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Peripheral sensory neurons clock genes are a molecular driver of pain rhythmicity

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Abstract

Pain intensity fluctuates across the day in patients, yet the cellular clock mechanisms that generate pain rhythmicity and their therapeutic potential remain poorly defined. Prior studies have established circadian variation in nociception and a requirement for intact clock function, but it has remained unclear the neurophysiological mechanisms which translate clock state into time-of-day changes in pain. The goal of this study was to define the clock component(s) and effector pathway(s) that drive pain rhythmicity and to test whether this axis can be exploited for analgesia.

We combined behavioural analyses under circadian conditions with genetic dissection of clock components in sensory neurons, electrophysiological interrogation of nociceptor excitability, and pharmacological modulation of the clock machinery. To establish translational relevance, we used human iPSC-derived nociceptors, including a human pain disease model, to test whether the same clock–excitability coupling operates in human cells.

We show that nociceptive behaviours exhibit robust circadian rhythmicity and that this rhythmicity depends on the molecular clock within peripheral sensory neurons. Loss of cryptochromes CRY1/CRY2 disrupts time-of-day differences in nociceptor excitability, identifying cryptochromes as essential clock effectors in the pain pathway.

Mechanistically, cryptochrome state controls voltage-gated sodium channel function, providing a direct link from clock machinery to nociceptor responsiveness.

Pharmacological stabilisation of cryptochromes using a small-molecule modulator suppresses sodium currents and reduces nociceptive responses across distinct pain paradigms, and this effect is abrogated when cryptochrome function is absent. In

synchronised human iPSC-derived nociceptors, we observe concordant clock-state-dependent changes in excitability, and cryptochrome stabilisation reduces hyperexcitability.

These findings identify a peripheral sensory neuron clock mechanism that drives pain rhythmicity through cryptochrome-dependent control of sodium channel function and highlight cryptochrome stabilisation as a non-opioid therapeutic strategy for pain disorders with diurnal symptom variation.

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Introduction

Whilst the diurnal variation of nociception and the temporal patterns of chronic pain disorders, including migraine, carpal tunnel syndrome and trigeminal neuralgia are well established,¹⁻⁴ the underlying mechanisms remain to be determined. The molecular clock arises from a transcription-translation feedback loop (TTFL),⁵ which is comprised of a number of key clock genes and their protein products: BMAL1, CLOCK, CRY(1&2), and PER(1&2). These proteins exert negative feedback on their own transcription. BMAL1 and

CLOCK bind to E-box elements in promoter regions, initiating the transcription of various genes, including *PER* and *CRY*. The translated *PER* and *CRY* proteins form heterodimers in the cytoplasm and translocate back into the nucleus to inhibit their own transcription by preventing the BMAL1 complex from binding to E-box elements. Deletion of any of these key genes (singular gene family i.e. *CRY* include both 1&2) results in the disruption of normal circadian rhythms and arrhythmia.⁶⁻¹³ However, the molecular and behavioural function of circadian rhythms exclusively in sensory ganglia¹⁴⁻¹⁶ is not well explored. The molecular circadian clock is a good candidate for driving diurnal variation in pain. The somata of nociceptors reside either in the dorsal root ganglia (DRG) or trigeminal ganglia (TG), ensheathed by satellite glial cells (SGCs), producing and regulating the repertoire of receptors and synaptic proteins at the peripheral and central nerve endings respectively. Hence the clock could regulate nociceptor responsiveness through controlling gene expression of these receptors or by regulating their function.

We have utilized mouse models and human induced pluripotent stem cells (hiPSC) derived neuronal models to investigate the mechanisms underlying diurnal variations in nociception and to identify a potential approach for harnessing these mechanisms to treat chronic pain disorders. Our work has revealed a conserved mechanism in mouse and human nociceptive neurons, whereby the cryptochromes determine time-dependent neuronal responsiveness through voltage gated sodium channels. This non-canonical function is highly potent and druggable through the use of cryptochrome stabilisers.

Materials and methods

Animals and circadian conditions

All procedures were performed under an approved UK Home Office Licence in accordance with the Animals (Scientific Procedures) Act 1986 and University of Oxford policies; the study conformed to ARRIVE guidelines. Adult mice (typically 2–3 months, 20–30 g) on a C57BL/6J background were housed in pathogen-free individually ventilated cages (≤ 5 /cage) with food and water ad libitum under 12:12 h light–dark (LD) cycles or transferred to constant darkness (DD) for circadian testing (≤ 8 days). Zeitgeber time (ZT) was defined with lights-on as ZT0. Under DD, mice were transferred to a light-tight chamber at the time lights would normally turn on, and circadian time (CT) was estimated from the prior LD schedule (no wheel-running or infrared activity monitoring was used). Behavioural testing for rhythmicity was performed across defined ZT/CT windows (typically ZT/CT 1–3, 6–9, 12–15, and in some experiments 18–21).

Genetically modified mouse lines

To dissect central versus peripheral clock contributions, transgenic lines were maintained on a C57BL/6J background and genotyped as detailed in the Supplementary Methods. Sensory-neuron clock disruption used Advillin-Cre-ERT2:Bmal1^{fl/fl} mice, generated by crossing Advillin-Cre-ERT2 (JAX 032027) with Bmal1^{fl/fl} (JAX 007668); recombination was induced with tamoxifen (100 mg/kg i.p. in corn oil) once daily for five consecutive days, with behavioural testing and sample collection performed 12–20 days from the first injection. Similarly, we generated Advillin-Cre-ERT2:Scn9a^{fl/fl} mice by crossing Advillin-Cre-ERT2 (JAX 032027) with Scn9a^{fl/fl} (gifted from John Wood, UCL, London, UK). SCN-targeted disruption used VIP-Cre crossed to Bmal1^{fl/fl} (VIP-Cre JAX 031628; Bmal1^{fl/fl} JAX 007668) to generate VIP-Cre:Bmal1 conditional knockouts. Cryptochrome loss-of-function employed Cry1^{-/-}:Cry2^{-/-} mice. Circadian reporter experiments used Per2-Luc knock-in mice. Additional sensory-line targeting included Scn10a-Cre:Bmal1 conditional knockout mice, as described in the Supplementary Methods.

Human iPSC lines and ethical approvals

Human iPSC lines were derived from skin biopsies using non-integrating Sendai virus following written informed consent with UK NHS REC approval (REC: 13/SC/0179 and 10/H0505/71) through the StemBANCC consortium. Two control lines (SBAD02, SBAD03) and one erythromelalgia patient line carrying SCN9A F1449V were used (full provenance in Supplementary Table S1).

Behavioural assays and pain models

Behavioural assays included von Frey mechanical testing (hindpaw and orofacial), plantar thermal thresholds (Hargreaves), and dynamic weight bearing (DWB). For rhythmicity experiments, mice were tested at defined ZT or CT windows under LD or DD as above. Detailed protocols (including acclimation/training, stimulus parameters, scoring, and threshold calculation) are provided in the Supplementary Methods. Animals were habituated to the experimental apparatus prior to performing assessments. Briefly, hindpaw mechanical thresholds were obtained using calibrated von Frey filaments and an up–down method; thermal thresholds were obtained using infrared radiant heat with repeated trials per paw; DWB assessed weight distribution as a percentage of body weight following standard filtering of posture scores.

For migraine-like pain, nitroglycerin (NTG; 1 mg/ml in 0.9% NaCl) was administered i.p. at 10 mg/kg either 2 h prior to behavioural testing (acute model) or as repeated injections around ZT12 on alternate days (six total injections over 12 days; chronic model), with assessment the following day. Treatment was randomly assigned and the experimenter was blinded to treatment and genotype. For post-operative pain, a unilateral partial medial meniscectomy (PMX) was performed under isoflurane anaesthesia in adult C57BL/6J mice after DWB acclimation and baseline measurement; KL001 (0.4 mg per animal; typical 22–25 g) or vehicle was administered i.p. 2–3 h prior to assessment, and DWB was analysed 48 h post-surgery with assessor blinding.

Pharmacology

KL001 (Sigma/Merck) was used for in vivo analgesia studies (0.4 mg per animal i.p. as above) and for in vitro assays (e.g., iPSC nociceptor MEA and patch-clamp preincubation paradigms) as detailed in the Supplementary Methods. PF05089771 was used as a Nav1.7 blocker in behavioural experiments as described in the Results and Supplementary Methods.

Sensory neuron and iPSC-derived nociceptor culture and synchronisation

Primary mouse trigeminal ganglion (TG) neurons were plated on ibidi dishes and maintained up to four days in vitro prior to electrophysiology. Human iPSC-derived sensory neurons were differentiated and matured beyond day 60–65 before functional assays. For circadian phase experiments in vitro, cultures were synchronised using forskolin (10 μ M, 1 h), and recordings were performed in defined post-synchronisation time windows corresponding to CT4 and CT16. Full differentiation, maintenance, and synchronisation procedures are described in the Supplementary Methods.

Patch-clamp electrophysiology

Whole-cell recordings from mouse primary TG neurons and iPSC-derived nociceptors were performed under continuous perfusion with oxygenated aCSF (95% O₂/5% CO₂). Recordings used a MultiClamp 700B amplifier with Digidata 1550 and were analysed in Clampfit. For sodium currents, a CsF/TEA-based internal solution was used; Nociceptors were maintained at a holding potential of -60mV, the membrane was hyperpolarised to -120mV for 100ms to remove inactivation of voltage gated sodium channels and then was depolarised to test potentials from -

70mV to +20mV for 1s in 10mV steps. Series resistance compensation and basic cell-quality criteria followed standard practice as detailed in the Supplementary Methods. Potassium leak currents were assessed using a voltage ramp protocol in the presence of TTX.

To assess time-of-day effects on central synaptic transmission, whole-cell recordings were also obtained from acute trigeminal nucleus caudalis (TNC) slices prepared from adult mice using an NMDG-based protective cutting solution, followed by recovery in oxygenated aCSF. Spontaneous EPSCs were recorded at -70 mV in the presence of picrotoxin using a CsCl-based internal solution, with glutamatergic origin confirmed by kynurenic acid. Event detection used WinEDR, and frequencies were calculated from defined time windows. Full slice preparation and recording parameters are provided in the Supplementary Methods.

Trigeminal ganglion slice calcium imaging

Acute TG slices were prepared by embedding dissected ganglia in low-melting agarose and cutting 210–320 μ m slices on a vibratome. Slices were loaded with Fura-2 AM (with pluronic acid and collagenase) and imaged after washout and recovery in oxygenated aCSF. Baseline recordings were followed by ligand application (e.g., ATP- γ S, capsaicin, menthol) at concentrations specified in the Supplementary Methods.

Multi-electrode array recordings in iPSC-derived nociceptors

iPSC-derived nociceptors were plated onto Matrigel-coated 48-well MEA plates (Axion Cytoview MEA 48) at day 12 post-induction and matured with standard maintenance; AraC was used briefly to limit mitotic non-neuronal cells, and rat astrocytes were added to support neuronal health. MEA recordings (Axion) were acquired at 37°C using short baseline recordings followed by compound application; spike detection used AxIS (threshold >6 SD above noise). Mean firing rates were derived using AxIS/AxISmetrics, with downstream pooling of active electrodes and analysis in MATLAB as described in the Supplementary Methods.

