Original Research

Suprachiasmatic nucleus neurons display endogenous resistance to excitotoxicity

Kathleen Bottum[1], Emily Poon[2], Benjamin Haley[2], Sumedha Karmarkar[3] and Shelley A Tischkau[3]

[1]Southern Illinois School of Medicine, Department of Medicine, Division of Internal Medicine and Psychiatry, Springfield, IL; [2]College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, IL; [3]Southern Illinois University School of Medicine, Department of Pharmacology, Springfield, IL, USA

Corresponding author: Assistant Professor Shelley A Tischkau, Department of Pharmacology, Southern Illinois University School of Medicine, PO Box 19636, 801 N Rutledge, Springfield, IL 62794-9636, USA. Email: stischkau@siumed.edu

Abstract

A comprehensive understanding of neuroprotective pathways is essential to progress in the battle against numerous neurodegenerative conditions. The hypothalamic suprachiasmatic nucleus (SCN) is endogenously resistant to glutamate (Glu) excitotoxicity *in vivo*. This study was designed to determine whether immortalized SCN neurons (SCN2.2 cells) retain this characteristic. We first established that SCN2.2 cells retained the ability to respond to Glu. SCN2.2 cells expressed *N*-methyl-D-aspartate (NMDA) receptor subtypes NR1 and NR2A/2B, suggesting the presence of functional receptors. mRNA for the NMDA receptor subunits NR2A and NR2B were higher in the SCN2.2 than in the control hypothalamic neurons (GT1-7). Specific NMDA receptor antagonists (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate and D-(−)-2-amino-5-phosphonovaleric acid blocked Glu-induced activation of gene expression. SCN2.2 cells were resistant to Glu excitotoxicity compared with GT1-7 neurons as assessed with a mitochondrial function assay, cell death by trypan blue exclusion and apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling. SCN2.2 resistance to Glu excitoxicity was retained in the presence of the broad spectrum Glu transport inhibitor, L-trans-pyrrolidine-2,4 dicarboxylate, excluding glial Glu uptake as a major neuroprotective mechanism. Collectively, these observations demonstrate endogenous neuroprotection in SCN2.2 cells; this cell line is resistant to excitotoxicity under conditions that are toxic to other immortalized cell lines. Thus, the SCN2.2 cell line may provide insights into the molecular mechanisms that confer endogenous neuroprotection in the SCN.

Keywords: NMDA receptor, glutamate, excitotoxicity, neurotoxicity, apoptosis, suprachiasmatic nucleus

Experimental Biology and Medicine 2010; **235**: 237–246. DOI: 10.1258/ebm.2009.009244

Introduction

Neurodegenerative processes underlie many brain illnesses such as stroke, Parkinson's disease and multiple forms of dementia.[1,2] Although the illnesses differ in many respects, common neurodegenerative processes, such as toxicity of the neurotransmitter glutamate (Glu), increased intracellular calcium and activation of cell death pathways, play a unifying role in these illnesses.[1,2] To date, although many of the cellular and molecular mechanisms of neurotoxicity have been delineated, little progress has been made in reversing these processes to treat neurodegenerative diseases.

Certain regions of the brain display natural resistance to neurotoxicity. For example, the CA3 region of the hippocampus is protected relative to the more vulnerable CA1 region.[3] A better understanding of the pathways employed by consistently neuroprotected brain regions might allow these mechanisms to be harnessed therapeutically to produce long-lasting neuroprotection in susceptible regions of the brain. We propose the suprachiasmatic nucleus (SCN) of the hypothalamus as a good model system in this pursuit.

The SCN controls the body's circadian rhythms.[4] A critical aspect of SCN function is the processing of light information, garnered from the environment and used to align the endogenous body clock to the daily cycle of light and darkness. Mechanistically, stimulation of the retino-hypothalamic tract by light striking the retina causes release of Glu onto the SCN.[5,6] Thus, the SCN is regularly

exposed to Glu,[7] an excitatory neurotransmitter associated with neurotoxicity.[8,9]

The resistance of the SCN *in vivo* to glutamatergic neurotoxins was first established in the 1980s.[10,11] Although commonly used to lesion various brain regions, kainic acid, *N*-methyl-D-aspartate (NMDA) and Glu are not sufficiently toxic to lesion the SCN; the SCN must be electrically or mechanically lesioned.[10–13] This observation was extended to an *in vitro* brain slice system in the 1990s using a cell swelling measurement of neuronal damage.[14] In acute brain slices the hippocampus, cortex and neostriatum were all susceptible to neurotoxic insult, but the SCN was completely resistant to these neurotoxins. Other work involving acute SCN brain slices routinely uses 1–10 mmol/L Glu to mimic physiologic light signals without any adverse effects to the health of the slice.[15–17] In contrast, 0.1–10 mmol/L is commonly used as a toxic stimulus in other brain regions.[18,19] Thus, although Glu neurotoxicity has not previously been formally investigated in the SCN, evidence suggests that the SCN is a site of naturally occurring excitotoxic resistance.

To substantiate the hypothesis that the SCN is protected against Glu in conditions lethal to other neurons, we chose the simplicity and ease of manipulation provided by the immortalized SCN2.2 cell line. This cell line is well established as a valid cell line model of the SCN.[20,21] It secretes peptides in a circadian rhythm identical to the intact SCN.[20] Implantation of SCN2.2 cells into an arrhythmic, SCN-lesioned animal restores circadian rhythms.[22] Thus the SCN2.2 cell line retains many of the functional properties of the intact SCN. We hypothesized that it would also show resistance to excitotoxicity similar to what has previously been observed in SCN *in vivo*.

The immortalized hypothalamic neuronal cell line GT1-7 served as our neuronal control. GT1-7 cells, derived from gonadotropin-releasing hormone (GnRH) secreting neurons, maintain the ability to secrete GnRH in response to depolarization[23] and have been previously used in neurotoxicity studies. Prior work on this cell line demonstrates that up to 80% of GT1-7 cells appear apoptotic within 24 h of exposure to toxic stimuli.[24] Overexpression of bcl-2 prevented apoptosis in GT1-7 cells.[24,25] Furthermore, the ryanodine receptor antagonist, dantrolene, is neuroprotective in GT1-7 cells, similar to the effects of dantrolene in an *in vivo* model of ischemia.[26,27] Estrogen, conversely, rendered GT1-7 cells more susceptible to Glu, and this effect is reversed by estrogen receptor antagonists.[19] All of these prior studies, combined with the anatomical proximity of native GnRH neurons to the SCN, supported our use of this cell line as a neuronal control for SCN resistance to excitotoxicity.

We hypothesized that the SCN exhibits naturally occurring neuroprotection against the excitotoxic effects of Glu. We examined Glu-induced excitotoxicity in SCN2.2 cells. Overall cell damage and death, as well as apoptotic activity, were determined. These studies provide an important basis for other mechanistic investigations of neuroprotective mechanisms in the SCN and their subsequent application to other brain regions. A thorough understanding of endogenous neuroprotective mechanisms will provide direction for future clinical work designed to develop neuroprotective strategies applicable to many regions of the brain. This approach might finally provide effective protection against the substantial morbidity and mortality produced by neurodegenerative processes.

