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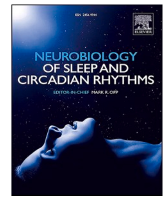

Activation of mGluR1 negatively modulates glutamate-induced phase shifts of the circadian pacemaker in the mouse suprachiasmatic nucleus

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ABSTRACT

In mammals, photic information delivered to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT) plays a crucial role in synchronizing the master circadian clock located in the SCN to the solar cycle. It is well known that glutamate released from the RHT terminals initiates the synchronizing process by activating ionotropic glutamate receptors (iGluRs) on retinorecipient SCN neurons. The potential role of metabotropic glutamate receptors (mGluRs) in modulating this signaling pathway has received less attention. In this study, using extracellular single-unit recordings in mouse SCN slices, we investigated the possible roles of the $G_{q/11}$ protein-coupled mGluRs, mGluR1 and mGluR5, in photic resetting. We found that mGluR1 activation in the early night produced phase advances in neural activity rhythms in the SCN, while activation in the late night produced phase delays. In contrast, mGluR5 activation had no significant effect on the phase of these rhythms. Interestingly, mGluR1 activation antagonized phase shifts induced by glutamate through a mechanism that was dependent upon $Ca_V1.3$ L-type voltage-gated Ca^{2+} channels (VGCCs). While both mGluR1-evoked phase delays and advances were inhibited by knockout (KO) of $Ca_V1.3$ L-type VGCCs, different signaling pathways appeared to be involved in mediating these effects, with mGluR1 working via protein kinase G in the early night and via protein kinase A signaling in the late night. We conclude that, in the mouse SCN, mGluR1s function to negatively modulate glutamate-evoked phase shifts.

1. Introduction

In mammals, the master circadian clock drives daily rhythms in diverse endocrine, physiological and behavioral processes, and it is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Welsh et al., 2010; Slat et al., 2013; Pauls et al., 2016; Buijs et al., 2021). This clock receives photic information, which plays a dominant role in synchronizing the internal pacemaker to the solar cycle. This occurs via the retinohypothalamic tract (RHT), a direct monosynaptic projection from retinal ganglion cells to the SCN, and via the geniculo-hypothalamic tract, an indirect projection from the intergeniculate leaflet of the lateral geniculate nuclei of the thalamus (Golombek and Rosenstein, 2010; Ashton et al., 2022).

Both *in vivo* and *in vitro* studies conducted in rodents have provided strong evidence that glutamate released from RHT terminals acts on N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, which are ionotropic glutamate receptors (iGluRs) that mediate the synchronizing effects of light. In rat hypothalamic slices, electrical stimulation of the optic nerve induces glutamate release (Liou et al., 1986) and elicits excitatory postsynaptic potentials in SCN neurons that are blocked by iGluR antagonists (Kim and Dudek, 1991). In the mouse and hamster, light-induced phase shifts in circadian motor activity rhythms are blocked by iGluR antagonists applied systemically or centrally (Colwell et al., 1990; Colwell et al., 1991; Colwell and Menaker, 1992; Rea et al., 1993). The local infusion of AMPA into the mouse SCN *in vivo* during the subjective night causes a phase delay in wheel-running activity, an effect that is eliminated by the co-administration of an AMPA receptor antagonist (Mizoro et al., 2010). Meanwhile, glutamate or NMDA applied to rat SCN slices causes phase shifts in circadian neural activity rhythms that are similar in phase-response relationship to the shifts of circadian motor activity rhythms elicited by light *in vivo* (Summer et al., 1984) and are blocked by iGluR antagonists (Ding et al., 1994).

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Abbreviations

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	one-way analysis of variance
DHPG	(S)-3,5-dihydroxyphenylglycine
DMSO	dimethyl sulfoxide
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid
iGluRs	ionotropic glutamate receptors
KO	knockout
mGluRs	metabotropic glutamate receptors
NMDA	N-methyl-D-aspartate
MPEP	2-methyl-6-(phenylethynyl)pyridine
PKA	protein kinase A
PKG	protein kinase G
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
t-ACPD	(±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid
VGCC	voltage-gated Ca^{2+} channel
ZT	zeitgeber time

Previous work suggests that metabotropic glutamate receptors (mGluRs) in the SCN may also have roles in modulating the effects of light on the SCN. A variety of studies using *in situ* hybridization and immunohistochemical techniques have demonstrated that mGluRs are expressed in the rodent SCN (Shigemoto et al., 1992; Ohishi et al., 1993a, 1993b; Gannon and Rea, 1994; van den Pol, 1994; Mick et al., 1995; Romano et al., 1995; Ghosh et al., 1997). Interestingly, mGluR1 is distributed on perikaryal and dendritic membranes, sometimes associated with synapses and sometimes not (van den Pol, 1994). Physiological analysis indicates that broad spectrum mGluR agonists inhibited kainate- or NMDA-evoked Ca^{2+} responses in rat SCN neurons (Haak, 1999). Furthermore, behavioral pharmacology experiments have found that mGluR agonists can modulate light-evoked phase shifts in hamster circadian activity rhythms (Haak et al., 2006; Gannon and Millan, 2011). Although these data from anatomical and pharmacological studies point to the possibility that mGluRs have some modulatory role (s) for glutamate-mediated photic resetting of the circadian clock, the question of whether mGluRs impact neural activity rhythms recorded in the SCN has not been addressed. In the current study, we tested the hypothesis that glutamate acts on mGluRs to alter the phase of the circadian pacemaker in the SCN and, in doing so, it may modulate the pacemaker-synchronizing effect of light mediated by iGluRs. We also examined the role of $Ca_V1.3$ L-type voltage-gated Ca^{2+} channels (VGCCs) and the signaling pathways involved in mediating these effects.

