ABSTRACT

Following sudden failure of the Na⁺/K⁺ATPase pump, the acute neuronal swelling and dendritic damage that occurs within minutes of stroke onset are consequences of anoxic depolarization (AD). The AD front in our protocol is imaged as an increase in tissue light transmittance (LT) propagating across gray matter of the hippocampal slice preparation. Under current clamp in the single neuron, AD is recorded as a sharp depolarization within 6 min of O₂/glucose deprivation (OGD). Simultaneous imaging and current clamp recordings show that the increased LT front and sudden depolarization are coincident. AD onset in CA1 hippocampal neurons is delayed in slices pretreated with 10 μM dibucaine (dib), a local anaesthetic understood to block voltage-gated sodium channels, and 10 to 100 μM carbetapentane (CP), a sigma1 receptor agonist.

We examined if changes to single cell excitability could explain how dibucaine and CP work to inhibit AD. Pretreatment of slices with dibucaine for 30 min had no effect upon resting membrane potential, or whole cell input resistance (n=11). However dib pretreatment consistently raised spike threshold, decreased AP frequency and increased the fast afterhyperpolarization (fAHP). Orthodromic and antidromic APs were also eliminated within 15 min. Intracellular dibucaine application in addition to similar effects upon intrinsic electrophysiological properties reduced the peak potential of the fast AD while extending the time until the persistent depolarization of AD reached zero.

In contrast, 30 -100 μM CP had no effect upon orthodromic or antidromic responses, probably because unlike dibucaine it did not markedly raise spike threshold. Also unlike dibucaine, the fAHP was eliminated while the slow AHP was accentuated, resulting in a lowering of the AP frequency during steady depolarization. Both drugs appear to inhibit AD onset by
reducing cortical excitability at the level of the single pyramidal neuron, but through strikingly different mechanisms.

Our simultaneous imaging and single cell recording under current clamp allowed for further examination of potential cell recovery after AD in CA1 neurons and astrocytes, as well as confirmation of AD generation in the CA3 region. Indirect evidence for a more robust AD generation and propagation was evident in the transverse slices of dorsal hippocampal CA3 region compared to coronal slices. AD in astrocytes was lower in amplitude and more prolonged, as well as often displaying recovery to near resting potential. This supported our previous imaging experiments showing that astrocytes quickly recover their volume post-AD.
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**ABBREVIATIONS**

- \([X]_i\) intracellular concentration of X
- \([X]_e\) extracellular concentration of X
- A Ampere
- 4-AP 4-aminopyridine
- aCSF: Artificial cerebrospinal fluid
- AD anoxic depolarization
- AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- AHP afterhyperpolarization
- AIW Axon Imaging Workbench
- ATP adenosine triphosphate
- AP action potential
- BK large conductance calcium activation potassium channel
- BTX batrachotoxin
- CA Cornu Ammonus
- \(Ca^{2+}\) calcium ion
- °C degrees Celsius
- CaCl\(_2\) calcium chloride
- Cl\(^-\) chloride ion
- CO\(_2\) carbon dioxide
- CP carbetapentane
- DAP depolarized afterpotential
- DC direct current
- DRG dorsal root ganglion
- EIPA 5-(N-ethyl-N-isopropyl) amiloride
- EGTA Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
- EPSP excitatory post synaptic potential
- GABA gamma-amino-butyric acid
- H\(^+\) hydrogen ion
- HSD hypoxic spreading depression
- HVAC high-voltage activated calcium currents
- Hz Hertz
- \(I_A\) rapidly activating, rapidly inactivating potassium current
- \(I_D\) slowly inactivating potassium current
- IOS intrinsic optical signal
- kDa kilodalton
- K\(^+\) potassium ion
- KCl potassium chloride
- \(K_{ATP}\) ATP-sensitive potassium channels
- LA local anaesthetic
- LT light transmittance
- MgSO\(_4\) magnesium sulphate
- \(\mu\) micro
- m milli
- M moles per litre
- Min minute

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viii
mf  mossy fiber
N₂  nitrogen
Na⁺  sodium ion
NaCl  sodium chloride
NaHCO₃  sodium bicarbonate
NaHPO₄  sodium phosphate
Na⁺/K⁺ATPase  sodium-potassium ATPase pump
NCKX  sodium calcium potassium exchanger
NCX  sodium calcium exchanger
NMDA  N-methyl-D-aspartic acid
O₂  oxygen
OGD  oxygen glucose deprivation
P  pico
P12  post-natal day 12
PYR  pyramidal layer
PID  peri-infarct depolarization
RAD  st. radiatum
QX-314  Lidocaine-N-ethyl-bromide
SD  spreading depression
SK  small conductance calcium activated potassium channels
St.  stratum
tPA  Tissue plasminogen activator
TEA  Tetroethylammonium
TTX  tetrodotoxin
V  Volt
σ  sigma
σR  sigma receptor
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Chapter 1: INTRODUCTION

I. IMPORTANCE OF STUDYING STROKE

According to the Heart and Stroke Foundation of Canada, stroke is the third leading cause of death consisting of 7% of all deaths in Canada and costing over $2.7 billion a year. Presently, the only approved therapy for stroke treatment involves thrombolytic agents such as tissue plasminogen activator (tPA). However their narrow therapeutic window and contraindications with hemorrhagic stroke limits their clinical use to less than 5% of patients (Weinberger et al., 2003). In order to develop new therapies for stroke, it is important to study the mechanisms underlying the initial neuronal damage. It is understood that brain slices can undergo a massive wave of depolarization within a few minutes following simulated stroke in vivo, although evidence of cell death doesn’t occur until hours later (Lipton, 1999). In slices, this anoxic depolarization (AD) leads to permanent loss of membrane potential in a similar manner to in vivo work (Tanaka et al., 1997).

Neuronal death following stroke is categorized as ischemic cell change (ICC) and is one of the three categories of necrotic cell death (Martin et al., 1998). In ICC the cell shows shrinkage of the nucleus and cytoplasm, and contains many swollen vacuoles that used to be mitochondria, golgi or endoplasmic reticulum. This can occur within hours after global ischemia in the most susceptible areas of the brain (Lipton, 1999). However, this necrosis is just the observed ‘decaying’ events after the cell dies, but not necessarily the actual time of cell death. It is important to understand the upstream events prior to necrosis to be able to pinpoint early mechanisms of cell dysfunction that could prove essential in testing new treatment options for stroke patients.
II. STROKE MODELS

A. In Vivo

In vivo models of ischemia can be categorized as either global or focal ischemia. A global ischemic event deprives the entire brain of all oxygen and nutrients (primarily glucose) required to sustain the survival of its cells of which circulatory arrest or drowning are both causes. Focal ischemia is more representative of brain embolism or thrombosis in a cerebral artery leading to either transient or permanent reduction in blood flow to a specific region of the brain. Focal ischemia, unlike global ischemia may form an ‘ischemic penumbra’ surrounding the ischemic core, characterized by metabolically active but functionally silent neuronal activity (Lipton, 1999).

In a wide range of animal models, one can occlude some or all of the 4 major blood vessels leading to the brain resulting in either focal or global ischemia. Recirculation can be induced through reopening of occluded blood vessels. Global ischemia can also be modeled through cardiac and/or circulatory arrest, though difficulties arise in reestablishing blood flow. Focal ischemia generally involves the occlusion of the middle cerebral artery at different locations along its length for anywhere between 5 minutes to 3 hours (Lipton, 1999).

Ten minutes of complete ischemia in vivo in rats leads to a complete destruction of CA1 neurons, although much of the death occurs in a delayed fashion within a one week period (Crumrine et al., 1997). Alternately only 5 minutes of ischemia in the gerbil results in complete CA1 cell loss within the same time period (Crain et al., 1988), indicating varying susceptibilities of the same neurons within different animal models.
As stated earlier, unlike global ischemia which leads to a consistent pattern of cell death throughout the brain, focal models of ischemia produce different effects within the core and penumbra of the lesion caused by differences in regional blood flow. Within the ischemic core, ATP levels drop to ~10-25% of their original values within a few minutes of the reduction in blood flow (Crumrine et al., 1997). Such reduction in ATP has been shown to initiate anoxic depolarization (AD), characterized by a large increase in extracellular K⁺ concentration surrounding both neuronal and glial components (Gido et al., 1997) and decrease in extracellular Na⁺ and Ca²⁺ concentration (Obrenovitch et al., 1988). Concomitant with this increase in extracellular K⁺ level are large reductions in extracellular Na⁺, Ca²⁺ and Cl⁻ concentrations as these ions rush into the cell, purportedly taking water with them by an unknown transmembrane pathway and so causing acute neuronal swelling and damage (Andrew et al., 2007).

During the hours following stroke onset (Hartings et al., 2003) recurrent peri-infarct depolarizations (PIDs) spread from the ischemic core out into the penumbra (Nedergaard & Astrup, 1986; Nedergaard & Hansen, 1993) likely due to ATP levels falling to ~50% (as opposed to the 10-25% ATP reduction within the ischemic core) (Folbergrova et al., 1992). While ATP levels are not low enough to support anoxic depolarization, PIDs can in theory also damage tissue because the recurrence of these waves depletes the energy of penumbral neurons, eventually preventing them from fully regaining their membrane potential. PIDS can further propagate out into healthy brain tissue, where they are recorded as spreading depression (SD) events. SD can be considered a shorter version of PID which does not promote neuronal injury (Nedergaard & Hansen, 1993). In support of this scenario, animal studies reveal that AD generation is the major determinant of ischemic brain damage (Kaminogo et al., 1998). Furthermore, PID propagation increases both growth rate (Hartings et al., 2003) and volume of the ischemic territory in rodent
models (Busch et al., 1996; Takano et al., 1996; Back et al., 1996). Lesion volume in human (Schiemanck et al., 2005b; Schiemanck et al., 2005a) and rodent (Alexis et al., 1996) stroke subjects correlates with functional deficits.

Unlike AD, but similar to SD, PIDs are reported in some studies to be blocked by NMDA receptor antagonists (Mies et al., 1994; Back et al., 1996). However this is contentious given the difficulty in distinguishing PIDs from SD events in vivo. Glutamate receptor activation is not required for the generation or propagation of AD in slice models (Anderson et al., 2005; Jarvis et al., 2001; Muller & Somjen, 2000b; Tanaka et al., 1997) and in vivo (Hernandez-Caceres et al., 1987; Nellgard & Wieloch, 1992; Murphy et al., 2008; Lipton, 1999).

Recently, two important findings have strengthened the PID story. First PIDs have been recorded from patients post head trauma (Fabricius et al., 2006) and post stroke (Dohmen et al., 2008). Second, PIDs have been imaged coursing along the surface of mouse neocortex following stroke (Farkas et al., 2008).

B. Studying Stroke with In Vitro Brain Slices

Slice models of ischemia have been quite valuable in the study of stroke, in that greater control exists over a number of variables not possible in vivo. For example, anaesthetics required of in vivo models can improve the outcome of simulated stroke on their own (Hans & Bonhomme, 2001; Sasaki et al., 2005). Consistent local cerebral blood flow after arterial occlusion is difficult to maintain in vivo (Blomqvist et al., 1984) and can drop by a few degrees during global ischemia (Brambrink et al., 1999). Controlling these parameters is straightforward using brain slices. Also drug delivery in slices simply involves dissolving compounds in the bath solution and is independent of the blood brain barrier. Finally electrophysiological measurements
are easier with slice models without the movement caused by pulmonary and cardiovascular effects. Consistent fluid flow and temperature also facilitates repeatable results with the slice preparation.

Oxygen and glucose deprivation (OGD), whereby oxygen is replaced with nitrogen in combination with removing glucose in the bathing medium, has been shown to induce AD in slices (Tanaka et al., 1997; Joshi & Andrew, 2001; Dong et al., 1988) in a similar matter and time frame to in vivo models of global ischemia (Murphy et al., 2008). Many of the connections between neurons are maintained in a slice model compared to the intact brain, as well as the effects of surrounding glia.

Disadvantages exist when using brain slices to observe effects of ischemia. First, the damage due to OGD is typical of short global ischemia, as protocols for eliciting focal stroke have not been developed. Any effects that occur are assumed to be similar to the central core of in vivo stroke models. The penumbra is a region of high interest that cannot as yet be exploited in slice preparations. Second, the continuous superfusion of aCSF and OGD removes harmful byproducts produced by simulated ischemia such as lactate or reactive oxidative species that can damage cells if allowed to accumulate as in vivo. Third, decapitation and slicing procedure may disrupt energy balance that the tissue may have already generated a first AD or SD event. Fourth, the loss of the blood brain barrier is a drawback for drug testing. Finally, while the 400 micron slice typically preserves synaptic contacts, certain cells or regions within the hippocampus may preferentially lose connections due to the inherent three-dimensional structure not preserved by any slice within a single plane.
C. Studying Stroke Using Isolated Neurons

Acutely dissociated cells or cultured neurons present a number of difficulties in relation to in vivo work, especially the altered metabolic state of cultured neurons from those within the living brain or even in brain slices (Lipton & Lobner, 1990). Acutely dissociated cells contain a soma, with a ‘stump’ of an axon and minimal amounts of proximal dendrites, thereby missing any effects of simulated ischemia due to the extensive dendritic and axonal network observed within both in vivo and slice models. Simulated ischemia with cultured cells cannot just include OGD as with in vivo or slice models, because of the significantly increased time required to observe effects (minutes in slice models to hours in cultured neurons). Due to a larger extracellular space and much lower density of neurons compared to slices, only trace amounts of oxygen are needed for survival. More likely though, the surviving neurons are selected for their ability to grow under harsh conditions. In fact, cultured cells can survive for much longer (1 to 2 hours) in OGD than neurons in vivo or in slices (just a few minutes). Additional measures are often used to better stress the neurons, such as addition of cyanide or dithionite, adding glutamate as an ischemic medium or increasing [K⁺]ₑ. It is noteworthy that cultured neurons have not been shown to undergo a fast AD typical of slices or in vivo, only a slowly developing depolarization. However acutely dissociated cortical neurons, which are more like neurons in situ, can generate an AD-like depolarization (Thompson et al., 2006). Thus AD may be an inherent feature of single pyramidal neurons. Theoretical models of AD have been created to address the issue of whether the single neuron can generate the depolarizing effect of AD, of which they conclude that AD or SD can occur within the single neuron (Somjen et al., 2008) (Kager et al., 2002).
D. The Hippocampal Slice Preparation

The hippocampal slice has been extensively studied in a range of protocols, so much electrophysiological and biochemical data are available addressing the effects of anoxia alone or with reduced glucose levels. Furthermore, its laminar organization makes it is easier to assess damage within cell bodies, dendrites, afferent and efferent connections.

