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Master's Thesis

探討生理時鐘中區精胺酸血管加壓素的神經連結迴路

Mapping the functional circuitry of AVP neurons in the central clock

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Mapping the functional circuitry of AVP neurons in the central clock

本論文係 梁啟雯 (姓名) r10b21019 (學號) 在國立臺灣大學生命科學系完成之碩(博)士學位論文，於民國 113 年 2 月 6 日承下列考試委員審查通過及口試及格，特此證明。

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摘要

生理時鐘調控我們的行為週期和身體的機制，例如代謝作用和內分泌系統的調節。我們的日常作息，代謝作用以及睡眠週期都是按照 24 小時為一周期。腦部的下視丘的上視神經交叉核 （suprachiasmatic nuclei, SCN）是哺乳動物的生理時鐘的主要調控中心。為了與外界的光暗週期同步，光的訊息會通過視網膜上的內發性感光視網膜神經節細胞（intrinsically photosensitive retinal ganglion cells, ipRGCs）傳遞到上視神經交叉核。在收到光的刺激後，ipRGCs 會釋放麩氨酸（glutamate）到上視神經交叉核以傳遞訊息。ipRGCs 和上視神經交叉核的連接也透過追踪特定基因表現的研究中得以證實。上視神經交叉核的構造可分為腹外側（ventral lateral）和背外側（dorsal medial），並由多種神經細胞所構成。在眾多神經細胞中，對於生理時鐘的形成有著重要影響的是表現血管活性腸肽（vascular intestinal peptide, VIP）和精胺酸血管加壓素（arginine vasopressin peptide, AVP）。腹外側主要由表現 VIP 的神經細胞組成，而背外側由表現 AVP 的神經細胞所分佈。在過去的研究中，視神經交叉核的神經同步以及訊息的整合是由於表現 VIP 的神經細胞接受 ipRGCs 所傳遞的光訊號。之後生理時鐘的訊息會由表現 AVP 的神經傳到其他腦區。然而，單一 ipRGCs 追踪法讓我們發現 ipRGCs 並非如一般認知僅限傳遞到表現 VIP 的神經細胞，實際上 ipRGCs 會分佈到整個上視神經交叉核。現如今的研究中，上視神經交叉核的神經細胞之間如何運作產生生理時鐘仍然處於探討的階段。於是我們使用鈣離子影像記錄方式，發現上視神經交叉核中表現 AVP 的神經細胞在河魨毒素（tetrodotoxin, TTX）下（動作電位在其他神經中受阻截）依然會受到麩氨酸，N-甲基-D-天門冬胺酸受體（N-methyl-D-aspartate receptor, NMDA）和代谢型谷氨酸（metabotropic glutamate, mGlu）的促效劑所活化。因此我們推測 ipRGCs 的訊息會被上視神經交叉核中表現 AVP 的神經細胞所接受。為了證實這項猜想，我們把表現 AVP 的神經細胞攜帶感知鈣離子濃度的熒光蛋白---GCaMP7f，並利用光調控的方式激發 ipRGCs，並觀察神經細胞的變化。實驗結果顯示 AVP 的神經細胞會接受來自 ipRGC 的訊息並表現出多種鈣離子濃度變

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化的反應，因此 AVP 的神經細胞可能會透過 ipRGC 受到外界的刺激進而影響生理時鐘。

關鍵字：生理時鐘；上視神經交叉核；精胺酸血管加壓素；內發性感光視網膜神經節細胞；感知鈣離子濃度的熒光蛋白

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Abstract

Circadian rhythms modulate our daily activity patterns and other body mechanisms, such as metabolic and neuroendocrine rhythms. It controls our daily behavior, metabolism, and sleep-wake cycle for a period of about 24 hours. Suprachiasmatic nucleus (SCN) in the hypothalamus is known as the central clock that controls circadian rhythms in mammals. The external light signal could entrain SCN through intrinsically photosensitive retinal ganglion cells (ipRGCs) by releasing glutamate. The ipRGC is a type of neurons in the retinal of mammalian eyes. The innervation of ipRGCs to SCN has been revealed by using genetic tracing techniques and contributes to the synchronization of the circadian rhythm. SCN is a heterogenous structure that contains different neurons, while the networking of circadian rhythm is prevalently related to AVP and VIP neurons. The shell region of SCN mainly consists of AVP neurons, while VIP neurons are major in the core region. In previous studies, VIP neurons are suggested as light signal-receiving neurons from ipRGCs, and AVP neurons are the primary output neurons for the SCN. However, in single ipRGC tracing study suggested that ipRGCs innervation is not limited to VIP neurons specifically but throughout the whole SCN. In addition, the mechanisms of communication within SCN neurons are still unrevealed in recent studies. By using in vitro calcium imaging, here we showed that glutamate, NMDA, and metabotropic glutamate agonists activated AVP neurons in SCN under the application of TTX to block multi-synaptic activation. To know whether ipRGC and AVP neurons in SCN are a monosynaptic pathway, we use a calcium sensor, GCaMP7f, to image the neural activity in SCN and stimulated by optogenetic simultaneously. The results show that

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AVP neurons receive signals from ipRGCs and exhibit different responses. Our results suggested that AVP neurons may also receive direct ipRGC input from the retina for circadian clock regulation.

Keyword: circadian rhythms; suprachiasmatic nucleus; arginine vasopressin peptide (AVP); intrinsically photosensitive retinal ganglion cells (ipRGCs); GCaMP7f

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Chapter I

Introduction

1.1 Intrinsically photosensitive retinal ganglion cell (ipRGC)

Visual information is received by retinal ganglion cells (RGCs) through photoreceptors, rods, and cones. The RGCs have the characteristic of long axons that extend to the brain region and form the optic chiasm and optic tract. In 1927, the light stimulus caused pupil constriction in mice that are coneless and rodless, suggesting the existence of a third type of photoreceptor (Keeler et al., 1927). Then, a group of retinal cells with melanopsin, a blue light–sensitive photopigment, was discovered to project from the retinal to the suprachiasmatic nuclei of the hypothalamus (Provencio et al., 2000). These retinal ganglion cells were activated by light even without synaptic input from rods and cones, and are now known as intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson et al., 2002). IpRGCs possess intrinsic phototransduction capabilities and transmit light information to non-image-forming brain regions. This non-image-forming system operates in parallel with an image-forming visual system that is derived from rods and cones (Berson et al., 2002, Hattar et al., 2002). Through the expression of photopigment, opsin-like protein, melanopsin, ipRGCs receive light signals and innervate to the central clock in our brain region, which influences circadian rhythm, sleep-wake cycle, and learning function (Gooley et al., 2001, H Wässle, 2004, S Hattar et al., 2003, Rachel Jones, 2005). The photic properties and projection of ipRGCs underscore their role in synchronizing the circadian clock with environmental time.

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1.1.1 The properties of ipRGCs

In the retina, intrinsically photosensitive retinal ganglion cells (ipRGCs) contain the light-sensitive photopigment melanopsin. These cells send light signals to the brain through the retinohypothalamic tract (RHT) (Gooley et al., 2001, Provencio I., et al., 2000). Earlier studies have demonstrated the selective expression of melanopsin RNA in the retinal ganglion cells of the retinohypothalamic tract (RHT), as confirmed through retrograde labeling and in situ hybridization techniques (Gooley et al., 2001). In 1998, melanopsin was in the photosensitive melanophores of Xenopus skin by Provencio and his colleagues. The frog responds to light due to these melanophores and reorganizes pigment granules within cells to change its coloration. By in situ hybridization, the melanopsin-encoded mRNA is expressed in other photosensitive tissues, for instance, the retina and iris (Provencio et al., 1998). Immunohistochemical approaches have further identified the expression of melanopsin protein in retinal ganglion cells that contain pituitary adenylate cyclase-activating peptide (PACAP), a known marker and neurotransmitter of the retinohypothalamic tract (RHT) (Hannibal et al., 2002). The RHT, which innervates the circadian pacemaker, the suprachiasmatic nucleus (SCN), underscores the significance of melanopsin in entraining our brain to the environmental light-dark cycle (Moore RY et al., 2001, Moore RY et al., 1995). To investigate the roles of melanopsin-containing ipRGCs in photoentrainment, researchers disabled ipRGCs, rods, and cones in triple-knockout mice. These mice exhibited a loss of pupillary light response and failed to entrain their circadian rhythms. Contrarily, coneless and rodless mice still respond to light by phase-shifting while the elimination of eyes has

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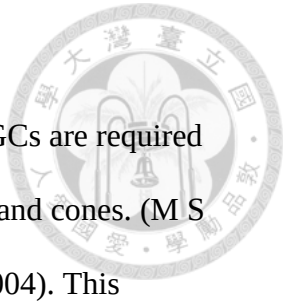

abolished their circadian rhythm. Thus, melanopsin-expressing ipRGCs are required for photoentrainment to mediate the circadian clock, alongside rods and cones. (M S Freedman et al., 1999, Hattar S et al., 2003, R. Lane Brown et al., 2004). This challenges the belief that rods and cones are the sole photosensitive photoreceptors, highlighting the critical role of melanopsin-ipRGCs in mediating the circadian clock. Recent studies have identified at least five distinct ipRGCs subtypes (M1, M2, M3, M4, and M5) with varying physiological properties (TM Schmidt et al., 2011). Among those subtypes, M1 ipRGCs innervate dominantly to non-image-forming centers in the brain region, including the SCN, while M2 ipRGCs exhibit less input (AD Güler, et al, 2008). In terms of membrane resistance, spike frequencies, firing patterns of action potentials, spike threshold, and other characteristics, M1 ipRGCs are notably distinct from other ipRGCs. It is likely to reflect their specialized functions in non-image-forming vision (Caiping Hu et al., 2013). M1 ipRGCs demonstrate a notable projection to non-image-forming visual nuclei such as the SCN, underscoring their pivotal role in circadian regulation. (Berson DM et al., 2002, S Hattar et al., 2006).

1.1.2 The projection of ipRGCs to the brain region

RGCs (retinal ganglion cells) play significant roles in visual information regarding cones and rods. Among retinal ganglion cells (RGCs), intrinsically photosensitive retinal ganglion cells (ipRGCs) constitute a distinct class that responds to light independently of rod and cone input. The crucial component in ipRGCs is melanopsin protein, encoded by the Opn4 gene, which are highly expressed in M1 ipRGCs. This discovery prompted researchers to develop immunohistochemical and genetic tools

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targeting melanopsin protein to trace the distribution of ipRGCs. M1 ipRGCs, as revealed by subsequent studies, project to various brain regions, including the suprachiasmatic nucleus (SCN), intergeniculate leaflet (IGL), ventral lateral geniculate nucleus (vLGN), the pupillary light reflex (OPN), perihabenular nucleus (pHb), ventrolateral preoptic area (VLPO), lateral hypothalamic area (LH), peri-supraoptic nucleus (pSON), IGL, anterior hypothalamus (AH), and subparaventricular zone (SPZ) (Marcos L et al., 2020).

Previous studies indicate that M1 ipRGCs significantly contribute to the input received by the SCN, IGL, and habenular region (S Hatter et al., 2006). The synchronization of the circadian rhythm in the SCN is mediated by photic input from ipRGCs through the retinohypothalamic tract (R Y Moore et al., 1972). This retinal light input serves to entrain the SCN, driving circadian rhythms in mammals (Abrahamson EE and Moore RY, 2001). Despite the dense retinal innervation pattern observed in the SCN, challenges persist in distinguishing the specific cell types within the SCN that receive ipRGC input (M Lokshin et al., 2015). As a result, clarity is yet to be achieved regarding the identity of SCN neurons influenced by ipRGCs.

1.2 Suprachiasmatic nucleus

The suprachiasmatic nucleus (SCN), located on both sides of the third ventricle just above the optic chiasm, serves as a multi-oscillatory network and functions as the central pacemaker situated in the anteroventral hypothalamus. In mammals, the SCN is synchronized to the light-dark cycle through photic information received from intrinsically photosensitive retinal ganglion cells (ipRGCs). When the light/dark cycle

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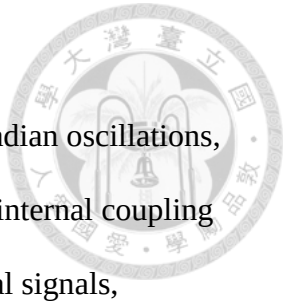

is eliminated, the SCN exhibits the expression of self-sustained circadian oscillations, resulting in a circadian rhythm lasting approximately 24 hours. The internal coupling within the SCN translates photic information into neural and humoral signals, subsequently transmitting them to other brain regions to regulate the rhythmicity of behavior and physiology (Lowrey PL et al., 2004, DK Welsh et al., 2010). The SCN, consisting of approximately 20,000 neurons, is anatomically divided into two regions: the core and shell. The ventral core region serves as the recipient of retinal input, while the dorsal shell region receives input from the core and outputs signals from the SCN (Abrahamson et al., 2001). Despite extensive study, the specific region within the SCN that receives photic input from ipRGCs remains a subject of ongoing research, contributing to the continual exploration of the intricacies of circadian regulation.

1.2.1 Population Neurons of SCN

The suprachiasmatic nucleus (SCN) is a heterogeneous structure composed of different neurons, categorized into core and shell subdivisions. The core region, situated in the ventral area, encompasses neurons expressing vasoactive intestinal polypeptide (VIP), calretinin, neurotensin (NT), and gastrin-releasing peptide (GRP). In contrast, the shell region, located dorsally, consists mainly of arginine vasopressin (AVP) neurons, alongside angiotensin II and met-enkephalin (Abrahamson et al., 2001). Most of the SCN neurons will release gamma amino butyric acid (GABA) and produce other neurons as well. For instance, some SCN neurons will release GABA and AVP. (Vallath Reghunandanan and Rajalaxmy Reghunandanan, 2006). It is widely acknowledged that VIP neurons in the core region receive light input, fostering

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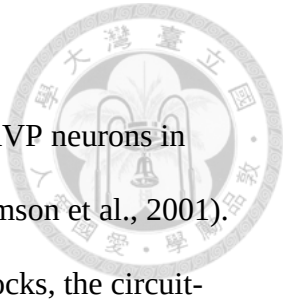

synchronization and networking among SCN neurons. Ultimately, AVP neurons in the shell region serve as the principal output from the SCN (Abrahamson et al., 2001). While individual SCN neurons possess intrinsic cell-autonomous clocks, the circuit-based mechanism orchestrates an effective oscillator, fostering sustained synchronization among individual cellular clocks.

