NOTE TO USERS

Electrophysiological Assessment of Chronic Cytokine-Mediated Inflammation in Rat Organotypic Hippocampal Slice Culture

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1. Abstract

Chronic neuroinflammation was studied using whole-cell patch-clamp technique in rat organotypic hippocampal slice culture through exposure to lipopolysaccharide (LPS), an inflammatory stimulus that acts primarily by the induction of pro-inflammatory mediators known as cytokines. Seven-day exposure to LPS (100 ng/ml) resulted in significant changes in both intrinsic and synaptic properties of CA1 pyramidal neurons, which included decreases in membrane resistance and action potential (AP) discharge frequency; and potentiations of evoked monosynaptic excitatory post-synaptic current (EPSC) and inhibitory post-synaptic potential (IPSP) amplitudes. Changes in inhibitory synaptic transmission were suppressed when cultures were co-incubated with the interleukin-1 receptor antagonist (IL-1Ra, 50 ng/ml), suggesting that the cytokine IL-1 has an integral role in mediating this effect. Collectively, these data show that chronic inflammatory treatment produces important changes in CA1 neuronal properties, and that the effect on GABAergic transmission appears to be mediated by IL-1.

Résumé

La neuroinflammation chronique a été étudiée via la technique du patch-clamp sur cellule entière, en exposant des tranches organotypiques d'hippocampe de rat en culture au lipolysaccharide (LPS), un stimulus inflammatoire qui agit principalement par l'induction de molécules inflammatoires, les cytokines. Sept jours d'exposition au LPS (100 ng/ml) ont induit des modifications significatives des propriétés intrinsèques et synaptiques des neurones pyramidaux CA1. Cela inclut une diminution de la résistance membranaire et de la fréquence de la décharge en potentiels d'action, ainsi qu'une potentialisation des courants évoqués post-synaptiques monosynaptiques excitateurs et des potentiels post-synaptiques inhibiteurs. Les changements synaptiques inhibitrices sont supprimés lorsque les cultures sont co-incubées avec l'antagoniste du récepteur à l'interleukin-1 (IL-1Ra, 50 ng/ml), ce qui suggère qu'IL-1 joue un rôle significatif dans la médiation de cet effet. Collectivement, ces données montrent qu'un traitement inflammatoire chronique produit d'importantes modifications des propriétés neuronales en CA1, et que l'effet sur la transmission GABAergique semble être dépendante de l'IL-1.

2. Introduction

2.1 The Brain and Immune Signaling

Traditionally, the central nervous system (CNS) has been considered to be an immunologically privileged area of the body. The blood-brain barrier was thought to provide almost complete protection from both pathogen exposure and the subsequent increased activity of peripheral immune mediators, both of which could be detrimental to normal CNS function. However, more recent evidence now suggests that certain circulating mediators can indeed gain access to the brain and trigger a number of CNS-mediated physiological responses, some of which involve the brain's own immune system (reviewed in Becher *et al.*, 2000).

This activation of the brain's immune response is mediated by the actions of non-neuronal or glial cells, namely microglia and astrocytes (Becher *et al.*, 2000). Microglia are similar to peripheral monocytes with respect to their role in the immune response in the brain (Ling & Wong, 1993). After CNS infection, exposure to pro-inflammatory stimuli, injury, or exposure to blood-derived cells, microglia are swiftly activated to perform a variety of innate immune functions (reviewed in Aloisi, 2001). This activation is graded, and is manifested by morphological changes, hypertrophy, and proliferation of additional microglial cells capable of secreting a range of immunological mediators such as cytokines and chemokines (Kreutzberg, 1996). These cells are also capable of generating reactive oxygen species (ROSs) such as nitric oxide (NO), and other downstream products of inflammation (Chao *et al.*, 1992).

Astrocytes are the second major class of immunoresponsive cells in the CNS, and have an important role in the regulation of CNS environment and neuronal homeostasis.

In addition to this normal physiological role, these cells are also responsive to factors influencing microglial activation such as injury and inflammation, albeit along a later time frame than microglia (reviewed in Piehl & Lidman, 2001). Activation of astrocytes leads to hypertrophy, proliferation, and migration to sites of injury or antigen infiltration (Dong & Benveniste, 2001). Additionally, these cells may serve to restrict inflammatory signaling in the CNS (Gold *et al.*, 1996).

The cellular responses described above illustrate the reaction of the CNS to injury or immune challenge such as that presented with an inflammatory agent. Glial cell activation fulfills an important role in the initiation of the responses cumulatively known as neuroinflammation. Characterized by complement activation, acute phase protein recruitment and the generation of reactive oxygen species, neuroinflammation is a vital immune response component primarily mediated by cytokines (see table 1). In fact, pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor α (TNFα) are capable of generating potent immunological responses by themselves.

Function	Microglia	Astrocytes
Activation	(+): IFNγ, IL-1β	(+): LIF, IL-6
Migration	(-): TGFβ, M-CSF, GM-CSF	(+) TGFβ
NO Production	(+): IFNγ, IL-1β, TNFα (-): TGFβ, IL-4, IL-10	(+): IFNγ, IL-1β, TNFα (-): TGFβ, IL-4, IL-10
Prostaglandin production	(+): IL-3, M-CSF, GM-CSF (-): TGFβ, IL-4, IL-10	(+): IL-1, IL-6, TNFα
Shape change		(+): IL-1β, IL-6

Table 1: Cytokine modulation of microglia and astrocytes. (+) indicates that the property is stimulated by the given cytokines, (-) indicates inhibition. Adapted from Xiao & Link, 1998.

2.2 Cytokines as Immunomodulators in the Brain

The importance of cytokine signaling is underlined when it is considered that a variety of neurons across many regions of the brain express receptors to these molecules (Vitkovic *et al.*, 2000; also see table 2). Neuroinflammatory conditions, and upregulated cytokine expression, manifest in a variety of disease states and experimental models. For example, the dopaminergic regions of the striatum in postmortem Parkinsonian brains show increased levels of the pro-inflammatory cytokines IL-1β and IL-6 accompanying neuronal degeneration (Mogi *et al.*, 1994). Multiple sclerosis is known to be a product of chronic immune system dysfunction, involving an upregulation of the proinflammatory cytokines IL-1β and TNFα (reviewed in Merrill & Benveniste, 1996), among other changes. Neuroinflammation also accompanies Alzheimer's disease (reviewed by Weiner & Selkoe, 2002). Beta-amyloid protein (Aβ), a major pathological marker of Alzheimer's disease, is capable of complement activation and attracting microglia, which become activated and establish chronic inflammatory conditions around senile plaques (reviewed in Eikelenboom & Veerhuis, 1999).

Family	Cytokines	Sources	Actions
Interleukins (pro-inflammatory)	IL-1α, IL-1β, IL-6	microglia, astrocytes, neurons	multiple pro-inflammatory mechanisms and sites of action
Interleukins (anti-inflammatory)	IL-4, IL-10	microglia, astrocytes	multiple anti-inflammatory mechanisms and sites
Tumour Necrosis Factors	TNFα, TNFβ	microglia, astrocytes, neurons	similar to IL-1, also induces tumour cytotoxicity
Interferons	IFNα, IFNβ, IFNγ	microglia, astrocytes, infiltrating cells	regulate cell growth; IFNγ is primary immunoregulator of this family

Table 2: Some cytokine families and examples from the CNS. For more extensive information, see McGeer & McGeer, 1997.

Involved in all the pathologies mentioned above, IL-1β was among the first pro-inflammatory cytokines discovered, and its actions are some of the most extensively studied therein (Rothwell & Luheshi, 2000). Any inflammatory treatment in brain tissue generally leads to an elevation in the level of this cytokine. For example, treatment with several inflammatory agents produces a strong induction of IL-1 in microglia (Vincent *et al.*, 2002). IL-1b expression is also elevated in such conditions as ischemia and excitotoxicity (Rothwell *et al.*, 1997; Pearson *et al.*, 1999). Acute brain insults such as these can induce microglia to increase IL-1 mRNA expression within minutes, and increase protein expression within 1-2 hours (Liu *et al.*, 1993; Ianotti *et al.*, 1993; Taupin *et al.*, 1993).

IL-1 exerts its effects through two agonist molecules, IL-1α and IL-1β. IL-1β is the primary intercellular signaling component, and its actions are opposed by the endogenous competitive IL-1 receptor antagonist (IL-1Ra). IL-1β signals are transduced through the IL-1 receptor subtype 1, or IL-1RI (Sims *et al.*, 1988). A second, non-transducing receptor IL-1RII exists in both membrane-bound and soluble forms, and serves as an additional inhibitory mechanism for IL-1 signaling (McMahan *et al.*, 1991). A transmembrane accessory protein, IL-1RAcP, is also required for IL-1 signal transduction (Greenfeder *et al.*, 1995).

In the brain, activated microglia serve as the primary source of IL-1β (Hanisch, 2002). IL-1RI is found primarily on astrocytes, although some expression is also seen in microglia and neurons (Pinteaux *et al.*, 2002; French *et al.*, 1999). Inflammatory stimulation also results in the secretion of IL-6 from microglia and astrocytes, which is dependent on IL-1RI activation (Parker *et al.*, 2002). IL-6 has been shown previously to

be a key inflammatory modulator in the periphery (Turnbull *et al.,* 2003), and is likely involved in direct cytokine signaling to neurons (Nelson *et al.*, 2002).

The key role of cytokines in inflammatory signaling has been established through the application of exogenous cytokines, and also through the induction of endogenous cytokines through the actions of pro-inflammatory substances. One of the best-known is lipopolysaccharide (LPS), a glycolipid derived from gram-negative bacterial cell wall. Also known as endotoxin, this agent has been recognized for many years to be capable of inducing inflammatory responses such as sepsis after systemic administration. It is among the most potent known activators of pro-inflammatory cytokine signaling in both chronic and acute models *in vivo* and *in vitro* (reviewed in Heumann & Roger, 2002).

2.3 Electrophysiological Approaches in Studies of Inflammation

Electrophysiological techniques remain among the best methods to directly assess the functional properties of neurons. Changes in basic neuronal responses to direct current injection, known as intrinsic properties, can have serious and important consequences on neuronal function. These properties include membrane resistance, action potential discharge frequency, and the magnitudes and latencies of non-synaptic currents. Acute inflammatory treatments induced by IL-1β application have been shown to modulate intrinsic neuronal properties such as spike frequency generation in dissociated subfornical organ neurons (Desson & Ferguson, 2003). Mice with genetically elevated levels of IL-6 show attenuations in endogenous firing and increased inhibitory synaptic responses in the cerebellum (Nelson *et al.*, 1999), and organotypic cerebellar cultures exposed chronically to this cytokine show reductions in action potential discharge frequency (Nelson *et al.*, 2002).

In addition to intrinsic properties, potential changes in synaptic properties of neurons must be considered. Alterations in the connectivity between neurons can produce major changes in CNS properties. One important synaptic property is plasticity, the ability of neurons to adjust synaptic strength based on stimulation properties. The two most common measures of plasticity examined in neurons are long-term potentiation (LTP) and long-term depression (LTD). LTP is the strengthening of the synaptic connections between neurons after high-frequency stimulation to the presynaptic neurons, and thought to be dependent on NMDA receptor activation. LTD is the opposite, a decrease in synaptic efficacy between two neurons, and the two phenomena are thought to be cellular events related to learning and memory.

Population recordings taken from the CA1 region of the hippocampus show that both LTP and LTD induction are inhibited by acute application of LPS, perhaps by a Ca^{2+}-dependent mechanism (Jo *et al.*, 2001). NMDA-dependent currents were also found to be inhibited by this treatment. In CA1-subiculum plasticity, acute LPS treatment attenuated paired pulse facilitation, shortened magnitude and duration of LTP, and shortened duration of LTD (Commins *et al.*, 2001).

