Supplementary Materials

Characterization of slice cultures grown in serum-free media. Characterization of slice cultures using serum-free media included their growth and use after 21 days *in vitro*, a point where culture development could be expected to mature to conditions most like their age-matched counterparts *in vivo* (for review see Kunkler et al., 2005).

We first examined whether elevated glucose was essential to neuronal content since after Pozzo Miller and coworkers (1994), we have grown serum-based cultures in 42 mM glucose. This might seem excessive when compared to the normal blood or cerebrospinal fluid levels of 6 mM seen *in vivo* or that used for Ringer's solution superfusion of acute brain slices (Dingledine, 1984). However, western blot analyses revealed significantly reduced neuronal NeuN levels at 6 mM glucose in serum-free media compared to control cultures grown in horse serum-based media (at 42 mM glucose) (Fig. S1). Furthermore, glial fibrillary acidic protein (GFAP) levels were significantly increased at 6 and 15 mM glucose, indicative of reactive change. This impact of reduced (i.e., 6 and 15 mM) glucose also was seen in cultures exposed to altered glucose levels from 21-42 days *in vitro* (data not shown). Microglia, characterized using digital image quantification of immunostaining (Caggiano and Kraig, 1996) for CD11b, showed no difference between slice cultures grown in horse serum or serum-free media. Thus 6 and 15 mM glucose is not sufficient for optimal growth and maintenance of slice cultures grown in serum-free media (as defined here). Rather, use of 24-42 mM glucose seems most appropriate for slice cultures. This glucose range has been established in previous studies (Noraberg et al., 1999; Pozzo Miller et al., 1994). Use of 42 mM glucose is further supported by evidence of improved function of slice cultures used in media at this glucose level (see Figure S5).

