexander, Ortmeyer, Johnson, & Hansen, 2003; Watkinson & Gordon, 1993). Given the better characterization of the mouse genome, many neuroscientists have now begun adopting mouse models (Deacon, Croucher, & Rawlins, 2002).

With the genetic variability of primary laboratory species brought under nearly total control, the other notable source of potential behavioral variability comes from environmental

interactions during development. To control for this, individual research subjects used for neuroscience and biomedical research are typically raised in impoverished environments, which are thought to limit variability from ontogenetic influence (Lewejohann *et al*., 2006; Gartner, 1999; Eskola, Lauhikari, Voipio, Laitinen, & Nevalainen, 1999; although see Baumans, 1997).

The high degree of genetic and behavioral control afforded by the dominant mouse model (and similar models more commonly used in neuroscience featuring rats and rhesus monkeys) allows for careful experimental control. Further, the replicability, accessibility, and relative low cost associated with this approach have all contributed to massive research productivity. The value, both realized and potential, of such a carefully managed and expedient research model should not be understated. It is in large part the functionality of the mouse model that has led to its supplanting nearly every other animal model commonly used in biomedical research (Sixth Report…, 2010).

Concerns With Common Laboratory Models

Despite the myriad benefits of traditional laboratory models (including as used in neuroscience), there is increasing support for the position that they go too far in limiting subject variability, in terms of both genetics and behavior. Indeed, the idea that one genetically and developmentally controlled animal might effectively stand in for members of any other species has been widely accepted *de facto*, with remarkably little critical assessment, and in the face of meaningful evidence to the contrary. By drastically under-

representing real-world behavioral and biological variation within research subjects, relevant phenotypic idiosyncrasies are unaccounted for. Indeed, emerging evidence suggests that the mouse model problematically discounts meaningful variability, limiting applicability of some results. In one recent and high-profile example at the intersection of the bio- and neurosciences, the use of mouse models for studying pain has been called into question. Research into nociception had already indicated that mechanisms for pain susceptibility vary markedly across different mouse species (LaCroix-Fralish, Austin, Zheng, Levitin, & Mogil, 2011), and even between sexes in the same strain (Mogil *et al*., 1997). Despite this, the large majority of pain research has been conducted on Black 6 mouse males. A recent high-profile report (Seok *et al*., 2013) suggests that the mouse model of inflammation, regardless of strain used, is manifestly inapplicable to humans. This is just one example highlighting the degree to which quite basic biological features may differ between animal strains and species.

Some limits of common laboratory models may be particularly relevant in the field of cognitive neuroscience. A raft of new evidence indicates subjects bred and raised in typical laboratory conditions may have persistent cognitive abnormalities and deficits across a wide range of domains in comparison to animals with more enriched environments during development and less constrained genetic profiles (Abolins, Pocock, Hafalla, Riley, & Viney, 2011; Martin, Ji, Maudsley, & Mattson, 2010; Kikusui, Nakamura & Mori, 2008; Laviola, Hannan, Macri, Solinas & Jaber, 2008; Balcombe, 2006; Wahlsten *et al*., 2003; Fernandez-Teruel, Escorihuela, Castellano, Gonzalez, & Tobena, 1997). Some degree of skepticism should be leveled at strong claims about cognitive and behavioral effects predicated on

results from models with atypical and uncharacterized cognitive deficits. Note that similar concerns have been raised regarding cognitive neuroscience's other dominant research animal: the undergraduate student (see Henrich, Heine, & Norenzayan, 2010 and accompanying peer commentary). The lack of diversity in undergraduate research subjects is increasingly acknowledged to be manifestly problematic for the generalization of findings, and yet this subject pool offers a far more diverse population than common laboratory animals, both genetically and developmentally. Calls for increased diversity in human subjects have been echoed, although less forcefully, by those agitating for more diversity in animal research subjects (*e.g.*, Hunter, 2012), but there has been little united effort on this front.

In brief, as more broadly in the biosciences, most animal models used for neuroscience research are heavily constrained, potentially undermining the generalizability of some findings. Alternative animal models for neuroscience research may better represent real-world variability, and may feature animals with more species-typical developmental courses, allowing for more meaningful representations of general brain function and behavior across diverse populations. It is true that, for many research questions, one might be reasonably satisfied that the traditional animal models are sufficient to provide an answer, despite their limitations. Even in these cases, alternative models allow for meaningful replication and confirmation of previous data gathered using traditional approaches. Potential benefits are discussed in more detail below.

Benefits of Alternative Models for Cognitive Neuroscience

1. Diversity

From the empirical standpoint, their allowance for a realistic representation of population diversity is one of the most compelling arguments for alternative animal models for research. More diverse populations of research animals (both genetically and in terms of developmental course) will better represent the diversity in wild populations (including humans). Strong findings drawing on more representative models will tend to be more broadly applicable.

Of course, this increased diversity will, by necessity, be coupled with increased "noise" in the data. This may require alternative models that allow for larger sample sizes, or increased focus on replication. Alternatively, the noise itself may be of value, as increasingly recognized by psychologists interested in individual cognitive and behavioral variability in humans (*e.g.*, Kanai & Rees, 2011; Vogel & Awh, 2008) and non-human animals (*e.g.*, Biro & Stamps, 2010; Koolhaas, Boer, Coppens, & Buwalda, 2010). If one accepts the possibility that an experimental treatment might interact with the diversity of the population of interest, then much of the "noise" in data acquired from more variable subject pools may be of great interest. Put simply, the difficulty of accounting for variability in experimental design is not, in-of-itself, an acceptable rationale for disregarding variability in a research population.

It is true to an extent that general brain anatomy and physiology are heavily conserved within mammals, and thus, in many cases, likely quite consistent across diverse populations (Aboitiz & Montiel, 2012). However, the linkages from brain to behavior can be quite

idiosyncratic, both intra- and inter-population, and thus are likely underrepresented by traditional animal models. For example, prefrontal dopaminergic circuits are involved in cognitive and behavioral control in all mammals studied, but the relative "tuning" of these circuits in different individuals can produce drastically different responses to similar stimuli (Cools, 2008). One must also be wary of generalizing findings in one species to another, even closely related, species. For example, despite largely conserved function, some behavioral tasks that are heavily dependent on hippocampal formation in humans may not be at all in non-human primates and other animals (Squire, Wixted, & Clark, 2007). This finding does not suggest that the hippocampus functions in a drastically different manner in humans and other primates; rather, it suggests that seemingly identical behavioral tasks can be processed in quite different ways in different species depending on a great many factors.

Aside from general issues of under-representing genetic and behavioral variability, there is further reason to be skeptical of laboratory models' ability to stand in for wild populations. There is evidence showing markedly different behavioral patterns in wild and domesticated exemplars of even the same or closely related species (*e.g.*, Holmes, Parmigiani, Ferrari, Palanza, & Rodgers, 2000; Blanchard, Flannely, & Blanchard, 1986). Measurable differences in the brains of wild and captive animals have also been measured in a number of species, including rats (Kruska, 1988), pigs (Plogmann and Kruska, 2008) and foxes (Trut, Oskina, & Kharlamova, 2009). The very breeding that makes laboratory animals tractable may lessen their value as model species.

In brief, the extent to which findings from traditional laboratory models can be reasonably generalized to other cases should not be taken for granted. Inferences from pre-existing behavioral data across species may be expedient, and, in certain instances parsimonious, but parsimony is a poor substitute for obtaining representative data when possible.

2. Normative Development

While developmental course contributes to population-level diversity, as mentioned above, some degree of environmental enrichment appears necessary in many species to allow individuals to develop typical cognitive functionality. Alternative models that allow for a more typical, or at least less restricted, course of development than in standard laboratory situations will tend to produce research subjects with more fully developed faculties that are more representative of the full range of function found in wild populations (including humans). Substantial evidence indicates that environmental enrichment during development broadly enhances cognitive capability (see Leggio *et al*., 2005 for review). Given that the bulk of studies showing the cognitive benefits of enrichment use traditional laboratory models, such findings might be more accurately framed as demonstrating partial mitigation of the atypical cognitive deficits caused by impoverished rearing environments, rather than cognitive enhancement. Environmental enrichment has also been shown to increase resilience and lead to conserved cognitive function following neural insult (relative, again, to laboratory animals' likely reduced baselines) (Pereira *et al*.2007). Findings such as these call into question how broadly representative the cognitive faculties of traditional

laboratory animals are, and also challenge the generalizability of patterns of impairment following neural insults in these animals.

Of course, none of these points should come as a surprise to psychologists, as there is extensive evidence that early experience actively shapes the brain, privileging certain connections and leading to the preferential pruning of others (O'Leary, 1992). This means that, in a very real sense, early experience can fundamentally alter the structure and function of the brain. In an early, extreme example of this phenomenon, cats raised without any experience of vertical lines were not, in a real sense, subsequently able to see them (Blakemore & Cooper, 1970). More subtle effects can also be produced with less drastic developmental restrictions. For example, social isolation during development in rats leads to alterations in frontal and hippocampal function, and may affect mood (Lapiz *et al*., 2003). Put more informally, an animal's early experience literally shapes its brain and constricts later cognitive functionality.

The impact of environment on the brain extends beyond early ontogeny as well—a large body of work indicates that relatively small changes in environmental enrichment can have quite strong, short-term effects on behavior, either negatively or positively (Mohammed *et al*., 2002). Similarly, a growing body of work indicates that enrichment can have rapid and dynamic effects on neurogenesis in adults, particularly in the hippocampus (Kemperman, Khun, and Gage, 2009).

3. Flexibility in Selecting a Model

Aside from questions of variability and validity, alternative models allow one to tailor a research approach to the hypothesis being assessed. For example, a dog is likely more amenable to the training required for unrestrained magnetic resonance imaging (MRI) than is a mouse (Berns, Brooks, & Spivak, 2012). Mice are more amenable to maze-based spatial testing than are most primates (Murray, Davidson, Gaffan, Olton, & Suomi, 1989). Primates are obviously a better choice for testing involving fine-grained motor manipulations than are dogs (*e.g.*, Isomura, Harukuni, Takekawa, Aizawa, & Fukai, 2009). When research models becomes as pervasive and all-encompassing as those predominately used in cognitive neuroscience, there is a real danger of restricting the questions asked to those that the models can answer—this can happen because the model is what is available to most researchers, but can also result from funding sources being unwilling to support non-traditional approaches (as dissussed in Nicholson & Ioannidis, 2012).

Developing Alternative Animal Models

It is of course well and good to trumpet the value of alternative models, and, increasingly, even scientists firmly embedded in the establishment give some credence to related concerns (see, for example, new funding initiatives, like the National Science Foundation's program to support "transformative research"). But the next step is to identify and develop particular alternative models and demonstrate some degree of viability there-with. In the most basic sense, this might involve merely adapting other species with desired characteristics to the mold established by the mouse model, as has been done in cognitive

neuroscience with prairie voles to study autism (Curtis, Hood, Chen, Cobb, & Wallace, 2010) and finches for the study of vocal flexibility (Scharff & Nottebohm, 1991). Such taxonomic adaptations might address the need for animals with different neurocognitive makeup, but will generally not address worries about genetic diversity and developmental course. There is also the risk that adapting a species to the traditional laboratory model may alter the features that first made them appealing alternatives.

The approach I followed in conducting the research contained in this dissertation, and the approach I advocate for here, is seeking out and developing natural models for cognitive neuroscience research. No matter how much might be learned from controlled laboratory populations, they do not fully represent wild populations. There are, of course, logistical hurdles to the use of wild populations in research. Many wild populations simply aren't accessible. Some species are carefully protected by laws far more strict than those governing the treatment of laboratory rodents. Many wild animals are difficult to work with. Even when accessible, many of these species are large, expensive, potentially dangerous, and there aren't always well established husbandry and care practices. Despite these hurdles, there are a number of promising populations that are accessible, and could, with the appropriate research partners, be handled lawfully, humanely, and safely. A cursory broad catalogue might include: most animals undergoing rehabilitation, animals being managed in wild or semi-wild habitats, and recent acquisitions from the wild at zoos and aquaria. Animals from these populations will have relatively broad genetic variability and broadly typical and rich developmental courses. Further, a meaningful subset of many if not most wild populations will have been naturally exposed to a variety of agents that can

differentially affect the brain and cognition. In such cases, one can conduct research with a wild population that has been, in essence, naturally sorted into control and experimental groups. There is no shortage of natural phenomena that cause predictable changes to the brain, *e.g.*, PCBs (Boucher, Muckle, & Bastien, 2009), lead (Yorifuji, Debes, Weihe & Grandjean, 2011), and parasitic toxoplasmosis infection (Gulinello *et al*., 2010). There are a host of potential models that have yet to be explored or exploited.

Sea Lions With Domoic Acid Toxicosis as an Alternative Research Model

As discussed previously, the research described in this dissertation features a novel animal model: wild CSLs with naturally occurring exposure to DA and resultant hippocampal damage. The approach I've taken represents one feasible alternative to the use of more traditional laboratory approaches, and does not suffer from the core deficiencies of common animal models discussed above. Because my subjects were drawn from a wild population, they presented with broad genetic diversity and variable life histories; thus, my results should not be confounded by lack of representative phenotypic variability, and, aside from the toxic exposure (which is the primary independent variable in this research), my subjects should have had a broadly species-typical course of neuro-development. Of course, as previously noted, this likely means the resultant data was somewhat "noisy." This was of limited concern in the present research as, through collaboration with veterinary scientists, rehabilitation workers, ecologists, and researchers in human psychology and neuroscience, I was able to collect a large body of data, both in behavior and brain imaging, on over 100

animals. In addition, I was able to ask a series of targeted questions using this research model, regarding both the particular impact of DA on marine life and the role of the hippocampus in supporting specific types of memory.

There are also focused benefits to studying wild animals with naturally occurring neurotoxic exposure. To wit, my research provides detailed, directly applicable information regarding the impact of DA on a heavily affected population. This can be invaluable to veterinarians, conservationists, and rehabilitation workers in making informed decisions in managing these animals. Much as one would need to characterize the effect of a hepatotoxin on liver function to understand its impact on an exposed population, understanding the behavioral sequelae to neurological damage is a necessary step in describing and addressing its negative effects. A more traditional laboratory approach to understanding the impact of DA on marine life (and one being pursued (*e.g.*, Adams, Doucette, James & Ryan, 2009)) would be to expose laboratory animals to DA and categorize the effects. Data obtained this way can be valuable, but one may be able to gather more immediately relevant information by directly studying the target species. Use of natural models may also allow even more direct applied benefit—in the case of the current research, we were able to design and test behavioral diagnostic assays for DA toxicosis using the very population needing to be diagnosed.

There is another benefit, of more humanistic concern, to naturalistic models for studying brain and behavior—they may meet a substantially higher ethical standard than traditional laboratory approaches. Because task-based fMRI is still very difficult with non-human

animals, the primary methods for using animals to study the role of different brain areas in cognition are invasive, including direct recording via implanted electrodes or physically damaging certain areas of an animal's brain and then measuring resultant changes in behavior. Most animal subjects used in such studies must be "sacrificed" in order to address the research question being asked. At the least, natural models for brain research could serve as a supplement to targeted lesion studies—in some cases, brain damage has already occurred in nature, and the extent can be determined *in vivo* using low-risk MRI. In addition, experimental treatment approaches may be more justifiable when used with a subject already suffering from the affliction of interest.

While, for most researchers, ethical concerns may place a distant second to issues of pragmatic expediency, they are an inescapable sub-current of all animal research, one openly acknowledged by the institutions regulating such experimentation. Indeed, every reputable researcher conducting animal experimentation at a US institution does so under USDA laws, which stipulate that "…the principal investigator considers alternatives to any procedure likely to produce pain to or distress in an experimental animal." This is echoed in the guidelines of all Institutional Animal Care and Use Committee guidelines, which require that experiments should be conducted so as to avoid all unnecessary suffering and injury to the animals. If one takes these exhortations seriously, one *must* consider alternative animal models to those traditionally employed.

In summation, the research described in this dissertation, comprising a series of cognitive neuroscience studies using wild CSLs with naturally occurring neurotoxic exposure to DA,

represents one viable alternative to the laboratory animal models traditionally used in cognitive neuroscience. By using subjects from a wild population, I was able to study the effects of neurotoxic exposure and resultant hippocampal damage in a large sample of genetically diverse, large-brained animals who are traditionally under-studied, and who are likely more meaningfully representative of typical wild populations (including humans) than are common laboratory animal strains. My findings have applied relevance in veterinary and rehabilitation settings, and theoretical relevance in cognitive neuroscience. Although my specific results must be interpreted rather narrowly in their specific fields, the general success of this project argues for the viability and value of alternative approaches to comparative research in cognitive neuroscience.

CHAPTER 1

Rapid Behavioral Diagnosis of Domoic Acid Toxicosis in California Sea Lions

NOTE: This first chapter consists of a complete manuscript, "Rapid Behavioural Diagnosis of Domoic Acid Toxicosis in California Sea Lions," published in *Biology Letters* (Cook, Reichmuth, & Gulland, 2011). In addition, appendices providing raw diagnostic data on the subjects, raw behavioral data, and additional statistical analysis have been included here. This short manuscript concerns a diagnostic behavioral assay and was targeted at a veterinary and rehabilitation audience. Although some data were available regarding brain damage from structural imaging and post-mortem examination, these data were not available for all subjects, and so were not discussed in-depth. All data were collected during my time as a doctoral student in Psychology at UCSC.

As briefly discussed in the manuscript, an auditory habituation measure was selected primarily for pragmatic purpose. The goal of this experiment was to develop and test a simple behavioral measure that might reasonably be implemented in a busy veterinary/rehabilitation setting on animals of both sexes, all different ages, and with a wide range of physical ailments. Given this, we opted to avoid any tasks involving training or food reward. Previous evidence suggested that habituation of an exploratory response elicited by objects in a free-field in rodents is heavily dependent on hippocampal integrity (Eacott & Meltzer, 2004; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002). In rather extensive pilot work with a number of young stranded CSLs, conducted both at The Marine Mammal

Center (TMMC) and at Long Marine Lab (LML) during the summer of 2010, I determined that CSLs do not reliably respond to novel objects in a free-field. Some existing work with humans indicated that habituation to non-aversive auditory stimuli was partially dependent on the hippocampus, and behavioral measures of habituation to auditory tones had been successfully implemented in field research with primates and captive studies of seals (Gotz & Janik, 2010). Pilot work I conducted at TMMC using auditory stimuli indicated a robust and reliable orienting response to a "looming stimulus" (see paper), so this was selected as the primary stimulus. The stimulus itself, the presentation cycle (one tone every 1-15 seconds) and the source level (~85 dB) were selected in effort to avoid startle responses and subsequent sensitization to the signal. The length of the exposure series was also restricted in effort to develop a practical measure for actual use.

Given the brevity of the manuscript, little time was spent discussing dishabituation measures (*i.e.*, responsivity following initial habituation). These data are discussed in Appendix 1.6.

Abstract

DA is a neurotoxic metabolite of widely occurring algal blooms that has caused multiple marine animal stranding events. Exposure to high doses of DA, a glutamate agonist, may lead to persistent medial temporal seizures and damage to the hippocampus. CSLs are among the most visible and frequent mammalian victims of DA poisoning, but rapid, reliable diagnosis in a clinical setting has proven difficult due to the fast clearance of the toxin from the blood stream. Here we show that the behavioral orienting responses of stranded CSLs diagnosed with DA toxicosis habituate more slowly to a series of non-aversive auditory stimuli than do those of CSLs with no apparent neurological deficits. A signal detection analysis based on these habituation measures was able to correctly identify 50% of subjects with DA toxicosis while correctly rejecting ~93% of controls, suggesting potential diagnostic merit.

Introduction

Over the last decade, marine mammal stranding events coincident with large blooms of *Pseudonitschzia australis* have become increasingly common (Goldstein *et al*., 2008). The factors producing this increase are complicated, but likely include interactions between marine mammal feeding and migratory patterns and location and timing of blooms (Bargu *et al*., 2009). Some types of *Pseudonitschzia* diatoms produce DA, a glutamate agonist with high affinity for AMPA and kainate receptors (Qiu *et al*., 2006). DA is cleared from the body rapidly (Maucher & Ramsdell, 2005), but persistent excitotoxic effects frequently result in neuronal degradation, particularly in the hippocampus and surrounding medial temporal region (Berman *et al*., 2002). Such neuronal necrosis is particularly acute in the dentate gyrus and hippocampal sectors CA4, CA3, and CA1 (Silvagni *et al*., 2005; Colman *et al*., 2005).

CSLs have been particularly visible victims of DA exposure and toxicosis. In magnetic resonance images (MRI) of the brain of 42 CSLs diagnosed with chronic DA toxicosis at TMMC in Sausalito, CA, 41 showed detectable hippocampal atrophy, ranging from mild to severe (Goldstein *et al*., 2008). Seventy of 89 animals with chronic DA toxicosis that died during the same period exhibited gross hippocampal lesions at necropsy, most commonly in sector CA3 and the dentate gyrus.

Diagnosing DA toxicosis in a clinical setting is generally a haphazard or time-intensive and expensive endeavor (Gulland *et al*., 2002). Direct diagnosis from blood sampling is rare as DA is cleared from the blood stream within 48 hours (Truelove & Iverson, 1994), and

animals are often not accessible for treatment until days after exposure (Goldstein *et al*., 2008). At TMMC, live animals are typically suspected of DA-poisoning on the basis of epidemiology and an initial clinical neurological examination—greater reliability of diagnosis relies on post-hoc assessment involving laboratory estimation of DA content of urine or feces, and analysis of the brain, either by post-mortem histology or MRI. Both electroencephalography (EEG), which identifies patterns of seizures, and MRI, which can identify significant neuronal necrosis and atrophy in the hippocampus, are effective but relatively slow and expensive diagnostic tools, and require sedation. Many cases of DA toxicosis are only accurately diagnosed during post-mortem examination by histological examination of the hippocampus (Silvagni *et al*., 2005). Diagnosis determines course of treatment and prognosis, and factors into veterinary decisions to release or euthanize stranded CSLs. Therefore, improved methods for *in vivo* diagnosis are needed.

This first attempt at devising a simple and empirically grounded behavioral diagnostic assay of DA toxicosis depends on habituation of an orienting response to non-aversive auditory stimuli. As hippocampal necrosis is a common consequence of DA toxicosis in CSLs, and hippocampal damage has been shown to slow habituation rates of unrestricted exploratory behavior in a range of species (Honey *et al*., 2007; Yamaguchi *et al*., 2004; Sokolov, 1990), a metric based on habituation holds diagnostic promise. Further, the habituation of an orienting response can be measured through observation, requiring no invasive or aversive procedures (Teufel *et al*., 2007). Here we present the results of a behavioral assessment designed to be sensitive to hippocampal damage in order to augment diagnosis of DA toxicosis in CSLs.

Materials and Methods

Forty-two CSLs undergoing rehabilitation at TMMC were sampled in this study (see Appendix 1.2). Effort was made to test all available admitted CSLs, regardless of diagnosis, during the study period. After testing was complete, subjects were assessed using veterinary clinical criteria wholly independent of performance in testing. Twelve CSLs were diagnosed with DA toxicosis and 27 CSLs, forthwith referred to as "controls," were evaluated as having no signs of DA toxicosis or other neurological abnormalities. A positive diagnosis of DA toxicosis was based either on clinical signs of seizures and ataxia that resolved following diuresis and sedation (as described by Gulland *et al*. (2002)) *and* presence of DA in urine or feces; *or* on detection of an abonormal hippocampus through MRI or post-mortem histology. Three CSLs had indeterminate diagnoses and were not included in the final sample.

The behavioral assessments were conducted in a quiet pen. Following acclimation, each CSL was exposed to a series of auditory stimuli in four sequential test phases (see Appendix 1.1). The testing sequence was designed to examine initial habituation of orienting responses to novel auditory stimuli, and to probe recovery of response, or dishabituation, following manipulations of spatial presentation, recovery interval, and stimulus type. Each test phase comprised successive presentations of one of two sounds from one of two diametrically opposite locations.

During testing, an experimenter, blind to subject diagnosis, observed subject behavior and coded responses in real time from closed circuit video. An orienting response emitted

following stimulus onset and within 0.5 seconds of stimulus offset was considered a positive response. Orienting was defined as a noticeable change in the angle of the subject's head toward the source of the stimulus in the vertical or horizontal plane. During each of the four testing phases, the auditory stimulus was presented on a fixed, semi-random schedule at intervals of 5–15 seconds until the subject habituated, at which point the next phase began. "Habituation" was defined as no observable orientation to three consecutive stimuli. The experimenter's real-time assessment of habituation was used during testing and for subsequent analysis. These scores were later validated by two independent observers who viewed the videotaped recordings of each session.

Exposures to habituation were compared between CSLs with DA toxicosis and controls for each of the four test phases using T-Tests with Bonferroni corrections for repeated measures. Test phases showing a significant difference between these groups were then further subjected to signal detection analysis employing receiver operating characteristic (ROC) curves. ROC curves assess a metric's likelihood of producing a correct positive diagnosis relative to the likelihood of a false positive diagnosis over a range of diagnostic sensitivity thresholds. Thresholds used here were the number of exposures prior to habituation in a particular test phase.

Results

CSLs with DA toxicosis took significantly more exposures to habituate in the first test phase than did controls (Figure 1)—there were no significant differences observed in test phases

2–4 (see Appendix 1.5). Agreement between the experimenter's initial coding of orienting behavior across all subjects and exposures and that of the post-hoc observers was 85% and 86%.

