EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

European Journal of Neuroscience, Vol. 45, pp. 1111–1123, 2017 doi:10.1111/ejn.13553

BEHAVIOURAL NEUROSCIENCE

NF-κB signalling is involved in immune-modulation, but not basal functioning, of the mouse suprachiasmatic circadian clock

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Keywords: ageing, circadian, neuroimmune, NF-κB, PER2, suprachiasmatic

Edited by Rae Silver
Received 1 November 2016, revised 22 February 2017, accepted 23 February 2017

Abstract

Circadian rhythms are recurring near-24 hour patterns driven by an endogenous circadian timekeeping system. The master pacemaker in this system is the hypothalamic suprachiasmatic nucleus (SCN). Recently interest has been drawn to how the SCN clock responds to immune system stimulation. A major signalling component in the immune system is nuclear factor (NF)-κB. In the present study we examined the role of NF-κB in SCN function. Whilst serum shocked fibroblasts showed rhythmic nuclear localisation of p65 and p65-dependent transcription, there were no circadian changes in the SCN in expression of the NF-κB components p65, c-Rel, p-IκB or p-IKK. Chronic treatment with the NF-κB inhibitor PDTC did not impact on circadian or diurnal rhythms. Phase-shifting light pulses did not impact on SCN expression of p65, and PDTC treatment did not attenuate the behavioural or molecular response to light pulses. Peripheral treatment with lipopolysaccharide resulted in increased NF-κB component expression in the SCN. *In vitro* experiments with SCN slice cultures showed that treatment with NF-κB inhibitors did not markedly alter rhythmic changes in PER2::LUC expression. Further, SCN slices from *nf-κb::luc* mice did not show any evidence for circadian rhythms in NF-κB-mediated transcription. Experiments utilising older mice (~16 months old) showed that SCN treatment *in vitro* with PDTC resulted in increased amplitude of rhythmic PER2::LUC expression, and LPS treatment resulted in altered PER2::LUC rhythm acrophase. Overall, we interpret our results as providing evidence for the involvement of NF-κB in the suprachiasmatic circadian clock following immune stimulation, but not under basal conditions.

Introduction

Circadian rhythms are recurring patterns in physiological, biochemical, metabolic, behavioural or other parameters that cycle approximately every 24 h and persist in the absence of external time cues (Reppert & Weaver, 2002). Such internally generated rhythms are the products of a complex circadian timing system, with a master pacemaker located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus synchronising semi-autonomous circadian pacemakers throughout the CNS and the periphery in order to impose a daily temporal architecture on mammalian physiology and behaviour (Antle & Silver, 2005). These circadian rhythms are in turn normally entrained to appropriate environmental signals (zeitgebers), the principal one being light (Golombek & Rosenstein, 2010). There is a direct glutamatergic innervation of the SCN arising from a subset of intrinsically photosensitive retinal ganglion cells that express the photopigment melanopsin that convey photic information to the SCN (Lucas *et al.*, 2014). At a molecular level, the workings of the circadian clock comprises a series of interlocking feedback/feedforward transcriptional loops whose core components are a panel of canonical clock genes and their protein products (Albrecht, 2012). These clock genes in turn regulate the temporal expression of a wide range of clock-controlled genes in a tissue specific manner (Zhang *et al.*, 2014), and such regulation is ultimately translated into physiological and behavioural rhythms.

An area of considerable research in previous years addresses the signalling input pathways that convey events at the SCN neuronal membrane (such as ligand-receptor binding events) to the core nuclear clockworks. To date several signalling pathways have been identified as playing such key roles, including those involving mitogen activated protein kinases (Coogan & Piggins, 2004), protein kinase C (Bonsall & Lall, 2013) and salt-inducible kinase 1 (Jagannath *et al.*, 2013). These signallers are believed to alter the activity of transcriptions factors such as CREB, which then bind to promoter sequences on clock genes to alter their expression (Lee *et al.*, 2010). One pathway that has not been systemically investigated in

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terms of regulation of suprachiasmatic clock function involves the transcription factor nuclear factor–κB (NF-κB) pathway. NF-κB is composed of dimers of members of the Rel family, including p50, p52, p65 (also known as RelA), c-Rel and Rel-B, with p65-p50 being the most common dimeric complex (Ghosh & Hayden, 2012). Under basal condition, NF-κB component proteins are held in the cytoplasm in complex with members of the inhibitor IκB protein family. When bound to IκBs, one nuclear localisation signal on NF-κB components is obscured. Upon activation, IκB is phosphorylated, then ubiquitinated and finally proteolysed, allowing NF-κB dimers free access to the nucleus to regulate transcription of genes containing κ-B binding sites (Moynagh, 2005). NF-κB has been shown to be activated following ligand binding to Toll-like receptors (TLRs), and receptors for the pro-inflammatory cytokines interleukin-1 and tumour necrosis factor (TNF), amongst others (Lawrence, 2009). Although originally described in the peripheral immune system, NF-κB has been implicated in CNS function, with roles described in synaptic plasticity, neurogenesis and learning and memory (Crampton & O'Keeffe, 2013; Snow *et al.*, 2014; Kaltschmidt & Kaltschmidt, 2015), as well as its better defined roles in neuroinflammation and neurodegeneration (Malek *et al.*, 2007). However, recent studies have raised questions as to whether NF-κB activity is constitutive in young adult neurons in the basal state (Herkenham *et al.*, 2011; Listwak *et al.*, 2013; Zhang *et al.*, 2013), indicating that activity in glia or endothelia may be linked to CNS processes linked to NK-κB activity.

Given that stimulation of the peripheral immune system can alter circadian timing (e.g. Marpegan *et al.*, 2004; Coogan & Wyse, 2008; Paladino *et al.*, 2014), and that the SCN appears to be responsive to such stimulation (Beynon & Coogan, 2010), it is of interest to ask the role that NF-κB signalling might play in the SCN circadian clock and resetting of circadian rhythms. One study in hamsters indicated that NF-κB signalling was involved in photic resetting of behavioural rhythms (Marpegan *et al.*, 2004). Previous studies in peripheral cell types have indicated that NF-κB interacts with core clock gene products (Bellet *et al.*, 2012; Narasimamurthy *et al.*, 2012; Spengler *et al.*, 2012; Curtis *et al.*, 2015), although given the differences in neuronal expression it is not clear whether such interactions may occur in the master SCN clock. In order to address this we have carried out a study in which we examined the expression of NF-κB pathway components in the SCN across the circadian cycle and following photic and immune stimulation, as well as examining the consequences of pharmacological blockade of the NF-κB pathway on circadian rhythms *in vivo* and *in vitro*.

Materials and methods

Fibroblast cell culture and serum shock

The immortalised mouse fibroblast NIH/3T3 cell line was cultured in Dulbecco's Modified Eagle medium (DMEM) with Glutamax-1, 4.5 g/L D-Glucose and pyruvate (Gibco, UK) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). All medium was pre-warmed to 37 °C. After reaching 80% confluency (approximately every 3 days), the cells were passaged by washing with phosphate – buffered saline (Gibco) and then exposed to a 5 min incubation with Trypsin-EDTA solution (0.5 g/L trypsin and 0.2 g/L EDTA in PBS) until cells became detached. Trypsin was deactivated with DMEM and cells re-suspended vigorously so a single cell suspension was achieved. Cells were then transferred to a 75 cm^2 tissue culture flask containing fresh cell culture medium at a 1 : 10 dilution. Serum shock experiments were carried out according to the protocol by Balsalobre *et al.* (1998). NIH/3T3 cells in log phase growth were washed with PBS, trypsinised and resuspended in10% DMEM and seeded into culture dishes at a density of 1×10^5 10 cm^2. Upon reaching confluency medium was replaced with serum rich medium [DMEM supplemented with 50% Horse serum (GIBCO)] time = 0 and after 2-h incubation medium was replaced with serum free DMEM. Nuclear and Cytoplasmic protein extractions were performed every 4 h up to 72 h after serum treatment using a Nuclear Extract Kit (Active Motif, Belgium). For sham shocked controls, serum free cells NIH/3T3 cells were seeded into culture dishes at a density of 1×10^5 10 cm^2 in 10%DMEM and upon reaching confluency media was replaced with serum free DMEM (time = 0) and subsequent nuclear and cytoplasmic extractions undertaken every 4 h for 48 h using Active Motifs Nuclear Extract Kit.