CA1 pyramidal neurons are highly vulnerable to ischemia and anoxia (Kreisman et al., 2000; Schmidt-Kastner & Freund, 1991; Lipton, 1999). It was originally assumed that this selective vulnerability of CA1 neurons was due to a high density of glutamate receptors and high degree of excitatory synaptic input. However AD onset and propagation are not affected by chemical block of synaptic transmission with either zero extracellular calcium or by glutamate receptor antagonists (Jarvis et al., 2001; Obeidat et al., 2000; Obrenovitch et al., 2000).

Even within the hippocampal slice, selective vulnerability exists with respect to ischemia. CA1 as well as neurons within the hilus of the dentate gyrus appear to be the most susceptible to neuronal damage due to OGD, whereas CA3 and the lower blade of the dentate gyrus appear either resistant of less susceptible to damages (Kreisman et al., 2000). The CA3 region resists LT changes both in interface slices caused by anoxia (Kreisman et al., 2000; Muller & Somjen, 1999) or caused by OGD in submerged slices (Jarvis et al., 2001; Obeidat & Andrew, 1998). CA3 pyramidal neurons also appear to be more resistant than CA1 neurons to in vivo ischemia based on histological studies in a number of in vivo animal models and protocols (Kirino et al., 1985; Blomqvist & Wieloch, 1985; Schmidt-Kastner & Freund, 1991; Kirino & Sano, 1984; Jorgensen et al., 1989; Petito & Pulsinelli, 1984; Wu et al., 2005)
III. ANOXIC DEPOLARIZATION

A. General Attributes

Due to the brain’s high metabolic demand of up to 25% of the body’s basal metabolic rate, in addition to a near exclusive dependence on glucose for its energy demands, vulnerability to ischemia is much greater than other tissues of the body. For example, heart and kidney cells can survive ischemia for upwards of 40 minutes (Lee et al., 2000). With minimal glucose stores and no mechanism for O₂ storage (unlike the presence of myoglobin in heart muscle cells) neurons can die within ten minutes of simulated ischemia. Within seconds of global ischemia, brain activity measured with electroencephalography abruptly ceases. Anaerobic metabolism can still occur, but is not sufficient to maintain ATP levels in the brain (Katsura et al., 1992). ATP depletion leads to failure of the Na⁺/K⁺ATPase pump, initiating a cascade of ion fluxes that rapidly propagates throughout all grey matter in the higher brain. Unlike most cells, the brain undergoes this energy draining ‘anoxic depolarization’ within 1-2 minutes of ischemia, reducing even further the stores of essential ATP generated by glucose and oxygen.

B. Ion Fluxes Underlying the AD

Shortly after onset of ischemia, an extracellular slow increase in [K⁺] from 3 to 8-10 mM is evident in many in vivo or in vitro animal models prior to the onset of AD (Tasker, 1999). This is generally attributed to an increase in activity of K<sub>ATP</sub> channels that open upon intracellular ATP depletion (Hansen, 1985) and/or Ca<sup>2+</sup> activated K<sup>+</sup> channels opening as intracellular Ca<sup>2+</sup> levels gradually increase prior to AD (Lipton & Lobner, 1990). Once extracellular [K⁺] reaches 10-12 mM, a rapid ionic shift occurs, whereby extracellular [K⁺] suddenly climbs to 50-60 mM, and extracellular [Na⁺] drops from 130 mM to about 50 mM. Extracellular [Ca<sup>2+</sup>] also decreases
(from ~2 mM to 0.1 mM) as does $[\text{Cl}^-]_e$ (Lipton, 1999). The release of various neurotransmitters such as glutamate (Rossi et al., 2000) and dopamine into the extracellular space is a direct result of the depolarization following the ischemic insult, but how much this contributes to damage is highly debatable. The exact mechanism of ion and water entry into the cell is unclear. Water is proposed to follow $\text{Na}^+$ and $\text{Cl}^-$ into the neuron causing swelling by an as yet unknown mechanism. Ionic movement occurs through known voltage gated $\text{Na}^+$, $\text{Ca}^{2+}$ and $\text{K}^+$ channels, as well as ligand gated channels such as NMDA and AMPA receptors. There may be an as yet unidentified channel(s) that open up only during metabolic stress. Complicating matters is the fact that an abundance of ion exchangers exist within many neurons of the brain such as $\text{Na}^+/\text{Ca}^{2+}$ (NCX) $\text{Na}^+$/H$^+$, $\text{Na}^+\text{Ca}^{2+}$/K$^+$ (NCKX) which are dependent on $\text{Na}^+$ concentrations, and in fact reverse operation with the large inward flux of $\text{Na}^+$ during AD.

**C. Initiation and Propagation of AD**

Presently, different theories exist as to initiating mechanisms of AD such as Grafstein’s potassium hypothesis and Van Harrevelds dual glutamate hypothesis (Somjen, 2001). Grafstein’s hypothesis, original describing spreading depression, dictates that a rise in extracellular potassium depolarizes cells thereby promoting further depolarization and subsequent release of K$^+$ in neighboring cells. This culminates in a ‘wave’ of depolarization across tissues (Grafstein 1956). In the ischemic brain, this increase in extracellular K$^+$ can be attributed to failure of the $\text{Na}^+$/K$^+$ATPase due to low ATP levels. In spreading depression, an increase in extracellular K$^+$ doesn’t always precede the negative membrane shift, leading to Van Harreveld’s dual hypothesis that SD can be caused by an increase in extracellular K$^+$ or glutamate (Van Harreveld, 1978). In AD, unlike SD, an increase in potassium always precedes the negative voltage shift indicative of AD. Studies indicating that glutamate blockers can block SD but not AD led further credence to
the rise in extracellular potassium, not glutamate, as an initiating factor in AD (Anderson et al., 2005).

While both neurons and astrocytes undergo AD, astrocytes have generally been assigned a passive role in AD and SD (Walz, 1997; Somjen, 2001). The rise in external K⁺ concentration caused by neuronal depolarization could result in a depolarization by astrocytes due solely to their inherently high permeability to K⁺ (Somjen, 2001). Intracellular recordings of purported astrocytes indicate a reduced depolarization that appears to plateau around the predicted Nernst potential, taking into account the new increased levels of extracellular K⁺ during AD (Muller & Somjen, 2000a).

D. Mechanisms of AD Block or Delay

AD initiation and propagation can be prevented by utilizing various blockers of known ion channels. On two occasions, authors have claimed complete block of AD (or other names such as ‘terminal depolarization’ or ‘hypoxic spreading depression’) with a ‘cocktail’ of blockers. Rossi et al. (2000) utilized a number of NMDA and AMPA and GABA_A antagonists to prevent AD in young animals (P-12) in hypothermic conditions (31°C) induced by 20 minutes of simulated ischemia in brain slices. With control cells initiating AD at around 11 minutes, the blockers only delay AD by just less than 100%. Other studies have demonstrated AD delays of over 100% by pretreatment with sigma receptors ligands (Anderson et al., 2005) and Na⁺ channel blockers (LoPachin et al., 2001; Muller & Somjen, 2000b).

Muller and Somjen (1998) also claimed to have blocked what they term ‘hypoxic spreading depression’ (HSD) with a combination of Na⁺, Ca²⁺ channel blockers and, AMPA and NMDA receptor blocker pretreatment for 30 minutes in interface slices. These ‘soups’ of blockers
reflect the non-specific increase in membrane conductance indicative of AD. While no fast ‘HSD’
occurred in CA1 pyramidal cells recorded intracellularly, neurons still underwent a slow
depolarization to from resting level to ~-40 mV. Since glucose levels remained high during the
experiment it is difficult to compare to AD where both oxygen and glucose are removed.

AD delay has been demonstrated by blocking Na⁺ entry into cells (Urenjak &
Obrenovitch, 1996; Weber & Taylor, 1994; Taylor et al., 1999) as well as under conditions that
slow ATP depletion like hypoglycemia, creatine supplementation, barbiturate anaesthetics and
hypothermia (Lipton, 1999). Other successful attempts at AD delay employed adenosine (A3)
receptor antagonists (Pugliese et al., 2007) or AMPA receptor and Ca²⁺ channel blockers.
However the mechanism behind individual delay is still obscure.

Modifying extracellular ions generally had no effect on AD latency other than reducing
Ca²⁺ from 2 mM to 0.25 mM, which slightly delayed AD. Even reducing extracellular Na⁺ from
~145 mM to 28 mM did not significantly alter AD onset (Tanaka et al., 1997) indicating that
massive Na⁺ influx is not a requirement for AD. The amplitude of the fast AD as well as peak
potential can be modified by low extracellular Na⁺ and Ca²⁺ concentration (Yamamoto et al.,
1997), indicating the late depolarization involves Na⁺ and Ca²⁺ currents.

IV. THE HIPPOCAMPAL FORMATION

A Hippocampus Proper

The hippocampal formation in cross section appears as two interlocking curved cortical
structures (or ‘C’s) with only 3 layers, as opposed to the 6 seen within the neocortex (Fig 1A).
One ‘C’ comprises the ‘Cornu Ammonus’ (Ammon’s Horn) or CA regions (CA1, CA2 and CA3)
that comprise the hippocampus proper. The other ‘C’ forms the dentate gyrus and hilus and is not officially part of the hippocampus. Hence the term ‘hippocampal formation’ is used to describe all CA regions, dentate gyrus, entorhinal cortex and subiculum (Aitken et al., 1991).

Viewed under a light microscope, the hippocampal formation appears as a series of adjacent cortical stripes. From the outside in within the CA subfields they are: the alveus, st. oriens (OR), st. pyramidale (PYR), st. radiatum (RAD), and st. lacunosum molecular (LM) (Fig. 1A). The alveus is a dark band of white matter comprising of pyramidal cell axons connecting primarily to the fimbria or fornix and comprises the main output of the hippocampus. The pyramidal layer contains the principal cell bodies of the hippocampus. CA1 Pyramidal cells posses two dendritic regions; apical dendrites which are a main component of the deeper st. radiatum and st. lacunosum molecular layers, and basal dentrites which comprise the more superficial st. oriens. CA3 pyramidal neurons have larger cell bodies and possess thicker and shorter dentritic trees than CA1. Schaffer collaterals travel within both regions to synapse with both apical and basal dendrites. A small lighter band forms the deepest layer of the CA subfields consisting of a less dense area of apical dentrites called the st. lacunosum molecular. Estimated cell numbers reach over 300 000 CA1, and over 400 000 CA3 neurons in the rat brain (Amaral et al., 1990).

The CA2 boundary is easy to distinguish from CA1 by the obvious transition to the larger cell bodies of CA2 (20 µm vs. 40 µm measured at the base) (Mercer et. al., 2007). The CA2/CA3 boundary is indistinct viewed with a light microscope, due to similar cell body at their base of 40 to 60 µm but are easy to distinguish by electrophysiological properties.
B. Dentate Gyrus of the Hippocampal Formation

The dentate gyrus is made up of over a million granule cells and ~30,000 interneurons contained within 3 distinct layers (Amaral & Witter, 1989). The deepest layer is the polymorphic layer comprising the distinctive axons of granule cells called mossy fibers, along with various interneurons. Relatively small granule cell bodies (diameter of ~7 µm) are the main components of the st. granulosum cell layer and a large number of GABAergic basket cells (Gaarskjaer, 1986; Gaarskjaer, 1985). The most superficial layer contains the granule cell dendritic tree forming the st. molecular. It receives axons from the entorhinal cortex making up the perforant path. Unlike the CA regions there is but one dendritic region, as granule cells do not possess both apical and basal dendrites.

C. Basic Circuitry of the Hippocampal Formation

Information flow through the hippocampus is mainly uni-directional with the basic circuit involving three synapses in a circuit or loop (Andersen et al., 1966). Initially, axons within layer II of the entorhinal cortex (perforant fibers) synapse with granule cells of the dentate gyrus and CA1 pyramidal neurons to form part of the perforant path (Witter et al., 1989) (Fig. 1A). Second, mossy fibers from the dentate gyrus synapse with pyramidal cells within the st. lucidum of the CA3 subfield. Finally CA3 pyramidal neurons send Schaeffer collateral axons to CA1 pyramidal neurons (Amaral & Witter, 1989). Information travelling along the trisynaptic pathway is therefore excitatory since both glutamatergic pyramidal cells and granule cells are involved (Andersen et al., 2000). CA1 neurons don’t project back to CA3, nor do CA3 neurons project back to granule cells (Fig 1A)
The original trisynaptic loop has been expanded to include CA1 axons branching out to the subiculum then back to the entorhinal cortex (Witter et al., 1989), as well as pyramidal neurons from layer III of the entorhinal cortex synapsing directly with CA1 and CA3 neurons. CA3 neurons also send fibers to the subiculum and entorhinal cortex, as well as recurrent collateral fibers back to other CA3 neurons.

V. PRINCIPLE CURRENTS IN PYRAMIDAL NEURONS

Currents related to AP generation are well characterized within CA1 pyramidal neurons. Observing changes within these currents caused by drug pretreatment with known inhibitors of AD can provide insight as to how CNS excitability is reduced. The components of the AP waveform involve an initial depolarization that opens voltage-gated Na\(^+\) channels contributing to the rising phase. A delayed opening of various K\(^+\) and Ca\(^{2+}\) channels also occurs with this initial depolarization and contributes to the falling phase. After AP generation there is a pronounced afterhyperpolarization (AHP) or depolarization (DAP) until baseline is reached. Reducing AHPs increases excitability, whereas increasing AHPs tends to reduce excitability.

