As long as our brain is a mystery, the universe - the reflection of the structure of the brain - will also be a mystery.

- Santiago Ramón y Cajal (1852-1934)

University of Alberta

Neurosilence: Intracerebral applications of protein synthesis inhibitors eliminate neural activity

by

Arjun Vishnu Sharma

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Psychology

Fall 2010
Edmonton, Alberta

Examining Committee

Dr. Clayton T. Dickson, Psychology, Physiology, and Centre for Neuroscience

Dr. Jeremy B. Caplan, Psychology and Centre for Neuroscience

Dr. Marcia Spetch, Psychology

Dr. Declan Ali, Biological Sciences and Centre for Neuroscience

Dedication

To my mother and father, who have so selflessly devoted their lives to providing me with every opportunity to explore and learn that I or anyone could ever have dreamed of. To my brother, who has served as an example of what can be achieved with focus. And finally to the memory of Doug Moore, who shone a light on this path of study, and whose teachings have illuminated every step of the way.

Abstract

The acquisition of a behavioural response (learning) and the later retrieval of this response (memory) are separated by an endogenous biological process which consolidates the temporary neural changes initiated by training. Intracerebral infusions of stimulants to the hippocampus potentiate this process and infusions of protein synthesis inhibitors (PSIs) impair it. A tacit assumption regarding the application of PSIs is that they have no effect upon spontaneous brain electrical activity; however, given their documented non-specific side effects, this idea was re-evaluated under controlled conditions. Hippocampal recordings were made in urethane anaesthetized rats before and after unilateral hippocampal infusions of the PSIs anisomycin and cycloheximide. Infusions suppressed local field potentials, eliminated sink/source alternations and silenced multiunit activity without affecting the contralateral hippocampus. This suppression was correlated with the degree of protein synthesis inhibition. These results present a serious confound for all results obtained using anisomycin and cycloheximide to test memory consolidation.

Acknowledgment

I would like to acknowledge the time, energy, bad jokes, and unrelenting faith that my advisor, Dr. Clayton T. Dickson has given me. I would also like to acknowledge the helpfulness and willingness to participate in the defence from my committee: Dr. Declan Ali, Dr. Jeremy Caplan, and Dr. Marcia Spetch. I owe a great debt of gratitude to Dr. Frank Nargang, who allowed me space and resources in his lab without which this thesis would not be possible. I would also like to thank Caitlin Hughes and Greg Silasi for technical assistance, as well as everyone in the Brain Rhythms Lab (both past and present) for managing to put a smile on my face every day. Finally, I would like to thank my family and friends; the encouragement and support I received throughout this learning experience are things I was infinitely lucky to have and couldn't have done without.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

2,4-dinitrophenol (DNP)

5-hydroxytryptamine (5-HT)

Actinomycin D (ACD)

Adenosine triphosphate (ATP)

Anisomycin (ANI)

Anterior-posterior (AP)

Calcium Ion (Ca^{2+})

Charge-coupled device (CCD)

Chloramphenicol (CAP)

Cornu ammonis (CA)

Current source density (CSD)

Cycloheximide (CHX)

Dentate gyrus (DG)

Di-n-butyl phthalate in xylene (DPX)

Double distilled water (ddH2O)

Deoxyribonucleic Acid (DNA)

Early phase long term potentiation (E-LTP)

Electroencephalograph (EEG)

Evoked potential (EP)

Excitatory postsynaptic potential (EPSP)

Gamma-aminobutyric acid (GABA)

Hippocampus (HPC)

Hippocampus contralateral to infusion (cHPC)

Hippocampus ipsilateral to infusion (iHPC)

Intelligence quotient (IQ)

Intracerebral (i.c.)

Late phase long term potentiation (L-LTP)

Local field potential (LFP)

Long term potentiation (LTP)

Medial-lateral (ML)

Messenger ribonucleic acid (mRNA)

Minimum alveolar concentration (MAC)

Multiprobe (MP)

Multiunit activity (MUA)

N-methyl-D-aspartic acid (NMDA)

Neocortex (nCTX)

Oligonucleotide (OGN)

Phosphate buffered saline (PBS)

Potassium ion (K^+)

Protein synthesis inhibitor (PSI)

Puromycin (PURO)

Region of interest (ROI)

Resistor-capacitor (RC)

Root mean square (RMS)

Slow oscillation (SO)

Sodium ion (Na^+)

Stratum granulosum (SGran)

Stratum lacunosum moleculare (SLM)

Stratum moleculare (SMol)

Stratum oriens (SOr)

Stratum pyramidale (SPyr)

Stratum radiatum (SRad)

Supraoptic nucleus (SON)

Tetrodotoxin (TTX)

Introduction

Psychology as a discipline is concerned with understanding the nature of behaviour and its causes. This is done in an empirical manner, usually by experimentally altering the nature of the organism's experience and observing the resulting behaviour. In its infancy, the field of psychology clearly recognized the fact that the organ mediating the behaviours of interest was the brain. Thus, it was proposed that changes within the brain initiated by experience are ultimately responsible for changes in behavioural output (James, 1890). A phenomenon which is central to almost all aspects of the experience of any organism is memory, and thus it has been discussed, philosophized about and studied for many years.

Memory and the brain

William James suggested that memory consisted of a number of distinct processes: the after image, primary memory, and secondary memory (James, 1890). The after image was an exceedingly brief stage of memory which decayed very rapidly; however, the main components of the memory model were considered to be primary and secondary memory. These latter concepts can be likened to the more modern ideas of working (short-term) memory (on the order of seconds to a minute) and long term memory (on the order of hours to years), respectively. Though James was not the first person to hypothesize these different stages of memory, he was among the first psychologists who put forth the idea that these abilities were supported by modification of the brain through experience; that is, by virtue of the brain being plastic. Independently corroborating this idea of the brain's involvement in memory processes was

Ramón y Cajal, who proposed that neurons were the basic units of brain communication and that they carry out this function using specialized endings called synapses. He further stated that these synaptic elements were not static and in fact modifiable (Rudy, 2008b, pp. 9-10). It is this proposal which has fuelled subsequent neuroscientific investigations into the brain mechanisms of learning and memory.

Synaptic plasticity and long-term potentiation

Cajal's idea that synaptic connections were modifiable in their strength (known as synaptic plasticity) fit nicely with the belief at the turn of the 19th century that new neurons are not produced in adult brains and that memory must be the result of connections between existing cells being modified. In the middle of the 20th century, Donald Hebb proposed a potential mechanism of plasticity popularized by Cajal:

> "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." (Hebb, 1949, p. 62)

This idea is known as Hebbian Theory and is often summed up with the saying, "cells that fire together, wire together." Hebb's idea was important, and it was later verified experimentally with the discovery of the phenomenon known as long term potentiation (LTP).

LTP was first discovered in 1966 by Terje Lømo and subsequently characterized in 1973 by Timothy Bliss and Terje Lømo, studying memory in the

rabbit hippocampus (HPC) (Bliss & Lomo, 1973; Lomo, 2003). They stimulated cells in the perforant pathway which terminated in the dentate gyrus (DG), and recorded excitatory postsynaptic potentials (EPSPs) in the DG. Single pulse stimulation of the perforant path would yield reliable and consistent amplitude EPSPs, but interestingly, if high frequency stimulation was transiently applied to the perforant pathway and then single pulses were re-administered, the resulting EPSPs in the DG would be greatly increased in both strength and duration. This finding was the first empirical evidence for functional, long-lasting synaptic potentiation and was dubbed LTP. Since then, much work has focused on LTP as a potential mechanism for memory, as they share many characteristics: LTP induction requires a certain threshold of stimulation before the phenomenon occurs, just as memory requires a specific set of stimuli. As well, LTP has been shown to be associative; pairing weak and strong stimuli can induce LTP, in a similar way to how pairing stimuli can induce Pavlovian conditioning and how memory can be considered to be associative.

A very important commonality between LTP and memory is that they can be separated into different temporal stages; this is well demonstrated by phenomena known as early and late phase LTP. The main distinction between early phase LTP (E-LTP) and late phase LTP (L-LTP) is that the former does not require protein synthesis while the latter does. E-LTP is mediated by NMDA receptor-dependent Ca^{2+} modulation. The NMDA receptor stimulation comes about by high frequency neural activity, tetanic stimulation, or by pairing pre and post synaptic activity. This phase of LTP can be likened to learning in that if it is not maintained or consolidated, it will fade away with time. L-LTP is required for this maintenance and it is assumed that during this stage newly synthesized

proteins are needed to maintain the initial changes by E-LTP. This protein synthesis process affects both presynaptic and postsynaptic cells by enhancing communication between them, and is analogous to strengthening an association between two external events, as in the case of Pavlovian conditioning. Thus, E-LTP and L-LTP can be likened to learning and long term memory, respectively.

Temporal stages of memory

The time dependence of memory, from a psychological perspective, can be seen from the temporal separation between the learning (acquisition) of a behavioural trait and its maintained expression (retrieval). The retrieval of information implies storage, and it is this process of retrieval that constitutes the behavioural expression of memory. Although the creation of memory is initiated by the learning episode, a time dependent and behaviourally passive process is involved in solidifying, or consolidating, the memory. The Roman rhetorician Quintilian had noted this very early on in his *Institutio Oratoria*:

> "It is a curious fact, of which the reason is not obvious, that the interval of a single night will greatly increase the strength of the memory, whether this be due to the fact that it has rested from the labour, the fatigue of which constituted the obstacle to success, or whether it be that the power of recollection, which is the most important element of memory, undergoes a process of ripening and maturing during the time which intervenes." (Quintilianus, antiquity; as translated by Butler, 1920, p. 237)

Perhaps the first empirical evidence for the time dependence of memory was shown by the psychologist Hermann Ebbinghaus, who studied retention of nonsense syllables (Ebbinghaus, 1885; as cited by Rudy, 2008b, pp. 3-5). From

this work he constructed a now famous forgetting curve which showed that the rate of decay of memory after a single learning experience was exponential. However, his work also showed that how information was presented was crucial. Repeated presentation of information to be remembered was critical to long term memory retention from short term memory. He also found that the time interval between the repeated presentations of information was highly important. If not enough time was allowed between repetitions, memory did not appreciably improve. If presentations were evenly spaced, then memory was greatly improved. This suggested that some endogenous process was occurring during the intervening periods and that this process was critical to consolidating memories.

Shortly after this, Müller and Pilzecker (1900, as cited in McGaugh, 2000) constructed the pioneering (and still influential) hypothesis of memory consolidation from their work on interference with learning. They had participants learn a list of syllables and found that if a new list was presented to participants shortly after the first, that memory for the first list was negatively affected. This effect faded with time; that is, the more time that passed before the presentation of the second list, the less detriment there was to memory for the first list. From these findings, they postulated that after a learning event, the brain perseverates on what was learned and that if this process is disturbed, memory is not properly consolidated.

This temporal staging of memory was also demonstrated in a different manner in a clinical setting. Théodule Ribot hypothesized about the time dependence of memory strength after looking at patients with brain pathologies (Ribot, 1882; as cited by Rudy, 2008b, pp. 7-8). From his work, Ribot found that

newer memories are much more vulnerable to disruption than are older memories from observing that his patients would lose their most recent memories first and their oldest or most 'entrenched,' last. This idea has come to be known as Ribot's Law. Further clinical evidence underscoring the time dependence of memory consolidation came from experiments with patients undergoing electroconvulsive stimulation to treat mental disorder. After treatment, the patients had no recollection of the events which had occurred just prior to their treatment. This effect has since been shown to exist in a number of controlled studies (Fraser, O'Carroll, & Ebmeier, 2008; Rami-Gonzalez, et al., 2001; Squire, 1977) and coupled with Müller and Pilzecker (1900)'s findings demonstrates that time and normal brain activity are critical factors in consolidating memory.

Support for the existence of an endogenous consolidation process also comes from studies utilizing pharmacological manipulations. If a stimulant is administered to a rat after learning a task (i.e. during the period of endogenous consolidation), then a facilitation of memory can be seen (Davis & Squire, 1984; Flood, Jarvik, Bennett, Orme, & Rosenzweig, 1977; McGaugh, 2000). Similarly, if brain activity is depressed during this period, then memory is adversely affected (Ambrogi Lorenzini, Baldi, Bucherelli, Sacchetti, & Tassoni, 1999; Biedenkapp & Rudy, 2009; Packard & McGaugh, 1996).

Memory systems in the brain

In addition to different stages of memory, it has been acknowledged that different types of memory exist – explicit and implicit. Explicit (or declarative) memory refers to that which can be consciously recalled, while implicit (or procedural) memory involves memories which are not consciously recalled. That

these different types of memory represent the function of different brain regions was demonstrated by the patient H.M., who had a bilateral temporal lobectomy to treat an epileptic disorder. The surgical removal involved two thirds of his HPC, while leaving the rest of his brain intact. Following neurosurgery, while showing no deficits in IQ, his memory was severely impaired. He was found to have a severe anterograde amnesia (the inability to form new explicit memories) as well as a graded form of retrograde amnesia (the inability to recall past explicit memories) (Scoville & Milner, 1957). Remarkably, although H.M. could not form new explicit memories, his ability to learn and perform procedural tasks (which utilize implicit memory) was largely unaffected (Corkin, 2002). This dissociation was further emphasized by the fact that even though H.M.'s performance on a procedural task increased with repeated training trials, he could not remember ever learning the task. Thus, the medial temporal lobe (and more specifically the hippocampus) appears to be paramount for explicit memory.

Similar data has been derived from animal experiments. Indeed, it appears that damage to the cortico-hippocampal-cortical circuit through the medial temporal lobe elicits memory disorders for explicit forms of knowledge in all organisms (Dickson, 2010). In fact, this circuit has been dubbed as the brain's hardware for explicit memories. In this circuit, the medial temporal lobe (including the HPC) receives highly processed multimodal information from the cortical mantle. From there, intra-hippocampal processing takes place via the trisynaptic pathway, with the processed information being sent back to the cortex.

The trisynaptic pathway includes entorhinal cortex layer II cells, which after receiving inputs from the cortex, send information to the granule cells of the DG by the perforant path. These cells then connect to the pyramidal cells in the

CA3 subfield of the HPC through the mossy fibre pathway. CA3 pyramids, in turn, send an output to the pyramidal neurons of CA1, specifically to the lamina known as stratum radiatum (SRad). After this, pyramidal cells in CA1 project directly and indirectly (via the subiculum) to deep layers of entorhinal cortex, which then project back to the cortex. There is also a more direct route known as the temporoammonic pathway, in which layer III cells of the entorhinal cortex feed directly to stratum lacunosum moleculare (SLM) in the CA1 subfield.