Figure 1

Responsiveness Across Auditory Phases

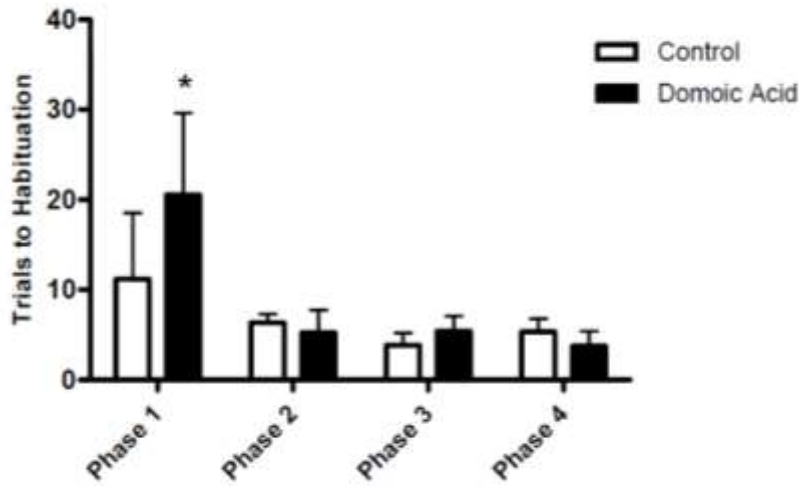

Figure 1: Mean number of exposures prior to habituation for subjects diagnosed with DA toxicosis and controls in four test phases: initial exposure, exposure following a spatial shift, exposure following a delay, and exposure following a stimulus shift. Error bars represent standard deviation. There was a significant difference between exposures to habituation for CSLs with and without DA toxicosis in the first ($p < 0.001$) but not the second, third, or fourth test phases ($p > 0.05$, T-tests with Bonferroni correction for repeated sampling).

A receiver operating characteristic (ROC) curve was computed using independent diagnosis of DA toxicosis and the number of exposures to habituation in phase 1 (Figure 2). Area under the curve was 0.82, suggesting a good diagnostic metric.

Figure 2

ROC Curve Based on Exposures Prior to Habituation in Test Phase 1

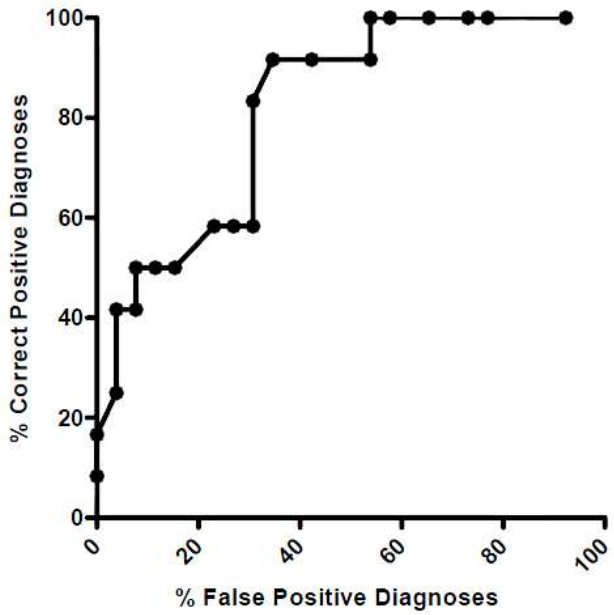

Figure 2: This ROC curve represents the diagnostic effectiveness of a metric based on exposures prior to habituation in the first test phase. The likelihood of producing a correct positive diagnosis (represented on the y axis) versus the likelihood of a false positive diagnosis (represented on the x axis) was computed across a range of sensitivity thresholds. These thresholds were defined as the number of exposures prior to habituation above which an animal was considered to have DA toxicosis. For the ROC curve, diagnosis was considered correct if it matched the independent veterinary assessment, and incorrect if it did not. The area under the curve was 0.82 and $p < .002$.

Discussion

The notable tendency of CSLs with DA toxicosis to habituate more slowly to a non-aversive auditory stimulus could be explained by the presence of hippocampal damage in these subjects (but see Appendix 1.4). There were no significant differences in responsiveness between subject groups in phases 2–4, suggesting that the spatial, delay, and stimulus manipulations did not have differential effects on animals with DA toxicosis. More generally, this result indicates that these dishabituation measures were relatively insensitive to confirmed or presumed hippocampal damage.

Quantitative behavioral diagnostics are rarely used in veterinary clinical settings, but in this case ROC analysis of exposures to habituation in the first test phase indicates that habituation is a promising measure to augment current diagnostic approaches to DA toxicosis. ROC analysis produces a ratio of correct positive diagnoses to false positive diagnoses across a range of thresholds (see Appendix 1.3). In the case of DA toxicosis in CSLs, false diagnosis of DA toxicosis could lead to an otherwise healthy CSL's being euthanized (as recommended in Thomas *et al*., 2009), so ultimately, a conservative diagnostic threshold of >22 exposures prior to habituation was selected for this study. Using this threshold, the habituation measure correctly identified 50% of CSLs with DA toxicosis while falsely diagnosing only 7% of control subjects. This auditory response test can serve as a rapid, inexpensive, and logistically easy diagnostic test for hippocampal damage available to most practitioners in the absence of advanced and expensive clinical diagnostics such as

MRI or EEG. This represents a novel and applicable behavioral approach to diagnosis of a neurological disorder in a veterinary setting.

Further refinement of the procedure is ongoing in concert with a study of CSLs with DA toxicosis in which all subjects are undergoing MR brain imaging. This may improve an already effective diagnostic measure, and will indicate whether the behavioral assay discussed here is sensitive to hippocampal damage as suspected or to other sequelae of DA toxicosis.

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Chapter 2

Behavioral Diagnosis of Domoic Acid Toxicosis in Wild California Sea Lions: Contributions of Epilepsy and Medial Temporal Lobe Damage

Note: This second chapter comprises a draft manuscript formatted for submission to *PLoS One*. This journal was selected primarily for its broad accessibility to divergent research communities. Because the study discussed herein may be of interest to professionals in a number of different disciplines (behavioral and brain sciences, rehabilitation, veterinary science, biological sciences), many of whom may not have institutional access to journal data bases, an open-access journal was an appealing target. The manuscript making up this chapter has been written with a broad audience in mind, so care is taken to synthesize the approach and findings in a palatable and intuitive manner for readers who might find some of the technical specifics in the methods section unfamiliar.

The research described in this manuscript was a direct follow-up to the work described in Chapter 1 of this dissertation. The finding from the previous diagnostic study that *post-hoc* veterinary diagnoses were strongly predicted by auditory habituation measures needed replication before being implemented in a veterinary setting. In addition, we were unable to obtain MR images for the majority of subjects in the first study—in this second study, by opportunistically including subjects taking part in separate, funded projects involving MRI, we were able to obtain behavioral and brain imaging data on all of the CSLs in our large subject pool. This allowed us to directly test hypotheses about the relationship between the behavioral measure and the degree of DA-related brain damage in the CSLs studied. The

possible relationship between epilepsy and habituation of an orienting response—which was neglected in the manuscript included here as Chapter1—was also examined here. Because subjects for this study were opportunistically selected from two different ongoing experiments, two slightly different protocols were used for brain imaging, and two separate locations were used for testing. This is discussed in the chapter below, where I use the terms “Period 1” and “Period 2” to refer to the two different periods of data collection.

Abstract

Large numbers of California sea lions (CSLs) (*Zalophus californianus*) strand in distress each year with domoic acid (DA) toxicosis—a neurological condition resulting from exposure to a metabolite of an algae occurring commonly of the coast of California. DA toxicosis is a damaging condition, as toxic exposure causes chronic epilepsy and progressive hippocampal damage. To date, rapid and reliable diagnosis of this condition in CSLs has proven difficult, due to the absence of easily identified bio-markers. Rapid, accurate diagnoses are needed, as they may contribute to the efficacy of treatment in this population. Toward that end, we previously developed a non-invasive behavioral assay probing responsivity to an auditory stimulus (Cook, Reichmuth, & Gulland, 2011). Here, we replicated the original behavioral finding using a wholly automated procedure well suited for a rehabilitation environment. Stranded CSLs with and without DA toxicosis were exposed to 54 repetitions of an auditory stimulus, and their total number of behavioral orienting responses were tallied. As in the prior study, responsivity was a strong predictor of DA toxicosis, with affected CSLs responding significantly more than control animals. In the rehabilitation setting, diagnosis of DA toxicosis relies on both subjective assessment of hippocampal morphology from magnetic resonance imaging (MRI) or histology and observed epilepsy—here we examined the relationship of both factors with responsivity. Neither subjective, categorical assessment of hippocampal damage by a radiologist nor volumetric measurements of the hippocampus and parahippocampus from MRI were predictive of responsivity. In addition, in the current study, the radiologist's assessment of hippocampal damage was not predictive of measured hippocampal volume. However, we found presence of epilepsy to be a strong predictor of

responsivity, both in the current study and, retrospectively, in the initial study from 2011. A basic responsivity measure, obtained from a rapid, automated auditory exposure assay, has diagnostic efficacy for DA toxicosis in a rehabilitation stetting. The success of this measure may be dependent on features of domoic-acid-related epilepsy, as opposed to gross brain insult.

Introduction

Domoic Acid

Beginning in the late 1990s, each year a large number of CSLs have come to shore in distress (*i.e.,* "stranded") as a result of toxic exposure to DA, a metabolite of an algal species called *Pseudonitschizia australis* that blooms commonly off the California coast (Scholin *et al*., 2000). DA is a glutamate agonist, and exposure can lead to chronic epilepsy and hippocampal and parahippocampal damage (Goldstein *et al*., 2008). Incidence of neurotoxic exposure to DA in CSLs has generally increased along with the frequency of DA-producing algal blooms (Jeffery, Barlow, Moizer, Paul, & Boyle, 2004). Prior to 1998, neurological symptoms were rarely documented in stranded CSLs in central California; between 1998 and 2006, however, over 20% of CSLs admitted to The Marine Mammal Center (TMMC) stranding facility in Sausalito, CA exhibited neurological signs consistent with toxic exposure to DA. The mortality rate among these animals, even with treatment, was 40% (Goldstein *et al*., 2008).

Notably, two clinical variants of the effects of toxic exposure to DA have been described in CSLs: acute DA toxicosis and chronic DA toxicosis. As described by Gulland *et al*. (2002), acute DA toxicosis involves seizures, ataxia, and other neurological symptoms, and is generally understood to be a result of recent high-dose exposure to DA in the wild. Chronic DA toxicosis, defined by Goldstein *et al*. (2008), is characterized by chronic epilepsy caused by one or more toxic exposures to DA, and presents as intermittent seizures, inappetance, hippocampal atrophy, and expression of a range of abnormal behaviors. While animals

suffering from acute DA toxicosis have tended to strand in close temporal proximity to a toxic bloom event (although this is complicated, see Bargu, Silver, Goldstein, Roberts, & Gulland, 2010), animals with chronic DA toxicosis strand year round, without any clear temporal relationship between blooms and stranding. It's important to note that these two conditions are not mutually exclusive—a CSL that strands following an acute exposure may end up manifesting chronic symptoms if she survives. Following the initial surge in the late 1990s, numbers of acute cases have remained generally stable year-to-year, while chronic cases have been increasing (Goldstein *et al*., 2008).

Need for Diagnostic Measures

Due to the large number of animals stranding with probable DA toxicosis, rapid and reliable methods for diagnosing the condition are needed—however, these have been difficult to develop (Gulland *et al*., 2002). Because DA clears the body very quickly following exposure, diagnosis based on tissue, fecal, or urine sampling is unreliable (Truelove & Iverson, 1994). Further, there is laboratory evidence that an animal may be exposed at relatively low doses without developing detectable symptoms (Tryphonas, Truelove, & Iverson, 1990; Iverson *et al*., 1988). The most reliable biological sequela for DA toxicosis yet identified is hippocampal atrophy, present in a large portion of afflicted animals (Goldstein, 2008), but this can be identified *in vivo* only by time consuming and expensive brain imaging techniques such as MRI. A further complication is that volumetrically distinct, gross hippocampal lesions may not manifest immediately following initial toxic exposure, but rather over time as the result of the chronic epileptic condition triggered by the initial exposure (Silvagni, Lowenstine,

Spraker, Lipscomb, & Gulland, 2005; Goldstein *et al*., 2008). This suggests that magnetic resonance imaging (MRI), while able to identify hippocampal pathology closely linked with chronic DA toxicosis, is of little use in identifying animals with early phase/acute symptomatology. Further, although qualitative assessments of hippocampal damage have been clearly linked with DA toxicosis, no prior empirical work has linked diagnoses of DA toxicosis with quantitative measures of brain damage.

Attempts to devise better diagnostic assays continue. Although no single, clear, easily testable bio-marker for DA toxicosis has been discovered, a recent hematological study (Neely *et al*., 2012) showed promise in using machine learning algorithms to assess serum peptide patterns in blood for diagnostic value in CSLs with early, acute exposure to DA. The assay was able to establish criteria with sensitivity of 100% and specificity of 60% or sensitivity of 30% and specificity of 100%, a marked improvement over previous attempts at developing biological assays for acute DA toxicosis, clearly meriting further exploration.

In our own work, we have taken an alternative approach, exploring behavioral assays for diagnosing DA toxicosis. Given that DA causes reliable neurological changes, it stands to reason that it should cause reliable behavioral changes. Behavioral assays may be rapid, inexpensive, and non-invasive, and could serve as useful adjuncts to biological and/or imaging-based diagnostic tests. Indeed, current diagnosis of DA toxicosis in the rehabilitation setting relies heavily on presentation of behavioral abnormalities, including seizures, ataxia, and head weaving (Gulland *et al*., 2002). However, these symptoms have not been empirically linked to DA toxicosis in a quantitative manner. Any standardized

behavioral diagnostic test to be used on a large group of animals in a stranding and rehabilitation setting must be reliable, rapid, and easy to score. A recent study of ours showed significant promise (Cook, Reichmuth, & Gulland, 2011). We exposed CSLs to repeated auditory stimuli and measured number of orienting responses prior to a pre-set habituation criterion. This was then assessed against best-case, independent, post-hoc veterinary diagnoses of all subjects. Animals judged DA positive following *post-hoc* diagnoses were twice as responsive as animals judged to have no neurological symptoms. Further, the results suggested a response criterion could be used with this exposure protocol to generate an effective diagnostic tool, with 50% sensitivity (correct positive diagnosis rate) and 93% specificity (correct negative diagnosis rate). Because the procedure used required real-time monitoring of response to determine when a habituation threshold had been met, it was not optimally suited for use in a rehabilitation setting.

At the time of the initial study, we hypothesized that the difference in responsivity between CSLs with DA toxicosis and controls was being driven by hippocampal pathology, but, as we were unable to obtain MRI data on most of the study's subjects, this could not be verified. There was also some evidence that epilepsy might have driven the increased responsivity observed in this behavioral assay. Although the role of the hippocampal formation in driving responsivity to stimuli is complicated and not fully understood, a great deal of evidence from parallel fields converges on the fact that the hippocampal formation does play a role in responsivity, habituation, and orienting to certain types of stimuli. There are fewer data on the link between epilepsy and responsivity, but some intriguing evidence suggests a connection.

Neurological Sequelae to Domoic Acid Toxicosis and Responsivity

As mentioned previously, the primary neurological symptoms observed with DA toxicosis are damage to the hippocampal formation and epilepsy (Goldstein *et al*., 2008). Evidence suggests both factors may be related to responsivity. Responsivity to stimuli is of course inversely related to habituation measures, and both are linked to behavioral and attentional orienting and exploration. There is strong evidence that attentional orienting and exploration and related habituation are at least partially driven by a distributed brain network including the hippocampus and parahippocampus (Friedman, Goldman, Stern, & Brown 2009; Stoppel *et al*., 2009; Bucci & Burwell, 2004; Yamaguchi, Hale, D'Esposito, & Knight, 2004; Acquas, Wilson, & Fibiger, 1996; Knight, 1996; Sokolov, 1990). In addition, prior evidence shows that experimental animals with hippocampal lesions tend to show increased responsivity/impaired habituation (Foreman & Stevens, 1987; Douglas & Isaacson, 1964), although this may be due in part to the general behavioral hyperresponsivity triggered by damage to any number of brain regions (Viggiano, 2008; Nadel, 1968).

While there are few data directly addressing the role of epilepsy in responsivity and habituation, increased autonomic response to neutral stimuli have been observed in humans with epilepsy (Bear, Schenk, & Benson, 1981) as have increases in orienting responses in humans (Rogozeza, Florea-Ciocoiu, & Constantinovici, 1983) and cats (Rogozea & Florea-Ciocoiu, 1976). There is also significant evidence indicating that hippocampal hyperexcitability can be a long-term product of epilepsy (Milgram, Yearwood, Khurgel, Ivy, & Racine, 1991), which might drive increased behavioral responsivity, and thus slowed

habituation, to stimuli processed by temporal lobe networks. This seems intuitive, given the role of neural disinhibition in temporal lobe epilepsy (Esclapez, Hirsch, Ben-Ari, & Bernard, 1999; Sloviter, 1987). Additionally, there is evidence of altered functional connectivity across a broad range of brain networks accompanying medial temporal epilepsy (Liao *et al*., 2011; Zhang *et al*., 2009; Zhang *et al*., 2009). There are most likely cognitive changes accompanying epilepsy that are dissociable from the proximal effects of related brain lesions. However, there is some reason to believe that at least some findings of increased behavioral excitability in humans with epilepsy could be due to comorbid psychiatric disorders that commonly co-present with epilepsy (Gaitatzis, Trimble, & Sander, 2004; Mungas, 1982).

In brief, there is some reason to believe that both hippocampal lesions and epilepsy resultant from DA could impact resposnsivity.

Current Study

In the current study, we sought to replicate our original finding of increased responsivity in animals with DA toxicosis using an automated procedure more suited for a rehabilitation setting. We also collected additional diagnostic data to further explore the relationship between hippocampal damage, epilepsy, and responsivity. We collected auditory habituation data on 27 CSLs undergoing rehabilitation: 13 with chronic DA toxicosis and 14 control animals with no apparent neurological symptoms. Each subject in the current study received structural MRI. Resultant brain images were assessed for damage to the hippocampal formation subjectively by a veterinary radiologist and quantitatively by

volumetric measures computed from manual tracing of the hippocampus and parahippocampus. Each CSL in the study was also observed throughout treatment for behavioral incidence of seizure activity.

The behavioral assay in this study comprised two consecutive phases. Phase 1 was similar to the approach used in the earlier study (Cook, Reichmuth, & Gulland, 2011), but, where the previous study had required a subjective assessment of habituation in real time, the stimulus presentation schedule in the current study was fully automated, to facilitate possible use of the assay in real-world rehabilitation settings. In Phase 1 of the current experiment, each subject received 54 tones alternating between two stimulus sources, regardless of responsivity.

The second experimental phase represented an attempt to produce a more sensitive measure of hippocampal-dependent function than raw responsivity, which is likely dependent on a number of unrelated factors. In Phase 2, each subject received another 54 tones, but this time alternating between one familiar and one novel stimulus source. Our hypothesis, based off similar studies with rodents using object exploration (Eacott & Norman, 2004; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002; Ennaceur, Aggleton, & Fray, 1997), was that CSLs with hippocampal damage would show a weaker differential response to the novel stimulus source than would control subjects. The hippocampus is understood to bind in memory stimuli and the context in which they occurred (Ranganath, 2010), so disruption of hippocampal function could result in impaired memory for location/stimulus bindings, which, in turn could make incongruous pairings less salient.

Because ratio of response to novel versus familiar location should control for general responsiveness (which could be driven by any number of diverse factors), our hope was that this measure could increase diagnostic resolution.

For all subjects, total responsivity in Phase 1 and relative responsivity to the novel versus familiar location in Phase 2 were assessed against post-hoc veterinary diagnosis for diagnostic efficacy. In addition, behavioral results from both phases were compared with observed epilepsy and with subjective and quantitative assessments of damage to the hippocampal formation.

Methods

Subjects

Twenty-seven wild CSLs undergoing rehabilitation following stranding took part in this study (see Appendix 2.1). Thirteen subjects received a diagnosis of chronic DA toxicosis, and 14 subjects were considered neurologically normal (*i.e.*, no evidence of any neurological symptoms, domoic-acid related or other) and served as controls. All subjects were acquired from TMMC in Sausalito, California. Subjects were of both sexes and ranged in age from nutritionally mature pups to sexually mature adults. Diagnostic and behavioral data were collected on each of the 27 subjects, but due to scanner error, two of the control subjects' MRI data could not be used, limiting combined MRI and behavioral analysis to 25 animals (13 subjects diagnosed with DA toxicosis and 12 controls). Initial sample size was 29, but two subjects were excluded from the study *a priori*, one because of equipment failure during behavioral testing, the other because of a diagnosis of acute DA toxisosis, as opposed to

chronic. Data for this study were collected during two separate periods (referred to below as “Period 1” and “Period 2”).

Period 1. Between July 2010 and November 2011, 16 CSLs were tested at University of California Santa Cruz’s Long Marine Laboratory (LML). These 16 subjects were all part of a separate study examining the neurocognitive impact of toxic DA exposure, and undertook testing for the current study following about two weeks of behavioral procedures conducted at LML (discussed in Chapter 3 of this dissertation). In brief, these procedures comprised a number of food-based spatial memory assays, and did not involve auditory stimuli or exposure to the testing enclosure used in the current study. One of the criteria for initial transport to LML was that each animal was judged responsive and willing to eat, so all subjects had undergone some rehabilitation at TMMC prior to their stay at LML. The mean time spent in captivity for each of these subjects, from arrival at TMMC to testing at LML, was 37 days (standard deviation ±16.6 days).

Period 2. Between August and November 2012, 11 CSLs were tested at TMMC (where they had been housed while undergoing rehabilitation). These subjects were part of an independent functional brain imaging study, but did not take part in the previous behavioral study described above, and had not been housed at LML. All subjects were judged responsive and willing to eat prior to testing. The mean time spent from arrival at TMMC to testing at TMMC for these subjects was 30.3 days (standard deviation ±20.6 days).

During both periods of data collection, subjects were selected opportunistically from the available patient population at TMMC by veterinary staff according to simple criteria based

on responsiveness and willingness to eat, irrespective of assumed neurological status. The experimenters were blind to the subjects' respective medical conditions and diagnoses during data collection. Because subjects were sampled from a rehabilitation population, there was a range of potentially overlapping medical conditions represented among them, including malnutrition, infection, physical trauma, and DA toxicosis.

Apparatus

Subjects in Period 1 were tested at the LML facility in a cement-floored, dry pen measuring 3.5 x 3.5 m. The pen was surrounded by chain-link fence on three sides with a cement wall along the fourth side. Individual CSLs were transported to the pen for testing from their home pool via carrier crate, the same crate used to transport them for other purposes. The transport time was less than 5 min. Subjects were released into the pen and the crate was removed prior to testing. Subjects had not been in this pen prior to testing.

Subjects in Period 2 were tested at the TMMC facility in a cement-floored, dry pen measuring 3 x 3 m that was similar to that used at LML. The pen was surrounded on three sides by chain-link fence, with a wooden wall along the fourth side. Subjects were transported to the pen from their home pool via carrier crate, and transport in the crate took less than 5 min. Subjects were released into the dry pen and the crate was removed prior to testing. The majority of subjects had not been in this pen prior to the current study.

In both facilities, the configuration of equipment was similar (Figure 3). Two Advent AV570 amplified speakers were placed outside of the pen prior to the subject's arrival. Speakers were placed on the ground, facing into the pen from the two corners on either side of the

entry gate (defined as locations “A” and “B”). Following an initial presentation phase and delay, one of the speakers (“B”) was moved to a new location (location “C”) for a second testing phase, facing in from a corner adjacent to its original position. The other speaker (“A”) remained in the same location for both testing phases. The speakers were wired to a computer in a control room out of sight of the subjects. The testing conducted at LML was filmed with Sony HD DCR-SR68 Handycam placed on a tripod set up beside the pen. Filming at TMMC was via the same camcorder attached to a tripod fixed above one of the pen’s corners. There was no extraneous activity near the pens during testing.

Figure 3

Testing Arrangement for Auditory Exposures

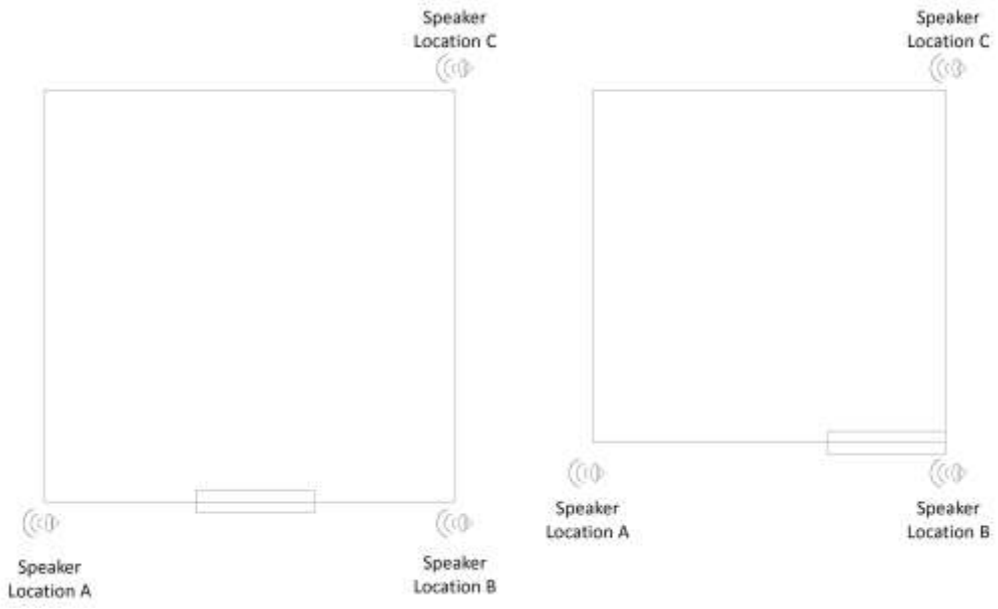

Figure 3: Schematic detailing testing arrangement for data collection in both Period 1 (left) and Period 2 (right).

The auditory stimulus used for this study was a "looming tone," which has some of the auditory characteristics of a rapidly approaching object (Ghazanfar, Neuhoff, & Logothetis, 2002). The tone was 750 ms, with the majority of its energy centered at 1 kHz—within the range of hearing for CSLs (Schusterman, 1974). The tone rose in intensity exponentially from 65 to 85 dB re 20μPa at 1 m. The digital stimulus was sent to the Advent speaker from a laptop computer operated by the experimenter.