Statistics and software

Sample sizes, definition of n , randomisation and blinding (where applicable), and statistical tests used for each experiment are reported in the figure legends and/or Supplementary Methods. Analyses were performed using GraphPad Prism and MATLAB alongside specialised acquisition/analysis software (AxIS/AxISmetrics, Clampex/Clampfit, WinEDR) as detailed in the

Supplement. Unless otherwise stated, tests were two-sided with significance thresholds indicated in figure legends.

Results

Nociception in male and female mice is circadian

Diurnal variations of sensory thresholds have been reported in wild-type naïve rodents via inflammatory,^{16,17} thermal,^{18,19} and postoperative²⁰ pain assays, although baseline diurnal variances were not always observed. These previous experiments have been conducted under 12:12 Light-Dark (LD) cycles. To confirm that nociceptive rhythmicity is circadian, and not just driven by LD cycle, we tested mice in constant darkness (DD). Regular daily fluctuations of mechanical nociception were observed in both LD and DD conditions, in hind paws and orofacial regions in naïve animals (Supplementary Fig. S1 A-B). Zeitgeber time (ZT) refers to a standardized way of measuring time in relation to external light cues that synchronise the internal circadian clock; lights-on is defined as ZT0. Under DD, circadian time (CT) was used because animals free-run on their endogenous circadian cycle, with CT0 marking the onset of the subjective day (equivalent to ZT0). For hind paw mechanical sensitivity, the 50% withdrawal threshold was lowest during the early light phase (ZT0–3), increased to the highest tolerance during the early hours of darkness (ZT12–15), and then declined again (ZT18–21). Similarly, the orofacial nociceptive response to mechanical stimulation (0.4 g von Frey filament) followed the same variation across tested time points, with significantly heightened nociception scores during the early day and reaching a nadir at ZT12–15 (Fig. 1A). No sex differences were observed in the temporal pattern of mechanical pain sensitivity for hind paw (Fig. 1B; Supplementary Fig. S1H). However, females orofacial pain score was significantly higher over male at ZT0–3 and ZT6–9 (Fig. 1A). The mice also showed similar variation in response to heat in the Hargreaves test (Supplementary Fig. S1C), similar to previous reports¹⁹. The variation in pain sensitivity was highly consistent over several days of repeated testing (Fig. 1B; Supplementary Fig. S1C).

Sensory neurons and glia show synchronised clock oscillations

We then sought to investigate the molecular circadian clock in sensory ganglia, where nociceptor somata reside, and whether this may be responsible for behavioural nociceptive rhythmicity. *Per2-Luciferase (Luc)* knock-in mice were utilized to monitor the bioluminescence associated with real-time *Per2* expression. TG and suprachiasmatic nuclei (SCN) were dissected from PER2::LUC mice kept in LD conditions and

bioluminescence was recorded every 30 minutes for approximately 5 days with no media change (Supplementary Fig. S1G). As previously reported, both tissues displayed strong rhythm of *Per2* promoter activities with SCN having more pronounced peak amplitudes and sustained rhythm, aligning with its role as a central oscillator.^{21,22} The *Per2* oscillations in TG, on the other hand, had a shifted peak by approximately 3h compared to the SCN. Transcript levels of *Per1*, *Per2*, and *Bmal1* were confirmed from TG samples dissected every 4h during one day for 6 time points in total (ZT: 0, 4, 8, 12, 16, 20). Clear antiphase oscillations were observed for *Bmal1* on the positive arm and two *Per2* genes on the negative arm (Supplementary Fig. S1E). The peak of *Bmal1* and trough of *Per2* matched the peak of behavioural sensitivity (ZT1-4); and trough of *Bmal1* and peak of *Per2* matched the trough of behavioural sensitivity (ZT12-16). In the following sections we refer to these two time points as active (ZT12-16) and inactive (ZT1-4) phases for the mice (illustrated in graphic abstract). We further confirmed that glial and neuronal components each expressed a pronounced and closely concordant *Per2* rhythm (Supplementary Fig. S1D,F). Fractionation was confirmed by enrichment of glial marker *Gfap* and neuronal gene *Trpv1*, neither of which was oscillating.

The peripheral sensory neuron clock drives diurnal variation in nociception

Having established circadian rhythmicity in nociceptive sensitivities and the presence of the molecular clock in peripheral sensory cells, we sought to establish a functional link by using *Cry1*^{-/-};*Cry2*^{-/-} dKO mice that have no molecular or behavioural circadian rhythm.^{6,23} We found that global deletion of the clock substantially attenuated the amplitude of variation in mechanical nociceptive responses and thresholds, with loss of a significant difference between the peak and trough of sensitivity (Fig. 1C,F; Supplementary Fig. S2). In contrast, when we selectively ablated the clock in VIP neurons through floxed *Bmal1* deletion in *VIP-Cre* mice (Supplementary Fig. S3), and thereby ablating the SCN clock,²⁴ we found diurnal pain behaviours were retained (Fig. 1D,G), suggesting that the central circadian pacemaker in the SCN does not directly drive pain behaviours. To specifically examine the role of the local clock in peripheral sensory neurons, we used a primarily sensory neuron promoter *Advillin-Cre-ERT2* mouse crossed with a floxed *Bmal1* mouse.²⁵ Following tamoxifen injection, inducing *Bmal1* deletion in peripheral sensory neurons (Supplementary Fig. S4), we found that the *Bmal1* knock out mice had significantly different orofacial pain score across two time points compared to control littermates, in particular there was no diurnal differences in nociception in both orofacial and hind paw von Frey (Fig. 1E,H). Since *advillin* is expressed in neural crest derived cells, we crossed a *Scn10a-Cre* mouse, which drives Cre expression in Nav1.8 expressing nociceptors with the

1 floxed *Bmal1* mouse to produce *Scn10a-Cre:Bmal1^{-/-}* mice. We again found that loss of the
 2 local clock resulted in abolition of the diurnal variation in sensory thresholds
 3 (Supplementary Fig. S5). Hence, the TTFL in primary sensory neurons are required for
 4 diurnal pain rhythms.

5 Neurophysiological responses of peripheral sensory neurons are clock 6 dependent

7 Next, we explored the contribution of circadian clock mechanisms to neurophysiological
 8 responses of primary nociceptive afferents that underlie nociceptive behavioural
 9 responses. In acute TG slices, we used Fura2-AM ratiometric calcium imaging to assess
 10 responses to capsaicin, menthol, or ATP γ S. In wildtype animals the responses to these
 11 compounds displayed clear diurnal patterns, with a greater proportion of cells responding
 12 at ZT4 (inactive phase) compared to ZT16 (active phase) (Fig. 2A). In *Cry1^{-/-}:Cry2^{-/-}* mice,
 13 these diurnal patterns with ATP γ S and Menthol were disrupted (Fig. 2D). The loss of CRY did
 14 not alter the response pattern for capsaicin, suggesting that capsaicin calcium responses
 15 through TRPV1 are not regulated by the cryptochromes. Next, using chip multi-electrode
 16 arrays (chip-MEA), we recorded spontaneous firing from TG slices and, unexpectedly, we
 17 found that spontaneous/basal activity was greater at ZT16 (Fig. 2B-C). However,
 18 application of ATP γ S led to significantly greater increase in firing activity only at ZT4, but not
 19 ZT16. We also investigated the central synaptic activity of primary nociceptors by
 20 performing patch clamp recordings from cell bodies of layers I/II in acute slice of the
 21 trigeminal nucleus caudalis (TNC). Again, we found that the frequency of spontaneous
 22 excitatory postsynaptic currents (sEPSC) was significantly higher at ZT16 compared to ZT4
 23 in wildtype animals and this pattern was absent in *Cry1^{-/-}:Cry2^{-/-}* mice (Fig. 2E-F) consistent
 24 with results from the chip-MEA assays.

25 Neurophysiological responses of primary sensory neurons are regulated by the expression
 26 of ligand-gated ion channels including TRP channels and voltage gated ion channels,
 27 particularly potassium and sodium channel. We performed RT-qPCR on several genes,
 28 including those encoding receptors for the ligands we tested by calcium imaging. TG were
 29 dissected from animals over 6 time-points in 24 hours and we found that *Trpv1*, *Trpa1*,
 30 *Trpm8*, *P2x4*, and *P2x5* did not show significant diurnal variation (Supplementary Fig. S6).
 31 This suggests the observed diurnal variation in calcium responses to capsaicin, ATP γ S and
 32 menthol (Fig. 2A) is not due to circadian regulation of transcription of these genes.
 33 However, the expression of *Kcnk2*, *Kcnk10*, *Kcnk18*, *Scn8a*, *Scn9a*, *Nr1d2*, *Nr1d1*, and
 34 *Kcna1* showed significant diurnal expression (Supplementary Fig. S6). This pattern was lost
 35 in the *Cry1^{-/-}:Cry2^{-/-}* mice, confirming the role of the TTFL in driving the gene expression
 36 oscillations of these ion channel genes.

Cryptochrome proteins regulate voltage-gated sodium channel currents

Functional activity of proteins is not always concordant with gene expression levels,²⁶ and therefore, we investigated whether ionic currents exhibit diurnal variation. Primary TG cultures, synchronized with forskolin (Supplementary Fig. S7A-D) were used to investigate nociceptor properties and ionic currents at CT4 (active phase) and CT16 (inactive phase), matching the time points when mice show lowered or increased pain sensitivity respectively (Fig. 3A). There is no light cue in cell culture and CT was used as time measurement for time post chemical synchronization. The CT used here is different to CT in DD of behaviour data. We found that resting membrane potential did not show diurnal variation (Fig. 3B), in contrast to tissues such as the SCN.²⁷ The rheobase was slightly elevated at the CT16 timepoint (Supplementary Fig. S8A), but the increase was not significant (Fig. 3C). Despite the differences in gene expression levels, the leak potassium current between the two time points was not significantly different (Fig. 3D).

Strikingly, the voltage-gated sodium channel (VGSC) currents were much larger at the CT16 time-point in comparison to CT4 time-point (Fig. 3E; Supplementary Fig. S9A). The larger sodium conductance at inactive phase aligned well with the greater responsiveness of sensory neurons to noxious ligands and the greater behavioural nociceptive sensitivity at this time. This difference in sodium current between time points was present in both TTX sensitive and insensitive current, with a larger TTX sensitive current during CT16 (Fig. 3F). This suggests that the circadian effector mechanism regulates multiple different types of VGSCs, which may explain the potency of circadian effects observed in the behavioural experiments (Fig. 1A,B). To further confirm that the difference in sodium current was not arising from an acute response to forskolin, we measured currents at CT28 i.e. 28 hours after FK stimulation (Supplementary Fig. S11). As with CT4, this also showed a significantly reduced current compared to CT16.