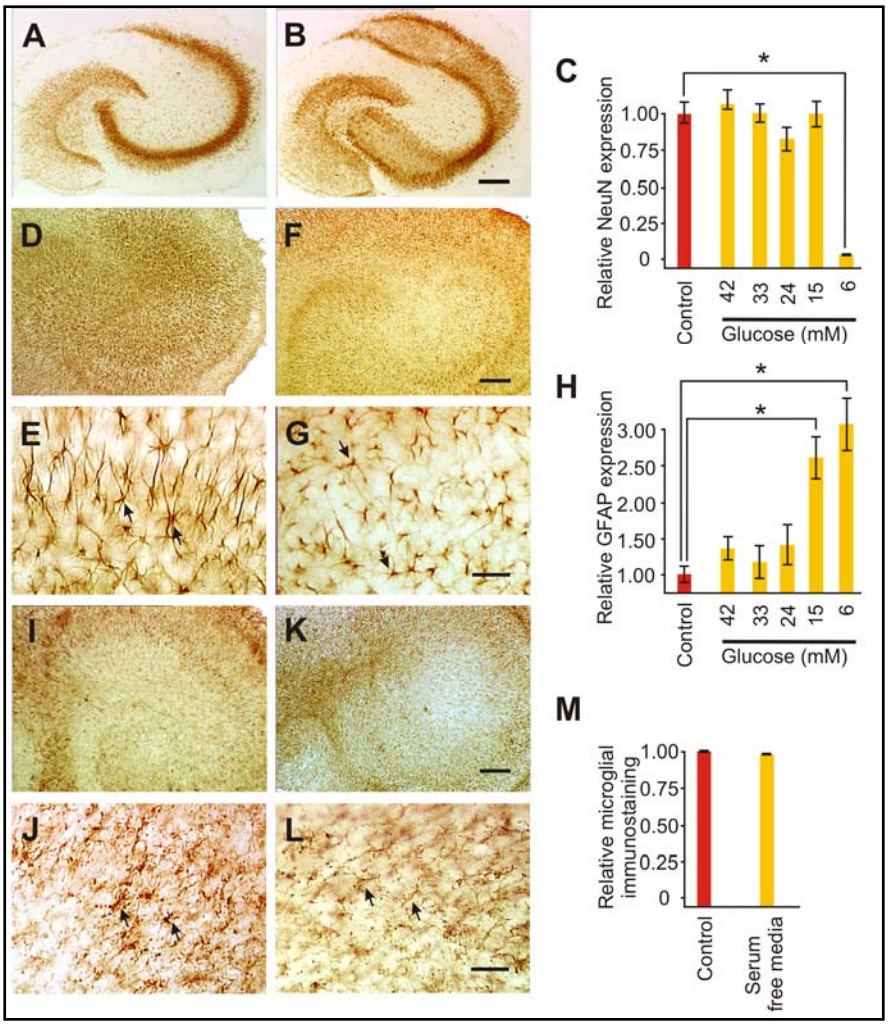

Figure S1. Effect of growth media glucose concentration on slice cultures. (A-C) NeuN immuno labeling was used to visualize principal neuron cytoarchitecture in slice cultures grown in horse serum (A) or serum-free media (B) and to quantify neuronal density via NeuN expression (Aptowicz et al., 2003) (C) in cultures grown in serum-free media (yellow) containing varying glucose concentrations. Only cultures grown in 6 mM glucose showed significantly less neuronal content ($P<0.001$; alpha=1.00) compared to cultures grown in horse serum (at 42 mM glucose (red/control)). (D-H) Astrocytic immunostaining and western blot quantifications for GFAP confirmed that 6 and 15 mM glucose triggered significantly increased ($P<0.001$; alpha=1.00) GFAP expression consistent with reactive astrocytic change. Images show low power GFAP immunostaining of cultures grown in horse serum (D) and serum-free media (F) and high power correlates (E and G). The arrows here and for microglia below point to exemplary cells. Cultures grown at 24-42 mM glucose in serum-free media (yellow) were not different from control cultures (red) grown in horse serum and 42 mM glucose (H). (I-M) Microglial characterization compared horse serum-based media (red) to serum-free equivalent (yellow) at 42 mM glucose. Semi quantitative immunostaining was used to quantify microglial levels (Caggiano and Kraig, 1996) and showed no significant ($P<0.170$; alpha=0.159) difference (paired t-test). Calibration bars are 250 μm (for 6.3 x images shown in A, B, D, F, I, K and 50 μm for 40x images shown in E, G, J and L.

[Relative levels (n=6/group) were: (C) 1.10±0.15 (42 mM), 0.98±0.13 (33 mM), 0.83±0.16 (24 mM), 0.89±0.17 (15 mM) and 0.02±0.00 (6 mM) versus 1.00±0.15 (control) for neurons; (H) 1.34±0.31 (42 mM), 1.16±0.45 (33 mM), 1.40±0.54 (24 mM), 2.61±0.53 (15 mM) and 3.04±0.67 (6 mM) versus control (1.00±0.20) for astrocytes; and 0.98±0.14 (serum-free media) versus 1.00±0.01 (control, horse serum-based media) for microglia].

Since neurons in primary culture may survive better at reduced oxygen tension (Brewer and Cotman, 1989), we tested whether oxygen would have a similar affect on slice cultures grown in serum-free media (Figure S2). We found that 10% oxygen led to significantly reduced principal neuron content, especially in the CA2 and dentate areas while 60% oxygen led to wide spread non selective and significant loss of neurons consistent with the deleterious affect of elevated oxygen tension on neuronal function (Pomper et al, 2006). Forty percent oxygen was no different from control (20% oxygen) but conceivably would generate increased reactive oxygen species and epileptiform activity (Pomper et. al., 2006) so 20% oxygen was used throughout experiments.

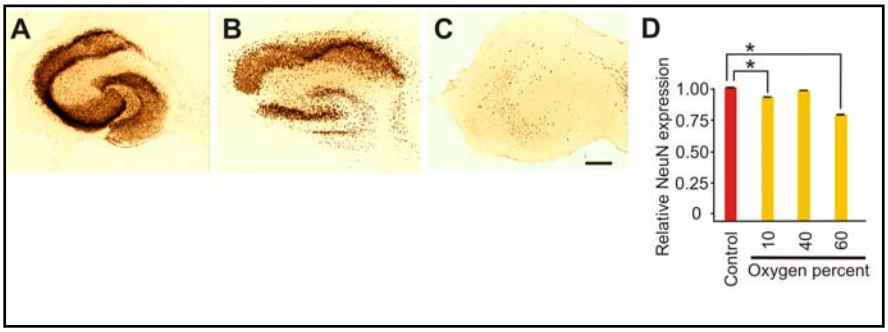

Figure S2. Effect of growth media oxygen on slice cultures. Images show typical principal neuron hippocampal slice cytoarchitecture at 21 days *in vitro* from cultures grown in serum-free media at various oxygen levels. (A) Neuronal pattern at 20% oxygen (control). (B) 10% oxygen led to selective a loss of CA2 and dentate area neurons. (C) Sixty percent oxygen led to a significant reduction in neuronal content (measured via semi-quantitative immunostaining for NeuN (D) as previously described (Caggiano and Kraig, 1996)). No difference was seen at 40% oxygen. Calibration bar is 250 μm.