Western blotting

Protein concentrations in cytoplasmic and nuclear fractions were determined using a detergent compatible protein assay kit, DC Protein Assay (Bio-Rad Laboratories, UK). Lysates were mixed with Laemmli Lysis Buffer (Sigma, UK) in a 1 : 1 ratio at a concentration of 5 μg protein and incubated for 5 min at 95–100 °C in a heat block. Samples were briefly spun down and proteins in the extract were separated by gel electrophoresis using an 8 or 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), depending on the size of the protein being detected. Five microlitre of precision plus protein all blue standard (Bio-Rad Laboratories) was loaded into the gel system as a protein marker. Following this Immun-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad) were immersed in 100% methanol, and incubated with fibre pads, blot paper (Bio-Rad) and polyacrylamide gels, for 15 min in transfer buffer pre-cooled to 4 °C. Proteins were electrotransferred onto the membrane using Trans-blot system (Bio-Rad, UK) at 400 mA for 1 h at 4 °C. After transfer the membranes (blots) were washed with TBS/T (Tris Buffered Saline containing 0.1% (v/v) Tween 20) for 5 min and non-specific binding was blocked by exposing the membranes to 3% dry milk (1.5 g milk and 50 mL TBS/T; Sigma), for 1 h at room temperature with mild agitation. Subsequent washes with TBS/T for 5 min were followed by incubation with primary antibody for 90 min at room temperature with mild agitation. The primary antibodies used in this experiment were p65 subunit of the NF-κB complex (sc-372, used at 1 : 1000, rabbit polyclonal; Santa Cruz Biotechnology, USA) and the nuclear matrix protein p84 (mouse monoclonal; abcam, UK, ab487, used at 1 : 1000) used as a nuclear marker and loading control. Membranes (blots) were washed 2 × 10 min with TBS/T then incubated with horseradish peroxidase-conjugated secondary antibody (goat polyclonal to rabbit IgG; abcam, ab6721, used at 1 : 2000 and rabbit polyclonal to mouse IgG; abcam, ab6728, used at 1 : 5000) for 45 min at room temperature with mild agitation. Membranes were subsequently washed 3 × 10 min with TBS/T. For protein detection peroxide solution and luminol/enhancer solution (Immun-star WesternC Chemiluminescent kit; Bio-Rad, UK) were mixed in a 1 : 1 ratio and incubated for 5 min at room temperature with gentle agitation. The proteins were visualised using ChemiDoc XRS (Bio-Rad) after 15 s to a 2-min exposure. Protein banding was densitometrically assessed using the Scion Image _ 4.2 software (Scion Corporation, MA, USA). Stain density of bands was analysed by measurement of the uncalibrated optical density [OD = log10(255/(255 – grey value, where the grey value is calculated for each pixel in the selection and score

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0–255))]. All values of target proteins were normalised to a standard nuclear matrix protein p84 followed by normalisation to control.

NF-κB p65 transcription factor assay

Nuclear extract samples were diluted in 20 μL of complete lysis buffer at a concentration of 5 μg. Thirty microlitre of Complete Binding Buffer was added to each well (containing an oligonucleotide with the NF-κB consensus site 5′ –GGACTTTCC-3′ immobilised to it) followed by 20 μL of sample diluted in complete lysis buffer, a blank (20 μL complete lysis buffer only) and a positive control Jurkat Nuclear extract (Active Motif, Belgium) were also added all in duplicate. The plate was then incubated for 1 h at room temperature (RT) with mild agitation (150 rpm on a rocking platform) and covered with an adhesive seal. Each well was subsequently washed three times with 200 μL 1× wash buffer. 100 μL of diluted NFκB primary antibody (1 : 1000 dilution in 1× antibody binding buffer) was added to each well and the plate covered and incubated for 1 h at RT without agitation. Wells were washed three times with 200 μL 1× wash buffer as previously described. This is followed by the addition of 100 μL of diluted HRP-conjugated secondary antibody (1 : 1000 dilution in 1× antibody binding buffer) for 1 h at RT without agitation. The wells were washed four times with 200 μL 1× wash buffer as described previously and 100 μL of pre-warmed developing solution added. The plate was incubated for 2 min at RT away from direct sunlight until the samples turned to a medium to dark blue. 100 μL of stop solution was added, which in the presence of acid turns the blue colour yellow and absorbance was read at 460 nm on POLARstar OMEGA plate reader (BMG labtech, Germany).

Behavioural circadian rhythm monitoring

Male C57Bl/6 mice (8–14 weeks old) obtained from Charles River Laboratories (Kent, UK) were used throughout this study apart from experiments for luciferase-reporter analysis (detailed below). All experiments were approved by the Research Ethics Committee, National University of Ireland Maynooth and licensed by the Irish Department of Health and Children and were carried out in accordance with European Union directive 2010/63. In total, 304 mice were used in this study. Animals were housed in a conventional facility, and as such were not specific pathogen free.

For the analysis of circadian wheel running behaviour, animals were singly housed in polypropylene cages equipped with running wheels (11 cm diameter) with food and water available *ad libitum* and temperature held constant at 21 + 1 °C and humidity at 50 + 10%. Cages were then housed in an environmental isolation cabinet to allow for full control of the photic environment. Illumination was via a white light fluorescent source, with average illuminance at the level of the cage floor of 250 lux when lights were on. Bedding was changed every 14 days, and never in the period leading up to or after a pharmacological intervention. Wheel running was monitored via microswitches attached to the running wheels communicating with the Chronobiology Kit (Stanford Software Systems, CA, USA) to allow for production of actograms of wheel-running behaviour. Prior to any pharmacological intervention animals were allowed to free-run for 2 weeks to allow for analysis of stable baselines of circadian factors. Phase-shifts were rated by two to three independent observers blind to the treatments by means of the line-of-best-fit method through activity onsets for 10 days prior to and after any intervention. Phase shifts were calculated as the difference in the regression lines before and after the intervention, calculated on the day following the intervention. Period length and rhythm strength were obtained from Poisson Periodograms in the Chronobiology kit.

For experiments examining the photic induction of NF-κB components in the SCN, animals were group housed in cages of five under a 12 : 12 light : dark cycle before being transferred into constant darkness (DD) for two cycles, with light pulses delivered at CT15 (for this case 3 h after when the lights were going out in the colony under the previous LD cycle) or at CT22 for 30 min (150 lux). Animals were sampled prior to the light pulse, 5 or 30 min into the light pulse, or 30 min after the completion of the light pulse. For circadian analysis of NF-κB in the SCN, group housed animals were entrained to a 12 : 12 light : dark cycle prior to being released into DD and sampled every 4 h for 24 h on the third cycle of DD. For these experiments animals were sampled in DD under dim red light.

In vivo *drug treatments*

We utilised pyrrolidine dithiocarbamate (PDTC) as an inhibitor of NF-κB activity for *in vivo* experiments. PDTC is a blood-brain barrier permeant molecule that appears to have a number of modes of action in preventing NF-kB activation, including inhibition of IκB ubiquitin ligase activity (Nurmi *et al.*, 2004; Gupta *et al.*, 2010; Li *et al.*, 2012). PDTC is also an anti-oxidant and metal chelator, although it appears that it's NF-kB inhibitory action is independent of its action as an anti-oxidant (Hayakawya *et al.*, 2003). For behavioural analysis of phase shifts, single housed animals were initially entrained to a 12 : 12 light : dark cycles before being released into DD for 14 days. Animals then received an i.p. injection of PDTC (200 mg/kg; Sigma, Ireland; Anderson *et al.*, 2015b) or vehicle (1% DMSO in saline) at either CT14.5 or CT21.5. They were then exposed to a 30 min light pulse before being placed back in DD. 14 days after the first treatment animals were treated with the cross-over treatment and light-pulsed at the same CT as in the first treatment. For experiments utilising sub-chronic treatment with PDTC, PDTC (50 mg/kg/day; Ahtoniemi *et al.*, 2007) or vehicle (1% DMSO) was delivered via the drinking water for 14 days under a 12 : 12 light : dark cycle, and for another 14 days after animals were transferred into DD. PDTC or vehicle was renewed every 3 days. Daily doses were calculated on the basis of previously determined daily drinking water intake for individual animals. Treatment was then crossed over (vehicle to PDTC, or vice versa) and for a further 14 days in DD followed by 14 days following being placed back into the original L : D cycle. Water and drug were renewed every 3 days throughout this experiment. For experiments involving LPS treatment, animals received a single i.p. injection of 5 mg/kg LPS (serotype 0111.B4; Sigma) or sterile saline vehicle which occurred between zeitgeber time (ZT) 6 and ZT8, where ZT0 is defined as the time of lights on. Animals were sampled 24 h after the LPS treatment.

Immunohistochemistry

For immunohistochemical analysis of brain, animals were terminally anaesthetised with 0.1 mL sodium pentobarbitone and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M Phosphate Buffer. Brains were subsequently removed, post-fixed overnight and cryoprotected in 30% Sucrose (Sigma) for 24 h. 30 μm thick serial coronal sections were cut throughout the rostrocaudal extent of the SCN on a freezing stage microtome (Leica). All sections through the SCN were

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collected and divided in four series, obtaining five or six sections through the SCN in each series. Immunohistochemistry was by a standard Avidin-Biotin Complex/Nickel DAB colourimetric protocol, as previously described (Beynon *et al.*, 2009). A list of all of the primary antibodies used in this study is in Table S1. Lack of non-specific staining was confirmed by pre-incubation with peptides used to raise the primary antibodies. As much as feasible sections for the same antigen from different groups were reacted in parallel, and when this was not possible development of immunostaining was standardised between runs (e.g. same amount of time developing in Ni-DAB) to minimise as much as possible inter-run variability. For analysis of immunostained sections photomicrographs of the mid-rostrocaudal level of the SCN were taken using a digital camera connected to an Olympus BX-51 light microscope equipped with an image analysis digital system (ImageJ 1.43; NIH, USA). Between three and six images were evaluated for each individual animal and region to give a mean value for each animal. Immunoreactive cells in each region of interest were quantified using either image analysis software or number of immunoreactive cells quantified by an observer (for immediate early genes). The observer was blinded to the experimental procedure during optical density measurements or quantification of immunoreactive cells per SCN. The difference in integrated optical density (IOD) of antibody immunosignal in the SCN or the number of immunoreactive cells that displayed clear nuclear staining in the SCN of all animal groups was evaluated for quantitative analysis. p65, as part of the NF-κB complex, when activated translocates to the nucleus and as such we quantified the number of cells that displayed clear nuclear p65 staining as well as quantitating the IOD for p65 immunostaining (Beynon & Coogan, 2010). The light intensity was kept constant while all measurements were taken to standardise IOD measurements for analysis.