Procedure

The behavioral testing procedure involved exposing each animal to repeated presentations of the auditory stimulus across two automated test phases—animals were not observed during testing and all results were scored from video after testing was complete. After being brought to the test enclosure, each subject was given 3 min to explore the pen and acclimate. Following the acclimation period, sound presentation began. Sound presentation always followed an ABAB (or ACAC) pattern such that the stimulus presentation alternated repeatedly between the two speaker locations. In both experimental phases, subjects received the stimulus 54[1] times following a pre-set presentation schedule. In both experimental phases, all inter-stimulus intervals were between 0.2 and 7.5 s, semi-randomized such that the mean interval between sound presentations was 4 s.

In Phase 1, the sound stimulus was presented alternately from the initial speaker locations A and B. Following this presentation phase, an experimenter physically moved one of the speakers (B) to a new location (C). The other speaker (A) remained unmoved, but the experimenter walked over to this speaker and crouched next to it for the same amount of time required to move the previous speaker from B to C. After rearranging the speaker configuration, the experimenter retreated again to the out-of-sight control room, and

[1] Animals received between 54 and 66 exposures in each phase of each period, however, responses to only the first 54 were analyzed. This alteration was not experimentally motivated, but was rather an error in stimulus preparation prior to the beginning of data acquisition Period 2. This unintentional manipulation was irrelevant to final measures of responsivity in presentation Phase 1, which focused only on the first 54 tones, irrespective of testing period, and had no apparent impact on behavioral measures related to presentation Phase 2.

another 3 min delay began. During this interval, no sounds were presented. Following this interval, the second presentation phase began.

Phase 2 was structured the same as Phase 1, in that the auditory stimulus was again presented in an alternating pattern (ACAC), this time switching between the familiar (A) and novel (C) speaker locations. Both presentation phases were initiated based on time elapsed (3 min from subject entry to the testing pen in Phase 1, or 3 min from speaker position change in Phase 2) and were not dependent on subject behavior. Following Phase 2, the subject was re-crated and returned to its home pen.

Data Collection

Responsivity Data

Behavioral data were coded to specifically identify orienting responses to the individual stimulus presentations. Data were coded *post-hoc* from the video recordings by one analyst who was blind to subject diagnosis at coding. After each individual sound presentation, a decision was made by the coder regarding whether the animal had produced an orienting response to the stimulus or had not. This was defined as a binary choice—because either a response or no-response had to be coded for each stimulus presentation, the coder was allowed to watch each stimulus presentation event as many times as necessary to make a decision. Response criteria were the same as those used in Cook, Reichmuth, & Gulland (2011) and were as follows: A response was defined as a noticeable change in head orientation (> 5 degrees) toward the location of the speaker presenting the stimulus. The change in head orientation had to begin after the onset of the stimulus and before 0.5

seconds had elapsed following stimulus offset. The animal's head did not have to end up in direct alignment with the speaker presenting each stimulus for a response to be counted; rather, it merely needed to end up in closer angular alignment with the relevant speaker location than it had begun. Because the focus was on measuring orienting responses, head movements away from the speaker were ignored. Further, other apparent behavioral markers of response, such as body startle, were not considered. To allow for a measure of observer reliability, a second coder analyzed 10 of the 27 usable sessions—these were randomly selected. The second coder was also blind to subject diagnosis and followed the same scoring instructions. Absolute response numbers as determined by the first coder were then used in subsequent analysis.

Diagnostic Assessment

Each subject was also accorded a *post-hoc* diagnosis by the veterinary staff at TMMC. This diagnosis was made following final disposition of each subject. The deciding veterinarian was blind to the behavioral data collected in the present study and to the quantitative measures of hippocampal and parahippocampal volume acquired from MRI, but took into account all other available information on each subject. Although diagnosis was multi-factorial, subjective assessment of hippocampal damage by a veterinary radiologist and observed seizures were the two most important factors influencing diagnosis. Subjective assessments of the MR images were based on apparent hippocampal atrophy, visible to the naked eye, as discussed previously in Goldstein *et al*., 2008. Seizures were coded during the

subjects' time undergoing rehabilitation by opportunistic visual observation by those familiar with their behavioral presentation, as discussed previously in Gulland *et al*., 2002.

Other information relevant to diagnosis included any medical sampling (such as measures of DA in urine or fecal samples), observations made of the animal during its time at TMMC, final outcome for each subject (whether this was a successful release, death, or euthanasia), and post-mortem information when available. This diagnosis allowed for a discrete assessment of whether each animal was suffering from acute or chronic DA toxicosis or not, and it was this discrete diagnostic measure that was used in further analysis in the current study.

While both subjective assessment of hippocampal damage and observed seizures were important factors guiding veterinary diagnosis, they were also assessed independently in relation to the behavioral measures of responsivity in the current study. For this purpose, and because neither measure was precise, both factors were coded as discrete variables for further analysis. In the case of subjective assessment of hippocampal damage, animals were sorted into four groups: No apparent damage, unilateral left damage, unilateral right damage, or bilateral damage. In the case of observed seizures, animals were sorted into two groups: No observed seizures or one or more observed seizures.

MRI Data

To quantify potential hippocampal and parahippocampal damage related to DA toxicosis, each subject in the study underwent structural MRI of the brain. Resulting images were used by the veterinary radiologist to make subjective assessments of hippocampal damage, as

discussed above. They were also used to compute quantitative measures of hippocampal and parahippocampal volume.

Subjects were imaged at the veterinary specialty clinic AnimalScan in Redwood City, CA, on a 1.5 T Siemens Magnetom Symphony scanner. During scanning, subjects' heads were placed in a CP extremity coil, selected to the optimize signal-to-noise ratio, and standard turbo Spin Echo (TSE) T2-weighted scans were obtained in an oblique plane perpendicular to the long axis of the hippocampus. This imaging orientation contributes to the ease of assessing hippocampal damage in CSLs (Montie *et al*., 2009; Goldstein *et al*., 2008). The oblique scans obtained from subjects evaluated during Period 1 were acquired with the following parameters: TR = 5470 ms, TE = 14 ms, FOV = 160 x 160 mm, slice thickness = 2.0 mm, voxel size = 0.625 mm x 0.695 mm x 2 mm.

Because animals evaluated in Period 2 were being imaged opportunistically during a separate imaging protocol, time constraints mandated increasing slice thickness on the oblique imaging sequence to limit acquisition time. This resulted in decreasing the number of slices obtained by about one third. The oblique scans obtained from subjects evaluated during Period 2 were acquired with the following parameters: TR = 3950 ms, TE = 98 ms, FOV = 160 x 160 mm, slice thickness = 3.0 mm, voxel size = 0.625mm x 0.695mm x 3mm.

Manual tracing was conducted on the oblique MRI images to provide lateralized measures of hippocampal and parahippocampal volume relative to whole brain size in each animal. Brain region size can vary quite considerably with age—by taking a measure of region volume relative to the whole brain, this can be somewhat corrected for. Tracing was

conducted using Quanta2 software (UC Davis IDEA Lab, Alzheimer's Disease Center grant, NIH P30 AG010129). Tracings are conducted on each relevant image slice—a volumetric measure is computed simply by multiplying the traced area on each slice by the slice thickness. For each subject, the right and left hippocampus, right and left parahippocampus, and whole brain (minus the cerebellum) were traced. To correct for variation in brain region size due to natural variability in whole brain size, relative hippocampal and parahippocampal volumes were computed for each animal by dividing the absolute volumes of these structures by whole brain volume. These relative volume measurements, expressed as percentages, were then used in further analysis, and are referred to subsequently in this manuscript as "hippocampal volume" and "parahippocampal volume." The details of how these standardized measures were obtained are rather technical, but potentially of interest, given the paucity of prior anatomical work on sea lion brains. These details are provided in Appendix 2.3

Data Analysis

The primary hypotheses assessed were: 1. Veterinary diagnosis would predict Phase 1 responsivity; 2. Observed seizures and/or hippocampal or parahippocampal volume would predict Phase 1 responsivity; 3. Veterinary diagnosis would predict relative responsivity to the unfamiliar versus the familiar stimulus source location in Phase 2; 4. Observed seizures and/or hippocampal or parahippocampal volume would predict relative responsivity to the unfamiliar versus the familiar stimulus source location in Phase 2; 5. Veterinary diagnosis,

observed seizures, and subjective assessment of hippocampal damage would all be predictive of measured hippocampal volume.

Analyses with veterinary diagnosis, observed seizures, or subjective assessment of hippocampal damage as the categorical independent measure used standard two-tailed T-tests with Bonferroni corrections for repeated analyses. Analyses with hippocampal or parahippocampal volume as the continuous independent measure used standard linear regression analyses, again with Bonferroni corrections for repeated analyses.

Receiver Operating Characteristic (ROC) analysis was used to assess whether total responsivity in exposure Phase 1 might serve as meaningfully sensitive diagnostic measures compared against veterinary diagnosis. ROC curves assess a metric's likelihood of producing a correct positive diagnosis relative to the likelihood of a false positive diagnosis over a range of diagnostic sensitivity thresholds.

Significant effort was also taken to validate our tracing protocol. This is discussed in Appendix 2.4

Results

Phase 1 mean responsivity in subjects diagnosed with chronic DA toxicosis (32.4) was greater than Phase 1 mean responsivity in controls (22.6) ($P < 0.05$, $t = 2.36$, (Figure 4), see Appendix 2.2 for all subjects' response measures).

Figure 4

Phase 1 Responsivity in Control and Chronic DA Animals

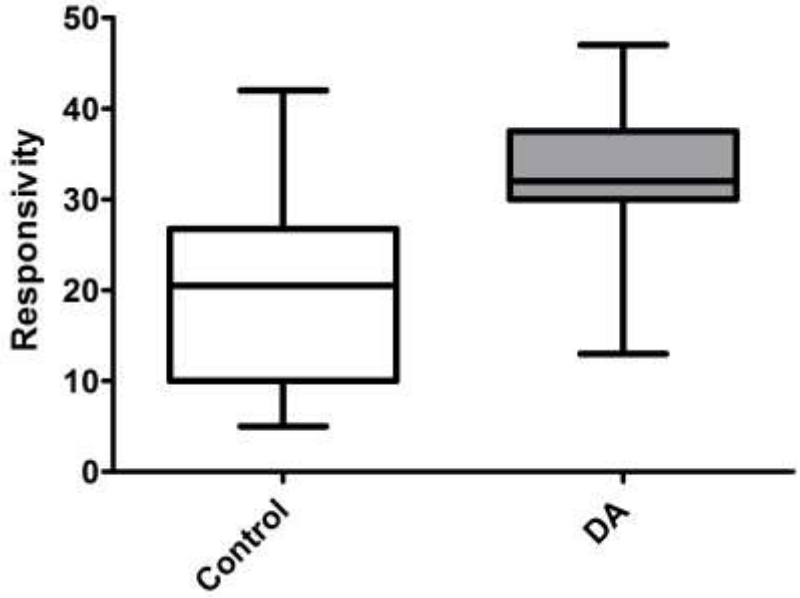

Figure 4: Mean responsivity (*i.e.*, number of total behavioral orienting responses) during Phase 1 exposure between controls and animals with DA toxicosis. Ranges represent 95% confidence intervals. DA subjects were significantly more responsive than controls.

Further, an ROC analysis of Phase 1 responsivity against post-hoc diagnosis (Figure 5) suggested that Phase 1 responsivity was a useful diagnostic measure in this population (area under the curve: 0.735, $P < 0.05$, see Appendix 2.6).

Figure 5

ROC Curve Representing Diagnostic Sensitivity of Phase 1 Responsivity

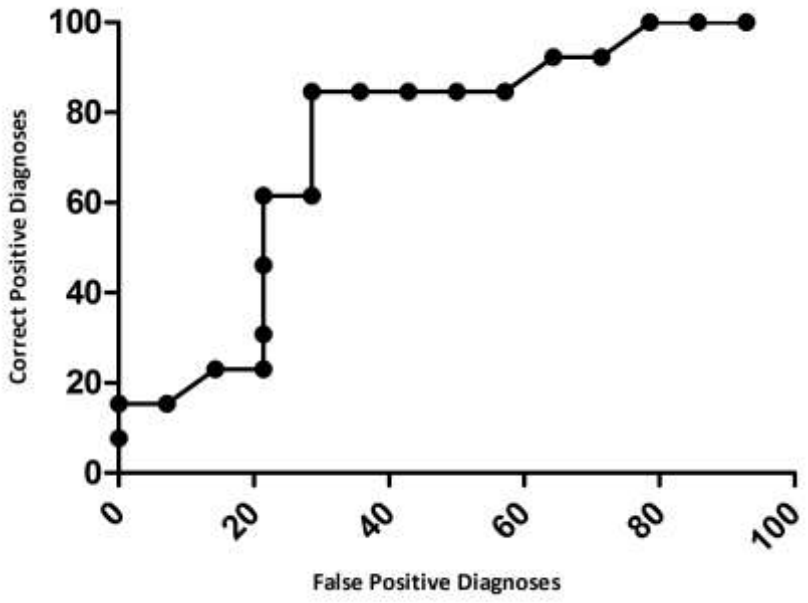

Figure 8: An ROC curve representing trade-off in correct positive diagnoses (y axis) versus correct rejections (x axis). Each point on the curve represents a diagnostic threshold, based on Phase 1 responsivity, with higher thresholds up and to the right.

Right hippocampal damage, left hippocampal damage, right parahippocampal damage, and left parahippocampal damage did not predict Phase 1 responsivity (all $P > 0.05$, Right hippocampus: $F = 0.86$, $Rsq = 0.036$; Left hippocampus: $F = 0.82$, $Rsq = 0.034$; Right parahippocampus: $F = 1.42$, $Rsq = 0.058$; Left parahippocampus: $F = 1.09$, $Rsq = 0.090$, see Appendix 2.5 for all subjects' regional brain volumes).

Epilepsy was observed in 7 of 27 subjects in the study. Animals observed to have seizures (Figure 9) had a significantly higher mean Phase 1 responsivity (36.1), than animals not observed to have had a seizure (25.2) ($P < 0.05$, $t = 2.45$).

Figure 6

Phase 1 Responsivity in Subjects With and Without Epilepsy

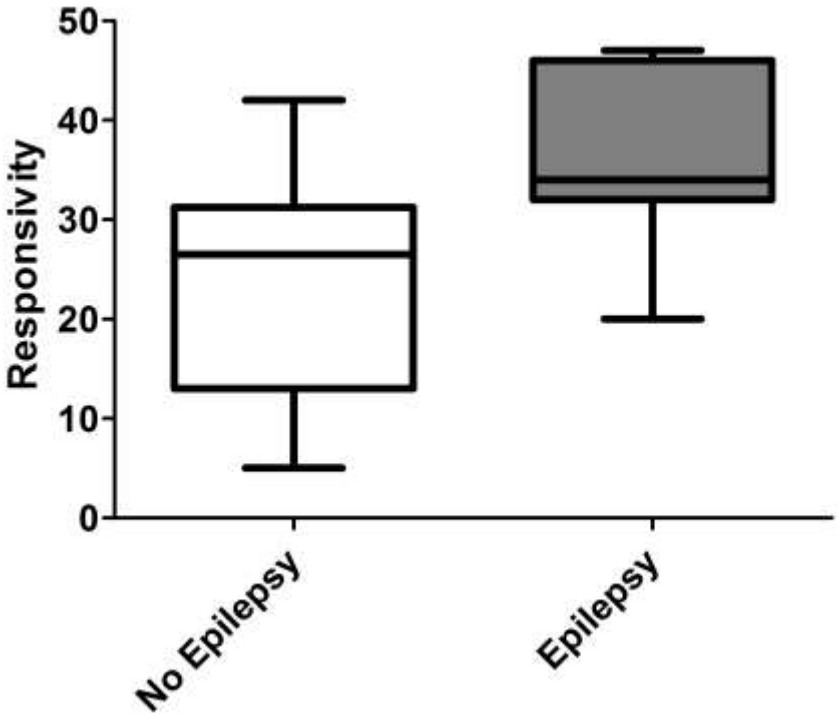

Figure 6: Mean responsivity in Phase 1 between subjects observed to have no seizures and subjects observed to have one or more seizures. Ranges shown represent 95% confidence intervals. Subjects with observed epilepsy were significantly more responsive than subjects not observed to have epilepsy.

Analysis restricted to control animals showed that mean response number was not different between the novel (7.18 responses) and familiar (7.0 responses) stimulus source locations during exposure Phase 2 ($P > 0.05$, $t = 0.07$).

Given this, it is likely trivial that mean relative responsivity to the unfamiliar versus familiar stimulus sources in the Exposure Phase 2 did not differ between animal with positive (1.17) and negative (1.48) veterinary diagnoses ($P > 0.05$, $t = 0.63$).

Mean (bilateral) hippocampal volumes were lower in animals diagnosed with DA toxicosis (0.0034) than in control animals (0.0039) ($P < 0.05$, $t = 2.15$, Figure 7). Mean (bilateral) parahippocampal volumes did not differ between animals diagnosed with DA toxicosis (0.0042) and control animals (0.0043) ($P > 0.05$, $t = 0.53$, Figure 7).

Figure 7

Hippocampal and Parahippocampal Volumes in Control and DA Animals

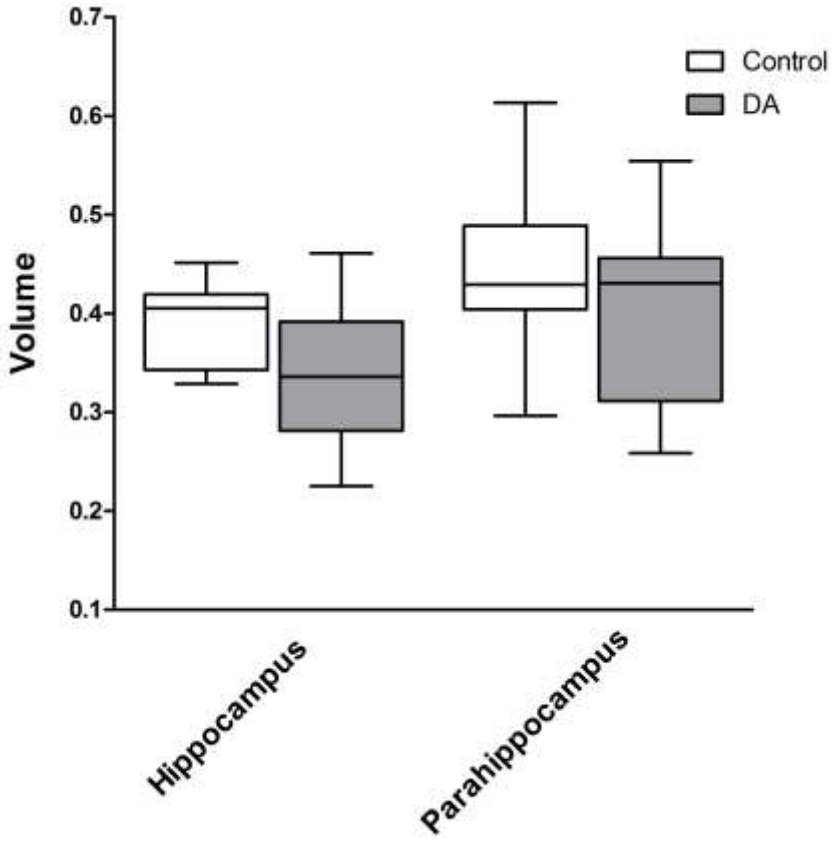

Figure 7: Mean right and left hippocampal and parahippocampal volumes between controls and subjects diagnosed with DA toxicosis. Ranges represent 95% confidence intervals. Hippocampal volumes were higher in control than in DA animals, but parahippocampal volumes did not differ.

Subjective assessment of MR images by a veterinary radiologist identified hippocampal

atrophy, unilateral or bilateral, in 15 of the 25 subjects for which full brain data were

obtained. Of the 15 animals determined to have atrophy, 3 had unilateral right damage, 2 had unilateral left damage, 6 had roughly equal bilateral damage, 2 had bilateral damage weighted more heavily to the right hippocampus, and 2 had bilateral damage weighted more heavily to the left hippocampus.

Mean hippocampal volume was no different in the 10 animals subjectively judged to have no hippocampal damage (0.376%) than in the 15 subjectively judged to have unilateral or bilateral damage(0.350%) ($P > 0.05$, $t = 1.01$). Mean right hippocampal was no different in the 11 animals subjectively judged to have unilateral right or bilateral damage (.172%) than in the 10 animals judged to have no damage (.187%) ($P > 0.05$, $t = 0.97$). Mean left hippocampal volume was, however, significantly less in the 10 animals subjectively judged to have unilateral left or bilateral damage (.155%) than in the 10 animals judged to have no damage (.189%) ($P < 0.05$, $t = 2.52$).

Parahippocampal damage was not assessed subjectively by a veterinary radiologist.

Mean (bilateral) hippocampal volume in the 7 animals observed to have one or more seizures (0.33%) was not different than in the 18 animals observed to have no seizures (0.37%) ($P > 0.05$, $t = 1.27$). Mean (bilateral) parahippocampal volume in the 7 animals observed to have one or more seizures (0.40%) was not different than in the 18 animals observed to have no seizures (0.43%) ($P > 0.05$, $t = 0.83$).

Discussion

In summation, we found that post-hoc veterinary diagnosis of chronic DA toxicosis in CSLs predicted responsivity to repeated auditory stimuli. Further, response criteria based off of these findings appear to have diagnostic merit for chronic DA toxicosis. Observed seizures also predicted responsivity, but volumetric measurements of hippocampus and parahippocampus did not. Neurologically healthy control animals did not respond differentially to a novel versus familiar stimulus source, and their response pattern did not differ from that of animals with chronic DA toxicosis. Veterinary diagnosis predicted hippocampal volume, but not parahippocampal volume. Subjective assessment of hippocampal damage by radiologist predicted left, but not right or total (bilateral) hippocampal volume. Observed seizures did not predict total (bilateral) hippocampal or parahippocampal volume.

The finding that control animals did not respond differentially to novel and familiar stimulus source locations in experimental Phase 2 suggests that the Phase 2 protocol is of no use in supporting diagnosis. This second assay could have failed for any number of reasons, including non-salience of the positional shift, or a disproportionate contribution of subject location to relative responsivity (*i.e.*, subjects may have been more likely to respond to the speaker they were closest to, as opposed to the speaker in the novel location). Given that this is a true null result, it is not discussed further, and the rest of the discussion focuses on the findings from experimental Phase 1.

The primary finding in this study—that best-practice, post-hoc veterinary diagnoses of chronic DA toxicosis are strongly predicted by a simple measure of gross responsivity in an untrained auditory exposure task—largely replicates a previous finding with a similar testing methodology (Cook, Reichmuth, & Gulland, 2011). Across two studies with 66 wild CSLs in total, this is a strong and likely reliable result. Further, ROC analysis suggests, in both studies, that a measure based on responsivity holds diagnostic merit.

In our previous work, we had hypothesized that the increased responsivity during initial exposure to auditory stimuli was due to hippocampal damage resultant from toxic exposure to DA toxicosis. In the current study, volumetric measurements of the hippocampus from MR images allowed us to look for a relationship between responsivity and regional brain damage—no such relationship was evident from our data. The volumetric measurements of hippocampal damage in the current study tracked with veterinary diagnosis, and, equivocally, with subjective assessment of hippocampal damage, suggesting they have at least some validity. This is especially the case considering the strong relationship between volumetric measurements of hippocampal damage and a number of behavioral measures in the work discussed in Chapter 3 of this dissertation.

Given the high concordance between veterinary diagnosis and initial responsivity, both in the current study and previously in Cook, Reichmuth, & Gulland (2011), and the non-predictiveness of hippocampal formation volume, it is likely that some feature of DA toxicosis incidental to extent of hippocampal and parahippocampal damage is acting to increase behavioral responsivity. The current findings implicate epilepsy.

Epilepsy is a core feature of a diagnosis of DA toxicosis, both in acute and chronic phases, and there is reason to believe medial temporal epilepsy could lead to a persistent increase in exploratory behavior and responsivity to environmental stimuli. In the current study, animals observed to have seizures were significantly more responsive than animals not observed to have seizures—this matches unreported data from Cook, Reichmuth, and Gulland (2011) (mean responsivity in animals observed to have seizures: 22, mean responsivity in animals not observed to have seizures: 11.8 ($P < .01$, $t = 3.24$)) It may be the case that some neurological feature of epilepsy, such as altered functional connectivity, is the proximal cause of increased responsivity in CSLs with DA toxicosis.

If epilepsy is the driving factor for increased responsivity, this could bear on how related diagnostic measures might be applied. If behavioral responsivity indeed tracks with epilepsy, not gross brain damage, it should serve as a useful diagnostic tool for identifying cases of both acute and chronic DA toxicosis. Such a measure would likely not predict severity of disease progression, but neither would it under-represent animals who had not manifested substantial gross brain pathology—a substantial concern with using brain damage as a diagnostic marker, since hippocampal damage as a result of DA toxicosis is likely progressive as a result of persistent epilepsy (Montie *et al*., 2012; Goldstein *et al*., 2008; Mathern *et al*., 1996).

The observational measure of seizure occurrence used in the current study likely has a low false positive rate, but, given that CSLs in rehabilitation are not observed 24 hours a day, a potentially substantial false negative rate. Reliable EEG measures of epilepsy have not been

obtained in CSLs, but would aid in confirming the biological basis of this reliable behavioral difference in responsivity to auditory stimuli found in animals with chronic DA toxicosis and controls.

In the current study, the strong finding of increased initial responsivity to repeated auditory stimuli in CSLs with chronic DA toxicosis, coupled with evidence that this is being driven by epilepsy, not brain damage, refines understanding of our previous findings (Cook, Reichmuth, & Gulland, 2011), and paves the way for implementation of a related diagnostic measure. Diagnostic efficacy of the responsivity measure was similar in the current study, featuring subjects with chronic DA toxicios, and in the previous study, which included subjects both with chronic and acute DA toxicosis. Lower or higher response thresholds could be selected depending on whether clinicians wish to minimize false positive or false negative diagnoses. In the current study, a threshold of 28.5 responses yielded the highest diagnostic resolution, with sensitivity (that's correct positive diagnoses) of 84.6% and specificity (correct negative diagnoses) of 71.4%.

