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감각기능의 주기적 리듬변화현상

Circadian Rhythm of Somatosensation

2013 년 8 월

서울대학교 대학원

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ABSTRACT

Circadian rhythm is now well established for a broad range of behavioural, psychological, and physiological variables. This is known to be regulated by master pacemaker in the brain. In recent years, the diurnal variations in a variety of syndromes associated with painful disease have received much scrutiny. The objective of the present study is to address the controversial issue over the existence of diurnal variations in pain perception. Behavioural analysis showed the diurnal variation of pain and mechanosensation in normal wild-type mice. Cultured dorsal root ganglia (DRG) neurons exhibited a temporal change of neuronal excitability with a dramatic increase of firing frequency in the afternoon, suggesting endogenous temporal control of somatosensory transduction. Analysis of DRG-specific ion channel identified temporal variations in expression of TRPV1 and Nav1.6 that may contribute to changes of firing frequencies of sensory DRG neurons. These findings suggest essential roles of peripheral sensory neurons in circadian variation of pain perception that is regulated by endogenous clock-controlled pathways. It raises a potential chronotherapeutic approaches to the treatment of pain.

Keywords : Circadian, Diurnal, Pain, Nav, Transient Receptor Potential (TRP)

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INTRODUCTION

1. The Circadian System

The Earth is a rhythmic environment. To compete effectively and therefore survive, our bodies have evolved a circadian system that helps to regulate a variety of innate biological functions and physiological behaviours for rhythmic control. Synchronisation of the endogenous circadian system to the 24-hour environmental cycle occurs by the combined actions of internal signals and environmental factors. The environmental time cues are collectively termed **zeitgebers** (German for "time givers"). Circadian system can be identified in most eukaryotic organisms as well as some prokaryotes, which reflect their critical role in organism's well-being [1].

1.1. CIRCADIAN CLOCK

Circadian rhythms (The term is from the Latin *circa*, "approximately," and *dies*, "day.") refer to any biological processes that exhibit 24-hour rhythmical patterns. In both central and peripheral systems, there are internal biological clocks that can free-run and continue to oscillate in the absence of external stimuli (light/dark, temperature and humidity variations). In the sensitive plant *Mimosa pudica*, the natural free-running period is about 22 hours [2]; in mice, it is about 23 hours; and in humans, it tends to be 24.5-25.5 hours [3].

Circadian rhythms are regulated through neuronal mechanisms coordinated by a master pacemaker, known as *suprachiasmatic nucleus (SCN)*, which resides within the hypothalamus [4, 5]. There are a number of clock genes and proteins (e.g. Bmal1, Per and Rev-erbα) that cycle in concentration and in spatial distribution and regulate the circadian cycle in the pacemaker neurons [6]. Ion channels have been reported to play a major role as molecular sensors in the regulation of rhythm [7].

1.2. CIRCADIAN PATTERN OF PAIN SENSITIVITY

Over many years, clinical studies have reported that a variety of syndromes associated with painful diseases show diurnal variations, in particular with thermal pain perception [8, 9]. However, there are also contradictory reports that observed no variation in heat pain sensitivity [10, 11]. Such inconsistency reflects one of the major problems inherent in this type of research; the question as to how the essentially subjective experience such as pain can be assessed objectively and reproducibly.

2. The Somatosensory System

Somatosensation involves the processing of mechanical, thermal, nociceptive and proprioceptive information which allows living organisms to perceive and respond to their physical environment. Primary afferent axons transmit such information to the dorsal horn of the spinal cord. Their cell-bodies are located in trigeminal ganglia and dorsal root ganglia (DRG), and project from around the body including the skin, sense

organs, and internal organs. The sizes of neuron cell bodies vary greatly, as do the type, number and length of their processes (Table 1).

Upon a sufficient stimulus, the electrical charge on the inside of the cell membrane changes from negative to positive. A wave of electrical charge travels along the entire length of the nerve fibre to a central nervous system. When each signal reaches specific cortical regions of the brain, it is processed to generate coherent perceptions and interpreted to produce appropriate physiological responses. A deregulation at any of these pathways may result in a failure of fundamental physiological function and may contribute to various symptoms of major human diseases.

The sensitivity of a sensory neuron to various changes and stimulus depends on the type of ion channels the neuron expresses; neuronal excitability depends on the spatial and temporal expression of several voltage-gated ion channels (see Chapter 3). A various sensory receptors are specialised to detect and distinguish between different types of stimuli which will be discussed in next few pages. A single stimulus usually activates many receptors, although figuring out exactly how physical force opens ion channel remains unanswered.

2.1. TOUCH

Most of the sensory receptors in the somatosensory system are **mechanoreceptors**, which are distributed throughout the body including the skin and deeper tissues [12].

They are sensitive to physical distortion such as bending or stretching and their range of functional roles involves monitoring skin contacts, blood pressure in vessels as well as in the heart and stretching of various internal organs (e.g. urinary bladder). The touch pathway, mediated by the cutaneous mechanoreceptors, is swift and uses the relatively large, myelinated Aβ fibers.

Mechanoreceptors vary in their preferred stimulus frequencies, pressures, and receptive field sizes [13]. They also vary in the persistence of their responses to long-lasting stimuli. These different mechanical sensitivities of mechanoreceptors mediate different sensations. When the mechanoreceptors are stimulated, energy is transferred to the nerve terminal in the middle, its membrane is deformed, and mechanosensitive channels open. Current flowing through the channels generates a receptor potential, which is depolarising. If the depolarisation is large enough, the axon will fire an action potential.

2.2. PAIN

The transduction of painful stimuli occurs in the free nerve endings of unmyelinated C fibers and lightly myelinated Aδ fibers that are activated by various types of stimuli that have a potential to cause tissue damage. The membranes of nociceptors contain ion channels which, when activated, causes the neuron to depolarise, generate action potentials, transmit pain signals to the brain and elicit the conscious experience of pain.

Nociceptors function via separate neural mechanisms and routes that are distinctive to those taken by mechanoreceptors. There are mechanical nociceptors, showing selective responses to strong pressure; thermal nociceptors, showing selective response to burning heat or extreme cold; and chemical nociceptors, showing selective responses to histamine and other chemicals.

It may be worth to note the difference between *pain* and *nociception*. Pain is the feeling, or the perception, of painful sensations, whereas nociception is the sensory process that provides the signals that trigger pain. In context, nociceptors may fire intense signals but it may or may not lead to pain sensation. Conversely, one could experience a terrible pain without activity of nociceptors.

3. Ion Channels

Ion channels are specialised to detect and distinguish between different types of stimuli hence form the molecular basis of neuronal activity. They mediate sensory transduction, which involves a conversion of sensory stimuli into electrical signals. Upon a sufficient trigger, ion channels open which depolarise the receptive fields. If this local depolarisation, called **the receptor potential**, is large enough, action potentials can be generated that propagate along axons towards the central nervous system.

In addition to their ability to recognise specific stimulus, ion channels also play an important role in the control of the electrophysiological properties of neurons. They

contribute not only to the resting membrane potential and membrane repolarisation, but also to the shape, duration, and frequency of action potentials. Studies have shown that different channels are predominantly expressed by different subpopulations of primary afferent neurons. Ultimately this forms functionally distinct subtypes of sensory neurons with specific threshold sensitivities and neuronal excitabilities.

3.1. VOLTAGE-GATED SODIUM CHANNEL

The voltage-gated sodium channels (VGSC) are primarily responsible for initiating the rapid upstroke of action potentials in neurons and play an important role in neuronal excitability [14]. VGSC gene family comprises nine homologous members *SCN1A* to *SCN11A*, which encode the sodium selective ion channels Nav1.1 to Nav1.9. The nine different VGSCs are differentially expressed and perform different functional roles (Table 2) [15, 16].

Disruption of VGSC function leads to a broad range of pathologies and they have been major targets of therapeutic drug development. The role of VGSCs in epilepsy and pain has been well established [17]. Of the nine sodium channel isoforms, Nav1.7-1.9 are predominantly expressed in small-diameter (<30μm) nociceptive DRG neurons and have been reported to contribute to nociception [18, 19]. In addition, there is increasing evidence of a role for VGSCs in other disorders such as anosmia [20].