Similar effects have been described *in vivo,* where chronic i.c.v. LPS induces impairments in LTP induction in the dentate gyrus (Hauss-Wegrzyniak *et al.*, 2002). A similar inhibition occurs after acute systemic LPS administration (Vereker *et al.*, 2000a). These authors also show that this effect and the accompanying neurodegeneration are partially dependent on caspase-1 activation. This protease is responsible for the activation of IL-1β by cleaving it from the inactive precursor pro-IL-1β (Thornberry *et al.*, 1992), and implies that the LPS-induced changes are dependent on IL-1β signals. Support for

this viewpoint is provided by experiments where acute IL-1β doses as low as 1 ng/mL have been shown to inhibit LTP induction between perforant path projections and dentate gyrus granular cells (Cunningham *et al.*, 1996). IL-1β inhibition of LTP in dentate gyrus is thought to be dependent on the activation of the protein kinases p38 MAPK and JNK (Coogan *et al.*, 1999; Vereker *et al.*, 2000b).

In addition to these alterations in plasticity, some baseline synaptic properties have been shown to be altered as a result of inflammatory treatments. $GABA_A$ currents are shown to be inhibited in both cultured single hippocampal neurons and organotypic cerebellar slice cultures after acute IL-1β application (Wang *et al.*, 2000; Pringle *et al.*, 1996). It has also been shown that IL-1β can increase synaptic inhibition (presumably GABAergic) in CA1 pyramidal neurons, although this is not true for CA3 neurons (Zeise *et al.*, 1992; 1997). Similar effects on the GABA system have been described in cortical preparations (Miller *et al.*, 1991).

Pro-inflammatory cytokines have also been shown to inhibit the glutamatergic system, for example the inhibition of NMDA-dependent field potentials in the dentate gyrus after IL-1 application *in vitro* (Coogan & O'Connor, 1997). Additionally, IL-1β can inhibit glutamate release from hippocampal synaptosomes taken from young rats (Murray *et al.*, 1997). Concurrent with this glutamatergic inhibition, acute IL-6 administration has been described to inhibit LTP in the CA1 region of the hippocampus (Tancredi *et al.*, 2000).

However, chronic treatments can result in different effects. For example, moderately high doses of IL-6 applied chronically to cerebellar organotypic cultures increase intracellular Ca^{2+} levels and influx from AMPA receptor activation (Nelson *et*

al., 2002). Chronic IL-6 treatment enhances response to NMDA activation in developing cerebellar granular cells in culture (Qiu *et al.*, 1998). These differential effects underline the crucial distinction that must be recognized between chronic and acute inflammatory treatments.

2.4 Rationale and Objectives

Considering the large body of research describing the modulation of electrophysiological properties in neuronal populations by acute inflammation and its downstream products, there has been very limited study of these properties in models of chronic inflammation. Acute immunological challenges such as the majority of those described above do not adequately model the potential changes that may result from chronic inflammation, both in disease conditions and in experimental models.

Furthermore, the majority of these studies examine the response of populations of neurons as opposed to single neurons themselves. While population studies are important to the understanding of neural function, examination of single neurons has so far been mostly neglected. Additionally, much attention has been paid to models of synaptic plasticity such as LTP and LTD while the effects of both acute and chronic inflammation on baseline synaptic and intrinsic properties remain largely undefined.

The current study was undertaken with the intention of addressing these shortcomings. The aim of this M.Sc. thesis was to use electrophysiological techniques to elucidate the effects of chronic inflammation on intrinsic neuronal properties and baseline synaptic transmission. The whole-cell patch clamp technique is among the best methods of examining the function of single neurons. An *in vitro* model was chosen for rigorous environmental control and convenience of the establishment of chronic inflammatory

signals. The static organotypic hippocampal slice culture (Stoppini *et al.*, 1991) was chosen specifically because organotypic slice cultures maintain both the glial cells necessary to mount an immune response, and the synaptic organization found *in vivo*, which makes them ideal preparations for studying synaptic properties in addition to the intrinsic properties of individual cultured neurons in the context of chronic inflammatory treatments.

Specific objectives were to:

- establish the existence of inflammatory conditions in the rat organotypic hippocampal slice culture after treatment with LPS through assessment of pro-inflammatory cytokine release by ELISA.

- examine the effects of chronic LPS treatment on intrinsic and synaptic properties in CA1 pyramidal neurons using the whole-cell patch-clamp technique.

- investigate the contribution of IL-1 receptor signaling to the effects on electrophysiological properties induced by LPS

3. Materials and Methods

3.1 Preparation of Organotypic Hippocampal Cultures

Cultures were prepared as described by Stoppini *et al.* (1991). Briefly, brains were dissected from neonatal [P(8-10)] rats under sterile conditions. Animal care and treatment procedures conformed to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care (see appendix). Horizontal sections (400 μm thick) were made using a vibrating microtome (Campden Instruments; London, UK) with a chilled bath consisting of Hank's Balanced Salt Solution (HBSS) supplemented with 12 g/L glucose, 25 μM HEPES buffer and a penicillin/streptomyosin mixture (100 units/ml and 100 μg respectively). Hippocampi were dissected from whole slices with the aid of a microscope. They were then rinsed through sterile dissecting media, followed by culture media. Slices were then placed on sterile circular sections of culture membranes and then placed onto membrane inserts (Millipore; Bedford, MA) pre-incubated with culture media for 30 minutes. Culture media is 50% Minimal Essential Medium, 25% heat-inactivated horse serum and 25% HBSS, supplemented with HEPES, glucose and antibiotics as per dissection medium. Media was changed 24 hours after culturing, and every 2-3 days thereafter. Slices are kept at 37°C with 95% CO_2 for the first 3-4 days, and then moved to similar conditions at 33°C. All media components were obtained from GIBCO (Grand Island, NY). Heat inactivation of serum was done by a 30 minute treatment in a water bath set to 50 degrees Celsius.

3.2 Culture Treatments

In all cases, cultures were exposed to treated media at 7 days in vitro (DIV). Sterile LPS and IL-1Ra were prepared, aliquotted and stored at –80°C, and added to control media as

needed. The final concentration of agents used was 100 ng/ml LPS and 50 ng/ml IL-1Ra. Culture media was changed 2, 4 and 7 days after beginning of treatment, and at concomitant times for control cultures. The collected media was aliquotted and kept at –80°C for later cytokine quantification.

3.3 ELISA Detection of Pro-Inflammatory Cytokines

To demonstrate the presence of inflammatory signaling, quantification of pro-inflammatory cytokines was carried out with standard ELISA kits used according to manufacturer's instructions. Briefly, the primary antibody was appropriately diluted in coating buffer (in mM: NaCl 140, KCl 2.7, KH2PO4 1.5, Na2HPO4 8.1) and incubated overnight at 4°C in 96-well plate (100 µl/well). Wells were rinsed 4 times between steps with wash dilution buffer (WDB, in mM: NaCl 500, NaH2PO4 2.5, Na2HPO4 7.5). Plates were then incubated 1 hr at 37°C with ovalbumin (0.1 % w/v in coating buffer as above) as a blocking step. Known concentrations of appropriate cytokine were serially diluted in culture medium and added to plate to generate a standard curve. A diluted sample along the standard curve was used for quality control. Undiluted 100 µl samples of culture media collected at 2, 4 and 7 DIV after LPS were added in duplicate to remaining wells, and plate is incubated 3 hrs at room temperature on shaker. Biotinylated secondary antibody is then diluted in WDB and 1% v/v sheep serum as per kit instructions, added to wells, and incubated 1 hr at room temperature on shaker. Avidin/HRP (Dako A/S, Denmark) was diluted 1:3000 v/v in WDB and added to wells, and incubated 30 minutes at room temperature on shaker. Tablets of OPD were diluted in substrate buffer (citric acid 34.7 mM, NaH2PO4 66.7 mM; adjusted to pH 5), supplemented with 0.4% v/v hydrogen peroxide (30%), and incubated in dark oven 15-30

minutes at 37°C. Excess H2SO4 (1 M) was used to stop the reaction, and absorbance was read at 490 nm using a Molecular Devices plate reader and analyzed with SOFTmax Pro 2.32 software (Molecular Devices). Primary and secondary antibodies were kind gifts from Steven Poole (NIBSC). Unless otherwise noted, all other components were obtained from Sigma-Aldrich (Oakville, Ont., Canada).

3.4 Electrophysiological Recordings

Electrophysiological recordings were taken between 14-15 DIV. Single slices were taken from culture and placed into the recording bath and perfused at 1-2 ml/min with ACSF (in mM: NaCl 126, NaHCO3 24, KCl 3, MgSO4 2, NaH2PO4 1.25, CaCl2 2, glucose 10), and bubbled with 95% CO2/5% O2. No LPS or IL-1Ra was included with ACSF during recordings. Slices equilibrated in ACSF for approximately 30 minutes before recordings were initiated. Whole-cell patch-clamp recordings in current- and voltage-clamp modes were taken from the somatic regions of individual pyramidal neurons in CA1. Neurons were visually identified using an upright Olympus microscope (Olympus; Melville, NY) equipped with Normarski optics and an infrared camera, and patched with glass micropipettes (5-8 MΩ) containing patch solution (in mM: potassium gluconate 144, MgCl2 3, EGTA 0.2, HEPES 10, ATP (as sodium salt) 2), filtered at 0.2 μm and stored at –80°C for later use. Neurons were recorded using a computer-controlled Visual Patch 500 amplifier and dedicated software (BioLogic; Claix, France). Recordings were saved to disk, and later analyzed using BioTools software, ClampFit 8.0, 8.2 and 9.0 (Axon Instruments Inc.; Union City, CA).

To examine if the neuron was healthy and able to fire after establishing whole-cell mode, neurons were exposed to a 200 pA depolarizing step. Neurons were rejected if

resting membrane potential was more positive than –50 mV, or did not discharge action potentials. Neurons were also rejected from all analyses if the spike amplitude was less than 60 mV, or AP duration was over 2.5 ms.

3.4.1 Electrophysiological Recordings of Intrinsic Properties

1. Basic Neuronal Properties: After determining that the neurons were healthy, the resting membrane potential (RMP) of the cells was recorded. RMP was determined within initial 30 s of patching to minimize the influence of patch solution on membrane potential. Action potential (AP) magnitude and duration were then characterized by a current-clamp protocol designed to elicit APs at threshold levels. Neurons were subjected to a series of 2 s depolarizations starting at 20 pA and increasing by 5 pA per stimulus until firing threshold was reached. The amplitude of action potentials was taken as the difference between membrane potential before and the peak amplitude of the spike, where duration was taken at one-half of total spike amplitude (figure 2b).

2. AHP current: The afterhyperpolarization, or AHP current, is activated by strong depolarization and can provide rectifying current in prolonged depolarizations and spike frequency accommodation (e.g. Powers et al., 2002; Stocker et al., 1999). Although examined in two protocols, prominent AHPs were not displayed in many neurons. This may have been due to the presence of potassium gluconate in the patch pipette, which is known to chelate Ca^{2+}, producing a reduction of the calcium-activated potassium current underlying AHPs (Stocker et al, 1999). Investigation is possible using potassium methylsulfate in place of gluconate in the pipette solution, as this salt does not reduce AHPs (Zhang et al., 1994).

3. I_h Currents: Another property measured was the hyperpolarization-activated potential named I_h. This is a mixed-cation (Na+/K+) current that depolarizes neurons once hyperpolarized and plays a role in counteracting inhibitory potentials. To evoke I_h, multiple hyperpolarizing current steps were injected while neurons were held manually by current clamp at a membrane potential near –60 mV. Neurons were hyperpolarized by a step of -120 pA for 4 s, and then returned to rest. The stimulus was repeated 14 times, 10 pA higher each time so that the final stimulus was +20 pA . Two measurements were taken: one before Ih onset (early), and one during Ih activation (late) (fig. 4a). I_h amplitude is measured as the difference between early and late membrane potential measurements.

4. Membrane resistances: Comparisons were also made between the early measured potentials (before I_h maximal activation) in control and in the LPS-treated neurons to assess membrane resistances. Linear regression was used to calculate the slope of a current-voltage graph in order to compare resistances between groups.

5. AP Frequency: Finally, it was investigated if the frequency of discharge in neurons from LPS-treated slices were different from controls. At a membrane potential of –60mV, neurons were depolarized by a series of 4 s duration current steps starting at 40 pA and increasing by 10 pA per step. Action potential firing frequency was calculated for the first and second halves of the depolarization, and for the entire stimulation.