[Significance levels were $P<0.001$ (alpha=1.00) for 10% and with relative levels of 0.88±0.01 (n=6), for 10% oxygen; 1.03±0.01 (n=6) for 40% oxygen and 0.78±0.02 (n=4) versus control 1.00±0.01 (n=7) at 20% oxygen.]

Antibiotics also can influence culture status. We found penicillin (25 U/mL) was not deleterious to cultures grown in horse serum (Kunkler and Kraig, 1997; 1998). However, we switched to use of gentamycin (Aptowicz et al. 2003, Kunkler et al., 2004, 2005) because of potential epileptigenic affects secondary to reduced inhibition from penicillin (Wong and Prince, 1979) that could confound interpretation of activity-dependent neuroprotective mechanisms. As

a result, we compared the use of penicillin and gentamycin in the serum-free media used here. We found that penicillin triggered a significant reduction if NeuN positive neurons (compared to gentamycin) prompting us to use the latter throughout these experiments (Figure S3).

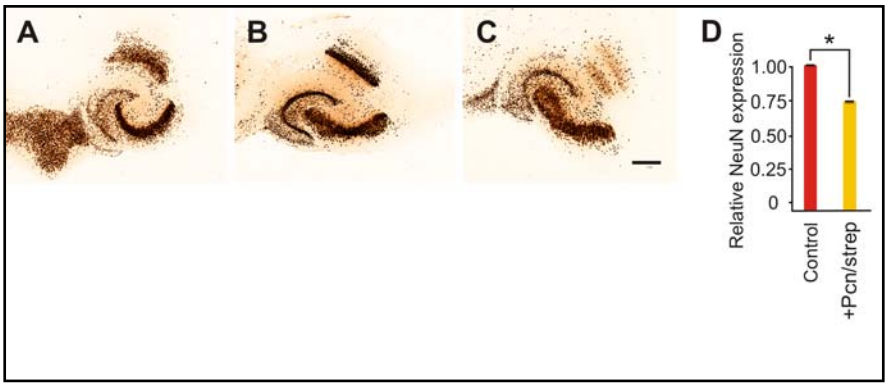

Figure S3. Effect of growth media penicillin on slice cultures. Typical NeuN immunostained images from slice cultures (n=6/group) are shown at 7 days (A), 14 days (B) and 21 days (C) *in vitro*. Neuronal content (using digital imaging semi quantitative analyses (Caggiano and Kraig, 1996)) showed that penicillin/streptomycin (Pcn/strep) triggered a significant reduction compared to gentamycin (control, see Figure 1 or S1 for comparative immunostaining) treated cultures at 7, 14, (data not shown) and 21 days (D) *in vitro*. Calibration bar is 250 μm.

[Specific significance level was $P<0.001$ (alpha=1.00) with relative NeuN levels (n=6/group) of 0.74±0.01 versus 1.00±0.03 (control)].

Optimization of slice culture recording conditions. We next optimized slice recording conditions so that non injurious activation of brain could be tested as a stimulus sufficient to preferentially generate increased expression of tumor necrosis factor alpha (TNF-α) in microglia. Spreading depression and learning share many similar biophysical and molecular physiological traits (Kunkler et al., 2005). In addition, both spreading depression and long-term potentiation (LTP), a cellular model of learning, can trigger neuroprotection in slice cultures (Kraig et al., 2005, 2006). The latter stimulus paradigm used chemical LTP since it is a tissue-wide activation of brain and thus allows easier detection of cell-specific changes (Otmakhov et al., 2004). However to date, we have noted that in some instances chemical LTP can induce low levels of pyramidal neuron loss days after induction. As a result, we used spreading depression here.

No injury is evident after spreading depression with slices grown in serum-based media (Kunkler and Kraig, 1998, Kunkler et al., 2004, 2005) or in serum-free media (Kraig et al, 2005, 2006). However, spreading depression-like events that occur with epileptiform activity in juvenile slice cultures are injurious (Pomper et al., 2006). Perhaps importantly, these authors used slice cultures maintained 13 days *in vitro* (i.e., "juvenile" hippocampal slice cultures). Instead, slice cultures used here were maintained at least 21 days *in vitro* (i.e., 28 days after birth) and so are "mature" since rats are weaned after 21 days. By 21 days *in vitro* synaptic physiology has fully matured *in vitro* (see Kunkler et al., 2005 for review). Furthermore, Pomper and colleagues used Pcn/strept, which conceivably could have enhanced epileptifom activity, and associated irreversible neuronal injury.