SCN PER2::LUC and nf-κb::luc in vitro *monitoring*

Expression of PER2 or NF-κB-mediated transcription was monitored in the SCN *in vivo* with the use of luciferase-based reporter constructs expressed in transgenic mice. For PER2::LUC monitoring we utilised a knock-in mouse line which expresses a PER2-LUC fusion protein (Yoo *et al.*, 2004). NF-κB-mediated transcription was monitored with the use of a transgenic line in which *luciferase* transcription is driven by a promoter sequence containing two NF-κB binding sites in front of a minimal *fos* promoter (Voll *et al.*, 2000). Both lines were obtained from The Jackson Laboratory (Maine, USA). PER2::LUC animals were maintained as homozygotes and *nf-κB::luc* animals as hemizygotes. Both lines were on a C57Bl/6 background and both male and female mice were used for slice preparation.

For SCN slice culture preparation, a protocol as previously described was followed (Hastings *et al.*, 2005). Briefly, mice were culled by cervical dislocation following halothane anaesthesia during the mid lights on period (ZT6-ZT8). Brains were rapidly removed and ice cooled before coronal 300 μm thick hypothalamic slices containing the SCN were prepared using a Vibroslice (Campden Insturments, UK). The SCN was then microdissected out under sterile conditions and transferred to interface-style Millicell culture inserts (Millipore, Ireland) placed in culture dishes sealed with a glass cover slip and containing 1.2 mL of sterile culture medium (DMEM supplemented with 3.5 g/L d-glucose, 0.035% sodium bicarbonate, 0.01 M HEPES buffer (all Sigma), 1000 μg/mL penicillin–streptomycin and 5% foetal bovine serum (both Gibco Invitrogen Ltd). Culture dishes were then placed in a light-tight incubator at 37 °C, with each individual culture placed under a photomultiplier tube (PMT) assemblies (H8259/R7518P; Hamamatsu, UK). Photon counts were integrated for 59 s every 1 min. Bioluminescence data were detrended offline by subtracting a 24 h running average from the raw data and smoothed with a 3 h running average (Guilding *et al.*, 2010). For drug treatments of PER2::LUC slices with NF-κB pathway inhibitors, PDTC (20 μm in 0.01% vehicle DMSO) or sulfasalazine [10 μm in 0.01% DMSO; Sulfasalazine inhibits NF-κB activation through blocking IκB kinase activity (Weber *et al.*, 2000)], or LPS as a NF-κB activator (100 ng/mL), were applied upon culture preparation and left in the culture medium for the duration of the recording.

Statistical analysis

Statistical analysis was carried out on SPSS (www.spss.com) on a PC. As appropriate to the individual experiment, data was either analysed via one-way ANOVA, between groups two-way ANOVA, between-within mixed ANOVAs, between groups *t*-tests, and paired *t*-tests as appropriate to the experimental design and data. *Post hoc* analysis of pairwise differences was via the Tukey HSD test. Assessment of circadian rhythmicity in independent samples (e.g. serum shocked fibroblasts, SCN examined by immunohistochemistry) was by CIRCWAVE V1.4 software (CircWave V1.4 by Hut, 2007, Groningen, The Netherlands) where peak phase and amplitude were assessed and a significant rhythm was deemed to be presented if the 95% confidence interval of the fitted curve's amplitude did not include 0. Circadian rhythmicity in PER2::LUC and *nf-κb::luc* was assessed with the Acro and Cosinor programmes (http://www.circadian.org/softwar.html).

Results

To initially gauge whether there may be an involvement of NF-κB signalling in the circadian system, we examined serum shocked fibroblasts, a well characterised cellular model of peripheral clocks. Here we found that serum shocked, but not sham treated cells, showed a significant circadian rhythm in the nuclear expression of p65 protein over 48 h ($P = 0.008$ via cosinor analysis for shocked cells; Fig. 1A). When we examined p65 driven transcriptional activity in these cells we also found there to be a significant rhythm in the serum shocked cells ($P = 0.009$ via cosinor analysis), but also found a significant rhythm in the sham treated cells ($P = 0.01$; Fig. 1B).

Given these initial observations we then examined expression of components of the NF-κB pathway in the SCN of mice in DD. We found that p65 expression, as measured either as optical density of the immunostaining (Fig. 2A) or as number of cells clearly expressing nuclear p65 (data not shown), did not vary with time in the SCN ($F_{5,12} = 1.23$, $P = 0.35$ via one-way ANOVA). Likewise, c-Rel-immunoreactivity also did not vary with time in the SCN ($F_{5,12} = 0.89$, $P = 0.53$; Fig. 2B). We then examined two upstream components of the pathway, phosphorylated-IKK and phosphorylated-IκB, and find that expression of these factors do not vary with time in the SCN ($F_{5,12} = 1.22$, $P = 0.35$; $F_{5,12} = 0.21$, $P = 0.95$ respectively, Fig. 2C and D). Further, none of these four antigens showed evidence of circadian rhythmicity as judged by co-sinor analysis. As a positive control, the clock gene product PER1 showed statistically significant cycling as expected in the SCN (Fig. S1).

To further investigate whether the NF-κB pathway may place a role in the ongoing running of the central circadian clock, mice were

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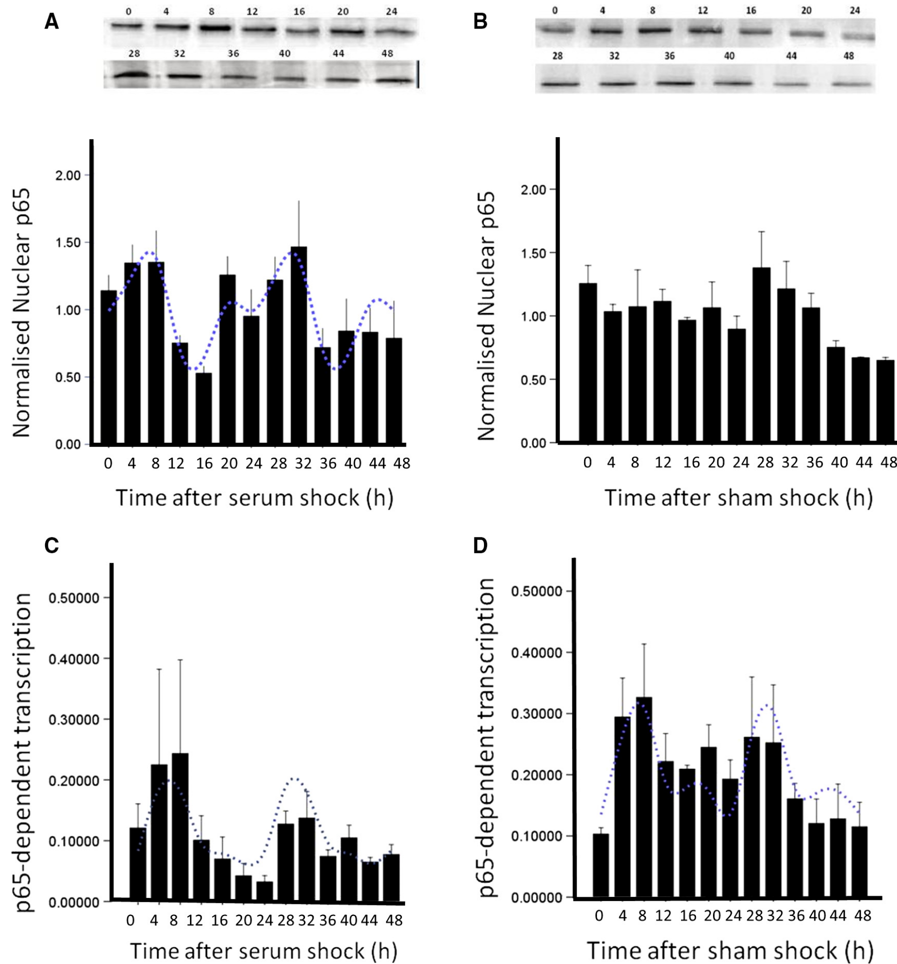

FIG. 1. Expression of p65 in the nuclear fraction of serum shocked (A) or sham-shocked (B) fibroblast over a 48 h time course after shock. (C) and (D) show the data for p65-dependent transcription. The dotted line shows the cosine wave of best significant fit (with a 12 h harmonic for A and D). Serum shocked cells show a significant circadian rhythm in expression, but sham-shocked cells do not (A and B). For p65 driven transcriptional activity there was circadian rhythmicity, as judged by co-sinor analysis, in both serum shocked (C) and sham-shocked cells (D). [Colour figure can be viewed at wileyonlinelibrary.com].