Materials and methods

Cell culture

SCN2.2 cells (generously provided by Dr David Ernest) were grown in Eagle's minimum essential medium (EMEM) supplemented with 2 mmol/L L-glutamine, 10,000 U/mL penicillin, 10,000 U/mL streptomycin, 250 μg/mL fungizone, and 10% fetal bovine serum (FBS) on laminin-coated tissue culture plates (1.5 μg/cm^2 laminin). GT1-7 cells (generously provided by Dr Pamela Mellon) were grown on uncoated plates (except as noted) in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 4.5 gm/L glucose, 2 mmol/L L-glutamine, 10,000 U/mL penicillin, 10,000 U/mL streptomycin, 250 μg/mL fungizone and 10% FBS.

Trypan blue exclusion assay

Adherent cells were removed from the plate surface by two-minute incubation in 0.05% trypsin. Media was saved so that dead or injured cells, which may float, can be included in the analysis. After staining with 0.4% trypan blue for 5–10 min, cells were counted manually. Dead cells stain blue; live cells exclude trypan blue and remain clear:

$$\%\text{Alive} = [\text{Clear cells}/(\text{Blue cells} + \text{Clear cells})] \times 100$$

Thiazolyl blue tetrazolium bromide assay

SCN2.2 and GT1-7 cells were grown on laminin-coated 24-well plates to encourage cell adherence to the surface and then incubated with Glu. Controls were not treated with Glu. After treatment, 250 μL of a 50 mg/mL thiazolyl blue tetrazolium bromide (MTT) solution was added to wells containing 1 mL of cell culture medium. After additional incubation for 4–8 h, MTT was replaced with 1 mL of dimethyl sulfoxide to dissolve MTT crystals in the presence of 125 μL of Sorensen's glycine buffer (0.1 mol/L glycine, 0.1 mol/L NaCl, pH 10.5). Plates were read immediately at 562 nm.

$$\%\text{Alive} = (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100$$

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

SCN2.2 and GT1-7 cells were incubated for 48 h in DMEM with 0 or 10 mmol/L Glu. Following incubation, the cells were scraped off the plate, fixed for 10 min in a fresh solution of 1% paraformaldehyde (made from methanol-free ultrapure EM grade stock) in phosphate-buffered saline,

and spread onto a microscope slide. Slides were postfixed in 2:1 ethanol:acetic acid for five minutes at −20°C. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed with the Apoptag Apotosis Detection System (Chemicon, Temecula, CA, USA) according to the manufacturer's specifications. Slides were mounted with 4′,6-diamidino-2-phenylindole in antifade solution. Negative controls included omission of the terminal deoxynucleotidyl transferase enzyme or the anti-digoxigenin antibody. Slides were viewed by two independent observers blinded to experimental conditions, and cells positive for fluorescein stained TUNEL cells as well as total cells were counted. Percent TUNEL-positive counts were averaged for the two observers.

$$\%\text{TUNEL positive} = (\text{TUNEL positive cells}/\text{Total cells}) \times 100$$

Time course experiments

SCN2.2 and GT1-7 cells were grown in 35 mm Petri dishes or 24-well plates. Cells were incubated with 10 mmol/L Glu for up to 120 h, with time points taken every eight hours. Cells were processed for either the trypan blue exclusion assay or the MTT assay.

Dose response experiments

SCN2.2 and GT1-7 cells were grown in 24-well plates. Cells were incubated for 48 h in the following concentrations of Glu: 0, 1, 5 and 10 mmol/L. Cell death was assessed using the MTT assay.

Quantitative polymerase chain reaction

RNA was isolated from SCN 2.2 and GT 1-7 cells with TRIzol as previously described.[28] RNA concentrations were calculated from the absorbance at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purity of RNA was estimated by the ratio of absorbances at 260/280 nm. In our hands, these ratios were consistently near 2, indicating the high quality of the samples.

cDNA was prepared from the isolated RNA using random primers in a MyCycler thermal cycler (BioRad, Hercules, CA, USA). A master mix consisting of 1 μL per sample of a 10 mmol/L dNTP mixture (containing 10 mmol/L dATP, 10 mmol/L dGTP, 10 mmol/L dCTP and 10 mmol/L dTTP at a neutral pH), 6 μg per sample random primers and enough diethylpyrocarbonate water to give a volume of 12 μL per sample was prepared. After addition of master mix, samples were heated to 70°C for five minutes to denature primers and dNTPs. Reverse transcriptase (RT) reaction was performed with MMLV enzyme (Promega, Madison, WI, USA) according to the manufacturer's protocol. Negative controls included omission of RNA or RT enzyme.

Quantitative polymerase chain reaction (PCR) was performed on a Cepheid thermocycler using SYBR Green (Applied Biosystems, Foster City, CA, USA). Primer sequences were obtained from the literature[29] or purchased from a commercial supplier (Qiagen, Valencia, CA, USA). The threshold cycle (C_t) was arbitrarily set in the linear phase of the logarithmic amplification curve, but held constant for all runs. PCR was performed as previously described[30] as modified from Bustin.[31] This standard curve method provides relative quantification, and eliminates much of the variability inherent in choosing a housekeeping gene or ribosomal RNA for normalization (see discussions in references[31,32]). For the standard curve, quantitative PCR was performed on cDNA prepared from 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 μg total RNA. The $\log_2$ (concentration) for each standard was plotted against the average C_t for the standard to form a standard curve for each primer set. Amplicon efficiency was calculated from the slope; efficiency $= 2^{-(1/\text{slope})}$ (see Table 1). NR2A and NR2B mRNA levels were normalized to NR1 levels for each cell type.

Immunoblot

Protein was prepared from SCN2.2 cells using Tissue Protein Extraction Reagent (T-PER) (Pierce, Rockford, IL, USA). Cells were scraped from a T25 tissue flask in tissue culture media, and pelleted. Proteins were extracted in 0.5 mL T-PER containing 1× protease inhibitor (Roche, Indianapolis, IN, USA). Centrifugation (4°C) at 10,000 rpm for five minutes removed cellular debris. Protein concentration in supernatants was determined by absorbance at 280 nm, using the ND-1000 spectrophotometer.

Twenty micrograms of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8% polyacrylamide gel) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk in TBS (50 mmol/L Tris-HCl, 150 mmol/L NaOH, pH 7.5) overnight at 4°C. The membrane was then incubated with rabbit anti-NR1 (Chemicon) diluted 1:100 to detect NR1 or rabbit anti-NR2A/B (Chemicon) diluted 1:200 to detect both NR2A and NR2B subunits. Donkey anti-rabbit horseradish peroxidase (HRP) secondary antibody (Applied BioReagents, Golden, CO, USA) diluted 1:5000 was then applied for one hour at room temperature. The blot was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Luciferase assay

Stably transfected SCN2.2 cells expressing luciferase reporter activity (SCN2.2.*cre-luc* and SCN2.2.*per1-luc*[21]) were grown to confluence on laminin-coated plates in EMEM with 10% FBS, 2.5 μg/mL fungizone and 100 μg/mL penicillin–streptomycin. At confluence, culture medium was replaced with serum-free medium (50% DMEM, 50% Ham's F-12, N-2 supplement, 2.5 μg/mL fungizone and 100 μg/mL penicillin–streptomycin) to promote differentiation. After 24 h in serum-free medium, cultures were shocked by exposure to 50% horse serum, 50% DMEM/50% Ham's F-12 for two hours. Following serum shock, cells were returned to serum-free medium. Twelve hours after serum shock, cultures were treated with Glu (10 mmol/L), the NMDA receptor antagonists

Table 1 Primers and quantitative polymerase chain reaction (PCR) amplification of *N*-methyl-D-aspartate receptor subunits R1, R2A and R2B of SCN2.2 and GT1-7 cells