Pomper and colleagues also show that slice recording Ringer's solution oxygen tension has a deleterious impact on synaptic function. Commonly *in vitro* brain preparations are perfused with standard Ringer's solution gassed with 95% oxygen (and 5% carbon dioxide) (Dingeldine, 1984). However, Pomper et al. show that 95% oxygen makes tissue hyperexcitable. Instead, these authors used 20% oxygen, a level that did not trigger hyperexcitable responses yet left the tissue normoxic. Since hyperexcitable tissue can prevent induction of spreading depression, we confirmed this oxygen tension impact on CA3 area field potentials (Figure S4) using a standard Ringer's solution (Kunkler and Kraig, 1998). Notably, field potential responses in slice cultures grown in serum-based media were less frequently hyperexcitable when exposed to Ringer's gassed with 95% oxygen (Kunkler and Kraig, 1998). This begins to suggest that slice cultures maintained in serum-free media (as defined here) are more excitable than those grown in serum-based media, a result we confirm below with induction of spreading depression.

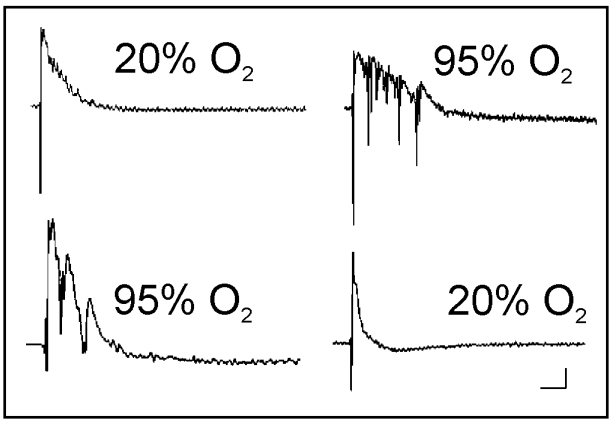

Figure S4. Slice culture hyperexcitability from elevated oxygen tension. A bipolar stimulating electrode was placed in the dentate gyrus to evoke field potentials in CA3 as previously described (Kunkler and Kraig, 1998). Responses show typical fast action potential (downward spike) and large field excitatory post synaptic potential (fEPSP, upward deflection) at 20% oxygen tension (top left). Ringer's solution oxygen was then changed to 95% and responses (after 15 min) became hyperexcitable as evidenced by multiple fast negative deflections riding on the fEPSP (top right). Recording conditions were then reversed beginning with 95% in a new slice culture. Again, responses were hyperexcitable (bottom left). After a 15 min transition to 20% oxygen, responses returned to normal (bottom right). Calibration bar is 2 mV (negative down) and 50 msec.

Finally, we tested the impact of glucose level and oxygen tension on spreading depression evoked by transient replacement of Ringer's chloride (Kunkler and Kraig, 1998, Kunkler et al., 2005). Results are shown in Figure S5 and show for the first time that spreading depression in mature slice cultures can be triggered by a single electrical pulse. Instead of requiring transient chloride substitution, when cultures are grown in serum-free media and this media is used for recordings, spreading depression is easily induced. This means that slices grown in serum-free media are more excitable than their counterparts grown in serum-based media. Furthermore, since a single electrical pulse can be sufficient to evoke spreading depression in hippocampus *in vivo*, this result provides further support that slice cultures grown in serum-free media more closely resemble their *in vivo* counterparts.

Spreading depression induction. Electrophysiological recordings and spreading depression were induced in slice cultures as described previously (Kunkler and Kraig, 1998, Kunkler et al., 2004, Kunkler et al., 2005) with modifications. Two sterile cotton strips (2 x 0.6 cm), each