The de novo protein synthesis hypothesis

Though evidence exists for an endogenous process of consolidation after learning, the manner in which this process helps to hardwire the changes induced by training in the brain are less clear. Different models abound, but of these, the *de novo* protein synthesis hypothesis appears to be the most accepted; in fact, it has become almost axiomatic in memory literature (Rudy, 2008a). What this model states is that the acquisition phase of learning not only initiates changes in neural circuits allowing novel behaviour, but also triggers the synthesis of new proteins which go on to change and stabilize connections and synaptic transmission between cells. This stability is thought to be the key to consolidation. Thus, available, already existing, pools of proteins are not considered in the memory consolidation process. In the framework of this hypothesis, the critical step in memory consolidation is the synthesis of new proteins. The *de novo* protein synthesis hypothesis is necessarily dependent upon the critical time period of endogenous processing that occurs after a learning event (Figure 1), as mentioned before. If a protein synthesis inhibitor (PSI) is given outside of the critical period, it has little to no effect on the memory trace of interest. Since infusing a PSI does not affect short term memory but

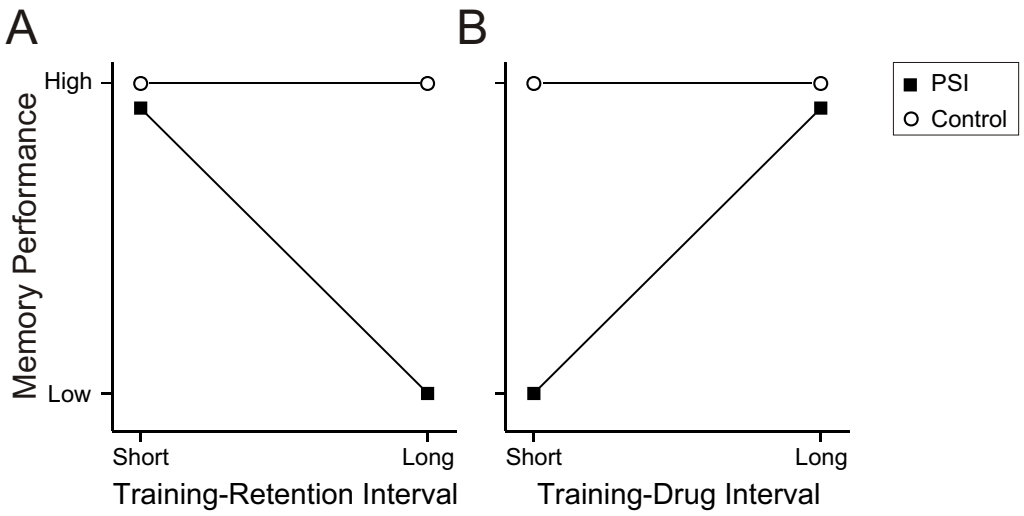

Figure 1. The critical period for post training consolidation. (A) PSI administration during or shortly after training does not impair short term memory performance but devastates long term memory. (B) PSI administration immediately following training but not after a longer delay (hours to days) impairs long term memory. Figure adapted from Rudy (2008).

inhibits long term memory as defined on the time scales reported above, strong evidence exists for the involvement of protein synthesis in memory processes. However, the implications of this hypothesis must be explored further.

This hypothesis has been one of the most actively investigated in the field of memory (Barraco & Stettner, 1976; Davis & Squire, 1984; Rainbow, 1979). These reviews detail a host of behavioural experiments in which both systemic and intracerebral (i.c.) applications showed detrimental effects on consolidation. Indeed, the first experiment leading to the intensive exploration of the *de novo* protein synthesis hypothesis suggested that i.c. applications were more reliable than systemic administration at impairing hippocampal dependent memories (J. B. Flexner, Flexner, & Stellar, 1963).

The use of other protein synthesis inhibitors to test memory

Since Flexner et al. (1963)'s initial study, a number of different PSIs have been used to test the *de novo* protein synthesis hypothesis; these include, for example: puromycin (PURO), acetoxycycloheximide, cycloheximide (CHX), and anisomycin (ANI). PURO was the drug first used in the investigation of the PSI-memory phenomenon, but was abandoned due to its inhibition of motor behaviour in animals (Davis & Squire, 1984). ANI and CHX were then used as the drugs of choice to sidestep these issues. All the used PSIs, however, do produce unwanted side effects when injected peripherally (Davis & Squire, 1984). PURO seems to be the PSI with the greatest number of side effects, even though it and all other relevant PSIs inhibit the same stage of protein synthesis. However, the method of inhibiting this stage varies between the PSIs (Grollman, 1967; Jimenez, Carrasco, & Vazquez, 1977; Nathans, 1964). The existence of

these side effects is highly significant for the consequences of memory studies, as the only empirical way to test memory in animals is through behavioural response. However, impairments in motor behaviour or general malaise will negatively affect performance in a non-specific manner. The resulting decrease in performance can be misattributed to amnesia, instead of the inability for the animal to perform the appropriate response. To sidestep this issue, many researchers have resorted to exclusively using i.c. injections of PSIs to mitigate the side effects obtained by administering these drugs systemically. Another benefit of this method of administration is that it seems to provide the most consistent amnestic effect (J. B. Flexner, et al., 1963). The most often used PSI in i.c. applications is undoubtedly ANI (followed distantly by CHX). This is because with both these drugs, sickness is less pronounced in animals and has an onset far from the time of injection (Davis & Squire, 1984) and presumably also because of their ease of availability.

Other biological effects of protein synthesis inhibitors

Beyond the actions of ANI on protein synthesis translation which are hypothesized to mediate its memory effects, there are a number of other properties it possesses which may also play a role in its memory-damaging abilities. For many years, ANI and other PSIs have been studied due to their ability to induce apoptotic (active cell death) pathways in cells (Adams, 2003; Iordanov, et al., 1997; Tsuchida, Kato, Yamada, & Kawamoto, 2002). If the amnestic effect of ANI and other PSIs can be attributed to the fact that they kill neurons in the brain, then the effect of PSIs are no different than permanently lesioning the given brain area. More recent reports also demonstrate other side effects of ANI; Canal, Chang, and Gold (2007) have shown that i.c. injections of

ANI result in vast increases (1,000-17,000%) in the extracellular concentrations of norepinephrine, dopamine and 5-HT. With effects such as this, it can be readily inferred that intercellular communication is greatly impaired, as well as the fact that rapid release of such great quantities of neurotransmitter may be the result of some insult to cell integrity in the area affected by ANI. Related to this, some literature points to the inhibition of catecholamine (adrenal gland hormones) synthesis in animals after administration of PSIs (L. B. Flexner & Goodman, 1975) and the lack of amnestic effects in adrenalectomized animals which are administered PSIs (Nakajima, 1975). Again, this suggests that mechanisms other than inhibition of the synthesis of new proteins may be part of the amnesia induced by these drugs.

Neural communication has also been shown to be impaired in other manners. Though PURO is known to induce disturbances in cerebral EEG activity, it has been claimed that ANI does not elicit this effect in animals (Davis & Squire, 1984). However, work conducted by Schwartz, Castellucci, and Kandel (1971), indicates that this may not necessarily be the case – when bathing a pacemaker neuron (a neuron with endogenous rhythmic activity) in ANI, the researchers found that over a period of hours, this activity waned. Although a similar effect was present in control situations, both effects were correlated with a decrease in the rate of amino acid incorporation into newly created proteins. However, this result appears to have gone unnoticed for a number of years. Kleim et al. (2003) have also found results which suggest that communication between cells may be disrupted. By injecting ANI and CHX into the motor cortex of rats, they found that when the motor maps of the rats were assessed by microstimulation, no movements could be evoked following the PSI treatment.

Motor maps are quite plastic and malleable, especially when fine motor skills are being learned (animals in the study were trained on a fine reaching task); therefore, Kleim et al. (2003) suggested that their findings were due to impaired plasticity in this region. However, when directly stimulating the brain, evoked potentials (EPs) were completely blocked following ANI treatment. This then leads to the question of whether electrical transmission between neurons is impaired.

Hypothesis: Protein synthesis is necessary for spontaneous neural activity

Intuitively it seems that if one is impairing cytosolic protein synthesis to the extent that no new proteins are being made at all, then basic functions other than simply memory consolidation should be impaired as well. Given that intercellular chemical communication (and even cell health) can be greatly impaired by the application of ANI, it also follows that electrical activity might be affected. Preliminary results point to this idea (Kleim, et al., 2003; Schwartz, et al., 1971), but the question has never been directly addressed using i.c. administration of PSIs known to impair memory. This is perhaps due to the implicit idea that cellular metabolism and neural activity are somehow decoupled, or are only very indirectly coupled. This may be due to the demonstration of Hodgkin and Huxley that when an axon is perfused with the correct ionic solution, action potentials can be generated (Hodgkin & Huxley, 1952). This would imply that neural transmission is a relatively independent process.

As a result of all of these factors, we elected to test the effects of i.c. application of ANI and CHX on spontaneously generated activity in the HPC. We have found that applications of concentrations of ANI which induce amnesia in

animals and significantly inhibit cerebral protein synthesis can completely silence activity generated in the HPC under urethane anaesthesia. We have also found that as protein synthesis inhibition increases, the suppression of neural activity also increases.

Methods

Data were obtained from 69 male Sprague-Dawley rats. The average weight of the rats was 255 ± 7 g. All experimentation conformed to the guidelines established by the Canadian Council on Animal Care and the methods were approved by the Biological Sciences Animal Policy and Welfare Committee of the University of Alberta.

Surgery, implantation, and recording

Animals were taken from their home cages and placed in a plexiglass chamber which was then filled with gaseous isoflurane mixed with medical grade oxygen at a minimum alveolar concentration (MAC) of 4.0. After loss of the righting reflex, animals were transferred to a nose cone delivering the isoflurane-oxygen mixture at MAC of 2.0-2.5; anaesthesia was maintained at this level. Surgical-level anaesthesia was assessed by a pinch to the hind paw – when no withdrawal reflex was present, the animal was judged to be at a sufficient level of anaesthesia for surgery. Animals were then implanted with a catheter containing urethane; once the catheter was inserted, isoflurane administration was stopped. An anaesthetic dose of urethane was then given (average ± standard error of the mean: 1.66 ±0.01 mg/kg) by administering a bolus of 0.01 ml at a time. Once fully anaesthetized, animals were transferred to a stereotaxic apparatus for electrode implantation.

Once in the stereotaxic apparatus, animals were maintained at a constant temperature of 37.0°C using a servo-driven system connected to a heating pad and rectal probe (Fine Science Tools; Vancouver, BC). Rats were given a subcutaneous injection of atropine methyl nitrate (0.06 mg/kg) to prevent

respiratory secretions. Lidocaine (2%) was injected under the scalp to ensure no discomfort to the animal.

Using stereotaxic coordinates measured from bregma, a monopolar electrode made of Teflon-coated stainless steel wire with a bare diameter of 125 µm (A-M Systems; Carlsborg, WA) was unilaterally implanted in the nCTX (AP, +1.2; ML, +2.1). Holes were made bilaterally in the HPC (AP, -3-3; ML, ±2.1) for electrode implantation; a monopolar electrode was inserted in to the HPC contralateral to the infusion site (cHPC) and a 16-channel linear multiprobe (MP) (Neuronexus Technologies; Ann Arbor, MI) with 100 µm spacing between contacts was inserted in the HPC ipsilateral to the infusion site (iHPC). In some experiments, a monopolar electrode was inserted in the iHPC using the same coordinates. Just posterior to the iHPC implantation site, a hole was drilled to allow access for a microinfusion cannula (AP, -4.0; ML, -2.6; DV, -3.0 to -3.5 mm) constructed from blunt ended 30-gauge stainless steel tubing soldered onto a similar short length 23-gauge stainless steel tube connector. After implantation, all monopolar recording electrodes were fixed to the skull using a jeweller's screw and dental acrylic.

Cannulae were attached to a 10 µl Hamilton syringe (Hamilton Company, Reno, NV) via a length of water filled PE 50 tubing (Becton Dickinson, Franklin Lakes, NJ). Infusate was loaded by negative pressure after first introducing a small air bubble at the cannula tip which could be visualized in the connecting tubing. The syringe was loaded into a microinfusion pump (Model 100, KD Scientific, Holliston, MA) and once all recording electrodes were inserted, the microinfusion cannula was lowered into the brain. Baseline pre-infusion recordings were then taken for a minimum of 10 minutes in all cases. Upon

completion of baseline recordings, animals were infused with either 1.0µl or 0.5µl of drug at a rate of 1µl/min. Verification of infusion was achieved by monitoring the smooth movement of the air bubble. Post infusion recordings were then taken, starting just prior to introduction of the infusate.

Single channel recordings were amplified at a gain of 1,000 and band pass filtered between 0.1 and 10kHz by a 4 channel AC amplifier (Model 1700; A-M Systems). MP recordings were amplified at a gain of 1,000 and filtered between 0.7Hz and 10kHz using a 16 channel head stage and amplifier system (Plexon; Dallas, TX). In all recordings, the two last channels of the 16 channel MP were unavailable due to limitations on the number of channels available on the A-D board (Molecular Devices; Union City, CA). All signals were digitized using the A-D board which was connected to a PC running AxoScope (Molecular Devices). All local field potential (LFP) signals were sampled at ≥ 1,000Hz after software controlled anti aliasing filtering at 500Hz. Multiunit activity was sampled at ≥ 10,000Hz.

Stimulation

In some experiments we tested the effect of ANI on evoked potential (EP) profiles elicited by stimulating the CA3 subfield in the cHPC. This was done by implanting a bipolar twisted wire (Teflon-coated stainless steel, 110µm bare diameter; A-M Systems) electrode to stimulate the CA3 region (AP, -3.5; ML, -3.5; DV, -3.0 to -4.0). Stimulation was elicited using an isolated constant current pulse generator (Model 2100; A-M Systems) with a 0.2 ms biphasic current pulse at an intensity range of 110-120 µA every 8 seconds. EPs were collected by recording at least 16 stimulation events and computing their average.

Stimulation and recording occurred prior to infusion as well as after infusion to evaluate the effect of infusions on EPs.

Radioactive treatment

In some experiments, after recording procedures were completed, we injected animals intravenously with ^{35}S labelled cysteine and methionine (11.05 µl/g bodyweight, totalling 2µCi/g). We then allowed 15 minutes for amino acid incorporation before perfusion.

Histology

Following electrophysiological experimentation, animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in buffer. Brains were extracted and placed in a solution of 4% paraformaldehyde, with the exception of those not used in radioactive experiments; these were placed in a solution of 30% sucrose and 4% formaldehyde. Brains were kept in these solutions at least overnight.