Gene	Species	Source of primers	Efficiency	R^2	Equation
NR1	Rat	Dracheva[29]	110%	0.9987	$Y = -0.9345X + 42.826$
NR2A	Rat	Dracheva[29]	96%	0.9609	$Y = -1.031X + 41.705$
NR2B	Rat	Dracheva[29]	98%	0.9657	$Y = -1.013X + 42.1$
NR1	Mouse	Qiagen QuantiTect	101%	0.9181	$Y = -0.993X + 44.032$
NR2A	Mouse	Qiagen QuantitTect	86%	0.9338	$Y = -1.1209X + 40.273$
NR2B	Mouse	Qiagen QuantitTect	Not detectable	Not detectable	Not detectable

Equations were generated from standard curve analysis of PCR products using 0.0625-4 μg RNA

(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, 1 μmol/L) and amino-5-phosphonovaleric acid (APV, 0.1 mmol/L),[7] Glu + MK-801, Glu + APV or media change alone (control). MK-801 and APV treatments began 20 min prior to 10 min Glu stimulation. Cells were returned to serum-free medium for six hours to allow accumulation of luciferase mRNA/protein. Cultures were frozen at -20°C until assay.

Extracts were prepared from frozen cultures by rocking for 20 min in 25 μL cell lysis buffer (CCLB, Promega) per cm^2 culture surface area. After centrifugation (2000 *g* for 5 min), supernatants were collected for luciferase and protein assays. Luciferase was measured on an MLX luminometer (Dynex technologies, Chantilly, VA, USA) using the Luciferase Assay System (Promega). Protein concentration was determined using the microtiter plate bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard. Data were calculated as relative luciferase activity/μg protein and reported as fold change from control cultures.

Inhibition of glial Glu transporters

SCN2.2 and GT1-7 cells were grown on 24-well plates precoated with laminin. Incubations were performed in DMEM alone, DMEM + 10 mmol/L Glu, DMEM + 250 mmol/L L-trans-pyrrolidine-2,4 dicarboxylate (PDC) or DMEM + 10 mmol/L Glu + 250 mmol/L PDC for 48 h. Cell death was assessed by the MTT assay.

Statistics

Data were expressed as the mean ± SEM. Repeating data were assessed by a one-way analysis of variance with Tukey's *post hoc* analysis using KaleidoGraph (Synergy Software, Reading, PA, USA). Paired means were evaluated with a paired *t*-test. Non-paired means were compared with a two sample, two tail *t*-test. All values are considered significant if $P < 0.05$.

Results

SCN2.2 cells have functional NMDA receptors

To assess the responsiveness of SCN2.2 cells to Glu, we examined the cell lines for the presence of functional Glu receptors. As shown in Table 1 and Figure 1A, PCR established the presence of mRNA for NMDA receptor subunits, NR1, NR2A and NR2B, in the SCN2.2 cell line, and NR1 and NR2A in the GT1-7 cell line. NR2B mRNA was consistently undetectable in the GT1-7 cells. mRNA for NR2A relative to NR1 was significantly higher in SCN2.2 cells than in GT1-7 cells ($P < 0.05$; Figure 1A). In each individual cell type, NR2A mRNA was expressed at a higher level than NR1 mRNA ($P < 0.05$ in SCN2.2 cells and $P < 0.01$ in GT1-7 cells; Figure 1A). In the SCN2.2 cells NR2B mRNA was expressed at a higher level than NR1 mRNA ($P < 0.001$; Figure 1A), whereas in GT1-7 cells NR2B mRNA was undetectable. Gel analysis indicates that each primer set amplified a product of appropriate size (Figure 1B,

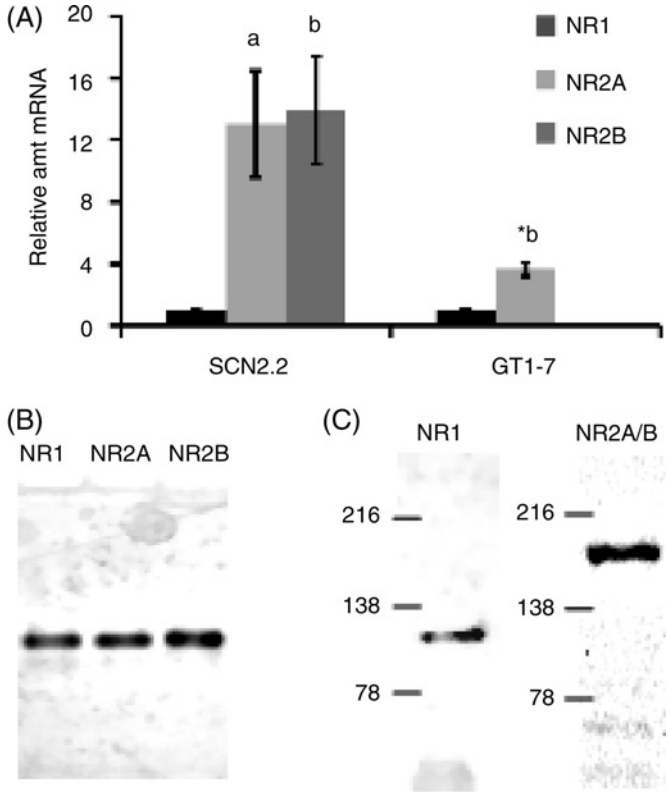

Figure 1 SCN2.2 cells express *N*-methyl-D-aspartate (NMDA) receptor protein. Quantitative polymerase chain reaction (PCR) for NMDA subunits NR1, NR2A and NR2B was performed on RNA from SCN 2.2 and GT1-7 cells. (A) Relative amounts of mRNA for each subunit relative to NR1 for SCN2.2 and GT1-7 cells. Each sample is the average of three experiments. '*' indicates $P < 0.05$ for mRNA in SCN versus GT, 'a' indicates $P < 0.05$ for gene compared with NR1 within same cell type, and 'b' indicates $P < 0.01$ for gene compared with NR1 within same cell type. (B) An agarose gel of the pure PCR products from SCN2.2 cells. (C) Immunoblot for NR1 and NR2A/2B from SCN2.2 cells. Experiments were repeated three times with similar results

data shown for SCN2.2 cells). Immunoblot analysis confirmed the presence of NMDA receptor subunit proteins in SCN2.2 cells (Figure 1C).

A luciferase reporter assay was used to assess whether SCN2.2 neurons respond to Glu. Expression of the circadian clock gene, Period1 (Per1), increases in response to Glu treatment, via a Cre-dependent mechanism.[17] In SCN2.2 neurons, expression of Cre-luciferase and Per1-luciferase constructs are stimulated by Glu.[21] Consistent with these prior findings, 10 mmol/L Glu induced a large increase in luciferase activity in SCN2.2 neurons transfected with Cre and Per1 constructs ($P < 0.005$; Figure 2). The NMDA receptor blockers, MK-801 (1 μmol/L) and APV (0.1 mmol/L) did not cause a significant change in luciferase activity when added to Cre-luciferase or Per1-luciferase transfected SCN2.2 cells alone. However, 1 μmol/L MK-801 and 0.1 mmol/L APV significantly attenuated the effect of 10 mmol/L Glu on luciferase activity (difference from control $P < 0.05$; Figure 2). Thus we are confident that the SCN2.2 cells express functional NMDA receptors and are capable of mounting an appropriate response to Glu.