Direct injection of depolarizing current into the cell body can also open voltage-gated sodium channels directly to generate the rising phase of the action potential (AP). The kinetics of the Na\(^+\) channel involves rapid activation within hundreds of microseconds, and then rapidly inactivating within a millisecond (Bean, 2007). Activation and inactivation kinetics of the voltage-gated sodium channel can be altered by many drugs including local anaesthetics such as dibucaine (Ragsdale et al., 1994). Voltage gated Ca\(^{2+}\) channels also open with depolarizing current; however they make little contribution to the rising phase of the AP due to slower
activation kinetics and lower conductances (Bean, 2007). The powerful coupling of internal calcium concentrations to potassium channels such as BK and SK channels are reflected in a net outward current. In fact blocking Ca\(^{2+}\) entry actually broadens APs (Bean, 2007) as well as eliminating afterhyperpolarizations (Storm, 1987) due to Ca\(^{2+}\) coupling to K\(^{+}\) currents.

The falling phase of AP generation, otherwise known as spike repolarization, is mostly accomplished by potassium currents mediated by Kv\(_4\) channels (I\(_A\) current) and Kv\(_1\) channels (I\(_D\) current) in hippocampal pyramidal cells (Hille et al 2001). I\(_A\) current refers to rapidly inactivating channels that can be blocked by 4-AP, whereas I\(_D\) inactivates slowly and is blocked by dendrotoxin. I\(_A\) can contribute to spike duration but I\(_D\) has very little effect (Mitterdorfer & Bean, 2002).

Large conductance Ca\(^{2+}\) activated K\(^{+}\) channels (BK) contribute to the fast AHP (fAHP) in pyramidal cells (Storm, 1990; Gu \textit{et al.}, 2007; Storm, 1987), whereas small conductance Ca\(^{2+}\) activated K\(^{+}\) channels (SK) underlie the sAHP (Sah, 1996). The recent observation of an apamin resistant current (i.e. not involving SK channels) also shows involvement in the generation of sAHP in hippocampal neurons (Stocker \textit{et al.}, 2004). A lower Ca\(^{2+}\) concentration of \(~300\) nM is required for activation of SK channels as opposed to \(~1-10\) µM for BK channels (Hille \textit{et al} 2001). L-Type Ca\(^{2+}\) channels are implicated as a route of Ca\(^{2+}\) entry for the sAHP due to blockage by dihydropyridine antagonists, while the route of Ca\(^{2+}\) entry is unknown for BK channels (Rascol \textit{et al.}, 1991). Presumably the slow AHP lasts as long as it takes to remove the extra [Ca\(^{2+}\)]\(_i\) (Hille 2001). This increased [Ca\(^{2+}\)]\(_i\) results in an enhanced sAHP through reduced Ca\(^{2+}\) extrusion along its electrochemical gradient (Hille 2001).

The slow AHP has a tendency to increase or summate with a succession of APs (Madison & Nicoll, 1984) whereby each AP adds more Ca\(^{2+}\) to increase the Ca\(^{2+}\) activated K\(^{+}\)
conductance to further slow down the discharge rate. Ca$^{2+}$ chelators have been shown to reduce the AHP and this corresponding accommodation (Madison & Nicoll, 1984).

In addition to slow and fast AHPs, hippocampal neurons also possess a depolarizing afterpotential (DAP) which also promotes spike firing and neuronal burst generation (Jensen et al., 1996). DAP currents are not fully understood, however a TTX-sensitive Na$^+$ current has been implicated in CA1 hippocampal neurons (Azouz et al. 1996). Increases in the DAP are observed with low $[\text{Ca}^{2+}]_e$ and high $[\text{K}^+]_e$ as well as the K$^+$ channel blockers TEA and 4-AP (Liu & Stan, 2004).

VI. INDIVIDUAL DRUG PROPERTIES

A. Local Anaesthetics Including Dibucaine

Dibucaine is one of the most potent local anaesthetics (LA) in terms of its activity on the batrachotoxin (BTX) binding site within the pore of the voltage gated sodium channel (Creveling et al., 1983). IC50 values range from 1.4 µM for dibucaine 5.4 µM for bupivacaine to 240 µM for lidocaine and 940 µM for benzocaine (Creveling et al., 1983). No observable binding occurs at the TTX binding site, nor within the scorpion toxin binding site (Postma & Catterall, 1984). LA affects on sodium channels are not fully understood, although a few well established properties have been demonstrated. Initially, the uncharged dibucaine molecule diffuses across the nerve membrane and equibrilates with its charged form inside the cell. The charged form then binds to the Na$^+$ channel near the BTX binding site on the intracellular side. A use-dependent block of sodium channels occurs with dibucaine, based on the more potent anaesthetic effects demonstrated in actively firing neurons. During firing, dibucaine can also enter the neuron
through the open sodium channels to bind cumulatively to the Na\(^+\) channel with each depolarizing pulse (Wakita et al., 1992). QX-314, a permanently charged lidocaine derivative can only act through an open channel block due to its impermeability to the neuronal membrane (Courtney, 1975). A further consequence of the use-dependent block leads is a tighter binding in the inactivated state of the Na\(^+\) channel than in either the resting or activated state (Kuroda et al., 2000).

Other actions attributed to LAs independent of Na\(^+\) channels, involve binding to voltage-dependent and voltage-independent K\(^+\) channels as recorded in dorsal root ganglions (DRG) at a much lower affinity (Brau et al., 1998; Brau et al., 1995; Olschewski et al., 1998). K\(_{\text{ATP}}\) channels in heart muscle cells (Olschewski et al., 1996; Olschewski et al., 1999) and oocytes (Yoneda 1993) as well as high voltage-activated Ca\(^{2+}\) currents in both central neurons (Xiong & Strichartz, 1998; Kansha et al., 1999) and DRGs (Sugiyama & Muteki, 1994; Hirota et al., 1997) also indicate modification by LAs. Notably, concentrations required for blockade in channels other than the Na\(^+\) channel are 5 to 15 times larger (Scholz, 2002).

**B. Carbetapentane**

The actions of the sigma receptors are poorly understood and less studied than that of LAs regarding an apparent neuroprotective role. The sigma receptor is a 26 k-Da protein with two known subtypes (\(\sigma\)1 and \(\sigma\)2) in a suggested single membrane spanning domain with no apparent G-protein coupled effects (Gundlach et al., 1986). The \(\sigma\)1 receptors are widely located within the rat CNS with high concentrations observed within neuronal cell bodies and dendrites of brainstem, hypothalamus and hippocampus, but not axon fibers or terminals (Hanner et al., 1996). Intracellular location includes both the plasma membrane and intracellular compartments such as within mitochondrial membranes and endoplasmic reticulum (Alonso et al., 2000). Sigma 1 has
been differentiated from σ2 by its high affinity for (+)-pentazocine and (+)-SKF-10,047 (Vilner and Bowen 2000), whereas σ2 shows a high affinity for ibogaine (Hanner et al., 1996). It should be noted that until a definitive answer regarding sigma receptors function, assigning a label of agonist or antagonist may be premature.

The function of sigma receptors have been difficult to elucidate due to a wide range of ligands showing affinity, although an ability to inhibit high voltage-activated Ca\(^{2+}\) currents (HVAC) has been implicated (Church & Fletcher, 1995). While affinity for HVACs seems to involve σ2 more than σ1 receptors, carbetapentane and other σ1 receptor agonists appear to better attenuate increases in [Ca\(^{2+}\)], due to simulated ischemia (Katnik et al., 2006). Other connections with sigma1 receptor activation include the ability to block delayed outwardly rectifying K\(^{+}\) currents (Wilke et al., 1999), BK channels as well as the M-current within cardiac neurons (Zhang & Cuevas, 2005).
Chapter 2: OBJECTIVES

I. To investigate the underlying electrophysiological properties of the local anesthetic dibucaine and the sigma receptor ligand carbetapentane in an effort to understand similarities or differences between two known drugs with the ability to delay anoxic depolarization.

II. To investigate possible synergistic or additive effects of dibucaine and carbetapentane.

III. To investigate whether dibucaine, when applied intracellularly, can alter the AD waveform in the single CA1 neuron as the AD front passes through the gray matter.

IV. To compare AD susceptibility and recovery ability by different cell types in the hippocampus.
Chapter 3: METHODS

I. HIPPOCAMPAL SLICE PREPARATION

Male Sprague-Dawley rats, 21-28 days old (Charles River, St. Constant, PQ) were cared for in accordance with the Canadian Council on Animal Care. They were housed in a controlled environment (22 ± 1°C, 12 h light: 12 h dark) with Purina rat chow and water supplied ad libitum. A rat was placed in a rodent restrainer and guillotined. The brain was excised within one minute and placed in ice-cold oxygenated (95% O₂ / 5% CO₂) high sucrose artificial cerebrospinal fluid (aCSF). Most slices were cut in the coronal plane (400 µm thick) using a vibrating blade microtome (Leica VT1000S) and taken from the middle third of the brain to include the dorsal hippocampus. Transverse slices from the lower third of the brain including the ventral hippocampus were utilized for orthodromic recordings as well as to compare CA3 AD propagation in different slice planes. Slices were transferred to a net submerged in a beaker of regular aCSF gassed with O₂/CO₂ at 22°C and slowly warmed to 30 ± 1°C over at least one hour prior to experimentation at 31°C for electrophysiological recordings. AD experiments were always performed at 35°C.

II. EXPERIMENTAL SOLUTIONS

The normal aCSF contained (in mM): NaCl 120, KCl 3.3, NaHCO₃ 26, MgSO₄ 1.3, NaH₂PO₄ 1.2, D-glucose 11, and CaCl₂ 1.8 (pH 7.3-7.4) and was superfused at a flow rate of 1-2 ml/min. High sucrose aCSF included 240 mM sucrose instead of NaCl. Prior to electrophysiological recording, individual slices were transferred to a submerged type chamber (RC-26, Warner Instruments) and held down with a slice anchor (SHD-26H, Warner Instruments)
without causing damage. A constant flow of 1-2 ml/min was maintained throughout the experiments. All valves were turned 20 s prior to recording times to reflect the ~20 s it took for new solutions to reach the slice chamber from the gravity perfusion system. Ischemia was simulated by either oxygen/glucose deprivation (OGD) or by addition of the Na⁺/K⁺ ATPase inhibitor ouabain (100 µM). For OGD, aCSF glucose was replaced with equimolar NaCl and the 95 % O₂/5 % CO₂ mixture gassing the aCSF was replaced with 95 % N₂/5 % CO₂.

Drugs were dissolved in double distilled water and applied by bath. They were the potent, long-acting local anaesthetic dibucaine hydrochloride and high affinity σ₁ agonist carbetapentane (citrate salt), both from Sigma-Aldrich.

The micropipette solution for intracellular electrophysiological recordings contained in (mM): K⁺Gluconate (130), KCl (10), MgCl₂ (1.1), Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (5), HEPES (10) Na⁺ATP (2) CaCL (0.1) pH to 7.3 with KOH. Occasionally dibucaine or lidocaine-N-ethyl-bromide (QX-314) was added to micropipette solution replacing equimolar K⁺gluconate.

III. SINGLE CELL ELECTROPHYSIOLOGY

Hippocampal neurons were ‘blind’ patch clamped with micropipettes pulled from borosilicate glass capillaries (1.2-mm ID, TW120F-4; World precision instruments ) to a resistance of 3-6 MΩ using a Flaming/Brown micropipette puller (P-87; Sutter Instrument Co., Novato CA, USA). All recordings were performed in whole cell current clamp using ‘Bridge’ mode of an Axoclamp 2A amplifier, sampled using a Digidata 1322A A/D converter (Axon Instruments) and analyzed with Clampex software (version 10.1). Low pass filtering occurred with an external Bessel filter (LPF 202a; Axon Instruments) at 10 Hz. Pipette junction potentials
were corrected just prior to achieving whole cell mode. Series resistance was compensated by
70%. Bath application of a drug was initiated only after a stable membrane potential was obtained
and 5 minutes had passed to allow for dialyzing the pipette solution into the cell. Electrophysiological
recordings were performed at 31°C for bath application of 30 minutes and compared with initial
recordings from the same cell. Initial drug free values of several electrophysiological parameters
were pooled and compared with the post-30 minute drug application for each drug and concentration.
After 30 minutes of drug application, temperature was slowly increased to 35°C over 10 minutes for
a total of 40 minute pretreatment before inducing simulated ischemia.

Our current pulse protocol was 9 sequential 1 s current steps in increments of 100 pA
ranging between with -500 and 300 pA and occurring every 6.5 seconds. Trains of action
potentials were elicited at depolarizing current levels and estimates of input resistance were
obtained by calculating the slope of the V-I curve at negative current levels using Campfit
software (version 10.1).

Orthodromic responses by CA1 pyramidal neurons were evoked by stimulating Schaffer
collaterals of CA3 axons within the st. radiatum using a concentric bipolar electrode (Rhodes
Electronics). An antidromic spike was elicited by stimulated within the alveus in close proximity
to the recorded CA1 neuron. A current pulse (0.5 ms in duration; 0.02 Hz) was evoked by a Grass
88 stimulator at a just-maximal level to produce a single spike. Stimulation was at 0.1 Hz to avoid
summating effects of slower currents activated by the AP and monitored until spike loss or until
the 30 minute drug treatment elapsed. After orthodromic spike loss, EPSP amplitude was
monitored at the same current strength and frequency.
IV. IMAGING LIGHT TRANSMITTANCE

Broad band changes in light transmittance in the near infrared range were monitored in real time during ischemia simulating medium. Cell swelling is imaged as an increase in LT during AD initiation and propagation in cortical brain slices (Obeidat & Andrew, 1998). Dendritic damage is imaged as decreased LT in the wake of the AD front that follows the LT increase. This LT reduction is the result of light scattering caused by the formation of dendritic beads (Polischuk et al., 1998; Andrew et al., 2002; Jarvis et al., 1999) which form within minutes of AD onset in brain slices (Obeidat et al., 2000) and intact brain (Zhang & Murphy, 2007). Similar beading is observed in vivo following focal ischemia (Hori & Carpenter, 1994). The slice was illuminated using a broadband, voltage regulated halogen light source (Fig. 1B) on an upright light microscope. The light traversed a band pass filter that transmitted red and near-infrared light (690 - 1000 nm). Video frames were acquired using a digital camera (Hamamatsu C4742-95) that was set to an exposure time of 0.050 s. Images were acquired and averaged at the equivalent of 32 frames per second and averaged utilizing Axon Imaging Workbench (AIW 6) software (Indec Biosystems). The first averaged image in a series served as a control (T₀), which was subtracted from each subsequent experimental image of that series (T) utilizing the ΔF/F₀ mode of the imaging software. The resulting series of subtracted images revealed changes in LT over time. The change (ΔT) was expressed as the digital intensity of the subtracted images using the formula ΔF/F₀ ≡ (F – F₀)/F₀. The change in light transmission was displayed using a pseudocolour intensity scale (Fig 1B). Zones of interest were selected to quantify and graphically display the experimental data off-line.