Brain slices for thionin staining and Fluoro Jade B analysis were obtained by first freezing tissue with compressed carbon dioxide and then slicing at a thickness of 48 µm using a rotary microtome (1320 Microtome; Leica, Vienna). A hole was punched in the ventral area of the right hemisphere to allow for differentiation between the hemispheres during later analysis. Slices were mounted on gel-coated slides and were allowed to dry for at least 24 hours before being used for further analysis. In experiments other than those used for autoradiography or Fluoro Jade B staining (see below) slices were stained with thionin and coverslipped for light microscopy analysis.

Slices were stained with Fluoro Jade B by first rinsing in double distilled water (ddH2O), then in ethanol (100%, 70%, and 30%). They were then rinsed in ddH2O for 1min before being incubated in 0.06% $KMnO_4$ for 15 minutes. Slides were again rinsed for 1min in ddH20. Slides were then transferred to a dark room where they were incubated in 0.001% Fluoro Jade B stock solution (25mg Fluoro Jade B powder in 250 mL ddH2O) for 30 minutes while being gently shaken. The slides were then rinsed in ddH2O for 1min and left to dry in a dark area overnight. After being dried, slides were washed in citrisolv and then coverslipped using DPX.

Fluoro Jade B stained slides were viewed under a microscope (DM5500B microscope; Leica) with a green fluorescent filter to visualize dead cells. Digital photomicrographs were obtained using a CCD camera (Retiga EXi Fast 1394; Qimaging, Surrey, BC) which were later imported into Corel PhotoPaint. Photographs were then colour-inverted and brightness equalized between them to allow for a fair comparison between different photographs.

Autoradiography

To obtain brain slices for autoradiography, tissue was cut using a vibratome (VibroSlice NVSL; World Precision Instruments, Sarasota, FL) at a thickness of 100 µm. As with microtome sectioning, a hole was punched in the ventral area of the right hemisphere to be able to identify it in further analysis. Slices were then mounted on gel-coated slides, but owing to their thickness, were allowed to dry for at least 36-48 hours before being used for further analysis. Slides were placed under Kodak BioMax MR-1 film (Perkin Elmer) and kept there for 24-36 hours. After this, the film was developed and scanned to TIFF images

using a scanner at 2400 DPI. Once scanned, individual slices were isolated from the scanned images using PhotoPaint (Corel; Ottawa, ON) and saved in TIFF format. These were straightened using Windows Live Photo Gallery (Microsoft; Redmond, WA). Slices were then visualized in ImageJ (National Institutes of Health; Bethesda, MD), with a region of interest (ROI) boundary drawn around each HPC in the slice and saved. In cases where protein synthesis suppression was to the extent that the iHPC could not be visualized, the ROI for the cHPC was flipped and used instead.

To analyze the effect of the injected drug, images of each brain slice from a given autoradiograph were converted to a grey scale. We measured light intensity values in the ROI. These values were obtained from a standard scale of 0 to 255, with 255 being bright and 0 being dark. In terms of autoradiograph analysis, a higher value on this scale indicated less amino acid incorporation (and thus less protein synthesis). To measure differences in protein synthesis between the two hemispheres, we analyzed both hemispheres at the coronal level of the recording site, as well as 2 slices anterior and posterior to the recording site. This gave us a total of 5 slices, which translated to a 500μm range. In order to specify the differences in the area and volume of the HPC affected by the infusion in each hemisphere, we calculated the percentage of the ROI which showed values of light intensity above a certain threshold for each slice within our 5 slice range. This was known as the percentage suppression measure. Using the threshold function, we assessed the maximum light intensity value that corresponded to white matter regions of the autoradiograph and that included the zone where the hole-punch was located that denoted the side ipsilateral to infusion. By adjusting this level we were able to find a threshold

value that would pick up only marginally small regions of white matter in the cHPC. Once a satisfactory level was reached, measurements of average light intensity values, as well as the number of supra-threshold pixels included in the ROI (expressed as a percentage) were taken. One-tailed paired t-tests computed in Origin (OriginLab; Northampton, MA) were used to calculate significant differences between the hemispheres. The above methods are summarized in Figure 2.

We also obtained a supplementary measure of protein synthesis suppression using light intensity values. By subtracting the average light intensity values of the cHPC from those found in the iHPC, we constructed a difference score for each slice. This was then averaged over our 5 slice range for each experiment to give a light intensity difference measure for each experiment.

In experiments where slices of the the entire dorsal HPC were available, we obtained a measure of the volume of the iHPC affected by the infused drugs. This was calculated by taking percentage inactivation measures over the entire dorsal iHPC. We then measured the number of slices in either the anterior or posterior direction that it took to reach 50% of the inactivation value obtained at the recording site.

Data analysis

Time and frequency domain analyses were computed from electrophysiological signals using code written in Matlab (The MathWorks; Natick, MA). These included autopower, cross-phase, coherence, and autocorrelation. Autopower and phase were then plotted using Origin for field

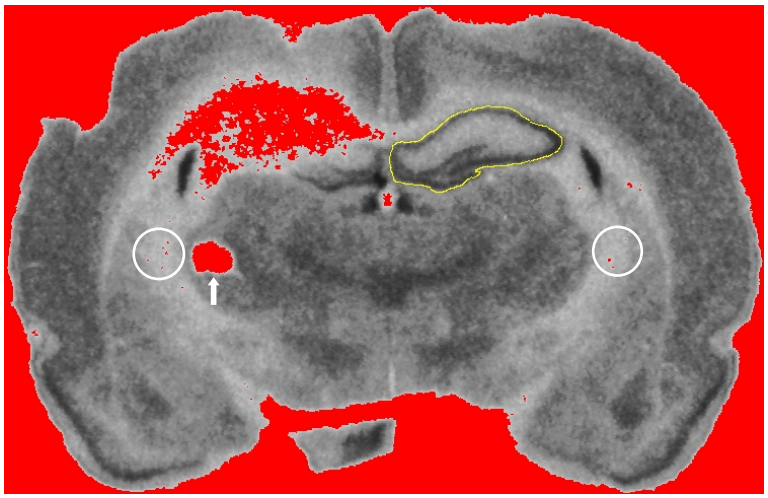

Figure 2. Example of autoradiographic analysis. Shown is a single whole brain slice autoradiograph with pixels demonstrating supra-threshold light intensity values indicated in red. Threshold level was adjusted to ensure that white matter in the lateral regions of each hemisphere (circled) was minimally marked. Arrow indicates hole punched into tissue to denote the hemisphere ipsilateral to infusion. Yellow outline bounds the region of interest (cHPC). Light intensity values and percentage of supra-threshold pixels were obtained from within the region of interest. The same region of interest (reversed) was used for both hemispheres.

signals and certain signal pair combinations. Segments of data no less than 60 seconds in length were used to compute spectra for SO activity; data segments of at least 30 seconds were used to compute spectra for theta. Spectra were estimated by using Welch's periodogram method, using a series of 6 second long, sequential Hanning windows with 2 second overlap. We computed spectral profiles from MP recordings using these same methods. Power values at the peak spectral frequency (the fundamental) were extracted and plotted by relative depth. Multiunit activity (MUA) was calculated from recordings by bandpass filtering between 500 and 3,000Hz using an RC single pole filter in ClampFit (Axon Instruments). MUA was analyzed by then computing the root mean square (RMS) of the filtered traces and plotting the resulting values using Origin. One-tailed paired t-tests comparing MUA pre and post infusion were then computed for both SO and theta in each experiment.

Current source density (CSD) analysis was conducted on spontaneous as well as averaged EP profiles from the probe. This analysis eliminates complications due to volume conducted potentials by estimating transmembrane current flow based on computations that follow the logic of previous work (J. A. Freeman & Nicholson, 1975; Ketchum & Haberly, 1993; Rodriguez & Haberly, 1989). The CSD was computed by estimating the second spatial derivative of voltage traces by using a three point difference on the voltage values across spatially adjacent traces. Thus, the differentiation grid size of the estimate is 300 μm, as this is the distance spanning 3 channels of the multiprobe. Mathematically:

$$CSD = [f(p_{i-1}) - 2f(p_i) + f(p_{i+1})]/d^2$$

Where $f(p_i)$ is the signal obtained from probe channel i (i = 2,3...13 for 14 channel recordings), and d is the distance between adjacent channels (0.1mm). For traces from each end of the probe (i= 1, 14), the differentiation grid was based only on the immediately adjacent channel (i=2, 13). We confirmed that the latter procedure yielded similar, if not identical, CSD results as the 3-point differentiation method by successively eliminating probe end channels, then re-computing and comparing results. CSD traces were also analysed using the spectral methods described above.

To evaluate the effect of the infused drugs on brain activity, ratios of post to pre infusion activity were computed for each hemisphere, giving a percentage value of the increase or decrease in activity as a result of infusion. In the case of the cHPC, only EEG power values were available; ratios were computed by first measuring the pre infusion fundamental and computing its base 10 value. Then the post infusion fundamental was measured and a ratio of log transformed values was computed by dividing post infusion activity by pre infusion activity. In the iHPC, similar ratios were computed by using EEG power; however, the RMS of the CSD was also used to compute these values. This was done by calculating the RMS of each channel across all frequencies for the full time course of the CSD. For post to pre ratios, a value greater than 1 would indicate an increase in power, whereas a value less than 1 would indicate a decrease in power. A value of 1 would indicate no change. Statistical analysis consisted of using one-tailed paired t-tests to measure any differences between the two hemispheres in LFP measures. One-tailed, one sample t-tests were also conducted on these ratios to determine whether the percentage change in activity (if any) after infusion was statistically significant. The rationale for using one-

tailed versus two-tailed tests was due to the existence of preliminary evidence suggesting an inhibitory effect of PSIs on spontaneous neural activity (Cohen, Ervin, & Barondes, 1966; Kleim, et al., 2003; Schwartz, et al., 1971). In addition, since a clear inhibitory effect on electrophysiological activity could be seen in real time during experiments, there was an *a priori* reason to use a one-tailed t-test.

For our correlation analysis, we calculated simple linear correlations and multiple regression values between protein synthesis suppression measures and neural activity measures using Microsoft Excel 2007 (Microsoft). To plot the correlations of percentage inactivation and light intensity difference values against neural activity, we divided our protein synthesis suppression measures into bins (low, medium, and high suppression) representing each 1/3rd of measures across the scale; in the case of percentage inactivation, we created bins corresponding to low through high in thirds across the full range (0-33.33, 33.33-66.67, 66.67-100%). For light intensity difference, we created similar bins. For both protein synthesis suppression measures, we then plotted the average value ± the SEM of our neural activity measures for each bin.

Solutions

Ethyl carbamate (urethane) was mixed in a near-saturated solution at a concentration of 0.8g/ml. ANI was initially dissolved in minimum 3 N HCl, brought to a final concentration of 100mg/ml with 0.5 M phosphate buffered saline (PBS) and pH balanced to 7.2 with equimolar NaOH. CHX was dissolved in a solution of 20% ethanol which was dissolved in 0.5 M PBS. Atropine methyl nitrate and lidocaine were dissolved in ddH2O. ANI, CHX, atropine methyl nitrate, lidocaine, and urethane were all purchased from Sigma-Aldrich (St. Louis,

MO). ^{35}S labeled cysteine and methionine solution (EasyTag EXPRESS ^{35}S Protein Labeling Mix) was purchased from Perkin Elmer (Waltham, MA). Fluoro Jade B was obtained from Chemicon (Temecula, CA).

Results

Electrode, cannula and multiprobe locations were confirmed using histological procedures. Figure 3 shows a summary of these placements. Ipsilateral and contralateral single electrode sites were found in the mid-apical dendritic layer of the CA1 region (ranging from below the pyramidal cell layer in SRad through to SLM) with the exception of a few cases (6 out of 41) which were found to be just dorsal of the pyramidal cell layer in stratum oriens (SOr) (see Figure 3A). Cannula tips were located in the same general regions as the electrode tips, just posterior to the recording locations. The majority of track terminations were located at the border of stratum pyramidale (SPyr) and SRad, with some exceptions (10 out of 24) ending in SOr (see Figure 3B). Multiprobe tracks were confirmed to fall in a plane that traversed the CA1 region of the HPC, passing through the hippocampal fissure and the DG ending in either stratum granulosum (SGran) or the hilar region of the DG (see Figure 3C). Only those experiments where the cannula tip penetrated past the CA1 pyramidal cell layer were used for subsequent analyses.

State alternations

As previously reported, rats maintained under urethane anaesthesia showed spontaneous alternations between one of two rhythmic brain states in the HPC - the activated state (theta) and the deactivated state (the slow oscillation [SO]) (Clement, et al., 2008; Dickson, 2010; Wolansky, Clement, Peters, Palczak, & Dickson, 2006). The theta oscillation was characterized by a highly rhythmic and stereotyped pattern of activity ranging between 3-4 Hz. During this state, activity at neocortical sites showed low voltage fast activity. SO consisted of large

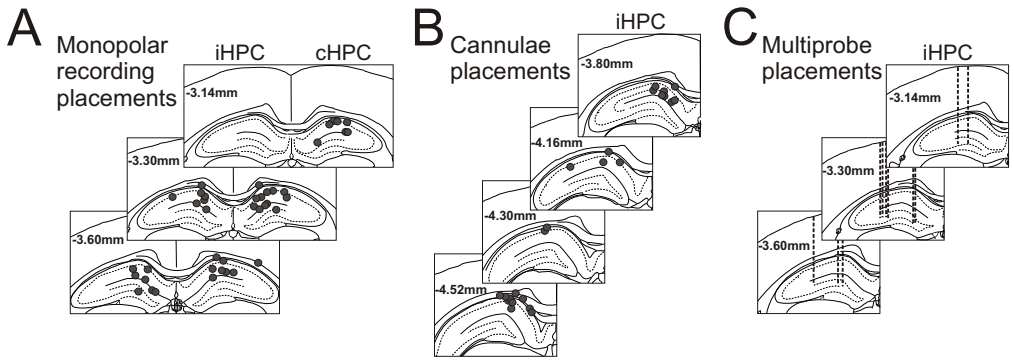

Figure 3. Histological Summary. (A) monopolar recording electrodes in the ipsilateral and contralateral cortex, (B) cannula tips, and (C) multiprobe tracks in ANI experiments with an infusion of 100µg in 1.0µl and thionin staining.

amplitude activity at a frequency of ~1Hz in both the nCTX and HPC. Examples of these signals are shown in Figure 4.

Anisomycin infusions eliminate spontaneous activity in the hippocampus

In our first set of experiments, we investigated the effect of ANI on spontaneously generated activity in the HPC. This was done by first unilaterally infusing 1µl of ANI at a dosage of 100µg in 1µl in the iHPC and measuring the effects upon the activity during both SO and theta states in the iHPC, cHPC, and nCTX using monopolar recording methods. We found that this infusion of ANI suppressed the recorded LFP in the iHPC, but did not affect LFPs recorded in either the nCTX or cHPC (Figure 5). This effect was pronounced and appeared maximal at 20-30 minutes; it was still present at this level 4 hours post infusion. No suppressive effect was found at any time point in either the cHPC or nCTX. Likewise, when infusing vehicle (PBS), no effects were observed at any of the three recording sites in the brain.