2. Materials and methods

2.1. Animals and housing

Male C57BL/6 mice (B6 mice, 4–8 weeks) bred in Korea Institute of Science and Technology were used for extracellular single-unit recording and Ca^{2+} imaging experiments. These mice served as the wild-type (WT) controls for these experiments. In addition, male knockout (KO) mice (4–8 weeks) for mGluR1 (Conquet et al., 1994) or $Ca_V1.3$ VGCC (Kim et al., 2015), having the genetic background of C57BL/6, were used in some of the electrophysiological experiments. Mice were housed in group cages (4–5/cage) in a temperature-controlled room (22–24 °C) with a 12 h:12 h light/dark cycle for ≥7 days. Zeitgeber time (ZT) 0:00 h was designated as the time of lights-on and ZT 12:00 h as the time of lights-off in the animal colony.

2.2. Brain slice preparation

Brain slices were prepared as described previously (Kim et al., 2015). In brief, the mouse was anesthetized with urethane (1.25 g/kg, i.p.) between ZT 11:00 h and 11:30 h, and the brain was quickly excised from the skull of the anesthetized mouse and submerged in 95% CO_2/5% O_2 saturated ice-cold artificial cerebrospinal fluid (ACSF), composed of (in mM) 124 NaCl, 1.3 $MgSO_4$, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2.4 $CaCl_2$ and 10 glucose. After being chilled in ice-cold ACSF for 1–2 min, the brain was trimmed to a block containing the hypothalamus. With the use of a vibrotome (7000smz-2, Campden Instruments, UK), slices (400-μm thickness) containing the SCN were cut in ice-cold ACSF in coronal orientation from the tissue block. The slices were kept in aerated (95% O_2/5% CO_2) ACSF for 30–60 min at room temperature before being transferred to a custom-made gas interface type electrophysiological recording chamber, which was superfused continuously with warm (35 °C) ACSF aerated with 95% O_2/5% CO_2 (pH 7.3–7.5).

2.3. Ca^{2+} imaging

Ca^{2+} imaging experiments were conducted with SCN cells dissociated as described before (Kim et al., 2015). Dissociated cells plated on Poly-D-lysine (Sigma-Aldrich, MO)-coated glass coverslips were loaded with fura-2 AM (Invitrogen, CA, USA) by incubating the coverslip in fura-2 AM (5 μM)-containing HEPES-buffered saline [composed of (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH 7.4] for 30 min at room temperature. Then, the coverslip was rinsed with HEPES-buffered saline and transferred to the Ca^{2+}-imaging chamber (RC-25; Warner Instrument, Hamden, CT, USA), which was superfused continuously (1–1.5 ml/min) with Mg^{2+}-free HEPES-buffered saline. The Ca^{2+} responses of dissociated SCN cells to a mixture of 100 μM glycine and 100 μM NMDA were used as a positive control in Ca^{2+} imaging tests; glycine acts as a co-agonist of NMDA receptor (Johnson and Ascher, 1987). Background subtracted intensity images at two excitation wavelengths (340 and 380 nm) were acquired using an EMCCD camera (Andor Technology, UK) and Axon Imaging Work Bench 5.1 image processing program (Indec Systems, CA, USA). Intracellular calcium concentration ($[Ca^{2+}]_i$) was expressed as the ratio of the fura-2 emission fluorescence intensities of 510 nm, excited at 340 and 380 nm.

2.4. Experimental treatment and extracellular single-unit recording

On the first day *in vitro*, the SCN slices were either treated with drugs or left untreated. The drugs were bath-applied separately or in combination for 30 min, the application starting at ZT 14:00 h or ZT 22:00 h. On the next day (i.e., second day *in vitro*), extracellular single-unit recordings were obtained from the SCN. Micropipettes (7–12 MΩ) pulled from borosilicate glass capillary and filled with ACSF were used as recording electrodes. Single-unit recordings were conducted without restricting the electrode tip to any particular region of the SCN. The voltage signals from recording electrodes were fed into a multiclamp 700A (Molecular Devices, CA, USA). The signals were digitized and sampled at 50-μs intervals with Digidata 1440A and pClamp 10.7 (Molecular Devices, CA, USA). The criteria used to identify single units were consistent waveform and spike amplitude. Six to eight single units were sampled every hour, each unit being recorded for a 1-min period. To detect the time-of-peak of circadian firing activity rhythm of SCN neurons, which is a reliable marker of the phase of circadian pacemaker (Chen et al., 1999), we plotted against ZT the mean firing rates of single units sampled for sequential 2-h periods with 1-h lags. In order to quantify the phase shift induced by a drug treatment, we compared the time-of-peak detected in the drug-treated slice with the average time-of-peak of control slices. The difference was taken as the amount of drug-induced phase shift.

2.5. Drugs

All drugs and chemicals used in the current study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Tocris Bioscience (Bristol, UK). The solutions of hydrophilic drugs were prepared with ACSF (electrophysiology) or Mg^{2+}-free HEPES-buffered saline (Ca^{2+} imaging), while the solutions of hydrophobic drugs were prepared by diluting their stock solutions with ACSF [stock solution solvent: dimethyl sulfoxide (DMSO), final concentration of DMSO: 0.01–0.003%]. Drugs used include (S)-3,5-dihydroxyphenylglycine (DHPG, 30 μM), gallein (100 μM), glutamate (100 μM), H89 (10 μM), KT5823 (0.5 μM), LY367385 (100 μM), 2-methyl-6-(phenylethynyl)pyridine (MPEP, 10 μM), nimodipine (6 μM).