3.4.2 Synaptic Responses in CA1 Pyramidal Neurons

1. Excitatory Synaptic Properties: Possible differences in excitatory postsynaptic responses to Schaffer collateral stimulation were investigated in pyramidal neurons from control and LPS-treated organotypic cultures. To elicit synaptic responses, continuous

currents (100 μs) were injected through a monopolar tungsten electrode (0.50 MΩ) placed in the middle of stratum radiatum near the CA2/CA1 interface to stimulate the Schaffer collateral inputs to CA1 pyramidal cells. Whole-cell patch recordings were made in both voltage- and current-clamp modes. Before addition of pharmacological agents to ACSF, synaptic stimulation of Schaffer collaterals elicited a short lasting excitatory post-synaptic potential (EPSP) immediately followed by the longer lasting inhibitory post-synaptic potential (IPSP).

Glutamatergic excitatory post-synaptic currents (EPSCs) were elicited in neurons held to -50 mV by voltage-clamp. GABAergic inhibitory events were blocked using 10 μM bicuculline. Pipette solution included QX-314 (5 mM; Sigma-Aldrich, Oakville, Ont.) to prevent action potential discharge during large EPSPs observed during synaptic stimulation under GABAergic blockade. Peak amplitudes were taken as the difference between the value at the peak and the mean membrane potential during the 25 s before the electrical stimulation. Although hippocampal glutamate release has been shown to be altered after acute systemic LPS treatment (Vereker et al., 2000), the effects of chronic LPS treatment on glutamatergic synapse properties are unknown. Increases in EPSC amplitude were compared between groups over increasing stimulus intensities (0-150 μA, +10 pA per stimulus level).

2. Inhibitory Synaptic Properties: Possible differences in inhibitory postsynaptic responses to Schaffer collateral stimulation were investigated in pyramidal neurons from control and LPS-treated slices. Changes in GABAergic inhibitory post-synaptic potentials (IPSPs) were then measured in CA1 pyramidal neurons while blocking the glutamatergic AMPA and NMDA receptors (DNQX, 20 μM and APV, 100 μM, respectively).

Although the effects of LPS on IPSPs are unknown, both decreases (Zeise et al., 1997) and increases (Luk et al., 1999) in inhibitory responses after acute IL-1β application have been reported. It remains unclear if chronic LPS treatment can affect IPSPs. Possible changes in IPSP amplitude were first examined in response to graded increasing intensity of synaptic stimulation. Measurements were taken at the peak of the IPSP response and for the mean membrane potential over the 25 ms before the stimulus to calculate amplitude. Stimuli were from 0 - 300 pA in amplitude, with 20 pA steps.

The reversal potential of IPSPs from Schaffer collateral stimulation were investigated in pyramidal neurons from control and LPS-treated slices. The reversal potential is an indication of the equilibrium potential of the ion mediating the response and previous experiments have shown that chloride is principally implicated (Streit et al., 1989). Reversal potentials of IPSPs are measured in current-clamp mode by applying synaptic stimulation below spike threshold at various holding potentials between –40 and –90 mV. Reversal potential found by calculating the slope of the line between the most positive membrane potential where stimulus produced a depolarization, and the most negative where stimulus produced a hyperpolarization. Based on the slope, the membrane potential at which IPSP amplitude would be zero was calculated and recorded.

3.5 Data Analysis

All data shown are expressed in terms of mean ± S.E.M. Statistical analyses were performed using GraphPad Prism software (v. 3.00, GraphPad Software, Inc.), and with Datasim statistical programming package (Drake Bradley; Bates College, Lewiston, ME). In instances where single measurements are taken from neurons in LPS-treated or control cultures (resting membrane potential, AP amplitude and duration, AHP latencies and

amplitudes, and chloride reversal potential), differences were demonstrated using an independent t-test. In instances where two groups are compared at multiple levels (IL-1β ELISA, Ih magnitude), data are analyzed using a two-way ANOVA with one-between subjects variable (LPS-treated or control) and one within-subjects variable (time point in ELISA, current level in Ih magnitude). All cases where control, LPS-treated, IL-1Ra-treated and LPS/IL-1Ra-treated neurons are compared (IL-6 ELISA, AP frequency, EPSC magnitude, and IPSP magnitude), a three-way ANOVA is used with two between-subjects variables (LPS exposure, IL-1Ra exposure) and one within-subjects variable (time point or current level). No post-hoc tests were required, as only two levels of any between-subjects variable are present in each analysis, and simple main effects were sufficient to detect significant differences. Significant differences were taken as cases where $p < 0.05$.

4. Results

4.1 LPS induces IL-1β release

The primary objectives of this study were to investigate whether exposure to chronic LPS induced changes in electrophysiological properties, and if so, whether these changes were mediated by IL-1β. To confirm that IL-1β was indeed induced by incubating cultures with LPS, an initial experiment was conducted to assess IL-1β levels in culture medium. As shown in figure 1, it was clearly demonstrated that this was the case. LPS treatment induced a dramatic potentiation in IL-1β release from cultures over the first 2 days (see figure 1), from 16.2 ± 8.6 pg/ml in controls (n = 5) to 264.7 ± 94.9 pg/ml (n = 6). A two-way ANOVA showed that this difference is highly significant ($p = 0.0004$). IL-1β levels returned to control levels after 2 days ($p = 0.909$ after 4 days, $p = 0.992$ after 7 days), and remained stable at this level.

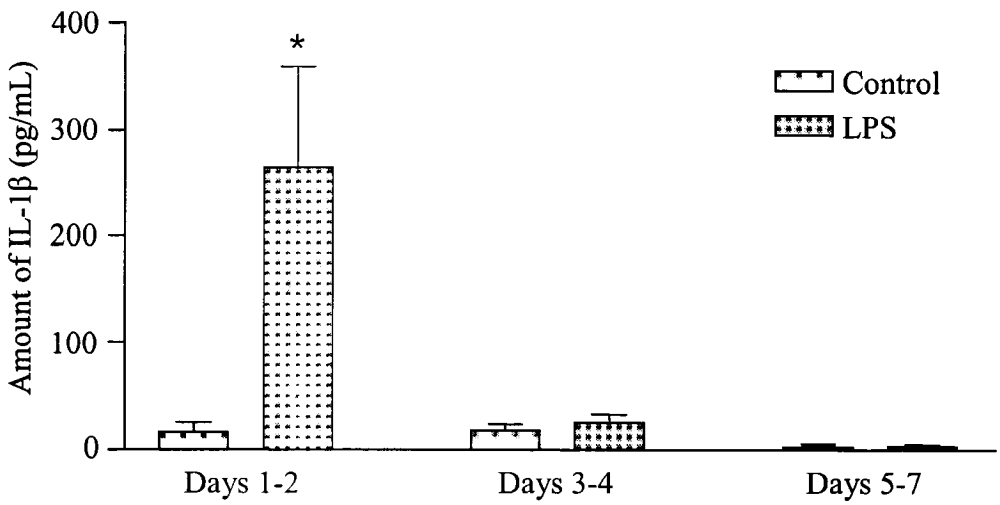

Figure 1: IL-1β release from organotypic hippocampal culture over 7-day exposure to LPS as measured by ELISA. Amount of IL-1β released into culture media over time periods specified is shown. LPS increased IL-1β release about 16-fold over the first two days of exposure ($p < 0.0001$), after which there was no difference from controls.

4.2 LPS induces no changes in basic neuronal properties

Chronic LPS exposure did not affect the basic properties of neurons from these cultures. Resting membrane potential for each neuron was recorded in both groups. As shown in figure 2a, neurons from LPS-exposed cultures showed a mean membrane potential of -59.0 ± 0.59 mV (n = 51), while control cultures had a mean of -58.0 ± 0.55 mV (n = 54). This difference was not found to be significant by studentized t-test (p = 0.214). Unless otherwise specified, data are given as mean ± S.E.M.

Action potential amplitude and duration were also characterized for these neurons. Amplitude was taken as the beginning of the rise period to the peak membrane potential, and duration was measured at half the calculated amplitude (figure 2b). Control neurons showed a mean AP amplitude of 79.8 ± 2.47 mV, and a mean duration of 2.2 ± 0.09 ms (n = 19). Similarly, LPS neurons showed a mean amplitude of 80.7 ± 1.52 mV, and a mean duration of 2.0 ± 0.06 ms (n = 22; figures 2c, 2d). A studentized t-test showed no significant difference between the two groups (p = 0.760, 0.085, respectively).

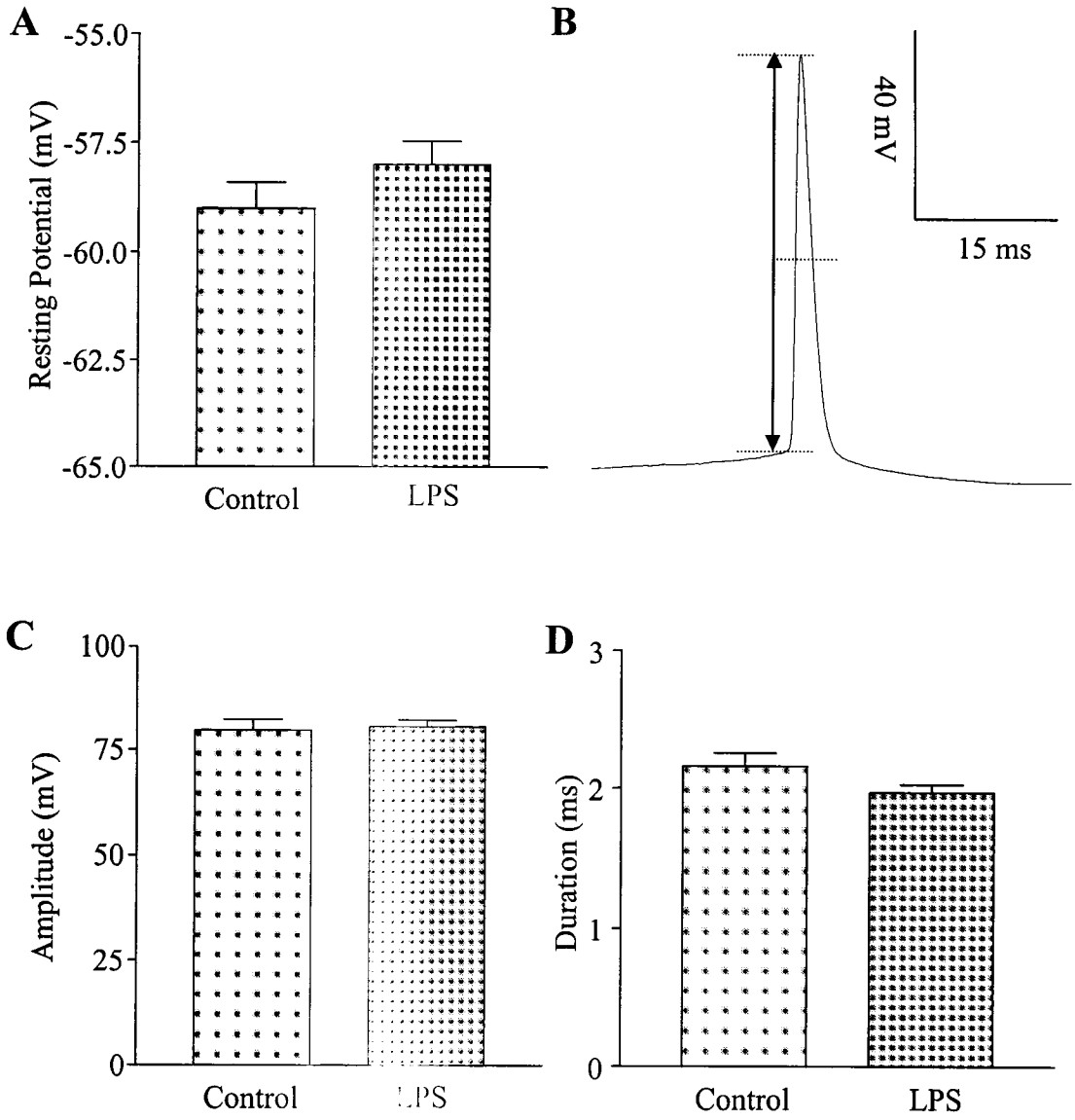

Figure 2: Chronic LPS does not affect basic neuronal properties. (**A**) Histogram of Resting membrane potential. Independent t-test reveals no significant difference ($p = 0.214$). (**B**) Representative trace of action potential displaying measurements of amplitude and duration. (**C**) Histogram of action potential amplitude in control and LPS-treated neurons. Independent t-test shows no significant difference between groups ($p = 0.760$). (**D**) Histogram of action potential duration. Again, no significant difference is detected between treatments ($p = 0.085$).