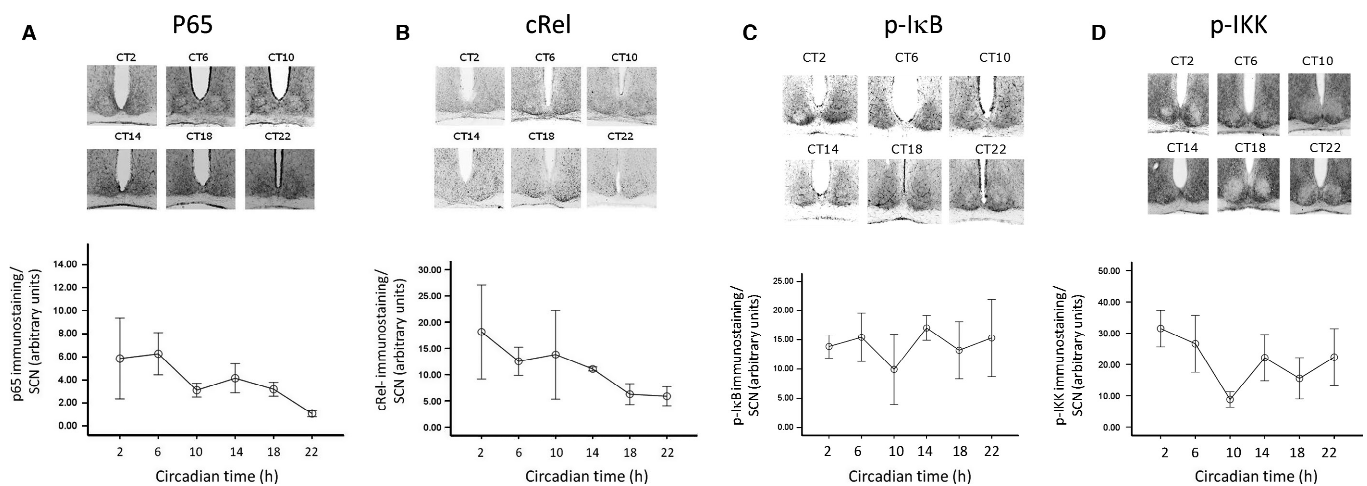

FIG. 2. Expression of p65 (A), cRel (B), p-IkB (C) and p-IKK (D) in the SCN across the circadian cycle from animals sampled in DD. N = 3–4 per time-point. There was no significant effect of time for SCN expression of any of these antigens. The scale bar on the photomicrographs represent 100 μm.

treated with vehicle and the NF-κB inhibitor PDTC chronically via drinking water under conditions of both LD and DD (Fig. 3A). Analysis of the locomotor rhythms under both LD and DD revealed no significant differences between vehicle and PDTC treatment: under LD, the phase angle of entrainment was not altered (Fig. 3B; P = 0.53 between vehicle and PDTC treatment via paired t-test). In

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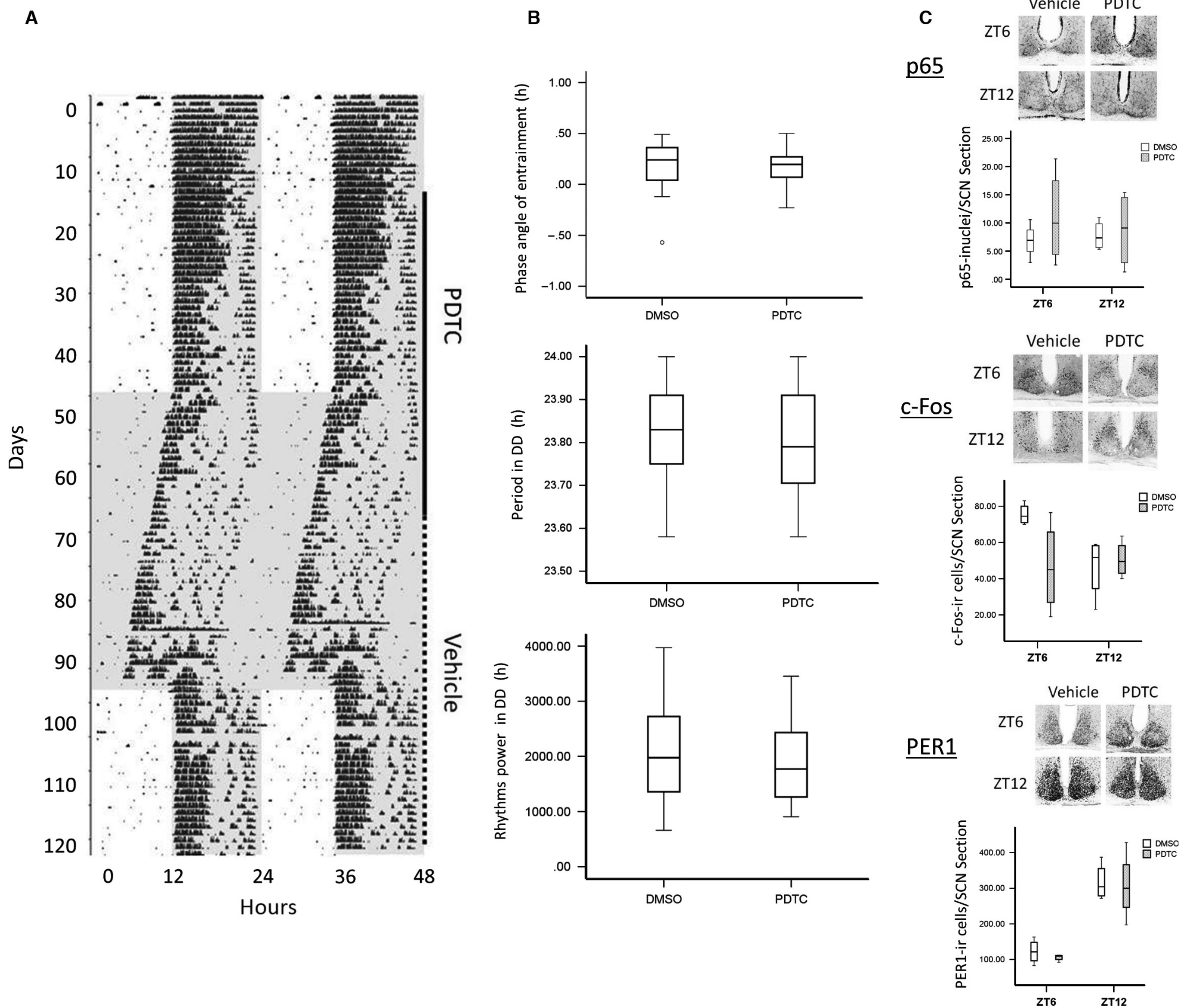

FIG. 3. Treatment with the NF-κB inhibitor PDTC does not impact on behavioural circadian wheel running rhythms. (A) Sample actogram showing the effects of PDTC (via drinking water at a dose of 50 mg/kg/day) and vehicle treatment on behavioural rhythms under light : dark (LD) and constant darkness (DD) conditions, where shading on the actogram indicates darkness; (B) Boxplots showing the effects of PDTC on phase angle of entrainment under LD, free running period in DD, and rhythm power in DD. There were no statistically significant differences in any of these measures between PDTC and vehicle treated ($N = 16$); (C) Effect of previous PDTC vs. vehicle treatment, shown as boxplots, on expression of p65, c-Fos and PER1 in the SCN during the lights-on phase (ZT6) and the start of lights-off phase (ZT12; chosen as this is the peak of PER1 expression in the SCN). There were no statistically significant effects of treatment or time x treatment interactions for these three antigens. $N = 4$ per group.

DD the period and rhythm power were not altered (Fig. 3B; $P = 0.52$ and $P = 0.52$ respectively between vehicle and PDTC treatment). We then examined the SCNs of animals treated with either PDTC or vehicle for their expression of p65, c-Fos and PER1 at ZT6 and ZT12 (Fig. 3C). These two time-points were chosen as they represent when the peak (ZT12) and near nadir (ZT6) of PER1 expression would be expected under a 12 : 12 L : D cycle (see Field *et al.*, 2000; and also Fig. S1 which shows the rhythmic expression of PER1 in the SCN in DD in our experiments). We found that there was no significant effect of drug treatment ($F_{1,15} = 0.667$, $P = 0.431$; $F_{1,13} = 0.600$, $P = 0.485$; $F_{1,16} = 2.39$, $P = 0.147$ respectively for p65, c-Fos and PER1), nor any significant drug x time-point interaction for any of the three antigens ($F_{1,15} = 0.667$, $P = 0.431$; $F_{1,13} = 0.600$, $P = 0.485$; $F_{1,16} = 2.39$, $P = 0.147$ respectively for p65, c-Fos and PER1). These results appear to indicate that the NF-κB pathway is not involved in the free-running of the SCN clock *in vivo*.