2.6. Statistical analysis

Numerical data are expressed as the mean ± SEM. Student *t*-test and Mann–Whitney Rank Sum test were used for the comparison of two independent data sets with and without normal distribution, respectively, while paired *t*-test was used for a paired dependent data set. One-way analysis of variance (ANOVA) and pairwise comparison with Newman-Keuls tests were performed to compare ≥3 independent data sets with normal distributions, while Kruskal-Wallis one-way ANOVA on ranks and pairwise comparison with Newman-Keuls tests were performed to compare ≥3 data sets without normal distributions. $P < 0.05$ (two-sided) was considered to be significant.

2.7. Study approval

The experimental procedures described above were approved by the Korea University College of Medicine Animal Research Policies Committee and Korea Institute of Science and Technology, while conforming to National Institutes of Health guidelines.

3. Results

3.1. The mGluR1/mGluR5 agonist DHPG phase-shifts the circadian pacemaker in the SCN

We first asked whether the application of the mGluR1/mGluR5 agonist DHPG affected circadian neural activity rhythms recorded in mouse SCN slices. As previously established (Ding et al., 1994; Kim et al., 2015), neural activity rhythms recorded from the SCN peaks at ZT 6 h (ZT 6.0 ± 0.3 h, n = 6). This value serves as a control and phase reference point for all other experiments. In SCN slices treated with DHPG (30 μM) in the early night (ZT 14 h), the peak of the rhythm was advanced by 2 h to ~ZT 4 h (Fig. 1A and I, n = 6). The same DHPG treatment applied in the late night (ZT 22 h) delayed the peak by 2 h to ~ZT 8 h (Fig. 1B and J, n = 5). The phase-shifting effects of DHPG are the opposite to those produced by glutamate (Fig. 1C, D, 1I and 1J), which causes delays in the early night and advances in the late night (Ding et al., 1994; Shibata et al., 1994; Kim et al., 2005; Mizoro et al., 2010). The phase shifting effects of DHPG were blocked by pretreatment of the SCN slice with the mGluR1 antagonist LY367385, but not by the mGluR5 antagonist MPEP (Fig. 1E–J). LY367385 alone had no significant effect on the phase of the SCN neuronal activity rhythm, and the peak of electrical activity in SCN slices treated with LY367385 (ZT 6.0 ± 0.0 h, n = 4) was not significantly different from that of untreated control slices (ZT 6.0 ± 0.3 h, n = 6) (P = 1.000, Mann-Whitney Rank Sum test). Thus, these experiments indicate that the mGluR group I agonist DHPG produces phase shifts in neural activity rhythms recorded in the SCN through a mechanism dependent upon mGluR1, but not mGluR5, activation.

3.2. $Ca_V1.3$ VGCCs play a crucial role in mGluR1-mediated phase shifts

Prior work has demonstrated that mGluR1 activation can modulate L-type VGCCs (Endoh, 2004; Zheng and Raman, 2011), and we sought to determine the role of these channels in mediating mGluR1-evoked phase shifts in the SCN. First, we examined the effects of DHPG (30 μM, focal application) on intracellular Ca^{2+} levels in acutely dissociated SCN cells in the subjective night. DHPG elicited Ca^{2+} transients that were comparable in magnitude to, and longer-lasting than, those evoked by NMDA (100 μM, focal application) (Fig. 2A). These DHPG-evoked Ca^{2+} transients were abolished when the cells were in Ca^{2+}-free medium containing EGTA (100 μM) (Fig. 2; 93.5 ± 2.4% blockade, n = 16). Next, we examined the impact of the L-type VGCC blocker nimodipine on phase shifts induced by DHPG. Pretreatment of the SCN slice with nimodipine (6 μM) completely blocked the DHPG-, as well as glutamate-, induced phase delays and advances (Fig. 3). Nimodipine alone had no significant effect on the phase of SCN neuronal activity rhythms (Nimodipine: ZT 6.2 ± 0.3 h, n = 6; Control: ZT 6.0 ± 0.3 h, n = 6; p = 0.699, Mann–Whitney Rank Sum Test). Finally, in order to determine the specific calcium channel responsible for DHPG-induced phase shifts, we utilized $Ca_V1.3$ L-type VGCC KO mice (Kim et al., 2015). The SCN neural activity rhythms were not impacted by the loss of $Ca_V1.3$ (KO: ZT 6.0 ± 0.3 h, n = 6; WT: ZT 6.0 ± 0.3 h, n = 6; p = 1.000, Mann–Whitney Rank Sum Test). However, the application of DHPG produced no phase shift in neural activity rhythms in SCN slices from the KO mice (Fig. 3). Our results indicate that Ca^{2+} influx through $Ca_V1.3$ L-type VGCCs is essential for the phase shifts induced by mGluR1 activation. This data suggests that mGluR1 activation leads to a Ca^{2+} influx from extracellular spaces and causes phase shifts through a mechanism completely dependent upon $Ca_V1.3$ L-type VGCCs.