4.3 LPS diminishes intrinsic excitability

The peak amplitudes and latencies of the sAHP and the fAHP from each neuron were measured as shown in figure 3a. Control neurons showed a mean fAHP amplitude of 2.68 ± 1.18 mV (n = 10), where LPS-treated neurons had a mean amplitude of 2.93 ± 0.53 mV (n = 9; fig. 3b). Latencies were also very similar between groups, with 122.8 ± 14.6 ms in control neurons and 109.4 ± 15.1 in the LPS-treated group (n = 6 and 9, respectively; fig. 3b). These values are not significantly different (p = 0.853 and 0.554, for amplitude and latency respectively). These similarities were also present in the sAHP measures, where mean amplitude was 0.90 ± 0.43 mV (n = 10) in controls and 0.56 ± 0.34 mV (n = 9) for LPS-treated neurons (fig. 3c). Latencies were also the same between groups (fig. 3c), with control neurons displaying their peak sAHP at 412.5 ± 58.7 ms compared to 395.9 ± 76.0 ms in LPS-treated neurons (n = 4 and 3 in control and LPS-treated neurons, respectively). Again, these differences were not found to be significant by t-test (p = 0.540 for amplitude and 0.867 for latency).

Membrane resistance to hyperpolarizing steps (fig. 4a) did not go unaffected by chronic LPS exposure. Regression analysis showed that the data were highly linear (r^2 = 0.998 in control, 0.999 in LPS), and therefore that using a single slope value to compare membrane resistances was valid. Membrane resistance in neurons from control cultures displayed a resistance of 267.8 ± 3.1 MΩ (n = 18), compared to 227.5 ± 2.2 MΩ in LPS-treated (n = 16; fig. 4b). Variance in membrane resistance reported here is standard deviation as opposed to S.E.M. The difference in membrane resistance was shown to be significant by t-test (p < 0.001). To ensure that the observed difference was not a result of the rectifying current I_h, amplitude of this current was measured at each stimulus level,

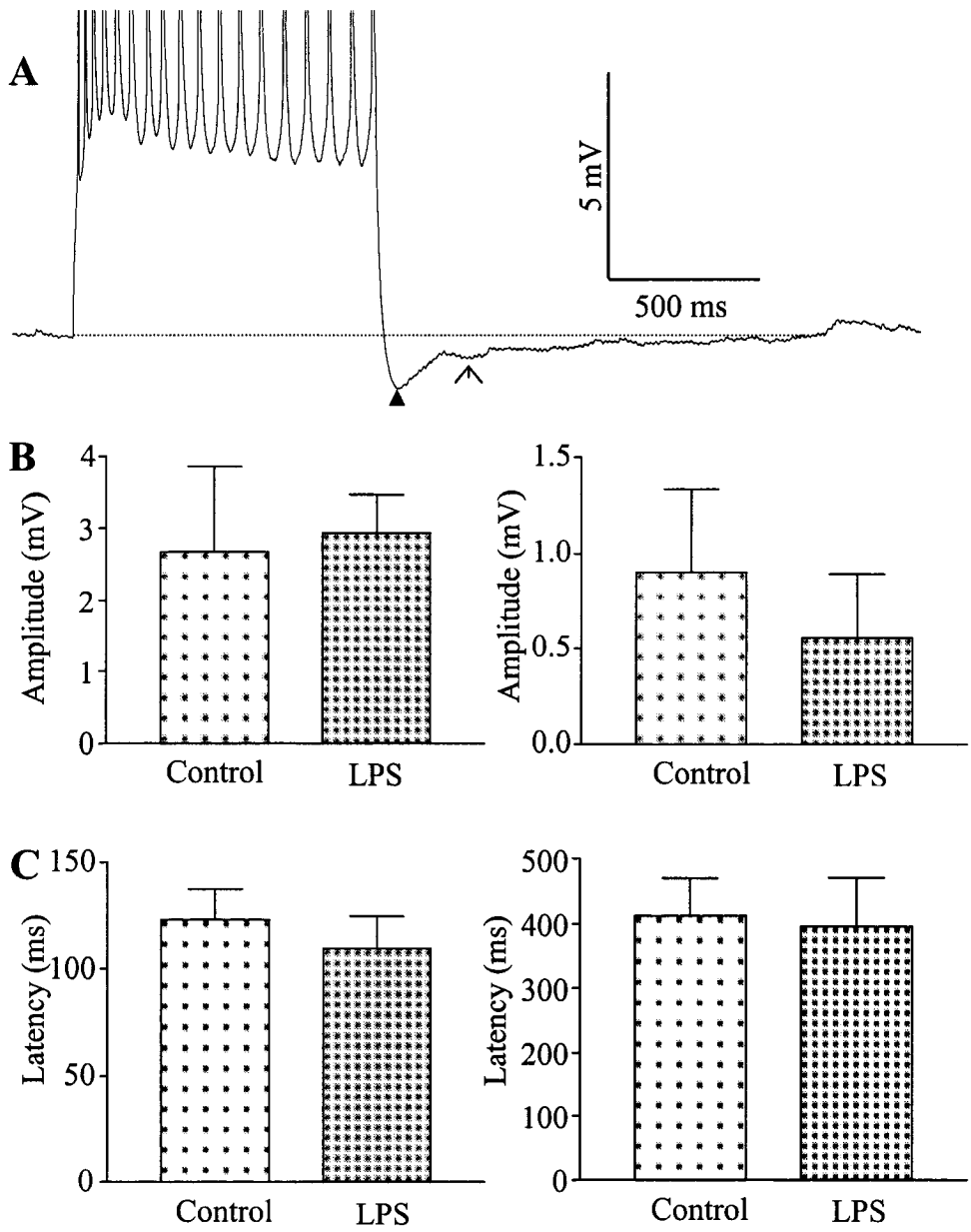

Figure 3: No effect of chronic LPS on afterhyperpolarization potential (AHP) properties. (A) Representative trace showing AHP (action potentials truncated). Measurements of fast AHP (fAHP) amplitude and latencies were taken at solid arrowhead, slow AHP (sAHP) at the open arrow. (**B**) Graphs depicting average amplitude of fAHP (left) and sAHP (right). Independent t-tests showed no significant difference between groups ($p = 0.853$ and 0.540, respectively). (**C**) Graphs depicting average latencies of fAHP and sAHP (as above). Again, no significant difference was detected between groups ($p = 0.554$ and 0.867).

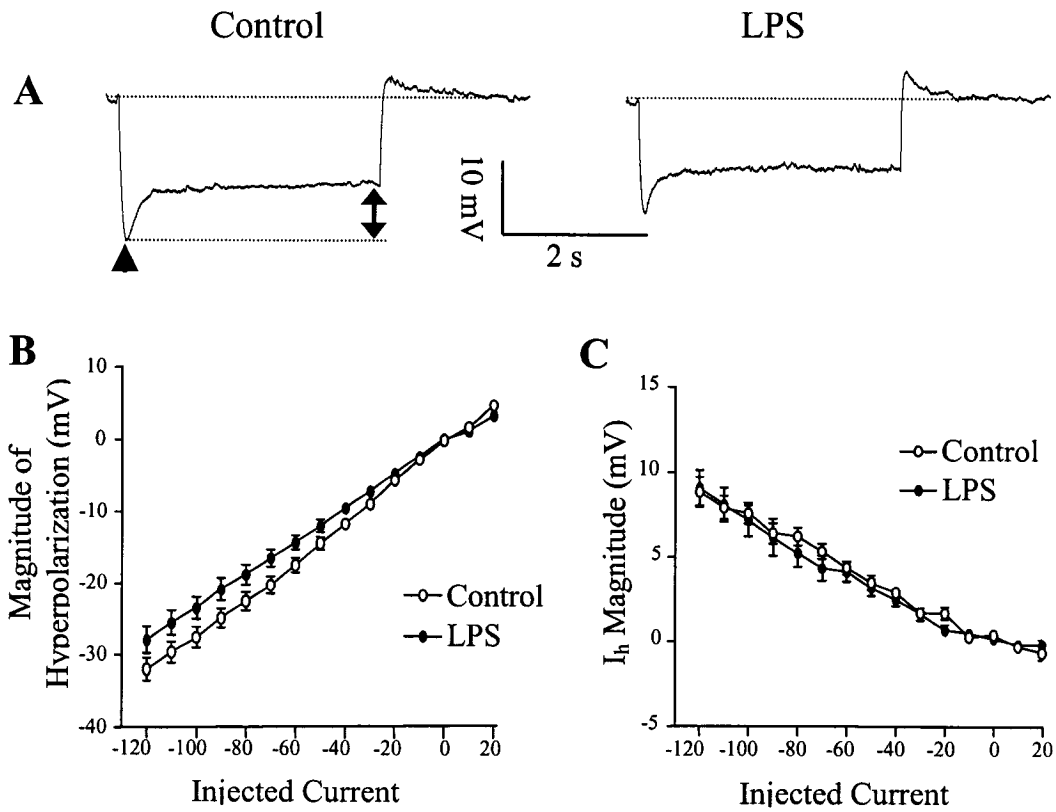

Figure 4: Effect of 7-day LPS exposure on membrane resistance. (**A**) Representative traces of membrane response from -60 mV to -60 pA hyperpolarizing pulse. Arrowhead represents where measure of amplitude taken, arrows show measurement of I_h current magnitude. (**B**) Current-voltage graph showing mean amplitude of membrane response to level of current injected in all neurons recorded. Linear regression shows slopes (membrane resistance) are significantly different (267.8 ± 3.1 MΩ in controls compared to 227.5 ± 2.2 MΩ in LPS-treated, $p < 0.05$). (**C**) Graph of mean I_h magnitude for all recorded neurons as a function of injected current. No significant difference is found between groups.

and the results analyzed by way of two-way ANOVA. This analysis showed no effect of LPS treatment on I_h magnitude (fig. 4c), showing that the difference in membrane resistances is not due to this particular confound.

Similarly, cultures treated with LPS show a difference in action potential discharge (see fig. 5a). Chronic LPS treatment significantly attenuated discharge frequency in response to stimuli from 80 to 140 pA, but not at higher or lower levels ($p < 0.05$; fig. 5b). This result indicates that LPS-exposed neurons are capable of achieving firing rates similar to those seen in control cultures, they require a higher current level to do so. A blockade of this effect was attempted using the IL-1 receptor antagonist IL-1Ra. This blockade was unsuccessful, causing a further reduction in AP discharge frequency, an attenuation that was also apparent when cultures were incubated with IL-1Ra alone. Simple main effects revealed the attenuation of firing frequency by IL-1Ra exposure was significant for stimuli of 100 pA and greater ($p < 0.05$). The ANOVA found no significant interaction between the effects of IL-1Ra exposure and LPS exposure ($p = 0.642$), suggesting that the two effects are independent and achieved through different mechanisms.