1.2.2 The coupling of SCN neurons

Each neuron within the suprachiasmatic nucleus (SCN) possesses intrinsic cell-autonomous clocks, essentially making them individual oscillators. The unique networking property of SCN neurons enables the coupling of these oscillators, leading to synchronization and the generation of a coherent circadian oscillation. In essence, SCN neurons collectively function as a circuit-based mechanism. Arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP), two key neuron types in the SCN, play pivotal roles in its function, exerting reciprocal effects on synchrony. AVP neurons, predominantly located in the shell regions, contribute to synchrony but with weaker effects. On the other hand, VIP neurons, primarily situated in the core regions that receive retinal input, perform a more significant role in mediating synchrony within the SCN. This reciprocal interaction between AVP and VIP neurons establishes a sustained and precise oscillatory network within the SCN. (Vallath Reghunandanan and Rajalaxmy Reghunandanan, 2006, Erik D. Herzog et al., 2017)

VIP neurons, predominantly distributed in the core regions of the SCN, have emerged as the most potent contributors to its functioning. Recent studies strongly support the pivotal role of VIP neurons in SCN coupling. Notably, the rhythmic release of VIP from the core region, acting on VPAC2 receptors distributed throughout the SCN, has

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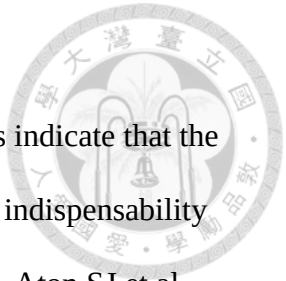

been identified. Additionally, experiments with mutant SCN cultures indicate that the application of a VIP agonist can rescue synchrony, underscoring the indispensability of VIP release for SCN neuron synchronization (Kallo I, et al., 2004, Aton SJ et al., 2005). A compelling hypothesis suggests that VIP neurons play a pivotal role in resetting the SCN's clock circuit in response to environmental light cues. The activation of VIP neurons by the retinohypothalamic tract (RHT) in the presence of light increases the release of VIP, resulting in a weakening of SCN synchrony. Intriguingly, at lower concentrations, VIP fosters the coupling of clock cells (Herzog et al., 2017). This hypothesis underscores the critical role of VIP neurons in responding to environmental light cues. Despite these insights, the intricate details governing the regulation of VIP release in the SCN to mediate the circadian clock remain to be fully resolved.

While VIP neurons have been extensively studied for their role in the SCN, it is essential to recognize the significance of AVP neurons in this circadian regulation network. Initially considered primarily as output neurons with limited impact on SCN, recent studies challenge this notion, shedding light on the crucial role of AVP neurons (Jin X et al., 1999). Contrary to previous beliefs, AVP neurons have emerged as key players in circadian rhythm alignment. Studies involving shifts in the light-dark cycle have demonstrated that a loss of AVP receptors leads to a shortened entrainment period in mice. Pharmacological blocking of AVP receptors (V1a and V1b) in the SCN accelerates recovery from jet lag in wild-type mice, underscoring the role of AVP neurons in circadian synchronization (Yamaguchi Y et al., 2013). These results suggest that AVP neurons are responsible for circadian rhythm alignment. The

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networking of SCN neurons is easier to reconstruct when AVP is abolished. Furthermore, cellular rhythmicity is reinforced, particularly in the SCN shell region—the primary distribution area of AVP neurons. The endogenous rhythmicity of clock genes, including Per1 and Per2 mRNA, is specifically expressed in the AVP region (Hamada T et al., 2001). These findings challenge the previous perception of AVP neurons as relatively inert, highlighting their substantial contribution to the synchronization of SCN neurons. To fully elucidate the intricate function of AVP neurons within the broader context of circadian regulation, further research is required.

Studies have shown that SCN neurons exhibit distinct firing rhythms in different regions, with a higher proportion of neurons expressing rhythmicity in the shell region compared to the core region (Nakamura W et al., 2001). This regional difference in rhythmicity may be attributed to the specific properties of neurons, particularly AVP and VIP, which are the dominant neuron types in both regions. The interaction between AVP and VIP is likely crucial for the coordination and synchronization of neurons within the SCN. Further investigation into this interplay could provide valuable insights into the regulatory mechanisms governing circadian rhythms in the SCN.

1.2.3 Molecular Clock

The circadian oscillations in mammals are governed by a molecular clock within cells, orchestrated through a transcriptional-translational feedback loop (TTFL) that spans a 24-hour period. Key components of this molecular clock include Period (Per) 1, Per2, Clock, Bmal1, Cryptochrome (Cry) 1, Cry2, Rev-erb alpha, and Rora. The

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heterodimer complexes formed by Bmal1 and Clock interact with E-box, initiating the transcription of Per1, Per2, Cry1, and Cry2 genes (J.S. Takahashi, 2017). The subsequent increase in PER and CRY proteins results in the formation of PER/CRY dimers, which, in turn, inhibit the transcription of their genes by blocking the activation mediated by Bmal1 and Clock. As PER and CRY proteins decline, this inhibition is relieved, initiating a new cycle approximately every 24 hours (Trey K Sato et al., 2006, C. Lee et al., 2001, Gekakis, N. et al., 1998). During the daytime, Clock-Bmal1 activation prompts the transcription of PER and CRY proteins in the afternoon. Subsequently, PER and CRY proteins accumulate in the evening, translocating into the nucleus at night. To inhibit their transcription effectively, the formed PER/CRY dimers interact with Clock-Bmal1 (Lee, C et al., 2001).

The molecular clock, indeed, is prevalent not only in the SCN but also in various brain regions, peripheral tissues, and organs. Peripheral tissues exhibit self-sustained circadian oscillations in explant cultures, indicating that the SCN serves more as a coordinator and synchronizer of cellular clocks rather than a master regulator of peripheral rhythms (Yoo et al., 2004; LeGates et al., 2014).

1.2.4 ipRGC and SCN

The connection between ipRGCs and SCN is facilitated by the retinohypothalamic tract (RHT). In response to photic information, neurotransmitters such as glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) are released at synaptic contact with SCN neurons. (Michael et al., 2006). Glutamate and PACAP play crucial roles in modulating circadian rhythmicity (DeVries et al., 1993). Moreover, studies indicate the release of the inhibitory neurotransmitter GABA from

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ipRGCs to SCN. Despite only 2% of ipRGC nerve terminals being Gad65 immunoreactive, Gad2 knockout mice exhibit insensitivity to decreases in environmental light levels and increased activity at lower light levels (Takuma Sonoda et al., 2020).

The increased firing rates of SCN neurons, induced by the release of glutamate and PACAP from ipRGCs, may contribute to the phase-shifting effects of environmental light. (Fernandez et al., 2016). VIP neurons, constituting approximately 10% of the SCN network, play a role in photoentrainment through innervation by ipRGCs (Abrahamson and Moore, 2001). SCN VIP neurons exhibit circadian rhythm and respond to light stimulation. Studies suggest that VIP neurons are crucial for transducing light signals and necessary for the resetting of daily rhythms (Jeff R. Jones et al., 2018; Cristina Mazuski et al., 2018). Despite the absence of VIP neurons, light-dark cycle synchronization persists in VIP-deficient mice, albeit with an 8-hour advanced shift (Colwell et al., 2003). This result indicates that VIP neurons are not the sole regulators of light entrainment in the SCN. Furthermore, VIP neurons can be influenced by other neurons. Activation of AVP neurons through optogenetics results in an elevation of calcium concentration in VIP neurons. In addition, there is also evidence indicating that AVP neurons play a critical role in SCN networking and circadian behavioral rhythm. The population of AVP neurons exhibits strong and stable coherence, peaking during the circadian daytime. The synchronization of AVP neurons during daytime suggests the acute response to a common input of either intrinsic or extrinsic origin, such as environmental light (Yusuke Tsuno et al., 2023; Adam Stowie et al., 2023).

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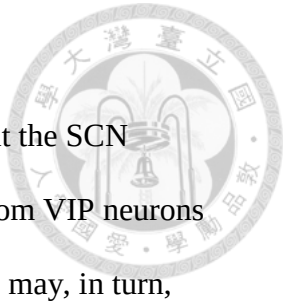

As ipRGC establishes connections with various cell types throughout the SCN (Fernandez et al., 2016), there may be additional neurons, distinct from VIP neurons that receive light information from ipRGC. The activation of ipRGC may, in turn, stimulate other neurons in response to light cues and subsequently activate VIP neurons.

1.3 Usage of Genetically Encoded Calcium Indicators (GECIs) - GCaMP

Upon stimulus, calcium ions are released into the cytoplasm from extracellular space or intracellular Ca2+ stores, thereby initiating intracellular signaling pathways. As Ca^{2+} transmits messages following the stimulation of the first messenger from outside, it is recognized as a second messenger. A notable characteristic of Ca^{2+} as a second messenger is its confinement to a small region after the stimulus. High-affinity Ca2+ binding sites in the cytoplasm facilitate the confinement, trapping, and limiting the diffusion of Ca2+. This feature is crucial for localizing responses in nerve cells (Makoto Endo, 2006).

Genetically encoded calcium indicators (GECIs) are employed to monitor neuronal firing patterns. These indicators emit light of a specific wavelength when bound to Ca^{2+}. Fluorescence resonance energy transfer (FRET)-based and single-fluorophore-based are two widely used GECIs. FRET-based indicators change light emission based on the distance between two fluorescent proteins, which decreases when calcium ions attach to them (Jihae Oh et al., 2019, Atsushi Miyawaki et al., 1997). On the other hand, single-fluorophore GECIs undergo a conformational change involving circularly permuted GFP (cpGFP) and calmodulin (CaM) when calcium ions bind to them. The GCaMPs family is a well-known single-fluorophore GECI, comprising

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cpGFP, CaM, and a calmodulin-binding peptide. The binding of Ca^{2+} to CaM alters its conformation and interactions with the calmodulin-binding peptide and cpGFP, modulating the fluorescence emission of GFP chromophores (Dana, H. et al., 2019). GCaMPs have been widely utilized to detect Ca^{2+} entry into neurons during neural activity (Tian, L et al., 2009).

Depolarization of neurons triggers calcium influx, leading to widespread calcium accumulations throughout the cell. This localized Ca^{2+} response offers the ability to discern whether a neuron has undergone an action potential, enabling the measurement of postsynaptic neuron responses through calcium imaging when stimulating a single neuron (Yuste, R et al., 1995; Kozloski, J et al., 2001). A prior study recorded synaptic activity in rat pyramidal neurons using calcium signals (Christine Grienberger al., 2012). Advancements in technology have given rise to a new class of indicators, such as GCaMPs, significantly enhancing their properties for neuronal recording with heightened sensitivity to calcium ion accumulation. These indicators facilitate the imaging of neuronal populations and the delineation of synaptic regions (Chen, T.-W. et al., 2013). Moreover, they can be employed to selectively image specific neuronal populations by crossing transgene mice expressing GCaMPs in a Cre recombinase-dependent manner with Cre recombinase driver mice. The resultant transgene mice offspring express GCaMPs in distinct cell populations (Liqun Luo et al., 2008; John G. Partridge, 2015). Within the GCaMPs family, GCaMP6 and GCaMP7 have emerged as prominent choices for detecting neuronal activity, particularly through two-photon microscopy or wide-field fluorescence imaging in recent years. GCaMP7, in particular, exhibits superior performance

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compared to GCaMP6, characterized by lower resting fluorescence and a higher signal-to-noise ratio (Sofroniew, N.J. et al., 2016; Dana, H. et al., 2019).

GECIs facilitate targeted recording of specific cell types and can be utilized for extended periods, with the defined neuronal population uniformly expressing GECIs, offering ample activity-dependent sensitivity and a broad fluorescence range. However, overexpression of GCaMPs within cells may lead to intracellular aggregation and subsequent apoptosis. To address this concern, an alternative strategy involves expressing GECIs in a confined area using a viral vector encoding GECIs. This viral vector can be delivered through intracerebral (IC) injection directly into the specific cell tissue within the brain region, as demonstrated in studies (Sverre Grødem et al., 2023).

1.4 Glutamate receptor

In the vertebrate nervous system, glutamate serves as a widely utilized excitatory neurotransmitter. Ionotropic receptors and metabotropic receptors are the two major types of glutamate receptors. Ionotropic receptors are further divided into three families based on their agonists: N-methyl-d-aspartate (NMDA) receptors, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, and kainate receptors (Y. Zhou et al., 2014). These ionotropic receptors become permeable to cations such as Na^+, K^+, and Ca^{2+} upon binding of glutamate. Activation of multiple ionotropic receptors leads to the generation of an action potential (Brian S. et al., 2000).

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As previously mentioned, ipRGCs release glutamate and PACAP to SCN regions through the retinohypothalamic tract (RHT). Consistent with this, significant glutamate immunohistochemistry is observed at the retinal nerve terminals (Hannibal J., 2002). A previous study further established glutamate as a photic input signal, supported by its location in RHT, release upon light stimulation, have similar effects on SCN neurons as light, and the elimination of these effects by glutamate antagonists (Hannibal J., 2002). Moreover, both ionotropic and metabotropic glutamate receptors have been identified in the SCN, underscoring the critical role of glutamate in circadian entrainment and its ability to induce circadian changes within the SCN. (Golombe DA et al., 2010). Additional evidence suggests that light-evoked responses from ipRGCs involve the activation of AMPA receptors (Jorge Alberto Perez-Leon et al., 2006). Consequently, the release of glutamate due to optic nerve stimulation activates SCN neurons, contributing to the mediation of the circadian rhythm.

Glutamate receptors are widely distributed throughout SCN cells and play a crucial role in modulating circadian rhythm in response to photic input. NMDA receptors, as one of the ionotropic glutamate receptor family, excite the majority of SCN cells when optic chiasm is stimulated (Mikkelsen JD et al., 1993, Kim YI et al., 1991). Approximately 94 percent of SCN neurons exhibit an increase in Ca^{2+} levels upon the application of NMDA. Notably, NMDA-induced calcium currents and transients in the SCN display rhythmicity, demonstrating circadian oscillation (Colwell CS, 2001, 2000). This implies the involvement of glutamate release in circadian changes.