SCN2.2 neurons are resistant to Glu excitotoxicity

The hypothesis that SCN neurons are protected against damage from Glu compared with other brain neurons was tested in the immortalized SCN2.2 cell line, which retains functional properties of the SCN.[20,33] The hypothalamic GT1-7 cell line, derived from embryonic GnRH, served as positive control.[23] Multiple independent measures were used to assess cell death, as indicators for specific stages of the death process.[34] Overall cell death from either apoptosis or necrosis was examined with the trypan blue exclusion assay,[27,35] whereas DNA damage associated with late stages of apoptosis was observed by TUNEL.[36,37] Early mitochondrial, endosomal and lysosomal damage in cell death causing metabolic dysfunction was measured with the MTT assay.[35]

Figure 3 displays the time course of cell death in SCN2.2 and GT1.7 cells exposed to 10 mmol/L Glu. A substantial proportion of SCN2.2 cells survived to the end of the incubation period, whereas GT1-7 cells were mostly dead at the

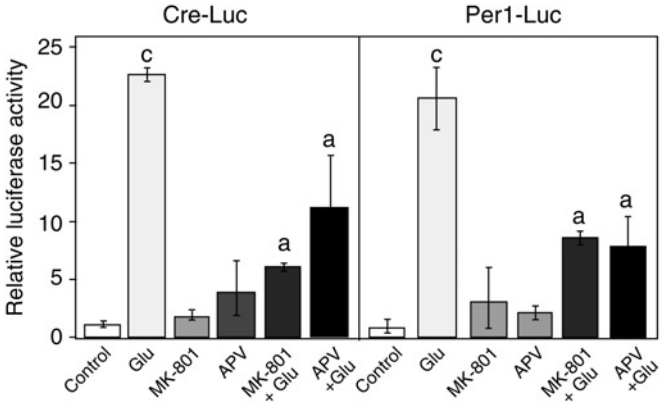

Figure 2 SCN2.2 cells show physiological response to glutamate (Glu). SCN2.2 cells bearing stably transfected luciferase reporters (Cre-Luc and Per1-Luc) were treated with 10 mmol/L Glu. Both Cre-Luc and Per1-Luc activity was increased by Glu treatment. This effect was significantly attenuated by the *N*-methyl-D-aspartate receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) at 1 μmol/L. Data were analyzed for differences from the control point with analysis of variance and *post hoc* Tukey analysis. N = 6 for each sample. 'a' indicates $P < 0.05$, 'c' indicates $P < 0.005$ compared with control. APV, D-(−)-2-amino-5-phosphonovaleric acid

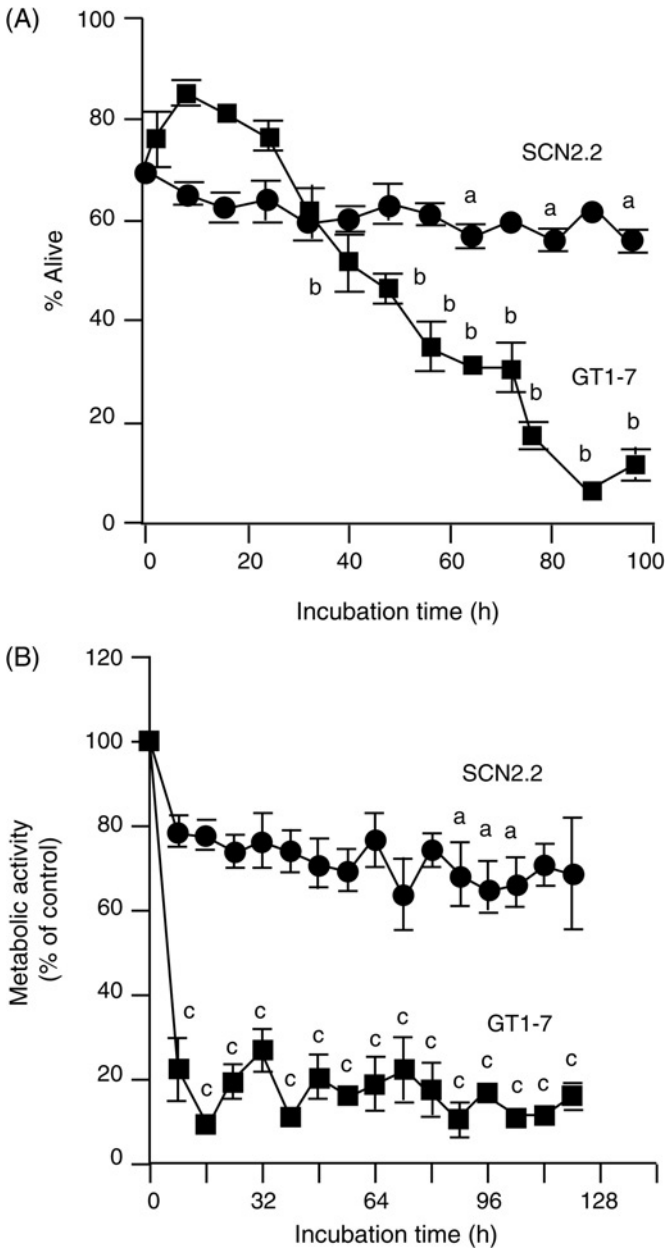

Figure 3 SCN2.2 cells survive glutamate (Glu) exposure as measured by trypan blue exclusion assay and thiazolyl blue tetrazolium bromide (MTT) assay. Cells were incubated with 10 mmol/L Glu. Time points were analyzed for differences from the control time point with analysis of variance and *post hoc* Tukey analysis. (A) At the appropriate time points, the cells were treated with trypan blue to determine cell viability. Dead cells take up the blue dye while live cells exclude the dye. $N = 3$ for each time-point. 'a' indicates $P < 0.05$; 'b' indicates $P < 0.01$. (B) Metabolic function was measured with the MTT assay; intact cells oxidize the dye while damaged cells do not. MTT is a marker of relatively early damage after exposure to Glu. Each point represents the average of three to five separate experiments; for each individual experiment $n = 4$. 'a' indicates $P < 0.05$; 'c' indicates $P < 0.005$

end of the experiment. A substantial reduction in survival was apparent in treated GT1-7 cells from 40 to 96 h after Glu exposure, as indicated by trypan blue exclusion ($P < 0.01$, Figure 3A). SCN2.2 cells exposed to Glu were not significantly different from unexposed SCN2.2 control cells at the majority of time-points.

In Figure 3B, metabolic damage as measured by the MTT assay was used as a surrogate for cell death in SCN2.2 and GT1-7 cells exposed to 10 mmol/L Glu. GT1-7 cells displayed significantly more cell damage for all time points after eight hours ($P < 0.005$). Although the SCN2.2 cells displayed some cell damage at all time points, only the 72, 88, 96 and 104 h time points were significantly different than the baseline value ($P < 0.05$).

Figure 4 reveals cell damage by MTT assay in SCN2.2 and GT1-7 neurons 48 h after exposure to 0, 1, 5 and 10 mmol/L Glu. With 5 and 10 mmol/L Glu the GT1-7 cells showed significantly more cell damage than control neurons exposed to no Glu ($P < 0.05$ and $P < 0.005$, respectively). SCN2.2 neurons were not significantly damaged at these Glu concentrations.