In *Cry1^{-/-};Cry2^{-/-}* mice, the diurnal variation was lost, and instead, these animals displayed increased sodium currents at both time points (Fig. 3G; Supplementary Fig. S9B). This again is consistent with the heightened, yet arrhythmic, pain sensitivity observed in *Cry1^{-/-};Cry2^{-/-}* mice (Fig. 1C,F). Taken together, this indicates that the diurnal variation of behavioural pain sensitivity is driven by voltage gated sodium conductance rather than changes in resting membrane potential, rheobase currents or leak potassium currents. Furthermore, although we found a diurnal pattern in VGSC gene expression (Supplementary Fig. S2), the gene expression peak was opposite to the sodium current peak, which may reflect the time required to produce protein and traffic the channel to the membrane. Therefore, we conclude that the large diurnal difference in sodium current is most likely to be regulated by Cry1 and Cry2 at the post-translational level.

Circadian synchronised human nociceptors exhibit diurnal neurophysiology

In order to bridge our findings from animal to human, we utilised hiPSC derived nociceptors differentiated and matured using our established protocols.²⁸ We successfully synchronised the nociceptors with either dexamethasone (Dex, 200nM 1h incubation) or forskolin (FK 10 μ M 1h incubation) post day 65 of differentiation. Rhythmic expression of *PER2* and *BMAL1* genes (Fig. 4B) and clock gene oscillations in hiPSC derived BMAL1:LUC reporter nociceptors, synchronised with Dex or FK (Fig. 4A), were confirmed using RT-qPCR and bioluminescence assay respectively. Induced oscillations has a period of 24h and quickly dampened after 48h, with FK inducing the most robust oscillations. We performed whole cell patching experiment at 4h-6h (active phase, pink band Fig.4A) or 16h-18h after FK synchronisation (inactive phase, purple band Fig.4A) and found sodium currents in hiPSC-derived sensory neurons significantly differed diurnally (Fig. 4C; Supplementary Fig. S9C), aligning very closely to the mouse nociceptor data and indicating more available VGSCs at the CT16 time-point (inactive phase).

Human iPSC nociceptors in culture exhibit spontaneous action potential firing due to active voltage gated sodium channels.²⁸ We predicted from the time-dependent variation in sodium current, spontaneous firing rates should also exhibit time-dependent variation post-forskolin synchronisation. This was confirmed using multi-well multielectrode array (MEA) recordings of hiPSC nociceptors (Fig. 4D), with mean firing of 2.266Hz at CT16 and 0.074Hz at CT4. Furthermore, the toxin OD1, which inhibits the fast inactivation of voltage-gated sodium channels, increased activity specifically at the CT4 time-point (Fig. 4E), suggesting that the reduced sodium currents observed at this time-points may be due to channel inactivation, which OD1 (50nM) is able to reverse. Conversely at the CT16 time-point, OD1 had no significant effect. Furthermore, application of ATP γ S (50 μ M) produced a significant increase in firing rates at the CT16 time point compared to non-significant response at the CT4 time point (Fig. 4F) – aligning with the electrophysiological responses we found in the mouse sensory neurons.

Cryptochrome stabilisation reduces sodium currents and nociceptive responses

Cry1 and 2 are transcriptional repressors of the TTFL. In comparison to the wildtype responses, *Cry1*^{-/-}: *Cry2*^{-/-} mice display increased pain sensitivity at ZT12-15 (Fig. 1C red box). *Bmal1* is a transcriptional activator of the clock, inducing a cascade of diurnal gene expression. In *Advillin-Cre:Bmal1*^{-/-} mice, pain sensitivity at ZT1-3 is reduced (Fig. 1E red box). We, therefore, hypothesized that stabilization of Cry1/Cry2 would reverse the

increased voltage dependent sodium currents at the CT16 time-point and thereby reduce nociceptive responses during the inactive phase. We used KL001 which was previously identified from a small molecule screen of CRY1/CRY2 stabilizers.²⁹ The application of KL001 to forskolin synchronized primary sensory neuron cultures resulted in a significant decrease of the sodium current (Fig. 5A; Supplementary Fig. S9A).

KL001 or vehicle control was administered intraperitoneally (i.p.) to wildtype mice at ZT0 and the effect of the drug on behavioural sensitivity was studied at ZT4, when mice have heightened nociceptive responses. We found that KL001 substantially decreased pain sensitivity in hind paw to von Frey stimulation (Supplementary Fig. S10D), and orofacial nociceptive responses (Fig. 5B). The effect of KL001 was not observed in the *Cry1^{-/-}:Cry2^{-/-}* mice (Fig. 5D) and primary *Cry1^{-/-}:Cry2^{-/-}* mice TG cultures (Fig. 5C; Supplementary Fig. S9B), confirming that KL001 effects are dependent *in vitro* and *in vivo* upon Cry1 and Cry2. To assess whether KL001 might be effective on human nociceptors, we applied KL001 to hiPSC-nociceptors cultured on MEA and found that KL001 rapidly (within an hour) and significantly suppressed firing activity (Supplementary Fig. S10A-B). We next pre-incubated forskolin synchronised hiPSC-nociceptors with KL001 for an hour before patch-clamp recordings to assess the effect on sodium current at the CT16 time-point. Results showed that KL001 substantially reduced the voltage gated sodium channel current to the level observed at the CT4 timepoint (Fig. 5E; Supplementary Fig. S9C), similar to the mouse nociceptor data (Fig. 5A).

Diurnal nociception is mediated by voltage gated sodium channels

Our results suggested that the diurnal variation of nociception was mediated through voltage gated sodium channels – in which case the heightened nociceptive responses at ZT4 should be reduced by blockade of sodium currents whilst there should be no significant effect at ZT16. In adult TG and DRG neurons, three different alpha subunits of VGSCs (Na_v1.7, Na_v1.8, Na_v1.9)^{30–32} are abundantly expressed, with Na_v1.7 being the most universal across C and A fibres.³² In the hiPSC-derived sensory neurons, Na_v1.7 is predominant with the differentiation protocol we used.^{33,34} Since KL001 was able to reduce sodium current of both mouse and human cultures, we targeted Na_v1.7 to test the hypothesis that VGSC are the key circadian effector. Wildtype mice were treated with either vehicle or the Na_v1.7 specific blocker (PF05089771, 75mg/kg i.p.). As predicted, mice treated with the Na_v1.7 blocker injections lost their diurnal variation in nociceptive responses (Fig. 5F). The loss of diurnal variation was due to suppression of responses at ZT4 to a level matching the responses seen at ZT16. We also determined the effect of Na_v1.7 deletion in sensory neurons using *Advillin-Cre-ERT2:Scn9a^{fl/m}* mice after tamoxifen

and again found a loss of diurnal variation in nociceptive responses (Supplementary Fig. S12).

Cryptochrome stabilisation with KL001 can ameliorate pain behaviours

To evaluate the therapeutic opportunity to harness this mechanism for chronic pain, we utilized a range of chronic pain models – the nitroglycerin (NTG) mouse migraine model,^{28,35} a post operative joint injury model (partial meniscectomy, PMX)³⁶ and erythromelalgia hiPSC patient cell line.^{28,37,38} We first assessed if circadian patterns persisted in a chronic pain model, using intraperitoneal (i.p.) injections of NTG, which is a well-established model of migraine pain. Mice were administered NTG (10mg/kg) at ZT12 day before behaviour assessment on alternate days for 12 days in total. As expected, responses to von Frey filaments and plantar thermal sensitivity measured using the Hargreaves method were exaggerated at each of the 3 time points tested compared to vehicle control after repeated NTG (at day 12), indicating peripheral sensitization for mechanical and thermal responses (Fig. 6A-B). Importantly, circadian variation of sensitized responses was also retained in these mice in both LD and DD conditions (Supplementary Fig. S1A,B). The magnitude of effect of NTG was similar at each time-point, but with an overall shift to greater nociceptive responses compared to vehicle treated mice (Fig. 6A-B). This suggests that the underlying mechanisms driving circadian variation in nociceptive responses are preserved in chronic pain states. We also noted that circadian effects are large, with the maximum difference between time-points (ZT0-3 vs ZT12-15) almost twice that induced by NTG (maximum difference between NTG and vehicle injected at each time point) (Fig. 6C), highlighting the potency of circadian mechanisms.

We next assessed whether KL001 could reduce pain response in the different pain models. KL001 or vehicle was administered to mice two hours before a single i.p. NTG injection (an acute migraine pain model) and found that mice injected with KL001 had significant pain reduction compared to the control group (Fig. 6D). We repeated this experiment, but now using the chronic NTG model induced by repeated NTG injections, where we again found KL001, but not vehicle, significantly reduced orofacial pain responses (Fig. 6E). To assess whether KL001 might have utility in other pain conditions, we utilised the acute post-operative phase following joint destabilisation by PMX. This involves surgical partial medial meniscectomy of the right knee with weight-bearing asymmetry being used as a measure of pain behaviour. One day after surgery, animals were administered either vehicle or KL001 at ZT1 and pain assessed at ZT4. In the vehicle injected animal, a significant difference in weight-bearing was observed for the injured leg, as expected. However, following KL001 treatment, weight bearing asymmetry was normalised, indicating KL001 was effective at

reducing pain in this post-operative pain model (Fig. 6G). Notably, the analgesic effect of a single dosage KL001 injection wore off within 24h.

Finally, to assess the effect of KL001 in a human pain model, we applied KL001 to nociceptors differentiated from a control hiPSC line and an erythromelgia patient hiPSC line (Table S1). As we and others have previously shown, the erythromelgia line (IEM-F1449V), in which there is a gain of function (GOF) mutation of $\text{Na}_v1.7$ sodium channel,^{28,37,38} showed significantly greater spontaneous weighted mean firing rates compared to the control line (Supplementary Fig. S10C). We found that addition of KL001 rapidly (within 1 hour) and potently reduced spontaneous firing in the erythromelgia patient line (Fig. 6F).

Discussion

In this study, we investigated the role and impact of the circadian transcriptional-translational feedback loop in sensory ganglia, examining its effects on cellular function and pain sensitivity behaviours across rodent and hiPSC sensory neuron models. Our data confirm the importance of the peripheral sensory clock in determining diurnal variations in nociception: in the absence of a peripheral sensory clock, diurnal oscillation in sensitivity is abolished. We thus identify a mechanistic pathway linking circadian time to nociceptor excitability through cryptochrome-dependent regulation of voltage-gated sodium channel (VGSC) currents, providing a direct route by which the sensory clock can shape diurnal pain behaviours.

Our data reveals that the molecular circadian clock in sensory neurons establishes diurnal differences in neurophysiological properties in both human and rodent sensory neurons. We found clock gene deletion had selective effects on functional responses. Whilst capsaicin induced calcium flux was unaltered, we found the large diurnal differences in currents mediated by VGSCs, involving both TTX-sensitive and TTX-resistant components was clock gene dependent. VGSCs underlie the depolarising upstroke of action potentials,^{39,40} are central to somatosensation and pain disorders,^{37,41-45} and the target for pain therapeutics.⁴⁶⁻⁴⁸ In this context, VGSC regulation provides a parsimonious effector mechanism by which the sensory clock can tune nociceptor responsiveness across the day.