3.3. Gβγ-protein kinase A (PKA) and Gβγ-protein kinase G (PKG) signal transduction pathways are involved in mGluR1-mediated phase shifts

Prior work has established that mGluR1 is a $G_{q/11}$ protein-coupled receptor, which, among other actions, activates second messenger cascades to evoke intracellular responses (Mao et al., 2022). We found that the Gβγ inhibitor gallein blocked both the phase advances and delays induced by DHPG (Fig. 4). Gβγ is well known to activate PKA (Marley et al., 1991; Maruko et al., 2005), and it may activate PKG too. Thus, we evaluated PKA and PKG inhibitors on the DHPG-evoked phase shifts. We found that the PKA inhibitor H89 blocked DHPG-evoked phase delays, but not phase advances (Fig. 4). In contrast, the PKG inhibitor KT5823 blocked DHPG-evoked phase advances, but not phase delays (Fig. 4). By themselves, gallein, H89, and KT5823 had no significant effect on the phase of SCN neuronal activity rhythms compared to untreated controls [gallein: ZT 6.5 ± 0.6 h, n = 4; P = 0.242, *t*-test; H89: ZT 5.8 ± 0.3 h, n = 4; P = 0.610, Mann-Whitney Rank Sum Test; KT5823: ZT 6.2 ± 0.2 h, n = 6; P = 0.669, Mann-Whitney Rank Sum Test]. Together, these results indicate that Gβγ-PKA signal transduction pathway is critical for mGluR1-evoked phase delays. In addition, the results raise the possibility that Gβγ and PKG are coupled to mediate mGluR1-evoked phase advances.

3.4. Activation of mGluR1 antagonizes glutamate-mediated phase shifts

Activation of mGluRs is known modulate the strength and timing of excitatory transmission in hippocampus (Cosgrove et al., 2011), striatum (Colwell et al., 1996), and perhaps even in the SCN (Haak, 1999). Therefore, in our final set of experiments, we sought to determine whether mGluR1 activation modulates glutamate-mediated phase shifts. Glutamate-induced phase shifts were completely blocked by DHPG pretreatment of the SCN slice (Fig. 5). Consistent with our prior findings, glutamate-induced phase delays were ~3 h and potentiated by the pretreatment of the mGluR1 blocker LY367385, mGluR1 KO, $Ca_V1.3$ KO, and the PKG inhibitor KT5823 (Fig. 5, left panel).

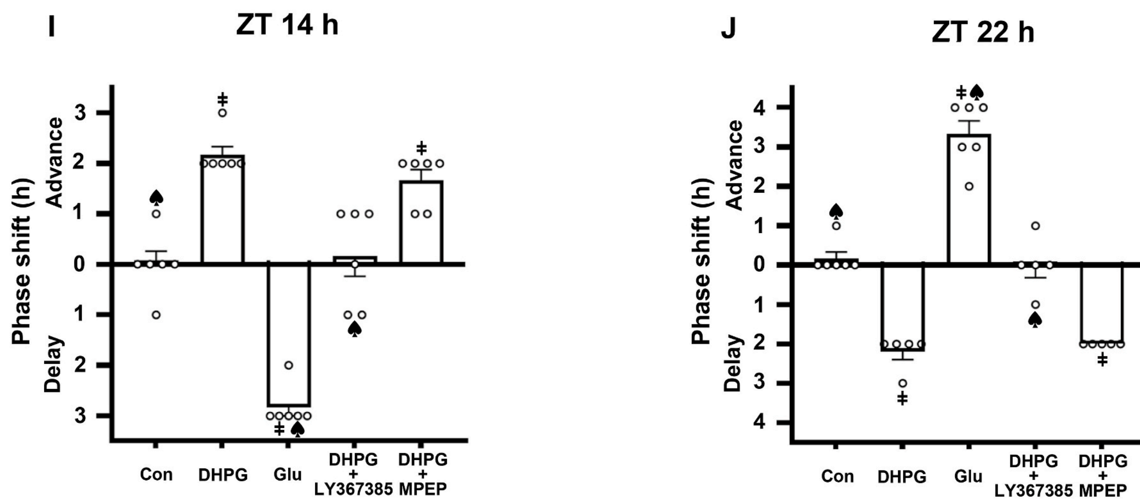

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Fig. 1. The mGluR agonist DHPG phase-shifts the circadian pacemaker in the SCN through a mechanism depend upon mGluR1. Neural activity rhythms recorded from SCN illustrating the phase shifting effects of the treatment (A–H). Each plot shows the representative result of 6 repeated experiments. The projected dark and light phases of the animal room are indicated with filled and open horizontal bars, respectively. The dashed vertical line in each plot indicates the average time of peak neural activity for control slices. The red arrow indicates the time of drug application (ZT 14 h or ZT 22 h). (I & J) Graphs summarizing the phase shifts evoked by experimental treatments on the time of peak of circadian neural activity rhythm. Data are expressed as the mean ± SEM. Newman-Keuls comparison tests were performed after Kruskal-Wallis one-way ANOVA ($P \leq 0.001$; I) or one-way ANOVA ($P \leq 0.001$; J). The results of pair-wise comparisons of the value of each experimental group with those of control (Con) and DHPG groups are denoted with daggers and spades, respectively. ‡, ♠: $P < 0.05$. DHPG, dihydroxyphenylglycine; LY367383, (S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid; Glu, glutamate; 2-Methyl-6-(phenylethynyl)pyridine hydrochloride, MPEP.