TUNEL is commonly used to measure the end result of apoptosis, degraded DNA.[37] Figures 5A–D illustrate typical TUNEL reactions for SCN2.2 and GT1-7 cells exposed or unexposed to 10 mmol/L Glu for 48 h. These data are quantified in Figure 5E, as indicated by averaged counts from two independent observers blinded to treatment conditions. Untreated SCN2.2 cells displayed increased basal levels of TUNEL-positive cells, but did not exhibit increased DNA damage after exposure to Glu. In contrast, TUNEL-positive cells were significantly increased in GT1-7 cells after Glu exposure ($P < 0.05$).

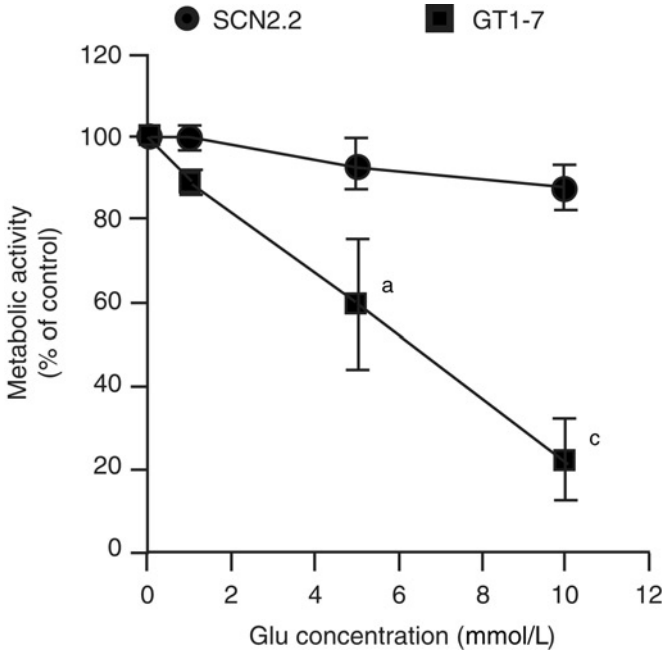

Figure 4 SCN2.2 cells incur less metabolic damage than GT1-7 cells in response to excitotoxic glutamate (Glu) concentrations. Cells were incubated with the indicated concentrations of Glu. Metabolic function was measured with the thiazolyl blue tetrazolium bromide (MTT) assay 48 h later. Each point represents the average of three to six separate experiments; for each individual experiment $n = 4$. Time-points were analyzed for differences from the control point with analysis of variance and *post hoc* Tukey analysis; 'a' indicates $P < 0.05$, 'c' indicates $P < 0.005$

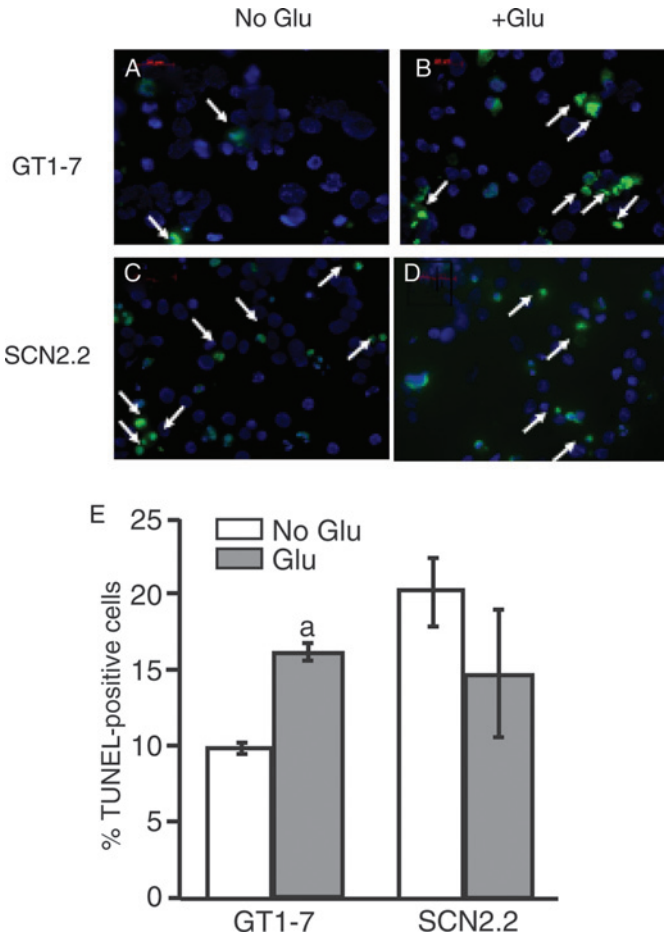

Figure 5 GT1-7 cells, but not SCN2.2 cells, undergo increased levels of DNA damage following glutamate (Glu) exposure. Cells were incubated with or without 10 mmol/L Glu for 24 h. Cells were fixed in 1% paraformaldehyde and processed for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Two independent observers, each blinded to experimental conditions, counted TUNEL-positive cells, and their results were averaged. $N = 3$ for each sample. (A–E) Samples of cell staining. (E) A quantitative summary of all experiments. Student's paired *t*-test was used to compare the Glu exposed to the unexposed cells; 'a' indicates $P < 0.05$

Excitotoxic resistance in SCN2.2 cells is not a function of Glu uptake by glia

Glu transporters, found primarily in glia, serve a protective function in Glu excitotoxicity by removing Glu from the extracellular environment.[38] We investigated the possibility that Glu uptake by glial cells might explain excitotoxic resistance in SCN2.2 cells by using PDC, a competitive inhibitor of all Glu transporters.[39–41] As shown in Figure 6, 250 mmol/L PDC alone did not cause cell death. In addition, inhibition of glial Glu uptake with 250 mmol/L PDC did not increase Glu excitotoxicity in SCN2.2 cells.

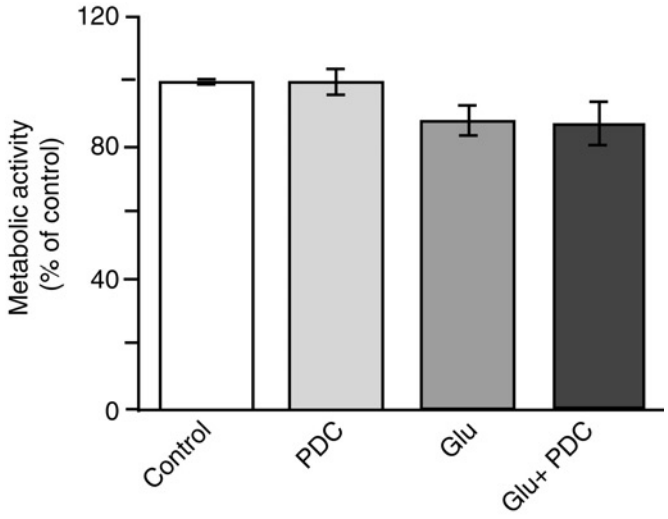

Figure 6 Inhibition of glutamate (Glu) transporters in SCN2.2 cells does not affect the response of SCN2.2 cells to Glu. SCN2.2 and GT1-7 cells were incubated for 48 h with control media, 10 mmol/L Glu, 250 mmol/L L-trans-pyrrolidine-2,4 dicarboxylate (PDC), or 10 mmol/L Glu + 250 mmol/L PDC. Cell death was measured by the thiazolyl blue tetrazolium bromide (MTT) assay. $N = 4$ for each sample