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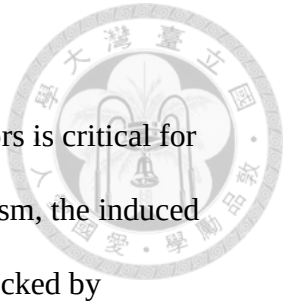

In addition to NMDA receptors, the activation of AMPA receptors is critical for the connectivity of SCN neurons. Upon the stimulation of optic chiasm, the induced excitatory postsynaptic currents (EPSCs) in SCN neurons can be blocked by antagonists of AMPA receptors (Michael G. Moldavan et al., 2018). The significance of glutamate from ipRGCs in circadian rhythm regulation is further underscored by experiments where vesicular glutamate transporter 2 (VGLUT2) in ipRGCs is ablated. VGLUT2 is essential for glutamate transmission from ipRGCs. Consequently, the conditional knockdown of VGLUT2 in ipRGCs leads to the silencing of glutamatergic signaling from ipRGCs, resulting in impaired circadian locomotor activity, photoentrainment, and negative masking responses to light. Negative masking indicates a reduction in locomotor activity in response to bright light (Nicole Purrier et al., 2014). In summary, the release of glutamate from stimulated ipRGCs modulates circadian rhythm through SCN neurons.

In conclusion, glutamate receptors are present in various brain regions, including the SCN. The transmission of glutamate from ipRGCs in response to photic input activates SCN neurons, playing a pivotal role in mediating circadian rhythm.

1.5 Optogenetics

The idea of optogenetics, proposed by Francis Crick in 1979, suggests that light can provide rapid spatiotemporal control for targeting specific neurons. This concept can be achieved by the photosensitive proteins that were discovered by microbiologists during that era. The flow of ion across the plasma membrane can be regulated by these photosensitive proteins and therefore modulate the neurons' activity (Jyotsna Joshi et al., 2020). Rhodopsins, consisting of opsins—a type of seven transmembrane-

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helix proteins—and a photosensitive chromophore called retinal, serve as photoreceptors through which all organisms receive light information from the environment. Based on their primary sequence and mode of action, the opsins are categorized as type I opsins and type II opsins. Type I opsins, discovered in archaea, eubacteria, fungi, and algae, are microbial opsins, while animals and humans are mainly type II opsins. Both of them have different ways of modulating transmembrane ion conductance. Type I proteins regulate transmembrane ion currents by light stimulation, such as light-driven ion pumps known as bacteriorhodopsins (BRs) and halorhodopsins (HRs), and light-driven ion channels, channelrhopsins (ChRs). On the contrary, type II proteins regulate transmembrane ion conductance indirectly, through the coupling of G-protein-based signal transduction pathways (Kato, H. E., et al., 2012, Govorunova, E. G. et al., 2017). By understanding the features of opsins, the concept of optogenetics can be brought to reality.

In optogenetics experiments, photo-sensitive proteins, such as channelrhodopsin and halorhodopsin are expressed in neurons to modulate the membrane potential or signaling pathway in response to light. These opsins can be restricted to specific types of neurons by genetic or anatomical approaches. For example, brain injection of viral vectors and expression of opsins by the specific promoter that is activated in certain types of neurons (Hamid Gholami Pourbadie et al., 2018). Previous studies show that channelrhodopsin (ChR2) expressing oocytes or mammalian cells have an increase of cytoplasmic Ca^{2+} concentration and are depolarized by tens of mV when exposed to blue light. Besides, light also induces electrophysiological responses of ChR2 expressing Purkinje fibers. This demonstrates that light-sensitive ion channel proteins

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can be used as a powerful tool in optogenetics experiments (Georg Nagel et al., 2003, Zaglia, T et al., 2015).

To investigate the firing patterns of the electrical activity of opsin-expressing intact cells and tissues, we can combine the optics system with genetically encoded voltage sensors or genetically encoded calcium sensors. This technique enables us to analyze the action potential of particular cells through the control of light (Park, S. A et al., 2014). The precise targeting of neurons can be achieved by two-photon (2P) optogenetics neuronal activation through holographic photostimulation. To imitate the spontaneous neuronal activity by photostimulation, an opsin with fast repolarization is needed to generate action potentials in high frequency. Among opsins used in optogenetic research, ChrimsonR is suggested to have fast repolarization between successive pulses (I-Wen Chen et al., 2019). In optogenetics experiments, microbial opsins are widely used due to their faster kinetics and relatively simple genetic engineering of a single component protein (Guru, A et al., 2015).

1.6 Brain expansion microscopy

Super-resolution microscopy is a valuable tool for surpassing the diffraction limit of light and investigating cellular structures at the nanometer scale. However, its application is constrained by the requirement for structures to be smaller than antibodies, crucial for biomolecule labeling. Super-resolution microscopy is limited to visualizing protein-specific antibodies rather than the biomolecules themselves (Andrew M Sydor et al., 2015). Moreover, many super-resolution techniques involve high costs and/or low imaging speeds. To address these limitations, expansion microscopy has been introduced, enabling scientists to achieve high resolution and

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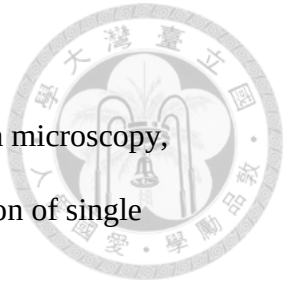

alleviate the crowding of proteins. The unique property of expansion microscopy, which involves expanding cells and tissues, allows for the observation of single molecules and nanoscale structures using conventional microscopes.

Expansion microscopy operates by physically magnifying the specimen isotropically, achieved by embedding it in a swellable polyelectrolyte hydrogel. Within this dense hydrogel, biomolecules undergo uniform expansion and separation, creating increased distances between them that can be resolved by diffraction-limited microscopes. For instance, a microscope with a diffraction limit of approximately 300nm could achieve a resolution ranging from (300/70~4.5) nm when the specimen is expanded 4.5 times in linear dimensions. Notably, biomolecules appear transparent after expansion due to the water content in the hydrogel, eliminating the need for time-consuming clearing techniques. Brain slices from mice expanded fourfold in an experiment and exhibited similar features to the original slices, suggesting minimal distortions resulting from the expansion process (Asmamaw T Wassie et al., 2018; Fei Chen et al., 2015).To anchor biomolecules and the polymer matrix, various anchoring agents, including proteins, nucleic acids, and lipids, have been employed and generalized. The continual development of anchoring strategies has expanded the versatility and applications of expansion microscopy (Gang Wen et al., 2023).

In general expansion microscopy, immunostaining plays a crucial role in labeling specific biomolecules for microscopic observation. The immunostaining procedure is contingent on the expansion microscopy protocol, involving both pre-labeling and post-labeling strategies. In the pre-labeling approach, specimens are stained before undergoing expansion, leading to fluorophore destruction during the expansion

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process and resulting in lower fluorescent signal intensity afterward (Tillberg, Paul et al., 2016). On the other hand, the post-labeling strategy, occurring after expansion, offers improved fluorescence intensity but may introduce errors like leakage due to expansion (Zwettler, Fabian U. et al., 2020). To address the decrease in signal-to-noise ratio, research has focused on enhancing fluorescence signals after expansion, with trifunctional molecules representing one such approach. These molecules can label the target unit, incorporate fluorophores, and anchor to the gel simultaneously (Wen, Gang et al., 2020).

The development of expansion microscopy has presented an alternative avenue for nanoscale imaging. With lower costs and relative efficiency, expansion microscopy stands out as a more feasible option compared to super-resolution microscopy.

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Statement of the Purpose

Circadian rhythms play a crucial role in regulating the daily behavior and endocrine system of most mammals, and their mediation occurs through the suprachiasmatic nucleus (SCN), the brain's pacemaker located in the hypothalamus. The synchronization between the intrinsic oscillation within the SCN and the external environment is facilitated by intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina. The light signals are received by these cells and conveyed to the SCN through the retinohypothalamic tract.

Previous studies have proposed that VIP neurons in the ventral region of the SCN act as the primary input neurons for ipRGCs. However, our recent findings challenge this assumption, revealing that the distribution of ipRGCs is not limited to the dorsal side but extends throughout the entire SCN. This opens up the possibility that neurons in other regions of the SCN might also receive input from ipRGCs.

Apart from VIP neurons, AVP neurons in the dorsal region are significant SCN neurons, recognized as output neurons projecting from the SCN to other hypothalamic regions. Intriguingly, the location of ipRGCs overlaps with AVP neurons, suggesting a potential functional connection between them. This thesis aims to substantiate the functional link between ipRGCs and AVP neurons in the SCN through the application of calcium imaging and photogenetics. Additionally, the study will analyze the response patterns of AVP neurons when subjected to ipRGC stimulation. Ultimately, we seek to unravel the mechanisms by which ipRGCs manipulate AVP neurons in the SCN.

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Chapter II

Method and Material

2.1 Animals

In this study, mice of mixed background (BL/6;129SvJ) were used for experiments. The AVP-Cre (AVP-IRES2-Cre) knock-in mice (obtained from Jackson lab) were injected (ML: +/-0.195 mm, AP: -0.45 mm, DV: -5.68 mm) with an adeno-associated viral vector conveying the genetically encoded calcium sensor pGP-AAV-syn-jGCaMP7f-WPRE (0.45μL, #104488 –AAV9 from Addgene) into the SCN. The transgene mice that specifically express GCaMP in AVP neurons were used for in vitro calcium imaging. The experiment was conducted on mice aged 3 weeks to 2 months and housed under a 12h light: 12h dark cycle. Besides, food and water were available ad libitum. All procedures described were conducted according to the guidelines for the Care and Use of Experimental Animal of National Taiwan University and were approved by the Ethics Committee for Animal Research of National Taiwan University.

For ipRGC tracing, we use Opn4-Cre mice crossed with Gad67-GFP mice. GFP would be expressed in GABAergic neurons, which are the main population neurons in SCN. The transgene mice were injected with td virus in both eyes and sacrificed after one month. Opn4 gene that encoded melanopsin expressed cre recombinase and was recognized by td virus.

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2.2 DNA isolation

The DNA extraction is prepared according to HotSHOT (Truett et al., 2000). About 0.2 cm tail snip is collected from each mouse and heated to 95°C for 30 minutes to 2 hours with 75μl lysis buffer (23mM NaOH/0.2mM EDTA). After lysis, the sample is applied to 75μl Tris-HCl (pH5.5) for neutralization. Then, we centrifuged the sample at 4000 rpm for 3 minutes. Finally, we stored the DNA sample at 4°C until PCR.

2.2.1 Polymerase chain reaction (PCR)

In this experiment, a pair of primers is used to detect AVP-Cre transgene. The forward primer of AVP-Cre transgenes is 5'- CTGCTGCTAAGGCTGGTACA-3', and the reverse primer is 5'- AGACCCCTAGGAATGCTGGT-3'.

2.3 Two-photon microscopic calcium imaging

Two-photon microscopic device (designed by Southport Co.) equipped with a 16X water immersion objective (Nikon, 0.8 NA, N16XLWD-PF objective, Nikon Instruments, America) was conducted for calcium imaging. To excite the wavelengths of green fluorescent proteins (GFP), 910 nm ultrafast pulsed laser (Mradian Femto sources co.) was used to generate the two-photon excitatory input. The laser power was set at 30-50 mW for imaging of calcium indicator. Fluorescent emissions of 575-585 nm were collected using two photomultipliers tubes (PMT) (H16201P-40, Hamamatsu), and recorded by channel 1. The scan parameters were set for 512×512 pixel of images (field of view, FOV: 512 × 512 μm; 23 frames per second) for image acquisition, and the input range was set between +/- 0.4 mV, recorded by custom-made software (designed by Southport Co.).

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Calcium imaging in acute brain slices is performed in a chamber under the microscope. A customized chamber is prepared for individual slices, and the oxygenated ACSF medium is continuously perfused (2 ml/ min) into the chamber at a maintained temperature (35-37 ℃). The medium temperature is controlled by a heater device (XH-W1209). Eventually, the brain slice will be recorded after 30 minutes of habituation. To activate ipRGC with ChrimsonR, we use excitation light power in the range of x to y mW.

2.3.1 Experimental design

The brain slice was recorded between ZT8 – ZT9. After incubation, the brain slice is moved to the recording platform with a perfusion device. Before starting the experiment, the slice was rested for 30 minutes in the chamber. First, the brain slice was recorded without light stimulation for 4 minutes as a control. Then light stimulation was applied in the next experiment for 4 minutes of recording. To investigate whether ipRGCs are directly connected to AVP neurons in SCN, we then applied 5µl TTX (1µM, T-550, Alomone labs) and 50µl 4-Aminopyridine (4AP, 100µM) in 50ml ACSF and replaced the control ACSF. The brain slice was perfused with ACSF containing TTX and 4AP for 10 minutes before recording started. After that, the brain slice that was bathed in the drug ACSF was recorded for 4 minutes with light stimulation or without light stimulation. After the application of TTX and 4AP, 50µl DNQX (20µM, TOCRIS, No. 0189) is applied in 50ml ACSF in the following experiment. DNQX is a widely used AMPA and kainate receptor antagonist. Then, the brain slice that was perfused in ACSF containing TTX, 4AP, and DNQX was recorded under the light stimulus or without light stimulus conditions. Eventually, the

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brain slice was perfused with origin ACSF for 20 minutes before the wash out experiment.

2.3.2 Optogenetics

The peak response of ChrimsonR is around 590 nm. To avoid the activation of GCaMP7f which is around 488nm, we use a 620 nm red LED to drive ChrimsonR. The light power of the LED measured at the brain slice was 10 μW. Besides, the flashing frequency and duration of LED light are controlled by Arduino programming language. In the process of the experiment, the LED is modulated to 10 Hz (a light pulse is 1 second long), 10 seconds 5 times at a regular interval of 30 seconds. The records will start at 30 seconds before the red light starts to flash and end at 30 seconds after the flashing finish. In sum, the whole recording duration takes 3 minutes and 50 seconds. Throughout the experiment, the LED is placed below the transparent recording chamber and the brain slice will be stimulated by red light.

2.3.3 Analysis

All images were analyzed by using the ImageJ. The recording images are merged into a single image and processed by Cellpose to circle neurons. The selected neurons are known as regions of interest (ROI). The average fluorescence of ROI was measured at an average of 20 image frames to improve signal-to-background noise by ImageJ. The frame images are also treated by alignment and motor correction through Matlab before further analysis. Then, we also circled a region without any neurons as background region to remove the photostimulation artifacts of image frames of the ROIs. In addition, the exponential drop is removed by the Matlab program to prevent

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the photo-bleaching problem. Besides, the highpass-filter was used to remove the effect of mechanical drift of each ROI. After all these processes, the mean fluorescence intensities of all ROIs were extracted by the Multi Measure plugin in ImageJ. Then, the relative percentage changes of fluorescence were computed for the calcium signal as ΔF/F0, while F0 is set to the average fluorescent intensity value measured from the trace over the whole recording. The ΔF/F0 of each ROI was calculated through the average of 5 trials and the mean of the whole recording, eventually presented as a heat map. Then, this data was plotted in 3D with principal component analysis (PCA) and categorized into different clusters by k-means clustering analysis. The stimulated ROI was identified by the threshold of the means of 20 seconds baseline before each light stimulation with 3 standard deviations.