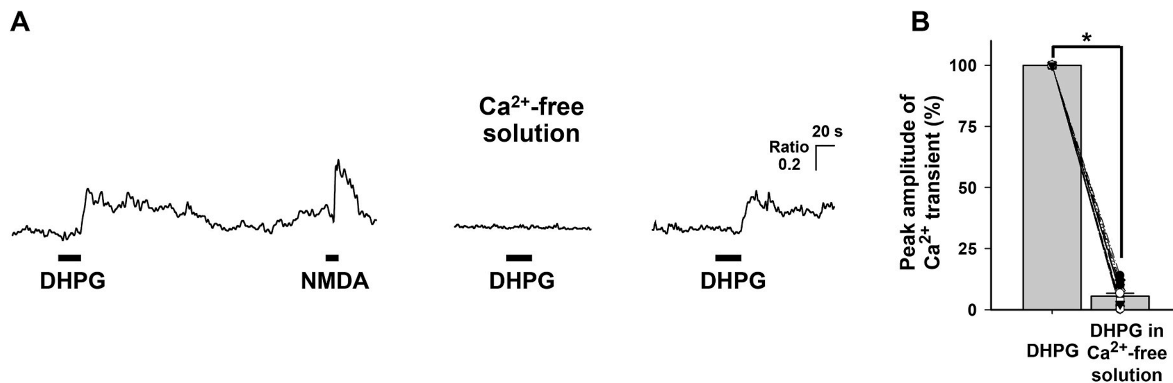

Fig. 2. DHPG-evokes increases in intracellular Ca^{2+} in SCN neurons through a mechanism dependent upon extracellular calcium. (A) Examples of DHPG-evoked Ca^{2+} responses under normal calcium and in a calcium-free solution. (B) Group data showing the % reduction in the peak amplitude of the DHPG-evoked calcium transient in the calcium-free solution. *: $P < 0.001$ (paired *t*-test).

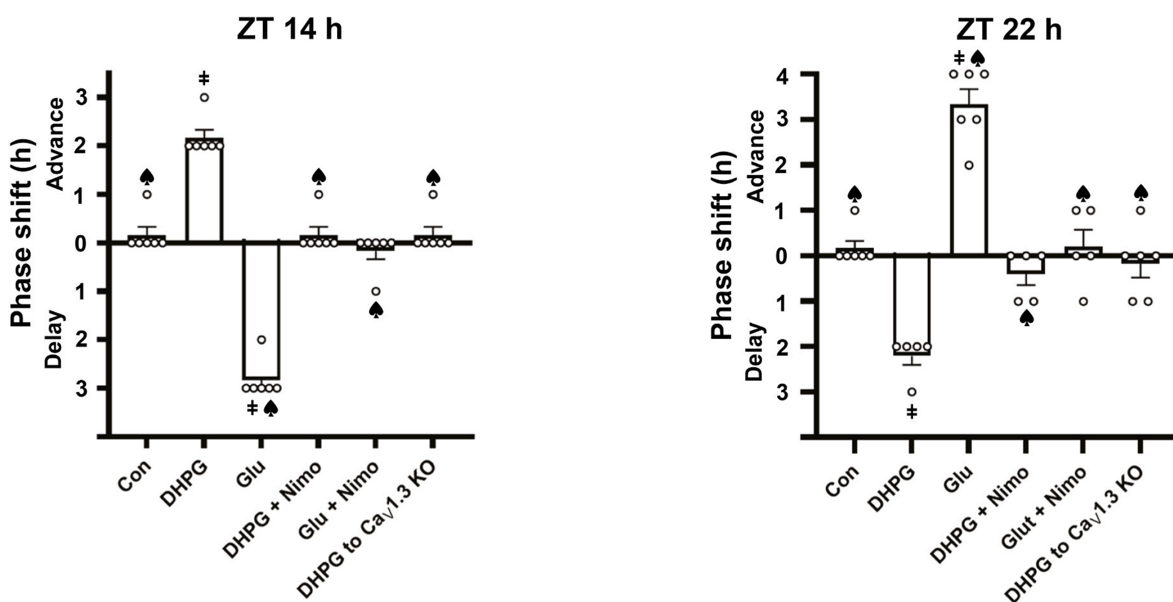

Fig. 3. DHPG-evoked phase shifts were blocked by the L-type Ca^{2+} channel blocker nimodipine and were not observed in $Ca_V1.3$ L-type VGCC KO mice. Nimodipine (6 μM) completely blocked the DHPG-, as well as glutamate-, induced phase shifts at ZT 14 h (left) and ZT 22 h (right). Nimodipine alone had no significant effect on the phase of SCN neuronal activity rhythm (data not shown). DHPG did not evoke phase shifts in brain slices from $Ca_V1.3$ L-type VGCC KO at either phase. Bar graphs show means ± SEM for each group. Individual data points are overlaid. Newman-Keuls comparison tests were performed after Kruskal-Wallis one-way ANOVA ($P \leq 0.001$; left panel) or one-way ANOVA ($P \leq 0.001$; right panel). The results of pair-wise comparisons of the value of each experimental group with those of control (Con) and DHPG groups are denoted with daggers and spades, respectively. ‡, ♠: $P < 0.05$. DHPG, dihydroxyphenylglycine; Glu, glutamate; Nimo, nimodipine.

Glutamate-induced phase advances were similarly potentiated by the pretreatment of the mGluR1 blocker LY367385, mGluR1 KO, $Ca_V1.3$ KO, and the PKA inhibitor H89 (Fig. 5, right panel). The mGluR1 KO exhibited similar SCN neuronal activity rhythms to WT mice which peaked around ZT 6 h (KO: ZT 5.8 ± 0.2 h, n = 6 *vs*. WT: ZT 6.0 ± 0.3 h, n = 6; p = 0.699, Mann–Whitney Rank Sum Test). Thus, collectively, these results indicate that the mGluR1 activation negatively modulates glutamate-induced phase shifts in the SCN.

