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Poly(I:C)-Induced Viral-Mimetic Inflammation Prolongs Zolpidem-Induced Loss of Righting Reflex in Mice

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Zolpidem is a non-benzodiazepine hypnotic that acts on γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors and is widely used to treat insomnia. Our previous studies demonstrated that inflammation induced by bacterial mimetics, such as lipopolysaccharide (LPS), prolongs zolpidem-induced loss of righting reflex (LORR) in mice, suggesting that systemic inflammatory states modulate GABAergic transmission. However, it remains unclear whether viral inflammation produces similar effects. In this study, we investigated the effect of polyinosinic:polycytidylic acid (poly(I:C)), a synthetic analog of viral double-stranded RNA, on the duration of zolpidem-induced LORR in mice. Poly(I:C) administration significantly prolonged zolpidem-induced LORR. However, this prolongation was attenuated by pretreatment with bicuculline, a $GABA_A$ receptor antagonist, and by bumetanide, an inhibitor of Na^+-K^+-$2Cl^-$ cotransporter isoform 1 (NKCC1). In addition, we examined the mRNA expression levels of $GABA_A$ receptor subunits, NKCC1, and K^+-Cl^- cotransporter isoform 2 (KCC2) in the hippocampi of poly(I:C)-treated mice. Poly(I:C) treatment did not significantly alter the mRNA expression of major $GABA_A$ receptor subunits but increased *Slc12a2* mRNA expression, which encodes NKCC1 in the hippocampus. Importantly, poly(I:C)-induced prolongation of LORR was attenuated by bumetanide, an NKCC1 inhibitor. In conclusion, these findings suggest that viral mimetic-induced inflammation prolongs zolpidem-induced LORR, possibly through alterations in intracellular chloride homeostasis.

Key words polyinosinic:polycytidylic acid, zolpidem, γ-aminobutyric acid type A receptor, Na^+-K^+-$2Cl^-$ cotransporters

INTRODUCTION

Zolpidem is a non-benzodiazepine hypnotic widely used for the treatment of insomnia because of its rapid onset of action and clinical efficacy.[1, 2] Although orexin receptor antagonists have become increasingly prevalent, benzodiazepine receptor agonists, including benzodiazepine hypnotics, still play an important role in insomnia management. In clinical practice, benzodiazepine receptor agonists have been associated with adverse neuropsychiatric events, including delirium, particularly in postoperative settings where systemic inflammation is frequently present.[3-5] Inflammation triggered by either bacterial or viral factors has emerged as a major modulator of central nervous system responses to pharmacological agents.

Viral infections produce a distinct, clinically relevant pattern of systemic inflammation. Polyinosinic:polycytidylic acid (poly(I:C)) is a synthetic double-stranded RNA that acts as a viral mimetic and induces robust neuroimmune responses via activation of Toll-like receptor 3 (TLR3).[6] Poly(I:C) administration elicits systemic and central cytokine responses, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), resulting in neuroinflammation.[7, 8] Poly(I:C) administration reduces the percentage of time spent in the open section of the zero maze, indicating anxiety-like behavior in mice.[9] Given the prevalence of viral infections and the increased recognition of post-viral neuropsychiatric syndromes, elucidating the mechanisms linking viral inflammation to altered central nervous system function is of scientific and clinical importance.

Our previous study revealed that lipopolysaccharide (LPS)-induced bacterial inflammation potentiates the sedative effects of γ-aminobutyric acid (GABA)-ergic drugs, including zolpidem, as evidenced by prolonged loss of righting reflex (LORR) duration in mice.[10] Notably, this enhanced sensitivity is thought to involve alterations in chloride homeostasis, particularly through changes in the expression or function of the neuronal chloride transporters Na^+-K^+-$2Cl^-$ cotransporter isoform 1 (NKCC1) and K^+-Cl^- cotransporter isoform 2 (KCC2), which are critical for regulating the inhibitory effects of GABA type A ($GABA_A$) receptors.