We next examined the role the NF-κB pathway may play in photic resetting of the SCN clock. We examined whether the NF-κB inhibitor PDTC would alter the phase-resetting effects of light. Animals were pre-treated with PDTC or vehicle 30 min prior to the presentation of a light pulse at CT15 or CT22. It was found that treatment with PDTC did not impact on the magnitude of the phase shifts elicited by a phase-delaying or -advancing light pulse ($P = 0.085$ and $P = 0.77$) via paired *t*-tests respectively for CT15 and CT22; Fig. 4A, B). We then examined the SCN for expression

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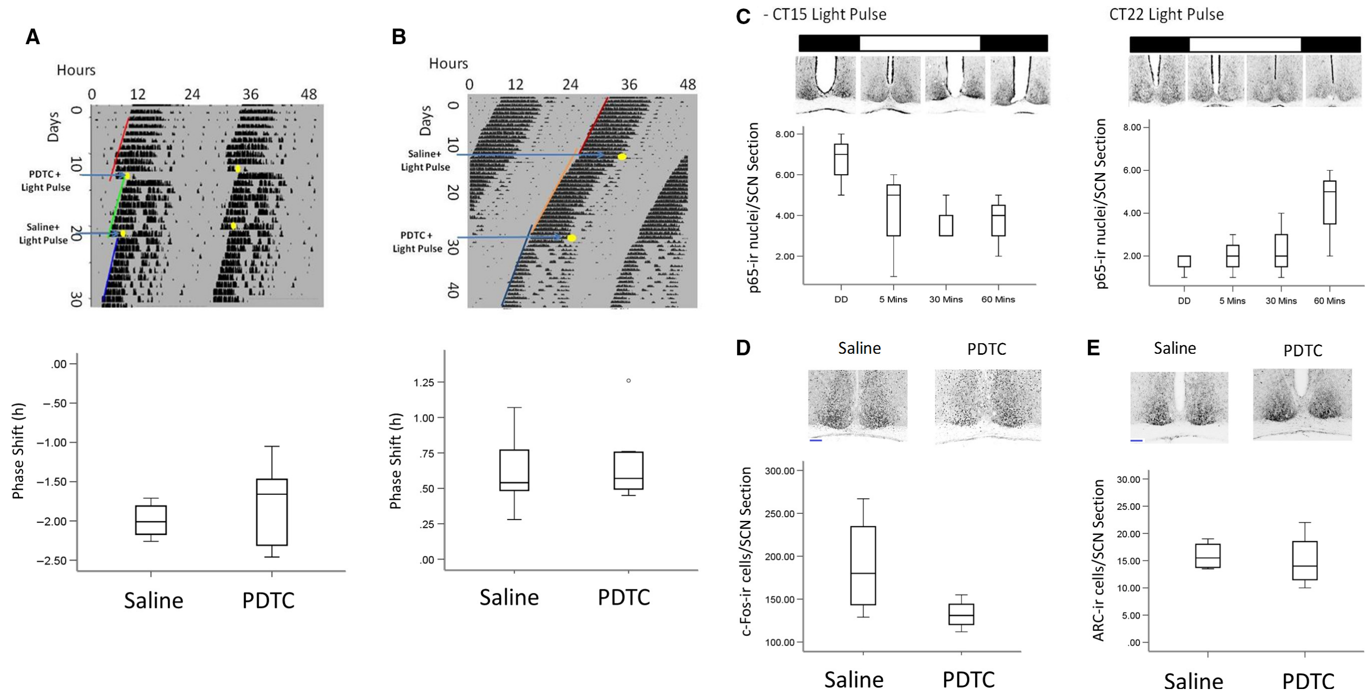

FIG. 4. Putative involvement of NF-κB in photic resetting of circadian rhythms. To examine whether acute inhibition of NF-κB would impact on subsequent light-induced phase shifts, mice were treated with i.p. PDTC (or vehicle) 30 min prior to a light pulse at CT 15 (A) or CT22 (B). In both cases, PDTC treatment was not associated with altered magnitude of phase shifts ($N = 8$ for each circadian phase; data shown as boxplots). Next the effect of light pulses on expression of p65 in the SCN was examined. (C) Light pulses at CT15 and CT 22 were not followed by changes in p65 in the SCN ($N = 3$ per timepoint, data shown as boxplots). (D) and (E) show the impact of PDTC treatment on light-induced expression of the immediate early genes c-Fos and ARC in the SCN at CT22. There was no difference of saline vs. PDTC treatment on expression of either of these antigens ($N = 4$ per group). [Colour figure can be viewed at wileyonlinelibrary.com].

of p65 after light-pulses at both CT15 and CT22. No change in p65 was found at 5, 30 or 60 min after the onset of the light pulse at either circadian phase (Fig. 4C and D; $P = 0.085$ and $P = 0.078$ via paired *t*-tests respectively for CT15 and CT22), although we confirmed that c-Fos-immunoreactivity was increased by these light pulses, as expected (data not shown). We then examined the effect of PDTC treatment on photic-induction of the immediate early genes c-Fos and ARC in the SCN after a light pulse at CT22 (these IEGs are induced in the SCN by photic stimulation during the subjective night; Kornhauser *et al.*, 1990; Nishimura *et al.*, 2003). We found that PDTC treatment had no effect on the magnitude of expression of either factors 1 h after the light pulse ($P = 0.13$ and $P = 0.37$ respectively for c-Fos and ARC induction via independent *t*-tests; Fig. 4D).

Having so far failed to find evidence for NF-κB involvement in the normal function of the SCN clock, we then examined whether there may be a role for NF-κB in mediating the circadian effects of peripheral immune challenge. Firstly we challenged animals with a 5 mg/kg dose of LPS and sampled the SCN 24 h later (Beynon & Coogan, 2010 demonstrated SCN responsivity at 24 h, but not 6 h following such a treatment). We found that LPS elicited a significant upregulation of p65 and p-IκB, and the IEG EGR-1 ($P = 0.036$, $P = 0.024$ and $P = 0.026$ respectively via independent *t*-test; Fig. 5A). c-Fos was also significantly upregulated by LPS challenge (data not shown), but c-Rel was not. We then examined whether there was a time-of-treatment effect of treatment on LPS-induced changes in the SCN, and to this end we treated animals with LPS (5 mg/kg) at either ZT6 or ZT18, sampling 24 h later. We find a significant main effect of treatment on p65 ($F_{1,17} = 6.55$, $P = 0.024$), but no time x treatment interaction ($F_{1,17} = 0.523$, $P = 0.483$). For p-IκB there was no significant effect of treatment on p-IκB ($F_{1,16} = 2.61$, $P = 0.132$), nor was there a significant time x treatment interaction ($F_{1,16} = 0.21$, $P = 0.654$; Fig. 5B).

Given that 5 mg/kg of LPS represents a substantial dose that elicits systemic inflammation, we examined the effects of treatment with LPS at a dose of 100 μg/kg given at CT15 to animals maintained in DD. We used this treatment as previous data has shown that such treatment elicits moderate phase-delays of the behavioural rhythm (Marpegan *et al.*, 2005; Anderson *et al.*, 2015a). From behavioural monitoring, we re-confirmed that LPS treatment at CT15 elicits a small, but statistically significant, phase delay of the circadian locomotor rhythm ($P = 0.011$ between LPS and saline treatments via paired *t*-test; Fig. 6A). We then examined effect of 100 μg/kg LPS treatment at CT15 on the SCN by sampling animals 4 h after treatment. We find that this lower dose LPS treatment resulted in increased p65 nuclear translocation ($P = 0.043$; Fig. 6B), but not increased p-IκB expression in the SCN ($P = 0.23$; Fig. 6C). We also report that the LPS treatment results in upregulation of c-Fos in the SCN (especially in the dorsolateral shell; $P = 0.02$; Fig. 6D) but exerted no effect on PER1 expression ($P = 0.22$; Fig. 6E).