4. Discussion

Glutamate released from the RHT activates iGluRs located on the membranes of SCN neurons to initiate the process of photic resetting of the circadian pacemaker in the SCN (Golombek and Rosenstein, 2010; Ashton et al., 2022). In the current study, we reveal that mGluR1 activation in the SCN in the early and late night not only induces phase shifts that are opposite in direction to those from iGluR activation, but also

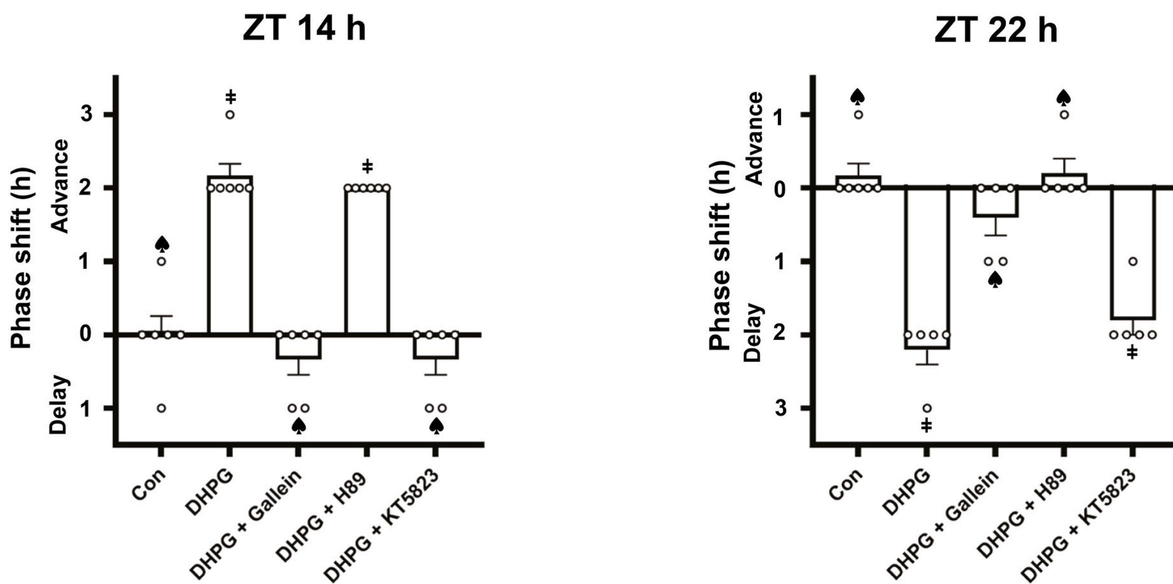

Fig. 4. Gβγ blocker abolished DHPG-evoked phase shifts, while kinase inhibitors had phase-dependent effects. In the early night (ZT 14 h), DHPG-evoked phase shifts were prevented by a Gβγ blocker (gallein), as well as a PKG inhibitor (KT5823). In the late night (ZT 22 h), DHPG-evoked phase shifts were prevented by a Gβγ blocker (gallein), as well as a PKA inhibitor (H89). Gallein, KT5823, and H89 alone had no significant effect on the phase of SCN neuronal activity rhythms (data not shown). Data are expressed as the mean ± SEM. Individual data points are overlaid. Newman-Keuls comparison tests were performed after Kruskal-Wallis one-way ANOVA ($P \leq 0.001$). The results of pair-wise comparisons of the value of each experimental group with those of control (Con) and DHPG groups are denoted with daggers and spades, respectively. ǂ, ♠: $P < 0.05$. DHPG, dihydroxyphenylglycine.