Although we observed diurnal gene expression of VGSCs, this did not align with the diurnal variation in sodium currents or nociceptive behavioural responses. Specifically, we find no diurnal variation in mRNA levels of *Scn10a* or *Scn11a*, and *Scn9a* shows higher mRNA expression at ZT16, whilst the current density is higher for both TTX-R and TTX-S channels

at ZT4. Together with the reported half-life of 18-26 hr,^{49,50} these findings argue against transcriptional regulation alone accounting for the observed circadian variation in sodium currents. Instead, the effects of KL001 on sodium currents, firing rates and pain behaviours were relatively rapid, consistent with post-translational control of VGSC function, for example through phosphorylation-dependent changes in channel gating properties,^{51,52} altered binding of VGSCs regulatory proteins^{53,54} or by altered membrane trafficking.^{55,56} This will require additional experiments to assess biophysical properties, cell-surface expression, endocytosis and plasma membrane recycling. Defining the molecular coupling between the clock machinery and VGSC regulation will be an important priority.

KL001 directly binds to both CRY1 and CRY2 to prevents ubiquitin mediated degradation.²⁹ The loss of effect of KL001 in the *Cry1*^{-/-}:*Cry2*^{-/-} mice underlines the specificity of its mode of action, and supports cryptochromes as the relevant clock effectors in this pathway. While cryptochromes have well-established nuclear repressor functions, knowledge of extranuclear interactions remains limited.⁵⁷ Nevertheless, regulation of neuronal firing through ion-channel mechanisms has been described in other systems^{58,59} supporting the possibility that cryptochromes can influence excitability through non-canonical mechanisms in neurons. We found that using either a Na_v1.7 antagonist or deleting Na_v1.7 in sensory neurons led to a loss of the diurnal pain behavioural responses, supporting a key role for this sodium channel in the mechanism.

Interestingly we saw a dissociation between baseline spontaneous firing activity and baseline synaptic activity in mouse in vitro preparations and their nociceptive behavioural responses. We found that spontaneous neuronal activity was increased at the time when animals showed the lowest nociceptive responses. This was not the case for hiPSC nociceptors and could be attributed to interspecies differences in receptor and channel composition between the two cultures.^{60,61} Notably, ligand-evoked responses and the magnitude of sodium currents were aligned and conserved in mouse and human sensory neurons, suggesting these measures more directly report the neurophysiological properties underlying behavioural pain responses.

Our work builds on previous studies of pain behaviours in both human and rodents,^{4,16,21} reporting diurnal modulation, with variability in whether baseline rhythms are observed.^{17,19,21,62} In contrast, we find robust oscillations at baseline and in a chronic pain model, and show these variations are maintained under constant darkness, confirming their circadian nature. The time-of-day modulation of nociceptive behaviour in our experiments was greater than that induced by nitroglycerin administration, suggesting either a strong circadian gating of this model or model-specific features; future studies will be required to establish generalisability across pain states. Differences across studies may

reflect housing and testing conditions, including repeated testing across multiple time points in a day. We minimised these confounds by ensuring habituation to experimental apparatus, housing mice in individually ventilated cages and testing on alternate days with no more than one time point per day. Standardising approaches to testing rhythmicity will be important for progress in this field.

This temporal pattern has clinical relevance across diverse pain states. Migraine attacks show temporal patterning,¹ carpal tunnel syndrome is characteristically associated with nocturnal pain and awakening,² and both labour pain and analgesic requirements vary between day and night.⁶³ These temporal patterns are likely to arise through multiple mechanisms, but our findings identify intrinsic circadian regulation of peripheral sensory neuron excitability as one potential contributor.

Sex differences in pain mechanisms are increasingly recognised.^{64–66} We found female mice to have increased responses compared to males for orofacial nociceptive responses but not hind paw mechanical nociceptive thresholds. Importantly circadian variation in nociception was present in both sexes despite these differences. Furthermore, KL001 was able to modulate nociceptive responses in male and female mice and to reduce firing activity in both male and female donor hiPSC derived nociceptors (Supplementary Fig. S10A-B).

Finally, these data support the therapeutic potential of targeting circadian mechanisms for pain disorders. Chronic pain remains a widely prevalent and highly disabling disorder. Current treatments and dosing schedules for chronic pain do not account for fluctuations in pain severity, which can result in inadequate pain control, adverse effects from over-dosing and long-term addiction.⁶⁷ All of these factors substantially impact quality of life.⁶⁸ We found that KL001 is potent in both mouse and human models of pain, consistent with a non-opioid analgesic strategy that acts through cryptochrome-dependent modulation of VGSC function rather than requiring broad resetting of circadian rhythms. Further interrogation of the CRY–VGSC coupling mechanism in sensory neurons may enable development of therapies that target peripheral sensory excitability while minimising disruption of circadian function in other tissues.

Data availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mohammed Zameel Cader (zameel.cader@ndcn.ox.ac.uk). Mouse lines and iPSC lines are available from the lead contact upon request with

completed and approved Materials Transfer Agreement. MATLAB analyse code, MEA data and any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

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Competing interests

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Supplementary material

Supplementary material is available at *Brain* online.

References

1. Fox AW, Davis RL. Migraine chronobiology. *Headache*. 1998;38(6):436-441. doi:10.1046/j.1526-4610.1998.3806436.x
2. Newman PH. Median Nerve Compression in the Carpal Tunnel. *Postgrad Med J*. 1948;24(271):264-269.
3. Tomson T, Ekbom K. Trigeminal neuralgia: time course of pain in relation to carbamazepine dosing. *Cephalalgia*. 1981;1(2):91-97. doi:10.1111/j.1468-2982.1981.tb00015.x
4. Kondratova AA, Kondratov RV. The circadian clock and pathology of the ageing brain. *Nat Rev Neurosci*. 2012;13(5):325-335. doi:10.1038/nrn3208
5. Logan RW, McClung CA. Rhythms of life: circadian disruption and brain disorders across the lifespan. *Nat Rev Neurosci*. 2019;20(1):49-65. doi:10.1038/s41583-018-0088-y
6. Edwards MD, Brancaccio M, Chesham JE, Maywood ES, Hastings MH. Rhythmic expression of cryptochrome induces the circadian clock of arrhythmic suprachiasmatic nuclei through arginine vasopressin signaling. *Proc Natl Acad Sci U S A*. 2016;113(10):2732-2737. doi:10.1073/pnas.1519044113
7. Kume K, Zylka MJ, Sriram S, et al. mCRY1 and mCRY2 Are Essential Components of the Negative Limb of the Circadian Clock Feedback Loop. *Cell*. 1999;98(2):193-205. doi:10.1016/S0092-8674(00)81014-4
8. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential Functions of *mPer1*, *mPer2*, and *mPer3* in the SCN Circadian Clock. *Neuron*. 2001;30(2):525-536. doi:10.1016/S0896-6273(01)00302-6
9. Gekakis N, Staknis D, Nguyen HB, et al. Role of the CLOCK Protein in the Mammalian Circadian Mechanism. *Science*. 1998;280(5369):1564-1569. doi:10.1126/science.280.5369.1564
10. Vitaterna MH, King DP, Chang AM, et al. Mutagenesis and Mapping of a Mouse Gene, Clock, Essential for Circadian Behavior. *Science*. 1994;264(5159):719-725.

- 1 11. Izumo M, Pejchal M, Schook AC, et al. Differential effects of light and feeding on circadian
2 organization of peripheral clocks in a forebrain Bmal1 mutant. *eLife*. 2014;3.
3 doi:10.7554/eLife.04617
- 4 12. Musiek ES, Lim MM, Yang G, et al. Circadian clock proteins regulate neuronal redox
5 homeostasis and neurodegeneration. *J Clin Invest*. 2013;123(12):5389-5400.
6 doi:10.1172/JCI70317
- 7 13. Börding T, Abdo AN, Maier B, Gabriel C, Kramer A. Generation of Human CRY1 and CRY2
8 Knockout Cells Using Duplex CRISPR/Cas9 Technology. *Frontiers in Physiology*.
9 2019;10:577. doi:10.3389/fphys.2019.00577
- 10 14. Halawani D, Wang Y, Ramakrishnan A, et al. Circadian clock regulator Bmal1 gates axon
11 regeneration via Tet3 epigenetics in mouse sensory neurons. *Nat Commun*. 2023;14(1):5165.
12 doi:10.1038/s41467-023-40816-7
- 13 15. Segal JP, Tresidder KA, Bhatt C, Gilron I, Ghasemlou N. Circadian control of pain and
14 neuroinflammation. *J Neurosci Res*. 2018;96(6):1002-1020. doi:10.1002/jnr.24150
- 15 16. Niir A, Ohno SN, Yamagata KA, et al. Diurnal Variation in Trigeminal Pain Sensitivity in
16 Mice. *Front Neurosci*. 2021;15. doi:10.3389/fnins.2021.703440
- 17 17. Zhang J, Li H, Teng H, et al. Regulation of Peripheral Clock to Oscillation of Substance P
18 Contributes to Circadian Inflammatory Pain. *Anesthesiology*. 2012;117(1):149-160.
19 doi:10.1097/ALN.0b013e31825b4fc1
- 20 18. Altug S, Uludag O, Tunctan B, Cakici I, Zengil H, Abacioglu N. Biological Time-Dependent
21 Difference in Effect of Peroxynitrite Demonstrated by the Mouse Hot Plate Pain Model.
22 *Chronobiology International*. 2006;23(3):583-591. doi:10.1080/07420520600650703
- 23 19. Choi YM, Jeong JH, Kim JS, Lee BC, Je HD, Sohn UD. Extremely low frequency magnetic
24 field exposure modulates the diurnal rhythm of the pain threshold in mice.
25 *Bioelectromagnetics*. 2003;24(3):206-210. doi:10.1002/bem.10094
- 26 20. Matsumiya LC, Sorge RE, Sotocinal SG, et al. Using the Mouse Grimace Scale to Reevaluate
27 the Efficacy of Postoperative Analgesics in Laboratory Mice. *Journal of the American*
28 *Association for Laboratory Animal Science : JAALAS*. 2012;51(1):42.