To complement out *in vivo* findings, we undertook a series of *in vitro* experiments utilising SCN slice cultures derived from PER2::LUC mice. We found that incubation of slices with PDTC (20 μM) did not alter the phase, period or amplitude of the rhythm of PER2::LUC ($P = 0.79$, $P = 0.93$ and $P = 0.35$ respectively) expression, although the mesor of the rhythm was significantly elevated in PDTC treated slices compared to vehicle control ($P = 0.001$); Fig. 7A). Very similar results were found in slices treated with another NF-κB inhibitor, sulfasalazine (10 μM; Fig. 7B), with no changes in rhythm period, acrophase or amplitude ($P = 0.94$, $P = 0.54$ and $P = 0.037$, respectively), but there was also not a statistically significant elevation in mesor in sulfasalazine

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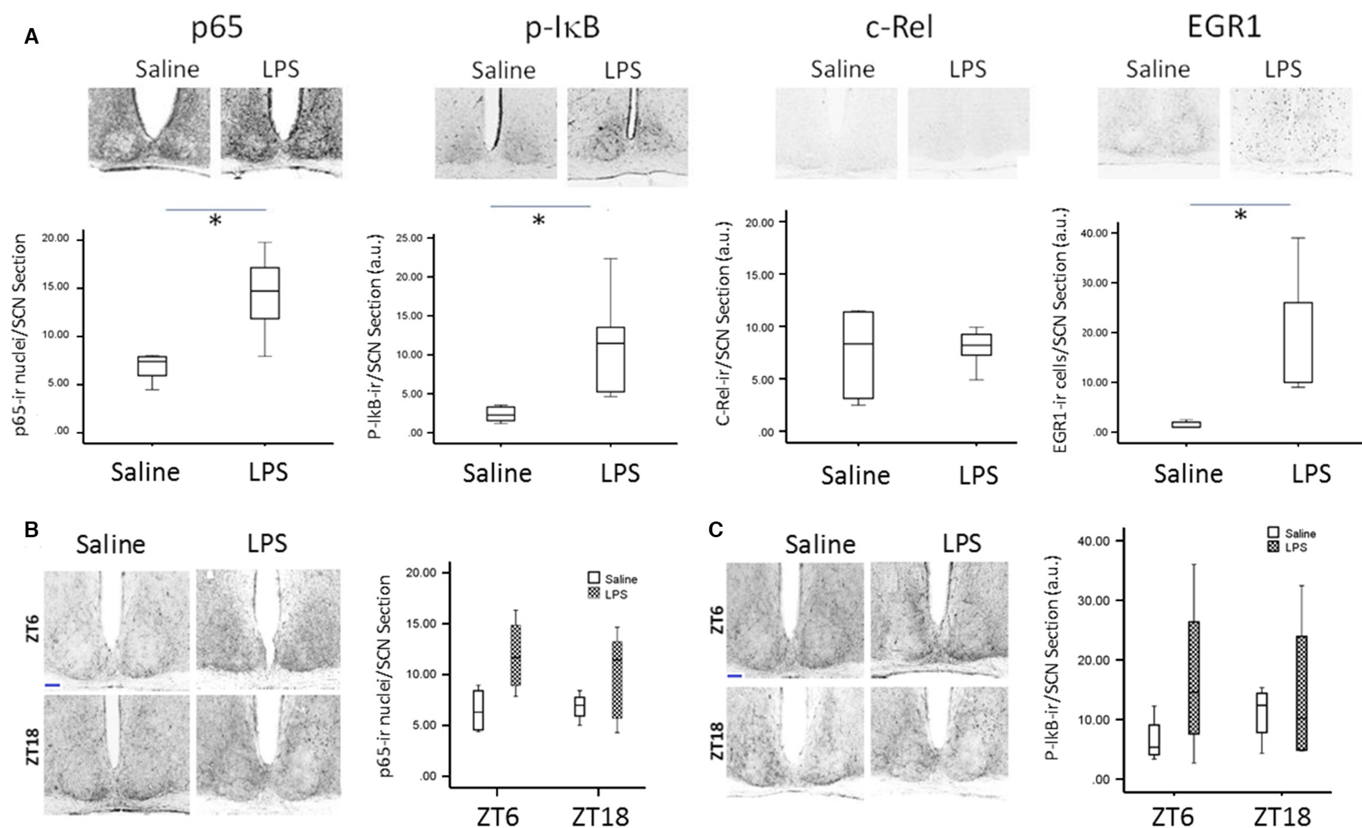

FIG. 5. Effects of LPS treatment on NF-κB component expression in the SCN. (A) Shows expression of p65, p-IκB, c-Rel and the immediate early gene EGR1 in the SCN 24 h after a 5 mg/kg LPS challenge. LPS treatment upregulated expression of p65, p-IκB and EGR1 (*$P < 0.05$), but not c-Rel in the SCN (n = 4–5 per group; data shown as boxplots). (B) and (C) show data from whether time-of-treatment (ZT6 vs. ZT18) impacts on the LPS-induction of p65 and p-IκB in the SCN. For p65 there was a main effect of treatment, but not a time x treatment interaction; for p-IκB, there was neither a main effect of treatment, nor a treatment x time interaction (N = 4–5 for each group per time point).

treated slices compared to control ($P = 0.07$). In a further set of slices, we utilised treatment with LPS (100 ng/mL) as an activator of the NF-κB pathway. Slices treated with LPS did not show statistically significant differences to vehicle treated slices in any of the chronometrics examined (Fig. 7C; $P = 0.53$, $P = 0.11$, $P = 0.13$ and $P = 0.12$ for period, acrophase, amplitude and mesor respectively). We then prepared SCN slices from *nf-kb::luc* mice and examined the spontaneous expression of NF-kB-mediated transcription across a number of days in culture. We find no evidence for circadian modulation of NF-κB-mediated transcription (Fig. 7D and E), although as a positive control we find that levels of NF-κB driven bioluminescence is higher in slices treated with LPS than vehicle ($P < 0.001$; Fig. 7F).

As the experiments described above indicated that NF-κB signalling may not play core roles in regulating SCN function under basal conditions in young animals, and given findings that NF-κB is implicated in altered hypothalamic functioning with age (Zhang *et al.*, 2013), we undertook experiments in older animals (~16 months) to examine any role for NF-κB in circadian function. Behavioural rhythms in these animals showed that ageing was associated with statistically significant decreased rhythm amplitude and decreased total running ($P = 0.04$ and $P = 0.01$ respectively; Fig. S2). When the SCN from these older animals was examined for p65 expression at ZT6 and ZT18, 2-way ANOVA shows no significant main effect of age ($F_{1,17} = 0.283$, $P = 0.601$) or time ($F_{1,17} = 1.36$, $P = 0.27$), nor was there a significant interaction between age and time ($F_{1,17} = 1.07$, $P = 0.31$; Fig. S2B). Next we examined the reactivity of SCN p65 to LPS (5 mg/kg) treatment, and found that LPS treatment resulted in a similar elevation of p65 compared to control to what was previously observed in young animals ($P = 0.005$ between LPS and saline treated groups). Finally, we prepared SCN slices from aged PER2::luc animals (~16 months old) and examined the effects of PDTC and LPS on bioluminescence rhythms. We find that PDTC treatment of slices results in a significant increase in rhythm amplitude and mesor ($P = 0.036$ and $P = 0.001$ respectively; Fig. S3A), whilst LPS treatment does not lead to significant changes in any chronometric (Fig. S3B).

Discussion

In this study we have examined what role the NF-κB pathway may play in the regulation of the murine suprachiasmatic circadian clock. We do not find evidence to support the contention that the NF-κB pathway plays a role in the free-running SCN clock *in vivo* in young, healthy animals, nor do we find evidence to support a role in photic resetting of the clock. Further, we do not find evidence that NF-κB plays a role in *in vitro* SCN clock function. However, in accordance with previous reports, we do find evidence for the neuroimmune regulation of NF-κB components in the SCN. Therefore, there appears to be a distinction in the conditions under which NF-κB signalling partakes in suprachiasmatic circadian function.

The role of NF-κB in circadian processes has previously been indicated in studies of specific peripheral cell types. Spengler *et al.* (2012) had previously demonstrated that the diurnal rhythm in

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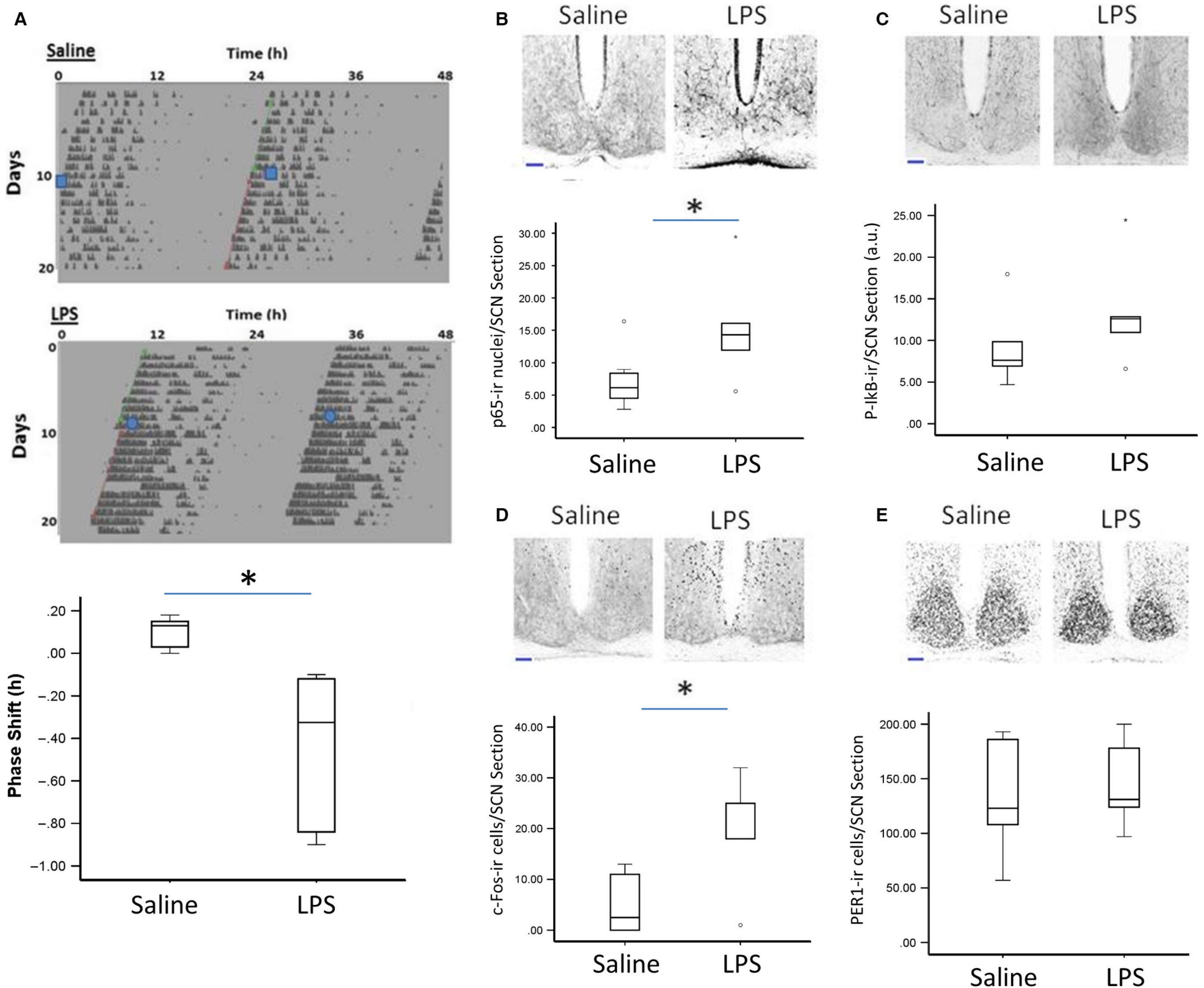