Discussion

These studies originated from the hypothesis that the SCN is endogenously protected against excitotoxic damage compared with other brain regions. Although to our knowledge the SCN has never been directly studied for its neuroprotective properties, previous work by other investigators indicates that the SCN is resistant to Glu and related compounds. Peterson and Moore[10] demonstrated that the SCN *in vivo* survives exposure to kainic acid that is lethal to the lateral hypothalamus and thalamus. Hastings *et al.*[11] demonstrated that the SCN *in vivo* was resistant to NMDA toxicity, in contrast to other regions of the anterior hypothalamus, which were susceptible. In fact in order to create SCN lesions *in vivo*, a surgical approach must be utilized.[42] Colwell and Levine exposed brain slices to NMDA, kainic acid, AMPA and Glu and measured cell swelling, a marker of later cell death. They showed that although these neurotoxins caused cell swelling in the hippocampus, cortex and neostriatum, the SCN was resistant.[14]

Prior work *in vivo* and in slices has shown that the SCN can survive and function after exposure to Glu at concentrations that are toxic to other brain regions. Injection of up to 1 mol/L Glu onto the SCN *in vivo* can stimulate brown fat thermogenesis, without any report of alteration or loss of circadian rhythms, which indicates that the SCN remains intact after high levels of Glu exposure.[13] A dose response curve revealed that 10 mmol/L was the lowest concentration required for maximal physiologic phase resetting in the SCN slice, and therefore this concentration is commonly used for SCN slice experiments.[15] Acute SCN slices from young adult animals survive at least three days in culture after exposure to 10 mmol/L Glu.[43,44] Thus, although it has never been the primary focus of attention and cell viability has not been specifically quantified, numerous studies have demonstrated the SCN's ability to withstand Glu, both *in vivo* and in the brain slice. We extend this work by demonstrating the ability of the immortalized SCN2.2 cell line *in vitro* to resist Glu neurotoxicity under conditions lethal to other immortalized neuronal cultures.

Our work supports other reports in the literature demonstrating that although the SCN is resistant to neurotoxic effects of Glu, it does contain a full repertoire of glutamatergic receptors, and can respond both *in vitro* and *in vivo* to Glu. The presence of mRNA coding for all NMDA receptor subunits (NR1, NR2A, NR2B, NR2C and NR2D) and two kainate-selective receptors (GluR5 and GluR6) has been demonstrated in the SCN.[45,46] NMDA microinjected into the SCN *in vivo* mimics the physiological effects of light.[47] Electrophysiological response of SCN neurons to NMDA and Glu has also been well established in slices.[15,48] Thus lack of neurotoxicity to Glu and NMDA is not because SCN neurons lack the ability to respond to these agents.

Several properties make the SCN an ideal model system for exploring naturally occurring neuroprotection. Brain slices from young adult animals provide increased physiological relevance, but still allow controlled study of the SCN.[16,17,49,50] SCN slices from adult animals can maintain circadian rhythms *in vitro* for several weeks.[51,52] Single unit recordings can be performed in SCN slices prepared from aged animals for at least 30 h *in vitro*.[53] The SCN can be easily located *in vivo*, facilitating microdialysis and histological studies of the SCN.[12] In addition, numerous pathways have been thoroughly explored in the SCN in the context of circadian rhythms. In particular, intracellular pathways activated by Glu, which are likely pertinent to neuroprotective strategies, have been widely investigated.[49] Finally, an immortalized cell line, SCN2.2, which retains many of the functional properties of the SCN, has been developed to facilitate *in vitro* SCN studies.[20–22]

We tested the SCN2.2 cell line for resistance to Glu excitotoxicity using Glu concentrations of 1–10 mmol/L. We chose this range because studies in SCN and hippocampal slices have used these levels.[15,54,55] Our results clearly indicate that the SCN2.2 cell line is protected against metabolic damage incurred by the neurotoxin Glu, as measured with the MTT assay. It is also protected against apoptosis as indicated by TUNEL. Finally, total cell death, as assessed by trypan blue exclusion, is substantially decreased after Glu treatment in the SCN2.2 cell line compared with the GT1-7 cell line. The SCN2.2 cell line produces both mRNA and protein for NMDA receptor subunits, and displays functional responses to Glu, which can be blocked by NMDA receptor blockers. In fact the SCN2.2 cells produce more mRNA for NR2A and NR2B than the control GT1-7 cells. Thus the SCN2.2 resistance to Glu excitotoxicity is not derived from an inability to respond to Glu. Although the SCN2.2 cell line does contain a small percentage of glial cells, these cells do not explain resistance of the SCN2.2 cell line to Glu; inhibition of the Glu transporter did not increase SCN2.2 cell death after Glu treatment. Taken together, these data conclusively establish naturally occurring excitotoxic resistance in the SCN2.2 cell line, which is likely similar to the SCN *in vivo*.

An unexpected finding in this study is that baseline levels of TUNEL are higher in the SCN2.2 cells than in the GT1-7

cells (Figure 5), despite the fact that Glu treatment did not alter the number of TUNEL-positive SCN2.2 cells. A similarly surprising finding in the literature is that cells that are artificially neuroprotected through preconditioning display higher levels of caspase 3, an indicator of apoptosis, as compared with control.[56–59] Furthermore, preconditioned cells show diminished activation of caspase 3 following toxin exposure, parallel to our results in SCN2.2 cells where baseline levels of TUNEL are higher, but do not increase with Glu treatment. The finding of increased baseline TUNEL levels in the SCN2.2 cells will need to be confirmed in SCN slices and *in vivo*, as it could be an artifact of the SCN2.2 cell culture system.

Mechanisms that underlie SCN resistance to excitotoxicity are beyond the scope of this work, but will be the subject of future investigation. Our results, which demonstrate increased NR2A and NR2B mRNA in the SCN2.2 cells compared with the GT1-7 cells, may provide one clue. NR2B often contributes to neurotoxic effects of NMDA receptors, while NR2A often exerts neuroprotective effects.[60–63] Our finding that the GT1-7 cells do not have measurable mRNA for the generally neurotoxic NR2B, whereas the SCN2.2 cells do express NR2B mRNA suggests that the ability of SCN2.2 cells to resist excitotoxicity is unlikely to be exclusively explained by alterations in NMDA receptor subunits. Because binding of Glu to the NMDA receptor is associated with an increase in intracellular calcium (Ca^{2+}), enhanced handling of intracellular Ca^{2+} flux by the SCN might provide protection. The SCN contains high levels of calbindin, a calcium binding buffer protein, which has been shown to be protective when transfected into neurons.[64] Calbindin serves as a Ca^{2+} buffer protein, and its presence may allow the SCN to maintain function in Glu conditions which in other cells produce toxic levels of Ca^{2+}. In addition, the SCN also has high levels of brain derived neurotrophic factor,[65,66] a neurotrophin that can protect cells, and it is also possible that its actions may explain the neuroprotection in the SCN.

Similar to the SCN *in vivo*, which is resistant to excitotoxicity compared with other regions of the brain, the immortalized SCN2.2 cells demonstrate increased resistance to excitotoxic stimuli compared with other neuronal cell lines. However, cell lines are generally more resistant to excitotoxicity. Because of its importance in cell cycle regulation, immortalized cells have higher levels of AkT activity. Activation of protein Kinase B/AkT/PI3 kinase signaling is associated with neuroprotection.[67] The AkT pathway is implicated in neuroprotective strategies engaged by numerous neuroprotective factors including, estrogens and selective estrogen receptor modulators,[68] brain-derived neurotrophic factor, insulin-like growth factor,[69] tumor necrosis factor[67] and nitric oxide.[70] Thus, it is possible that SCN2.2 cells have higher levels of AkT and this contributes to their relative neuroprotection compared with the other immortalized cell line used in these studies. Future studies will examine the role of this signaling pathway in SCN2.2 cells and in the SCN *in vivo*.