Simultaneous imaging and electrophysiology synchronized between the Clampex and Imaging Workbench software programs. Traces initiating in Clampex triggered AIW 6.0 image
acquisition. Graphing and statistical analyses of data were carried out using SigmaPlot for Windows (Jandel Scientific) or Microsoft Excel. Images were imported and figures were prepared using CorelDraw and Adobe Illustrator. The data from the imaging experiments were analyzed such that changes in LT of a given zone of interest were expressed as percent change in LT for that region, taken from the control image.

V. STATISTICAL ANALYSIS

Cells were included for further study if they had stable resting membrane potentials of at least -55 mV and series resistance and capacitive currents could be sufficiently compensated for. Recordings were terminated if access resistance increased over the course of the experiment that couldn’t be compensated for using the bridge balance. Statistical significance was determined by unpaired t-tests and all data is given as means +/- SD. Control data was pooled from initial recordings prior to drug application and compared with 30 minute pretreatment of each concentration and drug type.
Chapter 4: RESULTS

I. SIMULTANEOUS CA1 CURRENT CLAMP AND ΔLT IMAGING DURING OGD

Within 6 min of OGD, a wave of elevated LT coursed through the gray matter of the CA1 region and the dentate gyrus in the coronal brain slice that includes the dorsal portion of the hippocampus. Removing oxygen and glucose from the bathing medium evoked a front of increased light transmittance (LT) propagating at a rate of 2-4 mm/min across all grey matter of the dorsal hippocampus.

Initially, CA1 pyramidal neurons were recorded under magnification that only allowed visualization of the CA1 region, and a small part of the dentate gyrus. The front would rarely initiate focally, entering the field of view from the CA3 side regardless of the orientation of the slice in the flowing medium (Fig. 2A). On rare occasions, the AD could initiate at the site of the recording pipette placement. AD onset time in the CA1 region was measured as the point when the increased LT front passed by the recording electrode, which was 5:20 ± 1:21 min in 41 slices tested.

Under current clamp, more detailed observations could be made at the cellular level with regard to AD then could be detected with population-based extracellular recording or the imaging of LT change. The AD waveform was subdivided into a low prolonged early depolarization, a rapid depolarization (the fast AD) coincident with the front of LT and a later slow depolarization that ultimately approached 0 mV (Fig. 3).

We found that following OGD, CA1 pyramidal cells consistently underwent the fast AD that was temporally correlated with the passing elevated LT front of 28.8 ± 6.2 mV (n=13, Fig. 2B). The slope of the fast AD was relatively steep at 2.7 ± 1.5 mV/s. The fast AD transitions to a slower late depolarization (also called the persistent depolarization) at the peak potential of the
fast AD of -9.4 ± 4.6 mV. This late depolarization had a much shallower slope of 0.28 ± 0.16 mV/s than the fast AD and lasted 46.4 ± 31.3 seconds before fully depolarizing to near-zero membrane potential (Table 1). Inclusion of ATP within the recording pipette as a potential energy source could theoretically reduce the AD response of the recorded neuron, but AD was not enhanced in 6 neurons recorded without ATP in the pipette (data not shown).

II. INHIBITING AD OF CA1 NEURONS BY DRUG PRETREATMENT

A. Pretreatment with 1 µM or 10 µM Dibucaine (Dib)

An important initial component of AD is a massive influx of Na⁺ into neurons, so various attempts to inhibit AD have involved the use of sodium channel blockers (Lipton, 1999). Dibucaine has proved to be the most effective drug tested in our laboratory. AD onset was normally at 5:20 ± 1:21 min in untreated slices. Hippocampal slices exposed to 40 minutes of 10 µM dibucaine significantly delayed AD onset to 9:18 ± 1:51 min (p<0.001) in 9 slices tested (Fig. 4A, C). However, a lower pretreatment concentration of 1 µM dibucaine did not delay AD onset in CA1 pyramidal neurons (n=9) induced by 10 minutes of OGD. The average latency to AD onset was 5:16 ± 2:02 min (Fig. 4C).

B. Pretreatment with 10, 30 or 100 µM Carbetapentane (CP)

Another drug tested in our lab that delays the onset of AD is the σ1 agonist carbetapentane (CP). We reaffirmed that pretreatment of slices for 40 minutes with 10 µM or 30 µM CP significantly delayed AD onset time to 7:12 ± 1:46 (n=13, p<0.05) and 10:24 ± 1:34 (n=6, p<0.001) respectively, from a control value of 5:20 ± 1:21 min (Fig 4B, C). Increasing CP pretreatment to 100 µM for 40 min compromised slice health, so 20 minutes was used instead.
AD was significantly delayed, but less so than the lower concentration of 30 µM to 8:32 ± 0.47 in four slices (Fig. 4C), probably because of the reduced pretreatment time or toxic effects of the drug.

C. Combined Effects of Dibucaine and Carbetapentane on AD Onset

Based on work carried out previously in our laboratory, dibucaine and CP likely delay the onset of AD through different actions. Therefore we looked for any additive or synergistic effects of the two drugs. A low concentration of dibucaine (1 µM) that failed to delay OGD-induced AD was combined with a concentration of CP (10 µM) that significantly delayed AD onset. While this combined pretreatment delayed AD, it was not significantly longer than 10 µM CP alone (n=13, Fig. 4C). Increasing the pretreatment concentrations to 3 µM dib and 30 µM CP also increased the latency to AD onset 10:40 ± 0.48 (n=13) over control, but still failed to elicit a significant additive or synergistic effect compared to 30 µM CP alone (Fig. 4C).

III. INTRACELLULAR DRUG TREATMENT

A. Intracellular Exposure of Single CA1 Neurons to Sodium Channel Blockers

Bath-applied dibucaine is able to enter the intracellular compartment of neurons by slowly permeating the plasma membrane as well as through briefly opened Na⁺ channels. It binds with the channel on the intracellular side to block the pore. Alternately, we introduced 10 or 100 µM dibucaine through the recording pipette. This tested if the AD in the single neuron could be altered as the LT front passed through neighboring neurons and glia that were not exposed to the drug. We found that the intracellular dibucaine was not able to slow or stop the fast AD, either measured electrophysiologically or as an elevated LT wave front. However either concentration
lengthened the time available to the single neuron for recovery, presumably by slowing the progress of the late AD. Ten µM dib increased the late AD duration by over 50% to 73.2 ± 37.2 seconds (p<0.05) in 11 slices (Fig. 5B; Table 1) and 100 µM dib increased it to by over 250% to 124.5 ± 50.9 s (p<0.001) as tested in 14 slices (Fig. 5C). The higher concentration also reduced the fast AD amplitude and the peak potential of the AD as well as both the slope of the fast AD and late AD (Table 1).

Dibucaine’s primary pharmacological effects have been attributed to its binding within the voltage-gated sodium channel, but it is also reported to have poorly defined effects on the membrane. Therefore we compared it with QX-314, which is a permanently charged lidocaine derivative that cannot penetrate the plasma membrane. Inclusion within the pipette should reveal changes in AD quality caused by its block of voltage-gated Na⁺ channel conductance, rather than less specific effects upon the cell membrane. Surprisingly, no significant differences were found in the AD. Specifically, intracellular QX-314 did not reduce the duration of late AD or amplitude of the fast AD (Fig. 5D; Table 1). This provided evidence that dibucaine’s inhibiting affect upon AD progression in not necessarily through the classic blockade of voltage-gated Na⁺ channels.

B. Intracellular Exposure of Single Granule Cells to 10 µM Dibucaine

To examine if dibucaine’s effects upon AD were specific to CA1 pyramidal cells or were caused by a more general neuronal response, identical experiments were recorded during AD in granule cells with 10 µM dibucaine in the pipette. This concentration reduced fast AD amplitude from 23.3 ± 8.2 mV in 10 untreated granule cells to 15.8 ± 5.3 mV (n=13, p<0.01, Table 2). Moreover it reduced the peak potential of the fast AD from -11.4 ± 6.0 mV in untreated granule cells to -23.3 ± 6.3 mV (p<0.01, Fig. 6B). More importantly, the latency to reach zero potential following AD was increased by almost 200% to 132.7 ± 63.7 s compared to 44.2 ± 27.1 s
(p<0.001) in untreated granule cells (Table 2). Thus 10 µM dibucaine treatment slowed AD progression and decreased the amount of depolarization within individual granule cells in a similar manner to CA1 neurons.

IV. OUABAIN-INDUCED AD

A. AD Induction by 100 µM bath exposure to Ouabain

Adding 100 µM of ouabain to aCSF initiated a similar depolarization to OGD-induced AD by directly inhibiting the Na⁺/K⁺ ATPase pump on the extracellular side of the plasma membrane. Exposure resulted in a slightly delayed latency to onset compared to untreated slices exposed to OGD, but this difference was not statistically significant (6:21 ± 0:33 min with ouabain compared to 5:20 ± 1:21 min with OGD). Application of 100 µM ouabain also induced a slightly steeper fast AD slope of 3.9 ± 1.3 mV/s compared to CA1 (2.7 ± 1.5 mV/s) as well as an increased peak potential of -4.0 ± 1.8 mV compared to CA1 of -9.4 ± 4.6 mV (p<0.05, Table 3; Fig 7A). Therefore, while ouabain-induced AD was qualitatively similar to that induced by OGD, we found that it progressed slightly faster and was marginally more depolarizing.

B. Bath-Applied Dibucaine Inhibits Ouabain-Induced AD

In the current study, 10 µM dibucaine pretreatment for 40 min actually prevented AD in all 6 slices exposed to 10 minutes of 100 µM ouabain. This was more dramatic than the simple delay in onset induced by OGD. However with ouabain, the membrane potential did slowly depolarize from a resting level of around -65 mV to a mean level of -26 ± 5.5 mV by the end of the 10 minutes of ouabain exposure (Table 3; Fig 7B). This indicated that while the fastest part of
the AD was inhibited, dibucaine could still not prevent a slowly evolving depolarization resulting from failure of the Na⁺/K⁺ATPase pump.

C. Intracellularly Applied Drugs and Ouabain-Induced AD

Ten µM dibucaine bath-applied extracellularly blocked the fast AD evoked by 10 min of 100 µM ouabain exposure, but only delayed OGD-induced AD. One might expect intracellular application of dibucaine to also have a more profound effect upon AD induced by ouabain. However, inclusion of 100 µM dibucaine within the pipette failed to inhibit AD or improve recovery. Specifically there was neither reduction in the duration of the slow depolarization following the fast AD, nor a reduction in AD amplitude or peak potential (Table 3). Apparently dibucaine cannot reduce the more pronounced AD induced by ouabain from within a single cell, unlike AD evoked by OGD. Similar to OGD-induced AD, QX-314 again did not alter the AD waveform (n=4). This reinforces our finding that simply attempting to block AD in the single neuron does not affect AD progression through the population.

V. DRUG EFFECTS UPON INTRINSIC PROPERTIES OF CA1 NEURONS

A. Bath-Applied Dibucaine Effects

To help characterize how dibucaine may delay AD in its neuroprotective role, single CA1 neurons were whole-cell patched under current-clamp mode to measure changes in their intrinsic and synaptic electrophysiological properties. Current-evoked AP trains lasting one second revealed changes in AP threshold and waveform depending on the drug and its concentration (Fig. 8).
Neither resting membrane potential nor whole cell input resistance were altered after 30 minutes of bath pretreatment with either 1 or 10 µM dibucaine (Table 4). During depolarizing current injection, 1 µM dibucaine did not alter the fast afterhyperpolarization (fAHP), AP threshold, AP frequency or AP duration. A surprisingly small reduction in AP amplitude was noted (p<0.05) in 9 CA1 cells examined. Increasing the concentration of dibucaine to 10 µM enhanced the fAHP amplitude (measured relative to the AP threshold) from -3.4 ± 1.9 mV (measured in 45 CA1 neurons in aCSF) to -10.8 ± 3.7 mV (measured in 20 of those neurons exposed to 10 µM dibucaine) (p<0.001, Fig. 9A). The sAHP of the last AP in the train increased from -5.8 ± 1.8 mV to -15.8 ± 5.0 mV (p<0.01, Fig. 9B). The 10 µM dibucaine pretreatment also increased the AP threshold, shifting it from -47.5 ± 4.4 mV measured in 45 control CA1 neurons to -37.7 ± 4.5 mV measured in 20 treated CA1 neurons (p<0.001, Fig. 10). Corresponding reductions in AP frequency at all 3 current injection levels was likely caused by this increase in AP threshold (Fig. 11) as well as the aforementioned increase in the fast and slow AHP.

Interestingly, AP duration measured at half-AP amplitude was unchanged in the first AP in the train. However by the last AP at the highest current injection level, an increase from 1.2 ± 0.2 ms to 2.0 ± 1.7 ms (n=9, p<0.01) was observed (Table 5). This is perhaps facilitated by Na+ channel use-dependent block by dibucaine.