When comparing the peak power pre and post infusion, the reduction in LFP theta rhythm power in the iHPC due to ANI was significant (82.12 ± 5.46 % decrease, $p < 0.05$) at a time point of 90 minutes post infusion. Interestingly, although it was reduced in amplitude, SO power was not significantly reduced at an identical time point (23.67 ± 33.85 % decrease, $p = 0.28$). In the cHPC at 90 minutes post infusion, no reduction of power was found (theta: 40.45 ± 22.93 % increase, $p = 0.11$; SO: 63.20 ± 59.55 % increase, $p = 0.20$). Similarly, no effect was found in the nCTX at this time point either: 2.29 ± 6.62 %, $p = 0.38$. Results from all LFP experiments are summarized in Figure 6. Vehicle infusions also resulted in no significant reductions of power for ipsilateral theta (29.23 ± 11.74

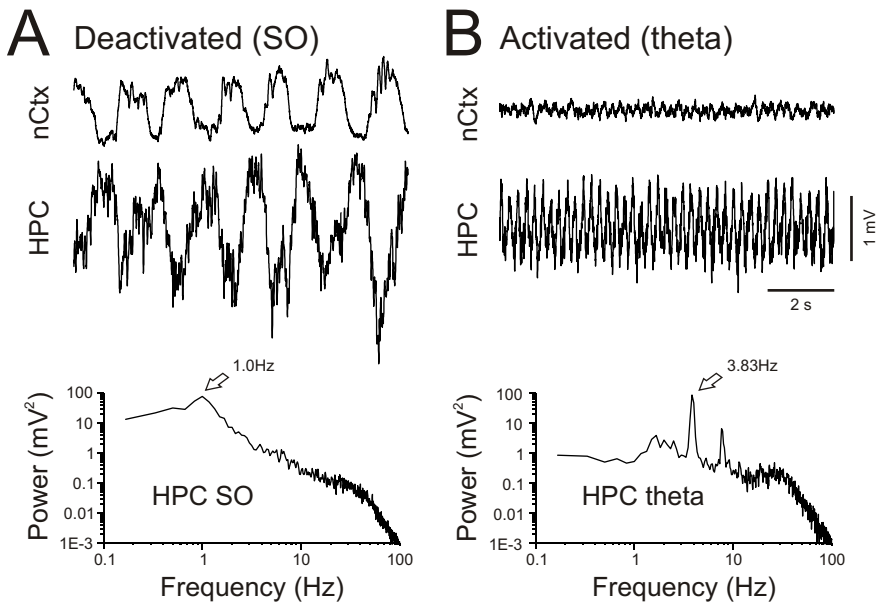

Figure 4. Examples of opposing states under urethane anaesthesia. Both raw traces of LFPs (upper panels) and power spectra (lower panels are shown.During deactivated states, the SO (A) is seen in both nCTX and HPC and shows a peak frequency of 1 Hz. During activated states, theta (B) is specific to the HPC with a peak frequency of 3-5 Hz. The nCTX shows low voltage fast activity during the activated state.

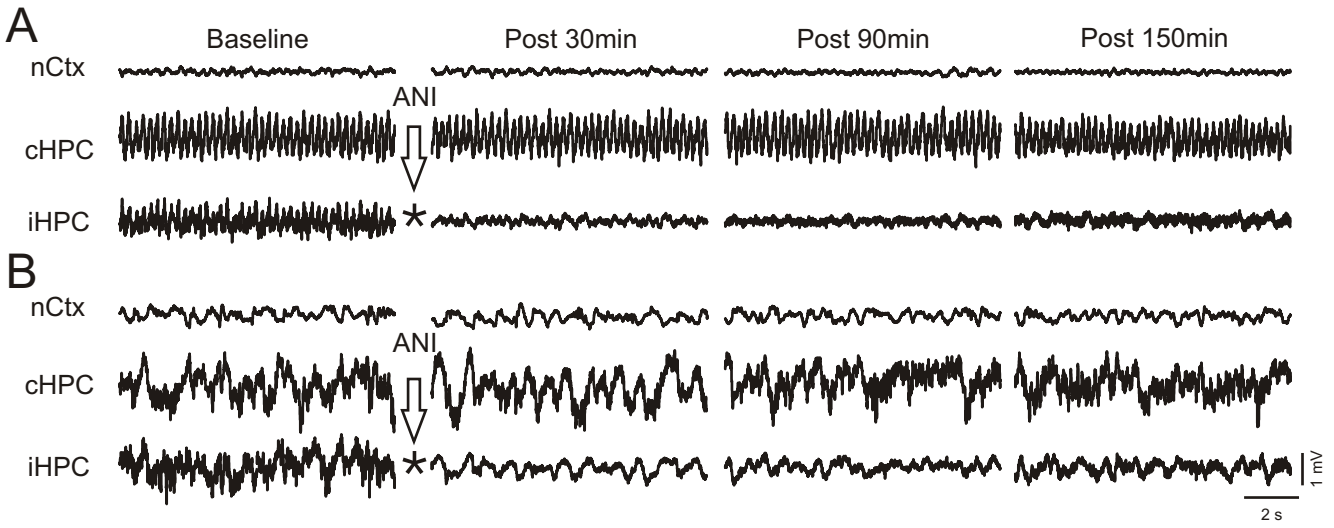

Figure 5. Effects of ANI infusions on local field potentials. (A) Theta state recordings showed that following ANI infusion (100µg in 1µl), the LFP of the iHPC was severely suppressed with a relatively fast onset and long duration. No effect was observed in either nCTX or cHPC activity. (B) SO state recordings showed a similar pattern of results.

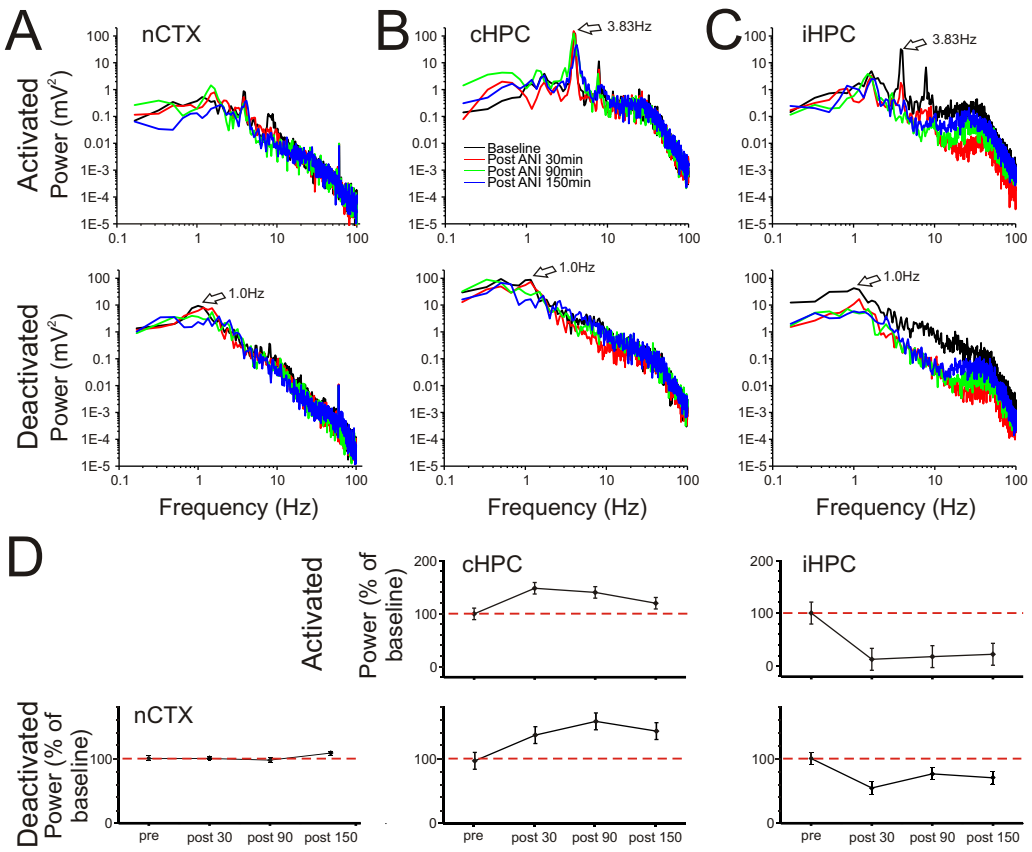

Figure 6. Effects of ANI infusions on local field potential spectra. No reductions in power are seen at either the peak frequency (or even across the rest of the spectrum) in either the nCTX (A) or cHPC (B) but are quite dramatic in the iHPC (C). (D) shows the average change of peak frequency power during activated (3-5Hz) and deactivated (1 Hz) states at all three sites. Only the iHPC shows a significant decrease following ANI infusions.

%, p = 0.12) or SO (135.27 ± 165.89 % increase, p = 0.28), contralateral theta (66.99 ± 12.34 %, p = 0.058) or SO (47.65 ± 21.61 %, p = 0.33), or neocortical SO (12.21 ± 3.44 %, p = 0.36).

Effects of anisomycin on laminar power profiles in the hippocampus

To further investigate these effects of ANI on spontaneous activity, we made MP recordings in the iHPC and constructed laminar power profiles of both SO and theta pre and post infusion. Pre infusion, theta and SO power profiles had similar shapes to our previous results (Nazer & Dickson, 2009; Schall, Kerber, & Dickson, 2008; Sharma, Wolansky, & Dickson, 2010; Wolansky, et al., 2006) – theta profiles had power maxima on either side of SPyr of the CA1 subfield with a phase reversal as well as a power minimum at SRad; the largest maximum was close to the level of the hippocampal fissure. During SO, there was no phase reversal in CA1 but there was a power maximum close to the hippocampal fissure, as with theta.

Post infusion of 100μg of ANI in 1μl, the laminar profile demonstrated a prominent disruption. Significant reductions were observed in power values themselves as well as a flattening of the shape of the power profile. This suggested that a reduction in active current generation was occurring following infusions across both states in the HPC (Figure 7). At the level of the hippocampal fissure the decrease in power was 85.28 ± 7.17 % ($p < 0.05$) and 54.79 ± 6.46 % ($p < 0.05$) for theta and SO, respectively. With PBS, this effect did not exist: theta: 0.20 ± 2.48 % increase, p = 0.47; SO: 14.20 ± 33.75 % increase, p = 0.37.

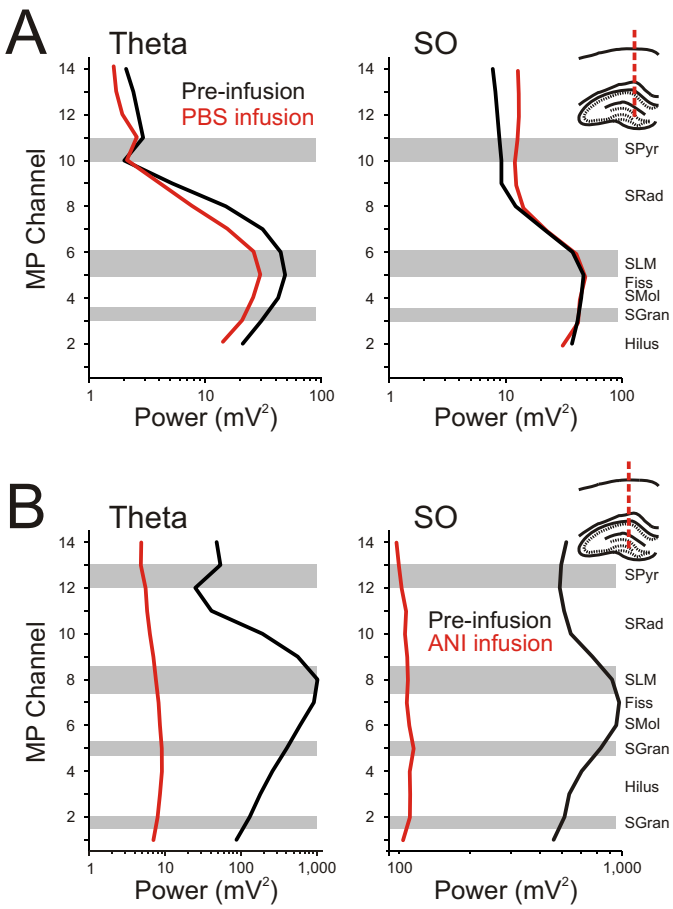

Figure 7. Spectral profile of theta and the SO before and after ANI infusion. Each line represents one laminar recording spanning 1.4 mm with the MP. Spectral analysis was performed on these signals to evaluate changes in the peak frequency power profiles as a result of ANI infusion. (A) Profiles for theta band and SO band power across depth show characteristic shapes and do not change as a result of PBS infusions. (B) Following ANI infusions, power profiles during both theta and SO states show a marked suppression in power in addition to a flattening of the characteristic shape found pre infusion. Schematic drawings indicate MP tracks. Black lines indicate the profile prior to infusion; Red lines are the profiles 90 minutes following infusion.

However, a major complication of using LFP traces to measure locally generated activity is that voltage recordings can be contaminated by volume conduction; that is, the propagation of signals generated at locations other than the recording site that travel through the extracellular space and are picked up by recording electrodes. To solve this problem, we performed CSD and MUA analyses using our MP recordings.

Effect of anisomycin on current source density analysis

We performed CSD analysis on profile recordings to elucidate the spatial distribution of current flow and to track changes occurring due to ANI infusions. Prior to infusion of 100µg of ANI in 1µl, strong sink/source alternations could be seen at the level of SLM in both theta and SO (Figure 8a). Following ANI infusions these alternations were effectively eliminated (Figure 8b). By computing the peak spectral power of the CSD at the level of SLM we were able to quantify the reduction of both the theta and SO signals. The reduction of theta power (average frequency: 3.64 ± 0.18 Hz) was 98.8 ± 1.03 %, $p < 0.05$, and the reduction of SO power (average frequency: 0.97 ± 0.078 Hz) was 99.15 ± 0.37 %, $p < 0.05$. PBS infusions had no effect on the CSD profile (theta: -130.8 ± 177.3 %, p = 0.30; SO: 31.11 ± 32.90 %, p = 0.26). These results show that when the issue of volume conduction is mitigated, the effects obtained from ANI infusions are even more striking than those found with LFP analysis.