3.2. TRP CHANNEL

There are 33 transient receptor potential (TRP) cation channel genes in mammals and can be subdivided into seven subfamilies on the basis of sequence similarity [21]. Functions of most TRP channels remain still unknown but some important findings showed that different TRP ion channels contribute to a wide variety of sensory transductions including mechanosensation and thermosensation [22-25]. TRPV1 and TRPA1 have been widely studied with regard to noxious temperature transduction for their potentials as therapeutic targets in acute inflammatory pain.

The well-known heat-sensing vanilloid receptor, TRPV1 is also activated by low pH, capsaicin (hot pepper active compound) and inflammation [26, 27]. TRPV1 is highly expressed in nociceptive fibres and have been implicated in nociception [28]. TRPA1, which has been shown to co-localise with TRPV1 in subpopulations of DRG, was first reported to be a sensor of painful cold stimuli [29]. Studies have also shown that TRPA1 was also involved in mechanical pain [30]; but TRPV1 was not.

3.3. PIEZO CHANNEL

Piezo1 and Piezo2 – also known as Fam38a and Fam38b, respectively – are recently identified ion channels for the rapidly adapting mechanosensitive cation current in mammals [31, 32]. Both Piezos are highly expressed in lung, bladder and colon; Piezo2 is highly expressed in sensory DRG neurons, which indicates its functional role in somatosensory mechanotransduction. In Drosophila, Piezo2 was reported to sense noxious

mechanical stimuli, but not noxious heat stimulus or touch [33]. However, our previous study with human Piezo2 showed a quite a contrary data which suggested its role in touch sensation and mechanical allodynia, but showed no responses to noxious mechanical stimulation or noxious heat [34].

	Aα	Aβ	Aδ	C
Diameter (μm)	13-20	6-12	1-5	0.2-1.5
Axons	myelinated	myelinated	myelinated	unmyelinated
Velocity (m/s)	80-120	35-75	5-30	0.5-2
Sensory functions	Prorioception of skeletal muscle	Mechanosensation of skin	Pain, temperature	Temperature, pain, itch

Table 1. Types of Primary Afferent Neurons.

Channel	Gene	Tissue Expressions	Disease Associations
NaV1.1	SCN1A	Cerebral Cortex, DRG, Sciatic Nerve, Heart	Dravet Syndrome; Generalised Epilepsy with Febrile Seizures Plus, Type 2
NaV1.2	SCN2A	Cerebral Cortex, DRG, Sciatic Nerve, Heart, Hippocampus	Epileptic Encephalopathy; Seizures
NaV1.3	SCN3A	Cerebral Cortex, DRG, Sciatic Nerve, Heart	Unclear
NaV1.4	SCN4A	Heart	Hereditary sodium channelopathies
NaV1.5	SCN5A	Brain, Skeletal muscle, Trigeminal V ganglion, Heart	Long QT Syndrome; Brugada Syndrome; Progressive Familial Heart Block
NaV1.6	SCN8A	Brain, Spinal Cord, Sciatic Nerve, DRG, Heart	Mental Retardation; Cerebellar Ataxia; Epileptic Encephalopathy
NaV1.7	SCN9A	Brain, Spinal Cord, Sciatic Nerve, DRG, Sympathetic Neurons, Cerebellum, Brainstem, Hypothalamus	Indifference to Pain, Congenital; Paroxysmal Extreme Pain Disorder; Dravet Syndrome; Generalised Epilepsy with Febrile Seizures Plus

NaV1.8	SCN10A	Corpus Striatum, DRG Trigeminal V ganglion	Unclear
NaV1.9	SCN11A	Cerebral Cortex, DRG, Brain, Spinal Cord, Trigenimal V Ganglion, Small Intestine	Unclear

Table 2. List of Sodium Channels.

MATERIALS AND METHODS

1. Behavioural Experiments

All behavioural tests were done using wild-type C57BL/6 male mice aged 6-8 weeks. Thermal sensitivity was measured using a Hargreaves apparatus [35]. The animal's hindpaw was exposed to an intense light beam (90℃) and mean paw withdrawal latency was calculated over an average of at least three trials. Mechanical sensitivity was assessed using von Frey filaments (Stoelting, Wood Dale, USA), and the 50% paw withdrawal threshold was calculated using the up-down method described by Chaplan *et al* [36]. Mice were observed individually and all experiments were performed in a blinded manner.

2. Adult Mouse DRG Culture

Adult mice DRG neurons from all spinal levels were dissected from wild-type C57BL/6 male mice aged 6-8 weeks killed by CO2 asphyxiation and subsequently digested in an enzyme mixture containing Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS), 5mM HEPES, 10mM glucose, collagenase type XI (5mg/ml, Sigma-Aldrich) and dispase (10mg/ml, GIBCO) at 37℃ for 40 minutes. After incubation, the ganglia were washed and mechanically triturated using a 1-ml Gilson pipette. The ganglia were centrifuged and resuspended in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco),

1% Glutamax, 1% penicillin/streptomycin (Gibco) and 100 ng/ml nerve growth factor (NGF) (Sigma-Aldrich). For electrophysiological recording, dissociated DRG neurons were plated onto 35-mm plastic dishes (Nunc, Denmark) coated with poly-L-Lysine (0.01mg/ml) and laminin (0.02mg/ml). Cultures were kept at 37℃ in 5% CO_2. Electrophysiology recordings were made at allocated times (see Figure 2.1)

3. Electrophysiology

Whole-cell current clamp recordings were done at room temperature using Axopatch 200B and Multiclamp 700 amplifiers (Axon Instruments, Molecular Devices Inc.). Neurons whose cell bodies were not in contact with those of other neurons were selected for recording. Pipettes were pulled from borosilicate glass capillaries using a P-97 micropipette puller (Sutter Instruments Co.) and fire polished by a MF 200 microforge (world Precision Instrument) to have resistance of 2-4 megaohms when filled with the pipette solution. The pipette solution contained (in mM): 140 KCl, 0.5 EGTA, 3 MgATP, 2 NaGTP and 10 HEPES (pH adjusted to 7.3 using KOH; osmolarity set to 300 mOsm with glucose). The bath solution contained (in mM): 140 NaCl, 4 KCl, 2 CaCl, 1 MgCl, 10 HEPES and 5 Glucose (pH corrected to 7.4 using NaOH; osmolarity to 320mOsm with glucose).

4. Data Acquisition and Analysis

Data were acquired using a Digidata 1322A and 1440A digitiser with PClamp 8.1 & 10

software (Molecular Devices). Data were low-pass filtered at 2-5kHz and sampled at 10-12.5kHz. Capacitive transients were cancelled with the amplifier circuitry; however, series resistances were not compensated. Voltages were not corrected for liquid junction potentials. Data analysis was performed using ClampFit 9.0, 10 and 10.2 (Axon Instruments, Molecular Devices Inc.), Office Excel (Microsoft Corp.), SigmaPlot 8 (Systat Software Inc.) and Prism version 4 (Graphpad Software, San Diego, USA). Neurons were excluded from analysis if the seal resistance or access resistance was unstable.

5. Total RNA Extraction and Reverse Transcription PCR

Mouse DRGs were excised at different times of the day and homogenized in Trizol (Invitrogen). Total RNA was isolated with RNeasy Mini Kit (Qiagen) or easy-spin Total RNA Extraction Kit (iNtRON Biotechnology Inc.) in accordance with the manufacturer's instructions. Reverse transcription was performed with 1μg of RNA by using SuperScript First-Strand Synthesis System Kit (Invitrogen) according to the manufacturer's instructions.

6. Microarray

Total RNA was extracted from DRG of wild-type mouse (n=6, all males) in the morning (7am) and in the afternoon (3pm) with RNeasy Mini Kit. 5μg of total RNA was transcribed to cDNA with One-Cycle cDNA Synthesis Kit. Biotin-labeled cRNA

were prepared with IVT Labeling Kit, and fragmented with Sample Cleanup Module. 6 Chips were hybridized with fragmented biotin-labeled cRNA (20μg for one chip). The expression of mRNA was detected with Affimetrix microarray (Mouse Genome 430 2.0 Array). Differentially-expressed genes were identified by comparing the fold change of expression of each gene between two time points [37]. Hybridisation and data analysis were performed by Microarray Services, Wolfson Institute of Biomedical Research, University College London.