Single APs were synaptically evoked in CA1 neurons by orthodromic stimulation of the CA3 Schaffer collaterals. A neuron could also be directly backfired from the alveus (antidromic stimulation). We did not test possible effects of 1 µM dibucaine. However after exposure to 10 µM dibucaine, orthodromic APs were lost within an average of 7.4 ± 2.4 min (n=8) as measured at 0.5 V above the of the initial voltage strength required to evoke the response (p<0.01, Fig. 12A). The AP threshold continued to increase over the following 5-10 minutes until spike failure.
Epsps continually decreased in amplitude over the 30 minute treatment from a maximum of 14.8 ± 5.0 mV at initial AP loss to 6.3 ± 5.0 mV (p<0.05, n=8). Antidromic spikes were more resistant to blockade, taking an average of 15.0 ± 14.6 min to fail at the initial stimulus strength (p<0.01, Fig. 12B). It still remained possible to evoke an antidromic AP with a slight (2-3 V) increase in voltage strength. After 30 minutes of pretreatment with 10 µM dibucaine, antidromic APs displayed slightly enhanced depolarizing after-potentials (DAP) at 3 and 5 ms of 13.4 ± 5.3 mV and 12.2 ± 3.6 mV respectively (Fig. 13). This was not statistically significant compared to drug-free aCSF (11.3 ± 2.5 mV and 10.9 ± 2.5 mV at 3 and 5 ms) as recorded in 24 CA1 neurons.

B. Bath-Applied Carbetapentane Effects

CP also reduced excitability in CA1 pyramidal neurons, although in markedly different ways than dibucaine. AP frequency was significantly reduced in 10 and 30 µM CP (p<0.05) and in 100 µM (p<0.001). Unlike dibucaine, this slower AP discharge was not caused by an increase in AP threshold because even at 100 µM, the initial threshold level was unchanged from that in regular aCSF (Fig 10). AP duration as measured at half amplitude increased in both 30 and 100 µM CP to 1.4 ± 0.9 ms and 2.0 ± 2.6 ms respectively (n=5) from a control value of 1.0 ± 0.1 ms (p<0.01). The threshold of the last AP in the train did increase after 30 minutes of 30 µM CP from -41.2 ± 4.0 mV to -34.3 ± 4.3 mV. In 10 µM CP it increased to -31.1 ± 4.0 (Fig 10). The fAHP was notably eliminated in the first AP of the train in all three concentrations, apparently facilitating spike train discharge. Conversely by the last AP in the train, the slow AHP amplitude was significantly increased to -11.2 ± 2.9 mV (p<0.01) in 10 µM CP and -10.6 ± 2.3 mV in 30 µM CP (n=11, p<0.01, Fig. 9B) from a control value of -5.8 ± 1.8 mV (n=25). The last AP in the train was not measured with 100 µM CP due to the insufficient generation AP discharge. Often only a single AP or AP doublet was observed.
When single APs were evoked by either antidromic or orthodromic stimulation, DAP amplitude measured at 3 and 5 ms was enhanced in 30 µM CP to 18.7 ± 5.3 mV and 15.5 ± 2.8 mV (p<0.01) respectively, from a control value of 11.3 ± 2.5 mV and 10.9 ± 2.5 mV (Fig. 13). CP at 100 µM enhanced the DAP to 31.9 ± 7.7 and 21.8 ± 3.9 mV as measured in 6 CA1 cells (p<0.01, Fig. 13). At the highest CP concentration, the orthodromic AP failed at ~30 minutes. Unlike dibucaine, it could still be generated at a higher voltage as tested in 3 CA1 cells. Also, unlike dibucaine, antidromic stimulation could generate an AP for the entire 30 minutes of CP exposure in 5 of 6 cells (30 µM) and 1 of 3 cells (100 µM CP). This provided strong evidence that the CP was altering spike-related currents in the soma-dendritic region but not in the axon.

**C. Combined Effects of Dibucaine and Carbetapentane**

Our observations that dibucaine and CP each reduced the excitability of CA1 neurons through different mechanisms prompted us to search for additive or synergistic effects of both drugs upon the excitability of single neurons. After 30 minutes of exposure to 1 µM dib combined with 10 µM of CP, the intrinsic electrophysiological properties were essentially similar to that of 10 µM CP alone, although the 1 µM dibucaine did tend to preserve the fAHP. Specifically in 10 µM CP alone, 11 of 12 cells demonstrated a complete loss of fAHP. However with 1 µM dibucaine included, a fAHP of -1.6 ± 1.9 mV was evident in 8 of 14 cells (Table 4). The inclusion of 3 µM dibucaine with 30 µM CP acted to raise the AP threshold of the first AP in the train to -38.8 ± 4.6 mV compared to -49.1 ± 2.8 mV in 30 µM alone (p<0.01, Fig. 9A). The increased threshold caused the expected reduction in AP frequency compared to 30 µM CP alone (p<0.001, Fig 11). Therefore the effects of the two drugs on these intrinsic CA1 properties appeared to be either slightly additive or just representing CP alone.
VI. AD INITIATION AND PROGRESSION IN CA3

The AD front entered the field of view from the CA3 side, so the objective lens magnification was reduced to include all 3 hippocampal subregions. CA3 neurons were simultaneously recorded to observe AD-related changes. Surprisingly, [because CA3 neurons are reported to be resistant to AD (Kreisman et al., 2000; Muller & Somjen, 1999)], a clear front of increasing light transmittance initiated focally within the CA3 st. pyramidal and propagated outward in all directions in 3 of 6 slices, entering both the CA1 region and the dentate gyrus via the hilus (Fig. 14A). These 3 slices displayed a fast AD concurrent with the passing LT front reaching the micropipette (Fig. 14B). In the other 3 slices, small focal increases in LT occurred, but without clear propagation throughout CA3 (Fig. 15A). Without a clear front of increased LT, no corresponding fast AD was observed, but rather a slow and gradual depolarization ultimately approaching 0 mV (Fig 15B).

Due to the curved 3-D orientation of the hippocampus, the whole rat brain must be sliced in different planes to cut cross-sectional hippocampal slices. Thus dorsal hippocampus was cut in the animal’s coronal plane and the ventral hippocampus was cut in the transverse plane. The latter yielded better cross-sectional slices of the CA3 region. In every transverse slice from which a healthy ventral CA3 recording was obtained, a reliable AD front propagated through the entire CA3 region to enter CA1 (Fig. 16A). These CA3 neurons underwent AD significantly earlier at 3:31 ± 1:33 min than CA1 neurons at 5:20 ± 1:21 min (Fig 17). Upon entering the CA1 region AD increased in velocity, with a more distinct LT front. Unlike the coronal slice, both the CA3 st. oriens, and st. radiatum displayed a clearly decreased LT within 10 minutes of the AD front passing. These observations each indicate that the AD in the CA3 region is more robust in transverse slices.
Under current clamp, CA3 pyramidal cells showed a less dramatic AD response, i.e. a shallower slope and less pronounced depolarization compared to CA1. While the fast AD amplitude in CA1 was $28.8 \pm 6.2$ mV ($n=13$), CA3 neurons displayed a lower amplitude of $11.2 \pm 5.4$ mV ($n=13$, $p<0.01$, 15B). Peak potential, as measured by the transition between the fast AD and slower late depolarization, was also lower in CA3 ($-27.2 \pm 6.6$ mV) compared with CA1 of $-9.4 \pm 4.6$ mV ($p<0.01$, Table 6).

**VII. RECOVERY POST-AD**

**A. CA1**

In our 21-28 day old rats, recovery of membrane potential post-AD occurred if the aCSF was introduced just as the wave of LT passed by the recording electrode (Fig 19A, B). This did not stop or slow the wave of propagation and the fast AD in the CA1 neuron was still recorded simultaneously with the imaging AD front. However the slow depolarization was usually attenuated, interrupted by an immediate return towards resting potential (or even a slightly more hyperpolarized state). Despite this, the slice continued to develop optical signs of damage. Specifically, there was a continued decrease in LT in dendritic regions (st molecular, and st. oriens) within the minutes of the AD front passing (Fig. 19A).

Despite the cell swelling induced during AD and post-AD, we were able to maintain high quality recordings long enough to induce and record a second AD event in 13 neurons (Fig. 20A). It was difficult to quantify the exact AD onset time or specific transition to slow depolarization in current clamp because the overall slope of the depolarization was much shallower than recorded with the first AD (Fig. 20B). A few recordings indicated a more AD-like fast depolarization could be generated following longer recovery times (upwards of 40 minutes) between AD events.
Interestingly, no propagating or general increase in LT was evident in any of these 13 slices during the second AD-like event. Presumably some of the cell swelling and dendritic beading underlying LT changes during the second AD remained from the first AD. Some of the slices did show a slight decrease in light transmittance post-AD in dendritic regions (Fig. 20A), suggesting further damage following the second AD.

C. Recovery by Other Cell Types

Apparently once AD initiates, a window exists between when the cell depolarizes to zero and the cell can be rescued by replenishing oxygen and glucose. However, defining the point when a neuron is irreparably damaged is difficult to assess because of the variability from cell to cell and slice to slice. For example in 4 cells (3 CA3 and 1 CA1) a full depolarization to zero occurred, albeit transiently, after AD had passed. However each cell was able to recover its full membrane potential and generate full action potentials.

We found that the most important measurement in terms of survivability is the time elapsed prior to reaching zero membrane potential, represented by the duration of the late AD. CA3 cells took over a minute longer to depolarize fully than did CA1 neurons (118.0 ± 36.4 seconds, p<0.001). Astrocytes maintained such a long plateau (>4min, Fig. 18A) that often the recording was lost prior to full depolarization probably because of the small size of the presumed astrocytes, the recording was often lost during AD or within the recovery time. However six astrocyte recordings displayed fully recovered membrane potential near resting levels following the introduction of aCSF a full two minutes after the passing AD front (Fig 18B). This was never observed with CA1 neurons, as they required reintroduction of aCSF a few seconds after the AD front passed. Likewise CA3 neurons required aCSF reintroduction within a minute.
Table 1: AD properties recorded in CA1 pyramidal cells

<table>
<thead>
<tr>
<th></th>
<th>Control OGD Ca1 (n=17)</th>
<th>10µM QX-314 (n=9)</th>
<th>10µM Dib (n=11)</th>
<th>100µM Dib (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD amp (mV)</td>
<td>28.8 ± 6.2</td>
<td>29.2 ± 6.0</td>
<td>26.1 ± 10.2</td>
<td>18.9 ± 6.9*</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-9.4 ± 4.6</td>
<td>-8.2 ± 5.4</td>
<td>-15.4 ± 11.0</td>
<td>-23.4 ± 11.2**</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>2.7 ± 1.5</td>
<td>3.1 ± 2.6</td>
<td>2.1 ± 1.6</td>
<td>1.5 ± 0.4*</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.28 ± 0.16</td>
<td>0.17 ± 0.13</td>
<td>0.18 ± 0.06</td>
<td>0.16 ± 0.04*</td>
</tr>
<tr>
<td>Duration of Late AD (s)</td>
<td>46.4 ± 31.3</td>
<td>58.8 ± 40.3</td>
<td>73.2 ± 37.2*</td>
<td>124.5 ± 50.9**</td>
</tr>
</tbody>
</table>

*p<0.05
**p<0.01
**Table 2: AD properties recorded in granule cells**

<table>
<thead>
<tr>
<th></th>
<th>Control OGD Granule (n=13)</th>
<th>10µM Dib (n=10)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD amp (mV)</td>
<td>23.3 ± 8.2</td>
<td>15.8 ± 5.3*</td>
<td>Reduced</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-11.4 ± 6.0</td>
<td>-23.3 ± 6.3**</td>
<td>Reduced</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>1.0 ± 0.6</td>
<td>0.75 ± 0.3</td>
<td>No Effect</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.29 ± 0.2</td>
<td>0.22 ± 0.1</td>
<td>No Effect</td>
</tr>
<tr>
<td>Duration of Late AD (s)</td>
<td>44.2 ± 27.1</td>
<td>132.7 ± 63.7**</td>
<td>Increased</td>
</tr>
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</table>

*p<0.05  
**p<0.01
Table 3: AD properties recorded with ouabain-induced AD

<table>
<thead>
<tr>
<th></th>
<th>Control OGD Ca1 (n=17)</th>
<th>Control Ouabain Ca1 (n=11)</th>
<th>10μM Dib Ouabain (n=6)</th>
<th>10μM QX-314 Ouabain (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD amp (mV)</td>
<td>28.8 ± 6.2</td>
<td>29.0 ± 6.6</td>
<td>24.8 ± 3.5</td>
<td>29.0 ± 8.5</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-9.4 ± 4.6</td>
<td>-4 ± 1.8*</td>
<td>-4.3 ± 2.4</td>
<td>-5.1 ± 0.5</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>2.7 ± 1.5</td>
<td>3.9 ± 1.3*</td>
<td>6.4 ± 3.0</td>
<td>8.0 ± 2.3</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.28 ± 0.16</td>
<td>0.18 ± 0.09</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.15</td>
</tr>
<tr>
<td>Duration of Late AD (s)</td>
<td>46.4 ± 31.3</td>
<td>25.75 ± 16.6</td>
<td>13.2 ± 7.2</td>
<td>10.16 ± 3.4</td>
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*p<0.05  
**p<0.01
Table 4: Intrinsic electrophysiological properties of the first AP in the train