In this study, we investigated the effect of poly(I:C)-induced inflammation on zolpidem-induced LORR and explored the associated changes in $GABA_A$ receptor subunits, NKCC1, KCC2, and cytokine profiles. In particular, we

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hypothesized that poly(I:C), similar to LPS, would prolong zolpidem-induced LORR by disrupting chloride regulation, thereby altering GABAergic inhibition. Overall, this study is anticipated to improve our understanding of the distinct and overlapping mechanisms by which bacterial and viral inflammation affect the pharmacodynamics of sedatives.

METHODS

Experimental Animals Animal experiments were conducted in accordance with the guidelines outlined in the Guide for Animal Experiments of Shujitsu University Advanced Science Research Center and were approved by the Animal Care and Use Committee of Shujitsu University (067-001). A total of 102 male ICR mice with an initial weight of 28–35 g were purchased from Jackson Laboratory Japan, Inc. (Yokohama, Japan) and randomly assigned to experimental groups. During the experiment, the mice were housed in a temperature-controlled room (temperature, 23 ± 1°C; humidity, ≈ 60%) under a 12-h/12-h light/dark cycle (lights on at 08:00) and housed 4 per cage. The animals had ad libitum access to standard laboratory food and water.

Drugs The following drugs were used in this study: poly(I:C) (HMW: InvivoGen, San Diego, CA, USA), zolpidem (ZOLPIDEM TARTRATE OD; Sawai Seiyaku Co., Tokyo, Japan), (+)-bicuculline (Sigma-Aldrich, St. Louis, MO, USA), and bumetanide (Sigma-Aldrich, St. Louis, MO, USA). (+)-Bicuculline was suspended in a 0.5% methylcellulose solution, and bumetanide was dissolved in 0.1 N NaOH. Mice were intraperitoneally (i.p.) injected with poly(I:C) and (+)-bicuculline at 10 mL/kg body weight and with zolpidem and bumetanide at 20 mL/kg body weight. Mice in the control group were administered the corresponding vehicle alone.

Behavioral Study Design for the Duration of Zolpidem-Induced LORR Behavioral experiments were conducted exclusively between 10:00 and 16:00. LORR was defined as the state in which the mice were lying on their back and unable to regain an upright position for at least 30 s. The duration of LORR was defined as the time from LORR onset to recovery of the righting reflex. The zolpidem dose required to induce LORR was determined based on previous study.[10] Mice were injected with poly(I:C) 24 h before the zolpidem-induced LORR test. To examine whether bumetanide-sensitive chloride transport mechanisms during the early phase of the poly(I:C)-induced inflammatory response contribute to the subsequent enhancement of zolpidem responsiveness, bumetanide was administered 30 min before poly(I:C) injection. Bicuculline was administered 30 min before zolpidem administration. Bumetanide and bicuculline doses were selected based on previous studies.[11, 12] Finally, the duration of LORR was calculated by subtracting the time of LORR onset from LORR recovery.[10, 11, 13, 14] Fig. 1 shows the schedule used for the behavioral studies.

Measurement of mRNA Expression of *Il6* (IL-6), *Tnf* (TNF-α), $GABA_A$ Receptor Subunits (*Gabra1*, *Gabrb2*, and *Gabrg2*), *Slc12a2* (NKCC1), and *Slc12a5* (KCC2) by Real-Time Quantitative Polymerase Chain Reaction Total RNA was extracted from the hippocampus of each animal using Maxwell RSC® SimplyRNA Tissue Kit (Promega, Madison, WI, USA). Total RNA (0.25 μg) was reverse-transcribed to generate cDNA using ReverTra Ace® quantitative polymerase chain reaction (qPCR) RT Master Mix (TOYOBO, Osaka, Japan).