7. Quantitative RT-PCR

Mouse DRGs were collected at different time points of a day (7am, 11am, 3pm, 7pm, 11pm and the following day 3am), total RNA was isolated, and cDNA was reverse-transcribed from mRNA. The resulting DRG cDNA samples were amplified with gene-specific primers designed with the analysis software Primer 3. The sequences of the primers used are shown in Table 3. Serial dilutions of cDNA were used to generate standard curve of threshold cycles versus the logarithms of concentration for the moderately expressed housekeeping gene.

Real-time quantitative PCR was performed on an eppendorf Cycler (BIO-RAD, CA) using iQ™ SYBR® Green Supermix (Invitrogen) following suppliers' instructions. PCR conditions were: 95℃ for 3 min pre-denaturation, followed by 40 cycles of 95℃ for 10 s and 60℃ for 20s. Each reaction was performed in three replicates on 96-well plates. Data were analysed by Cycler Software (BIO-RAD) and Prism version 4

(Graphpad Software, San Diego, USA) and reported as calibrated ratios normalized to GAPDH or β-actin.

8. Standard PCR

Mouse DRGs were collected at different time points of a day, total RNA was isolated, and cDNA was reverse-transcribed from mRNA. DRG cDNA samples were examined individually using DreamTaq Green PCR MasterMix (2X) (Thermo Scientific) according to the manufacturer's instructions. The resulting DRG cDNA samples were amplified with gene-specific primers. The sequences of the primers used are shown in Table 4. The primers specific for mouse Per2 and GAPDH were designed with the analysis software version 3 (Graphpad Software, San Diego, USA). Other primers were selected from references: Scn8a (Nav1.6) and Scn9a (Nav1.7) [20]; TRPA1 and TRPV1 [38].

PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized under an UV transiluminator. Amplicon sizes were determined by comparison with a DNA molecular weight marker (Fermentas, SM0333) that was routinely run on the gels. Controls with water were used to demonstrate the absence of contaminating genomic DNA.

9. Statistical Analysis

All values of data were expressed as means ± standard error of the mean (SEM). Results were illustrated and analysed using Graphpad Prism version 4 (Graphpad Software, San Diego, USA) [39]. Multiple groups were compared using one-way analysis or two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* analysis. Differences were considered statistically significant at $P < 0.05$.

Gene	NM	F/R	Sequence (5'→3')	Size
Mouse HMGB1	NM_010439.3	F	TAAAAAGCCGAGAGGCAAAA	144bp
		R	GCAGACATGGTCTTCCACCT	
Mouse Piezo1	NM_001037298.1	F	CTACAAATTCGGGCTGGAG	242bp
		R	TCCAGCGCCATGGATAGT	
Mouse Piezo2	NM_001039485.4	F	GCATAACCCTGTGCCAGATT	148bp
		R	CCAAGTAGCCCATGCAAAAT	
Mouse Per2	NM_011066.3	F	GCCGGAGCAAGGGACAGAC	92bp
		R	GGACTTGGGGAGAAGTCCA	
Mouse Rev-erb	NM_011584.4	F	GACATGACGACCCTGGACTC	123bp
		R	GCTGCCATTGGAGCTGTCAC	
Mouse GAPDH	NM_008084.2	F	TGCGACTTCAACAGCAACTC	200bp
		R	CTTGCTCAGTGTCCTTGCTG	
Mouse b-actin	NM_007393	F	TTCTTTGCAGCTCCTTCGTT	149bp
		R	ATGGAGGGGAATACAGCCC	

Table 3. Primers for Quantitative RT-PCR.

Gene	NM	F/R	Sequence (5'→3')	Size
Mouse Per2	NM_011066.3	F	GTCTTGACCTCCATGAAACCTC	212bp
		R	AGGGAACACACTGAGAGGATGT	
Mouse TRPA1	NM_177781.4	F	ATTGTTCTCATGAACTTACTGATTGGCT	186bp
		R	CCTGGGTCTATTTGGATACACGAT	
Mouse TRPV1	NM_001001445.1	F	TTGGATTTTCCACAGCCGTAGT	155bp
		R	GAACTTGAACAGCTCCAGACATGT	
Mouse Scn8a	NM_011323.3	F	ACGCCACAATTCGAACATGTCC	400bp
		R	CCTGGCTGATCTTACAGACGCA	
Mouse Scn9a	NM_018852.2	F	ACGGATGAATTCAAAAATGTACTTGCAG	402bp
		R	GTTCTCGTTGATCTTGCAAACACA	
Mouse GAPDH	NM_008084.2	F	GTGAAGGTCGGTGTGAACGGA	400bp
		R	CCCATCACAAACATGGGGGCA	

Table 4. Primers for Standard PCR.

RESULTS

1. Circadian rhythm of sensitivity thresholds

In order to systematically examine the diurnal variation of pain threshold, we examined the behavior of the normal wild-type mice and how they respond to various type of stimulus across the full 24-hour cycle. We conducted experiments at different time points with 4-hours intervals: 7am, 11am, 3pm, 7pm, 11pm and the following day at 3am.

Among the best established methods for investigating nociception in mice is the Hargreaves's test. We applied noxious stimulus using high intense heat and recorded the time taken for the mouse to withdraw its hindpaws as a withdrawal threshold. With a high consistency in the pattern of responding between individuals, sensitivity was found to increase in the evening (Fig. 1a). The change exhibited a circadian rhythm with a 24-hour cycle, with a maximum peak at 7pm and minimum peak at 7am.

Furthermore, we also examined the response to mechanical stimulus using von Frey filament applied to the hindpaws. Here we also observed time having a significant and very consistent effect on the responsiveness of the mice to the stimulus (Fig. 1b). There was a change of sensitivity thresholds with a 24-hour cycle with minimum and maximum peak at 7am and 7pm, respectively.

Using objectifiable methods such as Hargreaves test and von Frey test, together with a systematic experimental planning, we were able to assess the temporal variation of sensory perception in wild-type mice. From both of our consistent data, which also correlate with previous reports, we can safely come to a conclusion that mice are most sensitive to sensory inputs in the morning (7am).

2. Electrophysiological studies of mouse DRG neurons

Although light was the only environmental variable during our behavioural experiment, the observed change involves various physiological aspects of mice including change in their cognitive functions. Understanding how stimulus is detected at sensory endings and delivered to the brain for an actual perception hence resulting in a particular physiological response is just as a great challenge as how the stimulus is differently sensed – or perceived in the brain – depending on the time of the day. In this study, we focused on studying a temporal variation of neuronal characteristics and their influences on the change of sensitivity.

Electrophysiology is a widely used technique to study the functional properties of ion channels by examining the ionic currents that passes through them. In this study, we performed a whole-cell current-clamp recording to examine DRG activities. For our study, we designed an experimental time schedule that would allow us to examine the neuronal activity at different times of a day. It was also important to follow this promptly to remove all possible unknown variables that may influence the result (Fig.

2). Injections of depolarising currents were applied to examine action potential (AP) firing. Protocols were manipulated depending on the answer we were seeking.

First, we applied an injection of a current ramp – a gradual continuous increase of current – from -100pA to +1500pA for 2 seconds and measured the current threshold at which the first action potential was detected (Fig. 3a and 3b). We did not observe any oscillating pattern between morning and afternoon, but a gradual decline of the current threshold from approximately 400pA in consecutive recordings (Fig. 3c). Similar pattern was observed when we set the holding voltage to -60mV (Fig. 3d).

Next, we applied an injection of a current step – a step increase of current – from -100pA to +1900pA for 150ms and again measured the current threshold at which the first action potential was detected (Fig. 4a/b). Similar to the previous data, there was also a gradual declining pattern overall instead of an oscillating pattern between morning and afternoon (Fig. 4c). In this case, however, the starting current threshold was around much higher when we set the holding voltage to -60mV than normal voltage (Fig. 4d).