<table>
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<tr>
<th></th>
<th>Control (n=45)</th>
<th>10 µM CP (n=12)</th>
<th>30 µM CP (n=11)</th>
<th>100 µM CP (n=5)</th>
<th>1 µM Dib &amp; 10 µM CP (n=14)</th>
<th>3 µM Dib &amp; 30 µM CP (n=11)</th>
<th>10 µM Dib (n=20)</th>
<th>1 µM Dib (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-62.5 ± 3.0</td>
<td>-62.4 ± 3.2</td>
<td>-65.6 ± 3.1</td>
<td>-64.0 ± 2.6</td>
<td>-63.7 ± 4.8</td>
<td>-63.5 ± 3.7</td>
<td>-62.9 ± 3.3</td>
<td>-59.7 ± 3.0</td>
</tr>
<tr>
<td>fAHP (mV)</td>
<td>-3.4 ± 1.9</td>
<td>absent (11/12)*</td>
<td>absent*</td>
<td>absent*</td>
<td>-1.6 ± 1.9 (8/14 cells)*</td>
<td>Absent*</td>
<td>-10.8 ± 3.7**</td>
<td>-2.7 ± 1.7</td>
</tr>
<tr>
<td>AP Threshold (mV)</td>
<td>-47.5 ± 4.4</td>
<td>-47.7 ± 2.7</td>
<td>-49.1 ± 2.8</td>
<td>-47.0 ± 3.4</td>
<td>-47.4 ± 4.3</td>
<td>-38.8 ± 4.6**</td>
<td>-37.7 ± 4.5**</td>
<td>-46.0 ± 3.8</td>
</tr>
<tr>
<td>AP freq (0.1nA) (Hz)</td>
<td>12.7 ± 9.5</td>
<td>6.8 ± 4.4*</td>
<td>6.0 ± 2.1*</td>
<td>1.2 ± 1.3**</td>
<td>4.1 ± 5.4*</td>
<td>0.5 ± 0.8**</td>
<td>0.6 ± 1.5**</td>
<td>12.3 ± 9.5</td>
</tr>
<tr>
<td>AP freq (0.2nA) (Hz)</td>
<td>24.3 ± 9.5</td>
<td>13.2 ± 4.2**</td>
<td>9.4 ± 2.5**</td>
<td>1.6 ± 0.9**</td>
<td>8.9 ± 5.7**</td>
<td>2.3 ± 1.8**</td>
<td>3.6 ± 4.4**</td>
<td>34.2 ± 16.4</td>
</tr>
<tr>
<td>AP freq (0.3nA) (Hz)</td>
<td>31.5 ± 8.7</td>
<td>16.8 ± 5.1**</td>
<td>11.4 ± 2.8**</td>
<td>2.2 ± 1.1**</td>
<td>12.7 ± 5.6**</td>
<td>2.9 ± 2.3**</td>
<td>5.3 ± 4.3**</td>
<td>N/A</td>
</tr>
<tr>
<td>AP amp (mV)</td>
<td>89.6 ± 10.0</td>
<td>82.3 ± 14.4</td>
<td>74.9 ± 15.4*</td>
<td>62.5 ± 15.0**</td>
<td>81.9 ± 16.9</td>
<td>70.5 ± 10.2</td>
<td>76.9 ± 12.9</td>
<td>70 ± 21.5*</td>
</tr>
<tr>
<td>AP dur (1/2 amp) (ms)</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1.4 ± 0.9*</td>
<td>2.0 ± 2.6**</td>
<td>1.1 ± 1.3</td>
<td>1.3 ± 0.2*</td>
<td>1.0 ± 0.2</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Rm Ω</td>
<td>47.4 ± 8.4</td>
<td>45.8 ± 6.3</td>
<td>66.8 ± 17.2*</td>
<td>80.9 ± 16.9**</td>
<td>44.2 ± 8.6</td>
<td>57.5 ± 11.5</td>
<td>52.1 ± 13.4</td>
<td>46.6 ± 1.7</td>
</tr>
</tbody>
</table>

*p<0.05
**p<0.01
Table 5: Intrinsic electrophysiological properties in the last AP in the train

<table>
<thead>
<tr>
<th></th>
<th>Control (n=25)</th>
<th>10 µM CP (n=12)</th>
<th>30 µM CP (n=11)</th>
<th>100 µM CP (n=5)</th>
<th>1 µM Dib &amp; 10 µM CP (n=14)</th>
<th>3 µM Dib &amp; 30 µM CP (n=11)</th>
<th>10 µM Dib (n=9)</th>
<th>1 µM Dib (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAHP (mV)</td>
<td>-5.8 ± 1.8</td>
<td>-11.2 ± 2.9*</td>
<td>-10.6 ± 2.3**</td>
<td>N/A</td>
<td>-11.5 ± 4.2**</td>
<td>-17.3 ± 3.1**</td>
<td>-15.8 ± 5.0**</td>
<td>N/A</td>
</tr>
<tr>
<td>AP thresh (mV)</td>
<td>-41.2 ± 4.0</td>
<td>-31.1 ± 4.0**</td>
<td>-34.3 ± 4.3**</td>
<td>N/A</td>
<td>-34.1 ± 6.5**</td>
<td>-32.4 ± 3.1**</td>
<td>-28.3 ± 4.2**</td>
<td>N/A</td>
</tr>
<tr>
<td>AP amp (mV)</td>
<td>69.6 ± 9.5</td>
<td>50.1 ± 11.5**</td>
<td>47.4 ± 7.4**</td>
<td>N/A</td>
<td>54.4 ± 11.4*</td>
<td>51.21 ± 6.7**</td>
<td>43.9 ± 12.0**</td>
<td>N/A</td>
</tr>
<tr>
<td>AP dur (1/2 amp) (ms)</td>
<td>1.2 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>2.0 ± 0.5*</td>
<td>N/A</td>
<td>1.4 ± 0.4</td>
<td>1.6 ± 0.1*</td>
<td>2.0 ± 1.7**</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*p<0.05
**p<0.01
Table 6: AD properties recorded in different cell types

<table>
<thead>
<tr>
<th></th>
<th>Control OGD Ca1 (n=17)</th>
<th>CA3 Control (n=13)</th>
<th>Astrocyte Control (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD amp (mV)</td>
<td>28.8 ± 6.2</td>
<td>11.2 ± 5.4**</td>
<td>16.0 ± 6.5**</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-9.4 ± 4.6</td>
<td>-27.2 ± 6.6**</td>
<td>-27.3 ± 8.0**</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>1.6 ± 1.8</td>
<td>1.2 ± 1.4</td>
<td>0.7 ± 0.5**</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.25 ± 0.1</td>
<td>0.27 ± 0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Duration of Late AD (s)</td>
<td>46.4 ± 31.3</td>
<td>118.0 ± 36.4*</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*p<0.05

**p<0.01
Chapter 6: FIGURES
A) The electrophysiological setup consisted of patch clamp recording ‘blind’ from the CA1 hippocampal neurons in current clamp mode. Orthodromic (ortho.) action potentials of CA1 neurons were activated by stimulating the st. radiatum (RAD) near the CA3 region and antidromic (anti.) activation involved stimulating the alveus. Single cell recording in the granule cell layer (GC) was in the upper blade.

B) The imaging equipment consisted of a broad band halogen light source focused on the slice where light was transmitted, absorbed or reflected. Transmitted light was collected by the digital camera and processed by the camera controller. Increases in light transmittance (LT) were pseudocoloured blue-green-yellow and decreases in LT were pseudocoloured magenta.
A

B

Digital camera

Camera controller

DIGITIZED IMAGE

Dendritic damage

\[ \Delta T \%

Cell swelling

absorption

scatter

transmittance

brain slice

aCSF in

aCSF out

near infrared pass filter

wide band light source

ortho. stim.

alveus

pp

CA1

CA3

GC

subic.

Sch. collat.

Orthostim.

anti. stim.

OR

PYR

RAD

LM
Figure 2: Simultaneous imaging of LT and single cell current clamp in CA1 neurons

A) Pseudocoloured images demonstrating LT changes as a result of oxygen-glucose deprivation (OGD). The AD front is observed as a wave of increased LT propagating along the length of the CA1 region. Following in the wake of the AD front, is a reduced LT within the dendritic regions (st. oriens and st. radiatum) while the st. pyramidale continues to show increases in LT, even after aCSF is reintroduced shortly after the passing AD front.

B) Simultaneous single cell recordings under current clamp mode reveal a concurrent fast anoxic depolarization (AD) as the passing front of increased LT reaches the recording electrode.
A) Imaging AD in CA1 Region of Coronal Slice

B) Simultaneous Current Clamp Recording of CA1 Neuron
Figure 3: Components of AD trace

Individual components of the AD trace measured under current clamp in the single cell. The initiation of AD (A) is the estimated point where the initial slow depolarization transitions into a more rapid depolarization (in min: sec.). The peak potential (B) of the AD is the estimated point where the slope of the fast AD transitions into the slope of the late depolarization (in mV). The fast AD amplitude (C) was measured between the depolarization at the AD initiation and the peak potential (in mV). The duration of the late depolarization (D) was measured between the time corresponding to the peak potential and the time to reach a maximal depolarization (in sec.).
CA1 Components of the AD Trace

A: AD Initiation
B: Peak Potential of Fast AD (mV)
C: Fast AD Amplitude (mV)
D: Duration of Late AD
Figure 4: Drug effects on AD latency in CA1 hippocampal neurons

A) Representative trace of a single cell recording demonstrating the ability of a 40 minute pretreatment of 10 µM dibucaine (dib) to delay AD in a CA1 hippocampal neuron.

B) Representative trace of a single cell recording demonstrating the ability of a 40 minute pretreatment of 10 µM carbetapentane (CP) to delay AD in CA1 hippocampal neuron.

C) Mean AD onset times in control and drug treated slices by bath, measured as the increased LT front passed the recording electrode. Significant differences compared to control are induced by 10 to 100 µM CP, as well as 10 µM dib. No effect observed when combining 1 µM dib with 10 µM CP, compared to 10 µM CP alone. Adding 3 µM dib to 30 µM CP produced a simple additive effect.
A) 10 µM Dibucaine
0 mV
-68 mV

B) 10 µM Carbetapentane
0 mV
-62 mV

C) Drug Effects Upon AD Latency in CA1

- No effect

* p<0.01 compared to control

n=25 n=13 n=6 n=4 n=9 n=9 n=13 n=13

0:00 3:00 6:00 9:00 12:00

1 min

20 mV

Control 10µM CP 30µM CP 100µM CP 1µM Dib 10µM Dib 1µM Dib & 10µM CP 3µM Dib & 30µM CP
Figure 5: Intracellular sodium channel blocker effects on CA1 AD

Recordings during intracellular application of 10-100 µM dibucaine (B, C) within the recording pipette revealed differences compared to control (A) in the AD waveform in the single cell that were not related to fast AD onset. Intracellular application of membrane impermeable QX-314 (D) did not display the same changes in AD waveform. All three applications did not affect fast AD onset as the ΔLT front passed by.
Intracellular Sodium Channel Blocker Effects on CA1 AD

A) Control

B) 10 µM Dibucaaine

C) 100 µM Dibucaaine

D) 10 mM QX-314
Figure 6: Intracellular effects of dibucaine on granule cells

Representative traces of control (A) and 10 µM dibucaine (B) applied through the recording pipette revealed changes in the AD waveform caused by OGD that were unrelated to fast AD onset. As in Fig. 5, the most obvious drug effect is that the fast AD was attenuated, prolonging the late AD. Such a delay to reach complete depolarization is likely neuroprotective.
Intracellular Effects Dibucaine on Granule Cell AD

A) Control

-66 mV

B) 10 μM Dibucaine

-67 mV

20 mV

1 min
Figure 7: Ouabain induced AD in CA1 neurons

Control (A) and 40 minute pretreatment with 10 µM dibucaine applied by bath (B) during 10 minutes of 100 µM ouabain exposure. Control slices always undergo ouabain-induced AD, whereas dibucaine pretreated slices do not. Dibucaine pretreated cells still undergo a slow depolarization, indicating lack of a full protective effect.
A  Ouabain-Induced AD in CA1 Neurons

B  10μM Dibucaine & 100μM Ouabain in CA1
Figure 8: AP trains induced in CA1 neurons

Differences in AP trains from control (A) are revealed following initiation of a 1 second 100 pA depolarizing current pulse (arrow) Both 30 minute pretreatment by 10 µM dibucaine (B) as well as 1 µM dibucaine combined with 10 µM CP (C) required a 200 pA injection to fire APs. Dibucaine reduces AP frequency, apparently by raising the AP threshold, while CP reduces AP frequency without a corresponding increase in threshold (Table 4).
AP Trains in CA1 Neurons

A) Control

B) 10 µM Dibucaine

C) 1 µM Dibucaine and 10 µM Carbetapentane

D) 100 µM Carbetapentane
Figure 9: The AHP in CA1 neurons

A) The fast afterhyperpolarization (fAHP) is significantly increased relative to AP threshold in 10 µM dibucaine compared to CA1 neurons. The fAHP followed the first AP evoked by a 1 second depolarizing current injection pulse. Carbetapentane at 10 to 100 µM eliminated the fAHP in most cells, resulting in an accentuated after depolarization.

B) Compared to control, both dib and CP increases the slow AHP (sAHP) of the last AP in a one-second spike train. CP at 30 µM apparently inhibits the fAHP so strongly, that a DAP is revealed. AHP enhancement helps account for the slowed firing displayed in Figure 8.
A) fAHP of First AP in Train

Control 10 µM Dib 10 µM CP 100 µM CP

B) sAHP in the Last AP in Train

Control 10 µM Dib 10 µM CP 30 µM CP

AP Threshold

5 mV

3 ms

5 mV

25 ms
Figure 10: Drug effects upon AP threshold in CA1 Neurons.

Dibucaine pretreatment of with 10 µM dibucaine for 30 min significantly raises the AP threshold in both the first and the last AP of the train. The first AP threshold is raised to -37.7 ± 4.5 mV compared to -47.5 ± 4.4 mV in control CA1 neurons, whereas in the last AP threshold is raised to -28.3 ± 4.2 mV from -41.2 ± 4.0 mV. CP failed to raise the AP threshold in the first AP. However, by the last AP in the train the AP threshold is significantly increased for both 10 and 30 µM CP, further explaining why spiking slows during a one second spike train (Fig.8). No significant effects are observed when adding 1 µM dibucaine to 10 µM CP. However the addition of 3 µM dib to 30 µM significantly raised threshold in both the first and the last AP.
Drug Effects Upon AP Threshold

First AP

Threshold Voltage (mV)

Control 10 μM CP 30 μM CP 1 μM Dib 10 μM Dib 1 μM Dib & 10 μM CP 3 μM Dib & 30 μM CP

* p<0.05 compared to control
** p<0.05 compared to CP alone

No effect

45 12 11 20 9 14 11
25 12 11 9 9 14 11

63
Figure 11: Drug effects Upon AP firing frequency

AP firing frequency is significantly reduced in a concentration and stimulus dependent manner with 10 to 100 µM CP at all three levels of current injection (0.1, 0.2 and 0.3 nA). Likewise 10 µM dibucaine also reduced firing rate. Adding 1 µM dib to 10 µM CP does not further reduce AP frequency, while adding 3 µM dib to 30 µM CP significantly reduces frequency compared to 30 µM alone.
Drug Effects on AP Firing Frequency

*\( p < 0.05 \) compared to control
**\( p < 0.05 \) compared to CP alone
Figure 12: Effects of dibucaine on the ortho and antidromic spike response

Spike failure induced by 10 µM dibucaine pretreatment upon both orthodromic and antidromic stimulation. The orthodromically-evoked spike fails after an average of $7.4 \pm 2.4$ min, while it took about twice as long for the antidromic spike to fail ($15 \pm 4.6$ min). Dibucaine pretreatment apparently elicits greater affect at the synapse than the axon.
A) Orthodromic Stimulation

B) Antidromic Stimulation

20 mV

10 ms

Spike Failure in 10 µM Dibucaine
Figure 13: Drug effects on the depolarizing afterpotential (DAP)

A) Representative traces of antidromically-evoked APs in control, 100 µM CP and 10 µM dibucaine. The DAP was measured as the voltage amplitude relative to resting membrane potential at 3 and 5 ms after AP threshold.