Since membrane resistances were found to be different between LPS-treated and control neurons, it is reasonable to assume that voltage changes in control and LPS-treated neurons may not be equal between groups for the same level of current. Using the resistances calculated previously, it is demonstrable that an injection of 110 pA in controls is almost equal to a 130 pA depolarization in LPS-treated neurons. These two levels show a firing frequency of 7.6 ± 1.1 Hz ($n = 6$) and 5.2 ± 1.3 Hz ($n = 9$). These two values are not significantly difference by t-test, but are disparate enough to support the

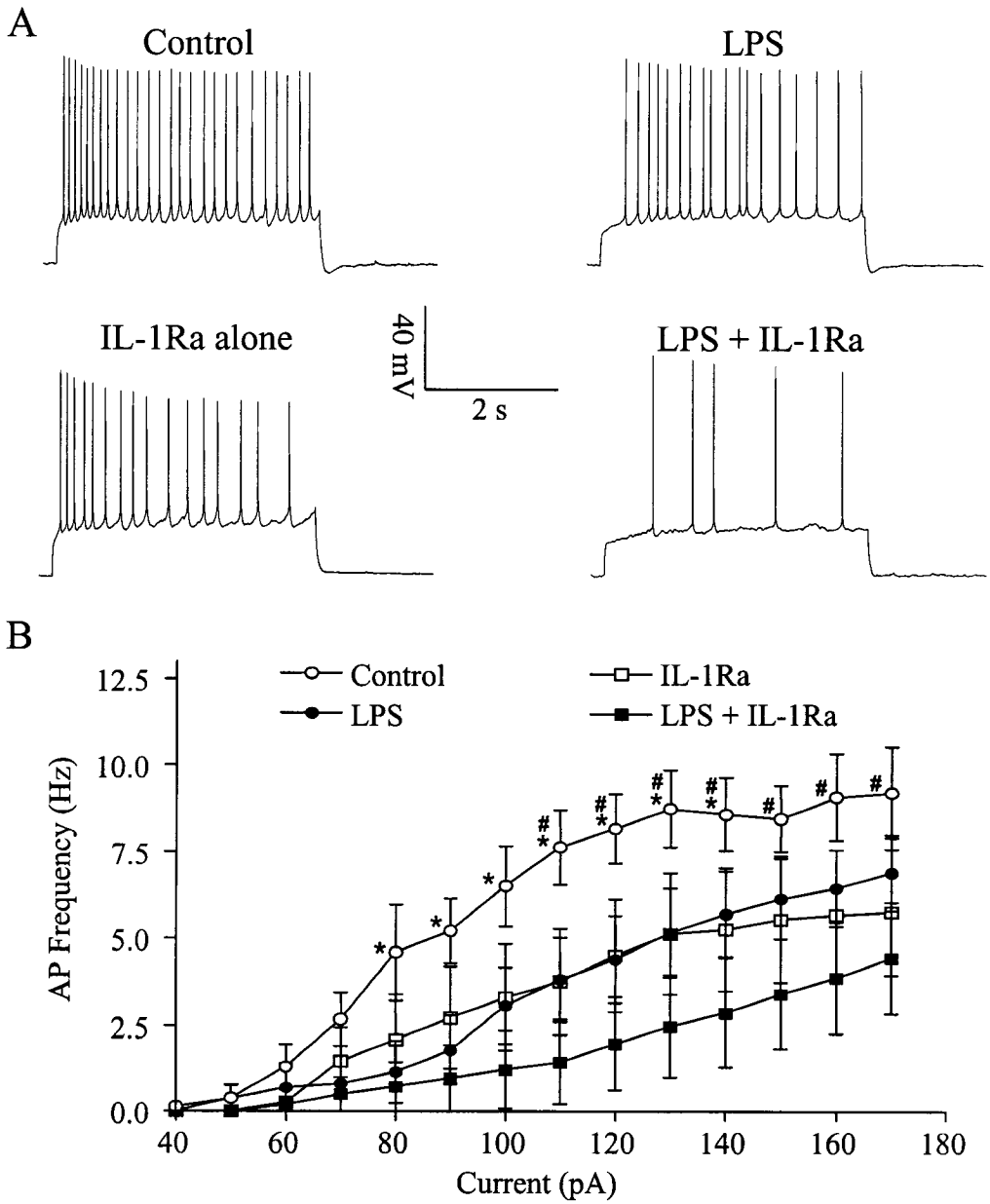

Figure 5: Effect of LPS and IL-1Ra on action potential firing frequency. (**A**) Representative traces showing firing frequencies in response to 110 pA depolarizing current for 4 s. (**B**) Graph of mean firing frequency ± S.E.M. for all four groups. (*) indicates points where a significant effect of LPS is found by three-way ANOVA ($p < 0.05$), where (#) indicates significant effect of IL-1Ra ($p < 0.05$). No interaction between LPS and IL-1Ra effects is detected ($p = 0.642$).

idea that the effect on frequency extends beyond the effect on membrane resistance. More substantially, analysis of threshold for action potential firing showed that threshold in control neurons was -38.0 ± 1.74 mV (n = 6) compared to -31.8 ± 1.06 mV in LPS-treated neurons (n = 9; data not shown). In IL-1Ra-treated neurons, threshold was -33.7 ± 2.0 mV (n = 6), and those co-incubated with IL-1Ra and LPS showed a mean threshold of 32.1 ± 1.9 mV (n = 7). These data were analyzed by two-way ANOVA, which indicated a significant effect of LPS ($p = 0.0268$), but no effect of IL-1Ra ($p = 0.239$) or interaction between the two factors ($p = 0.177$). This result demonstrates that action potential firing is elicited at more depolarized membrane potentials in neurons from LPS-treated cultures than in controls, and that chronic LPS exposure attenuates action potential generation independently of membrane resistance. Additionally, this result supports the independence of the IL-1Ra and LPS effects on action potential firing frequency.

4.4 LPS potentiates synaptic transmission

The synaptic properties of CA1 neurons were altered by chronic LPS treatment. Threeway ANOVA indicated a significant interaction of the effects of LPS, IL-1Ra and current level on EPSC amplitude normalized to 100%, where 100% represents the maximal response of the given neuron ($p < 0.0001$). Although maximal responses between groups were similar (see table 3), LPS-treated neurons displayed a saturated EPSC response at significantly lower stimulus levels than control neurons (fig. 6b). Simple main effects showed that EPSC magnitude was significantly potentiated by LPS exposure in all neurons at stimulus levels from 60 to 80 μA (average 58.3% closer to maximum amplitude; all ps < 0.05). LPS produced a potentiation in untreated neurons at stimulus levels from 30 to 80 pA (mean 45.0% closer to maximal response; all ps < 0.05), and from 60 to 100 pA in neurons co-incubated with IL-1Ra (average 66.9% closer to maximum; all ps < 0.05).

Simple main effects also showed significant inhibition of EPSC magnitude as a result of IL-1Ra treatment. This agent seemed to reverse the LPS effect found at the 30 to 60 pA stimulus levels (average 8% difference from controls, 39.8% further from maximum than LPS alone; all ps < 0.05). However, it also caused a significant decrease in EPSC magnitude in control neurons at stimulus levels from 60 to 100 pA (mean 50.8% further from saturation than control neurons; all ps < 0.05). These results indicate that LPS and IL-1Ra have independent, but opposite effects on EPSC magnitude.

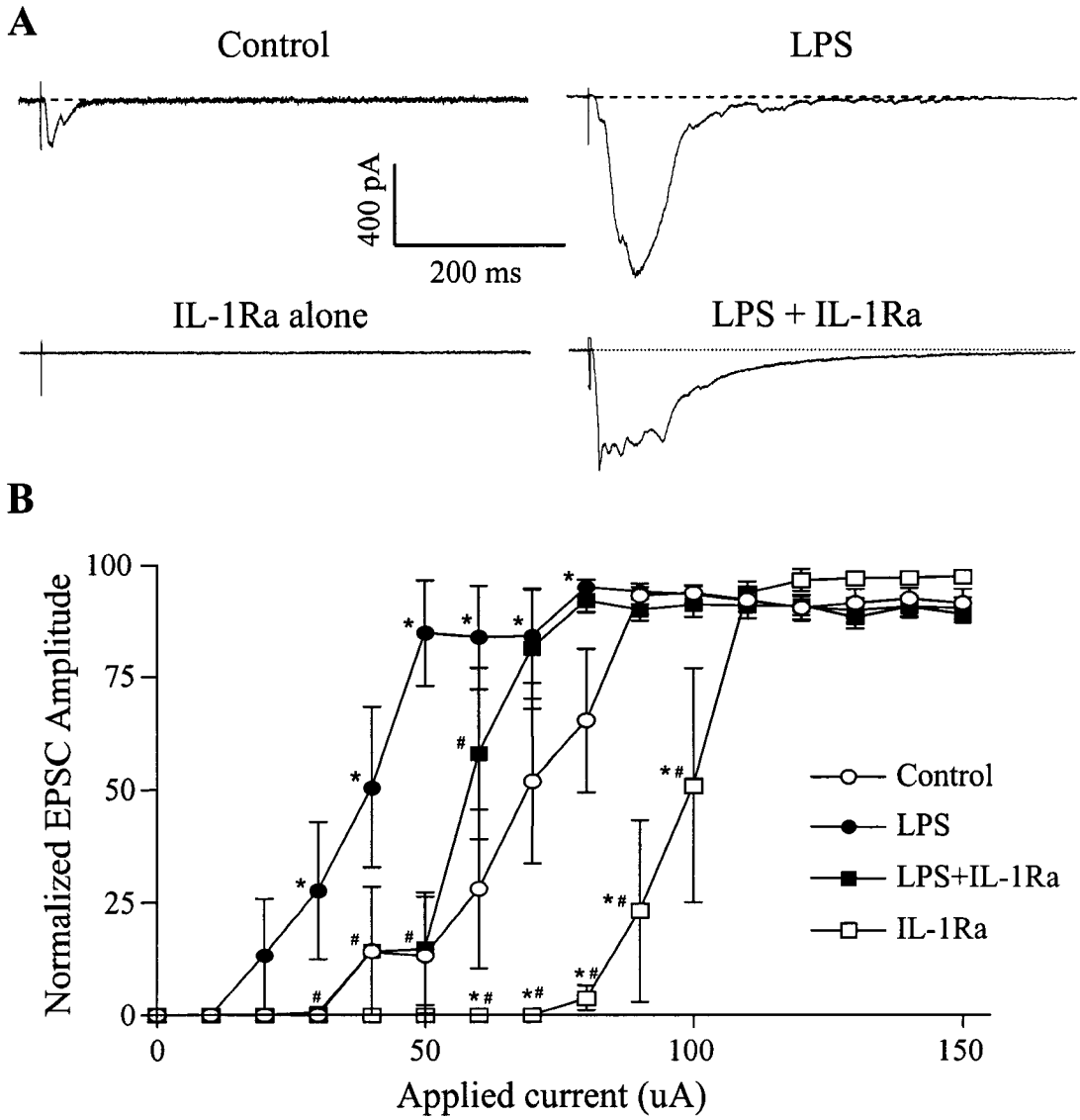

Figure 6: Chronic LPS treatment induces greater EPSC amplitude normalized to 100% at intermediate stimulus levels. (**A**) Representative voltage-clamp traces depicting EPSC amplitude from a holding potential of -50 mV in response to 120 pA stimulation to afferent fibres, as shown by arrow (stimulus artefacts truncated). (**B**) Mean normalized EPSC amplitude for all neurons recorded. Star indicates significant potentiation of EPSC induced by 7 days of 100 ng/ml LPS (all ps < 0.05). Hash indicates significant inhibition of EPSC amplitude due to IL-1Ra (50 ng/ml; all ps < 0.05).

Treatment	EPSC amplitude (pA)			IPSP amplitude (mV)		
	Maximum	S.E.M.	n	Maximum	S.E.M.	n
Control	1125.4	189.1	7	8.86	1.24	8
LPS	1193.7	262.7	8	9.93	1.46	7
LPS + IL-1Ra	872.2	127.0	7	7.40	1.61	7
IL-1Ra	724.2	224.2	4	9.47	2.55	6

Table 3: Mean maximum amplitudes of EPSCs and IPSPs in all recorded neurons, with standard error and number of subjects. No significant differences between groups were detected by threeway ANOVA.

Similarly, IPSPs in all groups examined had similar maximal amplitudes (see table 3). IPSPs evoked at intermediate stimulus levels showed augmentations in neurons from LPS-treated cultures at intermediate stimulus levels. Despite these differences, threeway ANOVA showed no significant interaction terms, although the threeway interaction between LPS, IL-1Ra and current level approached this level ($p = 0.0915$). In fact, as shown in figure 7b, analysis by simple main effects did show strong significant differences in IPSP saturation induced by LPS among neurons untreated with IL-1Ra at the 60 to 120 pA and 160 pA levels (average 20.5% closer to maximum, all $ps < 0.05$). No such effect was found for neurons treated with IL-1Ra, indicating that this potentiation was completely reversed. IPSP magnitude in neurons from cultures treated with IL-1Ra alone were not different from controls, showing a significant effect only on neurons treated with LPS at the 60 to 120 and 160 pA levels (all $ps < 0.05$). It is therefore likely that LPS effects on IPSP magnitude are the result of actions mediated by IL-1RI, which are blocked by excess IL-1Ra administration.