From these two observations, we concluded that there is no variation in the current threshold required for AP firing in DRG neurons. We suspected that the decline trend was observed due to technical issues of sustaining DRG culture in its best condition.

We then examined the firing frequency of DRG neurons over time. We applied an

injection of three currents in step – 500pA, 1000pA, and 1500pA– for one second and then counted a total number of action potentials detected in each step (Fig. 5). To our surprise, we observed a massive increase in number of action potential in the afternoon compared to the morning (Fig. 6). This oscillation was observed at all current steps, and both at normal membrane potential and at -60mV holding voltage. Here we also observed the gradual decline overall; the explanation for this is exactly same as before.

Because only two time points were measure in the present study, it is not possible to say whether neuronal excitabilities were highest – or lowest – at those time points. Although testing was not conducted across the full 24-hour cycle, these results clearly show the temporal variation of neuronal characteristics. This change in sensory neurons implies a critical change in our ability to sense external stimulus.

3. Genome-wide expression analysis of mouse DRG neurons

The neuronal excitability is mainly determined due to the spatial and temporal expression of several voltage-gated ion channels. In order to understand overall changes of gene expression, and to identify candidate genes that may play a role as molecular sensor, thus contributing to this change of neuronal characteristics, we performed genome-wide gene expression screening using microarray. Total RNAs were isolated from adult mouse DRG (n=6, males) in the morning (7am) and in the afternoon (3pm).

The initial analysis after a standard filtering process identified 9 significantly different genes (Table 5). Unfortunately, many of the names were unknown and some that were known were found to be irrelevant to our study. We could not find any transcripts encoding channels, receptors, GPCRs, kinases and other transcripts that were previously implicated in pain pathways or mechanosensation.

Microarray technology has its limitations and variables and the approaches used in the analysis of data can greatly affect the set of genes that are identified. Although undoubtedly most commonly used, fold-change is one of many choice of analysis methods that are used to identify such genes.

After a further analysis of looking through the entire list, we identified a gene called high mobility group box 1 (HMGB1) which is a DNA-binding protein that is highly expressed in DRG. Previous studies have shown HMGB1 to be contributing to pain hypersensitivity, tactile hyperalgesia and mechanical allodynia [40-42].

4. Expression levels of HMGB1 and Piezo.

In order to find out whether HMGB1 is involved in the sensitivity change, we examined its expression level across the full 24-hour cycle. Although microarray did not identify any change, Piezo is a candidate for sensing noxious stimulus that affects the pain sensitivity; we examined both Piezo1 and Piezo2. For positive controls, Per2 and Rev-Erbα were chosen as they are known to be clock genes that osciallate in its

expression over time. β-actin was used as a housekeeping gene.

The cDNA samples of adult mouse DRG were made at different time points with 4-hours intervals: 7am, 11am, 3pm, 7pm, 11pm and the following day at 3am. Although there was a change between that was statistically significant, quantitative RT-PCR analysis showed no expression change of HMGB1 (Fig. 7). Piezo1 and Piezo2 also showed no temporal variation, whilst Per2 and Rev-Erbα showing oscillations as expected.

A question was raised in regards to the choice of house-keeping gene used for analysis. Although β-actin is widely used for its high expression in all tissue types, we could not find any reference that confirms the consistency in their expression over time. Instead of b-actin, we compared the data to GAPDH; here we observed a disappearance of a change we observed earlier (Fig. 8). Overall trend did not change greatly but this finding raises a necessity to choosing house-keeping gene carefully for experiments.

5. Expression levels of Nav and TRP channels.

The change of neuronal characteristics is mainly due to the change of expression of ion channels. Amongst many, we have selectively studied ion channels that were reported to be expressed and function in mouse DRG neurons and mediate the role in pain regulation or mechanosensation. We have examined Nav1.6, Nav1.7, TRPA1 and TRPV1. Here again, Per2 and Rev-Erbα were used as positive controls.

In order to study the change of various channel expressions over time, standard polymerase chain reaction (PCR) was performed on cDNA extracted from mouse DRG at different time points (7am, 11am, 3pm, 7pm, 11pm and 3am) using sequence-specific primers (see Table 4). The expression of Per2 oscillated in its expression as expected (Fig. 9 and 10). In contrast, TRPA1, Nav1.7 and GAPDH did not change in its expression level. TRPV1 declined in its expression at 3pm. Nav1.6 increased in its expression over time. Nav1.7 remained fairly constant and so does GAPDH. Nav1.6 in particular correlate with our previous data showing increased AP frequency in the afternoon (Fig. 5 and 6). However, we also observed different expression pattern in the case of TRPV1.

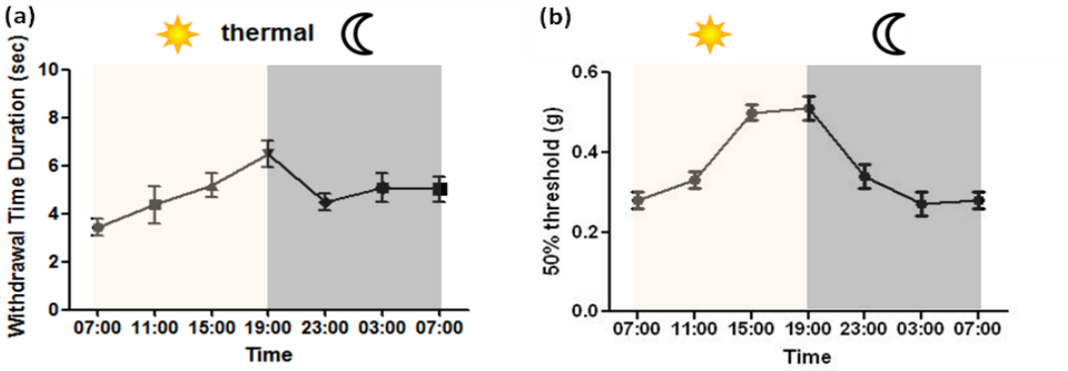

Figure 1. Behavioural responses of wild-type mice. Responses were examined at 7am, 11am, 3pm, 7pm, 11pm and 3am and 7am in the following day. **(a)** Thermal responses over time. Results were averaged (n = 16). (**b)** Mechanical responses over time. Results were averaged (n = 10).

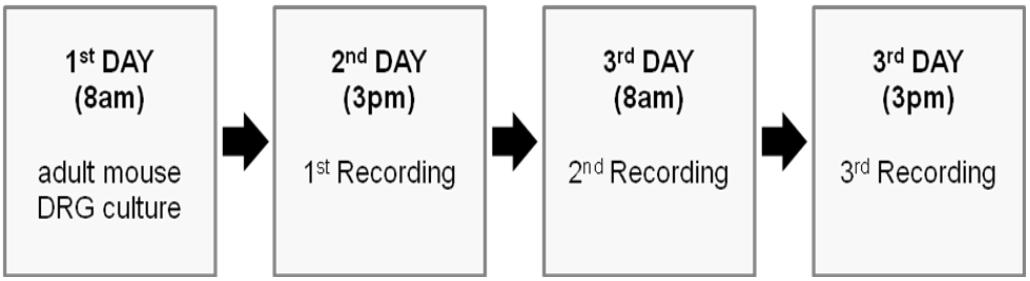

Figure 2. Experimental design for electrophysiological recordings. Adult mouse DRGs were cultured at exact same time in the morning and were incubated until allocated recording times. The first recording was made during the afternoon session on the second day from the DRG culture which gives more than 24-hours of incubation from the point of culture which is required for electrophysiological recording. The second recording was made during the morning session on the third day from the DRG culture. And the third, and the final, recordings were made during the afternoon session on the second day from the DRG culture.