B) CP significantly increases the DAP at both 3 and 5 ms in a concentration-dependent manner from 30 to 100 µM, whereas 10 µM dibucaine did not.
A) Drug Effects Upon the CA1 Antidromic Response

B) Drug Effects Upon DAP Amplitude of Orthodromic AP

Control    100 µM CP    10 µM Dib

V (mV)

3 ms 5 ms

3 ms 5 ms

3 ms 5 ms

Control    30 µM CP    100 µM CP    10 µM Dib

30 mV

1 ms

3 ms 5 ms

3 ms 5 ms

3 ms 5 ms

69
Figure 14: Simultaneous imaging recording in CA3 region of coronal slice

A) AD was observed in the CA3 region in 3 of 6 coronal slices. The AD front continued into both CA1 and dentate gyrus (white arrows). Unlike AD propagation in CA1, a clear reduction in LT is not evident in st. oriens or st. radiatum. However diffuse regions of reduced LT exist, indicating damage still occurring.

B) Simultaneous current clamp recording shows the fast AD corresponding with the propagating LT front reaching the recording electrode (red arrow). It is significantly reduced in amplitude compared to CA1 neurons. Similar to CA1 neurons, no recovery of membrane potential is observed with the reintroduction of aCSF after full depolarization.
A) Imaging AD Propagation in CA3 Region Of Coronal Slice

B) Simultaneous Current Clamp Recording of CA3 Neuron
Figure 15: Simultaneous imaging LT and current clamp recording with an undefined AD front

A) In the other 3 coronal slices where healthy CA3 recordings were obtained, no propagating AD front is observed throughout the whole CA3 subfield. Various small local increases in LT were however evident. The reduction in LT indicative of damage was similar to slices with propagating AD in that it did not clearly follow dendritic layers.

B) Simultaneous current clamp in CA3 neurons reveals a shallow slope with no clear demarcation between a fast AD event and the late AD. This transition is clearly evident in CA1 neurons or in CA3 neurons that undergo propagating AD.
A) Imaging AD in CA3 Region Of Coronal Slice: No Defined Front

B) Simultaneous Current Clamp Recording of CA3 Neuron
Figure 16: Simultaneous imaging LT and current clamp recording in CA3 region of transverse slices.

A) Improved orientation of CA3 neurons in the transverse slices presumably facilitates AD propagation (white arrows) in the more ventral CA3 region. Furthermore, a clear demarcation of dendritic layers displaying reduced LT was evident to indicate damage.

B) As in Figure 15, simultaneous current clamp recording (micropipette tip at red arrow) displays a corresponding fast AD event with reduced amplitude compared to CA1 neurons.
A) Imaging AD Propagation in CA3 Region Of Transverse Slice

B) Simultaneous Current Clamp Recording of CA3 Neuron
Figure 17: AD latency in different cell types

That CA3 neurons initiate AD prior to CA1 neurons is confirmed by a significantly faster AD latency of $3:31 \pm 1:33$ (min: sec) than either CA1 at $5:20 \pm 1:21$ or granule cells at $5:51 \pm 1:49$. 
AD Latency to Onset of Different Cell Types

* p<0.05 compared to CA1
Figure 18: AD initiation and recovery in astrocytes

A) The AD waveform is similar to CA1 and CA3 in that there is a corresponding fast AD with the passing LT front. Unlike neurons, there is a gradual depolarization with little subsequent depolarization. The open arrow indicates the removal of the micropipette from the cell to demonstrate an actual plateau of ~25 mV.

B) In 6 astrocytes, nearly full recovery is observed, even after the reintroduction of aCSF two minutes after the onset of the fast AD. The incomplete depolarization means that the astrocyte can quickly recover if aCSF is returned.
A) AD Recorded in Astrocyte

B) AD Initiation and Recovery in Astrocyte
Figure 19: AD initiation and recovery after AD in CA1 neurons

A) Full recovery of membrane potential or to a slightly hyperpolarized state can result when aCSF is reintroduced at the time of the wave of increased LT passing by the recording electrode.

B) OGD-induced LT changes do not appear different even if aCSF is reintroduced close to the time of the AD front passing by the recording electrode (red arrow). Even though the single CA1 neuron recovers membrane potential, the slice continues to display a reduction of LT indicative of dendritic beading and damage.
A) Initial AD in CA1 Neuron

B) CA1 Region: Imaging Initial AD
Figure 20: Imaging and electrophysiology of second AD in CA1

A) Even though full recovery of membrane potential occurs after AD passes through the slice, a second exposure to OGD fails to elicit either a focal or propagating increase in LT.

B) The second AD observed through current clamp recording does not display a fast AD or clear transition between the early slow depolarization and the late AD, making AD onset times difficult to determine.
A) CA1 Region: Imaging Second AD

B) Second AD in CA1 Neuron
Chapter 7: DISCUSSION

Anoxic depolarization (AD) has been imaged as a focally initiating, propagating increase in light transmittance (Anderson et al., 2005; Somjen, 2001; Obeidat & Andrew, 1998; Jarvis et al., 2001). Extracellular recording simultaneously with ΔLT imaging revealed a wave front of increased LT corresponding with sudden onset of an electrophysiological negative shift in extracellular D.C. potential representing sudden depolarization by the neuronal population (Jarvis et al., 2001; Muller & Somjen, 1999).

AD measurements in the single cell synchronized with LT imaging of the whole slice allows study of AD initiation and potential recovery better than either method on its own. Since a propagating AD front does not occur in damaged slice regions, a lack of AD front in CA3 region could indicate either neuroprotection or damage. Direct measurement of membrane potential resolves the issue. Furthermore, subtle differences in the waveform of the depolarization in different regions of the hippocampus can only be observed with simultaneous AD imaging and electrophysiology. Combining these techniques also helps clarify previous experiments in our lab concerning AD delay induced by both dibucaine and carbetapentane. The lower concentrations of both drugs that delayed AD also preserved synaptic function based on evoked population responses, suggesting that AD can be inhibited without undermining normal cortical function (Douglas et al 2005).
I. CORRELATION OF AD IMAGING WITH WHOLE CELL RECORDING

A. CA1

Until our study, the AD waveform recorded intracellularly in the single neuron has not been directly correlated with the passing AD front propagating through the population. AD onset latency times of ~5 min, based on the wave of LT passing the recording electrode in untreated slices in the CA1 hippocampus were in agreement with previous experimental protocols utilizing OGD and submerged slices (Kreisman et al., 2000; Tanaka et al., 1997). The fast AD correlated with this passing LT front. Occasionally, identifying precise time points was difficult because of limited visualization of the micropipette tip within the slice as well as a broad LT front. All neuronal subpopulations (CA1, CA3, granule cells, astrocytes) displayed this basic correlation, although each cell type demonstrated variations in their electrophysiological responses.

B. CA3

Slices that showed distinct cell layers in the CA3 region as visualized with a light microscope displayed fully propagating AD responses that entered both the hilus of the dentate gyrus and CA1. Transverse brain slices that included the ventral CA3 regions displayed the most distinct cell body layers as well as the clearest AD signal denoted by the distinct wave of increased LT.

Reasons for the robust AD front within the transversely cut CA3 region can be suggested. The CA3 cells may better survive the dissecting and slicing or they may be in a better 3-D alignment to propagate AD within the ventral hippocampus. Wu et al. (2005) have suggested dorsal CA3 slices are more vulnerable to the slicing procedure than the ventral region. Preferential damage in the dorsal region would reduce the likelihood of a robust AD front. Our
transverse slices containing ventral CA3 required a lower current threshold to evoke orthodromic APs in CA1 than coronal slices indicating an improved preservation of dendritic architecture (data not shown).

Coronal CA3 slices never demonstrated a clear reduction in LT after the AD front within its dendritic regions, while transverse slices did. Only half of the coronal slices produced a clear wave of increased LT that propagated throughout the full CA3 region, although all current-clamped cells fully depolarized to near zero potential within the same amount of time as slices with a clear propagating front. The conclusion is that the orientation of the CA3 population within the slice is important for the coordinated LT signal but does not affect whether each CA3 neuron goes through AD, which probably proceeds across the CA3 population in a less synchronized fashion.

C. Astrocytes

Correlation of the fast AD with the increased LT front also occurred in all astrocytes, but depolarization was incomplete. This has been previously observed by (Xie et al., 2008), but at room temperature (22ºC). Muller and Somjen (1998) only utilized anoxia alone instead of the more metabolically stressful OGD, so partial depolarization may not have been as surprising.

In our experiments, astrocytes maintained a plateau near 27 mV, which is similar to HSD effects in interface slices (Muller & Somjen, 1998). This is near the predicted Nernst potential based on a change of [K+]e from ~3 mM to ~50 mM. The late depolarization, which is apparently not generated in astrocytes, has been attributed to sustained influx of Na⁺ and Ca²⁺ in neurons (Yamamoto et al., 1997; Somjen, 2001). While astrocytes do possess Na⁺ and Ca²⁺ channels, their
numbers are greatly reduced (Sontheimer et al., 1994). This is probably one important reason why astrocytes recover better from AD-induced swelling (Risher et al. in press).

II. DRUG EFFECTS ON AD INITIATION

A. Bath Pretreatment of Dibucaine

Previous experiments in our lab showed that 1 µM dibucaine pretreatment delayed AD at the same time it preserved evoked synaptic function in both cortical and hippocampal neurons (Douglas et al. 2005). Present experiments did not detect this AD delay, possible because the pretreatment protocols varied slightly.

In 10 µM dibucaine, evoked field potentials were reduced by 80% (Douglas et al. 2005). Our current intracellular work reveals that both the orthodromic and antidromic AP was blocked and evoked EPSP amplitude was reduced significantly during the 30 minute pretreatment. It is likely that this resulted from an attenuated presynaptic AP, although this is not necessarily the only explanation. In any case, dibucaine’s inhibition of AD onset at 10 µM could result from suppression of AP firing as well as dampening synaptic input. Additional intracellular parameters were measured to better delineate how reducing cortical excitability may delay AD to provide neuroprotection.

Dibucaine’s best documented effect is its action on the purported LA binding site within the voltage gate Na⁺ channel (Kuroda et al., 2000; Ragsdale et al., 1994), although direct measurement of Na⁺ currents in pyramidal cells has not been shown. Lidocaine is a similar yet less potent LA that reduces the voltage-activated sodium current in isolated pyramidal neurons (Kaneda et al., 1989). Dibucaine has also been shown to interact with Ca²⁺ channels (Sugiyama & Muteki, 1994), as well as affecting the stability of plasma membranes (Kuroda et al., 1996).
Since no apparent change in resting membrane potential was evident in our experiments, an interaction by dibucaine with the persistent sodium current is unlikely. In contrast, TTX blocks the persistent sodium current and induces a 5-10 mV hyperpolarization (Xie et al., 1994). Part of the AD-delaying effect of TTX has been attributed to this increased hyperpolarization (Fung & Haddad, 1997; Muller & Somjen, 1999; Fung et al., 1999).

Dibucaine elicited a significant increase in the amplitude of the fast AHP in our experiments potentially through the known ability of LAs to block inward Na⁺ currents (Kuroda et al., 2000). With a reduced inward current, the delayed rectifier current comprises more of the total current to increase the fAHP. Aside from sodium channel effects on the fAHP, the activation of voltage-gated potassium channels and Ca²⁺ activated K⁺ currents could also lead to potentiated fAHP as reported in peripheral nerves (Scholz, 2002) and pyramidal neurons (Sah, 1996; Bekkers, 2000).

**B. Intracellular Application of Dibucaine**

Even if dibucaine was able to fully block the AD current when introduced in the single cell, the membrane would still depolarize considerably because of the increased [K⁺]ₑ released from the surrounding population of neurons undergoing AD. The fact that dibucaine was able to stop a portion of the fast AD, as well as a component of the late depolarization indicates that the single neuron itself does dictate much of the AD waveform. On the other hand, the Na⁺ channel blocker QX-314 did not alter either parameter, which was surprising. Apparently some component of AD was blocked by dibucaine that presumably did not involve voltage-gated sodium channels. While the current responsible for the fast AD is not known, it involves Na⁺, Cl⁻ and Ca²⁺ influx and K⁺ efflux (Czeh et al., 1993). The late depolarization has been attributed to Na⁺ and Ca²⁺ conductance (Yamamoto et al., 1997). Both dibucaine and QX-314 (Talbot &
Sayer, 1996) involve blockade of both Na\(^+\) and Ca\(^{2+}\) channels so it is difficult to identify a specific ion or channel affected by dibucaine, but not by QX-314. Without future experiments, it is difficult to determine what conductance, whether classic Na\(^+\) or Ca\(^{2+}\) channels or some as yet unknown channel is being affected by dibucaine.

Surprisingly, while ouabain-induced AD was easier to block with dibucaine pretreatment by bath, intracellular application had no effect. Perhaps the sustained ion flux after the fast AD due to ouabain is not based on Na\(^+\) and Ca\(^{2+}\), as it is with OGD. It is apparent that dibucaine cannot affect ouabain-induced AD in the single cell at the level of the soma, but given ouabain’s apparent differential effects in dendritic regions, it would be interesting to utilize dendritic recordings. One possible explanation is that ouabain completely shuts down the Na\(^+/\)K\(^+\) ATPase, whereas with OGD, there may be residual ATP to keep the Na\(^+/\)K\(^+\)ATPase running at a low level. Thus dibucaine can exert some small effect on the single neuron. Overall however, OGD is harder to block because it shuts down all energy-driven pumps as well as synaptic transmission. This is difficult to reconcile.