The findings discussed here strongly suggest that a simple behavioral assay measuring responsivity to a repeated auditory stimulus has high diagnostic efficacy for DA toxicosis in CSLs. Because the assay is rapid, completely automated, and can be coded post-hoc by neutral observers, it might feasibly be implemented in a rehabilitation setting. Although responsivity is not, by itself, a conclusive sign of DA toxicosis, it is a low-impact, evidence-based measure that addresses a real diagnostic need, and can serve to augment ongoing veterinary practice.

Chapter 3

Memory Deficits in Wild Sea Lions With Naturally Occurring Medial Temporal Lobe Damage as a Result of Toxic Exposure to Domoic Acid

NOTE: The body of Chapter 3 describes results from a study of memory in wild CSLs with naturally occurring brain damage as a result of DA toxicosis. It was written and formatted as a full-length article for the *Journal of Cognitive Neuroscience*. These data have specific relevance for veterinary and wildlife rehabilitation professionals, as well as psychologists and neuroscientists. However, given my emphasis on this work as a potential validator for non-traditional models to study brain and behavior, I would like to target it specifically to a journal widely read by researchers in the cognitive neurosciences, who may be less likely to encounter the work otherwise.

The work described here was conducted over three years, one subject at a time. Because each subject was available for only ~2 weeks, and could not be trained to associate humans with food, all training and testing had to be conducted rapidly and remotely. This required a quite novel approach for behavioral studies, much less ones featuring wild animals. As discussed briefly in the manuscript below, due to the slow pace of data acquisition, as well as the dearth of prior work on memory and the brain in CSLs, relatively conservative behavioral tests were selected. The goal was not to revolutionize understanding of the hippocampus (although this CSL subject group did allow for some relatively novel study parameters), but rather to understand the particular manifestation of DA in CSLs, expand

prior understanding of the hippocampus into a new species, and demonstrate the feasibility of cognitive neuroscience research with this alternative, naturalistic model.

Because this is written as a full-length article, it is comprehensive, and excludes very little relevant data. Much of the introduction and the imaging section of the methods will be redundant with Chapter 2.

Abstract

California sea lions (CSLs) in the wild are commonly exposed to domoic acid (DA), an algal neurotoxin that leads to chronic epilepsy and focal hippocampal damage. We obtained data from 30 wild CSLs undergoing rehabilitation, including 20 believed to be suffering from DA toxicosis, on two behavioral tasks—delayed alternation in a two-choice maze with a 7 and 20 second delay, and a once-daily foraging task. For each subject, performance on the delayed alternation task was measured relative to performance on corresponding non-delay trials, and was likely representative of episodic memory. Two behavioral measures were derived from the once-daily foraging task: across-session search time to the baited location, likely representative of long-term allocentric spatial memory, and within-session mean errors (*i.e.*, repeat visits to previously explored locations), likely representative of spatial working memory. Following behavioral testing, we acquired structural magnetic resonance imaging (MRI) data for each subject and used manual tracing to compute hippocampal and parahippocampal volumes relative to a measure of whole brain volume. Right hippocampal volume, but not left, predicted performance in the 7 second delayed alternation task and the within-session error measure from the once-daily foraging task. Right parahippocampal volume, but not left, was predictive of performance on the long-term allocentric spatial memory measure. Neither right nor left parahippocampal volume was predictive of the other behavioral measures. These findings represent the first direct data on hippocampal function in a marine mammal, and suggest that CSLs with brain damage resulting from DA toxicosis have impaired short-term episodic memory, spatial working memory, and long-

term allocentric spatial memory. This research represents one approach to developing alternative, naturalistic, and humane models for cognitive neuroscience research.

Introduction

Alternative Animal Models for Memory Research

Comparative research in cognitive neuroscience, as in the biomedical sciences more broadly, typically relies on highly controlled laboratory animal models (Bekris, Yu, Bird, & Tsuang, 2010; Sixth Report…, 2010; Lewejohann *et al*., 2006; Deacon, Croucher, & Rawlins, 2002; Wahlsten, 1972). Such models allow for careful experimental control and have been massively productive, but also suffer from some short-comings. Notably, the behavioral and genetic controls implemented in these models clearly lead to an underrepresentation of biological and behavioral variability in natural populations (including human populations) (Martin, Ji, Maudsley, & Mattson, 2010; Kikusi, Nakamura, & Mori, 2008; Leggio *et al*, 2005; Wahlstein *et al*., 2003; Holmes, Parmigiani, Ferrari, Palanza, & Rodgers, 2000; Kruska, 1988). While variability can obscure relevant results, it is also an inherent and undeniable feature of any natural population, and psychologists increasingly recognize the importance of grappling with subject variability in experimental design (Kanai & Rees, 2011; Koolhaas, Boer, Coppens, & Buwalda,2010; Henrich, Heine, & Norenzayan, 2010; Vogel & Awh, 2008). The dominant laboratory animal research models may also be hampered by overreliance on evidence from a limited number of species—while many brain processes are clearly conserved across species, some even quite ubiquitous mechanisms can vary drastically in important ways (*e.g.*, mechanisms governing pain, LaCroix-Fralish, Austin, Zheng, Levitin, & Mogil, 2011).

Typical laboratory animal models in cognitive neuroscience will continue to produce meaningful results. However, given their limitations, there is clearly a benefit to increased exploration of alternative models. Use of strains or species with broader ranges of genetic variability and more species-typical developmental courses could be particularly beneficial. In some cases, different results obtained from alternative models could call into question previously accepted findings, spurring further research to identify the source of the disparity (rearing, genetics, species-based, etc.). Complete or partial replications of findings with alternative models would also be of value, clarifying issues of likely effect size, and increasing the confidence with which results may be generalized to other species (including humans). Perhaps most importantly, on the applied side of cognitive neuroscience, increased diligence of this nature could reduce the costly and dangerous effects of human-inappropriate medical interventions derived from mouse and rat data (*e.g.*, van Meera, Kooijman, Gispen-de Wied, Moors, & Schellekens, 2012).

The relative value of alternative animal models to cognitive neuroscience will of course vary depending on the brain systems studied and the hypotheses of interest. Certain aspects of hippocampal functioning may be particularly poorly represented by traditional laboratory models. There are robust and well documented effects of environmental enrichment on hippocampal neurogenesis, showing that an impoverished environment greatly constrains this process (Kempermann, Gast, & Gage, 2002; Kempermann, Kuhn, & Gage, 1997), and evidence suggests this neurogenesis plays an important role in learning and memory (Deng, Aimone & Gage, 2010). Laboratory manipulations that interfere with hippocampal neurogenesis, including reduced environmental enrichment, adversely impact measures of

memory (Winocur, Becker, Luu, Rosenzweig, & Wotjtowicz, 2012; Nilsson, Perfilieva, Johansson, Orwar, and Eriksson, 1999). There is also strong evidence that environmental enrichment has hippocampus-specific neuro-protective effects (Young, Lawlor, Leone, Dragunow & During, 1999). In brief, traditional animal research models, with their reliance on restricted rearing conditions, almost certainly impair hippocampal function and increase sensitivity to certain types of insults. Studying hippocampal function using more diverse or representative animal models may yield new findings, or, at the least, help to validate previous research.

Here we present evidence that wild CSLs with naturally occurring hippocampal damage from environmental exposure to DA represent a potentially valuable model for understanding the function of medial temporal lobe networks in memory.

Hippocampal Damage in Wild Sea Lions as a Result of Domoic Acid Toxicosis

DA is a metabolite of algal diatoms that aggregate in frequent blooms off the California coast (Garrison, Conrad, Eilers & Waldron, 2004). An excitatory neurotoxin, DA targets glutamate receptors, and has a particularly high affinity for AMPA and kainate binding sites (Hampson & Manalo, 1999). There is a high concentration of AMPA and kainate receptors in the mammalian hippocampus, and DA acts to cause damage in these areas. The primary pathway for short-term damage is thought to be the influx of calcium ions into the synaptic cleft, triggered by DA's opening glutamate-specific ion-gated channels. The calcium ions in turn activate a number of enzymes that can contribute to excitotoxic damage (Pulido, 2008). DA has also been shown to specifically disrupt signaling in the dentate gyrus of the

hippocampus, leading to abnormal mossy fiber sprouting (Bernard, MacDonald, Gill, Ryan, & Tasker, 2007; Debonnel, Weiss, & Montigny, 1989). This in turn can lead to chronic epilepsy (Magloczky, 2010), which has been observed in rats, humans, and CSLs exposed to DA (Bernard, Ryan & Tasker, 2005; Silvagni, Lowenstine, Spraker, Lipscomb, & Gulland, 2005; Cendes, Andermann, Carpenter, Zatorre, & Cashman, 2004). Chronic temporal lobe epilepsy has in turn been shown in humans to lead to damage in the hippocampal and parahippocampal regions (Bernasconi *et al*., 2002), presumably through ischemic damage suffered during seizure activity (Liou, Clark, Henshall, Yin, & Chen, 2003). Hippocampal damage is a clear sequela to DA exposure in humans (Teitelbaum *et al*., 1990), rodents (Tryphonas, Truelove, Iverson, Todd & Nera, 1990), and CSLs (Goldstein *et al*. 2008). Interestingly, damage appears to be more severe in CSLs than in other species studied, although whether this is due to exposure profile or some species-specific biological factor remains unknown (Silvagni *et al*., 2005).

CSLs are now commonly exposed to DA in the wild, and many come to shore in distress (*i.e.*, "strand") as a result (Gulland *et al*., 2002). Between 1998 and 2006, over 20% of CSLs stranding and subsequently treated at The Marine Mammal Center (TMMC) in Sausalito, CA, were diagnosed with toxic exposure to DA (Goldstein *et al*., 2008). Among these animals, hippocampal damage was the most reliable measurable effect, identified via MRI in 41 of 42 diagnosed animals, and via post-mortem histological examination in 70 out of 89 diagnosed animals (Goldstein *et al*., 2008). As has been found in rodents, gross lesions in these CSLs are common in the CA3/CA1 axis of the hippocampus and the dentate gyrus, and extensive damage to other brain areas outside of the hippocampus is relatively rare, although

parahippocampal damage has also been observed. In essence, the most reliable gross neurological impact of DA exposure in CSLs is focal damage to the hippocampal formation.

Given the high rate of relatively restricted hippocampal damage in these animals (Goldstein *et al*., 2008), the growing body of normative data from MRI (Montie *et al*., 2009), and the demonstrated tractability of CSLs for behavioral research in long-term captivity (*e.g.*, Cook, Rouse, Wilson, & Reichmuth, 2013; Reichmuth, Kastak, & Schusterman, 2002; Gisiner & Schusterman, 1992), CSLs are an appropriate and feasible animal model for relatively large scale studies combining behavioral measures with quantitative assessments of neurological damage to brain areas concerning memory.

Because this mammalian population is representative of the biological variability and the enriched developmental environment experienced by wild populations, results from such study can serve as a meaningful comparison to the large body of data accrued from captive rodent models. An additional benefit to this type of natural, alternative model is that, in addition to providing increased understanding of target systems such as the hippocampus, results can also be used to inform practical decisions in veterinary and rehabilitation settings regarding how to treat and handle afflicted animals. CSLs may also be of particular interest for comparison to humans as a large-brained, long-lived, social species. Finally, because the brain damage has been incurred naturally, and can be characterized by *in vivo* MRI, this population allows for a particularly low-impact and humane avenue for brain lesion research, clearing a higher ethical bar than most similar studies with laboratory animals.

The availability for study of a large number of animals with a wide range of damage relatively restricted to the hippocampus, coupled with the tractability of CSLs and established neuroimaging protocols, combines to make CSLs with DA toxicosis a promising model for studying the function of the hippocampus.

The Hippocampus and Memory

Damage to the hippocampus in humans causes anterograde amnesia—the inability to produce new episodic memories (Scoville & Milner, 1957). While early attempts to model medial temporal amnesia in non-human animals originally produced divergent results (Teng, Stefanacci, Squire, & Zola, 2000; Mishkin, Malamut, & Bachevalier, 1984; Orbach, Milner, & Rasmussen, 1960), findings with both human and non-human animals have converged recently to produce a broadly coherent model of the functioning of the hippocampus and surrounding areas. Strong evidence suggests that the hippocampus serves in part to bind disparate streams of sensory information into coherent, contextual representations, and areas around the hippocampus serve to represent less contextually rich representations (Eichenbaum, Sauvage, Fortin, Komorowski, & Lipton, 2012). This understanding draws on evidence from fMRI studies with humans showing that the hippocampus is differentially involved in remembering the context of a prior stimulus, while surrounding areas are differentially involved in remembering the stimulus itself (Ranganath, 2010; Diana, Yonelinas, & Ranganath, 2007; Davachi, 2006), as well as evidence from non-human animals showing that restricted hippocampal lesions do not interfere with stimulus recognition, but

do interfere with tasks in which a particular stimulus must be remembered in relation to its initial context (Ennaceur, Aggleton, & Fray, 1997).

In a related line of studies, the hippocampus has been shown to play a crucial role in differentiating between separate but similar episodes, utilizing both "place" and "time" cells (MacDonald, Lepage, Eden & Eichenbaum, 2011; Eichenbaum, 2000; O'Keefe, 1976). This has been demonstrated elegantly in rodent models through delayed alternation tasks. While free-run left-right alternation in a two-choice T-Maze is not significantly impaired by hippocampal damage, *delayed* alternation, even with very short delay durations, is heavily impaired by hippocampal damage (Bannerman *et al*., 2001; Aggleton, Neave, Nagle, & Hunt, 1995). Single-cell recording measures collected during alternation tasks have shown that in free-running alternation hippocampal activity in the stem of the maze is dissociable between left and right turn trials. These patterns of activity are replicated in the hippocampus at the starting point of the maze during the delay in delayed alternation trials (Ainge, van der Weer, Langstrom, & Wood, 2007; Lee, Griffin, Zilli, Eichenbaum & Hasselmo, 2006).

In conjunction with current understanding of hippocampal networks, this observation has been used to convincingly argue that the hippocampus is involved in encoding experiential episodes and these episodes are retained during delay trials so as to guide subsequent behavior following the delay (Hasselmo, 2009; Eichenbaum & Lipton, 2008). That is, to successfully complete trials in delayed alternation, a subject needs to represent their most recent behavior during the delay so as not to repeat it. Because a subject will have gone

both right and left many times, accessing the correct episode may require retrieving a contextually bound episode involving temporal order, behavior, and location. Object recognition, which is spared with restricted hippocampal damage, serves limited use in delayed alternation, so can be isolated from other processes. Motor memory may also support performance in practiced, free-running versions of the task, but the delay interrupts this, allowing motor memory to be precluded from performance as well. From these factors, it seems performance in the delay condition of the alternation task likely relies on some form of contextually bound experiential memory. Despite some resistance (Suddendorf & Corballis, 2010; Tulving, 2005), "episodic memory" (or at the least, "episodic-like memory") is increasingly being attributed to non-human animals (Eichenbaum *et al*., 2012; Clayton & Russel, 2009), and may be demonstrated in tasks such as delayed alternation.

Delayed alternation tasks show reliable deficits accompanying hippocampal damage with even very short delays. While earlier understandings of hippocampal function implicated the brain structure exclusively in long-term memory (Milner, 1970), newer evidence indicates that bound contextual representations require the hippocampus, not only to retrieve such representations from the long-term store, but to maintain them in working memory (Voss, Warren, & Cohen, 2011; Shrager, Levy, Hopkins & Squire, 2008; Hartley *et al*., 2007; Olton, Becker, & Handelmann, 1979). This is in keeping with newer understandings of working memory, suggesting that it does not utilize a specific store, but rather is reliant on attentional mechanisms to manage domain-specific representation sites that work across a range of time scales (Jonides *et al*., 2008; Postle, 2006). In other words, the hippocampus

will be recruited for maintaining bound, ordered representations of events, regardless of the time scale.

In addition to contextual memory for events, the hippocampus has also been strongly linked with navigation, specifically with allocentric spatial representation (O'Keefe & Nadel, 1978). Navigation relying on a mental map anchored by external landmarks is inextricably linked with the hippocampus in a range of species (Paul, Magda & Abel, 2009; Gron, Wunderlich, Spitzer, Tomczak, & Riepe, 2000). Indeed, one of the more reliable findings in brain lesion studies is hippocampal-dependent impairment in the Morris water maze, which assesses an animal's ability to recall a location based only on relationships between external cues (D'Hooge & De Deyn, 2001; Morris 1984). Importantly, the hippocampus proper is not believed to be strongly involved in either egocentric spatial memory (Holdstock *et al*, 2000; Eichenbaum, Stewart, & Morris, 1990) or object recognition, both of which can inform navigation performance in certain contexts (Forwood, Winters & Bussey, 2004; Murray & Mishkin, 1998). It is likely that these intra and extra-hippocampal processes work roughly in parallel to support navigation in a complex environment (Burgess, 2006). Spatial working memory has also been strongly linked with the hippocampus—extensive findings indicate that rodents with hippocampal lesions have impaired spatial working memory. This is commonly measured in a radial maze, and operationalized as incorrect revisits to previously explored arms within a test session (Jonasson, 2005).

Current Study

In the current study, we used two distinct behavioral paradigms to attempt to broadly gauge the cognitive impact of naturally occurring hippocampal damage on wild CSLs: 1) a delayed alternation task in a two-choice maze, and 2) a once-daily foraging task. These tasks were selected to provide performance metrics of episodic memory, long-term allocentric spatial memory, and spatial working memory.

In the delayed alternation task, subjects were trained to perform a spatial alternation task on repeated trips through a two-choice maze, and then tested with delays of either 7 or 20 seconds between each trial. Performance on delay trials relative to matched no-delay trials was used as a measure of episodic memory. In the foraging task, subjects were given an opportunity to find a food reward in one of four locations once every 24 hours. The baited location was held stable within subjects. A measure of latency to the baited location across tested days was used to represent long-term allocentric spatial memory. A second measure taken from the foraging task—number of incorrect revisits to previously explored locations within test days—was used to assess spatial working memory.

Each subject also took part in *in vivo* structural MRI, allowing quantitative measures of hippocampal and parahippocampal volume via manual tracing and volumetric calculations. Due to the variability inherent in a natural population such as the one used here, we expected to also find a high degree of variability in extent of brain damage present across subjects. This afforded the opportunity to assess whether extent of damage correlated with extent of impairment on the two behavioral tests.

Our approach here is analogous to one utilizing traditional laboratory brain lesion models. However, by drawing subjects from a wild population with naturally occurring focal brain lesions, we were able to expand study of the hippocampus into a species with a high degree of both genetic and developmental variability.

Methods

Subjects

Subjects were 30 wild CSLs selected between April 2009 and November 2011 from available animals undergoing rehabilitation at TMMC in Sausalito, CA (see Appendix 3.1). Age and sex were not controlled—due to the demographics of CSLs stranding in the area covered by TMMC, ages were broadly mixed, and adult females predominated in the sample (8 males, 22 females).

Animals remained at University of California Santa Cruz's Long Marine Lab (LML) for the duration of testing—an average of two weeks, the shortest stay being 12 days, the longest 34, and were then returned to TMMC, following which they were taken for *in vivo* MRI to AnimalScan Imaging center in Redwood City.

All experimenters remained blind to the animals' status during behavioral testing and MRI analysis. Veterinary staff, however, were party to all available information about the subjects at time of selection, and were instructed to provide a 2-1 ratio of CSLs suspected of DA toxicosis to control individuals (subjects with no apparent neurological symptoms). Pre-

screening with MRI was not feasible, but, due to the reliably high rates of DA-afflicted CSLs presenting at TMMC throughout most recent years (Goldstein *et al*. 2008), we were fairly confident of constructing a sample with representative variability in hippocampal and parahippocampal atrophy. Secondary selection criteria required only that the animals not be receiving pharmaceutical treatment during the testing period, and that they be judged by the TMMC veterinary staff to be medically stable and reliably eating. Because subjects were drawn from a rehabilitation population, they presented with a wide range of physical afflictions, including trauma and wounds, infections, malnutrition, and cancers. After selection, subjects were transported from TMMC to LML, where they were kept for the duration of testing. In addition to the 30 subjects discussed here, a number of other animals who were transported to LML were unable to complete testing, for reason of either health or motivation. They were returned to TMMC, did not receive brain imaging, and are not addressed further in this paper.

At LML, animals were kept in an empty pool, 9 m in diameter, and 2 m deep (serving, essentially, as a pen). Within this pool, a smaller pool, 2.2 m in diameter and 0.6 m deep, was kept constantly full of water from a top-feed inflow pipe delivering fresh sea water. This smaller pool was accessible by a 2.3 m ramp descending from the pool lip, and by boxes (1.2 x 0.6 x 0.3 m) on either side of the ramp, functioning as stair steps up to the pool. CSLs are able climbers, and all subjects were able to enter and exit the smaller pool comfortably over the side at any point along the pool's perimeter.

All subjects were kept on a regular feeding schedule, as set by TMMC veterinarians, whether or not they were willing to participate in behavioral training and testing. Were animals willing to participate, all food was distributed in a testing or training context. Food provided was a mix of freshly thawed herring and capelin fish, which together provide the necessary fat and water content for CSLs. Daily vitamin supplements were also provided. The UCSC campus veterinarian oversaw animals during their time at LML, working with TMMC veterinary staff to monitor the animals' health. Experimenters were present only during the day, when they could monitor the animal from a control room adjacent to the large pool. An always-on web-cam allowed monitoring during off-hours.

While at LML, animals participated in training and testing 7 days a week, and, for continuity, all primary care, training, and testing was conducted by one experimenter with experience in marine mammal husbandry and operant conditioning training using positive (fish) reinforcement, with assistance as needed.

Behavioral Testing

General Procedure

Two behavioral testing protocols were conducted in the present study, a short-term, delayed alternation task, and a longer-term spatial foraging task. Reward used in training and testing was fish (either herring or capelin). Because all subjects were undergoing active rehabilitation, and were, potentially, candidates for release back into the wild, it was essential that all training and testing be conducted without direct human contact. Forming positive associations with humans (such as might be formed when repeatedly receiving food

reward directly from a handler) can negatively impact successful re-entry into the wild, leading to animals who are more likely to interact with humans, potentially in dangerous ways. Thus, the experimenters were out of sight when fish reward was delivered. All training and testing was conducted during daylight hours.

Delayed Alternation in a Two-Choice Maze

Maze Training

Initial training for the delayed alternation task began for each animal on his or her first day at LML, and proceeded as follows, with some small degree of variability. First, subjects were trained to repeatedly leave the small pool and walk down the ramp to the deck. This was done through successive approximation using fish reward. During training and testing sessions, the experimenter dispensed fish by throwing it into the pen from behind a blind, on deck next to the control room and opposite the small pool and inflow. All fish rewards were paired with a brief whistle tone, or "bridge." Each training or testing session began with the experimenter's throwing a fish into the small pool. Once the subject had eaten this fish, the experimenter threw a fish to the bottom of the ramp (this method of leading an animal through the environment by targeted reward dispensing is referred to as "baiting"). When the subject had walked down the ramp to receive the fish, another fish reward was thrown at the bottom of the ramp. Then, another fish was thrown into the small pool to prompt the animal's return. Once the animal had returned to the small pool, another fish was thrown at the base of the ramp, and so forth, to begin establishing a cycle wherein the

subject would repeatedly leave and return to the small pool (for a more detailed account of training see: Cook, Bernard, & Reichmuth, 2011).

Once the subject was reliably moving out of the pool and down the ramp for fish reward (usually within the first training session), the training criteria shifted. Now, after baiting the subject back into the small pool, before the experimenter would throw another fish at the bottom of the ramp, the subject had to first make an unprompted move toward the ramp. Once the subject would reliably swim toward the ramp after returning to the small pool, the criterion was again shifted, so that now the subject had to move toward the ramp and place a head or flipper on the ramp to be rewarded with a fish at the bottom of the ramp. In this way, training shifted gradually from baiting to operant conditioning, where each successive reward was contingent on the animal's making increased, unprompted progress toward the end-state of walking down the ramp to the deck unprompted.

When the subject was reliably walking down the ramp to the deck to receive fish reward without requiring any baiting (this generally required 2–3 training sessions), the next phase of training began. In this phase, the maze was put in place (Figure 8). The maze was a 2.4-meter-long wooden chute, 1.5 meters in height, that, when placed for training and testing sessions, extended directly from the end of the ramp descending from the subjects' small pool. When the maze was put in place, two side-walls were also placed along the ramp, such that an animal could not climb onto or off the side of the ramp. At the end of the chute on either side were two pairs of "saloon" doors, one pair on the left, one on the right. These opened outward such that an animal exiting via one of them would be moving

perpendicularly to the body of the maze's chute. Once through, the animal could not backtrack through these doors. These doors could be remotely opened or held closed by an experimenter through a system of ropes and pulleys. There were no return arms leading back from these doors to the beginning of the maze. The animals could thus return to the pool after exiting a door via a range of different trajectories. There was also a gate at the opening of the maze's chute, immediately at the bottom of the ramp, that could be opened up and inward by another rope and pulley system. During initial training and testing, this gate was always in the up (open) position.

Figure 8

Delayed Alternation Testing Arrangement

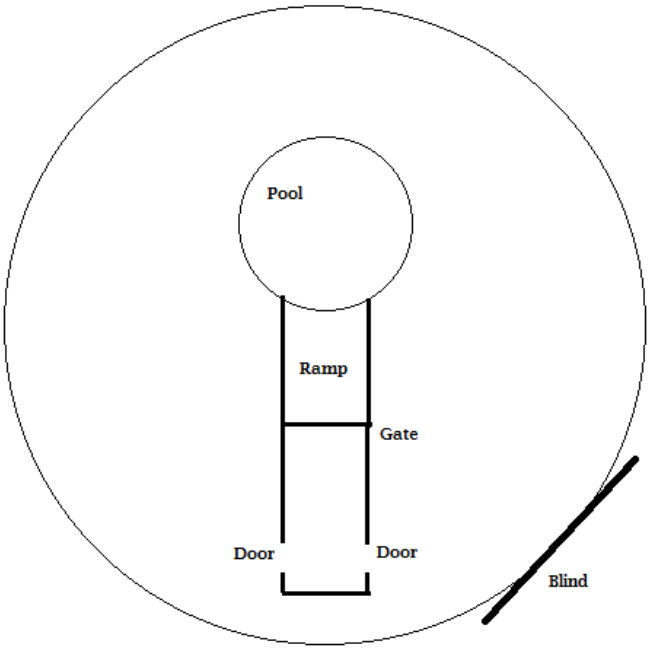

Figure 8: Overhead view of the experimental set-up for the delayed alternation testing.