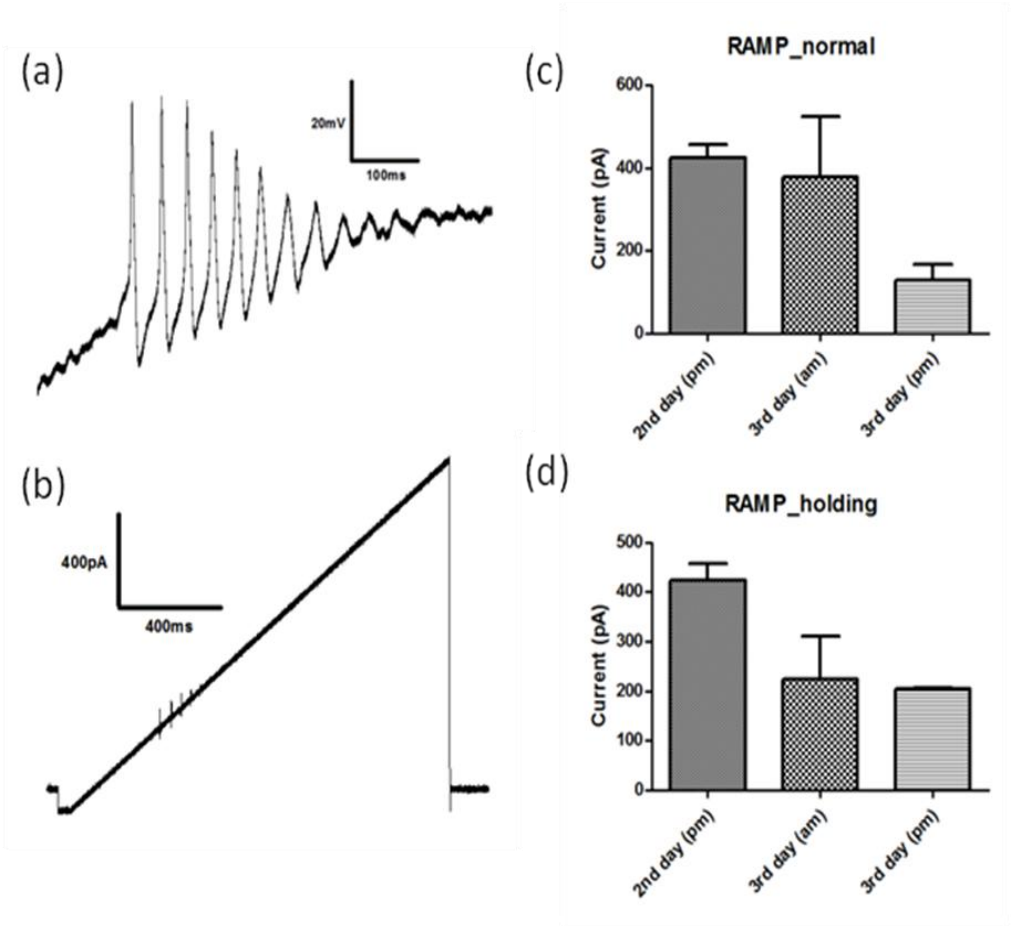

Figure 3. Current ramp recording. (a) Representative current trace of action potential (AP) firing. (b) Representative graph of an injection of a current ramp from -100pA to 1500pA for 2 seconds. (c) The quantitative representation of current thresholds (at normal membrane voltage). (d) The quantitative representation of current thresholds (at -60mV holding voltage). Currents were averaged for (c-d) (n = 15).

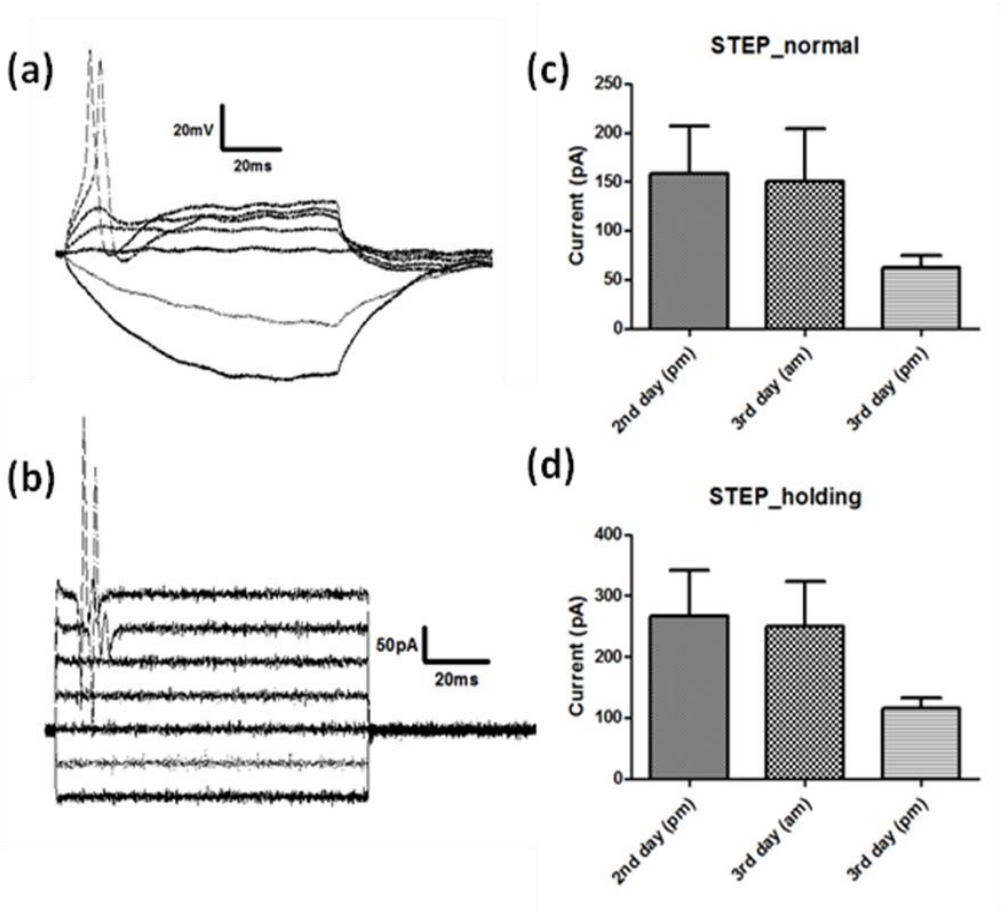

Figure 4. Current step recording. (a) Representative current trace of AP firing. (**b)** Representative graph of an injection of a current step from -100pA to 1900pA for 150 milliseconds. (c) The quantitative representation of current thresholds (at normal membrane voltage). (d) The quantitative representation of current thresholds (at -60mV holding voltage). Currents were averaged (n = 15).

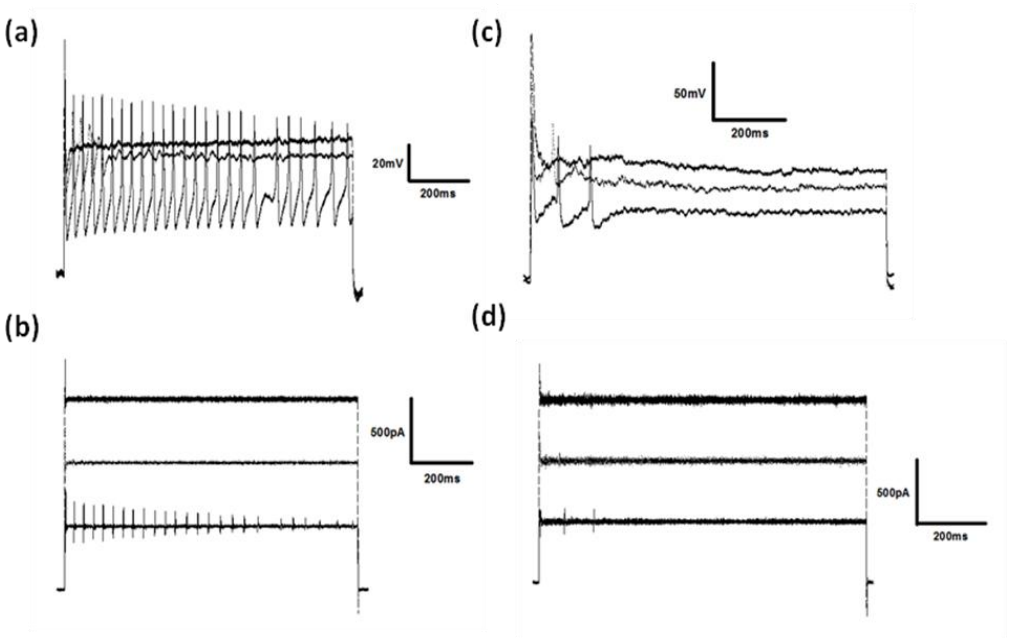

Figure 5. Example traces of firing frequency recording. Representative current trace of AP firing (a) in the afternoon (PM), and (c) in the morning (AM). Representative graph of an injection of a current step (b) in the afternoon (PM), and (d) in the morning (AM).