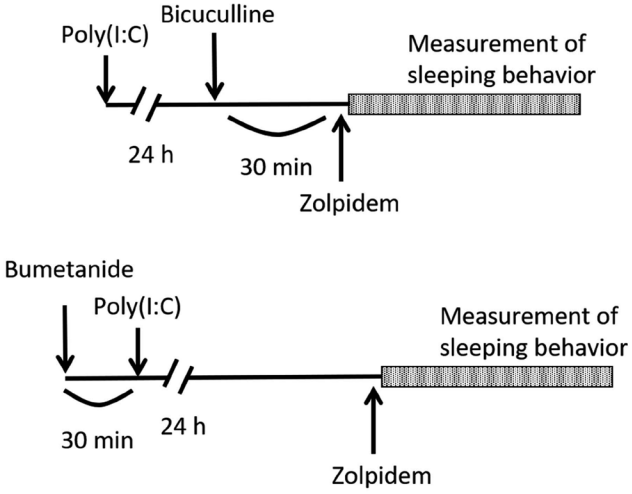

Fig. 1. Experimental Design for Behavioral Studies

Poly(I:C) was administered 24 h before the zolpidem-induced loss of righting reflex (LORR) test was performed. Bicuculline was administered 30 min before zolpidem administration. Bumetanide was administered 30 min before poly(I:C) treatment.

Real-time PCR amplification was performed using a QuantStudio®3 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences, target genes, and other related information are provided in Table 1. For SYBR Green-based assays, the PCR protocol consisted of an initial denaturation step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s. Upon completion of the reaction, specificity was confirmed using melting curve analysis. For TaqMan probe-based assays, PCR amplification was performed with an initial denaturation step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 1 s and annealing/extension at 60°C for 20 s. Fluorescence data were collected during the annealing/extension steps. To quantify the mRNA expression of the $GABA_A$ receptor subunits α1 (*Gabra1*), β2 (*Gabrb2*), and γ2 (*Gabrg2*), as well as *Il6*, *Tnf*, *Slc12a2*, and *Slc12a5*, forward and reverse primers and probes were designed and synthesized (Integrated DNA Technologies, Coralville, IA, USA). Finally, the mRNA levels of the target genes were calculated using the comparative cycle threshold (ΔΔCt) method and normalized to *Gapdh* expression.[15]

Statistical Analysis All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using Excel-Tokei ver. 7.0 (Esumi Co. Ltd., Tokyo, Japan). Significant differences were determined using a two-tailed Student's *t*-test for two-group comparisons and one-way analysis of variance followed by Dunnett's or Tukey's post hoc test for multi-group comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of Poly(I:C) Administration on *Il6* and *Tnf* mRNA expression in the hippocampus Poly(I:C) administration significantly increased *Il6* mRNA expression in the hippocampus of mice at 2 h ($F[3,20] = 20.67$, $p < 0.01$; Fig. 2A) and significantly upregulated *Tnf* mRNA expression at 2 and 5 h ($F[3,20] = 5.87$, $p < 0.01$; Fig. 2B).

Table 1. Oligonucleotide Sequences of the Primer Sets Used for Reverse Transcriptase–Polymerase Chain Reaction

(A) *Il6* and *Tnf*

Gene symbol	Accession number	Forward (5′ to 3′)	Reverse (5′ to 3′)	Amplicon (bp)	Position (5′ to 3′)
Il6	NM_031168.2	ccacttcacaagtcggaggctta	gcaagtgcatcatcgttgttcatac	112	218–329
Tnf	NM_013693.3	aagcctgtagcccacgtcgta	ggcaccactagttggttgtctttg	122	435–556
Gapdh	NM_001411840.1	tgcccccatgtttgtgatg	ggcatggactgtggtcatga	160	405–564