When the maze was initially put in place, both pairs of saloon doors were clipped open to allow the animal to freely exit without impediment. Regardless, many subjects had some apparent initial anxiety when first moving through the maze. If the subject was unwilling to come down the ramp now that the maze was in place, they were again baited with fish, thrown at the base of the ramp within the maze's chute. Further baiting was used to prompt them to continue moving through the maze and to exit through one of the side doors. This

baiting to prompt the animal to descend into the maze was stopped when the animal resumed descending the ramp without prompting. Throughout this period of training, to provide a balanced reinforcement history with both exits to the maze, fish reward was used to bait the animal out on alternating sides of the maze, such that, if on one trip through the maze the animal was baited out the left door, on the next trip through it would be baited out the right door. Fish reward was still provided when the animal returned to the small pool, and if, at this stage, the animal did not return unprompted, the animal was baited back to the small pool with fish reward (this was generally unnecessary by this point in training).

When the animal would reliably move through the maze in an alternating pattern prompted by baiting, and would return to the small pool after exiting each time without baiting, the next phase of training began. In this phase, baiting was no longer used to govern which door the animal used to exit. Instead, on each trip through the maze, one door was held closed by the experimenter, and the other was held open. In this way, an alternating pattern of maze traversal was prompted and reinforced, and a balanced reinforcement history was continued. When the subject was reliably moving through the maze in this alternating manner without any baiting required, the experimenter began opening the target doors slightly less on each successive trip through the maze (or "trial"), such that the animal began to have to actively push through the door to fully exit the maze. There was some variability in how long this phase extended, primarily dependent on how willing an animal was to push through the partially opened door. Some subjects had no apparent difficulty, while others seemed very hesitant to push through or even touch the doors. When the subject was

finally pushing through fully closed doors on both sides, the next phase of training (termed "free choice") began.

In the free choice phase, the experimenter no longer provided direct guidance to the animal to influence his or her decisions about which door to use to exit the maze. Instead, the experimenter simply rewarded correct choices and ignored incorrect choices. A "correct" choice was defined as the first door selected on any particular trial, regardless of which it was, and subsequently within a trial, the door opposite of that most recently selected. For example, if an animal selected the left door on its first trip through the maze within a training session, it would receive a fish reward. Now, to be rewarded again, the animal would have to select the right door. If the animal continued to select the left door, it would not be rewarded. Once the animal switched to the right door and received a reward, the left door was now the correct door, and no amount of traversals through the right door would be rewarded until the left had again been selected and rewarded. These reinforcement criteria were chosen such that the correct choice on any particular trial was always dependent on the animal's behavior on the most recent trial. Each animal then continued to learn, presumably building on its base of prompted alternating behavior, via selective reinforcement and operant conditioning to select alternating doors on each successive trip through the maze.

Two to four training (and later testing) sessions of 20 trials (a trial was any trip through the maze, whether it involved selecting the incorrect or correct door) were conducted each day. The criterion for moving onto delay testing was that the animal had to achieve 17/20 (85%)

correct door selections on two consecutive training sessions (because the first selection in a session was always counted as correct, this meant, functionally, that they had to produce 16/19 correct door selections on these two sessions). Once a subject met this testing criterion on the basic alternation task, pre-testing began.

Pre-testing and Testing

In pre-testing, the animal was given two 20-trial sessions of matched delay and no-delay trials. The delay was enforced by the aforementioned downward-hinged gate at the bottom of the ramp, which prevented entrance to the maze chute when closed. During each pre-testing session, subjects ran 10 regular free trials followed by 10 delay trials. On delay trials, the gate was lowered after the animal had exited through one of the doors. The gate was kept down for a very brief period during the first 10 delay trials, no more than 2 or 3 seconds from the time the animal stepped out of the small pool onto the ramp (the delay count began as soon as the animal's front flippers hit the surface of the ramp). This delay pre-testing was designed to keep the animals from abandoning the task when faced with the impediment of the door. This was a real concern, as subjects were never underfed and were not always highly food motivated. Further, at pre-testing, all had multiple days of practice running freely through the maze, and the imposition of the gate could cause a disruption of established behavior.

On the second pre-test session, 10 free trials were again followed by 10 delay trials. The delay during the second 10 delay trials was gradually increased from 2–3 seconds to 7 seconds by the last trial, which was the duration of the delay during initial test trials.

Following pre-testing, no animals were unwilling to continue participating when faced with the delays during actual testing.

Following pre-testing, testing proper began. During testing, four 20-trial sessions, each again comprising 10 free followed by 10 delay trials, were conducted with a fixed 7-second delay. Then, four 20-trial sessions, arranged in the same manner, were conducted with a 20-second delay. 20-second delay testing always followed 7-second delay testing, as the increased experience with the shorter delay likely contributed to the subjects' willingness to continue participating when later presented with the longer delay condition. The session design, comprising blocks of free running (no delay) followed by matched delay trials, enabled subsequent within subject, within session performance comparisons to be conducted.

Behavior during training and testing was coded post-hoc from video recording by one of three trained coders. Behavioral responses were discrete (exiting the maze from the left or right doors) and non-subjective, so inter-observer reliability ratings were judged unnecessary.

Once-Daily Foraging Task

In this test, four potential food locations (opaque plastic buckets) were lowered simultaneously via a system of ropes and pulleys into the sea lion's enclosure (Figure 9) once every 24 hours. The four locations were set roughly equidistant from the small pool, and did not change within or across subjects. Once lowered, buckets were arrayed along the

external wall of the large holding pool, with 3.7 m separating each bucket. For each subject, one, and only one, set location contained fish across all testing days. The amount of fish used in each exposure varied with animal size and appetite, but was set at roughly a sixth of the animal's daily diet, so as to constitute a very salient reward. The baited location was randomized across subjects. Each bucket was marked with fish scent prior to each presentation to control for the possibility of olfactory cuing. The buckets remained available until the subject had eaten all of the fish in the baited location and visited each of the buckets at least once, or until the subject had eaten all the fish and 5 minutes had passed from the initial point of presentation. The buckets were then removed simultaneously from the pen using the rope and pulley system.

Figure 9

Once-Daily Foraging Task Testing Arrangement

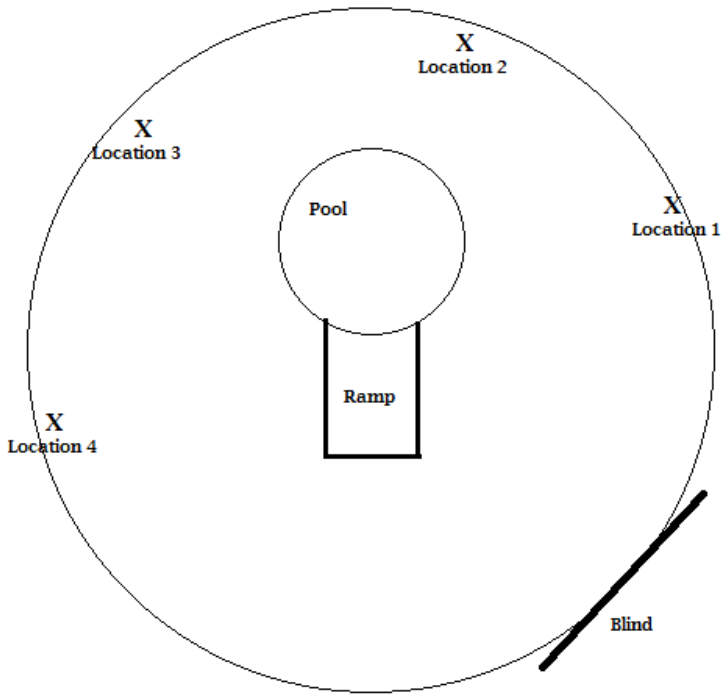

Figure 9: The experimental set-up for the once-daily foraging task.

To control the animal's location at the start of the task, each presentation began after fish were thrown into the small pool, and the animal had remained in or returned to the pool to eat them. In this way, a subject's relative distance to each of the four food locations was held roughly constant across testing, but their position within the small pool and body orientation were not controlled.

Eating fish out of buckets was not intuitive to CSLs during pilot work, so a familiarization phase was implemented for testing. During the first three days at LML, each subject was presented on multiple occasions a bucket filled with fish identical to the buckets used in the subsequent testing. If subjects did not approach and explore the bucket of their own volition they were prompted to do so through baiting—that is, fish were dropped into the bucket until the CSL investigated. This training was conducted at a neutral location, equidistant from the subsequent test locations, and was conducted between 1–3 times a day, dependent on a subject's predilection for approaching the bucket and finding the fish reward. Testing began at the end of the first day in which the animal first approached the training bucket and found and ate the fish without prompting. Despite this training, prompting to the buckets during the first and sometimes second test presentations of the buckets was still required. This was done first by raising and lowering the buckets repeatedly (and simultaneously) to draw attention. In most cases, this was adequate to trigger exploration. If not, the subject was baited to each location in turn by fish thrown next to each bucket location. Such baiting was only ever implemented on the first day of testing, and data from these sessions were not used in subsequent analyses.

Behavior during testing was coded post-hoc from video recording by one of three trained coders. An animal was judged to have visited a bucket at the point when he or she first brought his or her head within 12 inches of the rim of the bucket. Two separate behavioral measures were extracted from these data: whether or not a visit had occurred, and the time point at which it occurred (judged from the timing data encoded in the videos). Because of

the clear, simple criterion for coding a visit, interobserver reliability measures were judged unnecessary.

Brain Imaging

To characterize potential hippocampal and parahippocampal damage, each subject in this study underwent structural MRI. Animals were imaged at AnimalScan imaging facility in Redwood City, CA, on a 1.5 T Siemens Magnetom Symphony scanner. Animals' heads were placed in a CP extremity coil, selected to optimize signal-to-noise ratio. The primary use of the MRI data was to obtain quantitative, volumetric brain measurements. These measurements were conducted on the output from Turbo Spin Echo (TSE) T2-weighted scans obtained in an oblique plane perpendicular to the long axis of the hippocampus. This imaging orientation contributes to the ease of manual sectioning of the hippocampus (Montie *et al*., 2009; Goldstein *et al*. 2008).

The oblique scans were acquired with the following parameters: TR = 5470 ms, TE = 14 ms, FOV = 160 x 160 mm, slice thicknes = 2.0 mm, voxel size = 0.625 mm x 0.695 mm x 2 mm.

Manual tracing was conducted on the oblique images to determine measures of hippocampal and parahippocampal volume relative to whole brain size in each animal. Tracing was conducted using Quanta2 software (UC Davis IDEA Lab, Alzheimer's Disease Center grant, NIH P30 AG010129). For each subject, the right and left hippocampus, right and left parahippocampus, and whole brain (minus the cerebellum) were traced. These tracings were produced on individual brain slices using a mouse-driven pointer. Volumes were calculated by multiplying the traced area on each slice by the slice thickness. To

correct for variation in brain structure size due to natural variability in whole brain size, relative hippocampal and parahippocampal volumes were computed for each animal by dividing the absolute volumes of these structures by whole brain volume. These relative volume measurements, expressed as percentages, were then used in further analysis and are referred to subsequently in this manuscript as “hippocampal volume” and “parahippocampal volume.”

Tracing Parameters

Whole Brain Volume

An index of whole brain volume for each animal was acquired from the oblique scan sequences by tracing around the external boundary of the cortex on each slice as shown in Figure 10. We opted not to include the cerebellum in this measure because air/tissue interfaces around the middle and inner ear caused substantial signal drop-out, primarily in the cerebellum, for all subjects. Posterior to the midbrain structures, the cerebellum was clearly delineated from the cortex and sub-cortical structures, which were traced in completeness. Anterior to the posterior boundary of the mid-brain structures (defined here as the posterior boundary of the superior colliculi), it was difficult to differentiate the medial cerebellum from the lateral portions of the brain stem, and the superior portions of the brain stem from mid-brain and sub-cortical structures. To avoid unwanted variability, the inferior tracing boundary anterior to the midbrain structures followed the inferior boundary of the temporal lobes, connecting the medial-most portion of the left and right hippocampal gyrus with an interpolated straight line. This reliably excluded all cerebellar and brain-stem

tissue, and also necessarily excluded some degree of mid-brain tissue. Anterior to the anterior boundary of the midbrain structures, the inferior tracing boundary followed the inferior boundary of the temporal and frontal lobes, excluding extra-cortical cerebrospinal fluid (CSF) inferior to these boundaries, the brain stem, optic nerves, and the pituitary gland. At the most anterior portions of the brain, where the temporal and frontal lobes became discontiguous, each cortical area was traced separately. Superiorly, olfactory bulbs were included. This constrained measure of whole brain volume allowed for a relatively reliable measure that was not confounded by poor image quality in the cerebellar region.

Figure 10

Whole Brain Volume Tracing

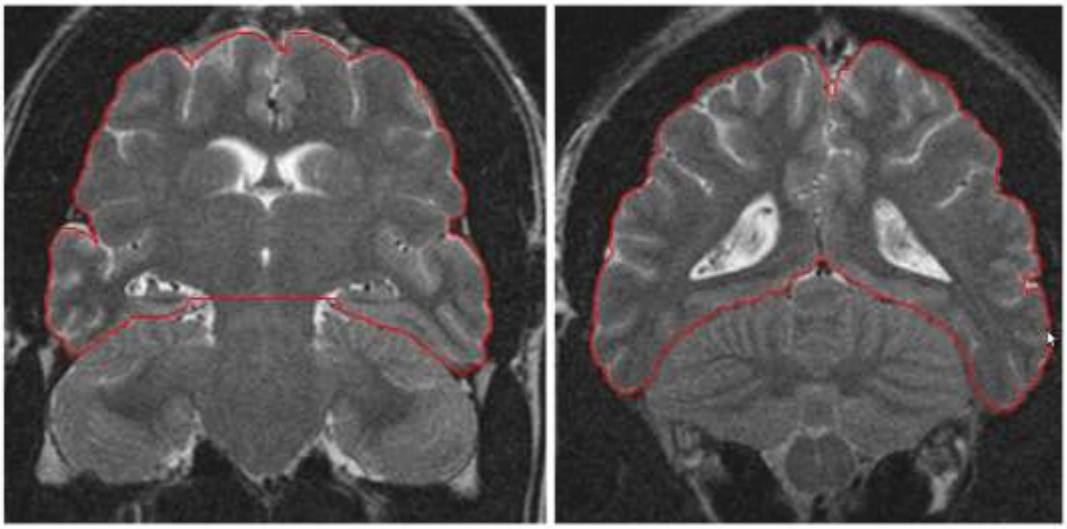

Figure 10: Manual tracings of whole brain volume on two representative slices, excluding cerebellum. The image on the left illustrates the method for excluding cerebellum and brainstem anteriorly—the inferior portion of the temporal lobes is traced, and the most medial points of the hippocampal gyruses are connected. The image on the right is more posterior, where the cerebellum could be more selectively excluded.

Hippocampus

Hippocampal tracing boundaries are illustrated in Figure 11. The anterior most MRI slice on which the hippocampus was traced was that on which the anterior boundary of the mid-brain could be seen. The mid-brain presented in these oblique images as two vertical pillars medial to the hippocampuses; anterior to these slices, the pons, which for CSLs extends beyond the midbrain anteriorly, is discontiguous with the rest of the brain. This boundary likely excluded part of the hippocampal head (or “pes”), but this was necessary due to the

difficulty of differentiating the pes from the amygdala on these sequences, and the lack of prior anatomical work establishing related segmentation criteria for this species. The posterior boundary of the hippocampus was defined as being on the last slice posteriorly on which the superior colliculi were visible. In most cases, this was just anterior to the first slice on which the corpus callosum could be seen as a continuous band, beyond which it was very difficult to differentiate hippocampal tissue from the fornix. Selection of standardized anterior and posterior boundaries for hippocampal tracing is very important, due to the high degree of variability introduced by ambiguity in these criteria (Jack, Theodore, Cook, & McCarthy, 1995). Use of the mid-brain formation allowed for a replicable criterion across subjects that was scalable to whole brain size.

The lateral boundaries of the hippocampus were defined on all slices by the temporal horns of the lateral ventricle, which were obvious on all subjects in this imaging orientation. The superior boundaries of the hippocampus were also defined by the lateral horns on more anterior slices. In the most posterior slices, where the CSL hippocampus appears to "flatten out," the superior boundaries were defined by the obvious transition from hippocampal gray matter to the white matter of superior structures. The medial boundaries of the hippocampus were clearly defined either by CSF or intra-cranial space. The inferior hippocampal boundaries were defined by the white matter separating the hippocampus from the parahippocampal gyrus. Tracing was directly superior to this white matter boundary (so excluding the white matter of the hippocampus where contiguous with the white matter of the parahippocampus), although white matter surrounding the hippocampus was included along the other boundaries. As in human brains imaged on 1.5 T

magnets, there were no clear anatomical boundaries visible to differentiate the CA of the hippocampus from the subiculum in our subjects' images, so part of the subiculum was necessarily included with the hippocampus. The inferior medial boundaries were established by connecting the medial-most point of the hippocampal gyrus to the medial most point of the hippocampus/parahippocampus white-matter boundary with a straight line. The inferior lateral boundaries followed the hippocampus/parahippocampus white-matter boundary—however, on some slices it was difficult to follow this all the way to the CSF of the medial horn of the lateral ventricle. In these cases, the last clear white matter laterally was connected to the most inferior point of the CSF of the lateral horn by a straight line.

Figure 11

Hippocampal Tracings

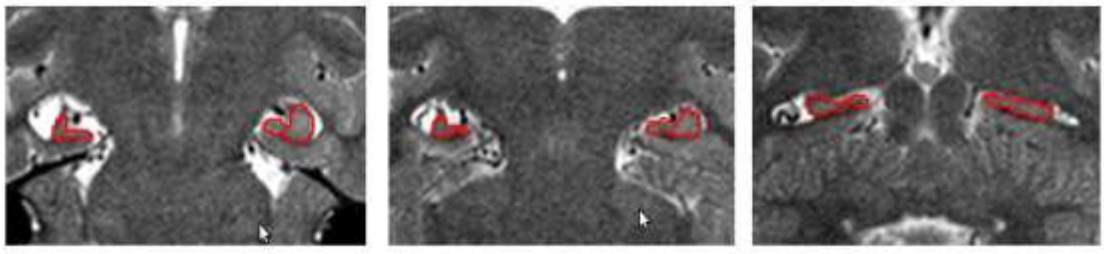

Figure 11: Three representative hippocampal tracings, anterior, mid, and posterior, from left to right.

Parahippocampus

The anterior and posterior boundaries of the parahippocampus (Figure 12) were defined identically to those used for hippocampal tracing, such that the parahippocampus was traced on all (and only) slices on which hippocampal tissue was traced. The criterion for the superior boundaries of the parahippocampus was simply the inferior most boundary of the white matter separating the hippocampus from the parahippocampus, such that there was no overlap in traced hippocampal and parahippocampal tissue. As in the hippocampal tracing, if this parahippocampal/hippocampal boundary was not clearly visible all the way to the CSF of the temporal horn, the last visible portion laterally was connected to the most inferior point of the CSF of the lateral horn by a straight line. The superior medial boundary of the parahippocampus mirrored the inferior medial boundary of the hippocampus—the medial-most point of the hippocampal gyrus was connected to the medial-most point of the parahippocampal/hippocampal white matter boundary by a straight line. The inferior boundary of the parahippocampus was always clearly visible as it bordered on intra-cranial space and/or CSF. The lateral boundaries of the parahippocampus were the most arbitrary. Most protocols for tracing the parahippocampus in humans rely on the collateral sulcus to serve as a marker of the lateral boundary. However, in our subjects, with these scan sequences, the collateral sulcus was either not visible or not present on many slices. Thus, we had to use another local feature to establish the lateral boundary. The lateral/inferior boundary of the hippocampus, where it meets with the superior boundary of the parahippocampus , was selected. We connected this point to the point on the parahippocampus directly inferior as per the absolute orientation of the brain in the Z axis—

this connection was drawn as a straight line. Using this point allowed a reliable local feature to determine the lateral boundary of the parahippocampus, but there is the possibility that this boundary may shift medially accompanying hippocampal atrophy, which could bias parahippocampal size lower in subjects with hippocampal atrophy. However, this point is approximately in alignment with the collateral sulcus on most slices where the latter structure was visible, and measurements of distance between different points on the medial boundary of the hippocampuses to the brain's midline on subjects with severe unilateral hippocampal atrophy suggested it is the more superior portions of the hippocampus that shift most medially with atrophy.

Figure 12

Parahippocampal Tracings

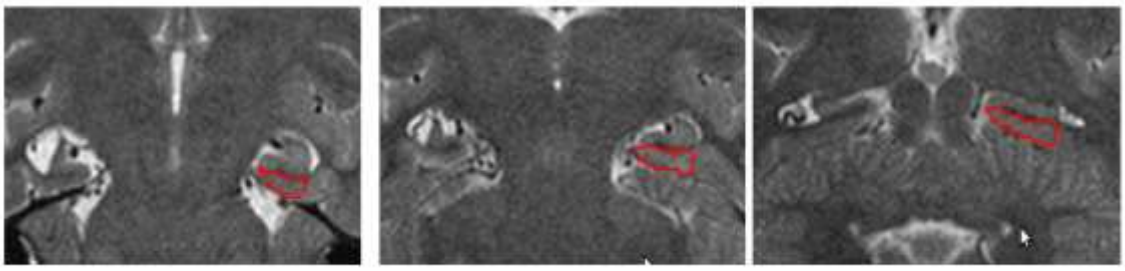

Figure 6: Three representative left parahippocampal gyrus tracings, anterior, mid, and posterior from left to right. For illustrative purposes, only left parahippocampus is traced here.

All images were also assessed by an experienced veterinary radiologist, who described any abnormalities qualitatively.

Data Analysis

MRI

A number of analytic approaches were used to validate the volumetric measures obtained from the MR images. Inter-observer reliability was computed using intra-comparison correlation of two independent coders' measurements of hippocampal and parahippocampal volumes. Criteria for whole-brain tracing were objective, and high variability dependent on rater was judged very unlikely.

To determine whether comparable damage was expressed in the left and right hippocampus and in the left and right parahippocampus, standard two-sided T tests were used to compare right and left hippocampal and parahippocampal volumes across all subjects. Linear Regression was used to determine whether hippocampal volume was predictive of parahippocampal volume. T-tests were also used to compare hippocampal and parahippocampal volumes between animals assessed by the radiologist to have damage or no damage to the right and left hippocampus, as well as to compare hippocampal and parahippocampal volumes between animals diagnosed by TMMC veterinarians with DA toxicosis and controls. Bonferroni corrections for repeated analyses were used throughout.

Delayed Alternation Performance Relative to Hippocampal and Parahippocampal Damage

Total number of training trials in the maze to reach the testing criterion was analyzed as a dependent variable in linear regressions against right and left hippocampal and parahippocampal volumes. In addition, difference in mean trials to learn the alternation task was compared via T test between subjects diagnosed with DA toxicosis and controls.

Performance on 7 and 20-second delay trials in the two-choice maze relative to matched non-delay trials (represented as a ratio of number of correct trials on delay trials over number of correct trials on matched non-delay trials) was examined as the dependent variable in linear regressions against right and left hippocampal and parahippocampal volumes as independent variables. By chance, two control subjects had striking cerebellar, but not hippocampal, pathology, allowing for an opportunistic assessment of a potential double dissociation: trials to acquire basic alternation and delay performance versus focal cerebellar and hippocampal damage.

Once-Daily Foraging Task

Long-Term Allocentric Spatial Memory

Due to variability in search patterns and the very low cost of making an “error” (that is, searching in a non-baited bucket), number of errors prior to finding the baited location across testing days was judged unlikely to be a useful measure. Instead, the primary behavioral measure used here was based on latency between stimulus presentation and the

subject's finding the correct (baited) bucket across successive testing days. To compute a measure representing improvement across subsequent days, a power curve was fit to these measures for each subject. The exponent measure of the equation describing each subject's curve was then used as a representation of improvement in task performance across testing days (referred to here as "across-session latency"). Animals whose speed to the correct bucket improved most quickly and markedly would have higher exponent values (corresponding, in essence, to a steeper/deeper curve), which we judged a reasonable approximation of improvement based on memory across testing days. These exponent values for each subject were then used as the dependent variable in linear regressions against right and left hippocampal and parahippocampal volumes.

Spatial Working Memory

A second measure was assessed from the once-daily foraging task—mean number of bucket revisits within test days (referred to here as within-session errors). This is comparable to incorrect arm revisits in radial maze paradigms, and should be emblematic of an animal's inability to recall where they have previously searched over a short period of time. These mean revisit values were then used as dependent variables in linear regressions against right and left hippocampal and parahippocampal volumes.

Finally to assess independence between the behavioral measures, the four primary behavioral measures (alternation performance with the 7 and 20-second delay, across-session latency, and within-session errors) for each subject were regressed against each other.

Results

MRI

Interobserver reliability for volumetric measurements, computed by intra-comparison correlation, was as follows: Right hippocampus: $r = 0.838$, $P < 0.0001$, Left hippocampus: $r = 0.904$, $P < 0.0001$, Right parahippocampus: $r = 0.884$, $P < 0.0001$, Left parahippocampus: $r = 0.706$, $P < 0.01$.

Descriptively, as measured by volumetrics, right relative hippocampal volumes in this sample ranged from 0.12% to 0.24%, with a mean of 0.18% and standard deviation of ±0.045%. Left hippocampal volumes ranged from 0.11% to 0.24%, with a mean of 0.19% and standard deviation of ±0.039%. Right and left hippocampal volumes were not different at the group level ($P > 0.05$, $t = 0.87$). See Appendix 3.4 for all subjects' regional brain volumes.