In our previous CSD analysis, the calculations of pre-post differences in spectral CSD power at SLM were dependent upon a single channel value at a specific frequency (the fundamental). However, we hypothesized that effects of ANI infusions would not necessarily be specific to a certain frequency or region

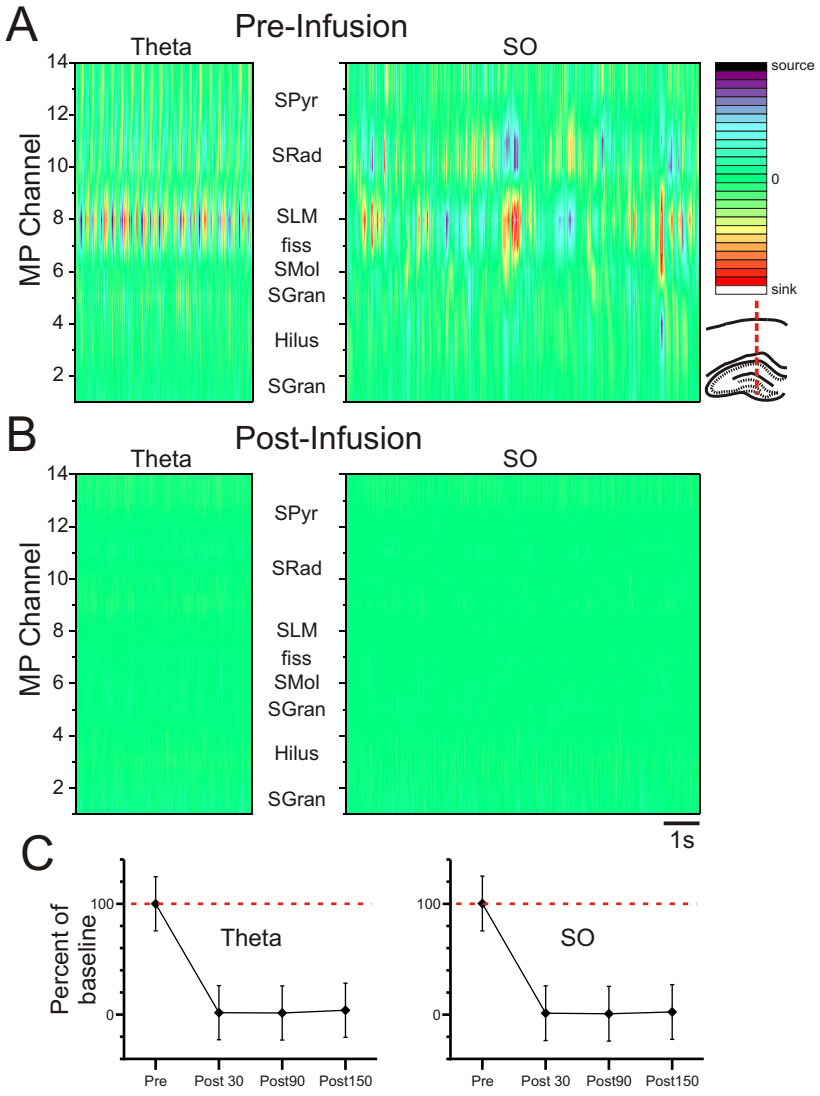

Figure 8. CSD analysis of theta and slow oscillatory activity before and after ANI infusion. (A) During theta and SO, prominent rhythmic alternations of sinks and sources are observed at the level of SLM of CA1 and paired with alternating sources and sinks at the levels of SRad. (B) After ANI infusion, the alternations appear to be eliminated. (C) CSD suppression pre ANI infusion and 3 time points after for theta and SO expressed as a percent of baseline power (mV). SPyr, stratum pyramidale; SRad, stratum radiatum; SLM, stratum lacunosum moleculare; fiss, fissure; SMol, stratum moleculare; SGran, stratum granulosum. CSD scale for SO: -25 to 25 mV/mm^2; CSD scale for theta: -15 to 15 mV/mm^2

within the HPC, so for future CSD analyses we employed the CSD RMS ratio as described in the methods section. In these experiments, we found a significant decrease in CSD RMS during both SO and theta: SO, 68.81 ± 8.77 % decrease, $p < 0.05$; theta, 68.99 ± 4.25 % decrease, $p < 0.05$ (Fig 9). To ensure our results could be equated with our previous findings, we also measured the decrease in power of our EEG measure. We found this to be significant for theta (89.85 ±19.29 % decrease, $p < 0.05$), but not SO (24.12 ± 10.65 % decrease, $p = 0.054$), though it approached statistical significance. This non-significant value for SO LFPs was likely due to volume conduction.

Effect of anisomycin on multiunit activity

In an initial set of experiments using the MP, after obtaining baseline recordings we would remove the MP from the brain and then infuse ANI in the same approximate location (although placing the cannula slightly offset from the original recording track) in order to 1) ensure local action of ANI and 2) mitigate any effects of pressure displacement of the probe on our MUA recordings. Five minutes post infusion, we removed the cannula and attempted to reposition the probe at the same depth previous to infusion. This protocol proved extremely difficult since we were unable to resolve any hippocampal cell layers by listening to MUA activity on the loud speaker, making the necessary fine ventral adjustments impossible. This suggested to us that in addition to suppression of LFP and CSD activity, ANI infusions were also inhibiting local unit activity.

In order to test this idea, we performed simultaneous MP recording and infusion procedures using a different protocol. We took care to ensure that probe displacement was minimized by angling the approach of the cannula, but still

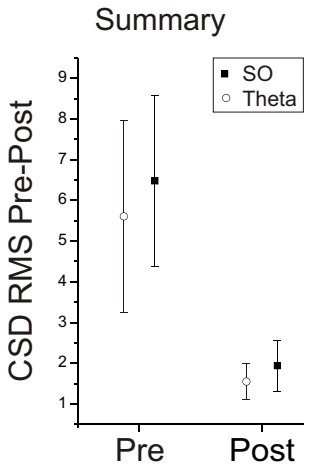

Figure 9. Effects of ANI on CSD RMS analysis. Summary of spontaneous CSD RMS for experiments in which 50ug of ANI in 0.5ul was administered demonstrates a significant suppression post infusion during both theta and SO.

having its tip close to the site of the MP. Indeed, vehicle (PBS) infusions using these methods did not appreciably alter MUA activity (n = 5). However, following ANI infusions, MUA at CA1 (SPyr) and DG (SGran) was almost entirely suppressed at a latency of 30 minutes (Fig 10). MUA RMS was significantly attenuated by ANI during SO ($t(12) = 2.36$, $p < 0.05$) and theta ($t(11) = 1.54$, $p = 0.07$), though the latter approached significance.

Effects of anisomycin on evoked potentials

Our data so far indicate a profound suppression of electrophysiological activity using ANI. To determine if this effect was also extended to afferent electrical stimulation we performed EP analysis by stimulating cHPC CA3 fibers projecting to CA1 in the iHPC. Before ANI infusion, we found a strong current sink generated in SRad of the iHPC when contralateral CA3 was stimulated, as well as prominent current sources in SPyr and SLM. However, following infusion these were completely eliminated (Figure 11). On average, there was a significant difference between the strength of the evoked sink at SRad prior to ANI infusion and the strength of the same source post infusion: $t(3)=8.73$, $p < 0.05$.

Anisomycin infusions do not induce cell death

As mentioned previously, it has been shown that applications of ANI can lead to cell death. To ensure that our electrophysiological results were not mediated by such effects, we investigated results obtained from infusions of 50µg ANI in 0.5µl by using Fluoro Jade B staining. Fluoro Jade B is known to stain cells dying by either apoptotic or necrotic mechanisms

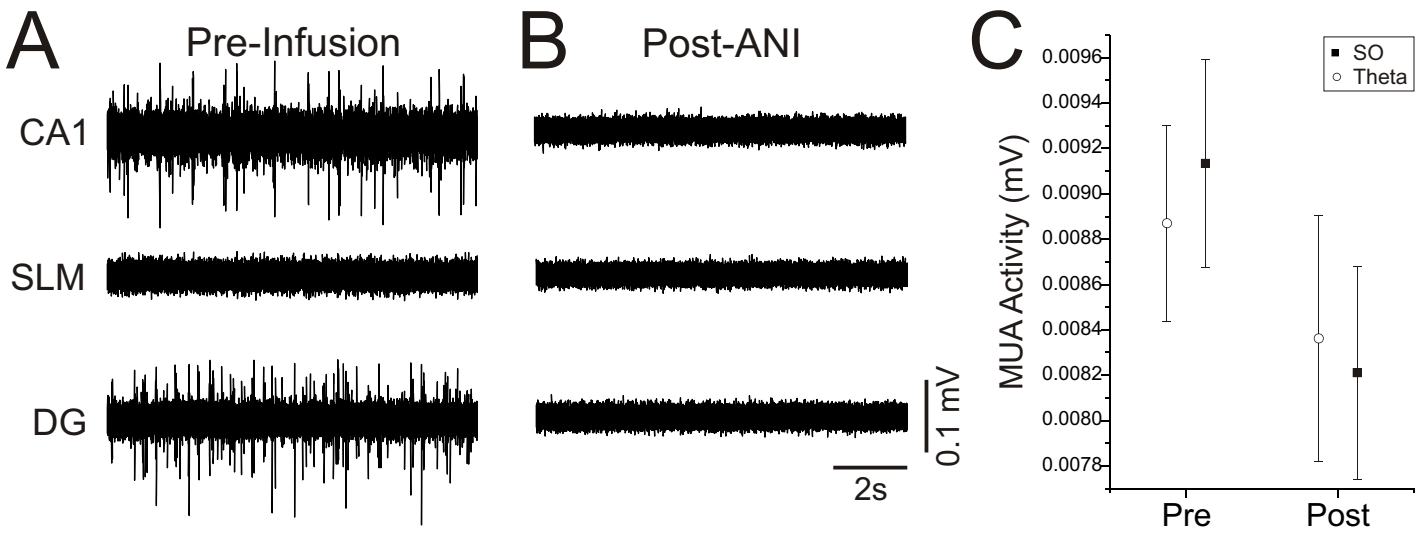

Figure 10. Effects of ANI on MUA activity. (A) Prior to infusion, high levels of MUA can be seen at cell layers in CA1 and the DG. (B) When ANI is infused into the HPC, a complete suppression of MUA activity is obvious in both cell layers, while there is little effect in the cell sparse SLM. (C) Summary of MUA RMS pre and post infusion across all experiments in which 50µg of ANI was infused in 0.5µl.

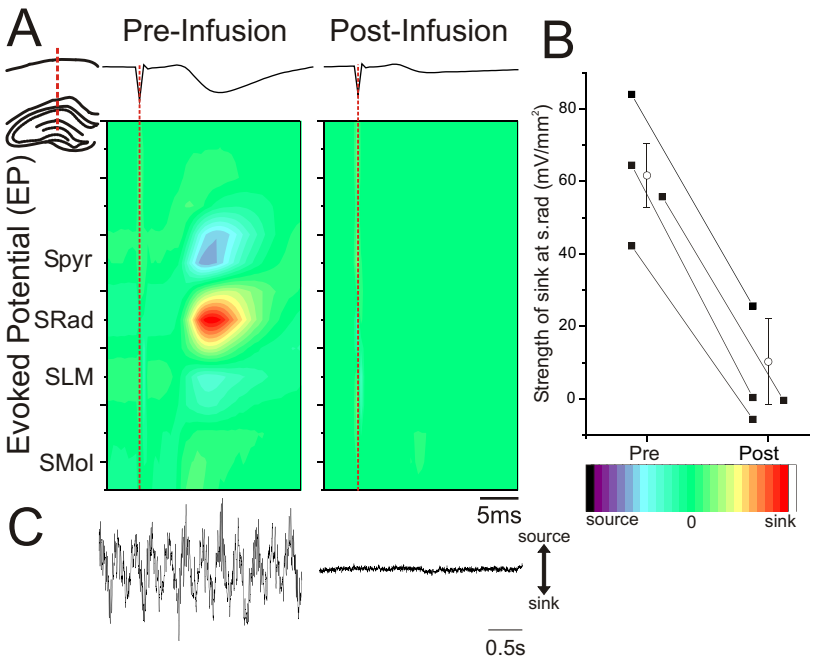

Figure 11. ANI infusions completely suppress both stimulus evoked and spontaneous CSD traces in the HPC. (A) EP CSD profile obtained via stimulation of contralateral CA3 shows a characteristic sink at the level of SRad before but not after ANI infusion. A single CSD trace at this level is displayed above the colour contour plot. (B) Summary of peak sink values from EP CSDs across all experiments demonstrates a significant suppression post infusion. (C) Spontaneous CSD trace at the level of SLM during the theta state in the same experiment shows a concomitant suppression of sink-source alternations following ANI. Scale for EP analysis: -56 to 56 mV/mm^2.

(Schmued & Hopkins, 2000). When Fluoro Jade B stained brain slices were visualized using fluorescence microscopy, we found no systematic positive staining in either the iHPC or cHPC (Figure 12). Positive controls for Fluoro Jade B analysis were obtained by stained cells in cortical regions abutting trephine holes in the skull, where some cell death is expected. This suggests that the electrophysiological suppression induced by ANI does not occur as a result of cell death.

Protein synthesis inhibitors unilaterally inhibit amino acid incorporation and neural activity

Given our obtained electrophysiological results, we hypothesized that protein synthesis itself may play an important role in maintaining spontaneous neural activity. To confirm that ANI was indeed specifically inhibiting protein synthesis in the iHPC, we performed our infusions as before, but following recordings we assessed the degree of amino acid incorporation using radiolabeled cysteine and methionine. This is a direct way to assay global *de novo* protein synthesis. In the 5 slices surrounding the recording site, we also calculated protein synthesis suppression by quantifying the percentage of the HPC which showed no amino acid incorporation below a given threshold. In our initial experiments, we found that 1µl infusions often could disrupt amino acid incorporation in the medial portion of the cHPC (though they did not affect electrophysiological activity), so we subsequently used half this volume (0.5µl).