2.3.4 Correlation analysis

To investigate the response of neurons after the application of TTX and 4AP under light stimulation, we will compare the synchronization of transient Ca^{2+} activities among individual AVP neurons under control and drug application with light stimulation. The values of ΔF/F0 of all ROIs in different conditions were cross-correlated with each other and a Pearson correlation coefficient (r) for each ROI-ROI interaction was generated. The population r values represent the synchrony level of transient Ca^{2+} activities among AVP neurons at different conditions. Then, the comparison of population r values was analyzed by using the Wilcoxon test and Mann-Whitney test.

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2.4.1 Eye Injection Surgery

At the age of 2 months, we injected the AAV2-Syn-ChrimsonR-tdTomato virus into both eyes of mice. The genotype of the mice was AVP $^{cre/+}$ and were sacrificed for the experiment after about 5 weeks. Before starting the surgery, the mouse was anesthetized with isoflurane. Then, we would use a 30-gauge needle to make an incision into the sclera. The virus solution was injected into the incision by using a glass needle (inner diameter of about 100 μm) attached to a 50 μl microliter Hamilton syringe at a rate of 4μm/min. Then, 1 μl of virus solution was injected into the vitreous and the needle was pulled out carefully. Finally, the mouse was placed in its home cage for recovery.

2.4.2 Acute Brain Slice

Optogenetics was combined with calcium imaging experiment to investigate the connection of neurons. The coronal section of brain slices that contained the suprachiasmatic nucleus were prepared from AVP $^{cre/+}$ mice with an injection of GCaMP7f and ChrimsonR at the age of 2 months. Injection of Urethane (1kg/6mL) is applied to mice for anesthetic and 8mL ice-cold N-methyl-D-glutamine (NMDG)-based artificial cerebrospinal fluid (ACSF) for perfusion. The ACSF medium is composed of the following: 92mM NMDG, 2.5Mm KCl, 30mM $NaHCO_3$, 1.25mM Na_2HPO_4, 10mM $MgSO_4$, 0.5mM $CaCl_2$, 25mM glucose, 20mM HEPES (all obtained from Sigma), pH 7.3 to 7.4, 308-316 mOsm. Then, we decapitated the mice and extracted the brain to immerse in ice-cold and oxygenated (95% O_2, 5%CO_2) NMDG-based ACSF. The brain was fixed in agarose and glued to a plate for brain slicing.

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Brain slices of 350μm were obtained by slicing-chamber at low temperature. When slices were prepared, they were transferred to a dish containing NMDG-based ACSF for 20 minutes at room temperature. Then, the brain slice was moved to the mixture of oxygenated HEPES-based ACSF and NMDG-based ACSF (1:1) at room temperature for 20 minutes. The HEPES-based ACSF contained the following components: 92mM NaCl, 2.5Mm KCl, 30mM $NaHCO_3$, 1.25mM Na_2HPO_4, 2mM $MgSO_4$, 2mM $CaCl_2$, 25mM glucose, 20mM HEPES. After that, the brain slice was incubated in HEPES-based ACSF for 45 to 60 minutes before transfer to a recording chamber. In the process of recording, the brain slice was perfused with ACSF continuously at 35 to 37 degrees Celsius at a rate of 1.5-2 ml/min. The ACSF for recording included the following components: 119mM NaCl, 2.5mM KCl, 26.2mM $NaHCO_3$, 1mM Na_2HPO_4, 1.3mM $MgSO_4$, 2.5mM $CaCl_2$, 11mM glucose.

2.4.3 Stereotaxic surgery

The stereotaxic surgery was performed on the AVP cre/+ mice at least 3 months old. The injection of AAV9-Syn-Flex-GCaMP7f in the SCN region of the transgenic mice induced the expression of GCaMP in AVP neurons in a specific region. The mouse was anesthetized with isoflurane (AthaneTM, 5% for induction, 1%-2% for maintenance) and fixed on stereotaxic by using ear bar and mouthpiece. Then, we would shave the hair on the mouse's head with a pet clipper and apply povidone-iodine solution on the skin's surface. After that, we will make an incision on the head area and clean it with 1% phosphate-buffered saline (PBS). To ensure the right position for the craniotomy hole, the bregma and lambda were modified to the same horizontal level. Two holes were drilled above the SCN (AP -0.42mm, ML -/+

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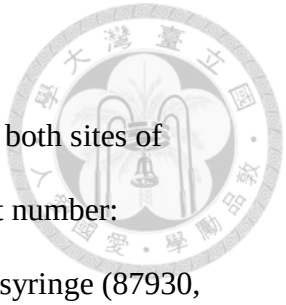

0.195mm, DV -5.68mm/from cortex). 450nl of virus was injected in both sites of SCN at a rate of 45nl/minute through a glass needle (Kwik-Fil^{TM}, lot number: 2605323, inner diameter about 20μm) that is attached to a 5μl microsyringe (87930, Hamilton) and controlled by a reversible nanoliter syringe pump (KDS-310-PLUS, KD Scientific). After the infusion of the virus, the glass needle remained in the brain for at least 5 minutes for the diffusion. When the injection was finished, the incision was closed with surgical suture (UNIK, black braided silk, 6/0, NO.0034) and housed individually under a 12:12 LD cycle for recovery. Finally, the mouse was sacrificed for experiments after 5 weeks.

2.5 Immunohistochemistry staining

After finishing recording, the brain slice was fixed with 4% paraformaldehyde (PFA) at 4°C overnight for AVP and GFP staining in the following experiments. Then, the brain slice was washed with phosphate-buffered saline (PBS) for 20 minutes at least 3 times. After that, the brain slice was incubated in a blocking solution for 2 hours. The blocking solution is composed of 0.9% Triton X-100, 0.1% goat serum, and 99% PBS. For AVP and GFP staining, we used vasopressin antibody (Rabbit, 1:1000, Immunostar) and GFP antibody (Chicken, 1:1000, Abcam) as primary antibodies. These primary antibodies were mixed with the blocking solution equally and incubated in the brain slice for 3 days at 4°C. After incubation, the brain slice was washed for 20 minutes at least 3 times and prepared for secondary antibody incubation. The blocking solution of secondary incubation was mixed with goat anti-chicken 488 (1:500, biotium) and donkey anti-rabbit 647 (1:500, biotum) in equal amounts. Then, the blocking solution with secondary antibodies for the brain

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incubation with duration of 2 hours. Finally, the section was mounted in rapiclear (SunJin Lab) after washed for 20 minutes at least 3 times.

For immunostaining on the brain slice of ipRGCs tracing, the brain slice was prepared in 200um and fixed in PFA overnight before staining. The staining protocol was the same as stated above. The ipRGC was stained in goat anti-chicken 633 (1:500, biotum), and AVP neurons were stained in goat anti-rabbit 568 (1:500, biotum).

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Chapter III

Results

3.1 Distribution of ipRGC to AVP neurons in SCN

We crossed Opn4$^{cre/+}$ mice with Gad67-GFP mice which will express GFP in GABAergic neurons. SCN region would be recognized by GFP as most of its neurons will release GABA. Subsequently, we administered a cre-dependent tdtomato virus into both eyes of the mice, enabling ipRGCs to express tdtomato and allowing their tracing to the SCN. Through immunostaining, we observed contacts of ipRGCs (red) with AVP neurons (magenta) in the SCN GABAergic neurons labeled by GFP (Fig. 1b). The histograms show the percentage of AVP neurons in contact with ipRGCs. On both sides of SCN region, about 4% of AVP neurons (40/850, left side; 42/968, right side) were found to be close to ipRGC. In contrast, at least 30% of AVP neurons that were colocalized to GABAergic neurons express more connection to ipRGCs (214/408, left; 169/551, right) (Fig. 1c). This strongly suggests the possibility of a direct anatomical connection between ipRGCs and SCN neurons, providing valuable insights into the neural circuitry involved in circadian regulation.

3.2 Activation of ipRGC induces different responses in AVP neurons

To investigate the potential innervation of AVP neurons in the SCN by ipRGCs, we conducted an experiment involving the expression of GCaMP7f in AVP neurons. Simultaneously, ipRGCs were infected with a virus carrying ChrimsonR, responsive to a 610nm wavelength of light. The results revealed two distinct patterns of response among AVP neurons. In the first group, constituting 53 percent of AVP neurons (group

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1), there was an initial increase in fluorescence intensity during the first light stimulation, followed by a subsequent decrease. In the second group (group 2), encompassing 47 percent of AVP neurons, there was a gradual augmentation of calcium activity with successive light stimulations (Fig. 2, 3). These distinct responses were evident when analyzing the mean intensity of the entire recording and the average intensity during each light stimulation. In the absence of the light-stimulating drug, 21 percent of AVP neurons were identified as activated during the light stimulation process. Notably, among the activated neurons, 21 percent belonged to group 1, while the majority (79 percent) fell into group 2 (Fig. 4). This suggests that a significant proportion of activated neurons exhibit a gradual increase in fluorescence intensity upon ipRGC activation, while a smaller subset responds only to the initial stimulation, decreasing their fluorescence intensity thereafter. These findings shed light on the diverse response patterns of AVP neurons to ipRGC stimulation and contribute to our understanding of the complex interactions within the SCN.

3.3 AVP neurons were activated in TTX and 4AP when ipRGCs were stimulated

To ascertain whether the pathway between ipRGCs and AVP neurons in the SCN is monosynaptic, we applied TTX and 4AP to the brain slice prior to light stimulation. TTX blocks sodium channels, impeding interneuronal function. The results demonstrated that, under light stimulation with TTX and 4AP, 24 percent of AVP neurons were activated. Among the stimulated AVP neurons, 50 percent belonged to group 1, and the other 50 percent to group 2, indicating that both types of AVP neurons were directly activated by ipRGCs in equal proportions (Fig. 5).

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Subsequently, we compared the activation patterns of neurons under normal light conditions to those responding in the presence of TTX and 4AP. Notably, 41 percent of AVP neurons were exclusively activated in light conditions, while 48 percent responded to ipRGCs only when TTX and 4AP were applied. Interestingly, a mere 11 percent of AVP neurons demonstrated activation in both scenarios. This suggests that the neurons activated in the presence of TTX and 4AP differed from those responding under normal light conditions. Furthermore, AVP neurons that were activated in both conditions predominantly belonged to group 2. Another 31 percent of neurons activated under TTX and 4AP conditions were group 2 neurons, whereas 50 percent were group 1 neurons (Fig.6). These findings provide insight into distinct activation patterns of AVP neurons in response to ipRGCs under different conditions.

3.4 Correlation coefficient of AVP neurons

The blockade of sodium channels by application of TTX eliminates action potentials in neurons, the anticipated outcome was a decrease in the correlation among SCN neurons in the experiment. To assess this, we computed the correlation coefficient between the fluorescence intensity of each ROI in the control and drug conditions. The results revealed a significantly higher correlation among SCN neurons in the control group compared to neurons in the TTX group without light stimulation. This suggests that TTX has indeed diminished the connectivity among neurons. However, when ipRGCs were activated, the stimulated neurons exhibited a higher correlation compared to other neurons that were not activated (Fig. 7). This observation

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underscores the impact of ipRGC activation in enhancing the connectivity among SCN neurons even under the influence of TTX.

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Chapter IV

Discussion

4.1 IpRGCs contact with AVP neurons in SCN

In previous studies, the distribution of ipRGCs has been traced by targeting the melanopsin protein, which is highly expressed in ipRGCs. These findings revealed the projection of ipRGCs to various brain regions, including the suprachiasmatic nucleus (SCN) (Marcos L et al., 2020). The SCN is a heterogeneous structure that can be categorized into core and shell regions based on the variety of neurons it contains. Neurons that are predominantly expressed in the core region consist of vasoactive intestinal polypeptide (VIP), calretinin, neurotensin (NT), and gastrin-releasing peptide (GRP), which are located in the ventral area. In contrast, arginine vasopressin (AVP) neurons, alongside angiotensin II and met-enkephalin, are primarily located in the shell region, situated in the dorsal area (Abrahamson et al., 2001).

Previous studies investigating the projection of ipRGCs to the SCN have revealed direct inputs from the retina to neurons in the core region of the SCN, such as GRP neurons and VIP neurons (M Tanaka et al., 1997, 1993; Y Ibata et al., 1989). However, there are also studies indicating that the projections of ipRGCs might not be limited to the core region but extend to other neurons in the SCN as well. Single ipRGC tracing studies have shown that the innervation of ipRGCs is distributed throughout the SCN (Fernandez et al., 2016). Our experiment, using immunostaining, demonstrated the contact between ipRGCs and AVP neurons in the SCN. The results indicated that a small number of AVP-expressing neurons are connected to ipRGCs. In

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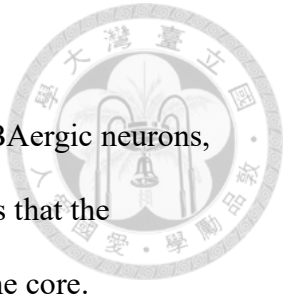

contrast, approximately 30% of AVP neurons co-localized with GABAergic neurons, suggesting potential connections with ipRGCs. This further confirms that the innervation of ipRGCs might extend to neurons in regions beyond the core.

Although SCN shell regions are predominantly composed of AVP neurons, a variety of other neurons are also co-expressed. These subtypes of neurons lack clear boundaries within the SCN and overlap with each other, leading to the expression of different neuropeptides. In addition to GABAergic neurons expressed throughout the SCN, some neurons co-express VIP and GRP, while others in the shell region express VIP and AVP together (H.J. Romijn et al., 1999; Jennifer A. Evans, 2016). The feature of co-expressing different peptides in SCN neurons may contribute to their functional activity. Therefore, AVP neurons that co-release GABA and contact ipRGCs might perform a vital role in the networking of SCN neurons. Additionally, the contacts between ipRGCs and AVP neurons in our results are consistent with studies indicating that AVP neurons might receive light input (Adam Stowie et al., 2023).