The SCN remains a compelling, yet completely unexplored model that can be exploited on multiple levels to reveal naturally occurring mechanisms of neuroprotection. The SCN has already been studied in the circadian field at varying levels of complexity, from the *in vitro* cell culture system, through the brain slice, to *in vivo* animal studies. Much is already known about the activation of SCN intracellular pathways in response to light and Glu.[49] Neuroprotection in the SCN is an intrinsic quality, which does not have to be experimentally created and then exploited in a limited time period. Thus investigations of the SCN may yield insights into neuroprotection that can be applied to therapeutic strategies for neurodegenerative disorders.

Author contributions: ST conceived the project; KB, EP, BH, SK and ST designed the experiments; KB and SJ supervised the experiments; KB, EP, BH, SK and ST performed the experiments; KB, BH, SK and ST analyzed the data; KB, EP and ST wrote the paper; KB, BH, SK, and ST revised the paper; KB and ST obtained financial support.

ACKNOWLEDGEMENTS

We gratefully acknowledge the expert technical assistance of Kelly Barry, Molly Comiskey, Caitlin Allen, Dr Richard Pamenter and Stacey Krager. Financial support was provided by the American Heart Association through a Fellow to Faculty Transition Award to KB (0375011N), and the University of Illinois Research Board through an award to SAT.

REFERENCES

1 Dong XX, Wang Y, Qin ZH. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin* 2009;**30**:379–87
2 Nakka VP, Gusain A, Mehta SL, Raghubir R. Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. *Mol Neurobiol* 2008;**37**:7–38
3 Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci* 1998;**18**:4914–28
4 Ebling F. The role of glutamate in the photic regulation of the suprachiasmatic nucleus. *Prog Neurobiol* 1996;**50**:109–32
5 Michel S, Itri J, Colwell CS. Excitatory mechanisms in the suprachiasmatic nucleus: the role of AMPA/KA glutamate receptors. *J Neurophysiol* 2002;**88**:817–28
6 Challet E. Minireview: entrainment of the suprachiasmatic clockwork in diurnal and nocturnal mammals. *Endocrinology* 2007;**148**:5648–55
7 Hamada T, Sonoda R, Watanabe A, Ono M, Shibata S, Watanabe S. NMDA induced glutamate release from the suprachiasmatic nucleus: an *in vitro* study in the rat. *Neurosci Lett* 1998;**256**:93–6
8 Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. *Brain Res Bull* 1998;**46**:281–309
9 Obrenovitch TP. High extracellular glutamate and neuronal death in neurological disorders. Cause, contribution or consequence? *Ann N Y Acad Sci* 1999;**890**:273–86
10 Peterson GM, Moore RY. Selective effects of kainic acid on diencephalic neurons. *Brain Res* 1980;**202**:165–82
11 Hastings MH, Roberts AC, Herbert J. Neurotoxic lesions of the anterior hypothalamus disrupt the photoperiodic but not the circadian system of the Syrian hamster. *Neuroendocrinology* 1985;**40**:316–24
12 Weaver DR. The suprachiasmatic nucleus: a 25-year retrospective. *J Biol Rhythms* 1998;**13**:100–12

13 Amir S, Shizgal P, Rompre PP. Glutamate injection into the suprachiasmatic nucleus stimulates brown fat thermogenesis in the rat. *Brain Res* 1989;**498**:140–4

14 Colwell CS, Levine MS. Glutamate receptor-induced toxicity in neostriatal cells. *Brain Res* 1996;**724**:205–12

15 Ding JM, Chen D, Weber ET, Faiman LE, Rea MA, Gillette MU. Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science* 1994;**266**:1713–7

16 Tischkau SA, Gallman EA, Buchanan GF, Gillette MU. Differential cAMP gating of glutamatergic signaling regulates long-term state changes in the suprachiasmatic circadian clock. *J Neurosci* 2000;**20**:7830–7

17 Tischkau SA, Mitchell JW, Tyan SH, Buchanan GF, Gillette MU. Ca^{2+}/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. *J Biol Chem* 2003;**278**:718–23

18 Rosenberg PA, Aizenman E. Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. *Neurosci Lett* 1989;**103**:162–8

19 Yang RC, Shih HC, Hsu HK, Chang HC, Hsu C. Estradiol enhances the neurotoxicity of glutamate in GT1-7 cells through an estrogen receptor-dependent mechanism. *Neurotoxicology* 2003;**24**:65–73

20 Earnest DJ, Liang FQ, DiGiorgio S, Gallagher M, Harvey B, Earnest B, Seigel G. Establishment and characterization of adenoviral E1A immortalized cell lines derived from the rat suprachiasmatic nucleus. *J Neurobiol* 1999;**39**:1–13

21 Hurst WJ, Mitchell JW, Gillette MU. Synchronization and phase-resetting by glutamate of an immortalized SCN cell line. *Biochem Biophys Res Commun* 2002;**298**:133–43

22 Earnest DJ, Liang FQ, Ratcliff M, Cassone VM. Immortal time: circadian clock properties of rat suprachiasmatic cell lines. *Science* 1999;**283**:693–5

23 Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* 1990;**5**:1–10

24 Bonfoco E, Zhivotovsky B, Rossi AD, Aguilar-Santelises M, Orrenius S, Lipton SA, Nicotera P. BCL-2 delay apoptosis and PARP cleavage induced by NO donors in GT1-7 cells. *Neuroreport* 1996;**8**:273–6

25 Srinivasan A, Foster LM, Testa MP, Ord T, Keane RW, Bredesen DE, Kayalar C. Bcl-2 expression in neural cells blocks activation of ICE/CED-3 family proteases during apoptosis. *J Neurosci* 1996;**16**:5654–60

26 Wei H, Perry DC. Dantrolene is cytoprotective in two models of neuronal cell death. *J Neurochem* 1996;**67**:2390–8

27 Wei H, Wei W, Bredesen DE, Perry DC. Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. *J Neurochem* 1998;**70**:2305–14

28 Mukai M, Tischkau SA. Effects of tryptophan photoproducts in the circadian timing system: searching for a physiological role for aryl hydrocarbon receptor. *Toxicol Sci* 2007;**95**:172–81

29 Dracheva S, Marras SA, Elhakem SL, Kramer FR, Davis KL, Haroutunian V. *N*-methyl-D-aspartic acid receptor expression in the dorsolateral prefrontal cortex of elderly patients with schizophrenia. *Am J Psychiatry* 2001;**158**:1400–10

30 Karman BN, Tischkau SA. Circadian clock gene expression in the ovary: effects of luteinizing hormone. *Biol Reprod* 2006;**75**:624–32

31 Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;**25**:169–93

32 Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001;**25**:386–401

33 Allen G, Rappe J, Earnest DJ, Cassone VM. Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. *J Neurosci* 2001;**21**:7937–43

34 Sgonc R, Gruber J. Apoptosis detection: an overview. *Exp Gerontol* 1998;**33**:525–33

35 Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem* 1997;**69**:581–93

36 Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;**119**:493–501

37 Labat-Moleur F, Guillermet C, Lorimier P, Robert C, Lantuejoul S, Brambilla E, Negoescu A. TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement. *J Histochem Cytochem* 1998;**46**:327–34