Descriptively, as measured by volumetrics, right parahippocampal volumes in this sample ranged from 0.13% to 0.27% with a mean of 0.20% and a standard deviation of ±0.042%. Left parahippocampal volumes ranged from 0.12% to 0.34% with a mean of 0.23% and a standard deviation of ±0.048%. Unlike with hippocampal volumes, right and left parahippocampal volumes were marginally distinct at the group level ($p = 0.054$, $t = 2.27$).

Parahippocampal volumes were significantly correlated with hippocampal volumes ($P < 0.001$, $F = 19.67$, $Rsq = 0.413$). This is not surprising, considering the root DA pathology related to damage in both areas. However, hippocampal volume predicted only 41% of

variance in the parahippocampus, suggesting that they are still meaningfully distinct measurements.

Because right and left hippocampal volumes showed a similar range and distribution, and were independent within subjects, further inference assessing differential relationships between right and left hippocampal volume and behavioral measures was judged to be warranted. Due to the large range of relative volume in both right and left parahippocampus, both were considered in further analysis, despite the range of volumes in the left parahippocampus being slightly larger.

Subjective assessment of MR images by a veterinary radiologist identified hippocampal atrophy, unilateral or bilateral, in 23 of the 30 subjects. Of the 23 animals determined to have atrophy, 6 had unilateral right damage, 2 had unilateral left damage, 6 had roughly equal bilateral damage, 7 had bilateral damage weighted more heavily to the right hippocampus, and 2 had bilateral damage weighted more heavily to the left hippocampus. These subjective measures broadly corresponded with the quantitative volumetric measurements.

Mean relative hippocampal volume was significantly greater in the 7 animals subjectively judged to have no hippocampal damage (0.449%) than in the 23 subjectively judged to have unilateral or bilateral damage(0.359%) (T test (two tailed), $P < 0.01$, $t = 3.62$).

Mean relative left hippocampal volume was significantly less in the 10 animals subjectively judged to have unilateral left or bilateral damage (.156%) than in the 7 animals judged to have no damage (.216%) ($P < 0.001$, $t = 4.15$).

Mean relative right hippocampal volume was significantly less in the 19 animals subjectively judged to have unilateral right or bilateral damage (.163%) than in the 7 animals judged to have no damage (.233%) ($P < 0.001$, $t = 4.64$).

Parahippocampal damage was not assessed subjectively by veterinary radiologist.

Hippocampal volume was also assessed against veterinary diagnoses. Mean relative right hippocampal volume was significantly less in the 20 animals diagnosed as having DA toxicosis (0.164%) than in the 10 control subjects (0.226%) ($P < 0.0001$, $t = 4.82$) Mean relative left hippocampal volume was not statistically different between the 20 animals diagnosed as having DA toxicosis (0.187%) and the 10 control subjects (0.210%) ($P > 0.05$, $t = 1.45$).

Mean difference between relative right parahippocampal volume was not statistically different between the 20 animals diagnosed as having DA toxicosis (0.191%) and the 10 control subjects (0.220%) ($P > 0.05$, $t = 1.84$). Mean relative left parahippocampal volume was not statistically different between the 20 animals diagnosed as having DA toxicosis (0.222%) and the 10 control subjects (0.236%) ($P > 0.05$, $t = 0.74$).

Given the concordance between between the subjective assessment of the veterinary radiologist and veterinarians and the volumetric measurements of hippocampal damage, these volumetric assessments appear to be a valid measurement of actual hippocampal pathology.

Behavioral Performance

Alternation trials required to meet testing criterion in the maze were not predicted by right or left hippocampal or parahippocampal volume (All $P > 0.05$, Right hippocampus: $F = 0.91$, $Rsq = 0.035$; Left hippocampus: $F = 0.14$, $Rsq = 0.006$; Right parahippocampus: $F = 0.067$, $Rsq = 0.003$; Left parahippocampus: $F = 0.81$, $Rsq = 0.031$). Nor were trials required to meet criterion different between subjects diagnosed with DA toxicosis (312.8) and controls (351.4) ($P > 0.05$, $t = 0.71$). See Appendix 3.2 for all subjects' alternation performance.

Right hippocampal volume was a moderate predictor of performance in the 7-second delay test ($P < 0.01$, $F = 10.55$, $Rsq = 0.274$, Figure 13).

Figure 13

7-Second Delay Performance as a Function of Right Hippocampal Volume

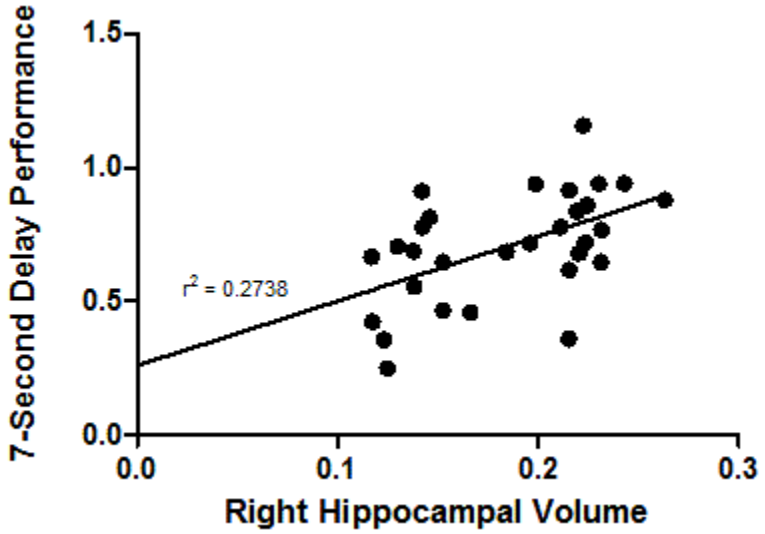

Figure 13: Ratio of performance in 7-second delay versus matched no-delay alternation trials on the y axis regressed against right hippocampal volume on the x axis. Right hippocampal volume was a significant predictor of 7-second delay performance.

Neither left hippocampal volume, right parahippocampal volume, or left parahippocampal volume predicted performance in the 7-second delay test (all $P > 0.05$; left hippocampus: $F = 1.11$, Rsq = 0.038; right parahippocampus: $F = 1.94$, Rsq = 0.065; left parahippocampus: $F = 0.27$, Rsq = 0.01).

Right hippocampal volume was trending toward marginal prediction of performance in the 20-second delay test ($P = 0.11$, $F = 3.92$, Rsq = 0.136, Figure 14).

Figure 14

20-Second Delay Performance as a Function of Right Hippocampal Volume

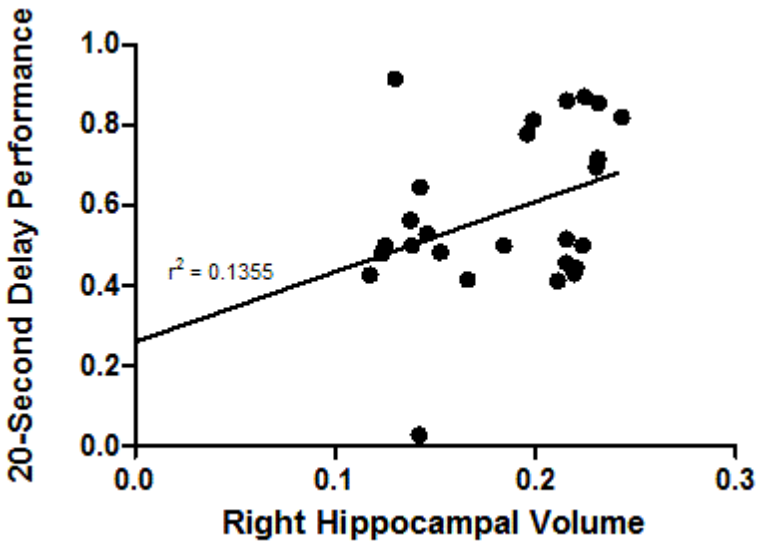

Figure 14: Ratio of performance in 20-second delay versus matched no-delay alternation trials on the y axis regressed against right hippocampal volume on the x axis. Right hippocampal volume was not a significant predictor of 20-second delay performance.

Neither left hippocampal volume, right parahippocampal volume, nor left parahippocampal volume predicted performance in the 20-second delay test (all P > 0.05; left hippocampus: F = 0.33, Rsq = 0.013; right parahippocampus: F = 0.03, Rsq = 0.001; left parahippocampus: F = 0.04, Rsq = 0.001).

The correlation of performance on the 7-second delay trials and 20-second delay trials within subject was not significant but appeared to be trending in that direction (P = 0.15, F = 3.45).

Mean trials to meet testing criterion in the alternation task was higher in the two subjects with cerebellar lesions (664.0) than in all other subjects (300.2) ($P < 0.01$, $t = 5.14$). However, mean performance on the 7-second delay task in the two subjects with cerebellar lesions (0.75) was not different than mean performance in the other subjects not diagnosed with DA toxicosis (0.88) ($P > 0.05$, $t = 0.99$). Mean performance on the 20-second delay task in the two subjects with cerebellar lesions (0.80) was not different than in the other subjects not diagnosed with DA toxicosis (0.69) ($P > 0.05$, $t = 0.76$).

Right hippocampal damage was a marginal predictor of across-session latency in the once-daily foraging task ($P = 0.08$, $F = 4.63$, $Rsq = 0.162$, Figure 15). See Appendix 3.3 for all subjects' once-daily foraging task performance

Figure 15

Across-Session Latency Regressed Against Right Hippocampal Volume

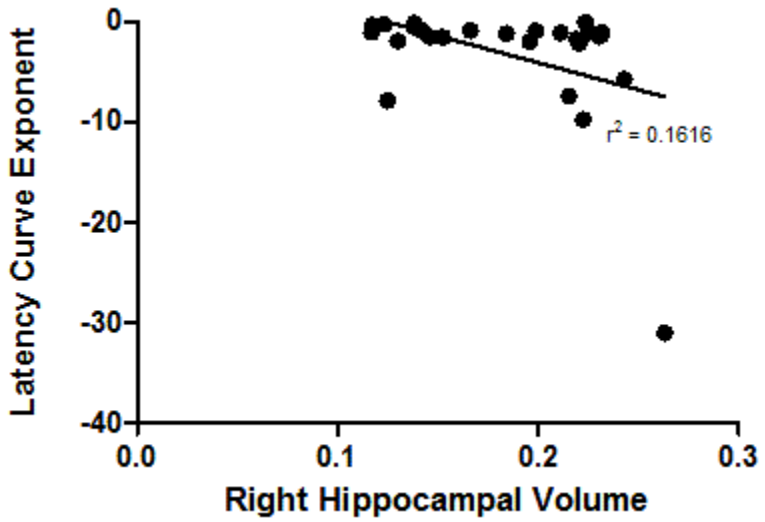

Figure 15: Across-session latency in the once-daily foraging task on the y axis regressed against right hippocampal volume on the x axis. Right hippocampal volume was not a significant predictor of across-session latency.

Right parahippocampal volume predicted across session latency on the once-daily foraging task ($P < 0.05$, $F = 5.70$, $Rsq = 0.192$, Figure 16).

Figure 16

Across-Session Latency Regressed Against Right Parahippocampal Volume

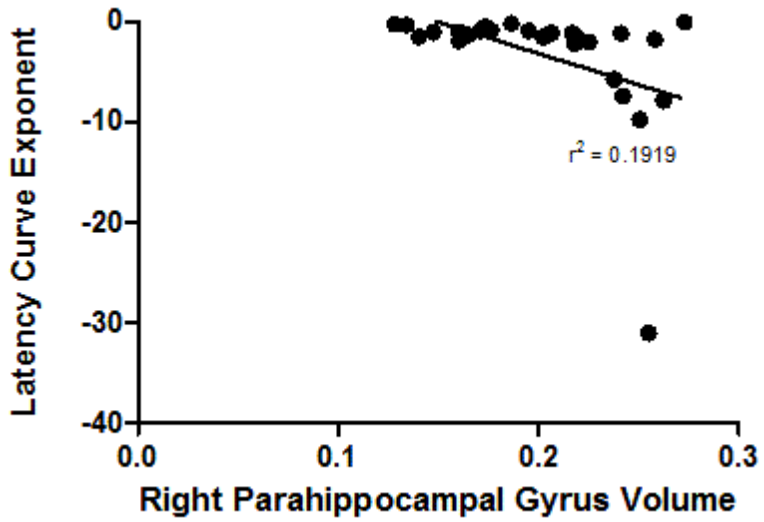

Figure 16: Across-session latency on the once-daily foraging task on the y axis regressed against right parahippocampal volume on the x axis. Right parahippocampal volume was a significant predictor of across-session latency.

Neither left hippocampal volume nor left parahippocampal volume predicted across session-performance on the once-daily foraging task (both $P > 0.05$; left hippocampus: $F = 1.70$, Rsq = 0. 066; left parahippocampus: $F = 0.66$, Rsq = 0.425).

Because both the hippocampus and parahippocampus were implicated by these regression trends, right and left hippocampus and parahippocampus were also used as independent variables in a multivariate regression against across-session latency. The multivariate model was not a significant predictor of across-session latency ($P > 0.05$, $F = 1.80$). Interestingly,

neither right hippocampal nor right parahippocampal volumes were individually significant contributors to the model (both P > 0.05, T values: Right hippocampus: 0.53, Right parahippocampus, 1.39).

Right hippocampal volume predicted within-session errors on the once-daily foraging task (P < 0.05, F = 7.02, Rsq = 0.226, Figure 17).

Figure 17

Within-Session Errors Regressed Against Right Hippocampal Volume

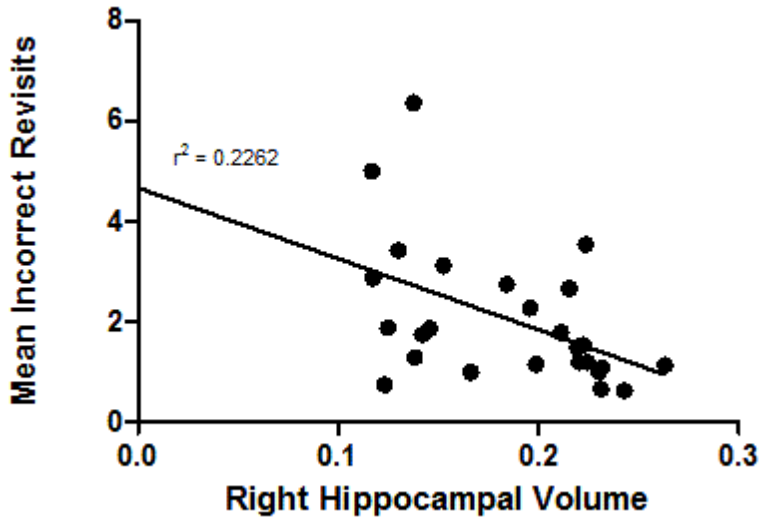

Figure 17: Mean within-session errors on the y axis regressed against right hippocampal volume on the x axis. Right hippocampal volume was a significant predictor of within-session errors.

Neither left hippocampal volume, right parahippocampal volume, nor left parahippocampal volume predicted within-session errors on the once-daily foraging task (All P > 0.05, Left

hippocampus: F = 0.025, Rsq = 0.001, Right parahippocampus: F = 0.40, Rsq = 0.128, Left parahippocampus: F = 0.50, Rsq = 0.021).

Performance on the 7 second delay alternation trials did not significantly correlate with across-session latency in the once-daily foraging task ($P > 0.05$, F = 0.73, Rsq = 0.029). Nor did ratio performance on the 20 second delay alternation trials correlate with across-session latency in the once-daily foraging task ($P > 0.05$, F = 0.097, Rsq = 0.004). Across-session latency in the once-daily foraging task did not correlate with within-session errors ($P > 0.05$, F = 0.81, Rsq = 0.033). Finally, within-session errors did not correlate with either 7-second delay performance ($P > 0.05$, F = 0.69, Rsq = 0.028) or 20-second delay performance ($P > 0.05$, F = 0.24, Rsq = 0.011).

Mean performance on the 7-second delay alternation trials was significantly lower in subjects diagnosed with DA toxicosis (0.629) than control subjects (0.857) ($P < .01$, t=3.30). Mean performance on the 20-second delay alternation trials was also significantly lower in subjects diagnosed with DA toxicosis (0.521) than control subjects (0.717) ($P < .01$, t = 2.56).

Mean across-session latency on the once-daily foraging task appeared to be lower in subjects diagnosed with DA toxicosis (-1.749) than control subjects (-6.685), but this was not significant ($P = 0.11$, t = 1.98).

Mean within-session errors in the once-daily foraging task were higher in the subjects diagnosed with DA toxicosis (2.50) than in the control subjects (1.07) ($P < 0.05$, t = 2.75).

Discussion

In the present study, volumetric assessments of hippocampal damage in a sample of 30 wild CSLs presenting with variable medial temporal lobe lesions were found to scale linearly with behavioral impairment on two separate measures: alternation in a two-choice maze with a 7-second delay, and within-session errors on a once-daily foraging task. Right parahippocampal damage was found to scale linearly with impairment on a third behavioral measure, across-session latency on a once-daily foraging task. Right hippocampal damage was also marginally predictive of impairment in across-session latency on the once-daily foraging task and the alternation task with a 20-second delay. In all these cases, effects were lateralized to the right hippocampal formation (which comprises the hippocampus and the parahippocampus).

Together, these findings suggest that, in wild CSLs, short-term episodic-like memory (as measured by delayed alternation), longer-term allocentric spatial memory (as measured by across-session latency in the once-daily foraging task) and spatial working memory (as measured by within-session errors on the once-daily foraging task) are all dependent on medial temporal lobe integrity, and at least partially lateralized to the right hippocampal formation. These results are largely in keeping with data obtained from more traditional laboratory models, and were obtained despite the increased noise likely present in this sample drawn from a diverse, wild subject population. Our results are the first to show homologous hippocampal function between a marine mammal species and species previously studied, and are among the first assessments of hippocampal function in a wild

population with naturally occurring lesions. As such, they also serve toward validating the generalizability of previous comparative research findings on hippocampal function outside of traditional laboratory models.

A relatively unique feature of our data was the variable spread of damage to the right and left hippocampus and parahippocampus across subjects—most studies using captive populations with laboratory-inflicted insults feature total ablation of a target area, usually bilateral. The apparent right lateralization of episodic, allocentric spatial, and spatial working memory function indicated by our data represents the first strong evidence of cognitive lateralization in a CSL. Extensive prior research indicates that long term allocentric spatial memory and spatial working memory are at least partially lateralized to the right hemisphere in humans (Jonides *et al*., 1996). However, evidence for the lateralization of spatial working memory in non-human animals is equivocal. While some evidence suggests differential contribution to spatial memory by the right hippocampus (*e.g.*, Mehta, Barnes, & MacNaughton, 1997; LaMendola & Bever, 1997), there is also evidence that spatial working memory is spared in rats as long as one hippocampus on either side is spared (Li, Matsumoto, & Watanabe, 1999). The majority of comparative studies involving hippocampal lesions feature bilateral damage, so there are limited data on this front.

In the current study, findings relating to parahippocampal volume were more equivocal than those featuring the hippocampus, although still of interest. As hypothesized, parahippocampal volume was not predictive of performance on the delayed alternation task, while right parahippocampal volume was predictive of across-session latency on the

once-daily foraging task. When local features are not fully controlled for and can thus predict reward location—as was necessarily the case in our testing paradigm—performance in spatial memory tasks may be bolstered by object recognition, which is dependent in part on perirhinal cortex, a sub-region of the parahippocampal gyrus. Right parahippocampal volume was significant and right hippocampal volume marginally significant when analyzed against across-session latency on the once-daily foraging task in individual regressions, but they were both highly non-significant when analyzed together as independent variables in a multiple regression. From this, it is clear that the contribution of these two volumetric measures to across-session latency did not covary strongly within subjects. This may indicate that different subjects used different strategies on the once-daily foraging task, and were either more reliant on distal cues or local feature recognition to solve this task, as opposed to relying equally on both mechanisms in parallel. Some prior evidence does point to differential contribution of different medial temporal subregions to similar spatial memory tasks (Vann, Brown, Erichsen, Aggleton, 2000).

Performance on the 7-second delayed alternation task was a marginally significant predictor of performance on the 20-second delayed alternation task. However, even though 7-second delay performance and within-session errors on the once-daily foraging task were both predicted by right hippocampal volume, and across-session latency on the once-daily foraging task was marginally predicted by right hippocampal volume, performance was not correlated between any of the three behavioral measures. This could be because each task was reliant on a different hippocampal subregion—resolution in our imaging data was not sufficient to differentiate subregions to address this question directly, and there is little data

addressing differential contribution of hippocampal subfields to tasks similar to those used here. However, it is important to note that correlation coefficients were moderately small for the comparison of each of these behavioral measures and right hippocampal volume, indicating that much of the variability in behavioral performance was due to factors external to hippocampal integrity.

In addition to their broad theoretical value, our findings also serve quite directly to increase understanding of DA-toxicosis related impairments in wild CSLs and, potentially, other affected species. As suggested by Goldstein *et al*. (2008), the importance of complex navigation to CSLs in the wild, and the prevalence of hippocampal damage in animals with DA toxicosis, could help explain the difficulty animals with DA toxicosis have in foraging. In the present study, we added explicit empirical support to this hypothesis, clearly demonstrating that impairment in long-term allocentric spatial memory and spatial working memory was substantial in CSLs with damage to the right hippocampal formation as a result of DA toxicosis. These findings add credence to anecdotal reports of abnormal navigational tendencies in CSLs with DA toxicosis (Thomas, Harvey, Goldstein, Barakos, & Gulland, 2009). We also showed hippocampal-dependent impairment in short-term episodic and spatial working memory—deficits in working memory may also negatively impact foraging over shorter time-scales (Winter & Stich, 2005; Prior & Gunturkun, 2001; Goss-Custard, 1977).

Practically, the strong lateralization of focal damage evidenced in the current study may be relevant for veterinary practitioners treating animals with DA toxicosis. MRI can provide accurate measures of hippocampal damage, and to the extent that memory tracks with right

hippocampal damage, animals with restricted left hippocampal damage may have a better prognosis. Of course, damage to the left hippocampus could impact behavioral measures not assessed in the current study.

In addition, in the current study veterinary diagnosis was a strong predictor of right hippocampal volume, performance on the delayed alternation tasks, and within-session errors in the once-daily foraging task. It was a marginal predictor of across-session latency in the once-daily foraging task. These data provide the first empirical evidence that veterinary diagnosis of DA toxicosis is related to quantitative measures of hippocampal damage and cognitive impairment.

In summation, the findings reported here hold both theoretical and practical value. They extend understanding of hippocampal function into a new mammal species, and do so using a novel, naturalistic model of hippocampal damage. They may also inform pragmatic decisions in the veterinary and rehabilitation setting. Further, despite the genetic and behavioral variability present in the wild population studied, findings largely mirrored previous work in rodents and primates. This serves to validate these previous findings, which is crucial given the reliance of many studies on impoverished and potentially un-representative rodent models.

Future studies with this population of animals could include wild tagging and tracking metrics that might better match brain data and laboratory behavioral measures with likely outcome for animals afflicted with DA toxicosis. In addition, functional brain imaging, such

as resting state fMRI or diffusion tensor imaging, might better characterize DA-related neural and behavioral deficits in CSLs at the network level.

General Conclusion

Together, the three studies presented in this dissertation provide a strong argument for the value of naturalistic models for studying the brain and behavior. The results of Chapter 1 showed that a simple, rapid behavioral measure could serve as a useful adjunct to veterinary diagnosis of DA toxicosis in a rehabilitation setting. Chapter 2 replicated this finding, and, through the addition of structural brain imaging, showed that auditory habituation in CSLs with DA toxicosis was most likely not being driven by brain damage, but rather some other neurological feature of the disease (potentially epilepsy). Chapter 3 quantified degree of behavioral impairment in CSLs with DA toxicosis across a delayed alternation task and within-session errors and across-session latency in a once-daily foraging task. These tasks draw on episodic memory, spatial working memory, and allocentric spatial memory, respectively, and impairments in all three measures were found to be related to damage to the right hippocampal formation. These findings have theoretical value specific to comparative understanding of hippocampal function. They also have applied value specific to veterinary practice and decision-making in a rehabilitation setting. As I repeatedly argued, I believe these findings also hold a broader theoretical value. They may serve to validate similar findings in more traditional laboratory models while at the same time indicating that brain and behavior research is feasible in a wild population of large, long-lived mammals undergoing rehabilitation, and, even more broadly, outside of the traditional laboratory setting.

Studies such as these will not replace traditional laboratory approaches any time in the near future—they are time consuming and logistically complex. Psychologists, in particular, ought not discount the power of expediency in governing trends in human behavior, and researchers are no exception to such influence. However, alternative approaches such as I've followed here allow for external validation and increased generalizability of previous findings, and may, in some instances, turn up quite different results than traditional laboratory approaches. It's no secret that contemporary biomedical research is slow and comparative results seldom translate to humans. A more measured, humane, and thoughtful approach for how to move forward will use a range of experimental tools and approaches, and must not be restricted exclusively to non-representative laboratory models, but should include opportunistic, naturalistic animal models as well. If emphases from funding agencies and universities shifted away from the low-risk/low-pay-off model now dominanting the field of behavioral neuroscience, a great many promising approaches to comparative research might begin to flourish.

Appendices

Chapter 1

Appendix 1.1: Supplementary Methods

The four experimental phases in this study comprised multiple presentations of one of two sounds, A and B, from one of two locations, 1 and 2. In Phase 1, to measure initial habituation, sound A was presented from location 1. In Phase 2, to measure dishabituation following a spatial shift, sound A was presented from location 2. In Phase 3, to measure dishabituation following a delay, sound A was again presented from location 2 following a 15-minute delay. In Phase 4, to measure dishabituation following a stimulus change, sound B was presented from location 2. Two different one-second auditory stimuli were used during testing: sound A was a "looming" tone increasing in both frequency and amplitude (Ghazanfar, Neuhoff, & Logothetis), and sound B was a complex stimulus shifting rapidly up and down in frequency. The energy of both calls was focused around 1 kHz, well within the range of hearing for CSLs in air (Schusterman, 1974). The peak amplitude of both sounds was ~90 dB re 20μPa at 1 meter. Due to the enclosure size, subjects were positioned within 4 meters of the sound source at all times.