To confirm the connection between AVP neurons and ipRGCs, super-resolution techniques such as expansion microscopy are needed to observe the synapses. Expansion microscopy offers high resolution and prevents crowded proteins. By using this technique, cells and tissues can be observed with conventional microscopes after expansion. Previous experiments have shown that expanded brain slices from mice exhibit similar features to the original slices with minimal distortions (Asmamaw T Wassie et al., 2018; Fei Chen et al., 2015). In future experiments, we plan to apply expansion microscopy to further explore the connection between ipRGCs and SCN neurons.

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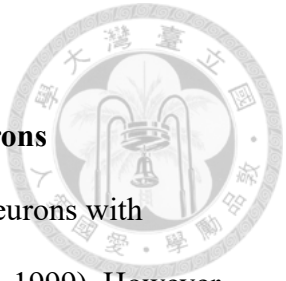

4.2 Activation of ipRGC induces different responses in AVP neurons

AVP neurons in the SCN have traditionally been viewed as output neurons with minimal impact on the synchronization of SCN neurons (Jin X et al., 1999). However, this statement is challenged by recent studies and suggests that AVP neurons play a crucial role in circadian rhythm alignment. Loss of AVP receptors has been shown to shorten the entrainment period in mice, and the networking of SCN neurons can be disrupted with the elimination of AVP neurons (Yamaguchi Y et al., 2013). Moreover, evidence points to the significance of AVP neurons in SCN networking and circadian behavioral rhythm regulation. Activation of AVP neurons can induce calcium concentration changes in VIP neurons, and AVP neurons demonstrate strong and stable coherence during the circadian daytime, indicating responsiveness to environmental light cues (Yusuke Tsuno et al., 2023; Adam Stowie et al., 2023). During our experiment involving GCaMP-expressing AVP neurons, we observed diverse response patterns of AVP neurons to ipRGC stimulation. One population of AVP neurons exhibited an initial increase in fluorescence intensity followed by a subsequent decrease during light stimulation, categorized as group 1. Another population of AVP neurons (group 2) showed a gradual increase in fluorescence intensity in response to light stimulation. Thus, activation of ipRGCs elicits two distinct types of responses in AVP neurons. These findings align with previous studies highlighting the critical roles of AVP neurons in the SCN.

The variability in the response of AVP neurons to ipRGC activation may stem from differences in neurotransmitter types. Ionotropic and metabotropic receptors are the two major types of neurotransmitter. Ionotropic receptors directly induce ion flow

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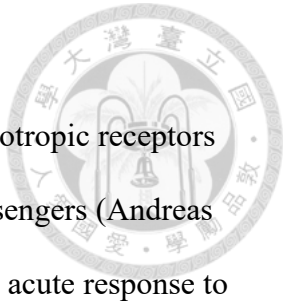

through ligand-gated ion channels upon ligand binding, while metabotropic receptors mediate ionic activity indirectly through G proteins and second messengers (Andreas Reiner et al., 2018; Bryan L Roth, 2019). AVP neurons exhibiting an acute response to initial stimulation may be associated with ionotropic glutamate receptors, which respond rapidly to stimulation. Conversely, metabotropic glutamate receptors, requiring multiple processes for activation, may correspond to AVP neurons that gradually increase stimulation in response to ipRGC stimulation. In our forthcoming experiments, we plan to use ionotropic and metabotropic glutamate antagonists to observe the activity of AVP neurons and gain further insights into their response mechanisms.

4.3 Direct innervation of ipRGC to AVP neurons

Present studies show that AVP neurons might be light-responsive neurons and play a vital role in the networking of SCN neurons. Evidence indicates that a population of AVP neurons in SCN expressed FOS, markers of light-induced gene expression (Casper Schwartz Riedel et al., 2024). In vivo recording experiment, jGCaMP7s signal in VIP neurons was increased when AVP neurons were stimulated by optogenetic, which represents the increase of calcium in VIP neurons (Yusuke Tsuno et al., 2023). These results reveal that AVP neurons can modulate other neurons in SCN and might be stimulated by light.

Based on our findings, there is at least 20% of AVP neurons exhibit activation in response to ipRGCs under the influence of TTX and 4AP. Further categorization of these activated AVP neurons reveals three distinct groups. The first group consists of

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AVP neurons exclusively responding to light stimulation without the application of TTX and 4AP. It is plausible that these neurons involve interneurons that are activated in response to ipRGC activation. In this pathway, ipRGCs activate interneurons, which in turn activate AVP neurons. To investigate the direct innervation of ipRGCs to AVP neurons, we apply TTX, by blocking neuronal connections, rendering these activated neurons unresponsive to ipRGC stimulation. In the second group, a population of AVP neurons is activated in both conditions, which is the application of TTX and 4AP under light stimulation, and light stimulation solely, suggesting a direct connection between these AVP neurons and ipRGCs. Lastly, the third group of AVP neurons only activates in the presence of TTX and 4AP under light stimulation, indicating the existence of a complex circuit within this particular population. These distinctions shed light on the diverse modes of interaction between ipRGCs and AVP neurons within the SCN. Additionally, the activated neurons exhibit two distinct types of light responses, categorized as stimulus-response and gradual response. Group 3 AVP neurons consist of gradual-response neurons, while group 2 neurons belong to gradual and stimulus-response neurons.

According to our experimental findings, AVP neurons have different responses in light conditions and the presence of TTX and 4AP. This suggests the possible involvement of an inhibitory loop in the connection between AVP neurons and ipRGCs (Fig. 9). During light stimulation without TTX, activated ipRGCs may trigger inhibitory interneurons, leading to the inhibition of AVP neuron activation. As a result, these neurons do not respond to light in normal conditions. However, when TTX is applied, inhibitory interneurons in the circuit are suppressed, allowing AVP neurons connected

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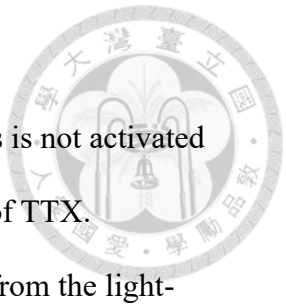

to ipRGCs to be activated. Consequently, this subset of AVP neurons is not activated in light conditions but responds to light stimulation in the presence of TTX. Therefore, another group of activated AVP neurons that is different from the light-conditioned AVP neurons arises when TTX is applied. This inhibitory loop may involve GABAergic neurons, which are widely expressed throughout the SCN (Michael A. Belenky et al., 2008). GABAergic neurons play a crucial role in synchronizing SCN neurons, as evidenced by studies showing that treatment with GABA can resynchronize clock cells in culture to the same circadian phases (Chen Liu and Steven M Reppert, 2000). Additionally, inhibiting GABA has been shown to lengthen the free-running period in culture, while GABA excitation also lengthens the period, suggesting that both excitatory and inhibitory GABA signaling are necessary to modulate circadian rhythm. Furthermore, studies have demonstrated that excitatory and inhibitory GABA signaling can have different effects on dorsal and ventral SCN regions, with excitatory GABA leading to stronger coherence and a shorter period in the dorsal SCN, and the opposite effect in the ventral SCN (Jihwan Myung et al., 2015). This suggests the presence of a repulsive coupling mechanism involving GABAergic neurons.

Supporting this notion, previous research has indicated the existence of excitatory and inhibitory connections within the GABAergic network, as observed through the analysis of neuronal firing patterns (G Mark Freeman Jr et al., 2013). In sum, these findings suggest that GABAergic neurons may indeed be crucial for the inhibitory loop proposed between AVP neurons and ipRGCs. In future experiments, applying GABA blockers could help determine the involvement of GABAergic neurons in the

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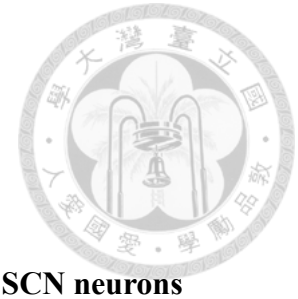
connection between ipRGCs and AVP neurons.



4.4 AVP neurons are involved in networking and synchronizing SCN neurons

The innervation of ipRGCs to AVP neurons was revealed in our experiments. Through brain slice immunostaining, we observe the proximity of ipRGCs to AVP neurons. While the resolution may not be sufficient to identify synaptic connections, this provides additional evidence of the connection between ipRGCs and AVP neurons in SCN. Furthermore, AVP neurons responded to the activation of ipRGC by optogenetics. Although there is a lack of a significant correlation coefficient between light-responsive neurons and non-responsive neurons, this may be attributed to the distinct response patterns exhibited by neurons during light stimulation. Consequently, when stimulated neurons display a reverse response, it contributes to a decrease in correlation between them. Therefore, ipRGCs are connected to the AVP neurons in SCN based on our observation.

To further support our statements, recent research has shed light on the critical roles of AVP neurons in the SCN in response to light stimulation. During the circadian daytime, the population of AVP neurons demonstrates stable synchronization, suggesting their responsiveness to environmental light cues (Adam Stowie et al., 2023). Additionally, the expression of light-induced gene markers in AVP neurons within the SCN provides further evidence supporting the notion of light modulation in these neurons (Casper Schwartz Riedel et al., 2024). Moreover, the activation of AVP neurons elicits changes in other neurons within the SCN. In vivo recording experiments have demonstrated alterations in calcium concentration in VIP neurons

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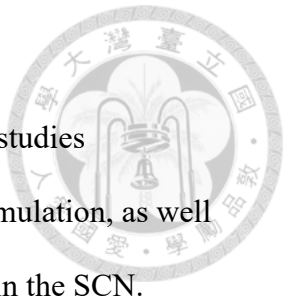

when AVP neurons are activated (Yusuke Tsuno et al., 2023). These studies underscore the close relationship between AVP neurons and light stimulation, as well as the importance of AVP neurons in influencing other neurons within the SCN.

The pivotal roles of AVP neurons in synchronizing SCN neurons have been elucidated through previous studies. For instance, the targeted deletion of Bmal1 specifically in AVP neurons in mice resulted in a faster reentrainment compared to control mice. This study revealed a reduction in the expression of circadian genes, including AVP, Prokineticin 2, and Rgs16, in the dorsal SCN of mutant mice. Similarly, studies involving double knockout of vasopressin V1a and V1b receptors in mice demonstrated an almost instantaneous reset of locomotor activity when entrained by light, underscoring the critical role of AVP signaling in circadian entrainment (Yoshiaki Yamaguchi et al., 2013). Additionally, hormonal regulation is involved by AVP neurons in the SCN. Inhibition of AVP receptors led to a decreased amplitude in SCN activity, which could be rescued by estrogen application, suggesting a connection between AVP neurons in the SCN and sex hormones (Lina Schlaeger et al., 2024). Moreover, the release of AVP in the SCN is modulated by melatonin, a pineal hormone involved in regulating the entrainment of daily rhythms, further emphasizing the involvement of AVP neurons in circadian modulation (Kazuto Watanabe et al., 1998). Overall, AVP neurons play a critical role in coupling neurons within the SCN and serve as interneurons involved in coordinating circadian rhythms regulated by various substances.

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Chapter V

Significance of work

It has been well known the suprachiasmatic nucleus (SCN) modulated circadian rhythm in mammals. However, the intricacies of neuronal networking within the SCN and its connections to intrinsically photosensitive retinal ganglion cells (ipRGCs) remain ambiguous. To explore the functional connections of ipRGCs within the SCN, we employ calcium imaging using GCaMP7f to observe neuronal responses in vitro. The firing patterns of neurons stimulated by optogenetics are recorded through two-photon microscopy. This technique allows us to precisely modulate the activation of ipRGCs while simultaneously observing the responses of neurons in the SCN. Furthermore, by applying a cre-dependent virus to transgenic mice, we can trace the innervation of ipRGCs to various neurons within the SCN. This method provides a unique opportunity to map the connections between ipRGCs and SCN neurons, revealing the neural circuits involved. Through these combined techniques, we can reveal the detailed connectivity and functional relationships between ipRGCs and SCN neurons, thereby enhancing our understanding of how light influences circadian rhythms.

In previous research, the distribution of ipRGCs to the SCN was not limited to the core region, as was assumed by earlier studies. Instead, ipRGCs are distributed throughout the entire SCN, including the shell region, which is predominated by AVP neurons. In my experiment, we found a close connection between ipRGCs and AVP neurons in the SCN. This connection is functional, as evidenced by the response of

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AVP neurons to the activation of ipRGCs through optogenetics. The responses of AVP neurons to ipRGC activation can be categorized into three groups: ipRGCs-stimulated AVP neurons involving interneurons, ipRGCs-stimulated AVP neurons under TTX, and AVP neurons response in ipRGCs stimulation and TTX conditions (direct innervation). The presence of different populations of AVP neurons responding to ipRGC stimulation under various conditions, including the application of TTX, suggests a complex interneuron networking within the SCN. Therefore, we propose an inhibitory loop that contributes to the connection between AVP neurons and ipRGCs. This loop likely involves GABAergic neurons, which are known to be widely expressed in the SCN and important for the synchronization and modulation of circadian rhythms.

In my future work, we will apply GABA antagonists to observe the response of AVP neurons under activation of ipRGCs. Additionally, we will use expansion microscopy to further confirm the physical connections between ipRGCs and AVP neurons. In sum, my study reveals novel insights into the innervation of ipRGCs to SCN neurons. The demonstrated connection between ipRGCs and AVP neurons elucidates the neuronal circuitry within the SCN and provides us with more information on the emergence and regulation of circadian rhythms. We can better understand how light influences circadian rhythms and how disruptions in these processes might lead to circadian rhythm disorders.