FIG. 6. Low-dose LPS treatment's impact on NF-κB in the SCN. (A) Treatment with 100 μg/kg LPS i.p. at CT15 induces a modest phase-delay compared to saline control treatment ($N = 6$). (B) SCN tissue examined 4 h after 100 μg/kg LPS treatment at CT15 showed that LPS treatment resulted in upregulation of p65 compared to saline control ($N = 6$ for each group; * = $P < 0.05$), but (C) did not result in any significant change in p-IκB SCN expression. (D) LPS treatment also upregulated SCN expression of c-Fos, but (E) did not significantly alter the expression of the clock gene product PER1. [Colour figure can be viewed at wileyonlinelibrary.com].

mortality associated with LPS-induced septic shock was coordinated with the whole-body response of NF-κB transcriptional activity in response to the LPS treatment. Further, such an effect could be understood in the light of CLOCK acting as a positive regulator of NF-κB in HEK cells, and NF-κB activity in liver and embryonic fibroblast being down regulated in *clock*$^{-/-}$ mice. Similarly, *cry* knockout mice show elevated constitutive NF-κB activity in fibroblasts via elevated phosphorylation of IκB and IKK (Narasimamurthy *et al.*, 2012). Potential for NF-κB to alter circadian clock gene expression is indicated by the finding that RelB suppresses circadian regulation of transcription by interacting with BMAL1 in fibroblasts; further, *relb*$^{-/-}$ fibroblasts have markedly altered circadian gene expression profiles (Bellet *et al.*, 2012). In intestinal epithelial cells, NF-κB-mediated transcription shows diurnal rhythmicity driven by the microbiome (Mukherji *et al.*, 2013). Such findings appear to be in agreement with our results from serum-shocked fibroblasts displaying circadian rhythmicity in nuclear p65 accumulation. The persistence of rhythmic p65-dependent transcription in sham-shocked fibroblasts may be a result of the serum free conditions used for the sham-shock acting on another component of the NF-κB system, such as P50. These results, together with previous findings, do suggest that NF-κB signalling plays a role in circadian timing in peripheral cells, and as such indicate the possibility that NF-κB could play a role in regulating clock processes in the CNS.

A small number of other studies have assessed NF-κB function in components of the circadian system. In the rat pineal gland it has been reported that NF-κB activity shows a sharp decrease at the onset of darkness in a light : dark cycle, and this decrease is driven by noradrenergic sympathetic input to the pineal (Cecon *et al.*, 2010; Villela *et al.*, 2014). In the hamster SCN, Marpegan *et al.* (2004) showed that whilst the SCN showed evidence of active NF-κB, there was no circadian or diurnal variation in the levels of nuclear p65 from SCN lysates. However, this study did report that report that treatment with PDTC attenuated photically-induced phase advances (but not delays). Our present results are in agreement with some of these previous findings, in that we find no evidence for

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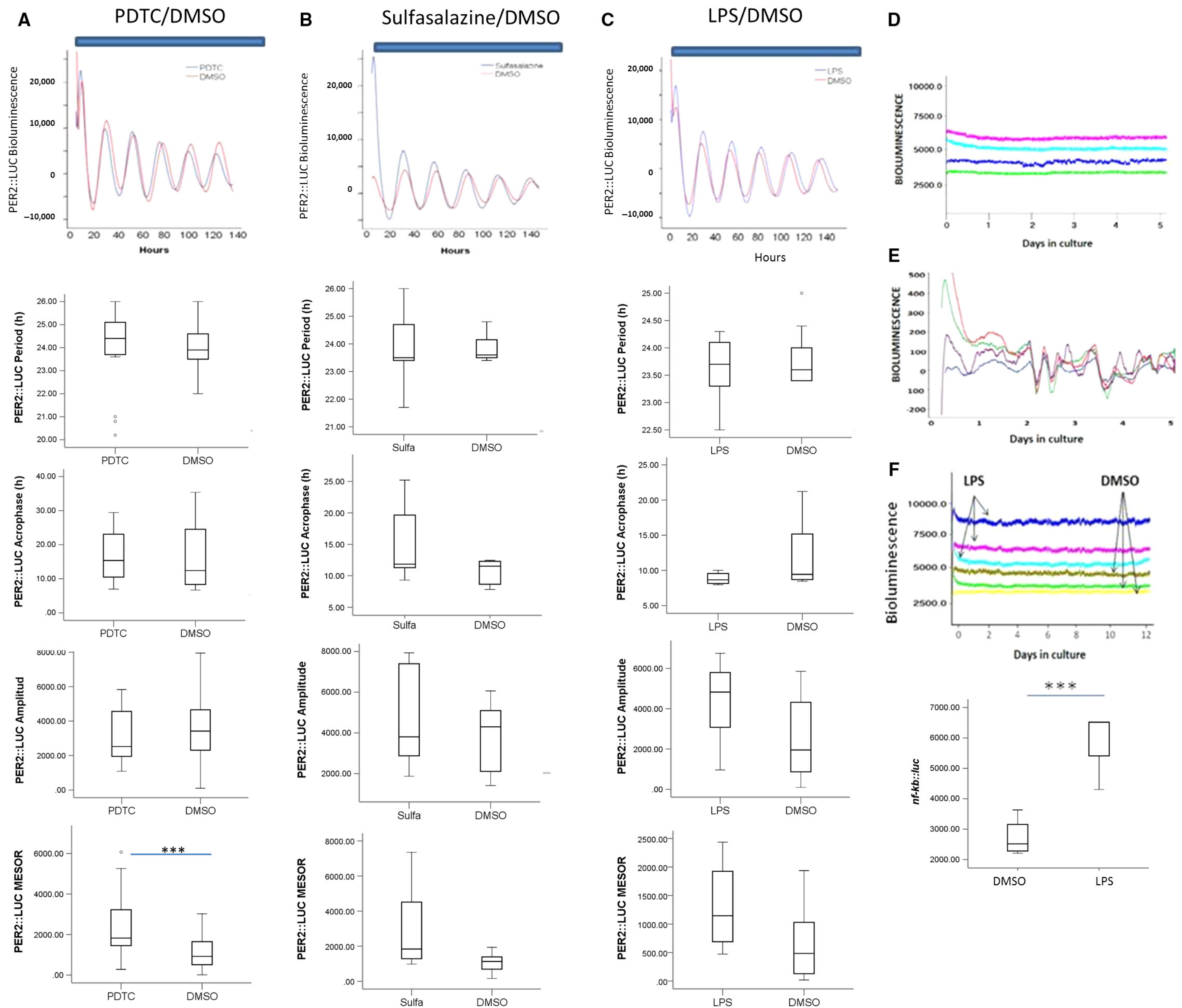

FIG. 7. Effects of NF-κB inhibition and activation on PER2::luc rhythms in the SCN *in vitro*. (A) Treatment of SCN slices with PDTC (20 μM; $N = 21$) elicited no statistically significant changes compared to vehicle treated slices ($N = 20$) in PER2::luc rhythm period, acrophase, or amplitude, although there was a significant increase in mesor in PDTC treated slices (** = $P < 0.01$). (B) Similarly, in slices treated with the NF-κB inhibitor sulfasalazine (10 μM; $N = 7$ for both treated and vehicle groups), there was no changes in period acrophase, amplitude or mesor. (C) Slices were treated LPS (100 ng/mL), as an activator of NF-kB ($N = 8$ for LPS and vehicle groups). LPS treatment was found to have no significant effects on PER2::Luc period, acrophase, amplitude or mesor. (D) Bioluminescence was monitored in SCN slices from *nfkb::luc* mice ($N = 14$). Raw bioluminescence traces from four slices are shown, as are detrended and smoothed data from the same slices (E), indicating that there is no evidence for any circadian modulation of *nfkb::luc* expression. (F) As a positive control, *nfkb::luc* slices were cultured in the presence of LPS. In these slices ($N = 7$), *nfkb::luc* signal was significantly stronger than in control slices (*** = $P < 0.001$). [Colour figure can be viewed at wileyonlinelibrary.com].