(B) *Gabra1*, *Gabrb2*, and *Gabrg2*

Gene symbol	Accession number	Forward (5′ to 3′)	Reverse (5′ to 3′)	Probe	Amplicon (bp)	Position (5′ to 3′)
Gabra1	NM_010250.5	tgagagctgaatgcccaatg	tctgctacaaccactgaacg	FAM/cctgcccac/ZEN/taaaattcggaagctatgc/IABkFQ	145	1,262–1,406
Gabrb2	NM_001362647.2	gtggtgattctcaagggtctaat	tggattcccgagtctcttct	FAM/tgaaagtaa/ZEN/ggaggtgggacacagc/IABkFQ	98	2,512–2,609
Gabrg2	NM_008073.4	gctacttcaccatccagacttac	ggcaggaacagcatccttat	FAM/attccctgc/ZEN/acactcatcgtggtc/IABkFQ	92	1,342–1,433
Gapdh	NM_001411840.1	tgcccccatgtttgtgatg	ggcatggactgtggtcatga	FAM/accaccaac/ZEN/tgcttagccccccctg/IABkFQ	160	405–564

(C) *Slc12a2* and *Slc12a5*

Gene symbol	Accession number	Forward (5′ to 3′)	Reverse (5′ to 3′)	Probe	Amplicon (bp)	Position (5′ to 3′)
Slc12a2	NM_009194.3	ctgccgagagtaaaggagttg	agagcatcacaccccaaatg	FAM/aacatgcag/ZEN/cggactaatacaccctt/IABkFQ	87	938–1,024
Slc12a5	NM_020333.2	atttcgctattaccactggactctct	cccggtactcgatgtacttataaatg	FAM/cctctgcct/ZEN/ggccctcatgttcat/ IABkFQ	137	2,185–2,321
Gapdh	NM_001411840.1	tgcccccatgtttgtgatg	ggcatggactgtggtcatga	FAM/accaccaac/ZEN/tgcttagccccccctg/IABkFQ	160	405–564

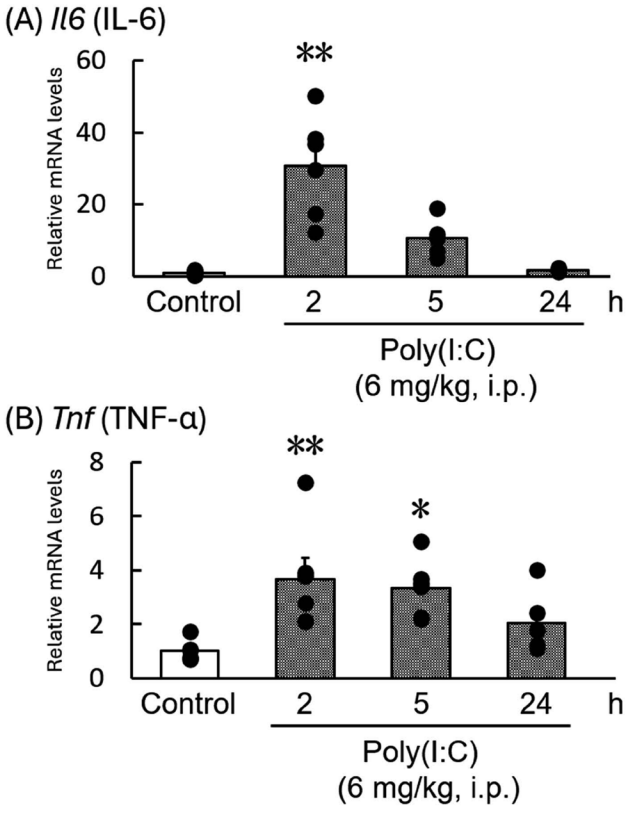

Fig. 2. Effects of Poly(I:C) on Hippocampal *Il6* (A) and *Tnf* (B) mRNA Expression in Mice.

Hippocampal *Il6* and *Tnf* mRNA expression was observed at 2, 5, and 24 h after poly(I:C) administration (6 mg/kg, i.p.). Data are expressed as mean ± standard error of mean (SEM; n = 6 for each group). Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparisons test. *$p < 0.05$ and **$p < 0.01$ indicate significant differences from the control group.