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Chapter VI

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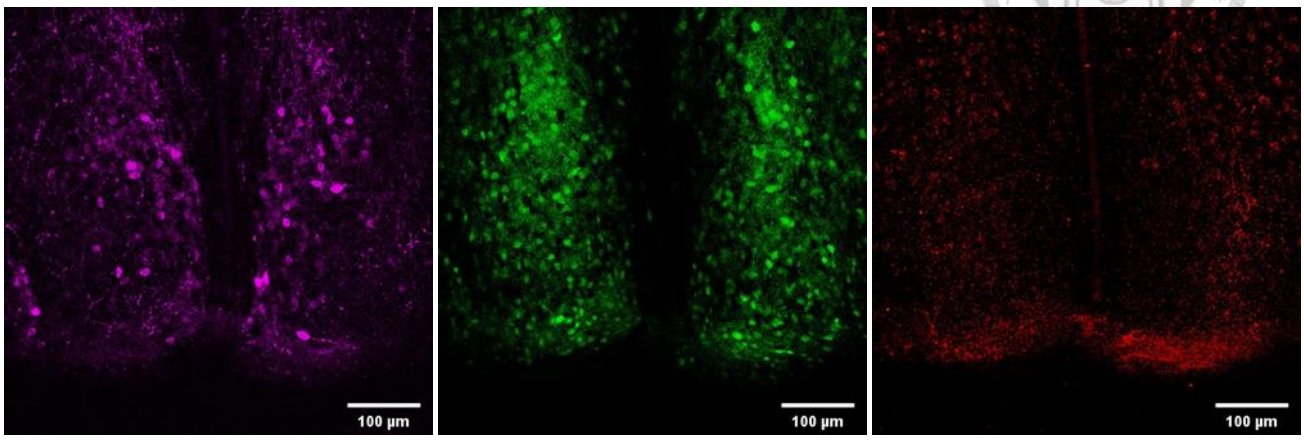

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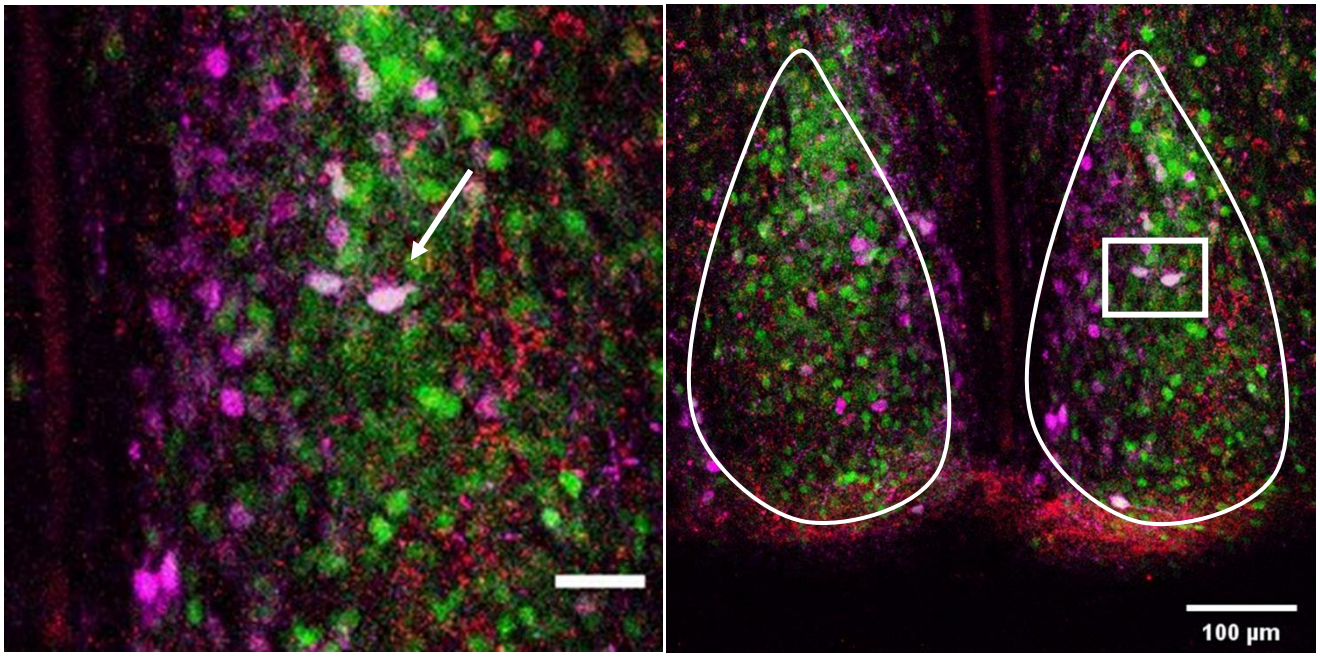

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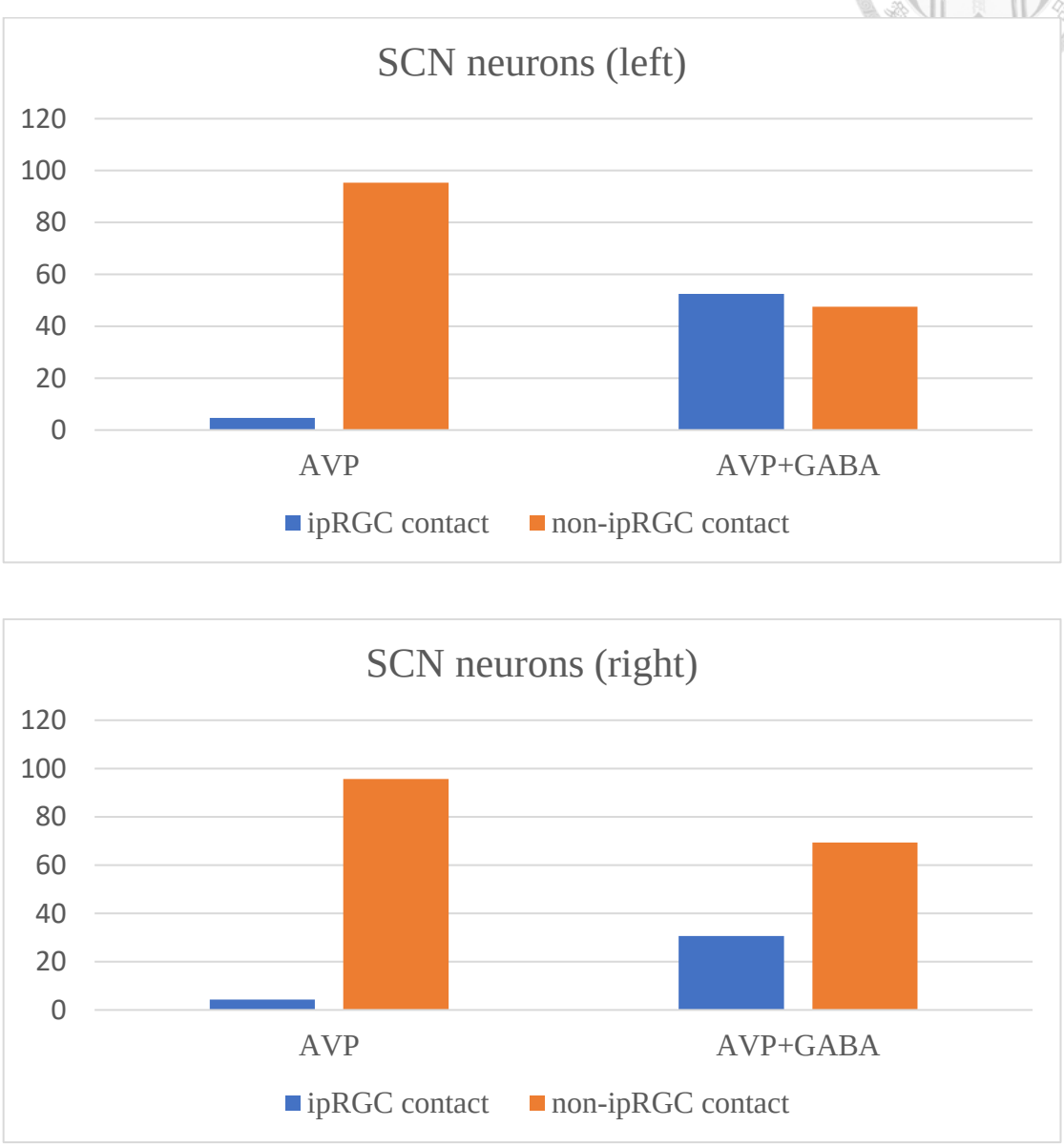

Figure 1. The representative distribution of ipRGCs to AVP neurons in SCN

(A) The GFP is expressed in the GABAergic neurons, which represent the SCN neurons. While magenta shows the staining of AVP neurons, red (Cholera Toxin Subunit B) indicates the expression of ipRGCs. (B) The white line circles the SCN

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region, while the white box shows the region of the enlarged picture. The white arrow points out the representative AVP neurons that are close to ipRGCs fiber and colocalized to GFP. (C) At least 20 percent of AVP neurons contact with ipRGC on both sides of SCN region. AVP neurons that are colocalized to GABAergic neurons exhibit a stronger connection to ipRGCs, compared to AVP neurons that express alone (n=1).

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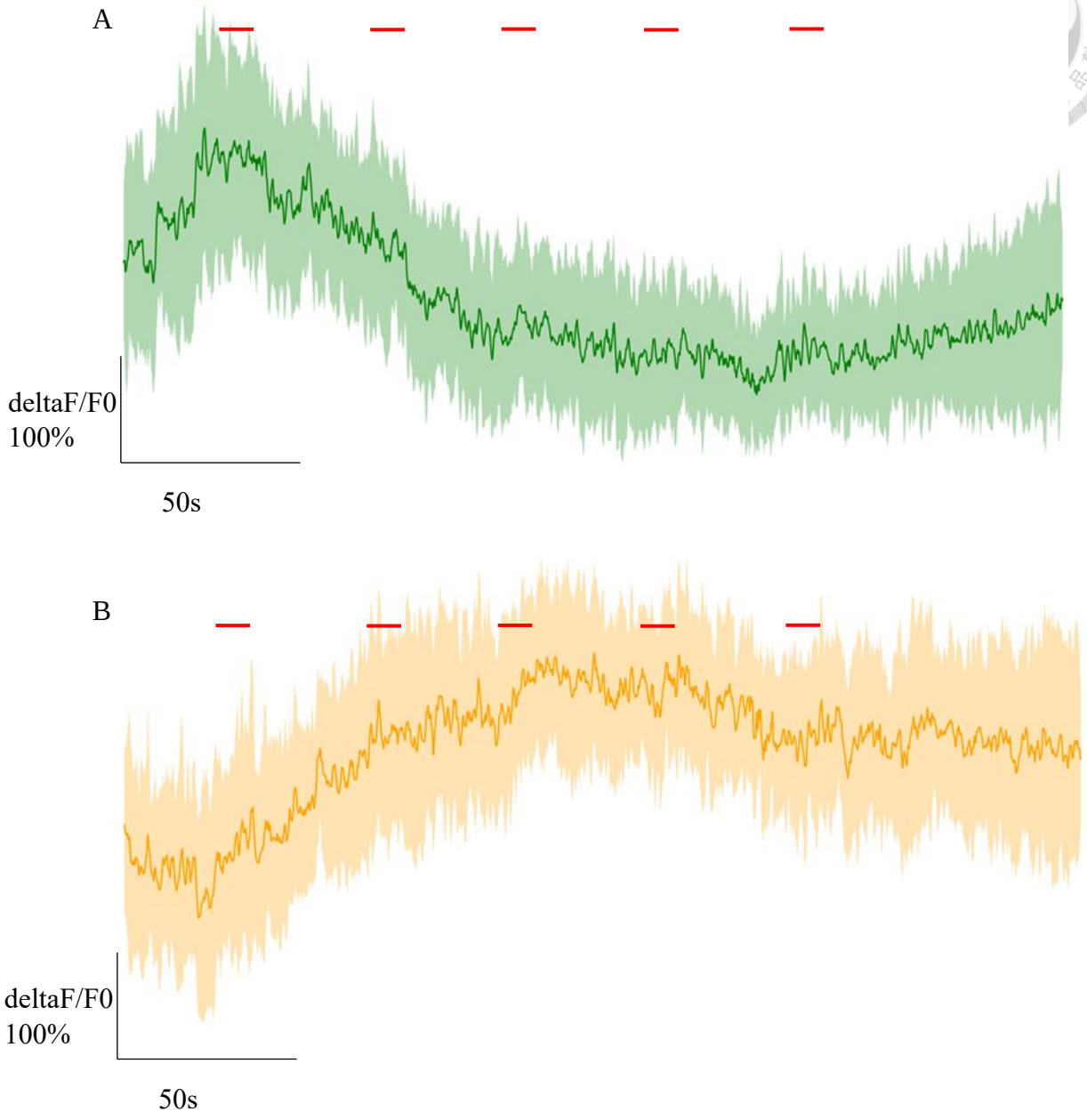

Figure 2. Activation of ipRGC induces different responses in AVP neurons

This is the result of the mean intensity of the entire recording. The red light indicates the light stimulation. There are two distinct patterns of response among AVP neurons. (A) In group 1, there was an initial increase in fluorescence intensity during the first

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light stimulation, followed by a subsequent decrease. (B) In the group 2, there was a gradual augmentation of calcium activity with successive light stimulations. (n=1)

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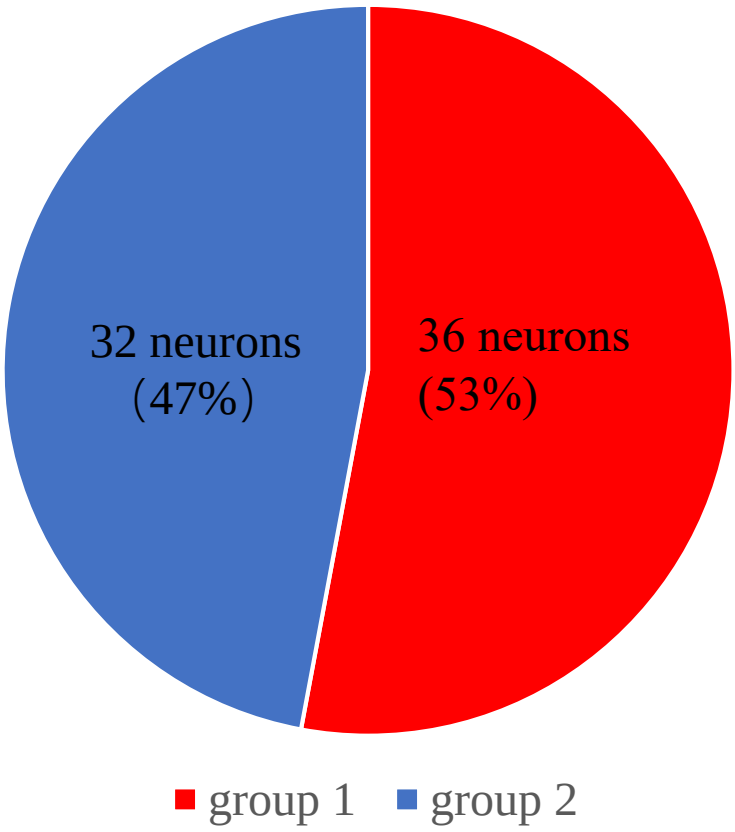

Figure 3. Response of AVP neurons under light stimulation can be differentiated into 2 groups.