Appendix 1.2: Subject Table

Name	Field #	Admit Date	Test Date	Age Class	Sex	DA	MRI	Histo	Epil
Guy	CSL-7686	7/13/08	7/29/08	Yearling	M	N	N	0	N
HH Harris	CSL-7773	7/23/08	7/29/08	Adult	F	N	0	Y	Y
hompy	CSL-7917	10/11/08	12/3/08	Subadult	F	N	0	0	N
Ox	CSL-8054	1/26/09	2/7/09	Adult	F	Y	0	Y	Y
Suntan	CSL-7626	5/4/08	5/30/09	Pup	M	N	0	0	N
Mr. G	CSL-7628	5/8/08	5/30/09	Pup	M	N	0	0	N
Rail	CSL-7633	5/13/08	5/30/09	Yearling	M	N	0	0	N
Gruffy Bert	CSL-7637	5/18/08	5/30/09	Yearling	M	N	0	0	N
Honddo	CSL-7654	5/29/08	6/8/09	Yearling	M	N	0	0	N
Yo-Daddy	CSL-7664	5/31/08	6/8/09	Pup	F	N	0	0	N
Apus	CSL-7672	6/3/08	6/8/09	Yearling	F	N	0	0	N
BT5	CSL-7671	6/3/08	6/8/09	Pup	F	N	0	0	N
Nally	CSL-7678	6/5/08	6/18/09	Yearling	M	N	0	0	N
Jablonski	CSL-7638	7/3/08	7/16/09	Yearling	M	N	0	0	N
San Tomas	CSL-7750	7/7/08	7/23/09	Adult	F	Y	Y	Y	N
Dano	CSL-7802	8/2/08	8/5/09	Adult	F	Y	0	0	Y
Laura Lee	CSL-7801	8/1/08	8/8/09	Juvenile	M	N	0	N	N
IVO	CSL-7804	8/2/08	8/8/09	Juvenile	M	N	0	0	N
Reefer	CSL-7803	8/2/08	8/8/09	Juvenile	M	N	0	0	N
Loyd	CSL-7805	8/3/08	8/8/09	Juvenile	M	N	0	0	N
Toaster	CSL-7780	7/24/08	8/14/09	Juvenile	M	N	0	0	N
Green Pepper	CSL-7785	7/26/08	8/14/09	Juvenile	M	N	0	0	N
Crazy Bill	CSL-7814	8/6/08	8/14/09	Juvenile	M	N	0	0	N
Morticia	CSL-7819	8/8/08	8/14/09	Adult	F	y	Y	Y	Y
Tallship	CSL-7788	7/28/08	8/21/09	Juvenile	M	N	0	0	N
Permit	CSL-7815	8/6/08	8/21/09	Juvenile	M	N	0	0	N
Tuna Dancer	CSL-7825	8/12/08	8/21/09	Juvenile	M	N	0	0	N
Minerva	CSL-7824	8/10/08	8/26/09	Juvenile	M	N	0	0	N
Sunnyside Up	CSL-7783	8/29/08	9/12/09	Yearling	M	N	0	0	N
Livewire	CSL-7887	9/5/08	9/12/09	Subadult	M	y	0	0	Y
Tamarra	CSL-7888	9/5/08	9/12/09	Subadult	M	y	0	0	Y
Thrasher	CSL-7831	9/2/08	10/16/09	Juvenile	M	N	0	0	N
Rupert	CSL-7975	10/17/08	10/24/09	Juvenile	M	N	N	N	N
Colver	CSL-7978	10/19/08	10/24/09	Subadult	M	Y	0	Y	Y
Gustina	CSL-8013	11/11/08	11/14/09	Juvenile	M	N	0	0	N

Name	Field #	Admit Date	Test Date	Age Class	Sex	DA	MRI	Histo	Epil
Virgy	CSL-8023	11/29/08	12/3/09	Subadult	F	Y	0	Y	Y
Sauvignon	CSL-8019	11/30/08	12/3/09	Yearling	F	Y	Y	Y	Y
Katiegee	CSL-9323	12/21/09	12/29/09	Adult	F	Y	0	Y	Y
Cameron Elias	CSL-9324	12/23/09	12/29/09	Adult	F	Y	0	Y	Y
Dr. Pep	CSL-9325	12/23/09	12/29/09	Adult	F	Y	Y	Y	Y
Crimson	CSL-8032	12/14/08	1/16/10	Pup	F	N	0	0	N
Skitter	CSL-8031	12/17/08	1/16/10	Pup	M	N	0	0	N

Appendix 1.2: Each subject tested, whether their data were excluded or not, is listed chronologically. Column categories are as follows. *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *Admit date*: The day each animal was brought from the wild to TMMC for rehabilitation. *Test date*: The day each animal undertook behavioral testing. *Age range*: Pup: < 1 year, Yearling: 1–2 years, Juvenile: 2–4 years, Subadult: 4–8 years, Adult: 5+ years. Age was determined by veterinary staff on the basis of size and secondary sex characteristics. *Sex:* M: Male, F: Female. Each animal was sexed by the veterinary staff on the basis of genital morphology. *DA*: Each animal's relevant veterinary diagnosis: N: No diagnosis of DA toxicosis, C: Chronic DA toxicosis, A: Acute DA toxicosis. Chronic and Acute diagnoses were collapsed in the initial publication of these data. Distinction between chronic and acute diagnoses is further discussed in Goldstein *et al*., 2008. In brief, animals stranding in groups following directly on large, documented blooms of DA-producing algae, who present with neurological symptoms such as seizures, are generally classified as "acute." Chronic animals strand year round, often alone, and their primary symptoms are epilepsy and hippocampal atrophy (as identified by MRI). *MRI*: 0: No MRI acquired, Y: An MRI was acquired, and hippocampal atrophy was observed by a veterinary radiologist, N: An MRI was acquired and no hippocampal atrophy was apparent. *Histo*: 0: No histopathology was acquired, Y: Signs of DA toxicosis were observed, N: No signs of DA toxicosis were observed. This was assessed on the basis of post-mortem brain sectioning. *Epil*: 0: No seizures were observed, Y: one or more seizures was observed. Measures of seizure were observational based on behavior

Appendix 1.3: Table of Correct and Incorrect Positive Diagnoses Across Responsivity Thresholds

Exposures to Habituation	% Hits	% False Alarms
> 2.5	100	93
> 4.5	100	78
> 5.5	100	74
> 6.5	100	67
> 7.5	100	59
> 8.5	100	56
> 9.5	92	56
> 10.5	92	44
> 11.5	92	37
> 12.5	83	33
> 14.0	58	33
> 15.5	58	30
> 17.0	58	26
> 18.5	50	19
> 20.5	50	15
> 22.5	50	7
> 23.5	42	7
> 24.5	42	4
> 26.0	25	4
> 27.5	17	0
> 33.0	8	0

Appendix 1.3: This table specifies the predicted diagnostic accuracy of different response criteria based off of performance of CSLs with and without DA toxicosis on the auditory habituation assay. percent of correct positive diagnoses/hits and incorrect positive diagnoses/false alarms across a range of possible diagnostic thresholds. Thresholds are set at different responsivity values during exposure Phase 1 such that a diagnostic threshold of >7.500 indicates that only animals responding more than 7 times will be given a diagnosis of DA toxicosis based on this measure.

Appendix 1.4: Discussion of Phase 1 Responsivity as a Predictor of Seizures

Although observed seizure activity was noted in the original publication of these data, it was not discussed as a possible driver of responsivity in the behavioral task. The assumption was that hippocampal integrity, which was not fully measured in all subjects, was the primary factor behind increased responsivity in subjects diagnosed with DA toxicosis. This hypothesis could not be tested, because MRI was available for only a subset of tested subjects. Given the replication in Chapter 2 of the primary finding here in Chapter 1 (increased Phase 1 responsivity in animals diagnosed with DA toxicosis), and further, the finding that hippocampal volume was NOT a predictor of Phase 1 responsivity in Chapter 2, it is likely that some other factor closely linked with DA is driving Phase 1 responsivity. As discussed in Chapter 2, medial temporal epilepsy is a candidate, being associated in prior studies with slower habituation of orienting responses. Although the data on seizures here is likely incomplete (the subjects were not observed 24 hours a day, so may well have had epilepsy without veterinary staff's being aware), there is still value in exploring the relationship between responsivity and observed seizures.

Mean Phase 1 responsivity was higher in subjects observed to have one or more seizures (22.0) than in animals observed to have no seizures (11.8) ($P < .01$, $t = 3.24$). Further ramifications of these findings are discussed more thoroughly in the body of Chapter 2.

Appendix 1.5: Subject Responsivity Table

Name	Field #	P1 Responses	P2 Responses	P3 Responses	P4 Responses
Apus	CSL-7672	1	8	0	0
BT5	CSL-7671	11	14	1	5
Cameron Elias	CSL-9324	13	0	4	0
Colver	CSL-7978	25	0	1	0
Crazy Bill	CSL-7814	18	13	3	3
Crimson	CSL-8032	18	17	25	9
Dano	CSL-7802	12	5	8	1
Dr. Pep	CSL-9325	13	2	6	2
Green Pepper	CSL-7785	10	3	2	8
Gruffy Bert	CSL-7637	16	4	4	6
Gustina	CSL-8013	4	0	0	0
Honddo	CSL-7654	12	2	0	8
Ivo	CSL-7804	4	1	0	7
Jablonski	CSL-7638	22	1	0	1
Katie G	CSL-9323	18	0	0	0
Laura Lee	CSL-7801	27	9	15	8
Livewire	CSL-7887	23	8	14	5
Loyd	CSL-7805	6	5	1	2
Minerva	CSL-7824	8	6	0	1
Morticia	CSL-7819	27	0	15	1
Mr. G	CSL-7628	6	7	1	6
Nally	CSL-7678	19	11	1	0
Ox	CSL-8054	38	10	1	0
Permit	CSL-7815	5	15	2	13
Rail	CSL-7633	10	11	7	4
Reefer	CSL-7803	11	4	2	8
Rupert	CSL-7975	7	0	3	11
San Tomas	CSL-7750	28	7	13	14
Sauvignon	CSL-8019	25	0	0	17
Skitter	CSL-8031	24	0	2	2
Sunnyside Up	CSL-7783	10	9	2	4
Suntan	CSL-7626	22	4	6	18
Tallship	CSL-7788	1	1	0	4
Tamarra	CSL-7888	13	8	1	5
Thrasher	CSL-7831	7	14	26	3
Toaster	CSL-7780	4	5	0	8

Name	Field #	P1 Responses	P2 Responses	P3 Responses	P4 Responses
Tuna Dancer	CSL-7825	15	5	1	11
Virgy	CSL-8023	9	30	2	0
Yo Daddy	CSL-7664	4	2	0	1

Appendix 1.5: Subjects are listed alphabetically with their total number of orienting responses prior to meeting the habituation criterion (three consecutive non-responses) in each of the four consecutive test conditions. Tests comprised Phases 1–4. There were two speaker locations (A and B) and two test stimuli (a looming tone and a frequency modulated elephant call). Column categories are as follows. *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *P1*: Number of responses during initial exposure to the looming tone from location A. *P2*: Number of responses during exposure to the looming tone from location B, beginning immediately after habituation criterion was met in P1. *P3*: Number of responses during exposure to the looming tone from location B, 15 minutes after the habituation criterion was met in P2. *P4*: Number of responses during exposure to the frequency-modulated elephant call from location B, beginning immediately after the habituation criterion was met in P3.

Appendix 1.6: Discussion of Phases 1-4 Dishabituation Results

The experimental design included four exposure series—response to the first showed a strong and highly significant differential responsivity between animals with a diagnosis of DA toxicosis and controls. However, there was no difference in responsivity between these groups on any of the next three exposure series, suggesting a difference in habituation rate, but no difference in dishabituation rate in this particular experimental protocol. This finding contrasts previous evidence in mice that hippocampal damage (likely accompanying DA toxicosis in CSLs) interferes with dishabituation following stimulus manipulation (*e.g.*, Kesner & Hunsaker, 2010). A common explanation for hippocampus-relevant dishabituation effects in mouse and rat studies is that the hippocampus binds stimuli and context in memory, so a novel stimulus/context pairing will be less salient to an animal with a damaged hippocampus. There may be many reasons we did not find such an effect here: *1*. There was no delay between Phase 1 and Phase 2. The same stimulus is presented in Phase 2 as in Phase 1, but from a different location. It may be that without time to forget, the stimulus remained equally salient to habituated animals with DA toxicosis and to controls, despite the DA animals having taken longer to habituate in Phase 1. It is also possible that the spatial shift was not as salient for auditory stimuli presented in a relatively small location as it would be for an actual object. *2*. There was a delay implemented between Phase 2 and Phase 3, but there was no change in location following the delay. The same stimulus was presented from the same location after the delay as before. It is likely that extra-hippocampal areas govern single-stimulus familiarity (Aggleton & Brown, 1999), so the delay

may not have led to differential salience of the signal between DA and control animals. Further, responsivity was very low for all animals in Phase 3, perhaps because the delay was long enough that many went to sleep, and perhaps because they were, by that point, generally habituated. *4*. The stimulus presented in Phase 4 was novel, but from a familiar location. One might have expected increased dishabitution accompanying hippocampal damage given the stimulus was being encountered for the first time, and there was some hint of increased responsivity in the DA animals (albeit not significant). Again, general habituation may have been high enough by this point that none of the animals were prone to much responsivity.

A nuanced awareness of the hippocampal literature would perhaps have suggested including a delay between Phase 1 and Phase 2 as in the experiment discussed in Chapter 2. However, we were looking for differential sensitivity to location change (Phase 1 to Phase 2), delay (Phase 2 to Phase 3), and stimulus change (Phase 3 to Phase 4), so isolated each of these components. In addition, hippocampal damage was not the only symptom of DA toxicosis, we were using auditory stimuli where most of the dishabituation/hippocampus literature used physical object/location pairings, and we were interested in whatever measure might be easiest to implement in a stranding setting.

Chapter 2

Appendix 2.1: Subject Tables for Data Collection Periods 1 and 2

Period 1 – 7/7/2010 to 11/5/2011

Name	Field #	Admit Date	Test Date	Age Class	Sex	DA	MRI	Epil
Contador	CSL-9752	7/7/10	8/6/10	Adult	F	C	YR	N
Philadelphia	CSL-9690	6/18/10	8/25/10	Adult	F	C	YL	N
BBQ	CSL-9724	6/25/10	9/10/10	Adult	F	C	N	Y
Bobble	CSL-9821	8/28/10	10/19/10	Adult	F	C	YLB	Y
Spearmint	CSL-9866	10/11/10	11/4/10	Adult	F	C	YLB	N
Graduate	CSL-9881	10/17/10	11/24/10	Adult	F	C	YR	Y
NotaSO	CSL-9923	2/16/11	3/13/11	Adult	F	C	YRB	N
Breezy	CSL-9931	3/31/11	5/1/11	Adult	F	C	YB	Y
Jessavila	CSL-9949	5/24/11	6/14/11	Subadult	F	C	YB	N
Midway	CSL-9988	7/2/11	7/21/11	Juvenile	M	N	YB	N
Tizer	CSL-10091	8/30/11	10/3/11	Adult	F	C	YB	Y
Carla	CSL-10121	9/12/11	10/19/11	Adult	F	N	YR	N
Christopher	CSL-10187	10/10/11	11/3/11	Juvenile	M	C	YRB	Y
Sarow	CSL-10170	10/4/11	11/24/11	Yearling	M	N	N	N
Saltystrike	CSL-10235	11/5/11	12/10/11	Adult	F	N	YB	N

Period 2 – 8/3/2012 to 9/26/2012

Name	Field #	Admit Date	Test Date	Age Class	Sex	DA	MRI	Epil
Banana	CSL-10385	8/3/12	8/17/12	Yearling	M	N	N	N
Wolverine	CSL-10381	8/2/12	8/17/12	Yearling	M	N	NA	N
Mr Peppy	CSL-10334	6/10/12	8/22/12	Yearling	F	N	N	N
Gulliver	CSL-10371	7/20/12	9/7/12	Yearling	F	N	N	N
Shareef	CSL-10392	8/8/12	9/8/12	Subadult	M	N	N	N
Nui Wahini	CSL-10397	8/15/12	9/9/12	Adult	F	N	YB	N
Devdichi	CSL-10413	9/2/12	9/12/12	Juvenile	M	N	YL	N
Kabebe	CSL-10398	8/16/12	9/20/12	Yearling	M	N	N	N
Clean Shores	CSL-10421	9/15/12	9/27/12	Subadult	M	C	N	Y
Wombat	CSL-10434	9/27/12	10/3/12	Subadult	F	N	N	N
Achop	CSL-10437	10/1/12	11/22/12	Subadult	F	N	NA	N
JJ	CSL-10433	9/26/12	11/22/12	Juvenile	M	N	N	N

Appendix 2.1: Subjects are grouped by Period 1 and Period 2 of data collection, and are listed in chronological order with relevant diagnostic data. Column categories are as follows. *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *Admit Date*: The day on which each subject was brought to TMMC from the wild. *Test Date*: The day on which each subject took part in behavioral testing. *Age Class*: Yearling: 1–2 years, Juvenile: 2–4 years, Subadult: 4–8 years, Adult: 5+ years. Age was estimated by veterinary staff on the basis of size and secondary sex characteristics. *Sex*: M: Male, F: Female. Sex was determined by veterinary staff on the basis of genital morphology. *DA*: N: No diagnosis of DA toxicosis, C: Chronic DA toxicosis, A: Acute DA toxicosis. Diagnoses were determined by veterinary staff. *MRI*: N: No hippocampal pathology, YB: Bilateral hippocampal pathology, YR: Unilateral right hippocampal pathology, YL: Unilateral left hippocampal pathology, YRB: Asymetric bilateral hippocampal pathology weighted to the right hippocampaus, YLB: Asymetric hippocampal pathology weighted to the left hippocampus. Seizures: N: No observed seizures while in captivity, Y: One or more observed seizures while in captivity. These designations were assigned by a veterinary radiologist.

Appendix 2.2: Subject Responsivity Table

Name	Field #	A1	B1	A2	C2	P1 Total	P2 Total	P2 - C2/A2
Achop	CSL-10437	18	23	18	19	41	37	1.06
Banana	CSL-10385	12	12	8	5	24	13	0.63
BBQ	CSL-9724	18	16	12	9	34	21	0.75
Bobble	CSL-9821	23	24	18	18	47	36	1.04
Breezy	CSL-9931	21	25	13	14	46	27	1.17
Carla	CSL-10121	5	11	2	3	16	5	1.5
Christopher	CSL-10187	18	14	9	10	32	19	1.11
Clean Shores	CSL-10421	13	20	12	9	33	21	0.75
Contador	CSL-9752	18	12	6	11	30	17	1.83
Devdichi	CSL-10413	6	3	4	10	9	14	2.5
Graduate	CSL-9881	13	7	5	7	20	12	1.45
Gulliver	CSL-10371	27	8	16	14	35	30	0.88
Jessavila	CSL-9949	15	18	15	16	33	31	1.07
JJ	CSL-10433	12	14	11	19	26	30	1.73
Kabebe	CSL-10398	7	6	3	0	13	3	0
Midway	CSL-9988	3	2	1	0	5	1	0
Mr Peppy	CSL-10334	15	12	16	11	27	27	0.69
Notaso	CSL-9923	14	18	13	11	32	24	0.88
Nui Wahini	CSL-10397	3	4	NA	NA	7	NA	NA
Philadelphia	CSL-9690	12	18	1	6	30	7	6
Salty Strike	CSL-10235	25	17	3	11	42	14	3.67
Sarow	CSL-10170	12	9	6	1	21	7	0.17
Schreef	CSL-10392	3	10	10	9	13	19	0.9
Spearmint	CSL-9866	15	15	8	12	30	20	1.5
Tizer	CSL-10091	21	20	14	12	41	26	0.86
Wolverine	CSL-10381	17	14	4	7	31	11	1.75
Wombat	CSL-10434	10	10	7	5	20	12	0.71

Appendix 2.2: Subjects are listed alphabetically with responses in each phase of behavioral testing. Column categories are as follows: *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *A1*: Total responses to location A in exposure Phase 1. *B1*: Total responses to location B in Exposure Phase 1. *A2*: Total responses to location A in exposure Phase 2 (following a 3 minute delay). *C2*: Total responses to the novel location C in exposure Phase 2

(following a 3 minute delay). *P1 Total*: Total responses in exposure Phase 1, summed across both location A and B. *P2 Total*: Total responses in exposure Phase 2, summed across both location A and C. *P2 – C2/A2*: Ratio of responses to the unfamiliar location C versus the familiar location A in exposure Phase 2.

Appendix 2.3: Manual Brain Region Tracing Protocol

Tracing Parameters

Right and left hippocampus and parahippocampus were traced in each subject on which viable MRI data were obtained. These tracings were produced on individual brain slices using a mouse-driven pointer. Volumes were calculated by multiplying the traced area on each slice by the slice thickness. Because the hippocampal and parahippocampal volumes were intended to serve as a measure of hippocampal or parahippocampal damage, and there is likely natural variability in the size of the hippocampal formation based both on developmental stage and brain size, volumes were corrected by taking a ratio of each animal's brain regions to an index of whole brain volume. These ratio measures were then used in subsequent analysis. Tracing parameters for whole brain and regional volumes were as follows.

Whole Brain Volume

An index of whole brain volume for each animal was acquired from the oblique scan sequences by tracing around the external boundary of the cortex on each slice as shown in Figure S2.1. We opted not to include the cerebellum in this measure because air/tissue interfaces around the middle and inner ear caused substantial signal drop-out, primarily in the cerebellum, for all subjects. Posterior to the midbrain structures, the cerebellum was clearly delineated from the cortex and sub-cortical structures, which were traced in completeness. Anterior to the posterior boundary of the mid-brain structures (defined here as the posterior boundary of the superior colliculi), it was difficult to differentiate the medial

cerebellum from the lateral portions of the brain stem, and the superior portions of the brain stem from mid-brain and sub-cortical structures. To avoid unwanted variability, the inferior tracing boundary anterior to the midbrain structures followed the inferior boundary of the temporal lobes, connecting the medial-most portion of the left and right hippocampal gyrus with an interpolated straight line. This reliably excluded all cerebellar and brain-stem tissue, and also necessarily excluded some degree of mid-brain tissue. Anterior to the anterior boundary of the midbrain structures, the inferior tracing boundary followed the inferior boundary of the temporal and frontal lobes, excluding extra-cortical cerebrospinal fluid (CSF) inferior to these boundaries, the brain stem, optic nerves, and the pituitary gland. At the most anterior portions of the brain, where the temporal and frontal lobes became discontiguous, each cortical area was traced separately. Superiorly, olfactory bulbs were included. This constrained measure of whole brain volume allowed for a relatively reliable measure that was not confounded by poor image quality in the cerebellar region.

Figure S2.1

Whole Brain Volume Tracing

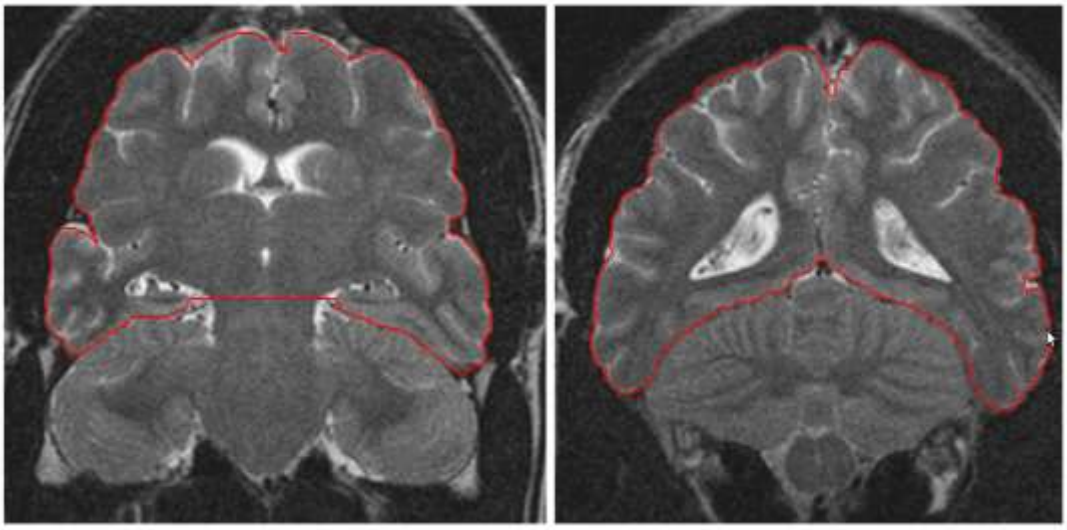

Figure S2.1: Manual tracings of whole brain volume on two representative slices, excluding cerebellum. The image on the left illustrates the method for excluding cerebellum and brainstem anteriorly—the inferior portion of the temporal lobes is traced, and the most medial points of the hippocampal gyruses are connected. The image on the right is more posterior, where the cerebellum could be more selectively excluded.

Hippocampus

Hippocampal tracing boundaries are illustrated in Figure S2.2. The anterior most MRI slice on which the hippocampus was traced was that on which the anterior boundary of the mid-brain could be seen. The mid-brain presented in these oblique images as two vertical pillars medial to the hippocampuses; anterior to these slices, the pons, which for CSLs extends beyond the midbrain anteriorly, is discontiguous with the rest of the brain. This boundary

likely excluded part of the hippocampal head (or “pes”), but this was necessary due to the difficulty of differentiating the pes from the amygdala on these sequences, and the lack of prior anatomical work establishing related segmentation criteria for this species. The posterior boundary of the hippocampus was defined as being on the last slice posteriorly on which the superior colliculi were visible. In most cases, this was just anterior to the first slice on which the corpus callosum could be seen as a continuous band, beyond which it was very difficult to differentiate hippocampal tissue from the fornix. Selection of standardized anterior and posterior boundaries for hippocampal tracing is very important, due to the high degree of variability introduced by ambiguity in these criteria (Jack, Theodore, Cook, & McCarthy, 1995). Use of the mid-brain formation allowed for a replicable criterion across subjects that was scalable to whole brain size.