C. Bath Pretreatment of Carbetapentane

Until this study, little was known about CP or other sigma receptor ligands mode of action on the intrinsic properties of CNS neurons. We observed a reduced neuronal excitability resulting from CP pretreatment in CA1 pyramidal neurons. However unlike dibucaine, CP did not induce an increase in AP threshold. Rather it was due to the increased sAHP. Since sigma 1 receptors have been found on both the plasma membrane and mitochondrial membrane of hippocampal neurons as well as on the postsynaptic dendrites (Alonso et al., 2000) it is possible the mode of action can be within those areas. No staining was observed on the axons of the presynaptic neurons (Alonso et al., 2000) so AD delay by CP , may not be at presynaptic axons.
The lack of a noticeable effect on the antidromically evoked AP confirms no sigma receptor ligand effect at the level of the axon.

While specific currents have not been observed in CA1 pyramidal neurons, sigma ligands have been shown to inhibit voltage-activated $K^+$ current in tumor cells (Wilke et al., 1999) and in oocytes expressing voltage-gated $K^+$ channels and sigma receptors (Aydar et al., 2002). Specific potassium channel effects observed in cardiac parasympathetic neurons include decreasing peak amplitude of delayed outwardly rectifying potassium currents and M current (Zhang & Cuevas, 2005). Calcium activated potassium channels such as large conductance (BK), small conductance (SK) and intermediate conductance (IK) underlie AHP in neurons and CP was shown to enhance the sAHP in our study. This would also explain the spike frequency adaptation that we observe (Sah, 1996). The spike broadening by CP is also suggestive of BK channel block as it is more pronounced by the last spike in the train, although delayed rectifying $K^+$ channel block can not be excluded.

While modulation of intracellular $Ca^{2+}$ has been linked to sigma receptor agonists, the authors attributed the action to sigma 2 receptor agonists (Zhang & Cuevas, 2002; Zhang & Cuevas, 2005), which have not shown the same degree of neuroprotection as sigma 1 agonists (Katnik et al., 2006; Anderson et al., 2005). Carbetapentane has been shown to possess a 30-fold increase in the binding of sigma 1 compared to sigma 2 receptors (Hirata et al., 2006).

Sigma receptor ligands (including CP) have previously demonstrated neuroprotection in cortical neurons. (Katnik et al., 2006) measured the increase in intracellular $Ca^{2+}$ under simulated ischemia instead of measuring AD. CP attenuated the increase in intracellular $Ca^{2+}$ due to sodium azide (to block aerobic metabolism) and glucose deprivation in cultured cortical neurons, attributed to direct preservation of intracellular $Ca^{2+}$ homeostasis (Katnik et al., 2006; Hayashi et
The IC50 value of 13 µM CP required to attenuate Ca\(^{2+}\) increases due to simulated ischemia in cultured neurons corresponds to values obtained in our experiments showing AD delay in brain slices.

**D. Dibucaine’s Role in Ouabain-induced AD Delay**

Low concentrations of dibucaine block AD during 10 minutes of ouabain exposure. However it had been unclear if the block maintained neurons at a normal resting membrane potential. Our current clamp experiments show that there is a slow depolarization that would not be detected with extracellular D.C. recording. Thus the lack of a D.C. shift makes it appear that AD was fully blocked when, in fact, the slower components were on-going. Dibucaine appears particularly effective then in dampening the fast AD.

Ouabain-induced AD appears more susceptible to blockade with equivalent drug concentrations (Anderson *et al.*, 2005) than OGD-induced AD. Increasing tissue osmolarity also reliably prevented ouabain-induced AD (Balestrino *et al.*, 1999). Also, blocking the Na\(^+/\)H\(^+\) exchanger with EIPA was able to prevent ouabain-induced AD, but not OGD-induced AD (Menna *et al.*, 2000)

Comparing dibucaine’s role in AD delay with a reduction in metabolic load seems to contradict its ability to delay AD induced by ouabain, which is assumed to be independent of ATP levels (Anderson *et al.*, 2005). However, a few assumptions must be made before such a leap can be made. First there can be no interactions, whether direct or allosteric, at the ouabain binding site by dibucaine. Second, it must be assumed that in hippocampal neurons, ouabain binds to all isoforms of Na\(^+/\)K\(^+\)ATPase channels present with sufficiently high affinity. Third, there must be a complete block of Na\(^+/\)K\(^+\)ATPase by ouabain.
Whether dibucaine interacts at the ouabain binding site is not known. However in rat cardiomyocytes, the similar local anaesthetic lidocaine affects ouabain binding (Maixent et al., 1998; Sterin-Borda et al., 2003). As for hippocampal neurons and high or low ouabain binding Na\(^+\)/K\(^+\)ATPase isoforms, 3 isoforms exist in neurons in relation to ouabain binding. Two high affinity binding sites, \(\alpha_2\) and \(\alpha_3\) (IC 50 of 23 nM and 460 nM respectively) and 1 low affinity binding site \(\alpha_1\) (IC50 ~320 µM) have been observed (Berrebi-Bertrand et al., 1990). Both the high and low affinity isoforms have been observed with immunocytological studies in the rat hippocampus, although \(\alpha_1\) staining was more prominent in the st radiatum (McGrail et al., 1991).

While the rise in [K\(^+\)]\(_e\) during ouabain-induced AD (Balestrino et al., 1999) is comparable to OGD-induced AD within the pyramidal cell layer (Lipton, 1999) and hypoxic SD (Muller & Somjen, 1999), it is within the st radiatum where differences arise. OGD-induced AD results in [K\(^+\)]\(_e\) increase of ~50 mM within dendritic layers (Lipton & Lobner, 1990; Muller & Somjen, 1999), yet ouabain-induced AD only reached 12-20 mM (Balestrino et al., 1999; Menna et al., 2000). This mirrors the predominance of low ouabain sensitivity isoform \(\alpha_1\) of the Na\(^+\)/K\(^+\)ATPase within the st radiatum (McGrail et al., 1991). While [K\(^-\)] was not measured during dibucaine pretreatment, dibucaine may have been able to stabilize the ion flux in dendritic, regions to partially preserve ATP levels and prevent AD.

### III. SIMILARITES BETWEEN DIBUCAINE AND CARBETAPENTANE

Our findings show that there is a reduced neuronal excitability induced by both dibucaine and carbetapentane as indicated by the reduction in AP frequency in response to current injection. Identifying the exact underlying mechanisms require voltage clamp work utilizing various channel blockers. Clearly dibucaine reduces excitability by increasing AP threshold through the
blockade of AP-associated sodium channels and CP reduces excitability by increasing the slow AHP. Subtle calcium or potassium channel effects cannot be excluded, as both can be inhibited by local anaesthetics (Hirota et al., 1997; Oda et al., 1992).

While both drugs delayed AD through different mechanisms of reduced cortical excitability, it is unlikely combining both drugs would delay AD any further without compromising the health of the slices. Even at 100 µM CP the health of the slice limited the AD delaying ability.

IV. NEURONAL RECOVERY FROM OGD

A. CA1

The ability of single CA1 neurons to recover membrane potential after AD depends on several factors. Tanaka et al (1997) did not observe recovery by adult CA1 neurons in submerged slices. However, Czeh et al 1993 demonstrated repeatable ‘hypoxic spreading depression’ (HSD) events within slices in an interface chamber. Interface slices have the advantage of a rapid diffusion rate from the oxygen-rich atmosphere compared to submerged slices which draw oxygen from the aCSF. More importantly, by only lowering O₂ and not glucose, the neurons still had a residual energy source and so were not truly ischemic. The 30 second lag of substrate diffusion from the superfusion system to the bath reported by (Tanaka et al., 1997) may account for the lack of recovery ability by delaying reintroduction of vital substrates required to sustain neuronal survival.

Our paradigm of simultaneous imaging with single cell electrophysiology in submerged slices gave us the advantage of reintroducing aCSF within this 30 second window. We were able to time the reintroduction of aCSF very soon after the fast AD, by observing the incoming AD
CA1 pyramidal neurons in our 21-28 day old rats were able to recovery full membrane potential after AD in the slice had passed the recording electrode. Accurate recording of whole cell resistance levels measured after recovery was difficult due to technical limitations, although in the cells that did not display a large increase in access resistance, whole cell input resistance levels were similar to levels measured prior to OGD exposure (data not shown). This indicates that the passing AD front, does not lead to permanent wholesale membrane leakage, at least in some cells.

In our experiments testing recovery of the orthodromic response in CA1 neurons, full APs could not be evoked at any time within the recovery interval (up to 40 min). EPSPs could eventually be evoked, but at reduced amplitude compared to pre-OGD. Antidromic APs could be re-activated once the neuron repolarized enough to restore Na\(^+\) channel activation. Perhaps a greater recovery interval would have led to complete AP or EPSP recovery. However it was difficult to maintain current clamp recordings from many minutes, especially during periods of slice swelling. Perhaps AD’s lasting effects cause irreparable harm to dendritic spines while preserving AP discharge. No slice showed a return to LT baseline in dendritic regions in recovered slices, further indicating synaptic damage from continuing dendritic beading. Since the current space clamp is incomplete past the proximal dendrites (Brown et al 1990), perhaps distal dendrites are susceptible to OGD and continue to bead, while the cell body can recover. Also, while dendritic beading has shown recovery following brief ischemia (Murphy et al., 2008) CA1 population recovery following AD in slices has not been observed. Since LT changes across dendritic regions of the entire slice do not return to baseline even when the single cell re-establishes its membrane potential, it seems probable that the recorded cell beads less than its neighbours.
That a good portion of the cortical slice does recover is demonstrated by our finding that a second AD event can be measured electrophysiologically upon re-exposure to OGD. A second AD event showed a less defined waveform, making AD onset and amplitude measurements difficult to measure. Unlike recovery by interface slices from anoxia alone (Czeh et al 1993), AD events in our preparation more accurately represented simulated ischemia and thus were less consistent and more damaging. Not enough cells in the slice recovered for subsequent full AD generation. If the dendrites maintained a beaded state they may not contribute a vital component of fast AD generation. The fact that the subsequent AD did not generate an initial swelling or a corresponding wave of increased light transmittance in any of the 13 slices is difficult to explain. Either the sensitivity of the recording was not able to pick up further LT changes, or AD causes unknown damage, not related to electrophysiological parameters.

B. CA3

CA3, like CA1 cells, were able to fully recover membrane potential with the reintroduction of aCSF following AD. Unlike CA1, the reintroduction of aCSF could occur within a longer interval after observing the fast AD. CA1 required close timing with the fast AD, whereas CA3 neurons could regain membrane potential within the duration of the late depolarization, almost a full minute after fast AD front passed by the recording electrode.

CA3 neurons recover from ischemia much better than CA1 cells, although it had been assumed due to not undergoing AD. Modifications must be made to explain the neuroprotection of CA3 neurons, now that AD can initiate and propagate in that region. The observed reduced fast AD amplitude, shallower fast AD slope and increased latency to 0 mV after AD in CA3 neurons compared to CA1 probably accounts for its improved recovery potential. However it is difficult to understand the exact mechanism of reduced AD response in CA3.
C. Astrocytes

Astrocytic recovery after AD caused by OGD has not been recorded in submerged slices due to OGD. Muller and Somjen (1999) showed recovery of membrane potential in presumed astrocytes following HSD. However they utilized anoxia instead of the more metabolically stressful OGD, so recovery was not unexpected. Other astrocyte recordings showed recovery but were performed at room temperature, where even adult CA1 pyramidal cells fully repolarize (Onitsuka et al., 1998). Again, recovery was not surprising.

We observed astrocytic recovery at any point during the extended plateau of incomplete depolarization once O$_2$ and glucose were restored. In contrast, CA1 neurons have a short time window after the fast AD before fully depolarizing and becoming unrecoverable. CA3 neurons, with their long late depolarization, are more easily salvaged if OGD is stopped before reaching 0 mV. Astrocytes show the greatest time period available for recovery once O$_2$ and glucose are restored. Our experiments demonstrate that an important determinant of recovery is the cell’s resistance to complete depolarization.

The fact that astrocytes are able to survive the AD with a more moderate loss in membrane potential is probably due to a number of factors. They have fewer voltage-dependent Na$^+$ and Ca$^{2+}$ channels that drive depolarization. They possess aquaporins, so they can dissipate intracellular water as it builds up and they have gap junctions which links them intracellularly in a network (Newman, 1986). The slow onset of AD and moderate prolonged depolarization helps explain how astrocytes so quickly recover from swelling following OGD as imaged with 2-photon laser scanning microscopy (Risher et al. in press).
Chapter 8: CONCLUSIONS AND FUTURE DIRECTIONS

Our findings show that dibucaine and carbetapentane alter the basic electrophysiological properties of pyramidal neurons in markedly different ways. Both drugs at moderate concentrations inhibit AD onset and both have been used clinically for other purposes. Importantly neither alters the resting membrane potential or whole cell input resistance of CA1 neurons but instead reduce the propensity for discharge. The obvious similarity in the two drugs from different classes involves the decreased cortical excitability. Regardless of the mode of action causing this decreased excitability, a delay in the onset of AD is not surprising considering the preservation of ATP levels that would be expected with application of both drugs. Future experiments directly measuring ATP levels under simulated ischemia (both OGD and ouabain) after pretreatment of CP and dibucaine will be necessary to confirm ATP preservation as the means of neuroprotection. A reasonable strategy would be to design a molecule that incorporates features of both molecules. This first requires identification of each pharmacophore which can be facilitated by previous work on AD inhibition by various ‘caines and sigma receptor ligands.

A second research area leading from this work is to carry out identical experiments using current clamp recording of magnocellular neuroendocrine cells (MNCs) while concurrently imaging light transmittance to confirm AD propagation through the hypothalamus. Prior work in the Andrew lab indicates that MNCs appear to be naturally neuroprotected in that they do not swell or bead despite AD coursing around the magnocellular nuclei. Current clamp recordings will document to what extent, if any MNCs depolarize in response to OGD. The longer-term goal is to determine the properties of MNCs that protect them from ischemia compared to the susceptible neurons of the cortex.
References