21. Han C, Lim JY, Koike N, et al. Regulation of headache response and transcriptomic network by the trigeminal ganglion clock. *Headache: The Journal of Head and Face Pain*. 2024;64(2):195-210. doi:10.1111/head.14670
22. Shirakawa Y, Ohno SN, Yamagata KA, et al. Circadian rhythm of PERIOD2::LUCIFERASE expression in the trigeminal ganglion of mice. *Front Neurosci*. 2023;17. doi:10.3389/fnins.2023.1142785
23. Horst GTJ van der, Muijtjens M, Kobayashi K, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*. 1999;398(6728):6728. doi:10.1038/19323
24. Todd WD, Venner A, Anaclet C, et al. Suprachiasmatic VIP neurons are required for normal circadian rhythmicity and comprised of molecularly distinct subpopulations. *Nat Commun*. 2020;11:4410. doi:10.1038/s41467-020-17197-2
25. J L, Ms M, J Z, et al. Temporal control of gene deletion in sensory ganglia using a tamoxifen-inducible Advillin-Cre-ERT2 recombinase mouse. *Mol Pain*. 2011;7:100-100. doi:10.1186/1744-8069-7-100
26. Mauvoisin D, Wang J, Jouffe C, et al. Circadian clock-dependent and -independent rhythmic proteomes implement distinct diurnal functions in mouse liver. *Proceedings of the National Academy of Sciences*. 2014;111(1):167-172. doi:10.1073/pnas.1314066111
27. Lalic T, Steponenaite A, Wei L, et al. TRESK is a key regulator of nocturnal suprachiasmatic nucleus dynamics and light adaptive responses. *Nat Commun*. 2020;11(1):4614. doi:10.1038/s41467-020-17978-9
28. Pettingill P, Weir GA, Wei T, et al. A causal role for TRESK loss of function in migraine mechanisms. *Brain*. Published online 2019. doi:10.1093/brain/awz342
29. Hirota T, Lee JW, St. John PC, et al. Identification of Small Molecule Activators of Cryptochrome. *Science*. 2012;337(6098):1094-1097. doi:10.1126/science.1223710
30. Rush AM, Cummins TR, Waxman SG. Multiple sodium channels and their roles in electrogenesis within dorsal root ganglion neurons. *J Physiol*. 2007;579(Pt 1):1-14. doi:10.1113/jphysiol.2006.121483

31. Wang J, Ou SW, Wang YJ. Distribution and function of voltage-gated sodium channels in the nervous system. *Channels*. 2017;11(6):534-554. doi:10.1080/19336950.2017.1380758
32. Fukuoka T, Noguchi K. Comparative study of voltage-gated sodium channel α -subunits in non-overlapping four neuronal populations in the rat dorsal root ganglion. *Neuroscience Research*. 2011;70(2):164-171. doi:10.1016/j.neures.2011.01.020
33. Hiranuma M, Okuda Y, Fujii Y, Richard JP, Watanabe T. Characterization of human iPSC-derived sensory neurons and their functional assessment using multi electrode array. *Sci Rep*. 2024;14(1):6011. doi:10.1038/s41598-024-55602-8
34. Young GT, Gutteridge A, Fox HD, et al. Characterizing Human Stem Cell-derived Sensory Neurons at the Single-cell Level Reveals Their Ion Channel Expression and Utility in Pain Research. *Mol Ther*. 2014;22(8):1530-1543. doi:10.1038/mt.2014.86
35. Farkas S, Bölcskei K, Markovics A, et al. Utility of different outcome measures for the nitroglycerin model of migraine in mice. *J Pharmacol Toxicol Methods*. 2016;77:33-44. doi:10.1016/j.vascn.2015.09.006
36. Drevet S, Favier B, Brun E, Gavazzi G, Lardy B. Mouse Models of Osteoarthritis: A Summary of Models and Outcomes Assessment. *Comp Med*. 2022;72(1):3-13. doi:10.30802/AALAS-CM-21-000043
37. Cao L, McDonnell A, Nitzsche A, et al. Pharmacological reversal of a pain phenotype in iPSC-derived sensory neurons and patients with inherited erythromelalgia. *Science Translational Medicine*. 2016;8(335):335ra56-335ra56. doi:10.1126/scitranslmed.aad7653
38. Alich TC, Röderer P, Szalontai B, et al. Bringing to light the physiological and pathological firing patterns of human induced pluripotent stem cell-derived neurons using optical recordings. *Front Cell Neurosci*. 2023;16. doi:10.3389/fncel.2022.1039957
39. Catterall WA. From Ionic Currents to Molecular Mechanisms: The Structure and Function of Voltage-Gated Sodium Channels. *Neuron*. 2000;26(1):13-25. doi:10.1016/S0896-6273(00)81133-2
40. Goldin AL, Barchi RL, Caldwell JH, et al. Nomenclature of Voltage-Gated Sodium Channels. *Neuron*. 2000;28(2):365-368. doi:10.1016/S0896-6273(00)00116-1

41. Faber CG, Lauria G, Merkies ISJ, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. *Proc Natl Acad Sci U S A*. 2012;109(47):19444-19449. doi:10.1073/pnas.1216080109
42. Hameed S. Nav1.7 and Nav1.8: Role in the pathophysiology of pain. *Mol Pain*. 2019;15:1744806919858801. doi:10.1177/1744806919858801
43. Shields SD, Deng L, Reese RM, et al. Insensitivity to Pain upon Adult-Onset Deletion of Nav1.7 or Its Blockade with Selective Inhibitors. *J Neurosci*. 2018;38(47):10180-10201. doi:10.1523/JNEUROSCI.1049-18.2018
44. Marchi M, D'Amato I, Andelic M, et al. Congenital insensitivity to pain: a novel mutation affecting a U12-type intron causes multiple aberrant splicing of SCN9A. *PAIN*. 2022;163(7):e882. doi:10.1097/j.pain.0000000000002535
45. Pokhilko A, Nash A, Cader MZ. Common transcriptional signatures of neuropathic pain. *Pain*. 2020;161(7):1542-1554. doi:10.1097/j.pain.0000000000001847
46. Dormer A, Narayanan M, Schentag J, et al. A Review of the Therapeutic Targeting of SCN9A and Nav1.7 for Pain Relief in Current Human Clinical Trials. *J Pain Res*. 2023;16:1487-1498. doi:10.2147/JPR.S388896
47. Fu W, Vasylyev D, Bi Y, et al. Nav1.7 as a chondrocyte regulator and therapeutic target for osteoarthritis. *Nature*. 2024;625(7995):557-565. doi:10.1038/s41586-023-06888-7
48. Emery EC, Luiz AP, Wood JN. Nav1.7 and other voltage-gated sodium channels as drug targets for pain relief. *Expert Opin Ther Targets*. 2016;20(8):975-983. doi:10.1517/14728222.2016.1162295
49. Waechter CJ, Schmidt JW, Catterall WA. Glycosylation is required for maintenance of functional sodium channels in neuroblastoma cells. *Journal of Biological Chemistry*. 1983;258(8):5117-5123. doi:10.1016/S0021-9258(18)32546-8
50. Sherman S, Chrivia J, Catterall W. Cyclic adenosine 3':5'-monophosphate and cytosolic calcium exert opposing effects on biosynthesis of tetrodotoxin-sensitive sodium channels in rat muscle cells. *J Neurosci*. 1985;5(6):1570-1576. doi:10.1523/JNEUROSCI.05-06-01570.1985

- 1 51. Kerth CM, Hautvast P, Körner J, Lampert A, Meents JE. Phosphorylation of a chronic pain
2 mutation in the voltage-gated sodium channel Nav1.7 increases voltage sensitivity. *Journal of*
3 *Biological Chemistry*. 2021;296:100227. doi:10.1074/jbc.RA120.014288
- 4 52. Vijayaragavan K, Boutjdir M, Chahine M. Modulation of Nav1.7 and Nav1.8 Peripheral Nerve
5 Sodium Channels by Protein Kinase A and Protein Kinase C. *Journal of Neurophysiology*.
6 2004;91(4):1556-1569. doi:10.1152/jn.00676.2003
- 7 53. Isom LL, De Jongh KS, Catterall WA. Auxiliary subunits of voltage-gated ion channels.
8 *Neuron*. 1994;12(6):1183-1194. doi:10.1016/0896-6273(94)90436-7
- 9 54. Salvage SC, Rahman T, Eagles DA, et al. The $\beta 3$ -subunit modulates the effect of venom
10 peptides ProTx-II and OD1 on NaV1.7 gating. *Journal of Cellular Physiology*.
11 2023;238(6):1354-1367. doi:10.1002/jcp.31018
- 12 55. Gomez K, Stratton HJ, Duran P, et al. Identification and targeting of a unique NaV1.7 domain
13 driving chronic pain. *Proceedings of the National Academy of Sciences*.
14 2023;120(32):e2217800120. doi:10.1073/pnas.2217800120
- 15 56. Cusdin FS, Clare JJ, Jackson AP. Trafficking and Cellular Distribution of Voltage-Gated
16 Sodium Channels. *Traffic*. 2008;9(1):17-26. doi:10.1111/j.1600-0854.2007.00673.x
- 17 57. Zhao M, Lu D, Chen M, Wu B. Introduction to Mammalian Circadian Clock System. In: Wu
18 B, Lu D, Dong D, eds. *Circadian Pharmacokinetics*. Springer; 2020:1-21. doi:10.1007/978-
19 981-15-8807-5_1
- 20 58. Fogle KJ, Parson KG, Dahm NA, Holmes TC. CRYPTOCHROME Is a Blue-Light Sensor
21 That Regulates Neuronal Firing Rate. *Science*. 2011;331(6023):1409-1413.
22 doi:10.1126/science.1199702
- 23 59. Fogle KJ, Baik LS, Houl JH, et al. CRYPTOCHROME-mediated phototransduction by
24 modulation of the potassium ion channel β -subunit redox sensor. *Proceedings of the National*
25 *Academy of Sciences*. 2015;112(7):2245-2250. doi:10.1073/pnas.1416586112
- 26 60. Rostock C, Schrenk-Siemens K, Pohle J, Siemens J. Human vs. Mouse Nociceptors –
27 Similarities and Differences. *Neuroscience*. 2018;387:13-27.
28 doi:10.1016/j.neuroscience.2017.11.047

61. Middleton SJ, Barry AM, Comini M, et al. Studying human nociceptors: from fundamentals to clinic. *Brain*. 2021;144(5):1312-1335. doi:10.1093/brain/awab048
62. Minett MS, Eijkelkamp N, Wood JN. Significant Determinants of Mouse Pain Behaviour. *PLoS One*. 2014;9(8):e104458. doi:10.1371/journal.pone.0104458
63. Knezevic NN, Nader A, Pirvulescu I, Pynadath A, Rahavard BB, Candido KD. Circadian pain patterns in human pain conditions – A systematic review. *Pain Pract*. 2023;23(1):94-109. doi:10.1111/papr.13149
64. Pieretti S, Di Giannuario A, Di Giovannandrea R, et al. Gender differences in pain and its relief. *Ann Ist Super Sanita*. 2016;52(2):184-189. doi:10.4415/ANN_16_02_09
65. Hurley RW, Adams MCB. Sex, Gender, and Pain: An Overview of a Complex Field. *Anesth Analg*. 2008;107(1):309-317. doi:10.1213/01.ane.0b013e31816ba437
66. Francis-Malavé AM, Martínez González S, Pichardo C, et al. Sex differences in pain-related behaviors and clinical progression of disease in mouse models of colonic pain. *Pain*. 2023;164(1):197-215. doi:10.1097/j.pain.0000000000002683
67. Savage SR, Kirsh KL, Passik SD. Challenges in Using Opioids to Treat Pain in Persons With Substance Use Disorders. *Addict Sci Clin Pract*. 2008;4(2):4-25.
68. Haack M, Simpson N, Sethna N, Kaur S, Mullington J. Sleep deficiency and chronic pain: potential underlying mechanisms and clinical implications. *Neuropsychopharmacology*. 2020;45(1):205-216. doi:10.1038/s41386-019-0439-z