The reversal potential of the chloride ion, the principal mediator of the GABAergic IPSP response (Streit *et al.*, 1989), was also examined in LPS-treated and control cultures (figure 8). Mean reversal potential of chloride in control cultures was

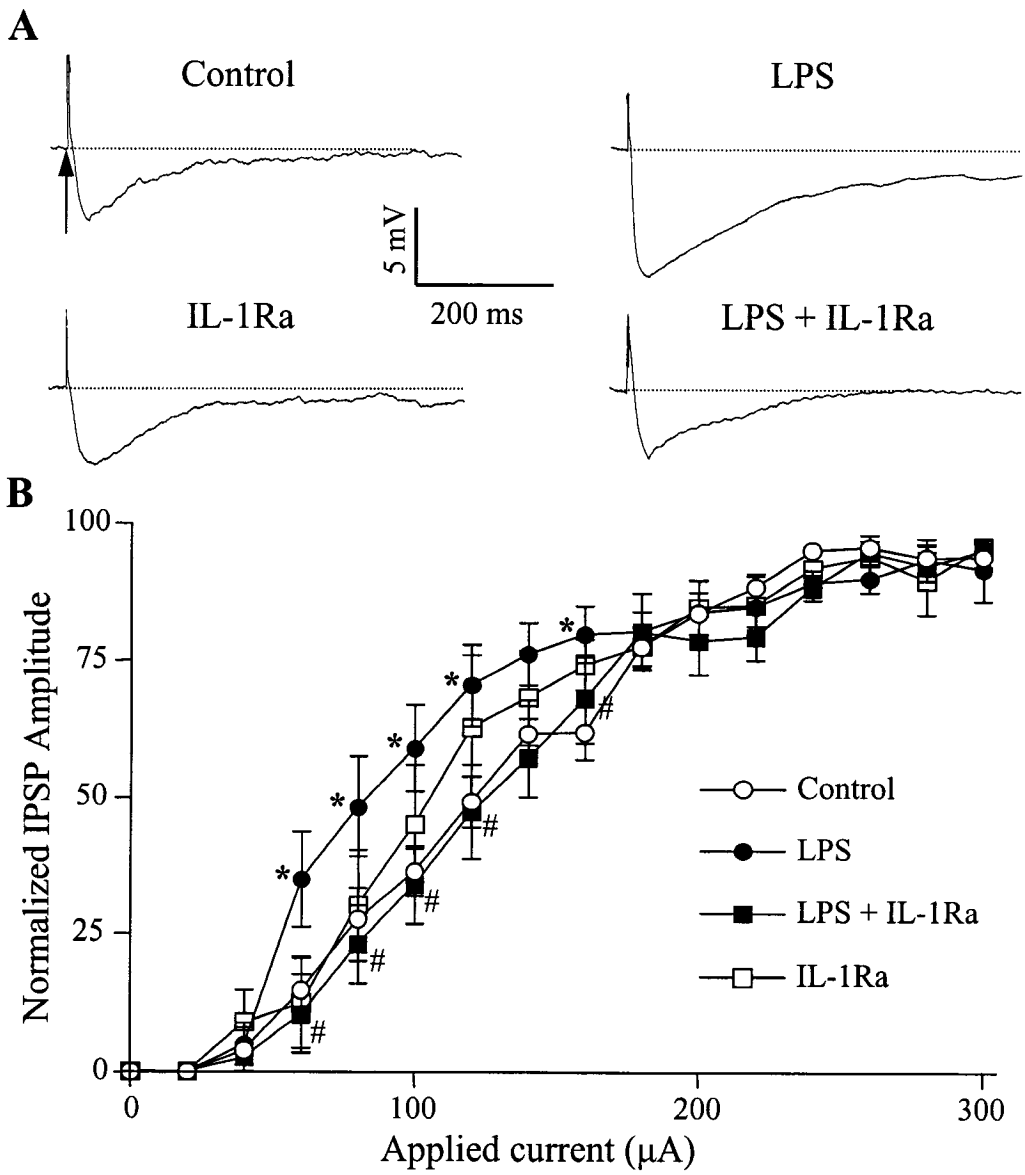

Figure 7: Chronic LPS treatment induces greater IPSP amplitude normalized to 100% at intermediate stimulus levels. (**A**) Representative current-clamp traces depicting IPSP amplitude from a holding potential of near -50 mV in response to 120 pA stimulation to afferent fibres, as shown by arrowhead (stimulus artefacts truncated). (**B**) Mean normalized IPSP amplitude for all neurons recorded. Star indicates significant potentiation of IPSP induced by 7 days of 100 ng/ml LPS (all ps < 0.05). Hash indicates significant reversal of LPS effect when co-incubated with IL-1Ra (50 ng/ml; all ps < 0.05).

71.3 ± 2.0 mV (n = 10), compared to 71.8 ± 2.0 mV in LPS-treated cultures (n = 11). This difference was not significant ($p = 0.853$). This result demonstrates that the observed change in IPSP magnitude is not a result of increased driving force on the ion mediating the response.

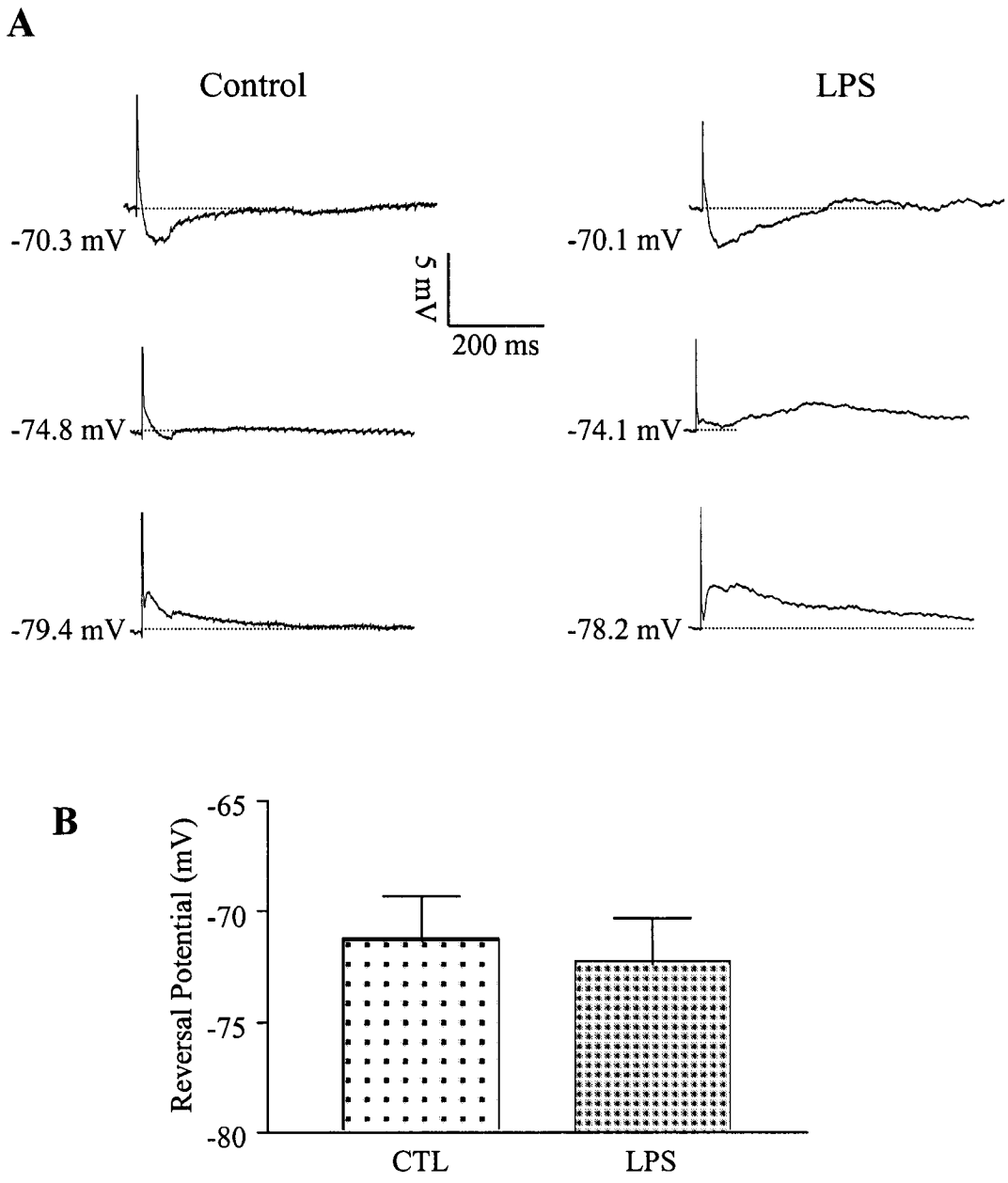

Figure 8: No effect of chronic LPS on chloride reversal potential. (**A**) Representative current clamp traces of responses to 300 μA stimuli at various membrane potentials as noted on left side of trace. Stimulus artefacts truncated. Note reversal of chloride-mediated change in potential. (**B**) Histogram of mean reversal potential for all neurons recorded. Mean calculated membrane potential in control neurons was -71.3 ± 2.0 mV (n = 10) compared to -71.9 ± 2.0 mV in LPS-treated neurons (n = 11). This is not a significant difference (p = 0.853 by independent t-test).

4.5 LPS induces IL-1-dependent IL-6 release

These results clearly demonstrated that chronic LPS administration has effects on electrophysiological properties of CA1 pyramidal neurons, and furthermore that some of these effects are IL-1-dependent. In the brain, IL-1β can act either directly, or through secondary mediators such as IL-6 (Luheshi, 1998). To examine the link between IL-1β and IL-6 in the inflammation of these cultures, a second ELISA was performed, this time for IL-6. This experiment was performed on cultures exposed to LPS, and those which were co-incubated with LPS and IL-1Ra, as well as their appropriate controls. As shown in figure 9, IL-6 levels in culture media showed a tenfold increase during the first two days of LPS exposure, from 75.2 ± 8.4 pg/ml in controls ($n = 6$) to 786.2 ± 163.2 pg/ml. Simple main effect analysis showed this increase to be highly significant ($p < 0.0001$). This release was significantly attenuated (206.5 ± 16.3 pg/ml) when cultures were co-incubated with IL-1Ra ($n = 6$; $p = 0.0001$). However, IL-1Ra did not abolish all IL-6 release from cultures, as LPS still induced a significant increase in the levels of this cytokine in comparison to cultures treated with IL-1Ra alone (21.7 ± 10.2 pg/ml; $n = 5$; $p = 0.0454$). No significant differences in IL-6 existed between groups after 2 days exposure to LPS ($p = 0.625$ at 4 days, $p = 0.611$ at 7 days).