The lateral boundaries of the hippocampus were defined on all slices by the temporal horns of the lateral ventricle, which were obvious on all subjects in this imaging orientation. The superior boundaries of the hippocampus were also defined by the lateral horns on more anterior slices. In the most posterior slices, where the CSL hippocampus appears to “flatten out,” the superior boundaries were defined by the obvious transition from hippocampal gray matter to the white matter of superior structures. The medial boundaries of the hippocampus were clearly defined either by CSF or intra-cranial space. The inferior hippocampal boundaries were defined by the white matter separating the hippocampus from the parahippocampal gyrus. Tracing was directly superior to this white matter boundary (so excluding the white matter of the hippocampus where contiguous with the white matter of the parahippocampus), although white matter surrounding the

hippocampus was included along the other boundaries. As in human brains imaged on 1.5 T magnets, there were no clear anatomical boundaries visible to differentiate the CA of the hippocampus from the subiculum in our subjects' images, so part of the subiculum was necessarily included with the hippocampus. The inferior medial boundaries were established by connecting the medial-most point of the hippocampal gyrus to the medial most point of the hippocampus/parahippocampus white-matter boundary with a straight line. The inferior lateral boundaries followed the hippocampus/parahippocampus white-matter boundary—however, on some slices it was difficult to follow this all the way to the CSF of the medial horn of the lateral ventricle. In these cases, the last clear white matter laterally was connected to the most inferior point of the CSF of the lateral horn by a straight line.

Figure S2.2

Hippocampal Tracings

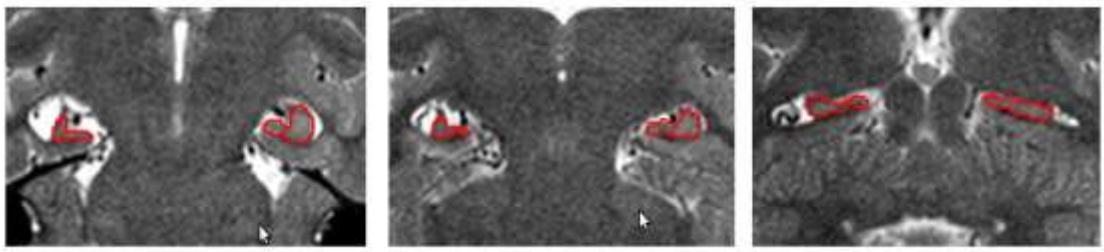

Figure S2.2: Three representative hippocampal tracings, anterior, mid, and posterior, from left to right.

Parahippocampus

The anterior and posterior boundaries of the parahippocampus (Figure S2.3) were defined identically to those used for hippocampal tracing, such that the parahippocampus was traced on all (and only) slices on which hippocampal tissue was traced. The criterion for the superior boundaries of the parahippocampus was simply the inferior most boundary of the white matter separating the hippocampus from the parahippocampus, such that there was no overlap in traced hippocampal and parahippocampal tissue. As in the hippocampal tracing, if this parahippocampal/hippocampal boundary was not clearly visible all the way to the CSF of the temporal horn, the last visible portion laterally was connected to the most inferior point of the CSF of the lateral horn by a straight line. The superior medial boundary of the parahippocampus mirrored the inferior medial boundary of the hippocampus—the medial-most point of the hippocampal gyrus was connected to the medial-most point of the parahippocampal/hippocampal white matter boundary by a straight line. The inferior boundary of the parahippocampus was always clearly visible as it bordered on intra-cranial space and/or CSF. The lateral boundaries of the parahippocampus were the most arbitrary. Most protocols for tracing the parahippocampus in humans rely on the collateral sulcus to serve as a marker of the lateral boundary. However, in our subjects, with these scan sequences, the collateral sulcus was either not visible or not present on many slices. Thus, we had to use another local feature to establish the lateral boundary. The lateral/inferior boundary of the hippocampus, where it meets with the superior boundary of the parahippocampus , was selected. We connected this point to the point on the parahippocampus directly inferior as per the absolute orientation of the brain in the Z axis—

this connection was drawn as a straight line. Using this point allowed a reliable local feature to determine the lateral boundary of the parahippocampus, but there is the possibility that this boundary may shift medially accompanying hippocampal atrophy, which could bias parahippocampal size lower in subjects with hippocampal atrophy. However, this point is approximately in alignment with the collateral sulcus on most slices where the latter structure was visible, and measurements of distance between different points on the medial boundary of the hippocampuses to the brain's midline on subjects with severe unilateral hippocampal atrophy suggested it is the more superior portions of the hippocampus that shift most medially with atrophy.

Figure S2.3

Parahippocampal Tracings

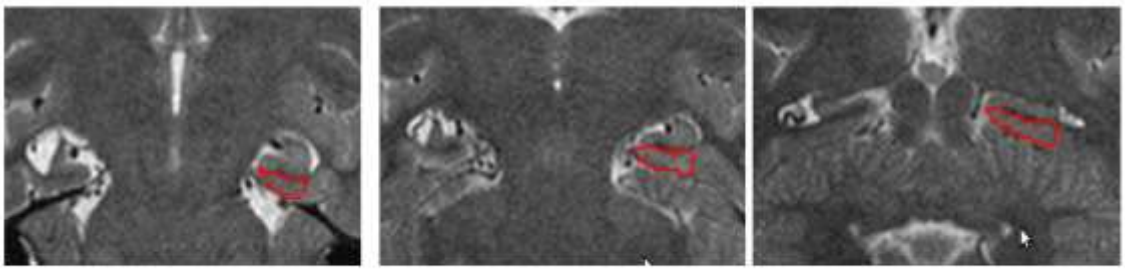

Figure S2.3: Three representative left parahippocampal gyrus tracings, anterior, mid, and posterior from left to right. For illustrative purposes, only left parahippocampus is traced here.

Appendix 2.4: Assessment of Tracing Protocol

Inter-observer reliability for the hippocampal and parahippocampal tracings was assessed by intra-comparison correlation between the two tracers' computed volumes. Criteria for whole-brain tracing were objective, and high variability dependent on rater was judged very unlikely.

Hippocampal volumes between data acquisition Period 1 (using thinner MRI slices) and data acquisition Period Two (using thicker slices) were compared via two-tailed T test. To determine whether comparable damage was expressed in the left and right hippocampus and in the left and right parahippocampus, T tests were used to compare right and left hippocampal volumes across all subjects and to compare right and left parahippocampal volumes across subjects.

MRI

Inter-observer reliability for volumetric measurements was assessed via intra-comparison correlations between two the tracings of two independent tracers. These were quite high, suggesting stable and replicable tracing parameters, and were as follows: Right hippocampus: $r = 0.838$, $P < 0.0001$, Left hippocampus: $r = 0.904$, $P < 0.0001$, Right parahippocampus: $r = 0.884$, $P < 0.0001$, Left parahippocampus: $r = 0.706$, $P < 0.01$.

There was no group difference in mean total hippocampal volumes between animals imaged during data acquisition Period 1 (0.373%) and Period 2 (0.344%) ($P > 0.05$, $t = 1.08$). Generally, it's believed that manual tracing on image series with thicker slices will lead to

higher variability in measures. However, in this case, the standard deviation of the hippocampal volumes in the second acquisition period (0.04%) was lower than that of the volumes in the first acquisition period (0.08%). This suggests there were no effects grossly biasing measurements from one imaging protocol in relation to the other, justifying collapsing across both acquisition periods for further analysis.

Descriptively, as measured by volumetrics, right hippocampal volumes in this sample ranged from 0.12% to 0.27%, with a mean of 0.18% and standard deviation of 0.039%. Left hippocampal volumes ranged from 0.10% to 0.24%, with a mean of 0.17% and standard deviation of 0.031 %. Right and left hippocampal volumes were not statistically distinct at the group level ($P > 0.05$, $t = 0.28$).

Descriptively, as measured by volumetrics, right parahippocampal volumes in this sample ranged from 0.13% to 0.30% with a mean of 0.20% and a standard deviation of 0.045%. Left parahippocampal volumes ranged from 0.12% to 0.31% with a mean of 0.21% and a standard deviation of 0.046%. As with hippocampal volumes, right and left parahippocampal volumes were not statistically distinct at the group level ($p > 0.05$, $t = 0.70$).

Parahippocampal volumes were significantly correlated with hippocampal volumes ($P < 0.01$, $F = 8.34$, $Rsq = 0.266$). This is not surprising, considering the root DA pathology related to damage in both areas. Hippocampal volume predicted just 26.6% of variance in the parahippocampal volume, however, suggesting that they are still meaningfully independent measurements.

Right and left hippocampal volumes showed a similar range and distribution. Similarly, right and left parahippocampal volumes showed a similar range and distribution, suggesting our sample broadly represented a range of medial temporal damage bilaterally, thus justifying further lateralization-based inferences.

Appendix 2.5: Regional Brain Volumes Table

Name	Field #	RHP	LHP	HP	RPHG	LPHG	PHG
Banana	CSL-10385	0.212	0.199	0.411	0.248	0.246	0.494
BBQ	CSL-9724	0.232	0.231	0.461	0.262	0.292	0.554
Bobble	CSL-9821	0.146	0.135	0.281	0.14	0.118	0.259
Breezy	CSL-9931	0.166	0.116	0.282	0.176	0.196	0.372
Carla	CSL-10121	0.23	0.221	0.451	0.219	0.277	0.495
Christopher	CSL-10187	0.211	0.244	0.455	0.206	0.224	0.431
Clean Shores	CSL-10421	0.127	0.173	0.3	0.188	0.234	0.422
Contador	CSL-9752	0.142	0.21	0.352	0.171	0.262	0.433
Devdichi	CSL-10413	0.174	0.155	0.329	0.232	0.227	0.459
Graduate	CSL-9881	0.152	0.185	0.337	0.202	0.231	0.433
Gulliver	CSL-10371	0.206	0.222	0.429	0.3	0.313	0.613
Jessavila	CSL-9949	0.13	0.126	0.256	0.16	0.17	0.33
JJ	CSL-10433	0.163	0.187	0.35	0.234	0.199	0.433
Kabebe	CSL-10398	0.168	0.185	0.353	0.133	0.163	0.296
Midway	CSL-9988	0.199	0.213	0.412	0.195	0.23	0.425
Mr Peppy	CSL-10334	0.207	0.196	0.403	0.253	0.22	0.474
Notaso	CSL-9923	0.138	0.198	0.336	0.186	0.267	0.454
Nui Wahini	CSL-10397	0.188	0.152	0.339	0.154	0.138	0.293
Philadelphia	CSL-9690	0.219	0.167	0.386	0.258	0.21	0.468
Salty Strike	CSL-10235	0.215	0.192	0.407	0.219	0.184	0.402
Sarow	CSL-10170	0.224	0.197	0.422	0.217	0.192	0.409
Schreef	CSL-10392	0.155	0.14	0.295	0.19	0.2	0.39
Spearmint	CSL-9866	0.271	0.182	0.453	0.242	0.195	0.437
Tizer	CSL-10091	0.117	0.108	0.225	0.134	0.148	0.282
Wombat	CSL-10434	0.178	0.162	0.34	0.209	0.213	0.422

Appendix 2.5: Subjects are listed alphabetically with brain volumes of right and left hippocampus and parahippocampus. All volumes are listed here as percentages relevant to an indexed measure of whole brain volume. Column categories are as follows: *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *RHP*: Right hippocampal volume as a percentage of whole brain volume. *LHP*: Left hippocampal volume as a percentage of whole brain volume. HP: Total hippocampal volume as a percentage of whole brain volume. *RPHG*: Right parhippocampal gyrus volume as a percentage of whole brain volume, *LPHG*: Left

parahippocampal gyrus volume as a percentage of whole brain volume, *PHG*: Total parahippocampal gyrus volume as a percentage of whole brain volume.

Appendix 2.6: Table of Correct and Incorrect Diagnoses Across Responsivity Thresholds

Responses	Hits (%)	False Alarms (%)
> 6.000	100	92.857
> 8.000	100	85.71
> 11.00	100	78.57
> 14.50	92.31	71.43
> 18.00	92.31	64.29
> 20.50	84.62	57.14
> 22.50	84.62	50
> 25.00	84.62	42.86
> 26.50	84.62	35.71
> 28.50	84.62	28.57
> 30.50	61.54	28.57
> 31.50	61.54	21.43
> 32.50	46.15	21.43
> 33.50	30.77	21.43
> 34.50	23.08	21.43
> 38.00	23.08	14.29
> 41.50	15.38	7.14
> 44.00	15.38	0
> 46.50	7.692	0

Appendix 2.6: This table lists correct positive diagnoses/hits and incorrect positive diagnoses/false alarms across a range of response thresholds. Response thresholds are such that, for example, a response threshold of > 8.000 indicates that only subjects responding 9 or more times would be diagnosed with DA toxicosis based on the measure.

Chapter 3

Appendix 3.1: Subject Table

Name	Field #	Admit Date	Test Date	Age Class	Sex	DA	MRI	Epil
Jetty Horn	CSL-8052	2/18/09	4/2/09-4/14/09	Pup	M	N	N	N
Anubis	CSL-8095	4/18/09	4/14/09-5/09/09	Pup	M	N	N	N
Sami Monkey	CSL-8105	5/3/09	5/12/09-5/26/09	Subadult	F	C	YRB	N
Snotball	CSL-8684	7/10/09	7/16/09-7/30/09	Adult	F	A	YRB	N
G-Dock	CSL-8181	6/28/09	8/08/09-9/13/09	Yearling	F	N	YB	N
Pepo	CSL-8883	8/4/09	8/30/09-9/14/09	Adult	F	C	YRB	N
B Flat	CSL-8722	9/13/09	9/30/09-10/12/09	Adult	F	C	N	N
ChaCha	CSL-9110	9/19/09	10/30/09-11/15/09	Yearling	M	N	N	N
Rodin	CSL-8973	10/23/09	1/13/10-2/25/10	Yearling	F	N	N	N
Dr. Pep	CSL-9325	12/23/09	1/15/10-1/30/10	Adult	F	C	YR	Y
Sally Angel	CSL-9336	3/1/10	3/10/10-3/24/10	Juvenile	M	N	YRB	Y
Epsen	CSL-9364	3/19/10	4/6/10-4/19/10	Yearling	M	N	N	N
Sephia	CSL-9597	6/2/10	6/16/10-7/1/10	Adult	F	C	YL	Y
Akbar	CSL-9679	6/16/10	7/5/10-7/20/10	Subadult	F	C	YR	Y
Contador	CSL-9752	7/7/10	7/21/10 -8/6/10	Adult	F	C	YR	N
Philadelphia	CSL-9690	6/18/10	8/9/10-8/25/10	Adult	F	C	YL	N
BBQ	CSL-9724	6/25/10	8/25/10-9/10/10	Adult	F	C	YRB	Y
Peridot	CSL-9807	8/17/10	9/16/10-10/2/10	Adult	F	C	YR	Y
Bobble	CSL-9821	8/28/10	10/5/10-10/19/10	Adult	F	C	YLB	Y
Spearmint	CSL-9866	10/11/10	10/19/10-11/4/10	Adult	F	C	YLB	N
Graduate	CSL-9881	10/17/10	11/11/10-11/24/10	Adult	F	C	YR	Y
NotaSO	CSL-9923	2/16/11	2/28/11-3/13/11	Adult	F	C	YRB	N
Breezy	CSL-9931	3/31/11	4/18/11-5/1/11	Adult	F	C	YB	Y
Jessavila	CSL-9949	5/24/11	6/3/11-6/14/11	Subadult	F	C	YB	N
Midway	CSL-9988	7/2/11	7/7/11-7/21/11	Juvenile	M	N	YB	N
Tizer	CSL-10091	8/30/11	9/19-11-10/03-11	Adult	F	C	YB	Y
Carla	CSL-10121	9/12/11	10/6/11-10/19/11	Adult	F	N	YR	N
Christopher	CSL-10187	10/10/11	10/19/11-11/03/11	Juvenile	M	C	YRB	Y
Sarow	CSL-10170	10/4/11	11/03/11-11/24/11	Yearling	M	N	N	N
Saltystrike	CSL-10235	11/5/11	11/27/11-12/10/11	Adult	F	N	YB	N

Appendix 3.1: Subjects are listed with relevant diagnostic values in the order they were tested. Column headers are as follows: *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *Admit Date*: The day each subject was brought to TMMC after stranding. *Test Date*: The range of dates each subject was at LML taking part in behavioral testing. *Age Class*: Pup: < 1 year, Yearling: 1–2 years, Juvenile: 2–4 years, Subadult: 4–8 years, Adult: 5+ years. Age was estimated by TMMC veterinary staff on the basis of size and secondary sexual characteristics. *Sex*: M: Male, F: Female. Sex was determined by TMMC veterinary staff on the basis of genital morphology. *DA*: N: No diagnosis of DA toxicosis, C: Chronic DA toxisosis, A: Acute DA toxicosis. Diagnoses were assigned by TMMC veterinary staff. MRI: N: No observed hippocampal pathology, YB: Bilateral hippocampal pathology, YR: Unilateral right hippocampal pathology, YL: Unilateral left hippocampal pathology, YBR: Asymmetrical bilateral hippocampal pathology weighted right, YBL: Asymmetrical bilateral hippocampal pathology weighted left. These determinations were made qualitatively by a veterinary radiologist. *Epil*: N: No observed seizures, Y: One or more observed seizures.

Appendix 3.2: Delayed Alternation Performance Table

Name	Field #	7norm	7del	20norm	20del	7d/ norm	20d/ norm	Delay/ norm
Akbar	CSL-9679	35	24	24	12	0.69	0.50	0.61
Anubis	CSL-8095	25	22	NA	NA	0.88	NA	0.88
BBQ	CSL-9274	30	23	24	20.5	0.77	0.85	0.81
B flat	CSL-8722	28	7	26	13	0.25	0.50	0.37
Bobble	CSL-9821	32	26	34	18	0.81	0.53	0.67
Breezy	CSL-9931	37	17	28.9	12	0.46	0.42	0.44
Carla	CSL-10121	33	31	36	25	0.94	0.69	0.81
Cha Cha	CSL-9110	35	22.6	27	19.4	0.65	0.72	0.68
Christopher	CSL-10187	36	28	34	14	0.78	0.41	0.60
Contador	CSL-9752	33.9	30.9	35	1	0.91	0.03	0.46
DrPep	CSL-9325	34	15.9	31	15	0.47	0.48	0.48
Epsen	CSL-9364	34	32	35	28.7	0.94	0.82	0.88
Gdock	CSL-8181	25	17	27	12	0.68	0.44	0.56
Graduate	CSL-9881	34	22	31	15	0.65	0.48	0.57
Jessavila	CSL-9949	34	24	35	32	0.71	0.91	0.81
Jettyhorn	CSL-8052	32	37	NA	NA	1.16	NA	1.16
Midway	CSL-9988	32	30	32	26	0.94	0.81	0.88
Notaso	CSL-9923	36	20	34	17	0.56	0.50	0.53
Pepo	CSL-8883	32	22	34	19.1	0.69	0.56	0.62
Peridot	CSL-9807	28	10	26	12.5	0.36	0.48	0.42
Philadelphia	CSL-9690	33	27.6	36	15.5	0.84	0.43	0.63
Rodin	CSL-8973	33.9	31	34.9	30	0.92	0.86	0.89
Sally Angel	CSL-9336	36	28	31	20	0.78	0.65	0.72
Salty Strike	CSL-10235	34	21	30	15.5	0.62	0.52	0.57
Sami Monkey	CSL-8105	30	20	NA	NA	0.67	NA	0.67
Sarow	CSL-10170	19.8	17	28	24.4	0.86	0.87	0.87
Sephia	CSL-9597	27.8	20	34	17	0.72	0.50	0.60
Snoball	CSL-8684	30	21.5	18	14	0.72	0.78	0.74
Spearmint	CSL-9866	32	11.6	30.9	14.1	0.36	0.46	0.41
Tizer	CSL-10091	33	14	31.9	13.6	0.42	0.43	0.43

Appendix 3.2: Subjects are listed alphabetically with behavioral data from the delayed alternation task. Column headers are as follows: *Name*: Informal designation assigned to each animal. *Field #*: The formal code assigned to each stranded CSL for the purpose of long term tracking. *7norm:* The number of correct responses out of 40 on the non-delay trials

matched with the 7-second delay trials. *7del*: The number of correct responses out of 40 on the 7-second delay trials. *20norm*: The number of correct responses out of 40 on the non-delay trials matched with the 20-second delay trials. *20del*: The number of correct responses out of 40 on the 20-second delay trials. On 7norm, 7del, 20norm, and 20del, Subjects who ran more or fewer than 40 trials had their total correct responses ratio corrected to 40. *7d/norm*: Performance ratio on the 7-second delay trials compared to matched non-delay trials. *20d/norm*: Performance ration on the 20-second delay trials compared to matched non-delay trials. *Delay/norm*: Performance ratio on all delay trials, 7- and 20-second, to the matched non-delay trials.

Appendix 3.3: Once-Daily Foraging Task Performance Table

Name	Field #	Across-Session Latency	Within-Session Errors
Akbar	CSL-9679	-1.18	2.75
Anubis	CSL-8095	-30.99	1.14
BBQ	CSL-9274	-1.04	1.09
B Flat	CSL-8722	-7.84	1.89
Bobble	CSL-9821	-1.51	1.88
Breezy	CSL-9931	-0.87	1.00
Carla	CSL-10121	-1.44	1.00
Cha Cha	CSL-9110	-1.36	0.67
Christopher	CSL-10187	-1.12	1.80
Contador	CSL-9752	-0.87	1.75
Epsen	CSL-9364	-5.74	0.64
G-Dock	CSL-8181	-2.17	1.20
Graduate	CSL-9881	-1.55	3.13
Jessavila	CSL-9949	-1.93	3.43
Jettyhorn	CSL-8052	-9.74	1.55
Midway	CSL-9988	-0.90	1.17
NotaSO	CSL-9923	-0.17	1.30
Pepo	CSL-8883	-0.52	6.36
Peridot	CSL-9807	-0.26	0.75
Philadelphia	CSL-9690	-1.76	1.50
Sami Monkey	CSL-8105	-1.05	5.00
Sarow	CSL-10170	-1.14	1.20
Sephia	CSL-9597	-0.04	3.55
Snotball	CSL-8684	-2.01	2.29
Spearmint	CSL-9866	-7.41	2.67
Tizer	CSL-10091	-0.35	2.88

Appendix 3.3: Subjects are listed alphabetically with performance measures from the once-daily foraging task. *Across Session Latency*: Latency to correct bucket on each test day was computed for each subject, and then each subject's sequential latency scores were fit to a power curve. The exponent function from the curve was taken as an approximation of acquisition speed. *Within-Session Errors*: Mean number of buckets visited on each testing day following the first visit to the baited bucket was computed for each subject. Revisits to the baited bucket were not counted.

Appendix 3.4: Regional Brain Volumes Table

Name	Field #	RHP	LHP	HP	RPHG	LPHG	PHG
Akbar	CSL-9679	0.184	0.237	0.421	0.241	0.264	0.505
Anubis	CSL-8095	0.263	0.239	0.502	0.255	0.244	0.499
BBQ	CSL-9724	0.232	0.229	0.461	0.160	0.275	0.436
B flat	CSL-8722	0.125	0.224	0.349	0.262	0.292	0.554
Bobble	CSL-9821	0.146	0.135	0.281	0.140	0.118	0.259
Breezy	CSL-9931	0.166	0.116	0.282	0.176	0.196	0.372
Carla	CSL-10121	0.230	0.221	0.451	0.219	0.277	0.495
Cha Cha	CSL-9110	0.231	0.207	0.439	0.165	0.163	0.328
Christopher	CSL-10187	0.211	0.244	0.455	0.206	0.224	0.431
Contador	CSL-9752	0.142	0.210	0.352	0.171	0.262	0.433
DrPep	CSL-9325	0.152	0.220	0.372	0.162	0.282	0.445
Epsen	CSL-9364	0.243	0.206	0.449	0.238	0.247	0.485
Gdock	CSL-8181	0.220	0.174	0.394	0.218	0.244	0.462
Graduate	CSL-9881	0.152	0.185	0.337	0.202	0.231	0.433
Jessavila	CSL-9949	0.130	0.126	0.256	0.160	0.170	0.330
Jettyhorn	CSL-8052	0.222	0.196	0.419	0.251	0.241	0.491
Midway	CSL-9988	0.199	0.213	0.412	0.195	0.230	0.425
Notaso	CSL-9923	0.138	0.198	0.336	0.186	0.267	0.454
Pepo	CSL-8883	0.138	0.235	0.372	0.173	0.240	0.414
Peridot	CSL-9807	0.123	0.211	0.334	0.128	0.244	0.372
Philadelphia	CSL-9690	0.219	0.167	0.386	0.258	0.210	0.468
Rodin	CSL-8973	0.216	0.235	0.451	0.222	0.338	0.559
Sally Angel	CSL-9336	0.142	0.142	0.285	0.172	0.167	0.339
Salty Strike	CSL-10235	0.215	0.192	0.407	0.219	0.184	0.402
Sami Monkey	CSL-8105	0.117	0.224	0.341	0.147	0.215	0.363
Sarow	CSL-10170	0.224	0.197	0.422	0.217	0.192	0.409
Sephia	CSL-9597	0.224	0.144	0.368	0.273	0.199	0.472
Snoball	CSL-8684	0.196	0.198	0.394	0.225	0.243	0.468
Spearmint	CSL-9866	0.215	0.182	0.453	0.242	0.195	0.437
Tizer	CSL-10091	0.117	0.108	0.225	0.134	0.148	0.282

Appendix 3.4: Subjects are listed alphabetically with regional brain volumes as computed by manual tracing from MRI. All volumes are listed here as percentages relevant to an indexed measure of whole brain volume. Column headers are as follows: *RHP*: Right hippocampal volume as a percentage of whole brain volume. *LHP*: Left hippocampal volume as a percentage of whole brain volume. *HP*: Total hippocampal volume as a percentage of

whole brain volume. *RPHG*: Right parahippocampal gyrus volume as a percentage of toal brain volume. *LPHG*: Left parahippocampal gyrus volume as a percentage of whole brain volume. *PHG*: Parahippcoampal gyrus volume as a percentage of whole brain volume.

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