38 Sattler R, Rothstein JD. Regulation and dysregulation of glutamate transporters. *Handb Exp Pharmacol* 2006;277–303

39 Bridges RJ, Stanley MS, Anderson MW, Cotman CW, Chamberlin AR. Conformationally defined neurotransmitter analogues. Selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate diastereomer. *J Med Chem* 1991;**34**:717–25

40 Balcar VJ. Na(+)-dependent high-affinity uptake of L-glutamate in cultured fibroblasts. *FEBS Lett* 1992;**300**:203–7

41 O'Shea RD, Fodera MV, Aprico K, Dehnes Y, Danbolt NC, Crawford D, Beart PM. Evaluation of drugs acting at glutamate transporters in organotypic hippocampal cultures: new evidence on substrates and blockers in excitotoxicity. *Neurochem Res* 2002;**27**:5–13

42 Locatelli A, Caldani M, Tessonneaud A, Cooper HM, Viguier-Martinez MC. A new surgical approach to the ram suprachiasmatic nuclei. *J Neurosci Methods* 1994;**51**:189–95

43 Ding JM, Faiman LE, Hurst WJ, Kuriashkina LR, Gillette MU. Resetting the biological clock: mediation of nocturnal CREB phosphorylation via light, glutamate, and nitric oxide. *J Neurosci* 1997;**17**:667–75

44 Franken P, Cao V, Heller HC, Miller JD. The glutamate induced phase shift in the SCN slice: a two pulse study. *Brain Res* 1999;**818**:34–40

45 O'Hara BF, Andretic R, Heller HC, Carter DB, Kilduff TS. GABAA, GABAC, and NMDA receptor subunit expression in the suprachiasmatic nucleus and other brain regions. *Brain Res Mol Brain Res* 1995;**28**:239–50

46 Moriya T, Takahashi S, Ikeda M, Suzuki-Yamashita K, Asai M, Kadotani H, Okamura H, Yoshioka T, Shibata S. *N*-methyl-D-aspartate receptor subtype 2C is not involved in circadian oscillation or photoic entrainment of the biological clock in mice. *J Neurosci Res* 2000;**61**:663–73

47 Novak CM, Albers HE. *N*-Methyl-D-aspartate microinjected into the suprachiasmatic nucleus mimics the phase-shifting effects of light in the diurnal Nile grass rat (*Arvicanthis niloticus*). *Brain Res* 2002;**951**:255–63

48 Pennartz CM, Hamstra R, Geurtsen AM. Enhanced NMDA receptor activity in retinal inputs to the rat suprachiasmatic nucleus during the subjective night. *J Physiol* 2001;**532**:181–94

49 Gillette MU, Mitchell JW. Signaling in the suprachiasmatic nucleus: selectively responsive and integrative. *Cell Tissue Res* 2002;**309**:99–107

50 Gillette MU, Tischkau SA. Suprachiasmatic nucleus: the brain's circadian clock. *Recent Prog Horm Res* 1999;**54**:33–58; discussion 58–39

51 Maywood ES, O'Neill JS, Chesham JE, Hastings MH. Minireview: the circadian clockwork of the suprachiasmatic nuclei – analysis of a cellular oscillator that drives endocrine rhythms. *Endocrinology* 2007;**148**:5624–34

52 Brown TM, Banks JR, Piggins HD. A novel suction electrode recording technique for monitoring circadian rhythms in single and multiunit discharge from brain slices. *J Neurosci Methods* 2006;**156**:173–81

53 Satinoff E, Li H, Tcheng TK, Liu C, McArthur AJ, Medanic M, Gillette MU. Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? *Am J Physiol* 1993;**265**:R1216–22

54 Molz S, Decker H, Dal-Cim T, Cremonez C, Cordova FM, Leal RB, Tasca CI. Glutamate-induced toxicity in hippocampal slices involves apoptotic features and p38 MAPK signaling. *Neurochem Res* 2008;**33**:27–36

55 Molz S, Decker H, Oliveira IJ, Souza DO, Tasca CI. Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevented by GMP. *Neurochem Res* 2005;**30**:83–9

56 Tremblay R, Chakravarthy B, Hewitt K, Tauskela J, Morley P, Atkinson T, Durkin JP. Transient NMDA receptor inactivation provides long-term protection to cultured cortical neurons from a variety of death signals. *J Neurosci* 2000;**20**:7183–92

57 McLaughlin B. The kinder side of killer proteases: caspase activation contributes to neuroprotection and CNS remodeling. *Apoptosis* 2004;**9**:111–21

58 McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E. Caspase 3 activation is essential for neuroprotection in preconditioning. *Proc Natl Acad Sci USA* 2003;**100**:715–20

59 Tanaka H, Yokota H, Jover T, Cappuccio I, Calderone A, Simionescu M, Bennett MV, Zukin RS. Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. *J Neurosci* 2004;**24**:2750–9

60 Chen M, Lu TJ, Chen XJ, Zhou Y, Chen Q, Feng XY, Xu L, Duan WH, Xiong ZQ. Differential roles of NMDA receptor subtypes in ischemic neuronal cell death and ischemic tolerance. *Stroke* 2008;**39**:3042–8
61 Liu Y, Wong TP, Aarts M, Rooyakkers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M, Craig AM, Wang YT. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both *in vitro* and *in vivo*. *J Neurosci* 2007;**27**:2846–57
62 von Engelhardt J, Coserea I, Pawlak V, Fuchs EC, Kohr G, Seeburg PH, Monyer H. Excitotoxicity *in vitro* by NR2A- and NR2B-containing NMDA receptors. *Neuropharmacology* 2007;**53**:10–7
63 Martel MA, Wyllie DJ, Hardingham GE. In developing hippocampal neurons, NR2B-containing *N*-methyl-D-aspartate receptors (NMDARs) can mediate signaling to neuronal survival and synaptic potentiation, as well as neuronal death. *Neuroscience* 2009;**158**:334–43
64 Costa MS, Britto LR. Calbindin immunoreactivity delineates the circadian visual centers of the brain of the common marmoset (*Callithrix jacchus*). *Brain Res Bull* 1997;**43**:369–73
65 Liang FQ, Walline R, Earnest DJ. Circadian rhythm of brain-derived neurotrophic factor in the rat suprachiasmatic nucleus. *Neurosci Lett* 1998;**242**:89–92
66 Liang FQ, Allen G, Earnest D. Role of brain-derived neurotrophic factor in the circadian regulation of the suprachiasmatic pacemaker by light. *J Neurosci* 2000;**20**:2978–87
67 Marchetti L, Klein M, Schlett K, Pfizenmaier K, Eisel UL. Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by *N*-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. *J Biol Chem* 2004;**279**:32869–81
68 Bourque M, Dluzen DE, Di Paolo T. Neuroprotective actions of sex steroids in Parkinson's disease. *Front Neuroendocrinol* 2009;**30**:142–57
69 Johnson-Farley NN, Patel K, Kim D, Cowen DS. Interaction of FGF-2 with IGF-1 and BDNF in stimulating Akt, ERK, and neuronal survival in hippocampal cultures. *Brain Res* 2007;**1154**:40–9
70 De Palma C, Falcone S, Panzeri C, Radice S, Bassi MT, Clementi E. Endothelial nitric oxide synthase overexpression by neuronal cells in neurodegeneration: a link between inflammation and neuroprotection. *J Neurochem* 2008;**106**:193–204

(Received August 14, 2009, Accepted December 7, 2009)