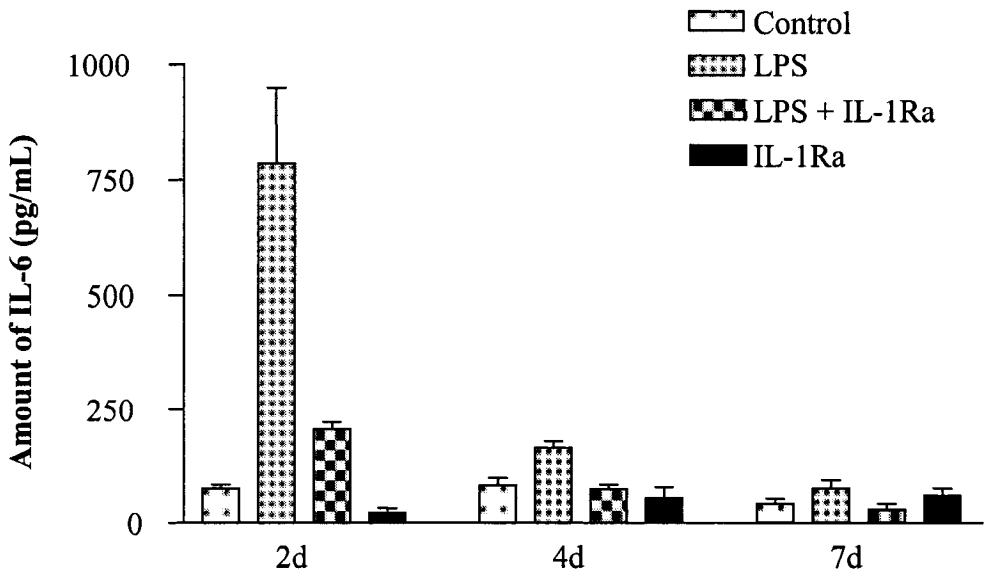

Figure 9: IL-6 release from organotypic hippocampal culture over 7-day exposure to LPS as measured by ELISA. Amount of IL-6 released into culture media over time periods specified is shown. LPS treatment significantly elevated IL-6 over the first two days cultures were treated with LPS, as indicated by stars ($p < 0.05$). Conicubation of LPS with IL-1Ra significantly attenuated IL-6 release, as indicated by hash ($p = 0.0001$).

5. Discussion

The current study was undertaken to examine the effects of chronic cytokine-mediated inflammation on baseline synaptic and intrinsic neuronal properties. Inflammatory conditions modeled in the organotypic hippocampal slice culture through seven days of exposure to 100 ng/ml LPS induced a dramatic increase in the levels of pro-inflammatory cytokines IL-1β and IL-6. This treatment also induced alterations in the electrophysiological properties of CA1 pyramidal neurons. As evidenced by a significant decrease in membrane resistance (fig. 4) and in frequency of action potential discharge (fig. 5), intrinsic neuronal excitability is attenuated after chronic inflammatory treatment. However, increased synaptic excitability also resulted from this treatment, as evidenced by a potentiation in normalized EPSC and IPSP magnitudes. Since the development of these cultures closely parallels that *in vivo* with respect to electrophysiological properties (De Simoni *et al.,* 2003), and LPS does indeed induce potent increases in pro-inflammatory cytokine release, this technique has proven to be a good model of chronic inflammation of the rat hippocampus. Although some argument may be made that cultures prepared at P(8) may differ significantly from those prepared at P(10), it must be noted that untreated neurons were also of a similar age, providing appropriate controls. Secondly, the requirements for proper development of the organotypic slice appear to be in place by P(5) (de Simoni *et al.*, 2003), suggesting any potential difference that may exist will be negligible after 14 days *in vitro*, when recordings were made.

5.1 LPS Induces Pro-Inflammatory Cytokine Release

The results of the current study indicate that LPS induces a dramatic rise in IL-1β release from the rat organotypic hippocampal slice culture. This is a primary modulator

of LPS effects (e.g. Vereker *et al.*, 2000a). Furthermore, LPS also induced a striking increase in IL-6 release into culture media, which was partially IL-1-dependent since it was significantly blocked by the IL-1R antagonist IL-1Ra. However, it should also be noted that the amount of IL-6 detected by ELISA in the co-incubation of LPS with IL-1Ra remained significantly elevated from control levels, despite being significantly lower than levels induced by LPS alone (fig. 1). This may be due to either direct induction of IL-6 release by LPS, or through the actions of other pro-inflammatory cytokines such as TNFα, which is also elicited by LPS administration (Nadeau & Rivest, 1999).

The concomitant upregulation of IL-1 and IL-6 induced by LPS treatment and the inhibition of IL-6 release when cultures were co-incubated with LPS and IL-1Ra suggest that some of the effects observed in the current study may be mediated by IL-6. Since IL-1Ra was able to reverse the effects of LPS on IPSP magnitude, and potentially has a role in EPSC modulation, it is possible that IL-6 is an important downstream modulator of the changes observed in chronic LPS exposure. This question would be well-addressed through the co-incubation of LPS with neutralizing antibodies or antiserum for IL-6, and the subsequent investigation of electrophysiological properties, specifically IPSP amplitude.

5.2 Chronic LPS Affects Intrinsic Properties of CA1 Pyramidal Neurons

Among the most fundamental intrinsic neuronal properties is the change in membrane potential caused by a given injection of current. This relationship is known as membrane resistance, and was demonstrated to be significantly different in neurons from LPS-treated cultures in comparison to controls (fig. 4b). This indicates that neurons exposed to chronic inflammatory conditions show less change in membrane potential

during an injection of hyperpolarizing current. It must be noted that this difference in membrane resistance may not be apparent for depolarizing currents, as voltage-gated currents intrinsic to the membrane generally render the membrane response to depolarization non-linear. Thus, the effect of chronic LPS on membrane response to depolarization may differ significantly from that reported here for hyperpolarizing current.

This difference in membrane resistance may be due to a number of mechanisms. One of the most obvious manners by which this could occur is an alteration in cell size, thus requiring a larger injection of current to produce an equal change in membrane potential. This has been shown in previous studies of morphological changes, such as increases in the number and/or length of dendrites (Liu *et al.*, 2000; Zhu, 2000), and may be apparent in neurons from LPS-treated cultures. Additionally, an increase in the number in potassium channels open at rest could produce this phenomenon (Lesage, 2003).

In addition to this modulation of membrane resistance, it was also found that chronic LPS significantly attenuated the frequency of AP discharge during a depolarizing step (fig. 5b), similar to results in other models of exposure to cytokines involved in the inflammatory response (Nelson *et al.*, 2002). There are a number of factors that may explain this change in firing frequency in neurons of LPS-treated slices. Firstly, it is clear that this effect is not dependent on AP duration, as this property is not significantly different between groups (fig. 2d).

AP frequency can be modulated by certain subliminal currents such as the AHP current. Inhibition of AHP leads to a shortening of the interspike interval, thus increasing

discharge frequency (Lancaster & Nicoll, 1987). However, AHP currents were investigated in two different paradigms, and neither showed any differences in AHP amplitude or peak latency (fig. 3). Though the current study used a pipette solution containing a high concentration of gluconate, a compound known to reduce AHP (Zhang *et al.*, 1994), the results nonetheless show that the AHP is an unlikely source of this difference.

The observed difference in membrane resistance must also account for some of the effect on AP discharge frequency shown in the current study. Since membrane potential changes less in response to hyperpolarizing currents, it must be assumed that a similar effect would underlie depolarizations, and would therefore account for some of the observed attenuations. Using the resistances calculated previously by linear regression (fig. 4b), Ohm's law indicates that an injection of 110 pA in controls produces a change in potential similar to a 130 pA depolarization in LPS-treated neurons. These two levels show a firing frequency of 7.6 ± 1.1 Hz (n = 6) and 5.2 ± 1.3 Hz (n = 9), respectively. Although these two values are not significantly different by t-test, they are disparate enough to suggest that the effect on frequency may extend beyond the effect on membrane resistance.

Other ionic currents, such as potassium shunts, could also be responsible for this attenuation. Early voltage-gated potassium currents such as I_A are active during the first several hundred milliseconds of depolarization, and can impede the membrane potential from reaching threshold levels, and thus delay AP generation (Byrne, 1980). However, analysis of AP frequency calculated for the first 2 s of depolarization did not have a

larger effect size than overall frequency, which would be the case if I_A were indeed the source of the decrease in firing frequency.

It was also investigated if IL-1β was responsible for the reduction of AP frequency observed. To assess this hypothesis, cultures were co-incubated with LPS and IL-1Ra. However, this treatment did not reverse the change observed in AP frequency during depolarization with chronic LPS incubation, and in fact seemed to further attenuate spike discharge (fig. 5b). Experiments where cultures were incubated with IL-1Ra alone confirmed that this agent induced a significant attenuation of firing frequency by itself. The effects of IL-1Ra and LPS were statistically independent of each other, implying that they occur by different mechanisms.

In addition, an analysis of AP firing threshold showed a significant effect of LPS, but no effect of IL-1Ra. This effect further supports the existence of LPS effects on AP generation from that of IL-1Ra, although the mechanisms of this effect remain to be determined. The fact that threshold is significantly higher in neurons from LPS-treated cultures could partly account for the attenuated firing frequency reported in this study. One important conclusion that can be drawn from this result is that this LPS effect is not IL-1β-dependent, and therefore likely mediated by other cytokines such as TNFα, or even anti-inflammatory cytokines upregulated by LPS treatment, such as IL-10 (Kitamura *et al.*, 2000). One potential species responsible for this change may be nitric oxide (NO), a compound known to be elevated during inflammation (DiGirolamo *et al.*, 2003). NO-dependent signaling has been previously shown to reduce voltage-dependent Na^+ channels through cGMP-dependent mechanisms, and through s-nitrosylation of voltage-gated sodium channels (Ahern *et al.*, 2002).

One interesting note is the observation of the reduction in AP firing in the presence of IL-1Ra alone. It has recently been demonstrated that IL-1Ra has agonist effects of its own, independent of IL-1 receptors (Loscher *et al.*, 2003). This as yet undefined mechanism may be responsible for the IL-1Ra effects on AP frequency shown in this study.

These results show that individual neurons exposed to chronic inflammation induced by seven days of 100 ng/ml LPS show reduced excitability through the decrease of membrane resistance and attenuation of action potential discharge frequency. Additionally, IL-1Ra has a similar but independent effect on AP discharge frequency. In LPS-treated cultures, these changes occur without the alterations of several other intrinsic electrophysiological properties. For example, membrane potential is not significantly different between groups (fig. 2a). This may indicate that cells are equally healthy between groups. Although cell death was not assessed in these studies, it is reasonable to assume that cells accepted for analysis were not in an apoptotic condition, as depolarized resting membrane potentials would be expected to accompany such a process. Similarly, dying cells would also exhibit smaller action potential amplitudes and shorter durations (Laiwand *et al.*, 1988). No such significant differences existed in either of these parameters in LPS-treated neurons compared to controls (fig. 2c, 2d). However, rejection criteria did bias the samples used towards uniformity of the AP, which does not allow for the conclusion that slices were equally healthy.

5.3 Chronic LPS Produces Increases in Synaptic Inhibition

Preliminary experiments designed to examine whether LPS exerts effects on synaptic properties indicated that an increase in polysynaptic IPSP amplitude was

induced by chronic inflammatory treatments. This result was pursued by an investigation of monosynaptic IPSP amplitude, isolated from the potential interference of glutamatergic events through the blockade of AMPA/kainate receptors (DNQX, 20 μM) and NMDA receptors (D,L-APV, 100 μM). Normalized monosynaptic IPSP amplitude was significantly potentiated by chronic LPS treatment at intermediate levels of stimulation (figure 7). Simple main effects indicated that LPS induced higher amplitude of IPSP response at intermediate stimulus levels, an effect that was blocked by the IL-1RI antagonist IL-1Ra. No effects on IPSP amplitude were observed when incubating IL-1Ra alone. Although the overall ANOVA indicates no three-way interaction, there are clearly interacting effects of IL-1Ra and LPS on IPSP magnitude that would be revealed after a slight increase in subject number. These results strongly support the idea that the effect of LPS on IPSPs is IL-1β-dependent. These changes in IPSP amplitude are not due to changes in $GABA_A$ receptor-mediated chloride influx since the chloride reversal potential is the same between groups (see figure 8).

Possible mechanisms for this change include an upregulation in postsynaptic $GABA_A$ receptor expression, an alteration in the receptor's affinity for GABA, and/or a presynaptic potentiation of GABA release. Further experiments investigating the nature of spontaneously released miniature IPSPs may help reveal whether the LPS-mediated increase in IPSP amplitude is presynaptic or postsynaptic in nature.

This effect on IPSP amplitude seems to be directly mediated by IL-1 because of the selective blockade achieved during IL-1Ra co-incubation with LPS. Furthermore, this result is similar to previous electrophysiological studies of GABAergic function after acute IL-1β application (Wang *et al.,* 2000; Zeise *et al.*, 1992). Although there are

conflicting reports on the effects of IL-1β in models of excitotoxicity (Pringle *et al.*, 2001; Ma *et al.*, 2003), these findings could represent a protective mechanism designed to limit cell death due to excitatory signaling during an inflammatory response.