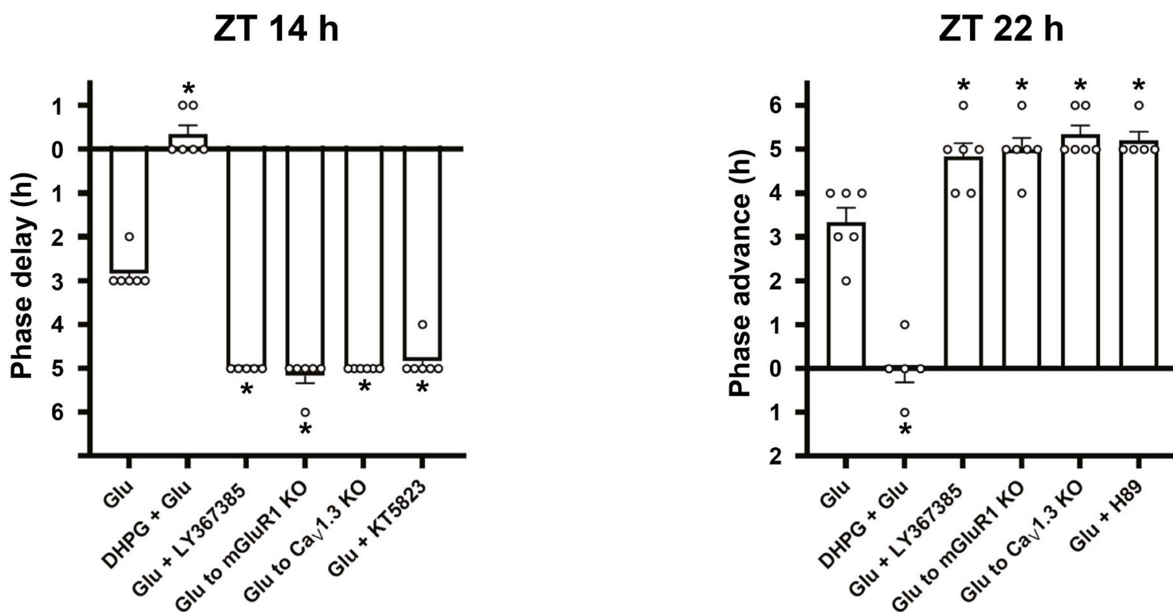

Fig. 5. Effects of DHPG, mGluR1 blocker (LY367385), mGluR1 KO, Cav1.3 L-type VGCC KO, KT5823, and H89 on glutamate-induced phase shifts of circadian neural activity rhythms in the SCN. DHPG inhibited glutamate-induced phase shifts at both ZT 14 h and ZT 22 h. In the early night (ZT 14 h), glutamate-induced phase delays were enhanced by the application the mGluR1 blocker as well as the PKG inhibitor. The drugs by themselves were without effect on phase (data not shown). Glutamate-induced phase delays were enhanced in mGluR1 KO and Cav1.3 KO mice compared to WT controls. In the late night (ZT 22 h), glutamate-induced phase advances were enhanced by the application the mGluR1 blocker as well as the PKA inhibitor. Glutamate-induced phase advances were enhanced in mGluR1 KO and Cav1.3 KO mice compared to WT controls. Data are expressed as mean ± SEM. Individual data points are overlaid. Newman-Keuls comparison tests were performed after Kruskal-Wallis one-way ANOVA ($P \leq 0.001$; left panel) or one-way ANOVA ($P \leq 0.001$; right panel). The results of pair-wise comparisons of the value of each experimental group with that of glutamate group (Glu) are denoted with asterisks ($P < 0.05$).

antagonizes the glutamate-induced phase shifts. Interestingly, the antagonism of mGluR1 signaling actually produced larger phase shifts in response to glutamate application. Therefore, mGluRs may act as a brake to limit the magnitude of the phase shifts.

In this study, we sought to identify the signaling pathways through which mGluR1 acts in the SCN. We found that $Ca_V1.3$ KO blocked the phase-shifting effect of DHPG, but potentiated glutamate-elicited phase shifts, while nimodipine at a concentration of 6 μM, which would block $Ca_V1.2$ and $Ca_V1.3$ L-type VGCCs by 70–100% (Xu and Lipscombe, 2001), prevented the occurrence of both the glutamate- and DHPG-induced phase shifts. Thus, these results strongly suggest that mGluR1-and iGluR-mediated phase shifts depend on $Ca_V1.3$ and $Ca_V1.2$

L-type VGCCs, respectively, for their occurrence. Meanwhile, in a previous study conducted with mice, we discovered that $Ca_V1.3$ L-type VGCCs were mainly expressed in the somata of SCN cells while $Ca_V1.2$ L-type VGCCs were located primarily in the processes (Kim et al., 2015). Perhaps, the mGluR1-mediated Ca^{2+} influx through $Ca_V1.3$ L-type VGCCs at the somata may lead to a significant rise in dendritic Ca^{2+} concentration to inhibit the Ca^{2+} influx at dendrites by inactivating local $Ca_V1.2$ L-type VGCCs (Haack and Rosenberg, 1994), hence negatively modulating iGluR-mediated phase shifts. Similarly, the mGluR1-mediated Ca^{2+} influx through $Ca_V1.3$ L-type VGCCs may transcriptionally activate the small G protein gem, which in turn moves to dendrites to inhibit $Ca_V1.2$ L-type VGCCs there (Matsuo et al., 2022) for the negative modulation of iGluR-mediated phase shifts.

Another interesting aspect of our results are the findings that the Gβγ-PKA pathway is crucial for the induction of phase delays by mGluR1 activation in the late night, whereas Gβγ and PKG, as independent factors or as a coupled system, are involved the induction of the phase advances by mGluR1 activation in the early night. These findings are consistent with prior work from the Gillette laboratory suggesting time-of-day variation in the signaling pathways through which glutamate produces phase shifts (Gillette and Tischkau, 1999). For example, this group provided evidence that activation of the cAMP/PKA gating pathway permits signal flow through the main photic resetting pathway downstream of iGluR, whereas, in the late night, it inhibits the signal flow (Tischkau et al., 2000). Recent work highlights the robust rhythms in intracellular cAMP found in the SCN network (Ono et al., 2023). Thus, it is possible that PKA activated from mGluR1 activation in the late night functions as the gating molecule that hinders the signal flow through the main photic resetting pathway downstream of iGluR, to antagonize the glutamate-mediated phase advances. Perhaps PKG activated by mGluR1 activation in the early night also would act as a gating molecule to inhibit the occurrence of iGluR-mediated phase delays. The relative roles of PKG and PKA signaling pathways gating light- or glutamate-induced phase shifts will be an interesting area for future research.

Recent work suggests that inhibition of glutamate-mediated phase delays by PKG in the early night may be achieved through its modulation of clock gene expression (Plano et al., 2021). That is, the expression of clock genes underlying the phase delays induced by iGluR activation at early night may be negatively modulated by PKG and/or by its effectors, such as protein phosphatase 2 A. Still, in the case of mGluR activation of PKA and PKG signaling, the downstream targets are not known.