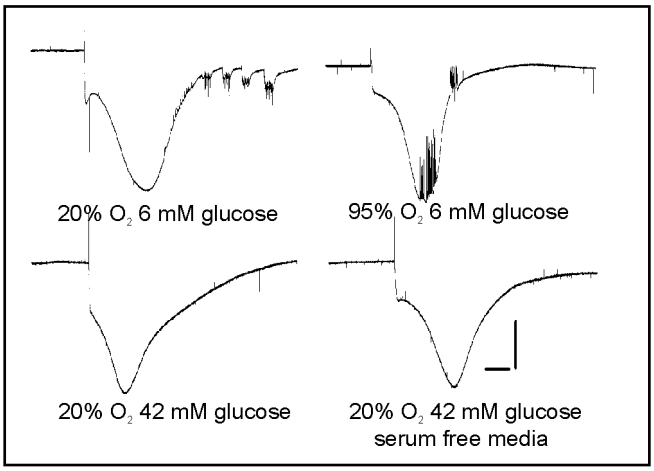

Figure S5. Spreading depression can be triggered by a single electrical pulse in serum-free media. Slice cultures grown to maturity in serum-based media require transient removal of chloride (i.e., by replacement with acetate) to induce spreading depression using Ringer's solutions with glucose levels of 6 mM (Kunkler and Kraig, 1998, Kunkler et al., 2005). However in some instances epileptiform-like activity occurs as spreading depression resolves (top left), an affect that can be enhanced by 95% oxygen (top right). Raising Ringer's glucose to 42 mM, like that used in growth media, stopped this excessive activity (lower left). This realization prompted us to try recording with serum-free media. We discovered that when recording in serum-free media, spreading depression could be triggered with a single electrical pulse to the dentate area and did not require anion substitution (lower right). Calibration bars are 10 mV (negative down) and 10 msec.

saturated with 2 mL of media, were then placed around the outer edge of the insert membrane. To stabilize the insert within the dish, Pharmed tubing (2.8 mm OD; Cole-Parmer; Vernon Hills, IL) was cut into 0.5 cm lengths and placed between the insert and the 35 mm dish. The dish was then covered tightly with polyvinyl chloride.

The insert was mounted in an open perfusion micro incubator (PDMI-2; Medical Systems, Greenvale, NY) and an inlet and outlet opening for media burned through the polyvinyl chloride cover using battery operated surgical cauterizing device. Serum-free media, adjusted to pH 7.3 to 7.4 with 5% CO_2/20% O_2/balanced N_2 and pre-warmed with a dual channel chamber system heater controller (model TC-344B; Warner Instruments Inc, Hamden, CT) to achieve a temperature of 36 °C in the micro incubator, was perfused under the insert at a rate of 1 to 2 mL/min. A third whole was burned in the polyvinyl covering above the slice culture to allow placement of a monopolar (or bipolar) stimulating electrode in the dentate gyrus and an interstitial microelectrode in the CA3 pyramidal neuronal layer. Evoked CA3 field potential

responses were optimized with stimulating pulses of 100 μs in duration at 0.5 Hz while increasing the current up to 10 μA. Spreading depression was induced every 10 minutes over one hr (7 total) with stimulating pulses of 100 ms duration (at 100 Hz and 50 to 100 μA) for < 250 ms. Slice cultures were returned to normal incubation conditions after recordings.

Cell-specific immunostaining. Slice cultures were processed for cytokine mRNA detection after 1 hr recovery. While remaining attached to the insert membrane, slice cultures were frozen on dry ice before being cut out of the insert and stored at -80 °C. Frozen cultures then were placed on a flat surface of frozen 20% sucrose and serial cryostat sections (12 μm) were cut and mounted on polyethylennaphtalate (PEN-foil) slides (Leica). Slides were stored less than one week at -80 °C before immunostaining, followed immediately by laser dissection. Alternatively, cultures processed for microglia harvesting were fixed for one hr in ice-cold 70% ethanol containing 150 mM sodium chloride and 20% sucrose prior to sectioning.

Sections were stained with cell-specific antibodies for neurons (NeuN, Chemicon, Temecula, CA), astrocytes (GFAP; Roche, Indianapolis, IN) or microglia (BSI-B_4, Sigma, St. Louis, MO). Sections for neuronal and astroglial immunostaining were allowed to come to room temperature for 10 min, fixed for one min in cooled 70% ethanol or acetone respectively, and rinsed 3 times in diethyl pyrocarbonate (DEPC)-treated phosphate buffered saline (PBS; 7.4 pH). Sections were then blocked in PBS containing 3% goat serum, 0.025% triton-X and 0.5 unit/μL RNasin inhibitor (Promega, Madison, WI) for 5 min prior to incubation in primary antibody (1:20) for 20 min. For GFAP immunostaining sections were rinsed 3 times in PBS, incubated for 20 min with goat-anti-mouse HRP conjugated IgG (1:20; Biosource, Camarillo, CA), and rinsed again in PBS. The immunoreactive product was visualized with the diaminobenzidine reaction