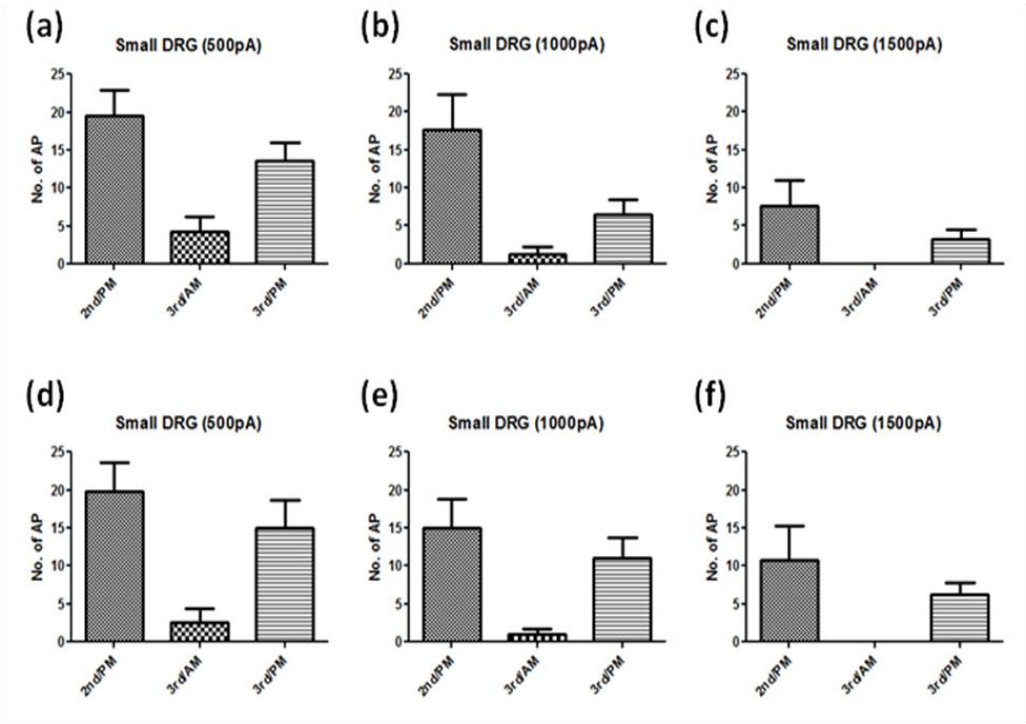

Figure 6. Firing frequencies of mouse DRG neurons. The quantitative presentation of firing frequency upon different currents (a-c) at a normal membrane potential and (d-f) at a -60mV holding voltage. Currents were averaged (n = 15).

Gene name	Function
Dbp /// Sphk2	D site of albumin promoter (albumin D-box) binding protein /// sphingosine kinase 2
Nr1d1 /// Thra	nuclear receptor subfamily 1, group D, member 1 /// thyroid hormone receptor, alpha
1700001F09Rik /// Gm10375 /// Gm3543 /// Gm3676	Unknown
Srsy	Unknown
Gm129	C1orf51 (chromosome 1 open reading frame 51)
10608382	Unknown
LOC100504240 /// LOC100504530 /// LOC100502692 /// LOC100504515	Unknown
LOC100504530	Unknown
LOC100504530 /// LOC100502692 /// LOC100504515	Unknown

Table 5. Outcome of Microarray Analysis. Microarray analysis of mouse DRG neuron identified nine transcripts that were found to be significant.

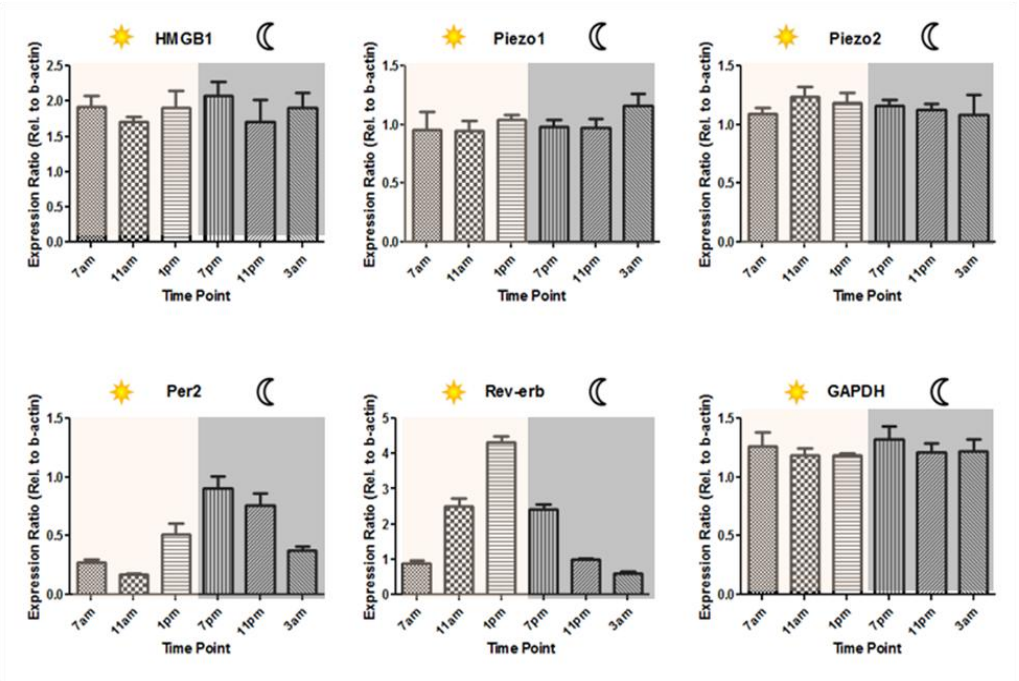

Figure 7. Quantitative RT-PCR analysis I. The variation of mRNA levels of HMGB1, Piezo1, Piezo2, Per2, Rev-erb, and GAPDH were measured from wild-type mouse DRG neurons. Times were 7am, 11am, 3pm, 7pm, 11pm and the following day at 3am. Data were normalised with β-actin. Each bar represents n=4 mice.