We confirmed that this dose suppressed neural activity in the iHPC. For CSD RMS there was a significant decrease in the iHPC as opposed to the cHPC: SO, 37.97 ±15.35% decrease, $p < 0.05$; theta, 47.71 ±11.79% decrease, $p < 0.05$

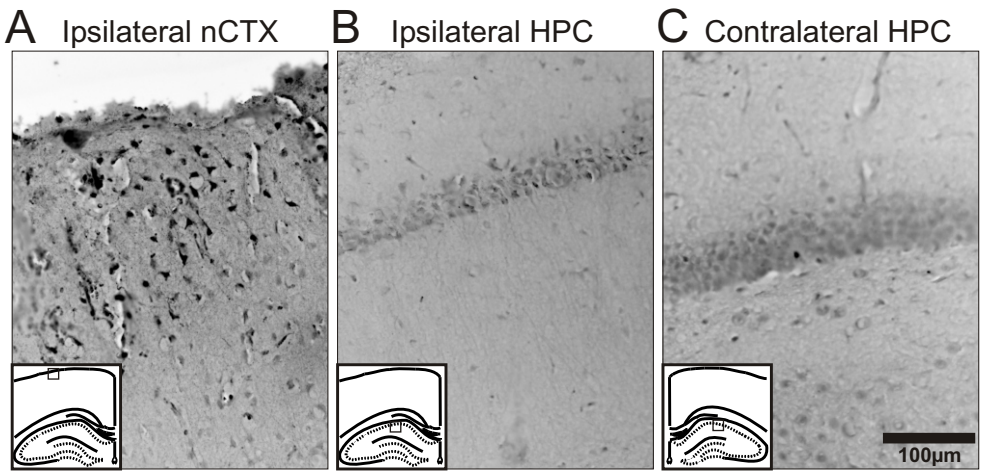

Figure 12. ANI infusions that suppress hippocampal activity do not elicit hippocampal cell death. When stained using Fluoro Jade B, positively stained (dead or dying) cells can be seen in superficial cortical regions damaged by skull trephination and pial damage (A). However, no systematic staining is observed in either iHPC (B) or cHPC (C) CA1 cell layers. In this experiment, CSD RMS analysis showed a 78.02 % decrease in activity in both SO and theta following ANI infusion (50µg in 0.5µl). Inset: locations from which images were obtained.

(Figure 13a). Accordingly, the autoradiographs showed striking differences between the two hemispheres – in the cHPC, anatomical features (i.e. cell layers) and laminar striations could be clearly seen, while in the iHPC a more uniform distribution of elevated light intensity values was observed. Using our threshold measure, the percentage inactivation of the iHPC was 72.58 ± 9.82% compared to only 7.80 ± 2.57% in the cHPC. These values were significantly different: $t(8)= -7.17$, $p < 0.05$. In contrast, vehicle infusions induced neither a decrease in amino acid incorporation ($t(3) = 1.83$, $p = 0.86$), nor CSD RMS activity: SO 5.03 ± 10.01% increase, $p = 0.51$; theta 0.16 ± 5.47% increase, $p = 0.51$. These results suggested that the effect of ANI on neural activity may be related to protein synthesis suppression.

To further assess the association between protein synthesis inhibition and electrophysiological suppression, we used CHX. We infused 20µg of CHX in 0.5µl using the same protocol as described above at. Similar dosages have been used in the past and have been found to show amnestic effects (Berman, Kesner, & Partlow, 1978; Eichenbaum, Quenon, Heacock, & Agranoff, 1976). Similarly to the case with ANI, CHX significantly suppressed both the CSD RMS ratio and MUA. In the CSD RMS post-pre ratio, we found a significant effect for SO (20.74 ± 4.71% decrease, $p < 0.05$) as well as for theta (16.81 ± 7.98% decrease, $p < 0.05$) as shown in Figure 13b. We found significant MUA inhibition both during SO ($t(5) = 4.59$, $p < 0.05$) and theta ($t(5) = 3.38$, $p < 0.05$). Using autoradiography, we found significant differences in the amount of amino acid incorporation suppression above our selected threshold between hemispheres: the iHPC percentage suppression was 73.54 ± 16.32%, which was significantly different than that found in the cHPC: 4.99 ± 1.69%; $t(5) = -4.48$, $p < 0.05$. These

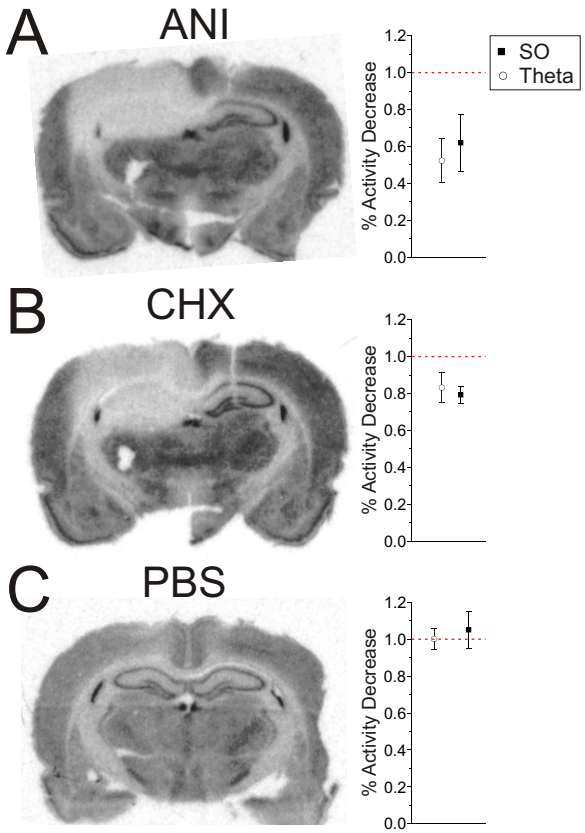

Figure 13. ANI and CHX infusions induce significant decreases in both amino acid incorporation and hippocampal CSD RMS. (A) Infusions of 50µg of ANI in 0.5µl significantly inhibit protein synthesis as well as CSD RMS when averaged over all experiments. (B) Infusions of 20µg of CHX in 0.5µl also significantly inhibited protein synthesis as well as CSD RMS values. (C) Infusions of PBS in to the same region demonstrate a lack of effect in amino acid incorporation and CSD RMS activity.

results further supported the notion that protein synthesis and neural activity may be related.

Effects of varying levels of protein synthesis suppression on neural activity

To make a systematic investigation of ANI's protein synthesis suppression effects, we infused 0.5µl of ANI in final dosages of 25, 12.5, and 0.5µg which we hypothesized would give varying levels of protein synthesis inhibition as well as neural activity suppression. Indeed, we found a dose dependent effect in our electrophysiological measures (Figure 14a) and a trend toward such an effect in measures of amino acid incorporation (Figure 14b). However, given the variability in the dose dependency curve of protein synthesis suppression, we decided to directly assess the relationship across all experiments by characterising electrophysiological suppression as a function of the degree of protein synthesis inhibition as measured by amino acid incorporation.

To accomplish this, we used 3 different protein synthesis measures: percentage of suppression in the recording zone, light intensity differences between hemispheres, and the volume of the dorsal iHPC affected by PSI infusion. We correlated each of these protein synthesis suppression measures with measures of electrophysiological inhibition, including CSD RMS and MUA. The volume of dorsal iHPC affected only correlated significantly with MUA during SO ($r(24) = 0.39$, $p < 0.05$). We then found that light intensity difference values did not significantly correlate with any of our electrophysiological measures. Next, we correlated our percentage inactivation measure with our electrophysiological measures and found that it significantly correlated with CSD

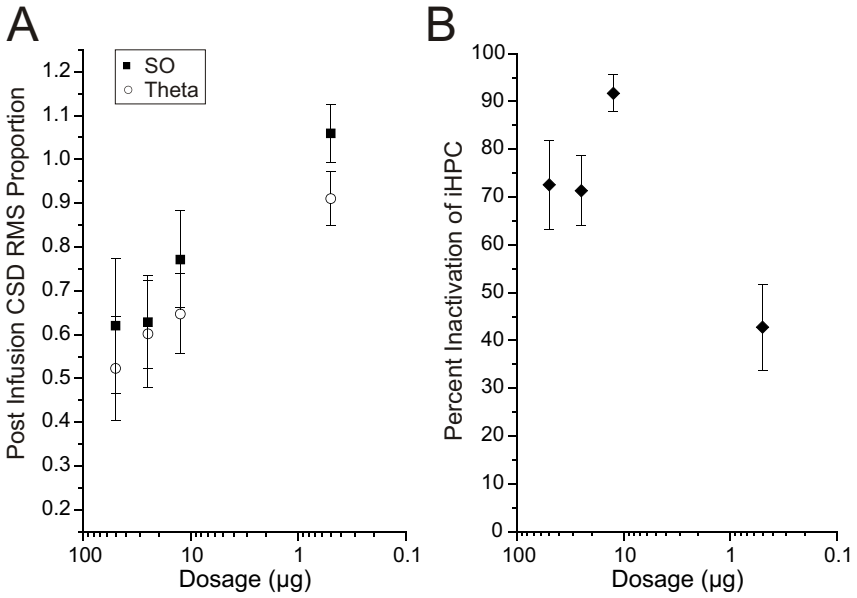

Figure 14. Neural suppression and protein synthesis suppression are roughly dose dependent. (A) As dosage of ANI is decreased, neural activity suppression is also decreased. (B) As dosage of ANI is decreased, autoradiographic analysis shows a general trend towards less suppression of protein synthesis. However, significant variability exists in this dose-dependence curve.

RMS during SO ($r(31) = -0.37$, $p < 0.05$) and theta ($r(31) = -0.49$, $p < 0.05$) and with MUA during both states as well: SO, $r(30) = 0.36$, $p < 0.05$; theta, $r(29) = 0.32$, $p < 0.05$.

Since the percentage inactivation measure is based on a threshold value and thus describes only if pixels are above or below this threshold value (not how far away they are from this threshold), we hypothesized that combining this measure with the light intensity difference (which does give information about the degree to which amino acid incorporation is occurring in a given area of tissue) would give a more accurate depiction of the relationship between protein synthesis and neural activity. Using the volume of tissue affected in addition to the light intensity difference and percentage inactivation measures would give a measurement of the degree to which a certain volume of tissue was affected. To investigate this idea, we used a multiple linear regression approach. After performing a stepwise analysis of the variables, we found that the best predictors of the relationship between protein synthesis and neural activity were percent inactivation followed by light intensity difference; multiple regression showed that these accounted significantly for the variance in the CSD RMS measure during both SO ($R^2 = 0.26$, $p < 0.05$) and theta ($R^2 = 0.60$, $p < 0.05$). The regression of percent inactivation and light intensity difference also significantly accounted for variance in MUA for SO ($R^2 = 0.22$, $p < 0.05$) but not for theta ($R^2 = 0.11$, $p = 0.22$). Overall, these results suggest that the degree of protein synthesis suppression and neural inactivation are indeed related, and that decreasing levels of amino acid incorporation result in more inactivation. Regression tables are shown in Table 1.

	R Square	*Adjusted R square*	*Multiple R*
CSD RMS SO	0.26	0.21	0.51
CSD RMS Theta	0.46	0.42	0.68
MUA SO	0.22	0.17	0.47
MUA Theta	0.11	0.04	0.33

Table 1. Table of values for all regressions.

This relationship can also be observed when grouping data in terms of protein synthesis suppression. We binned the data in terms of the degree of suppression of amino acid incorporation (low, medium, high) and evaluated the neural inactivation in each. In all cases, it was apparent that as the amount of protein synthesis suppression increased, so too did the amount of neural inactivation (Figure 15). This shows that the effects of ANI and CHX at behaviourally relevant concentrations disturb not only protein synthesis but also neural activity.

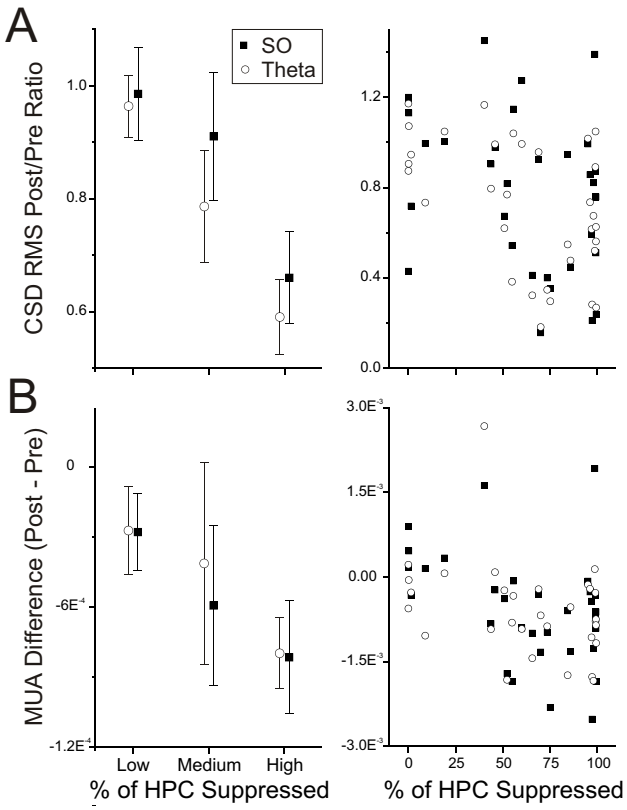

Figure 15. Correspondance of binned protein synthesis suppression measures and inhibition of neural activity. As protein synthesis inhibition increases, neural activity decreases. Error: SEM.

Discussion

Summary

Our findings clearly demonstrate that local applications of ANI profoundly depress spontaneous and evoked local field potential, CSD, and MUA in the HPC. These effects are rapid (peaking within 20-30 minutes) and long-lasting (>4-6 hrs). The abolition of hippocampal neural activity was not due to cell death and is more likely due to the specific effect of ANI on protein synthesis as similar effects were observed with CHX and there was a strong and significant relationship between the degree of neural suppression and the extent of protein synthesis inhibition as characterized by autoradiography.

The implications for these results are two fold: 1) The use of local applications of ANI in past, present and ongoing behavioural studies, is highly likely to be confounded by its suppressive effects on neural activity and 2) intact protein synthesis appears necessary for spontaneous and evoked neural activity.

Implications of the use of intracerebral anisomycin for memory research

In recent years, there has been a flood of criticism of the use of intracerebrally applied ANI. For example, Rudy (2008a) has noted a body of literature which indicates that ANI is used to induce apoptosis in cells – although this could explain its effect on neural activity measures we did not find any evidence for this in the present study, since we did not see any Fluoro Jade B staining in the iHPC. In addition, Canal et al. (2007) found that injections of ANI into the amygdala resulted in a 1,000-17,000% increase of extracellular neurotransmitters in that region. We do not know if this occurred in the present

study; however, it can be concluded that hippocampal function was severely impaired by our treatments, given our electrophysiological data. The previously used PSI, PURO, has been shown to silence neural activity when administered i.c. (Cohen, et al., 1966) and this has led to the discontinuation of its use in behavioural paradigms testing memory. However ANI has been staunchly defended as not producing such effects (Davis & Squire, 1984), although it should be noted that the classic study regarding a limited influence of ANI on neural activity acknowledged that fully functional protein synthesis (as measured by amino acid incorporation) was necessary for spontaneous activity (Schwartz, et al., 1971).