containing nickel ammonium sulfate (0.06%). The sections were then rinsed in PBS, counterstained with 1% neutral red, rinsed in PBS and DEPC-water and dried for 15 min prior to laser dissection. For NeuN immunostaining, sections were rinsed and incubated for 20 min with Cy3-conjugated Affinipure goat-anti-mouse IgG (Jackson Immuno Research, West Grove, PA). During the final 5 min of secondary incubation, YoYo-1 (1:200; Invitrogen) was added to the incubating solution for nuclear staining. Sections for microglia immunostaining were fixed for one min in 70% ethanol, rinsed and incubated for 2 hrs in PBS containing 0.5 unit/µl RNasin inhibitor and BSI-B_4 (1:100). The reaction was visualized and slides processed as described above for GFAP. All solutions and rinses were DEPC-treated to minimize the degradation of mRNA's.

Laser microdissection and RNA isolation. Laser microdissection was performed using a Leica AS LMD system (ver 6.4, Leica). Cells were dissected using a 63x objective and collected into the cap of a 500 µL microcentrifuge vial containing RLT buffer (RNeasy Micro Kit, Qiagen, Valencia, CA) supplemented with 1% β-mercaptoethanol. Cell-specific enriched samples were harvested using the following laser settings: aperture, 6; intensity, 45 and speed, 5. A 30 µm diameter region of interest was used to dissect neurons, while a 20 µm diameter region of interest was used to dissect astrocytes and microglia. Because the staining intensity of GFAP and GSI-B_4 labeled glia may vary between spreading depression-treated and sham controls, all immunolabeled cells located within a single visual field were collected. This random selection of cells, indiscriminate of their staining intensity, minimized a potential selection bias by the investigator (Burbach et al., 2004). Tissue collection was verified by inspecting the tube cap. Total RNA from 50 neurons, 100 astrocytes and 200 microglia were isolated using the RNeasy

Micro kit (Qiagen) according to the manufacture's recommendations. Preliminary studies determined the sample size of each cell population provided sufficient amounts of RNA to measure the housekeeper gene, β-actin. Samples were stored at -25 °C until processed for RT-PCR.

Rat TNF-α cDNA subcloning. Rat TNF-α cDNA was cloned from total RNA isolated from Conconavalin A (ConA) activated rat splenocytes. To isolate splenocytes, whole rat spleen was placed into a Dounce homogenizer and homogenized in 6 mL of ice-cold hepes-buffered saline. This procedure was repeated 2 additional times and the pooled homogenate was brought to 30 mL with ice-cold hepes buffered saline. The homogenate was placed on ice for 5 min after which time settled tissue debris was removed. The homogenate was underlain with 10 mL Ficoll and spun at 1800 rpm for 15 min. The buffy coat, containing the splenocytes, was removed to a clean 50 mL tube and the cells were washed 3X with ice-cold HBSS. Cells were resuspended in RPMI medium supplemented with 10% fetal bovine serum and 40 μg/mL gentamycin sulfate antibiotic. Cells were diluted to $1.6x10^6$ cell/mL and cultured overnight in 6 well clusters at 2 mL/well. The next day cultures were supplemented with 2 mL of medium containing 5 ug/mL of ConA. Cells were harvested after an additional 48 hrs in culture.

Total RNA was isolated from cultured splenocytes using the method of Chomczynski and Sacchi (1987). First strand cDNA synthesis was primed with 100 pM random hexamers using 200 U SuperScript II reverse transcriptase (Invitrogen) and 5 μg of total RNA in a 20 μL reaction mixture that was 500 μM in dNTPs (GE Healthcare Bio-Sciences, Uppsala, Sweden), 1 U RNasin / μL (Promega, Madison, WI), 10 μM in dithiothreitol and 1x in first strand buffer. Reaction proceeded at 42 ^oC for 50 min.