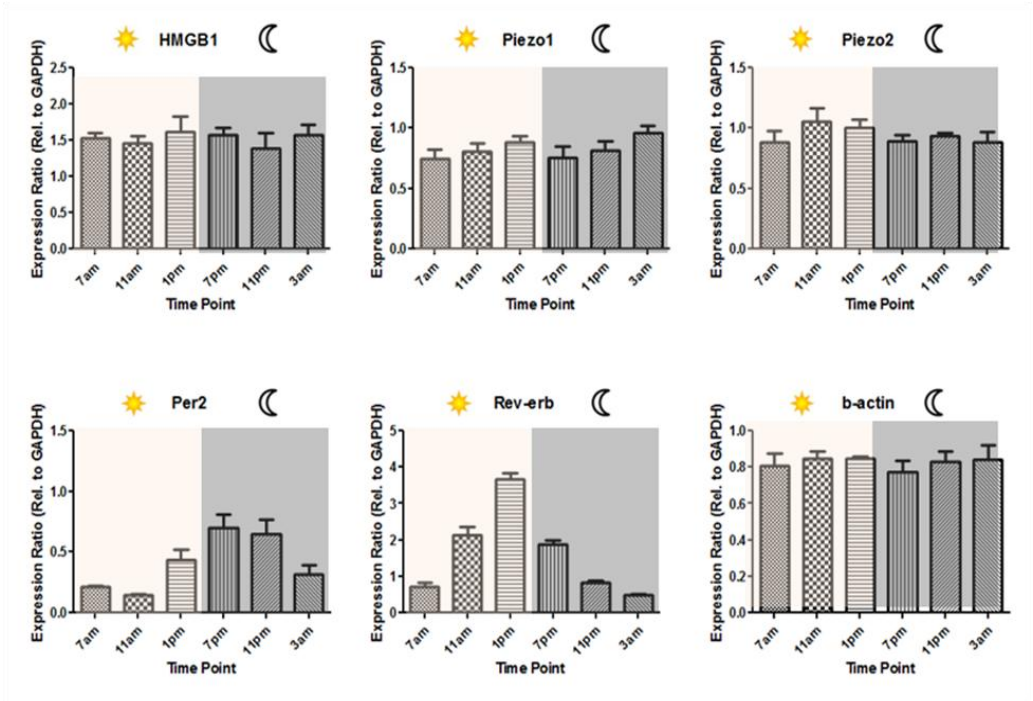

Figure 8. Quantitative RT-PCR analysis II. The variation of mRNA levels of HMGB1, Piezo1, Piezo2, Per2, Rev-erb, and β-actin were measured from wild-type mouse DRG neurons. Times were 7am, 11am, 3pm, 7pm, 11pm and the following day at 3am. Data were normalised with GAPDH. Each bar represents n=4 mice.

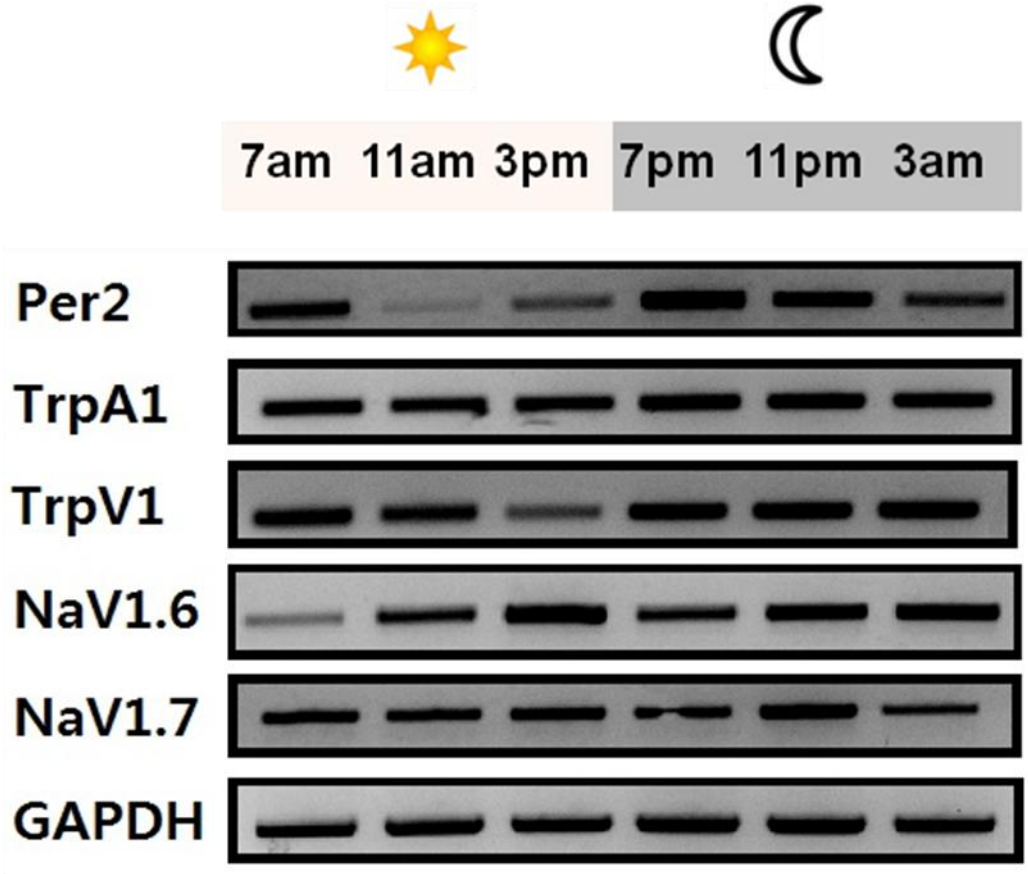

Figure 9. Gel image of PCR analysis. The variation of mRNA levels of Per2, TRPA1, TRPV1, Nav1.6, Nav1.7 and GAPDH were measured from wild-type mouse DRG neurons. Times were 7am, 11am, 3pm, 7pm, 11pm and the following day at 3am.

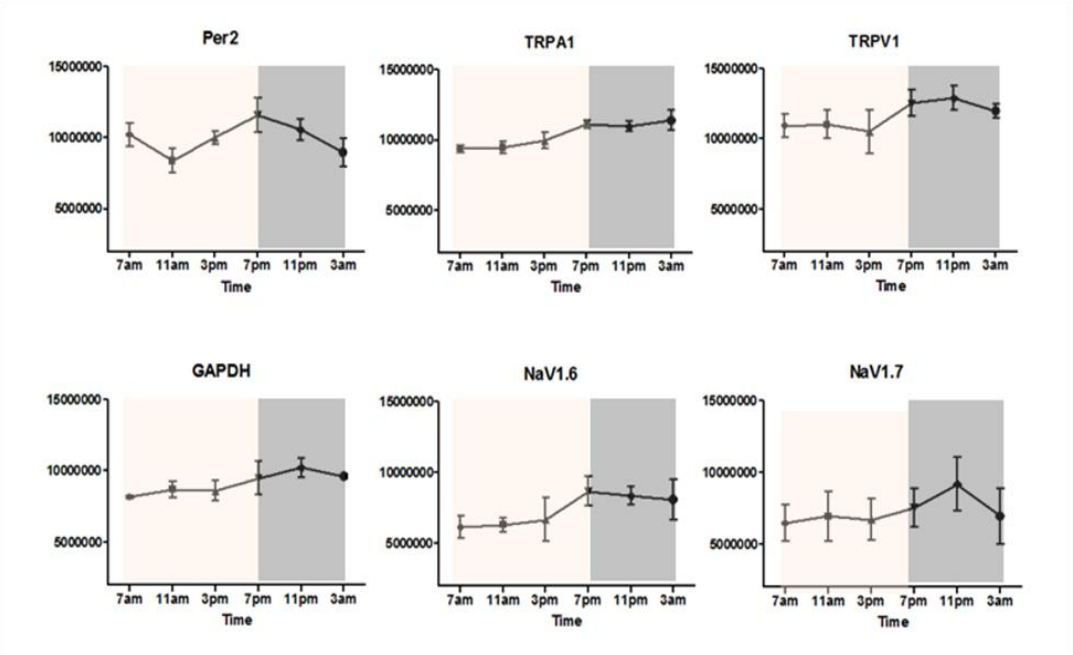

Figure 10. Quantitative representation of PCR analysis. The variation of mRNA levels of Per2, TRPA1, TRPV1, Nav1.6, Nav1.7 and GAPDH were measured from wild-type mouse DRG neurons. Times were 7am, 11am, 3pm, 7pm, 11pm and the following day at 3am. Data were averaged. Each bar represents n=4 mice.

DISCUSSION

In this study, we showed a clear diurnal variation of pain. This pattern of behaviour can be correlated with the degree of consciousness (the sleep/awake state). With the animals being awake during the dark period and sleeping mostly during the light period, the process from perception of both noxious and mechanical stimulation to an actual withdrawal motor response was quickest in the morning – approximately 7am – but relatively lower in the evening. Our data also correlates with previous study by Jhaveri *et al*, who examined time course of sleep patterns, locomotor activity and core body temperatures of C57BL/6J mice [43].

Although it is beyond our current study to examine the central mechanism of such clock-controlled activity, it is important to assess if this diurnal variation of pain sensitivity is regulated by the master circadian pacemaker in the hypothalamus. As a part of further study, we are currently in a process to examine circadian clock gene (bmal1) mutant mouse generated using advillin-cre mice.