5.4 Chronic LPS Produces Increases in Glutamatergic Synaptic Responses

Similar to the effects on the IPSP, preliminary results indicated that polysynaptic EPSC magnitudes were also potentiated by chronic inflammatory treatment. This result was followed by an examination of EPSC amplitude during $GABA_A$ receptor blockade (bicuculline, 10 μM) to remove potential contamination of EPSC by GABAergic events. Unlike acute LPS treatments, which have been shown to inhibit NMDA currents (Jo *et al.*, 2001), the chronic LPS treatment in the current study induced a potentiation of EPSC magnitude at lower stimulation levels, as shown in figure 6. This treatment induced a significant potentiation of normalized EPSC magnitude by LPS in all neurons at intermediate stimulus intensities, whereas IL-1Ra significantly attenuated this increase. IL-1Ra alone had similar effects, making it difficult to conclude that this LPS effect is dependent on IL-1.

However, this effect of IL-1Ra may reflect some role of IL-1β in normal glutamatergic transmission, as postulated by some researchers (e.g. Schneider *et al.*, 1998). If IL-1β had a direct role in baseline EPSC generation, an increase in IL-1RI signaling could lead to a concomitant increase in EPSC amplitude. This would account for both the potentiation seen during LPS administration, and for the IL-1Ra reversal of this change over the majority of stimulus levels where an LPS effect was observed. In addition, it would provide for the reduction in EPSC amplitude observed after incubation with IL-1Ra alone through the blockade of endogenous IL-1β release.

These agents could also modulate presynaptic glutamate release. Previous studies have shown glutamate release to be inhibited by acute LPS exposure (Vereker *et al.*, 2000b). This finding is not likely to extend to chronic LPS exposure, as it cannot account for increased the EPSC amplitude seen in the current study. However, acute application of IL-1Ra has also been shown to inhibit glutamate release from synaptosomes (Loscher *et al.*, 2003), and is a possible mechanism for its inhibition of EPSC magnitude. LPS and IL-1Ra could also modulate AMPA/kainate receptor sensitivity, by increasing or decreasing glutamate binding site properties.

This effect may involve the actions of other cytokines involved in the inflammatory response. Chronic infusion of IL-6 in cerebellum has been shown to potentiate EPSC magnitude, which may be due to an increase in either NMDA receptor-mediated responses (Qiu et al., 1998) or AMPA responses (Nelson et al., 2001). Alternatively, the cytokine TNFα may be implicated, as it has been shown to increase both AMPA receptor expression at the synapse and AMPA-mediated responses (Beattie et al., 2002). For these reasons, it would be interesting to examine AMPA receptor expression at the synapse through immunohistochemical techniques. Analysis of spontaneously elicited miniature EPSCs may decipher if AMPA or NMDA receptor-mediated responses are altered, and would give an indication of whether postsynaptic mechanisms are involved.

Taken together, the results of this study indicate that a significant decrease in intrinsic neuronal excitability results from chronic inflammatory treatments as short as seven days, and an increase in inhibitory monosynaptic responses. These two factors could combine to decrease glutamatergic activity, sensitizing postsynaptic receptors to

glutamate release, accounting for the potentiation in EPSC magnitude induced by this treatment. Furthermore, this dose of LPS only caused a significant induction of pro-inflammatory cytokines over the first two days of exposure. This brief elevation in immune signaling causes persistent changes that would have important ramifications to CNS function during inflammation or disease, and during recovery from immune challenges.

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Appendix: Animal Use Protocol

DH file

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #:
Investigator #:
Approval End Date:
Facility Committee:

☐ Pilot ☒ New Application ☐ Renewal of Protocol #

Title (must match the title of the funding source application): Acute actions of cytokines in molecularly and physiologically defined septohippocampal neurons.

1. Investigator Data:

Principal Investigator: Sylvain Williams Office #: Caton/2126
Department: Psychiatry Fax#: 762-3034
Address: 6875 Lasalle blvd, Verdun Email: wilsyl@douglas.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Work #:	Emergency #:
Sylvain Williams	761-6131 xt5937	768-3596
Marc Danik	761-6131 xt2372	849-1734

3. Funding Source:

External ☒	**Internal** ☐
Source (s): NSERC	Source (s):
Peer Reviewed: ☒ YES ☐ NO**	Peer Reviewed: ☐ YES ☐ NO**
Status : ☒ Awarded ☐ Pending	Status: ☐ Awarded ☐ Pending
Funding period: 04/2001-03/2004	Funding period:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	01-07-2001	or ongoing ☐
Expected Date of Completion of Animal Use (d/m/y):	30-03-2004	or ongoing ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator:	Date:

Approval Signatures:

Chair, Facility Animal Care Committee:		Date:
University Veterinarian:		Date:
Chair, Ethics Subcommittee(as per UACC policy):		Date:
Approved Period for Animal Use	Beginning:	Ending:

☐ This protocol has been approved with the modifications noted in Section 13.

4. Research Personnel and Qualifications: List the names of all individuals who will be in contact with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). Indicate any training received (e.g workshops, lectures, etc.). The PI certifies that all personnel listed here have suitable training and/or experience, or will be provided with the specific training which qualifies them to perform the procedures described in the protocol. Each person listed in this section must sign to indicate that s/he has read this protocol. (Space will expand as needed.)

Name	Classification	Training Information	Signature
Marc Danik	Research a.	McGill Workshop	
Carole Puma	Post-doc	McGill Workshop	
Florence Sotty	Post-doc	McGill Workshop	

** If an undergraduate student is involved , the role of the student and the supervision received must be described.

5. Summary (In language that will be understood by members of the general public)

a) Rationale: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

The data obtained with these experiments are vital to our understanding on how certain peptides called cytokines and chemokines work in the brain and how they modulate the electrical activity of neurons.

b) Specific Objectives of the Study: Summarize in point form the primary objectives of this study.

To characterize the intracellular pathways used by cytokines to modulate synaptic activity using electrophysiology and imaging.

c) Progress Report: If this is a renewal of an ongoing project, **briefly** summarize what was accomplished during the prior approval period and indicate if and how the current goals differ from those in the original application.

Not applicable

d) Summary of Procedures for Animal Use Report to the CCAC : Using **key words only,** list the procedures used (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). Refer to Appendix 1of the Guidelines for a more complete list of suggested key words.

sacrifice by decapitation, Tissue collection

6. Animals To Be Used

a) Purpose of Animal Use (Check one):

1. ☒ Studies of a fundamental nature/basic research
2. ☐ Studies for medical purposes relating to human/animal diseases/disorders
3. ☐ Regulatory testing
4. ☐ Development of products/appliances for human/veterinary medicine

b) Will the project involve breeding animals? NO☒ YES ☐
Will the project involve the generation of genetically altered animals? NO ☒ YES ☐
Will field studies be conducted? NO ☒ YES ☐

c) Description of Animals

	Species 1	Species 2	Species 3	Species 4	Species 5	Species 6
Species	Rat	Rat				
Supplier/Source	Charles Rivers	Charles Rivers				
Strain	Sprague-Dawley	Sprague-Dawley				
Sex	Mother (with pups)	pups				
Age/Wt		13-18 day old pups				
# To be purchased	50 litters/year	200				

# Produced by in-house breeding	0	0				
# Other (e.g.field studies)						
#needed at one time	0	1 pups /day				
# per cage	1 litter					
TOTAL# /YEAR	**50**	**200 pups**				

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

7. Justification of Animal Usage

a) Please justify the number of animals requested for each species described above, **based on the experimental objectives of the project.** Include information on experimental and control groups, # per group, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable (space will expand as needed).

1 pup will be sacrificed per day, this will enable the collection of a sufficient number of neurons per experiments. Approximately 200 experiments, hence 200 pups are needed to obtain the necessary results and reach statistical significance for each study. Therefore, approximately 200 pups are needed to complete one project per year.

Test Agents or Procedures	# of Animals and Species Per Group	Dosage and/or Route of Administration	# of endpoints	Other variables (i.e. sex, weight, genotypes,etc.)	**Total number of animals**
e.g. 2 Drugs	6 rats	.03, .05 mg/kg - IM, IP (4 variables)	1, 7, 10 days (3 variables)	Male, Female groups (2 variables)	2 x 6 x 4 x 3 x 2 = 288

b) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).

For this type of experiments no methods other than tissue from live animals are available

c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

Young Sprague-Dawley rats are used because almost all electrophysiological experimental procedures were developed with that specie.

8. Animal Husbandry and Care

a) Special cages NO ☒ YES ☐ Specify:

Special diet NO ☒ YES ☐ Specify:

Special handling NO ☒ YES ☐ Specify:

b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ Specify:

c)

Multiple institution facility housing: NO ☒ YES ☐

Indicate all facilities where animals will be housed:	Building:	Lehman animal housing facility, Douglas Hospital	Room No:
Indicate area(s) where animal use procedures will be conducted:	Building:	Lehman	Room No: 2125

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. **It is UACC policy that these SOPs be used when applicable.** Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/fgsr/rgo/animal/ . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection (UACC#1)	☐	Production of Monoclonal Antibodies (UACC#7)	☐
Anaesthesia (rodents) (UACC#2)	☐	Production of Polyclonal Antibodies(UACC#8)	☐
Analgesia (rodents/larger species) (UACC#3)	☐	Collection of Amphibian Oocytes (UACC#9)	☐
Breeding (transgenics/knockouts) (UACC#4)	☐	Rodent Surgery (UACC#10)	☐
Transgenic Generation (UACC#5)	☐	Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)	☐
Knockout/in Generation (UACC#6)	☐		☐

10. Description of Procedures

a) **For each experimental group, describe all procedures and techniques in the order in which they will be performed** - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. **If a procedure is covered by an SOP, write "as per SOP", no further detail is required.** Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.

Rats will be rapidly decapitated, the brain cooled and then quickly removed from skull. The brain is then cut into slices, and the slices incubated in an enzyme for cell dissociation. Individuals neurons are then recorded and imaged using electrophysiology and fluometers, respectively.

b) **Field Studies** – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.

Method of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:

Transportation and /or housing of animals in the field:

Special handling required:

Capture of non-target species, potential injury/mortality:

Will captured animals be released at or near the capture site YES ☐ NO ☐
If not, specify if they will be relocated to other locations and/or populations.

Describe any potential ecological disruption this study may cause:

It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

c) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort Table will expand as needed.

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency

d) Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed).

Species	Agent	Dosage (mg/kg)	Total volume (ml) per administration	Route	Frequency

e) Endpoints : 1) Experimental – for each experimental group indicate survival time .
2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).

Specify person(s) who will be responsible for animal monitoring and post-operative care
Name: **Phone#:**

f) Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical method of euthanasia without prior use of anaesthesia (justify here):

We will not use any anaesthesia to euthanize the young pups since these substances may alter the activity of the ionic channels we are measuring experimentally. Mothers and unused pups will be sacrificed in CO2 chamber.

Specify Species	
	☐ anaesthetic overdose, list agent/dose/route:
	☐ exsanguination with anaesthesia, list agent/dose/route:
pups	☒ decapitation without anaesthesia ☐ decapitation with anaesthesia,list agent/dose/route:
	☐ cervical dislocation
Mothers and unused pups	☒ CO_2 chamber
	☐ other (specify)
	☐ not applicable (explain)

11. Category of Invasiveness: B ☒ C ☐ D ☐ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments).* Please refer to this document for a more detailed description of categories:

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.

Category C: Studies or experiments involving minor stress or pain of short duration. These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.

Category D: Studies or experiments that involve moderate to severe distress or discomfort. These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals. **According to University policy, E level studies are not permitted.**

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. **A copy of these certificates must be attached, if applicable.**

No hazardous materials will be used in this study: ☒

a) Indicate which of the following will be used in animals:

☐Toxic chemicals ☐Radioisotopes ☐Carcinogens ☐Infectious agents ☐Transplantable tumours

b) Complete the following table for each agent to be used (use additional page as required).

Agent			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

c) After administration the animals will be housed in:

☐ the animal care facility
☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

d) Describe potential health risk (s) to humans or animals:

e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.