TNF-α cDNA was amplified from the reverse transcription products using PCR and primers that directed the amplification of the coding regions and introduced a 5'-flanking *BamHI* restriction site and a 3'-flanking *EcoRI* restriction site. Primer sequences are shown in Table SI. PCR reactions were carried out in a volume of 50 μL and consisted of 1xPCR buffer (10 mM Tris pH 8.3 and 50 mM KCL), 1.5 mM $MgCl_2$, 150 μM of dNTPs, 15 pmol each of forward and reverse primers, 5 μL of reverse transcription products and 1.25 U TAQ polymerase. Cycling parameters consisted of 30 seconds denaturation at 94 ^oC, 1 min annealing at 60 ^oC and 1 min extension at 72 ^oC and reactions proceeded for 30 cycles.

The contents of 4 identical PCR reactions were pooled and extracted once with 1:1:0.05 mixture of phenol: CHCl3: isoamyl alcohol (PCIA) and subsequently extracted 1x with $CHCl_3$. DNA was recovered by precipitation with sodium acetate, pH5.4, and ethanol. DNA pellets were washed 1x with 75% ethanol, air dried. Amplified cDNAs were dually digested for two hrs in a 120 μL digestion buffer containing 150 U of *BamHI* and 150 U *EcoRI*. The digest was extracted 1x with PCIA and 1x with $CHCl_3$ and DNA was recovered by ethanol precipitation. DNA pellets were washed 1x with 75% ethanol, air dried and resuspended in 15 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The *BamHI-EcoRI* digested PCR products were ligated into like-digested pBSKS+ vector (Stratagene). 10 μL of ligation product was used to transform DH5α cells (subcloning-efficiency, Invitrogen). Transformed bacteria were plated onto LB-Amp plates. Ten colonies from each transformation were grown up over-night in LB-Amp medium and mini-prep DNA was isolated and analyzed by *BamHI-EcoRI* digestion. Two positive clones were analyzed by DNA sequencing to verify sequence integrity.

Rat IL-1β and β-Actin, cDNAs. Full-length rat cDNA clones for β-Actin (Image Clone ID: 6920838) and IL-1β (Image Clone ID: 1783806) were purchased from Open Biosystems (Huntsville, AL). Clones were propagated on LB-agar plates supplemented with the appropriate antibiotic.

Plasmid preparation. To produce larger quantities of plasmid, single colonies were used to inoculate 75 mL of LB medium supplemented with the appropriate antibiotic and the cultures grown over-night. Plasmid DNA, isolated from bacteria using the Qiagen plasmid midi purification kit following the manufacturer's protocol (Qiagen) was resuspended in TE buffer and UV absorbance at 260/280 nm was used to determine concentration and purity. Plasmid concentration and purity were confirmed by electrophoresis on agarose gels and visualization of DNA by ethidium bromide staining.

cDNA and RNA standards. A 5 ng/μL working stock of plasmid in TE buffer was prepared to serve a DNA template. Standards for cDNA were serially diluted 1:10 in Tris buffer (10 mM, pH 8.0) down to a level 0.5 fg/μL and then diluted 1:2 thereafter. A combination standard was prepared by mixing equal volumes of each of the cytokine and β-actin cDNAs followed by a further 1:1 dilution in Tris buffer. As both the size of the vector and cDNA inserts are known, the molecular weight of each plasmid species was calculated. Using the molecular weight and mass of plasmid present, the copy number for each cDNA species present in the standard was calculated. Table S2 lists the copy number vs. mass used in the standard curves.

Real time quantitative PCR assay for cytokine gene expression. Cytokine gene expression was determined using a multiplexed TaqMan real-time RT-PCR strategy (Heid et al., 1996). For

slice culture-laser dissection cytokine gene expression, a three color multiplex assay was employed using a HEX labeled probe for detection of the house keeping gene β-Actin, FAM labeled probe for detection of IL-1β and Cy5 labeled probe for the detection of TNF-α.

Probe and primer design considerations. In general, primers were designed to have a Tm of 58 ^{o}C to 60 ^{o}C, to have a GC content of roughly 50%, to avoid GC runs on the 3' end, do not form self-dimers (primer-dimers), to be free of substantial internal secondary structure (free energy of 3 kcal/M or less), and result in an amplimer of around 80 base pairs (range 78 to 83 bp) that corresponds to a region of low sequence identity to other expressed rat genes. TaqMan probes were designed to have a Tm of around 6 ^{o}C higher than the Tm of the primers, an overall length of 20-30 bases, to have a GC content of 40% to 60%, to avoid long runs of any single nucleotide, free of substantial secondary structure (free energy of 6 kcal/mol or less), and the 5' end cannot be a G (results in lower FAM intensity). Probes were sited on the strand which had minimal G content and a placed within 3 bp of the flanking primer.