Our data also suggest endogenous temporal control of somatosensory transduction, responsive to different types of inputs, including noxious and mechanical stimulus. Cultured DRG neurons exhibited a temporal change of neuronal excitability with a dramatic increase of firing frequency in the afternoon. This was an interesting data since our behavioural data showed a decrease of sensitivity from 8am to 3pm. At the present, we do not have a clear answer that explains the direct relationship between

these two observations; further studies are necessary to comprehend the link between *in vivo* and *in vitro* studies.

Through screening a number of voltage-gated ion channels that are known to be expressed in mouse DRG neurons and to function as key molecular sensors of heat nociception or touch, we observed temporal variations in expressions of TRPV1 and Nav1.6. Nav1.6 exhibits a rapid activation and a fast inactivation with a recovery rate from inactivation approximately five times faster than Nav1.7 [44]; this suggests that neurons expressing Nav1.6 channels should exhibit higher firing frequencies than neurons expressing Nav1.7 channels. This argument supports our data that show a clear increased expression of Nav1.6 from 7am to 3pm and also explains the increased firing frequency from our *in vitro* study. TRPV1 is known to respond to hot temperatures above 43°C; its decreased expression at 3pm fits with the decreased sensitivity in mice, although it quickly returns to its high level of expression by 7pm whereas the decreased sensitivity continues further *in vivo*. Perhaps there is a time lapse between gene expression and physiological behaviours for rhythmic control.

A recent study by Lee and Montell showed TRPA1 in flies contributed to temperature control of circadian rhythm [45]. However, our study has shown that TRPA1 did not change in its expression level. One of the plausible explanations may be that the function of TRPA1 in LPNs differs to its function in sensory neurons. Furthermore, TRPA1 plays a different role in flies from mouse and other mammalian system.

Firing frequency is regulated by altering sodium channel expression patterns but also by modulating the activity of specific VGSCs. There are chemical modulators that are known to be responsible for the activation and sensitisation of nociceptors. Previous studies reported that the inactivation kinetics of Nav1.6 was modulated by calmodulin (CaM) and TRPV1 was modulated by Protein kinase C (PKC) [45, 46]. Mechanotransduction of cultured sensory neurons was also modulated by PKC and NGF [47]. These modulators may exhibit an endogenous rhythmic cycle of expression that influence functions of these ion channels thus resulting in the temporal variation of neuronal excitability.

It is highly possible that other channels, including Nav1.8 and Nav1.9, may also change in their expressions over time. We speculate that different spatial and temporal expressions channels collectively work to result in different functional outcomes of sensory transduction. Further studies are also required to confirm the actual involvement of channels to the diurnal variation of pain, *in vivo*.

In regards to our unanswered question over the link between our *in vivo* and *in vitro* studies, we also came to question ourselves why the neuronal excitability increase from 8am to 3pm during which mice fall a deep sleep. In attempt to understand this in an evolutionary perspective, the ability to detect the surrounding physical environment is crucial for living organism's fate of survival. In the case of mammals, all five senses are fully functional whilst awake; the body is able to sense without much difficulty. During a sleep, however, most of the senses are partially dysfunctional as their

functions are highly dependent on cognitive abilities. It makes sense, therefore, if the body has evolved to have an innate mechanism that can compensate this problem by giving alternative routes. In this study, we hypothesise that this compensation may be achieved by enhancing the neuronal excitability.

What remains uncertain, however, is exactly how different sensory modalities contribute to specific pain qualities *in vivo*. Furthermore, exactly how the variety of sensory information is melded into perception remains the Holy Grail of neuroscience. Much work lies ahead to discover answers to these questions, which is the exciting part of scientific research.

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국 문 초 록

일주기 생체 리듬은 행동적, 심리적, 또는 생리적인 것을 포함한 다양한 생명현상들과 관련된 것으로 잘 알려져 있다. 이러한 리듬은 우리 뇌에 존재하고 있는 master pacemaker에 의해 조절된다고 알려져 있다. 최근에 일중변동(circadian variation)이나 주간변동(diurnal variation)이 통증과 관련된 다양한 질병들과의 관련성이 제기되고 있으나 존재여부에 대한 논쟁이 여전히 많다. 본 연구는 이러한 통증인식과 주간변동간의 관련성 여부를 행동분석, 전기생리분석, 유전자분석을 통해 알아보고자 하였다. 마우스의 행동분석(behavioural analysis)을 통해 통증과 기계적 감각의 일주기 생체 리듬을 확인할 수 있었다. 배양된 척수후근절(dorsal root ganglion; DRG)에서 활동전위의 빈도가 오후에 증가하는 것을 확인함으로서 신경단위세포의 흥분도의 시간적 변화를 확인하였고 이것은 곧 체감각변환의 주기적 내재적 조절이 있음을 시사한다. DRG에 집중된 이온채널 유전자들을 분석해보았을 때 감각신경의 활동전위의 빈도를 조절할 수 있는 TRPV1과 Nav1.6채널의 시간적 변화에 따른 발현차이를 확인할 수 있었다. 이러한 발견은 통증인식의 일중변동에서의 말초감각신경의 중요한 역할을 시사하며 이것은 일주기 시계에 의해 조절되는 내재적 경로에 의한 것임을 알 수 있었다. 따라서 통증치료에 있어 시간요법적인 접근의 가능성을 시사한다.

주 요 어 : 일주기 생체 리듬, 통증, Transient Receptor Potential (TRP), NaV,

학 번 : 2011 – 23063

감사의 글

대학교 학부과정 그리고 석사과정을 통해 자연과학이라는 학문을 마음껏 배울 수 있었던 것은 제 인생에 가장 큰 행복이었습니다. 책과 논문을 통해 배우는 과정부터 직접 실험을 하고 모은 결과들을 정리하며 학위논문으로 적기까지. 이 모든 다양한 과정들을 통해 그 무엇보다 제 자신을 알아가는 가장 중요한 계기가 되었습니다. 과학을 배우면서 형성된 논리에 근거하는 사고방식은 제가 앞으로 살아가며 마주할 수많은 불확실한 상황 속에 변하지 않을 굳건한 반석이 될 것입니다.

우선 좋은 연구환경에서 마음껏 배우며 실험을 진행할 수 있도록 물심양면으로 지원해주시고 끊임없이 지도해주신 오우택 교수님께 진심으로 감사 드립니다. 처음 연구주제를 확실히 정하지 못하고 갈팡지팡할 때에 또 실험후반에 공황에 빠지는 듯 했을 때에도 교수님의 이끌어주심으로 끝까지 포기하지 않고 연구를 마감할 수 있었습니다. 너무나도 훌륭하신 교수님을 제 지도교수님으로 맞이한 것은 축복이었습니다. 진심으로 감사드리며 앞으로 제가 살아가는데 교수님께 배웠던 시간들 항상 기억하며 살겠습니다.

학자라는 끊임없는 길을 묵묵히 걸어가시는 박사님들. 정주영 박사님, 조하원 박사님, 양영덕 박사님, 양동진 박사님, 장용우 박사님 그리고 이성훈 박사님. 정말 진심으로 존경합니다. 박사님들을 보면 나이를 들어가는 것과 성숙해가는 것은 별개라는 것을 알겠습니다. 박사님들의 높이와 인품을 따라가기엔 저는 터무니 없이 부족하지만 더 많은 배움과 경험을 바탕으로 저

도 숙성이 잘된 사람이 되기를 바랍니다.

배움과 나눔의 귀한 시간을 함께하고 성장함의 소중한 기회를 허락해준 연구실 오빠언니들 그리고 동생들. 제선오빠. 병준오빠, 혜연언니, 형섭, 주영오빠, 단비언니, 희량언니, 규상오빠, 정은언니, 민영, 윤선언니, 지미, 정원, 소영, 주희 그리고 현석오빠까지. 모두들 감사합니다. 앞으로도 꾸준히 나누고 공유하길 바라며 여러분의 앞날이 축복과 행복으로 풍성하시길 진심으로 기원합니다.

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