Figure legends

Figure 1 Circadian variation in sensitivity thresholds of mice. Orofacial von Frey pain score between 4 time points of the day in female and male mice (**A**), $n = 13-17$ females and $n = 10-21$ males per time point, 2-way ANOVA (significance of sex $F_{(1, 117)} = 33.78$, $P < 0.0001$, Time points $F_{(3, 117)} = 22.10$, $P < 0.0001$ and interaction $F_{(3, 117)} = 3.89$, $P = 0.0108$). (**B**) von Frey hindpaw test showed steady and persistent differences in thresholds between the three time windows tested (ZT 0-3; ZT 6-9; ZT 12-15) over 3 days, $n = 3-17$ females and $n = 3-9$ males per time window, 2-way ANOVA (significance of time window $F_{(5, 94)} = 36.03$, $P < 0.0001$, no

significance between days $P=0.3357$ and no significance between sex $P=0.0844$). (C) Orofacial: Wildtype male ($n=14-18$ per time point) and $Cry1^{-/-};Cry2^{-/-}$ mice ($n=8-10$ per time point). 2-way ANOVA significance between genotype $F_{(1,46)}=28.62$, $P<0.0001$ for orofacial. Red box highlights mean orofacial response of $Cry1^{-/-};Cry2^{-/-}$ mice. (F) Hindpaw: Wildtype male ($n=9$ per time point) and $Cry1^{-/-};Cry2^{-/-}$ mice ($n=4$ per time point). WT ZT12-15 has significantly lower orofacial pain score than $Cry1^{-/-};Cry2^{-/-}$ mice ($P<0.0001$), and both time points in WT hindpaw significantly differ to $Cry1^{-/-};Cry2^{-/-}$ mice ($F_{(1,11)}=13.85$, $P=0.0034$). (D) *VIP-Cre* ($n=5-6$ per time point) and *VIP-Cre:Bmal^{fl/fl}* male mice ($n=6$ per time point). 2-way ANOVA showed a significant of time orofacial $F_{(1,19)}=61.92$, $P<0.0001$. For hindpaw (G): *VIP-Cre* ($n=6-7$ per time point) and *VIP-Cre:Bmal^{fl/fl}* male mice ($n=6$ per time point), significance was observed for time $F_{(1,21)}=23.23$, $P<0.0001$. (E) *Advillin-Cre-ERT2:Bmal^{fl/fl}* male mice induced with tamoxifen (TAM) ($n=7-11$ per time point) or vehicle ($n=7-8$ per time point). Red box highlights mean orofacial response of sensory neuron *Bmal1* knocked out mice. 2-way ANOVA showed significant interaction between time and genotype $F_{(1,30)}=9.445$, $P=0.0045$ for orofacial and significance in time $F_{(1,12)}=6.167$, $P=0.0288$ for hindpaw (H), *Advillin-Cre-ERT2:Bmal^{fl/fl}* male mice induced with tamoxifen (TAM) ($n=4$ per time point) or vehicle ($n=4$ per time point). Vehicle injected ZT1-3 has significantly higher orofacial pain score than TAM injected mice ($P<0.0001$). (A-H) All data are represented as mean $\pm$ SEM. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$, **** $P<0.0001$.

Figure 2 Circadian variation in excitability of sensory neurons in mice trigeminal ganglia (TG). Fura2-AM ratioimetric calcium imaging showed diurnal differences in the proportion of responsive (0.05 increase of ratio from baseline) neurons and glia cells to ATP-YS 50 μ M, capsaicin 10 μ M, menthol 200 μ M at ZT4 and ZT16 of wildtype male mice (A) or $Cry1^{-/-};Cry2^{-/-}$ male mice (D) TG slices (total number of neurons assessed (ZT4,ZT16): WT ATPys (29,43), Capsaicin (36,72), Menthol (24,56); Cry double KO ATPys (45,81), Capsaicin (87,96), Menthol (42,81)). Chi-square test with two-tail, * $P<0.05$, ** $P<0.005$, *** $P<0.0005$, **** $P<0.0001$ (B) Firing rate of primary male mouse TG neurons, before and after stimulation with ATP-YS 50 μ M, recorded from acute TG slices using multiple electrode arrays (MEA) at ZT4 and ZT16. Data log normalized. Two-way ANOVA * $P=0.0111$, *** $P=0.0001$, **** $P<0.0001$ (significance of treatment $F_{(1,1174)}=36.89$, $P<0.0001$ and interaction $F_{(1,1174)}=10.29$, $P=0.0014$). (C) MEA electrode activation after addition of ATP-YS 50 μ M per male mice TG slice between ZT4 and ZT16 (TG slices $n=11$). Unpaired two-tail t-test, * $P=0.0308$. (E) Patch clamp current traces and (F) frequency of spontaneous excitatory postsynaptic current (sEPSC) in Lamina I/II of trigeminal nucleus caudalis neurons from acute slices of wildtype male mice or $Cry1^{-/-};Cry2^{-/-}$ male mice at ZT4 and

ZT16. 2-way ANOVA showed significant interaction between time and genotype $F_{(2,81)}=3.226$, $P=0.0449$, and significance between time points in wildtype $**P=0.0025$.

Figure 3 Circadian variation in sodium current of sensory neurons in mice. (A) Schematic diagram illustrating the time dependent patching experiment, with colour coding for CT4 (red) and CT16 (purple) corresponding to time after chemical synchronization. **(B-D)** intrinsic neuronal properties in wildtype mouse primary nociceptors (mixed male and female pups) at CT4 and CT16 time points following 10 μ M forskolin synchronization - resting membrane potential ($n=23$) **(B)**, rheobase ($n=19$) **(C)**, and potassium leak current ($n=17$) **(D)**. Unpaired two-tail t-test. $*P\leq 0.05$. **(E)** patch clamp recordings of voltage dependent sodium currents in wildtype ($n=11$ per time point). Two-way ANOVA significance of interaction between time point and voltage $F_{(9,180)}=5.081$, $P<0.0001$. **(F)** Recording of TTX independent voltage gated sodium current ($n=9-15$ per time point). The shaded area highlights the TTX sensitive current when compared to the patching data without TTX. Two-way ANOVA significance between time point and voltage $F_{(9,198)}=3.487$, $P=0.0005$. **(G)** *Cry1*^{-/-}: *Cry2*^{-/-} ($n=7$ per time point) mouse primary nociceptors, following 10 μ M forskolin synchronization, at time points CT4 and CT16. No significant interaction for *Cry1*^{-/-}: *Cry2*^{-/-} $F_{(9,108)}=0.303$, $P=0.9724$.

Figure 4 Circadian variation in hiPSC-derived sensory neurons. (A) Bioluminescence data of *BMAL1:LUC* nociceptors (4 repeating wells per condition SBAD03 cell line). Line graph shows detrended group data (black dots) with a fitted damped sine wave to data (coloured lines) (FK fit: $R^2=0.7147$, RMSE=7.276, period= ~ 24 hr, peak=15.76hr, peak prominence=4.59; Dex fit: $R^2=0.6263$, RMSE=8.05, period= ~ 24 hr, peak=15.89hr, peak prominence=3.24; DMSO fit: $R^2=0.7012$, RMSE=4.982, period= ~ 24 hr, peak=17.5hr, peak prominence=1.45). **(B)** Gene expression of PER2 and BMAL1 in human nociceptor samples collected over 6 timepoints. Sine wave fitted to the data (*BMAL1*: $R^2=0.6431$, RMSE=0.1062; *PER2*: $R^2=0.597$, RMSE=0.1135). **(C)** patch clamp recordings of voltage dependent sodium currents in iPSC derived nociceptors (>day 60) from a healthy donor (SBAD03) following 10 μ M forskolin synchronization. Red circles represent CT4 (4h post FK sync $n=18$), purple circles represent CT16 (16h post FK sync $n=23$). Statistical significance calculated with two-way ANOVA **** $P<0.0001$. **(D)** Baseline extracellular electrode firing rate in log scale of nociceptor after synchronization with forskolin at 2 time points in presence of CNQX (10 μ M). Inactive electrodes not shown. 5 repeating wells per time point. Two tail t-test ** $P<0.005$. **(E)** Nociceptor response to OD1 (50nM) 1h post addition of the drug compared to the same electrodes at baseline in presence of CNQX at 2 time points. 5 repeating wells

per time point, two-way ANOVA *** $P < 0.0005$ (F) Nociceptor response to ATPyS (50 μ M) immediately post addition of the drug compared to the same electrode recording at baseline in presence of CNQX at 2 time points. 5 repeating wells per time point. All grouped data are mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$.

Figure 5 Exposure to KL001 decreases sodium current and reduce sensitivity. (A) patch clamp recordings of voltage dependent sodium currents in wildtype male mouse primary nociceptors, following 10 μ M forskolin synchronisation at CT16 time point with or without 20 μ M KL001. 2-way ANOVA showed significant interaction between time and current $F_{(9,153)} = 2.106$, $P = 0.0322$ (vehicle $n = 11$, KL001 $n = 8$, total animal = 5). (B) orofacial pain response at ZT4 in wildtype male mice i.p. injected with vehicle ($n = 5$) or 0.4mg KL001 ($n = 6$); unpaired two-tail t-test * $p \leq 0.05$, ** $P < 0.005$. (C) patch clamp recording of *Cry1*^{-/-}: *Cry2*^{-/-} male mice (vehicle $n = 14$ per time point, KL001 $n = 8$, total animal = 5). Interaction between time and voltage is not significant. (D) orofacial pain response of *Cry1*^{-/-}: *Cry2*^{-/-} with vehicle or KL001 i.p. (E) patch clamp recordings of voltage dependent sodium currents in iPSC derived nociceptors (>day 60) from a healthy donor (SBAD03) following 10 μ M forskolin synchronization. Purple circles represent CT16 (16h post FK sync $n = 23$), green circles represent CT16 with 1h preincubation of KL001 (20 μ M) at 37°C ($n = 12$). Statistical significance calculated with two-way ANOVA **** $P < 0.0001$. (F) Orofacial pain score measured from male wildtype mice i.p. injected with vehicle control or Na_v1.7 blocker (PF05089771, 75mg/kg) at ZT4 and ZT16 time points; $n = 4$ per group, significance measured by two-way ANOVA. All grouped data are mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$.

Figure 6 Analgesic properties of KL001 in disease models. Vertical bar graphs that show changes in latency of Hargreaves test (A) and change in threshold(g) of von Frey test (B) over 3 time points with/without treatment of NTG at day 12 of chronic experiment ($n = 18$ male mice). All values normalized to ZT6-9 time point. Red bar and green bar on top of graph show maximum difference between the 3 time points for control and NTG treatment for the two behaviour tests. (C) Bar graph show the maximum difference between sensitivity due to NTG treatment and Circadian regulation for Hargreaves test (A) and von Frey test (B) at day 12 of chronic experiment in male mice. (D) orofacial pain responses in wildtype male mice administered vehicle ($n = 6$) or 0.4mg KL001 ($n = 6$) in acute or (E) chronic NTG model from baseline (BL) to day 12 (D12); unpaired two-tail t-test *** $P < 0.0005$ and two-way ANOVA ** $P = 0.0011$. (F) MEA recording of spontaneous firing from mature (>day 60) nociceptors differentiated from an erythromelalgia patient iPSC line (IEM-F1449V)

1 treated with vehicle or KL001(20 μ M). Statistical significance calculated with two-tail t test.
2 $n=5$ repeating wells per condition. (**G**) Post-operative pain was assessed by dynamic weight
3 bearing (DWB) 24h following unilateral partial meniscectomy (PMX) in wildtype male mice.
4 KL001 injection significantly reduced pain 24h after administration of drug compared to
5 vehicle injected controls ($n=5$), significance by two-way ANOVA. The effect of KL001 had
6 reversed by 48h and pain behaviour was similar to controls. All grouped data are mean $\pm$ SEM,
7 $* P<0.05$, $** P<0.005$, $*** P<0.0005$, $**** P<0.0001$.