Under light stimulation without drugs, group 1 neurons include 47 percent of AVP neurons, while 53 percent of AVP neurons categorized as group 2 neurons.

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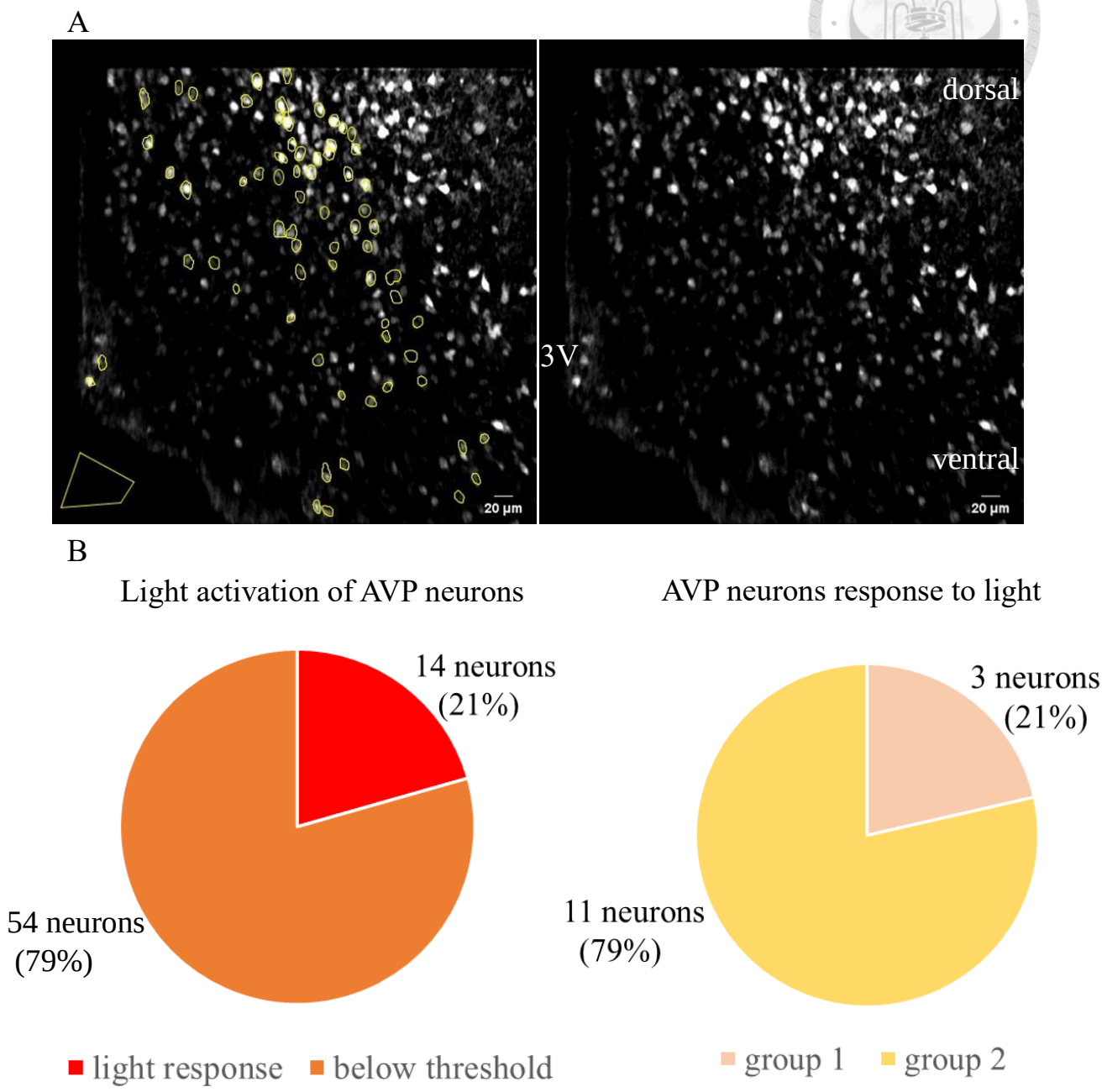

Figure 4. The majority of activated neurons under light conditions without drugs fell into group 2

(A) These show the recording picture of two-photon imaging with ROI and background (a quadrilateral). (B) The pie chart shows the percentage of activated ROI in light stimulation. Most of the AVP neurons that show light response are belonged to

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group 2. (n=1)

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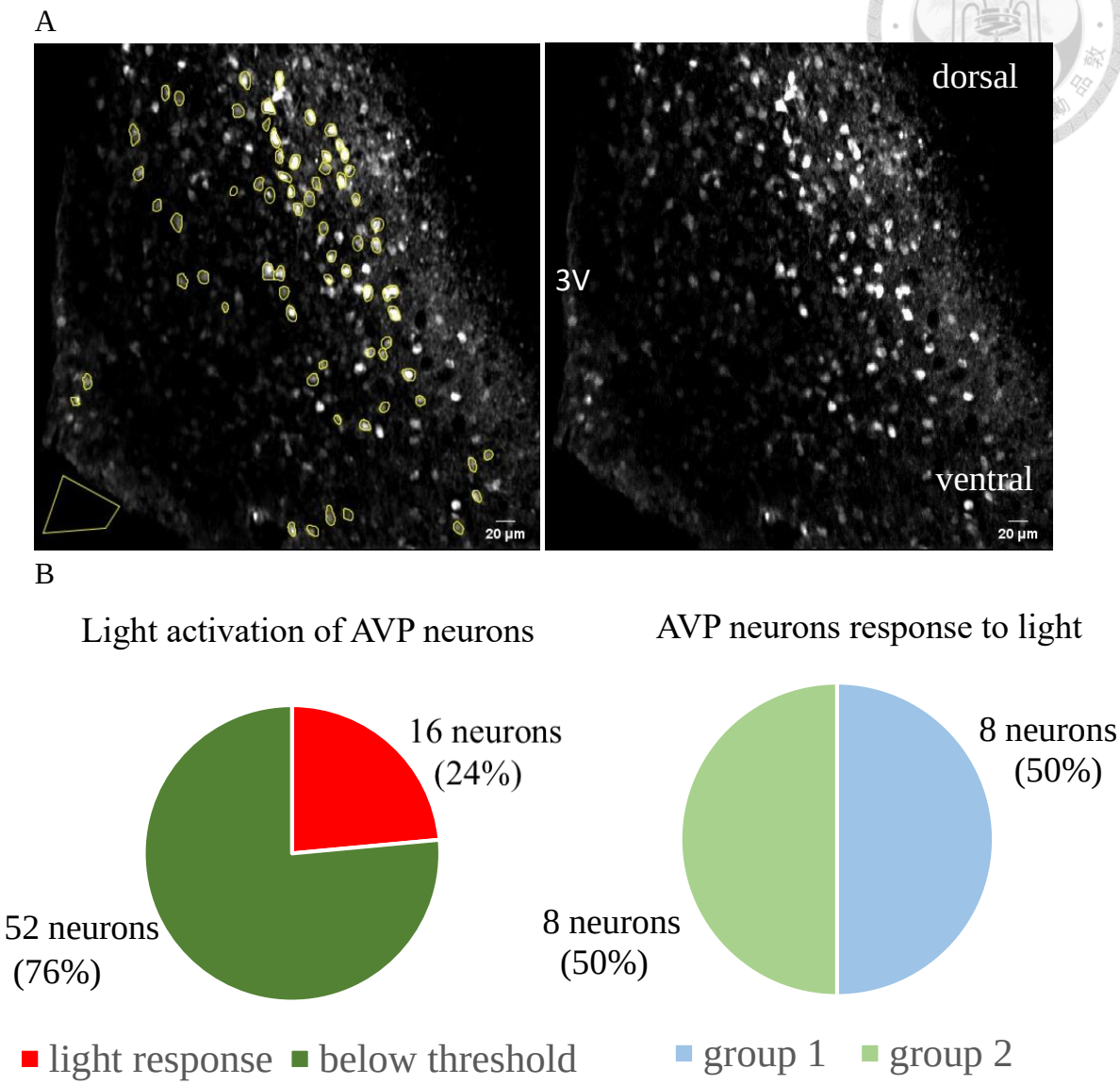

Figure 5. AVP neurons were activated in TTX and 4AP when ipRGCs were stimulated

(A) These show the recording picture of two-photon imaging with ROI and background (a quadrilateral). (B) The pie chart shows the percentage of activated ROI in light stimulation under TTX and 4AP. 24 percent of the AVP neurons show light response. The stimulated neurons belong to two groups equally. (n=1)

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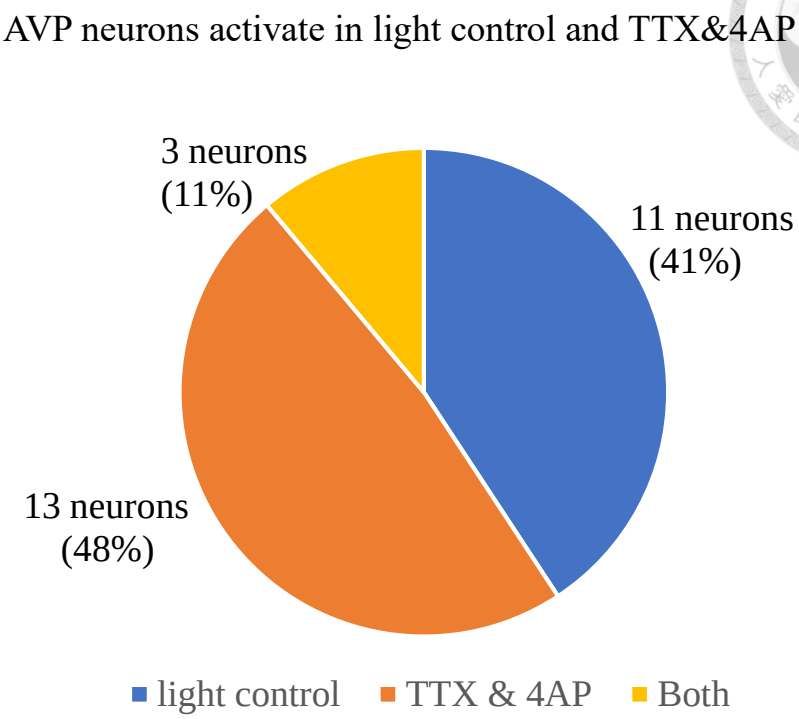

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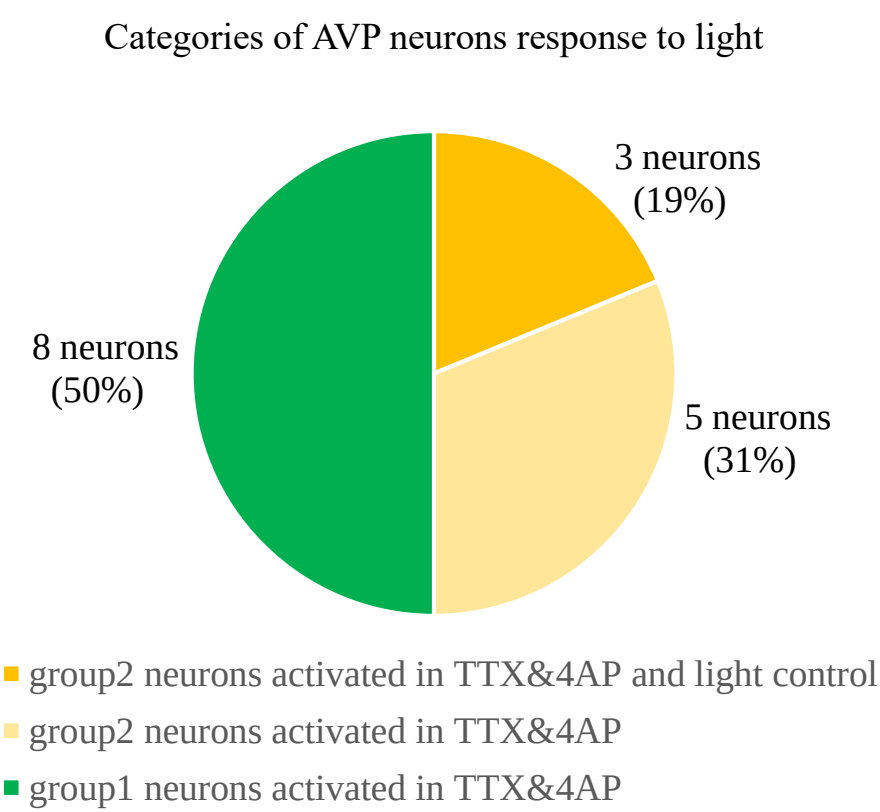

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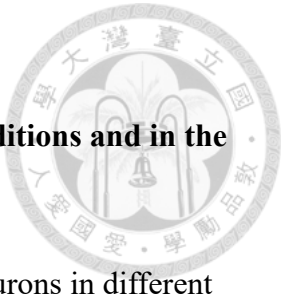

Figure 6. Activation patterns of neurons under normal light conditions and in the presence of TTX and 4AP.

The pie chart shows the comparison percentage of activated AVP neurons in different conditions. (A) A small population of AVP neurons was activated in both conditions. 41 percent of AVP neurons activated during light control solely, while 48 percent of AVP neurons responded under the drug condition only. (B) Group 2 neurons include AVP neurons that are activated in both conditions. Most activated AVP neurons under drug conditions belong to group 1.