Our results suggest that ANI (as well as CHX) induce a neural silencing effect that is strikingly similar to local infusions of agents such as the Na^+ channel blocker tetrodotoxin (TTX), the local anaesthetic lidocaine, or the GABAergic agonist muscimol (Ambrogi Lorenzini, et al., 1999; Chang & Gold, 2003; Klement, Past'alkova, & Fenton, 2005; Packard & McGaugh, 1996). Although our results are specific for the HPC, it is likely that similar effects would be observed in other regions of the brain. Interestingly, previous research using microstimulation of the motor cortex also showed that bupivicaine (a local anaesthetic) K^+/Na^+ channel blocker) induced a similar abolition of motor maps as local infusions of ANI and/or CHX, the difference being that the duration of effect of the PSIs (lasting at least 4 days) was substantially longer than for bupivicaine (lasting at least 20 min) (Kleim, et al., 2003). Although we recorded in some cases for time periods longer than 6 hours, we never saw a return to baseline following ANI infusions into the HPC. It would be of substantial interest to characterize the time frame of inhibition, perhaps using an unanaesthetized preparation. Wanisch and

Wotjak (2008) found that i.c. injections of 0.5 µl of ANI (totalling 62.5ug) induced significant levels of protein synthesis inhibition for up to 9 hours, though visible but non-significant effects still extended past that point.

Another interesting question that was not addressed by our study is whether the relationship between protein synthesis and neural activity is bidirectional. We have shown that inhibiting protein synthesis suppresses neural activity, but it would also be of interest to demonstrate that induced neural inactivation (for example using TTX) also inhibits amino acid incorporation into new proteins. Considering that elevated neural activity can induce the expression of immediate early genes (IEGs) whose protein products themselves control further transcription, this idea seems highly plausible.

Given that ANI blocked spontaneous and evoked activity during both activated (theta) and deactivated (SO) hippocampal states, it would appear that this effect is indiscriminate and affects neural activity in general. Since the states observed under urethane remarkably resemble natural sleep states (Clement, et al., 2008), this likely indicates that they can be generalized to the unanaesthetized animal. Interestingly, previous research has shown alterations of the sleep cycle when using systemic administration of ANI (Rojas-Ramirez, Aguilar-Jimenez, Posadas-Andrews, Bernal-Pedraza, & Drucker-Colin, 1977). This could have relevance for the memory consolidating properties of sleep itself (Diekelmann & Born, 2010; Marshall, Helgadottir, Molle, & Born, 2006).

In behavioural studies showing an amnestic effect of ANI, doses have ranged widely (Wanisch & Wotjak, 2008). It should be noted that the maximal dosages used in our study were either equivalent to or half the doses used in

behavioural studies. This suggests that the results on neural activity we have obtained would be expressed to a greater degree in other experiments which use higher doses of ANI. At a minimum, our findings present a serious confound for past, present and ongoing behavioural experiments using i.c. ANI infusions.

Can neural inactivation caused by protein synthesis inhibition explain prior behavioural results?

There have been a large number of studies using ANI and CHX to test the *de novo* protein synthesis hypothesis of memory, yielding a seemingly reliable finding: that protein synthesis is necessary for consolidation. However, our results suggest an alternative explanation: these behavioural results are due to the neural inactivation that ANI and CHX secondarily elicit. For those experiments in which ANI is applied directly following the training, it would stand to reason that this would be equivalent to inactivation lesions. However, the case in which drug is applied before or during training could be more problematic. For example, the assumption of many researchers would be that inactivation of the HPC would result in an inability to actually perform on a "hippocampal-dependent" task. Since this is typically not the case, the assumption is that the HPC is functioning normally.

However, Maren, Aharonov, & Fanselow (1997) showed a pattern of results which suggest that it is not necessarily the case that a classical "hippocampal-dependent task" is reliant solely on the HPC; they found that damage to the HPC before contextual conditioning produces no behavioural deficit when tested. However, when the HPC was damaged after training, a large amnestic effect was seen. They thus proposed that there is competition between

a hippocampal and non-hippocampal system to acquire this conditioning, with the hippocampal system overshadowing the non-hippocampal system. If the HPC is damaged before training, the non-hippocampal system acquires the memory and thus memory is preserved. If there is no damage to the brain during learning, the HPC acquires the memory without allowing the non-hippocampal system to acquire it; if the HPC is then inactivated before testing, the animal will show no memory. Sutherland, Sparks, & Lehmann (2010) have added to these observations by extensively reviewing the literature and suggesting that even when a non-hippocampal system has acquired a memory, if the HPC is active at the time of testing, it will inhibit the non-hippocampal system and the animal will thus be unable to retrieve the target memory, even though it still exists. The animal will be thought of as having amnesia, although this is not exactly the case. This conjecture has been directly tested by Biedenkapp & Rudy (2009), who confirmed these results through the use of muscimol to reversibly inactivate the HPC of rats.

Given our results, we feel that the amnestic effects of PSIs that have been described in past literature can be more accurately explained by the neural silencing induced by ANI and the phenomenon of overshadowing. Prior to training, ANI infusions that inactivate the HPC would prime the non-hippocampal system to acquire the “hippocampal-dependent” memory. During testing, when the HPC is back online, its influence would presumably inhibit or overshadow the non-HPC system. However, if the HPC was again inactivated before testing, the non-hippocampal system would be able to retrieve and express the relevant memory. By comparing the pattern of results obtained with TTX and ANI (with simultaneous electrophysiological recording) this could be tested directly.

Implications of current findings for the relationship of protein synthesis and neural activity

Is protein synthesis necessary for neural activity?

The results of the current study suggest that newly synthesized proteins are paramount for continued cell function. Though it has been found that newly synthesised protein products in the HPC of rats have average half lives ranging from 29 to 60 hours (Austin, Lowry, Brown, & Carter, 1972), suggesting that the longevity of existing proteins is extensive and certainly beyond the time scale of the neural suppression effects shown here, it is assumed that our results are tied to the high endogenous rate of spontaneous protein synthesis that is ongoing during even so-called baseline states. While the low turnover rate suggests that our measurements reflect true incorporation (and not metabolism) when observed, combined with the speed at which neural suppression occurs, it implies that the cellular requirement of newly synthesized proteins for proper physiological functioning is very high. In accordance with this, Wanisch & Wotjak (2008) found that 5 min after the systemic administration of ^{35}S labelled cysteine and methionine, protein synthesis could be observed in the HPC of mice. Given that our electrophysiological results were maximized 20 min after infusion of ANI, it is likely that the cellular inhibition of all protein synthesis by ANI is responsible for our obtained effect.

Indeed, preliminary empirical evidence suggesting that specific protein products are also necessary for signalling comes from the findings of Neumann, Kremarik, & Pittman (1995). They introduced antisense oligonucleotides (OGNs: short strands of nucleotides which can inhibit specific gene products from being

created by binding to complementary mRNA strands) directly onto cells in the supraoptic nucleus (SON) of rats. The OGNs were designed as transcriptional blocks for the neuropetides vasopressin and oxytocin, which are expressed in SON cells. What these researchers found was that presenting the antisense OGN for vasopressin to vasopressin-expressing neurons of the SON silenced the extracellular unit discharge from these neurons, and also suppressed the neurons' responsiveness to both antidromic and chemical stimulation. An identical effect was found for oxytocin-expressing neurons presented with antisense OGN for oxytocin. No effects were observed with scrambled OGNs and more convincingly, when antisense OGN for vasopressin was presented to an oxytocin-expressing neuron (and vice versa), no effect was observed. This suggests that specific protein products of a cell are a vital part of its inherent ability to function properly both on its own and within a network and that inhibiting just *one* protein product can have devastating effects on electrical activity and responsiveness.

Which protein products inhibited by protein synthesis inhibitors are necessary for healthy cell function and neural activity?

Since the inhibition of just one protein product can so drastically alter cell functioning, it can be reasonably concluded that the inhibition of all cytoplasmic proteins by PSIs such as ANI and CHX will also have a negative impact on all functions of the cell, and thus overall cell operation. Some crucial protein classes that would be affected by these manipulations would be enzymes, cytoskeletal proteins, signalling molecules, and membrane channels.

Enzymes are ubiquitous “helper” molecules that aid the function of other proteins. Indeed, even the simple maintenance of the resting membrane potential by the Na^+/K^+ pump requires enzymatic processes to occur for its continued function. This is true of almost every other cellular process as well. Given this, inhibiting *all* enzymatic products would likely affect not only signalling but global cell operation.

Cytoskeletal proteins (such as those that make up microtubules, neurofilaments, and microfilaments in addition to their membrane anchoring molecules) are integral for maintaining cell shape and for cellular transport. Given that these structural proteins are necessary for cell integrity, the delivery of other molecules throughout the cell, and in some cases, axon growth, it stands to reason that inhibiting their production would be detrimental to the cell.

Signalling molecules, which include intracellular 2^{nd} messengers as well as intercellular signalling molecules such as neurotransmitters, are integral to a neuron’s ability to be an active participant in any network (local or extended) and are critically reliant on functional cytoskeletal transport systems to be effective. Therefore, inhibiting these proteins from being made would leave the cell unable to receive, interpret, or send any signals to its neighbours, rendering it completely unable to communicate.

Perhaps more directly related to electrical signalling, membrane proteins may also be affected. Membrane ion channels and pumps are protein aggregates and their activity would be likely candidates for imposing the requirement of active processes in cell signalling. These channels are necessary for the excitable nature of neuronal cell membranes.

Protein synthesis in other cellular organelles

Processes that are carried out by ion pumps, most notably the Na^+/K^+ pump, which helps to maintain the resting membrane potential, are energy requiring and continuous. The continued function of these pumps is reliant upon the consumption of adenosine triphosphate (ATP), the energy currency of cells. This leads to the fact that ATP is another crucial candidate for neuronal function. Preliminary evidence does seem to lend credence to the idea that electrical activity is at least in some form dependent upon energy generation in the form of ATP (Kann & Kovacs, 2007). ATP is generated through the function of mitochondria, the power plants of cells, and requires mitochondrial protein synthesis to occur for its production.

ANI and CHX are purported to be specific for eukaryotic protein synthesis (Chan, Khan, Harvey, Merrick, & Pelletier, 2004; Grollman, 1967, 1968). Evidence exists suggesting that mitochondria are ancient prokaryotic organisms that evolved towards their current symbiotic relationship to eukaryotes (Sagan, 1967), suggesting that ANI and CHX should not affect mitochondria. However, since we still do not know the full range of effects that these drugs have on healthy cells, they may indeed have some consequence for mitochondrial protein synthesis which would then be relevant to both cellular metabolism and neural activity. It would be of great value to explicitly investigate this hypothesis. Indeed, specific inhibitors of mitochondrial protein synthesis have been found to induce amnesia in behavioural experiments in chicks as well as rats (F. M. Freeman & Young, 1999; Fride, Ben-Or, & Allweis, 1989; Gibbs & Ng, 1984). If ANI and/or CHX do affect mitochondrial protein synthesis, this may be a nonspecific effect or an extension of cytoplasmic protein synthesis inhibition; it

would then be necessary to assess the relationship between mitochondrial protein synthesis inhibition and neural activity so the results could be directly compared with those found using ANI and/or CHX.

Preliminary evidence of the involvement of both mitochondrial protein synthesis and the availability of oxygen for energy generation has come from recent work by Huchzermeyer et al. (2008), who showed that spontaneous gamma oscillations in CA3 of the HPC are critically dependent on both the partial pressure of oxygen and mitochondrial activity. However, further verification of the involvement of mitochondrial protein synthesis and energy production in the generation of spontaneous neural activity is necessary. A very basic test could be performed by infusing chloramphenicol (CAP), a mitochondrial PSI, into the HPC and observing spontaneous activity to see if it is negatively affected. However, CAP does not affect membrane gradients in mitochondria of neuroblastomas (Vayssiere, et al., 1992), which are important in the production of ATP. This issue could indicate a nonspecific effect of CAP on neural activity.

An alternative to CAP may be 2,4-dinitrophenol (DNP). DNP is known to specifically inhibit the function of oxidative phosphorylation (for which mitochondrial membrane gradients are critical), thus inhibiting ATP production through this mechanism (Chappell, 1964; F. M. Freeman & Young, 1999). Since DNP may interfere with processes that CAP fails to inhibit, it may be prudent to infuse both drugs together, as well as separately. In this manner, some of the prerequisite active processes needed for neural activity may be elucidated. However, not only the different types of protein synthesis are necessary to investigate, but also the different stages of cytoplasmic protein synthesis.

The importance of the different stages of protein synthesis

As mentioned, ANI and CHX inhibit protein synthesis by interfering with translation at ribosomal subunits 80 and 60, respectively (Grollman, 1967; Jimenez, et al., 1977; Obrig, Culp, McKeehan, & Hardesty, 1971). They specifically target the elongation process, acting on the enzyme peptidyl transferase. PURO, one of the initial PSIs used in memory research which is also known to block neural activity (Cohen, et al., 1966) also inhibits the translational step of protein synthesis by inducing premature termination of the polypeptide chain (Nathans, 1964). However, it would be of interest to test other translational inhibitors. This can be done quite simply by using the compound emetine, which is known to inhibit translation at the 40S subunit of the ribosome (Jimenez, et al., 1977). Emetine has been shown to elicit amnesia in both rats and bees in behavioural experiments (Lima, et al., 2009; Stollhoff & Eisenhardt, 2009), though it is not as widely used as ANI or CHX. Emetine could simply be used in place of ANI or CHX in the protocol used in the current study to understand its effect on neural activity. However, it is also critical to investigate the effects of interference with protein synthesis at other steps in its synthesis.

An important stage in protein synthesis which occurs before translation is transcription of DNA. A well known drug, actinomycin D (ACD), inhibits transcription by interfering with the activity of RNA polymerase (Sobell, 1985). This stops elongation of the RNA chain, rendering any subsequent steps in the protein synthesis process obsolete. If protein synthesis and neural activity are indeed related, blocking protein synthesis at its most basic step should silence neural activity. However, work in mammalian neuronal systems (Bramham & Wells, 2007; Steward & Schuman, 2001; Wang, et al., 2009) has indicated that

local protein synthesis can occur in dendrites and synaptic boutons using local mRNA templates. Following mRNA transcription from DNA it can remain intact and is not always immediately degraded, meaning that it can continuously participate in translation. This would suggest that neural activity would perhaps be inhibited after a much greater delay than that found in the current study; however, this remains to be empirically tested.

An alternative way to disrupt protein synthesis is to use an antisense OGN to interfere with RNA synthesis and function. Since antisense OGNs can either be used to block the expression of certain genes or to introduce errors into the mRNA sequence so that incorrect proteins (or no proteins at all) will be synthesized by the ribosome, they can be used in a much more specific manner to investigate the various stages of protein synthesis than the aforementioned drugs. This method of protein synthesis inhibition provides a finer level of control because it is gene specific. However the suggestion from previous studies (Neumann, et al., 1995) is that it can target activity by inhibiting an important and specific protein product.