8

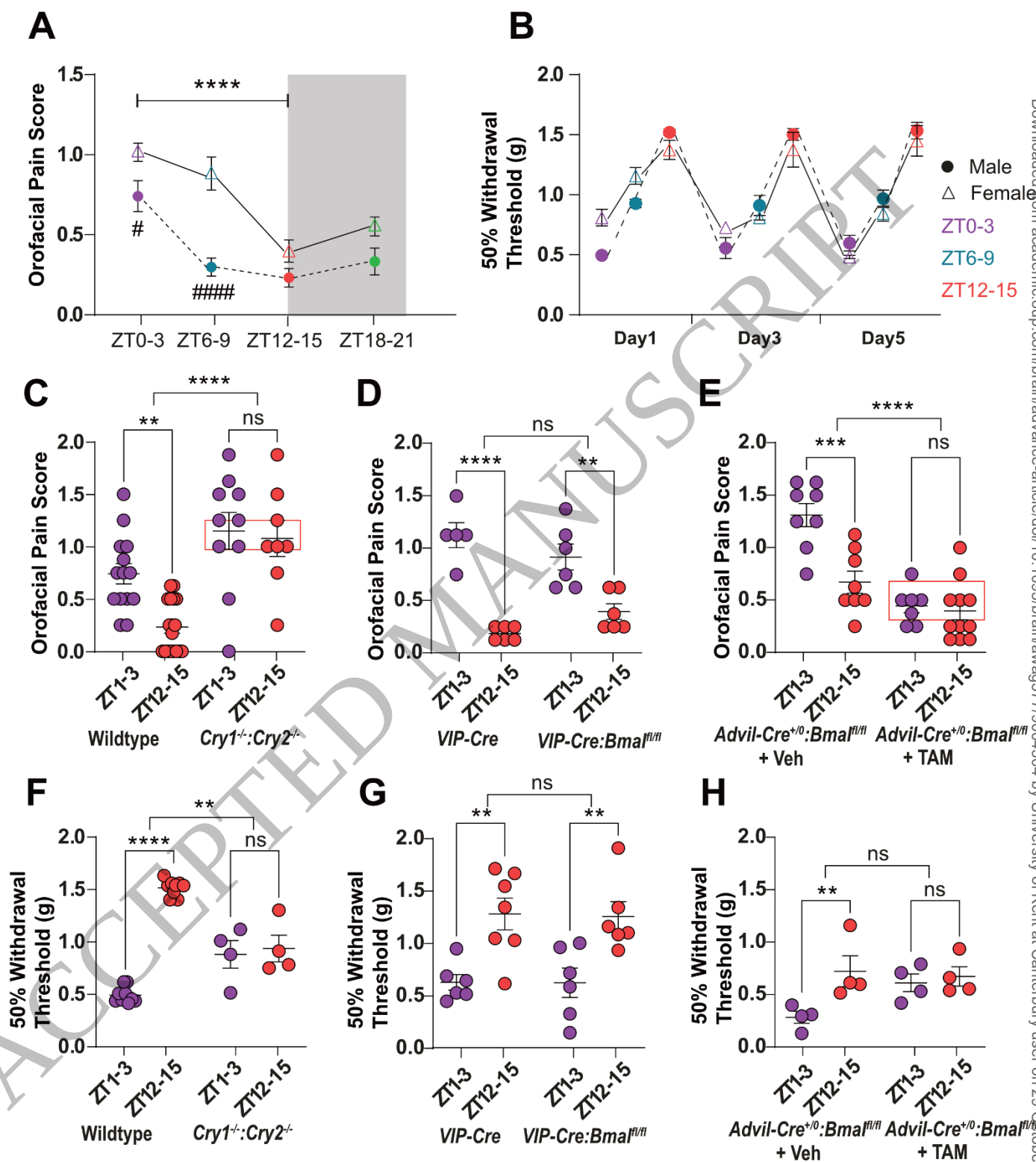


Figure 1
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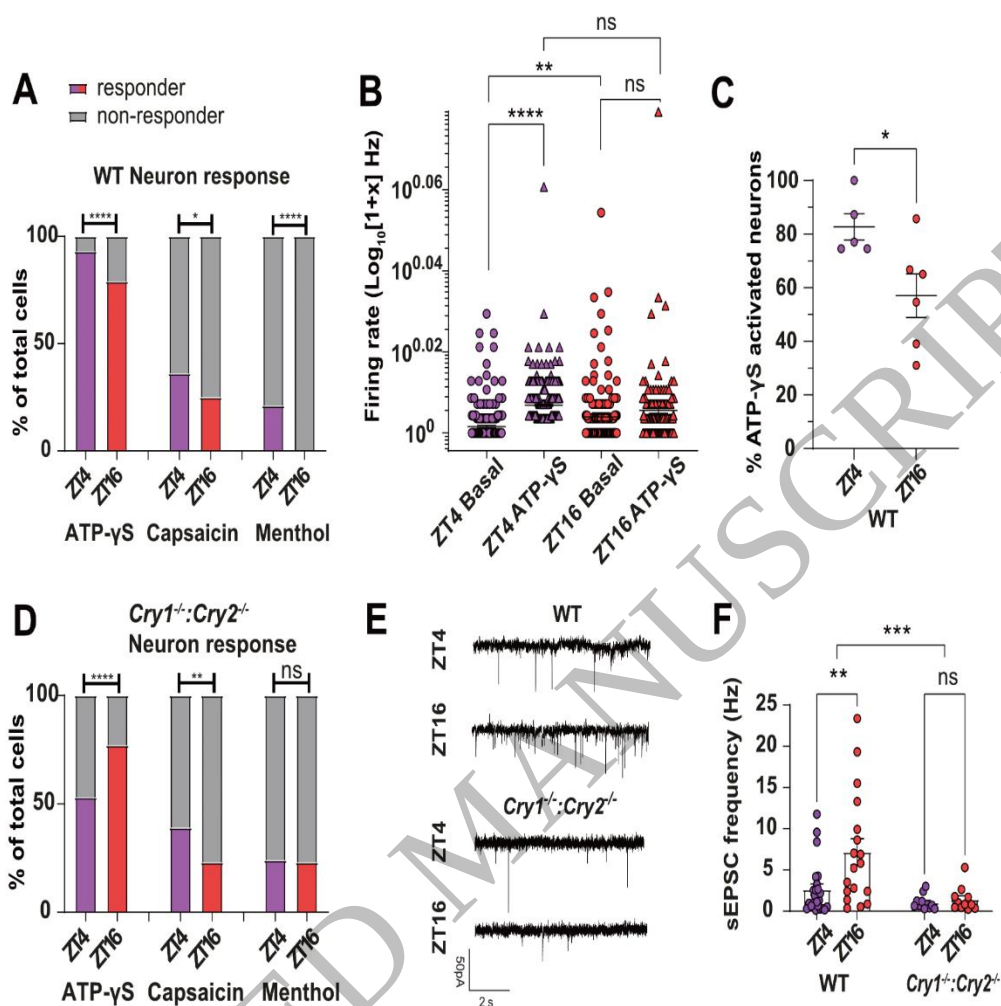


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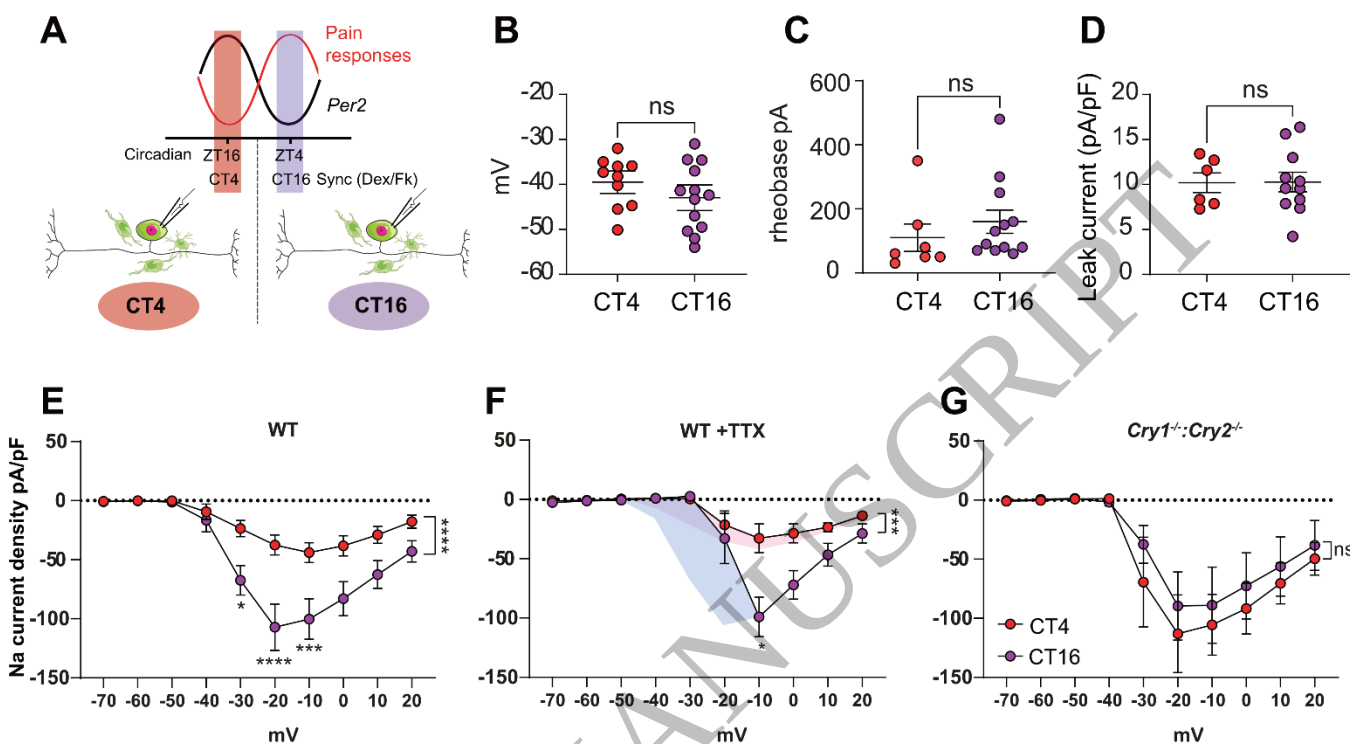