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Control group (without light stimulation and drug)

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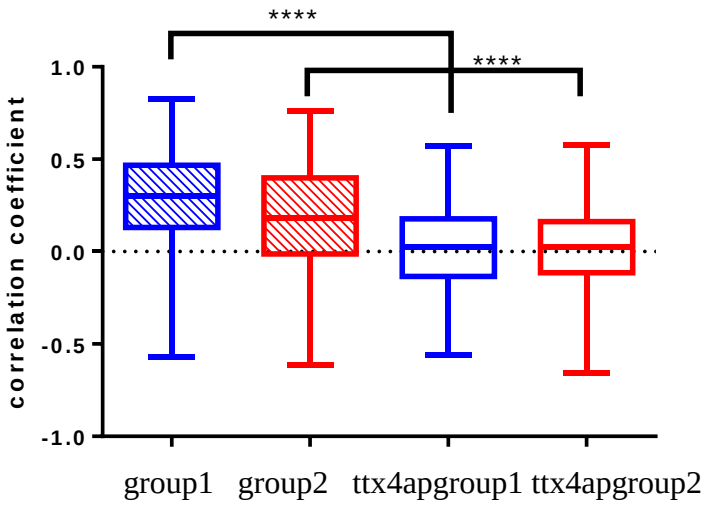

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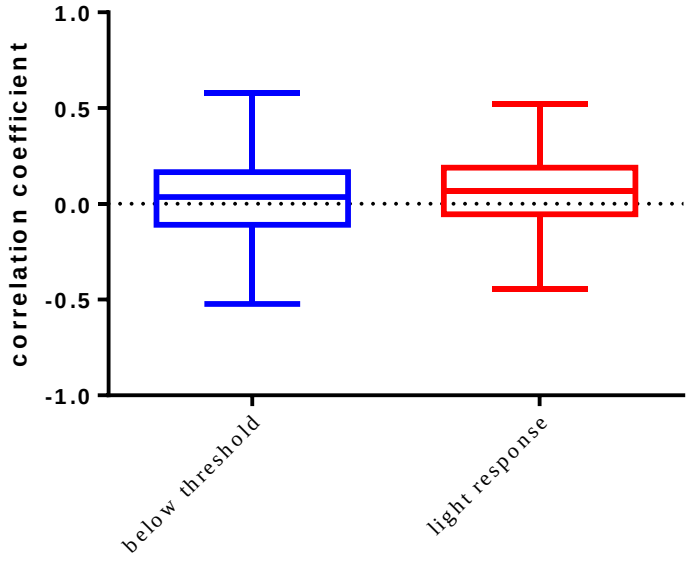

Figure 7. Correlation coefficient of AVP neurons

The plots show correlation coefficient of AVP neurons in different conditions. (A) The AVP neurons in control have a significant higher correlation compare to the neurons in TTX and 4AP (Wilcoxon matched-pairs signed rank test, P value< 0.0001). (B)

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AVP neurons that express light response have higher neurons' correlation than neurons that do not respond (Unpaired t test with Welch's correction, P value = 0.0655).

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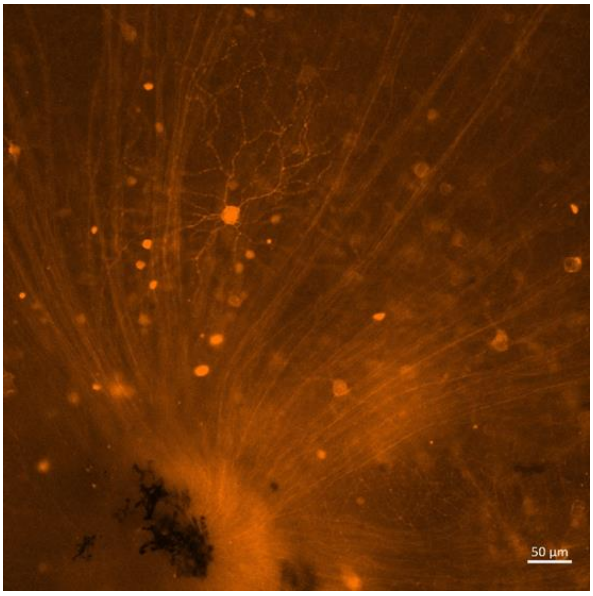

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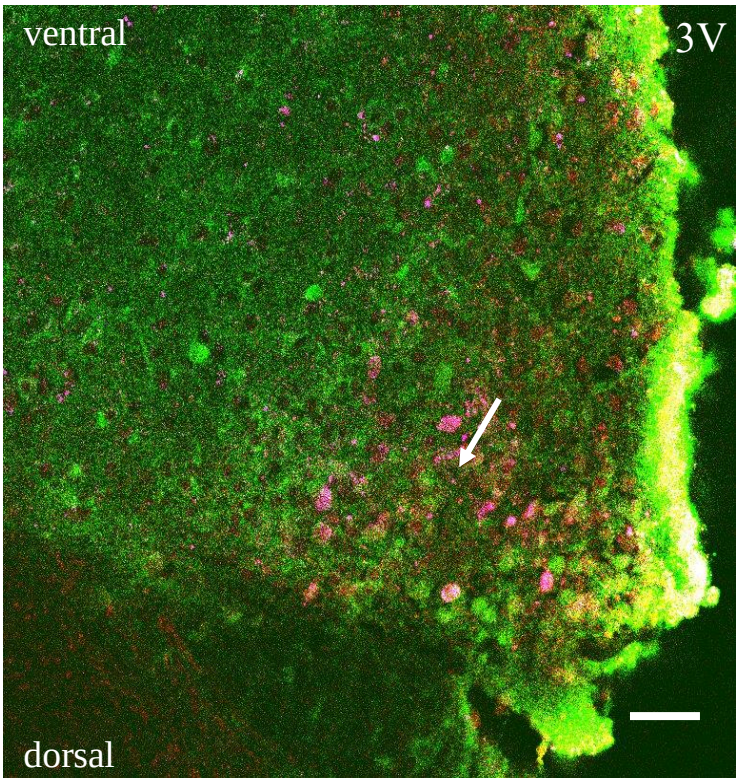

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Figure 8. Representative picture of AVP-Cre mice with GCaMP7f injection for calcium imaging recording

(A) It shows the retina with ChrimsonR-Tdtomato injection. (B) Red colour represents ipRGC, green represents the GCaMP, and magenta represents the AVP immunostaining after recording. The white bar indicates 40μm, and the white arrow points out one of the AVP antibodies colocalized to the GCaMP.

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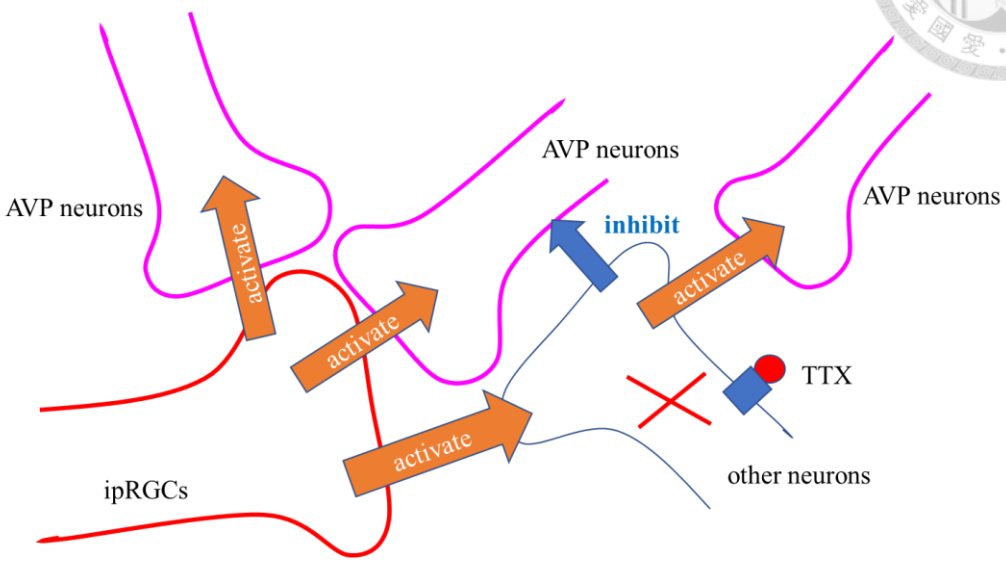

Figure 9. Representative picture of an inhibitory loop in ipRGCs and AVP neurons

We propose the idea of an inhibitory loop in the circuitry involving ipRGCs and AVP neurons in the SCN. Based on our findings, AVP neurons in response to ipRGC activation can be categorized into three distinct groups. First, a population of AVP neurons is activated through the activation of interneurons that are stimulated by ipRGCs. Second, a group of AVP neurons receives signals directly from ipRGCs. Third, AVP neurons are both activated by ipRGCs and receive inhibitory signals from other neurons. Under normal conditions, the inhibitory signals counterbalance the excitatory input from ipRGCs, resulting in no net activation of these AVP neurons. However, under TTX conditions, which block sodium channels and inhibit interneuronal activity, these AVP neurons will be activated.

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Appendix Abstract and Poster

Abstract of FENS 2024

Presentation Style: Poster

Abstract number: PS01-26AM-377

Presentation time: Wednesday, 26/6/2024, 11:30-13:00

IpRGCs Modulate Calcium Response of AVP neurons in Central Clock

Chi Wen Liong[1], But Yip Chun[2], Chi-Kuang Sun[2], Shih-Kuo Chen[1]

Circadian rhythms modulate our daily activity patterns and other body mechanisms, such as metabolic and neuroendocrine rhythms. In mammals, circadian rhythms are controlled by the central clock, suprachiasmatic nuclei (SCN). The external light signal could entrain SCN through intrinsically photosensitive retinal ganglion cells (ipRGCs). SCN contains different neurons, while AVP and VIP neurons are critical for the networking of circadian rhythm. AVP neurons are mainly expressed in shell region of SCN, while VIP neurons are expressed in core region. In a previous study, VIP neurons are suggested as light signal receiving neurons, and AVP neurons are the primary output neurons for the SCN. However, in single ipRGC tracing study suggested that ipRGCs innervation does not limit to VIP neurons specifically but throughout the whole SCN. In addition, the mechanisms of how SCN neurons communicate with each other's remain unknown. To know whether ipRGC and AVP neurons in SCN are a monosynaptic pathway, we use GCaMP6s, a calcium sensor, to image the neural activity in SCN under optogenetic stimulation. The results show that AVP neurons receive signals from ipRGCs and exhibit different responses. Our results suggested that AVP neurons may also receive direct ipRGC input from the retina for circadian clock regulation.

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IpRGCs Modulate Calcium Response of AVP neurons in Central Clock

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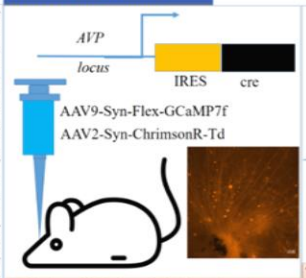
Presentation number: 850

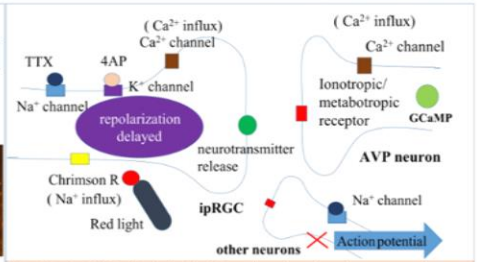
Abstract

Circadian rhythms modulate our daily activity patterns and other body mechanisms, such as metabolic and neuroendocrine rhythms. In mammals, circadian rhythms are controlled by the central clock, suprachiasmatic nuclei (SCN). The external light signal could entrain SCN through intrinsically photosensitive retinal ganglion cells (ipRGCs). SCN contains different neurons, while AVP and VIP neurons are critical for the networking of circadian rhythm. AVP neurons are mainly expressed in shell region of SCN, while VIP neurons are expressed in core region. In a previous study, VIP neurons are suggested as light signal receiving neurons, and AVP neurons are the primary output neurons for the SCN. However, in single ipRGC tracing study suggested that ipRGCs innervation does not limit to VIP neurons specifically but throughout the whole SCN. In addition, the mechanisms of how SCN neurons communicate with each other's remain unknown. To know whether ipRGC and AVP neurons in SCN are a monosynaptic pathway, we use GCaMP6s, a calcium sensor, to image the neural activity in SCN under optogenetic stimulation. The results show that AVP neurons receive signals from ipRGCs and exhibit different responses. Our results suggested that AVP neurons may also receive direct ipRGC input from the retina for circadian clock regulation.

Material and Method

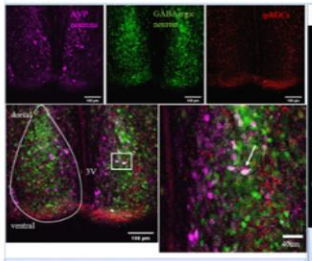


AAV virus with Chrimson R is injected on mice retina.AAV virus with GCaMP7f expressed in AVP neurons of transgene mice, AVP cre mice.



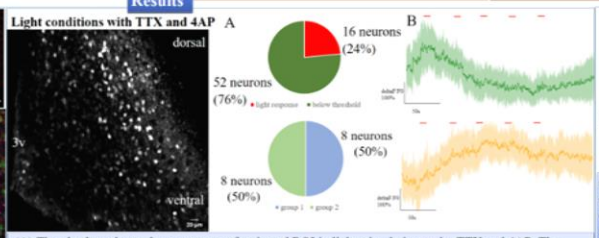
Tetrodotoxin (TTX) selectively block the sodium channels on the nerve membrane, action potentials of other neurons were blocked. 4AP, 4-aminopyridine, block selectively potassium channels in nerve membranes, delayed repolarization. Any light-evoked response that remains under TTX and 4AP, must be monosynaptic.

Results



The white line circles the SCN region, while the white box shows the region of the enlarged picture. The white arrow points out the representative AVP neurons that are close to ipRGCs fiber and colocalized to GFP.

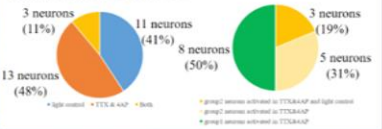
Light conditions with TTX and 4AP



(A) The pie chart shows the percentage of activated ROI in light stimulation under TTX and 4AP. The stimulated neurons belong to two groups equally. .

(B) This is the result of the mean intensity of the entire recording. The red light indicates the light stimulation. There are two distinct patterns of response among AVP neurons. In group 1, there was an initial increase in fluorescence intensity during the first light stimulation, followed by a subsequent decrease. In the group 2, there was a gradual augmentation of calcium activity with successive light stimulations.

Activation patterns of neurons under normal light conditions and in the presence of TTX and 4AP



The pie chart shows the comparison percentage of activated AVP neurons in different conditions. A small population of AVP neurons was activated in both conditions. Most activated AVP neurons under drug conditions belong to group 1.

Conclusion

1. **Activation of ipRGCs induce calcium response in a population of AVP neurons, and indicates a monosynaptic pathway.**
2. **Consistent with the previous studies (Fernandez et al., 2016), ipRGCs are not projected to restricted areas but throughout the SCN, include AVP neurons in shell region.**
3. **Connections and projections of ipRGCs in the SCN will reveal the mechanisms of circadian rhythm and findings concerning the central clock pathway.**

Acknowledgement

- The project was supported by the Taiwan Ministry of Science and Technology grant MOST 103-2628-B-002-001-MY3 and 111-2636-B-002-021 (to S.K.C.)
- Thank you to my advisor, and all my lab members who have supported me furthering my research.

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