rhythmic expression of any of the NF-κB components we examined in the SCN, nor do we find that ongoing treatment with PDTC alters either diurnal or circadian rhythms in the mouse. Further, PDTC treatment did not impact on the day/night rhythm in PER1 expression in SCN (although there is the caveat that only two time-points were examined in this experiment). Our experiments *in vitro* further failed in large part to show any significant effect of inhibition of the NF-κB pathway with either PDTC or sulfasalazine on PER2::LUC rhythms, or using LPS as an activator of the NF-kB pathway via TLR4. The effect of PDTC on mesor may reflect a mechanism through which NF-κB is upregulated as a by-product of slice preparation and/or culture, and which impacts on the rhythm adjusted mean level of PER2::luc expression. A lack of impact of LPS treatment on PER2::LUC SCN rhythms has previously been reported by Guenthner *et al.* (2009), and indicates that activation of NF-κB signalling via this mechanism does not impact on core clock processes. Further, in phase-resetting experiment with 100 μg LPS at CT15, we did not observe changes in PER1 after 4 h, although there may be subsequent modulation that would be revealed at different time-points. However, it is interesting to note that a previous study demonstrated that TNF-α treatment, which would also be expected to activate NF-κB, alters PER2::LUC rhythms in SCN astrocytes (Duhart *et al.*, 2013). Such an effect may be confined to glial cells, and differences between LPS and TNF-α treatment effects may also

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reflect differing signal transduction mechanisms. Finally in our experiments with *nf-kb::luc* SCN slice we observed no evidence for circadian regulation of NF-kB driven transcription in the SCN, although the *nf-kb::luc* signal was responsive to treatment with LPS. These results are in contrast to findings from *Drosophila* neurons, which show circadian rhythms in NF-κB driven luciferase activity (Tanenhaus *et al.*, 2012). There is the possibility that NF-κB driven transcriptional activity may show circadian rhythms in glial cells rather than neurones, and future work may address this question in SCN derived astrocytes and microglia.

We further find no evidence to support a role for NF-κB signalling in photic resetting of the clock. In other paradigms, NF-κB has been postulated to be regulated by glutamatergic transmission and to be involved in synaptic plasticity (Snow *et al.*, 2014). Given that the major route of transmission of photic information to the SCN is the mono-synaptic glutamatergic projections of the RHT (Abrahamson & Moore, 2001), it seems plausible that NF-κB signalling might be involved in photic resetting. However, we find no evidence to support this contention. Light-pulse did not impact on p65 in the SCN at either the delay or the advance portion of the photic phase-response curve, treatment with PDTC did not attenuate photically-induced phase delays or advances, and PDTC treatment also did not impact on photic induction of the IEGs c-Fos and ARC in the SCN. These findings differ from those of Marpegan *et al.* (2004) who report that i.c.v. injection of PDTC significantly attenuates photic phase advances in the hamster. The reason for the discrepancy between these finding is not clear; the modality of PDTC administration (i.p. vs. i.c.v) may be a factor, although several studies have demonstrated CNS effects of i.p. administered PDTC at the concentration used and in the timescale also that is relevant for the current experiments (e.g. Madrigal *et al.*, 2001; Shen *et al.*, 2003). There may be a species difference between hamsters and mice, for example in the magnitude of the photic phase advances (larger in hamsters) and differences in the photic phase response curves. Further, there is always a concern around specificity when using pharmacological inhibitors of biochemical pathways. PDTC has been used extensively in the past, and is a potent inhibitor of NF-κB activation (Gupta *et al.*, 2010). However, it is also an antioxidant, and also as an inhibitor of NF-κB signalling, suppresses transcription of inflammation-driven products, such as inducible nitric oxide synthase, and as such there is potential for off-target or indirect effects. However, given that we do not see evidence for nuclear translocation of p65 in the SCN following light pulses, we propose that PDTC does not attenuate phase shifts in the mouse because the NF-κB pathway is simply not involved in photic phase shifting in the mouse.

Given that NF-κB has been implicated in hypothalamic dysfunction with ageing (e.g. Zhang *et al.*, 2013), that circadian rhythms are impacted by age (Popa-Wagner *et al.*, 2017), and that the SCN shows evidence of neuroinflammation during normal ageing (Deng *et al.*, 2010), we examined whether NF-kB might play a role in the function of the SCN from older animals. Mice of the age used in the current study have previously been demonstrated to exhibit abnormalities in the circadian system (e.g. Wyse and Coogan, 2010). The current data do not suggest that ageing is associated with elevation of basal p65 expression in the SCN, or with changes in the reactivity of the SCN to peripheral LPS treatment. *In vitro* findings that PDTC treatment does increase PER2::LUC rhythm amplitude suggests that NF-κB could be implicated in the diminution of rhythm robustness that has been described in a number of models of healthy ageing (Popa-Wagner *et al.*, 2017), and the seeming discrepancy between *in vivo* and *in vitro* findings may be explained by activation of the NF-κB pathway as part of an inflammatory response during slice preparation and culture (Schneider *et al.*, 1998; Humpel, 2015). It will be of interest to examine if SCN NF-κB signalling is implicated in circadian dysfunction in models of diseases such as Alzheimer's disease, in which neuroinflammation is strongly implicated (Dá Mesquita *et al.*, 2016), and in which there are also marked circadian changes (Coogan *et al.*, 2013).

It may be surprising that given the previously postulated roles of NF-κB signalling in neurophysiological processes, such as synaptic plasticity (Salles *et al.*, 2014), that there appears to be so little a role for NF-κB in regulating SCN function, especially when findings from peripheral cell types indicate clear molecular mechanisms through which such regulation could occur. However, in the light of the current controversy over whether young, adult, healthy neurones express NF-κB under basal conditions (Listwak *et al.*, 2013; Zhang *et al.*, 2013), and findings that a number of antibodies previously used in the investigation of neuronal functions of NF-κB may not be reliable in terms of specificity (Herkenham *et al.*, 2011; Slotta *et al.*, 2014), a relatively straightforward explanation for the current results may be that SCN neurons simply do not express appreciable levels of NF-κB activity in neurons, and effects which are observed (e.g. following LPS treatment) may be associated with glial cells (as previously postulated by Leone *et al.*, 2006). It may be that NF-κB plays little role in neuronal function in the SCN under basal conditions, but that NF-κB signalling is involved in SCN glial function. Astrocytes and microglia have previously been demonstrated to express molecular circadian rhythms (Fonken *et al.*, 2015; Prolo *et al.*, 2005), and as such glia have previously been postulated as an interface for circadian-immune signalling (Leone *et al.*, 2006). Our current findings for involvement of NF-κB signalling in the SCN following immune challenge is congruent with this hypothesis. It will be of interest for future studies to directly address the role of NF-κB signalling in SCN glial response to immune activation and neuroinflammation with careful cellular and biochemical analysis.

In summary, we believe that our current experimental approach has been comprehensive, and that our findings are consistent in failing to demonstrate substantive evidence for involvement of NF-κB signalling in the SCN circadian clock under basal conditions, whilst also indicating that NF-kB signalling may be important in circadian-immune dialogue in the master SCN clock.

Supporting Information

Additional supporting information can be found in the online version of this article:
Fig. S1. Rhytmic Expresison of PER1 in the SCN in DD.
Fig. S2. Impact of ageing on p65 expression in the SCN.
Fig. S3. Impact of NF-κB inhibition and activation on PER2::LUC rhythms in SCN slice cultures prepared from older animals.
Table. S1. Primary antibodies used in the study.

Acknowledgements

We acknowledge financial support from Science Foundation Ireland (10RFP/NES2752).

Abbreviations

CT, Circadian time; DAB, Diaminobenzidine; DD, constant darkness; DMSO, dimethyl sulfoxide; IKK, IκB kinase; LD, Light : dark; LPS, lipopolysaccharide; NF-κB, Nuclear Factor-κB; PDTC, Pyrrolidine dithiocarbamate; PER, PERIOD; SCN, Suprachiasmatic nucleus; TLR, Toll-like receptor; TNF-α, Tumour necrosis factor-α; ZT, Zeitgeber time.

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Conflict of interest

The authors declare no conflicts of interest.

Author contribution

SOK co-designed, conducted and analysed *in vivo* and *in vitro* mouse experiments; ALB co-designed, undertook and analysed experiments in fibroblasts; JSD co-designed and oversaw experiments in fibroblasts; PNM contributed to experimental design; ANC conceived the project, co-designed all experiments, analysed data and was the primary author of the manuscript. All authors contributed to the preparation of the manuscript.

Data accessibility

All data presented in the current manuscript can be obtained from the corresponding author. Further, all data has been uploaded to the publisher's website.

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