For multiplexed PCR reactions, each primer pair must have compatible Tm's, i.e., maximum variation between pairs is constrained to 1 ^{o}C. Strict design criteria resulted in primer/probe sets with optimal performance at similar reaction conditions. Probe and primer design was facilitated by using the Beacon Designer software package (Ver 2.0, Biosoft International, Palo Alto, CA).

Probes and primers. The IL-1β probe and primers were designed according to Wang et al. (2000). The remaining primer and probe sets were designed using the criteria detailed above. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). Probes were

synthesized by either Integrated DNA Technologies or BioSource. Primers and probes are detailed in Table S3.

TaqMan real-time reverse transcriptase PCR assay. RT-PCR reactions were performed in 96 well plates using the RNA UltraSense One-Step RT-PCR with Platinum *Taq* system (Invitrogen, cat # 11732-927). Multiplex amplification of three targets required supplementation of the basic kit components with additional Mg^{2+}, dNTPs, and Taq polymerase. Each reaction contained 10 µL of 5x Reaction Mix supplemented with 3.0 µL of 50 mM $MgSO_4$ to bring reaction to 5.5 mM in Mg^{2+}, and 1.25 µL of 10 mM PCR Nucleotide Mix (Roche) to bring reaction to 440 mM in dNTPs. In addition, each reaction contained: 10 pM of each primer and probe set for IL-1β and TNF-α, 5 pM of each primer and probe for β-actin, 10 µL of RNA (or 10 µL cDNA controls, see Table 2), 2.5 µL of RT/Platinum *Taq* mix supplemented with 0.4 µL of 5 U/ µL of Platinum Taq polymerase (resulting in 4 U Taq/reaction) in DEPC treated water to a final volume of 50 µL. Thermocycling and data collection were performed using the iCycler thermal cycler fitted with the iCycler iQ detector (Bio-Rad, Hercules, CA) which is capable of simultaneous monitoring of probes labeled with FAM, HEX, Texas Red and Cy5. Reaction conditions consisted of an RT step of 30 min at 50 ^{o}C followed by 3 min at 95 ^{o}C during which time well-factors necessary to correct for background fluorescence were measured, the template is denatured and Platinum *Taq* is activated. Subsequently, 45 thermal cycles consisting of 15 s at 95 ^{o}C followed by 30 sec at 60 ^{o}C were performed. Fluorescence was measured during the 60 ^{o}C extension step. Cycle threshold values were recorded, converted to copy numbers using cDNA standard curves and results expressed initially as copy number ratios for cytokine/β-action x 1000. Then, to reduce variance and appropriately account for low or no induction of cytokine mRNA, results were converted to log (x+1) values before statistical considerations (Snedecor and Cochran (1989).

Table S1. Primers for TNF-α coding sequence amplification

TNFα-F	gcggccat**GGATCC**ATCAGCACAGAAAGCATGATCCG
TNFα-R	gcggccat**GAATTC**TCACAGAGCAATGACTCCAAAGTAG

Table S2. Cytokine Standards*

MASS	**COPY NUMBER**		
(fg)	**IL-1β**	**TNF-α**	**β-Actin**
625	172807.6	153793.2	105428.9
62.5	17280.8	15379.3	10542.9
6.25	1728.1	1537.9	1054.3
0.625	172.8	153.8	105.4
0.3125	86.4	76.9	52.7
0.125	34.6	30.8	21.1

*Cytokine cDNAs were serially diluted to produce standard curves. Copy number was calculated from mass of cDNA in standard.

Table S3. Primer and probe sequences used in this study

Target	Accession	Pos	Sequence 5' → 3'	T_m
IL-1β	M98820	F	CACCTCTCAAGCAGAGCACAG	58.8
		R	GGGTTCCATGGTGAAGTCAAC	57.1
		P	FAM-TGTCCCGACCATTGCTGTTTCCTAGG-BHQ1	64.2
TNF-α	X66539	F	ACCACGCTCTTCTGTCTACTGA	58.2
		R	CTGATGAGAGGGAGCCCATTTG	58.2
		P	Cy5-TTCTCCTCCTTGTTGGGACCGATCAC-BHQ2	64.9
β-Actin	NM_031144	F	TGTGGATTGGTGGCTCTATCCT	58.3
		R	GCCGGACTCATCGTACTCCT	58.1
		P	HEX-CTCACTGTCCACCTTCCAGCAGATGT-BHQ1	65.4

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