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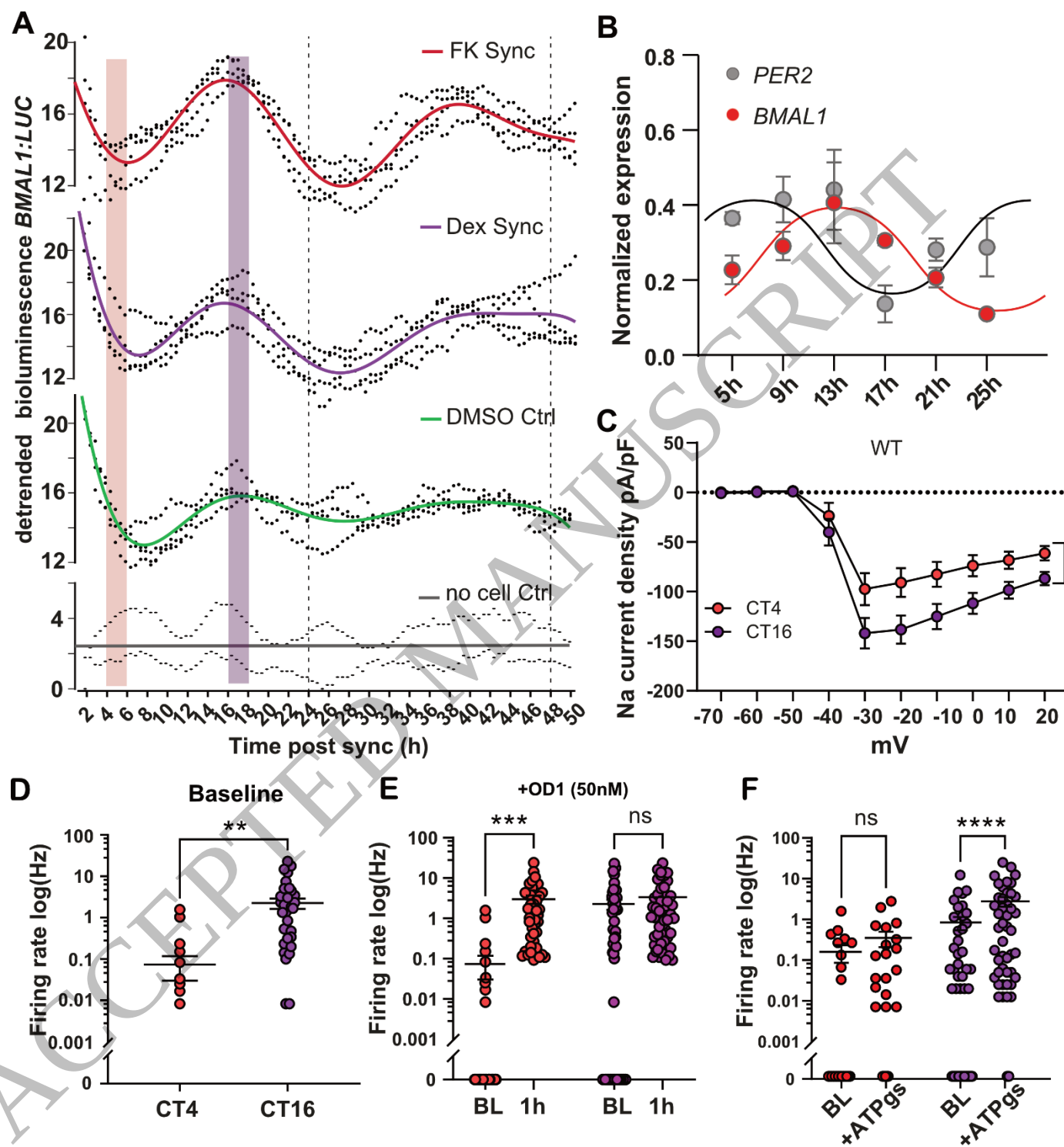


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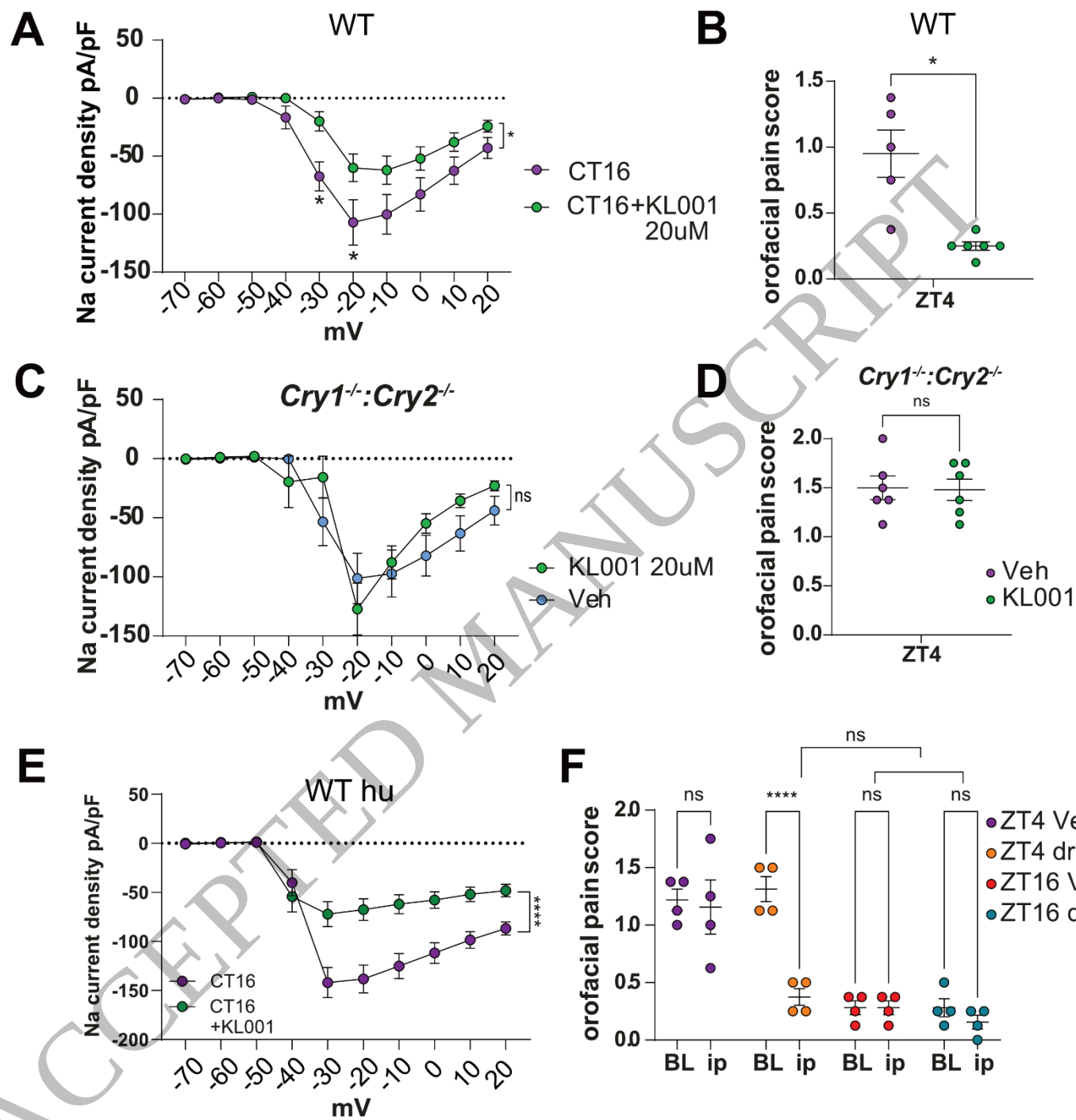


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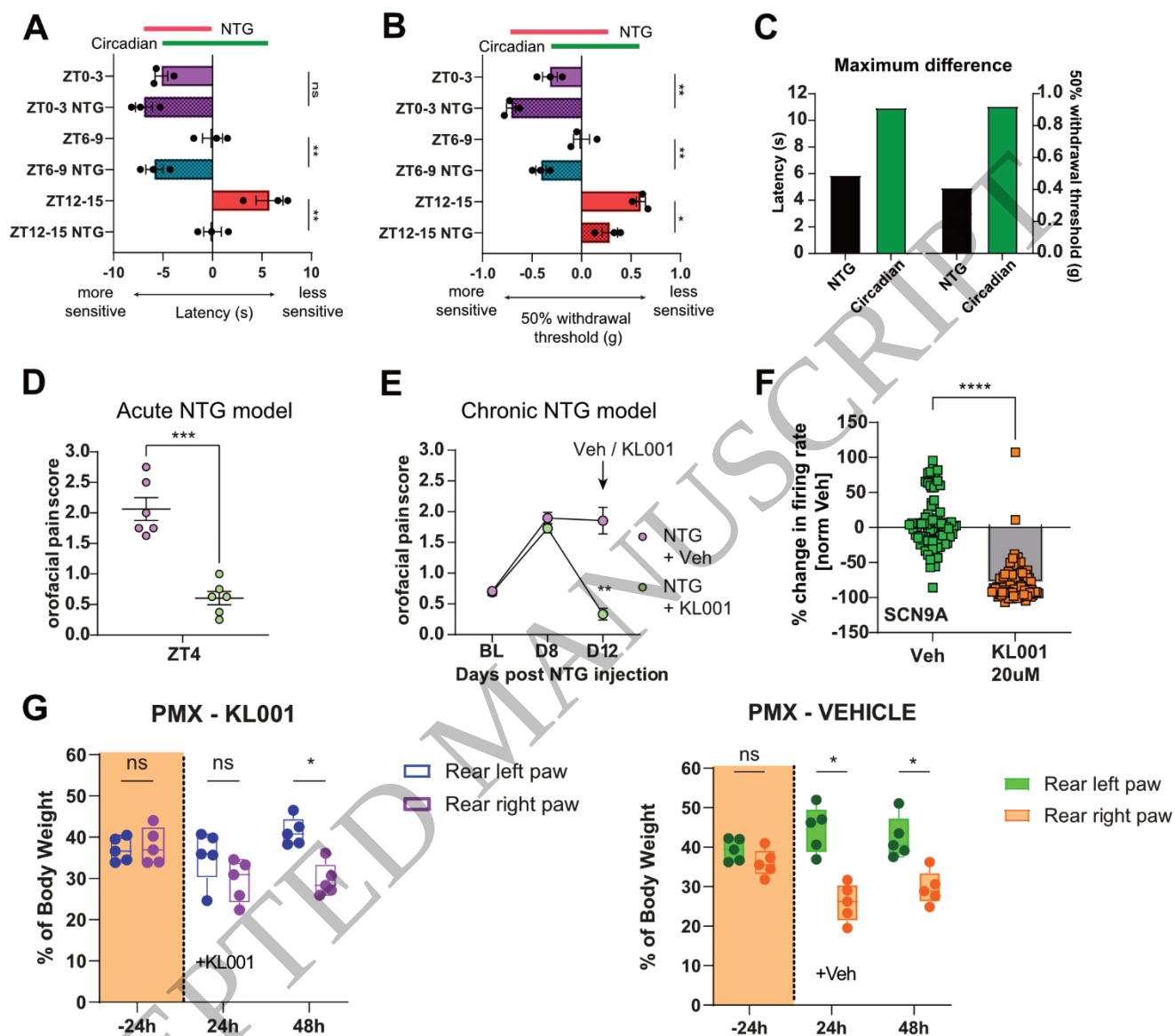


Figure 6
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