PKA and PKG activated by mGluR1 may antagonize iGluR-mediated phase shifts by regulating the functions of L-type VGCCs as well. In other neuronal systems, it has been shown that the phosphorylation of "PKA" site on the L-type VGCC potentiates the opening of this channel whereas "PKG" site phosphorylation inhibits the opening (Mahapatra et al., 2012). In SCN neurons, PKA activated from mGluR1 stimulation in the late night may help to open $Ca_V1.3$ L-type VGCCs so that Ca^{2+} influx through these channels may lead to the inhibition of $Ca_V1.2$ L-type VGCCs by causing the channel inactivation mechanism and/or transcriptionally activating the small G protein gem (see above). In the meantime, PKG activated from mGluR1 stimulation in the early night may inhibit directly $Ca_V1.2$ L-type VGCCs for the negative modulation of iGluR-mediated phase delays. This notion, however, does not fit with the reports that PKG negatively modulates not only $Ca_V1.2$ but also $Ca_V1.3$ L-type VGCCs (Mahapatra et al., 2012; Sandoval et al., 2017), channels identified to be crucial for the antagonistic effects of mGluR1 activation against iGluR-mediated phase shifts. Further studies will be needed to test this hypothesis.

The finding of the current study that mGluR1 activation limits the magnitude of glutamate-induced phase shifts is consistent with the observation made in a previous study that the non-selective mGluR agonist t-ACPD suppressed kainite- and NMDA-elicited increases in $[Ca^{2+}]_i$ in neurons of rat SCN explant cultures (Haak, 1999). Prior behavioral pharmacology studies have found evidence that mGluRs can potentiate or inhibit the magnitude of light effects on the circadian system of hamsters (Haak et al., 2006; Gannon and Millan, 2011). However, there are a number of differences between the *in vivo* and *in vitro* situation. Still, based on our data, we would expect that drugs selectively activating mGluR1 would reduce the magnitude of light-induced phase shifts of the circadian system while drugs that inhibit these receptors would enhance the magnitude of light-induced responses.

Among the limitations of the current study is that we did not identify the cellular location of the mGluR1s that are involved in the antagonism of glutamate-induced phase shifts. The results of our Ca^{2+} imaging experiments conducted with the use of acutely dissociated SCN cells, however, suggest that mGluR1s are located on postsynaptic cells. This notion is supported by an anatomical study of van den Pol (1994), which demonstrates that mGluR1s are distributed on perikaryal and dendritic membranes of SCN neurons, which are often associated with synapses. It is worth noting that mGluRs are well known to be trafficked (Scheefhals and MacGillavry, 2018; Bodzęta et al., 2021) and could well shift their cellular location with time-of-day. Another limitation of the current study is that mGluR pharmacology is complex. We carefully selected pharmacological agents and used concentrations based on the published literature. For example, the DHPG (30 μM) was used at a concentration commonly considered to be specific. We also demonstrated that the effects of DHPG at this concentration were selectively blocked by mGluR1 (but not mGluR5) antagonists. Still, we cannot completely exclude the possibility that the effects of DHPG or any of the drugs we tested were not specific. Future Ca^{2+}-imaging and/or electrophysiological studies showing DHPG concentration/dose-response relationship may provide further insights to this issue.

In this study, we did not attempt to identify the source of the glutamate that starts the cascades explored in this work. Our working assumption is that the glutamate is released from the intrinsically photosensitive retinal ganglion cells projecting to the SCN through the RHT. However, the role of astrocytes as a source of glutamate has to be considered (Parpura and Haydon, 2000; Angulo et al., 2004; Hamilton and Attwell, 2010; Mahmoud et al., 2019). For example, there is evidence that SCN astrocytes are active during circadian nighttime, when they suppress the activity of SCN neurons by regulating extracellular glutamate levels. This glutamatergic gliotransmission is sensed by neurons of the dorsal SCN via specific pre-synaptic NMDA receptor assemblies containing NR2C subunits (Brancaccio et al., 2017).

Glutamate release activates multiple receptors that interact with each other and thus, determines the response of the cell. Exploring these interactions is critical to developing an understanding of the functional consequences of synaptic transmission in the SCN, as well as in the rest of the nervous system (Reiner and Levitz, 2018; Burada et al., 2021). This functional complexity contributes to glutamatergic signaling complexity and provides the framework for biological plasticity. The mGluR modulation remains an appealing target for therapeutic interventions (Récasens et al., 2007), and a variety of potential modulators have been synthesized. While these agents may not have lived up to early expectations as therapeutic strategies in Fragile X syndrome (Stoppel et al., 2021; Grabb and Potter, 2022), these same compounds may find utility in other conditions including modulation of the impact of light on the circadian system.

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CRediT authorship contribution statement

Yoon Sik Kim: conceived the concept and the design of the study,

participated in data interpretation and wrote the manuscript, participated in data collection and analysis. **C Justin Lee:** conceived the concept and the design of the study. **Ji-Hyeon Kim:** participated in data collection and analysis. **Young-Beom Kim:** participated in data collection and analysis. **Christopher S. Colwell:** participated in data interpretation and wrote the manuscript, All of the authors approved the final manuscript for submission. **Yang In Kim:** conceived the concept and the design of the study, participated in data interpretation and wrote the manuscript.

Declaration of competing interest

We authors declare that there is no conflict of interest.

Data availability

Data will be made available on request.

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