Conclusions

Our results show that both neural activity and protein synthesis share a relationship; specifically, when protein synthesis is significantly inhibited using similar concentrations of PSIs to those used in behavioural experiments, neural activity is suppressed. To our knowledge, no studies have specifically and systematically addressed this relationship. Despite the fact that the *de novo* protein synthesis hypothesis in memory consolidation has biological validity, our definitive demonstration suggests a serious confound for previous research using

this model. State dependent activity and processing are also strongly tied to memory consolidation, especially during sleep (Diekelmann & Born, 2010; Marshall, et al., 2006).

What we have shown here is that PSIs which have long been considered to mediate their effects on memory solely through the inhibition of protein synthesis also have very pronounced effects on spontaneous activity in the HPC. This raises interesting questions of very nature of the relationship between protein synthesis and spontaneous neural activity. Beyond this, we found that certain dosages of these PSIs eliminate the effects of afferent stimulation to recording sites without inducing cell death, suggesting that the inherent capacity for neurons to generate any sort of electrical activity has been severely compromised. These results all seem to point towards a simple but important question: if cells cannot talk to each other, how can they ever decide what to remember?

References

Adams, J. M. (2003). Ways of dying: multiple pathways to apoptosis. *Genes Dev, 17*(20), 2481-2495.

Ambrogi Lorenzini, C. G., Baldi, E., Bucherelli, C., Sacchetti, B., & Tassoni, G. (1999). Neural topography and chronology of memory consolidation: a review of functional inactivation findings. *Neurobiol Learn Mem, 71*(1), 1-18.

Austin, L., Lowry, O. H., Brown, J. G., & Carter, J. G. (1972). The turnover of protein in discrete areas of rat brain. *Biochem J, 126*(2), 351-359.

Barraco, R. A., & Stettner, L. J. (1976). Antibiotics and memory. *Psychol Bull, 83*(2), 242-302.

Berman, R. F., Kesner, R. P., & Partlow, L. M. (1978). Passive avoidance impairment in rats following cycloheximide injection into the amygdala. *Brain Research, 158*(1), 171-188.

Biedenkapp, J. C., & Rudy, J. W. (2009). Hippocampal and extrahippocampal systems compete for control of contextual fear: role of ventral subiculum and amygdala. *Learn Mem, 16*(1), 38-45.

Bliss, T. V., & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol, 232*(2), 331-356.

Bramham, C. R., & Wells, D. G. (2007). Dendritic mRNA: transport, translation and function. *Nat Rev Neurosci, 8*(10), 776-789.

Canal, C. E., Chang, Q., & Gold, P. E. (2007). Amnesia produced by altered release of neurotransmitters after intraamygdala injections of a protein synthesis inhibitor. *Proc Natl Acad Sci U S A, 104*(30), 12500-12505.

Chan, J., Khan, S. N., Harvey, I., Merrick, W., & Pelletier, J. (2004). Eukaryotic protein synthesis inhibitors identified by comparison of cytotoxicity profiles. *RNA, 10*(3), 528-543.

Chang, Q., & Gold, P. E. (2003). Intra-hippocampal lidocaine injections impair acquisition of a place task and facilitate acquisition of a response task in rats. *Behav Brain Res, 144*(1-2), 19-24.

Chappell, J. B. (1964). The effects of 2,4-dinitrophenol on mitochondrial oxidations. *Biochem J, 90*(2), 237-248.

Clement, E. A., Richard, A., Thwaites, M., Ailon, J., Peters, S., & Dickson, C. T. (2008). Cyclic and sleep-like spontaneous alternations of brain state under urethane anaesthesia. *PLoS One, 3*(4), e2004.

Cohen, H. D., Ervin, F., & Barondes, S. H. (1966). Puromycin and cycloheximide: different effects on hippocampal electrical activity. *Science, 154*(756), 1557-1558.

Corkin, S. (2002). What's new with the amnesic patient H.M.? *Nat Rev Neurosci, 3*(2), 153-160.

Davis, H. P., & Squire, L. R. (1984). Protein synthesis and memory: a review. *Psychol Bull, 96*(3), 518-559.

Dickson, C. T. (2010). Ups and downs in the hippocampus: the influence of oscillatory sleep states on "neuroplasticity" at different time scales. *Behav Brain Res, 214*(1), 35-41.

Diekelmann, S., & Born, J. (2010). The memory function of sleep. *Nat Rev Neurosci, 11*(2), 114-126.

Ebbinghaus, H. (1885). *Ueber das Gedaechtnis*. Leipzig: Duncker & Humbolt.

Eichenbaum, H., Quenon, B. A., Heacock, A., & Agranoff, B. W. (1976). Differential behavioral and biochemical effects of regional injection of cycloheximide into mouse brain. *Brain Res, 101*(1), 171-176.

Flexner, J. B., Flexner, L. B., & Stellar, E. (1963). Memory in mice as affected by intracerebral puromycin. *Science, 141*, 57-59.

Flexner, L. B., & Goodman, R. H. (1975). Studies on memory: inhibitors of protein synthesis also inhibit catecholamine synthesis. *Proc Natl Acad Sci U S A, 72*(11), 4660-4663.

Flood, J. F., Jarvik, M. E., Bennett, E. L., Orme, A. E., & Rosenzweig, M. R. (1977). The effect of stimulants, depressants, and protein synthesis- inhibition on retention. *Behav Biol, 20*(2), 168-183.

Fraser, L. M., O'Carroll, R. E., & Ebmeier, K. P. (2008). The effect of electroconvulsive therapy on autobiographical memory: a systematic review. *J ECT, 24*(1), 10-17.

Freeman, F. M., & Young, I. G. (1999). Chloramphenicol-induced amnesia for passive avoidance training in the day-old chick. *Neurobiol Learn Mem, 71*(1), 80-93.

Freeman, J. A., & Nicholson, C. (1975). Experimental optimization of current source-density technique for anuran cerebellum. *J Neurophysiol, 38*(2), 369-382.

Fride, E., Ben-Or, S., & Allweis, C. (1989). Mitochondrial protein synthesis may be involved in long-term memory formation. *Pharmacol Biochem Behav, 32*(4), 873-878.

Gibbs, M. E., & Ng, K. T. (1984). Dual action of cycloheximide on memory formation in day-old chicks. *Behav Brain Res, 12*(1), 21-27.

Grollman, A. P. (1967). Inhibitors of protein biosynthesis. II. Mode of action of anisomycin. *J Biol Chem, 242*(13), 3226-3233.

Grollman, A. P. (1968). Inhibitors of protein biosynthesis. V. Effects of emetine on protein and nucleic acid biosynthesis in HeLa cells. *J Biol Chem, 243*(15), 4089-4094.

Hebb, D. O. (1949). *The Organization of Behavior*. New York: Wiley.

Hodgkin, A. L., & Huxley, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol, 117*(4), 500-544.

Huchzermeyer, C., Albus, K., Gabriel, H. J., Otahal, J., Taubenberger, N., Heinemann, U., et al. (2008). Gamma oscillations and spontaneous network activity in the hippocampus are highly sensitive to decreases in pO2 and concomitant changes in mitochondrial redox state. *J Neurosci, 28*(5), 1153-1162.

Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L., et al. (1997). Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol Cell Biol, 17*(6), 3373-3381.

James, W. (1890). *The Principles Of Psychology* (Vol. 1). New York: Henry Holt & Company.

Jimenez, A., Carrasco, L., & Vazquez, D. (1977). Enzymic and nonenzymic translocation by yeast polysomes. Site of action of a number of inhibitors. *Biochemistry, 16*(21), 4727-4730.

Kann, O., & Kovacs, R. (2007). Mitochondria and neuronal activity. *Am J Physiol Cell Physiol, 292*(2), C641-657.

Ketchum, K. L., & Haberly, L. B. (1993). Synaptic events that generate fast oscillations in piriform cortex. *J Neurosci, 13*(9), 3980-3985.

Kleim, J. A., Bruneau, R., Calder, K., Pocock, D., VandenBerg, P. M., MacDonald, E., et al. (2003). Functional organization of adult motor cortex is dependent upon continued protein synthesis. *Neuron, 40*(1), 167-176.

Klement, D., Past'alkova, E., & Fenton, A. A. (2005). Tetrodotoxin infusions into the dorsal hippocampus block non-locomotor place recognition. *Hippocampus, 15*(4), 460-471.

Lima, R. H., Rossato, J. I., Furini, C. R., Bevilaqua, L. R., Izquierdo, I., & Cammarota, M. (2009). Infusion of protein synthesis inhibitors in the entorhinal cortex blocks consolidation but not reconsolidation of object recognition memory. *Neurobiol Learn Mem, 91*(4), 466-472.

Lomo, T. (2003). The discovery of long-term potentiation. *Philos Trans R Soc Lond B Biol Sci, 358*(1432), 617-620.

Maren, S., Aharonov, G., & Fanselow, M. S. (1997). Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behav Brain Res, 88*(2), 261-274.

Marshall, L., Helgadottir, H., Molle, M., & Born, J. (2006). Boosting slow oscillations during sleep potentiates memory. *Nature, 444*(7119), 610-613.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science, 287*(5451), 248-251.

Müller, G. E., & Pilzecker, A. (1900). Experimentelle Beiträge zur Lehre vom Gedächtnis. *Z. Psychol. Ergänzungsband, 1*, 1-300.

Nakajima, S. (1975). Amnesic effect of cycloheximide in the mouse mediated by adrenocortical hormones. *J Comp Physiol Psychol, 88*(1), 378-385.

Nathans, D. (1964). Puromycin Inhibition of Protein Synthesis: Incorporation of Puromycin into Peptide Chains. *Proc Natl Acad Sci U S A, 51*, 585-592.

Nazer, F., & Dickson, C. T. (2009). Slow oscillation state facilitates epileptiform events in the hippocampus. *J Neurophysiol, 102*(3), 1880-1889.

Neumann, I., Kremarik, P., & Pittman, Q. J. (1995). Acute, sequence-specific effects of oxytocin and vasopressin antisense oligonucleotides on neuronal responses. *Neuroscience, 69*(4), 997-1003.

Obrig, T. G., Culp, W. J., McKeehan, W. L., & Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J Biol Chem, 246*(1), 174-181.

Packard, M. G., & McGaugh, J. L. (1996). Inactivation of hippocampus or caudate nucleus with lidocaine differentially affects expression of place and response learning. *Neurobiol Learn Mem, 65*(1), 65-72.

Quintilianus, M. F. (antiquity; as translated by Butler, 1920). *Institutio Oratoria* (H. E. Butler, Trans.). Cambridge: Harvard University Press.

Rainbow, T. C. (1979). Role of RNA and protein synthesis in memory formation. *Neurochem Res, 4*(3), 297-312.

Rami-Gonzalez, L., Bernardo, M., Boget, T., Salamero, M., Gil-Verona, J. A., & Junque, C. (2001). Subtypes of memory dysfunction associated with ECT: characteristics and neurobiological bases. *J ECT, 17*(2), 129-135.

Ribot, T. (1882). *Diseases of Memory*. New York: Appleton.

Rodriguez, R., & Haberly, L. B. (1989). Analysis of synaptic events in the opossum piriform cortex with improved current source-density techniques. *J Neurophysiol, 61*(4), 702-718.

Rojas-Ramirez, J. A., Aguilar-Jimenez, E., Posadas-Andrews, A., Bernal-Pedraza, J. G., & Drucker-Colin, R. R. (1977). The effects of various protein synthesis inhibitors on the sleep-wake cycle of rats. *Psychopharmacology (Berl), 53*(2), 147-150.

Rudy, J. W. (2008a). Is there a baby in the bathwater? Maybe: some methodological issues for the de novo protein synthesis hypothesis. *Neurobiol Learn Mem, 89*(3), 219-224.

Rudy, J. W. (2008b). *The Neurobiology of Learning and Memory*. Sunderland: Sinauer Associates, Ltd.

Sagan, L. (1967). On the origin of mitosing cells. *J Theor Biol, 14*(3), 255-274.

Schall, K. P., Kerber, J., & Dickson, C. T. (2008). Rhythmic constraints on hippocampal processing: state and phase-related fluctuations of synaptic excitability during theta and the slow oscillation. *J Neurophysiol, 99*(2), 888-899.

Schmued, L. C., & Hopkins, K. J. (2000). Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res, 874*(2), 123-130.

Schwartz, J. H., Castellucci, V. F., & Kandel, E. R. (1971). Functioning of identified neurons and synapses in abdominal ganglion of Aplysia in absence of protein synthesis. *J Neurophysiol, 34*(6), 939-953.

Scoville, W. B., & Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiatry, 20*(1), 11-21.

Sharma, A. V., Wolansky, T., & Dickson, C. T. (2010). A comparison of sleeplike slow oscillations in the hippocampus under ketamine and urethane anesthesia. *J Neurophysiol, 104*(2), 932-939.

Sobell, H. M. (1985). Actinomycin and DNA transcription. *Proc Natl Acad Sci U S A, 82*(16), 5328-5331.

Squire, L. R. (1977). ECT and memory loss. *Am J Psychiatry, 134*(9), 997-1001.

Steward, O., & Schuman, E. M. (2001). Protein synthesis at synaptic sites on dendrites. *Annu Rev Neurosci, 24*, 299-325.

Stollhoff, N., & Eisenhardt, D. (2009). Consolidation of an extinction memory depends on the unconditioned stimulus magnitude previously experienced during training. *J Neurosci, 29*(30), 9644-9650.

Sutherland, R. J., Sparks, F. T., & Lehmann, H. (2010). Hippocampus and retrograde amnesia in the rat model: a modest proposal for the situation of systems consolidation. *Neuropsychologia, 48*(8), 2357-2369.

Tsuchida, T., Kato, T., Yamada, A., & Kawamoto, K. (2002). Cycloheximide induces apoptosis of astrocytes. *Pathol Int, 52*(3), 181-185.

Vayssiere, J. L., Cordeau-Lossouarn, L., Larcher, J. C., Basseville, M., Gros, F., & Croizat, B. (1992). Participation of the mitochondrial genome in the differentiation of neuroblastoma cells. *In Vitro Cell Dev Biol, 28A*(11-12), 763-772.

Wang, D. O., Kim, S. M., Zhao, Y., Hwang, H., Miura, S. K., Sossin, W. S., et al. (2009). Synapse- and stimulus-specific local translation during long-term neuronal plasticity. *Science, 324*(5934), 1536-1540.

Wanisch, K., & Wotjak, C. T. (2008). Time course and efficiency of protein synthesis inhibition following intracerebral and systemic anisomycin treatment. *Neurobiol Learn Mem, 90*(3), 485-494.

Wolansky, T., Clement, E. A., Peters, S. R., Palczak, M. A., & Dickson, C. T. (2006). Hippocampal slow oscillation: a novel EEG state and its coordination with ongoing neocortical activity. *J Neurosci, 26*(23), 6213-6229.