Table 2. Effects of Poly(I:C) on the Duration of Zolpidem-Induced Loss of Righting Reflex (LORR) in Mice

Drugs	Duration of loss of righting reflex (min)
Control	34.3 ± 2.7
Poly(I:C)	80.1 ± 10.5 **

Poly(I:C) (6 mg/kg, i.p.) was injected 1 day before the zolpidem-induced LORR test. Zolpidem was administered intraperitoneally at a dose of 75 mg/kg. Data are expressed as mean ± standard error of mean (SEM; n = 6 for each group). Data were analyzed using an unpaired Student's t-test. **$p < 0.01$ indicates a significant difference from the control group.

Effects of Bicuculline on Poly(I:C)-Mediated Prolongation of Zolpidem-Induced LORR In this study, we measured the duration of zolpidem-induced LORR 24 h after poly(I:C) administration. Notably, poly(I:C) administration significantly prolonged the duration of zolpidem-induced LORR (Table 2). In addition, we examined whether $GABA_A$ receptors were involved in the poly(I:C)-induced prolongation of zolpidem-induced LORR using bicuculline, a $GABA_A$ receptor antagonist. To ensure the reliability of the results, we used a subeffective dose of bicuculline that did not affect zolpidem-induced LORR under control conditions, as demonstrated in our previous studies.[10, 11] Bicuculline treatment significantly reduced the duration of LORR in poly(I:C)-treated mice ($F[2,15] = 5.13$, $p < 0.05$; Fig. 3).

Effects of Poly(I:C) on $GABA_A$ Receptor Subunits in the Hippocampus In this study, we examined the effect of poly(I:C) on the mRNA expression of $GABA_A$ receptor subunits that constitute benzodiazepine-sensitive receptor complexes in the hippocampus. Poly(I:C) administration did not produce statistically detectable changes in the hippocampal mRNA expression of the $GABA_A$ receptor subunits (*Gabra1, Gabrb2,* and *Gabrg2*) 24 h after treatment (*Gabra1*: $F[3,12] = 1.20$, $p = 0.35$; *Gabrb2*: $F[3,12] = 1.33$, $p = 0.31$; *Gabrg2*: $F[3,12] = 1.47$, $p = 0.274$; Fig. 4).

Effects of Poly(I:C) and Bumetanide on the Duration of Zolpidem-Induced LORR To investigate the role of neu-

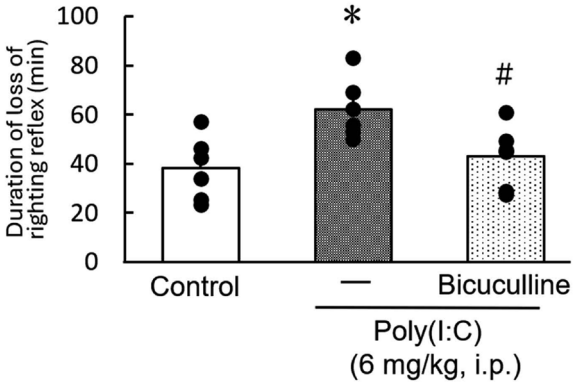

Fig. 3. Effects of Bicuculline on the Duration of Zolpidem-Induced Loss of Righting Reflex (LORR) in Poly(I:C)-Treated Mice.

Poly(I:C) (6 mg/kg, i.p.) was injected 1 day before the zolpidem-induced LORR test. Bicuculline (3 mg/kg, i.p.) was administered to poly(I:C)-treated mice 30 min before administering zolpidem (75 mg/kg, i.p.). Data are expressed as mean ± standard error of mean (SEM; n = 6 for each group). Data were analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test. *$p < 0.05$ indicates a significant difference from the control group, and #$p < 0.05$ indicates a significant difference from the poly(I:C) group.

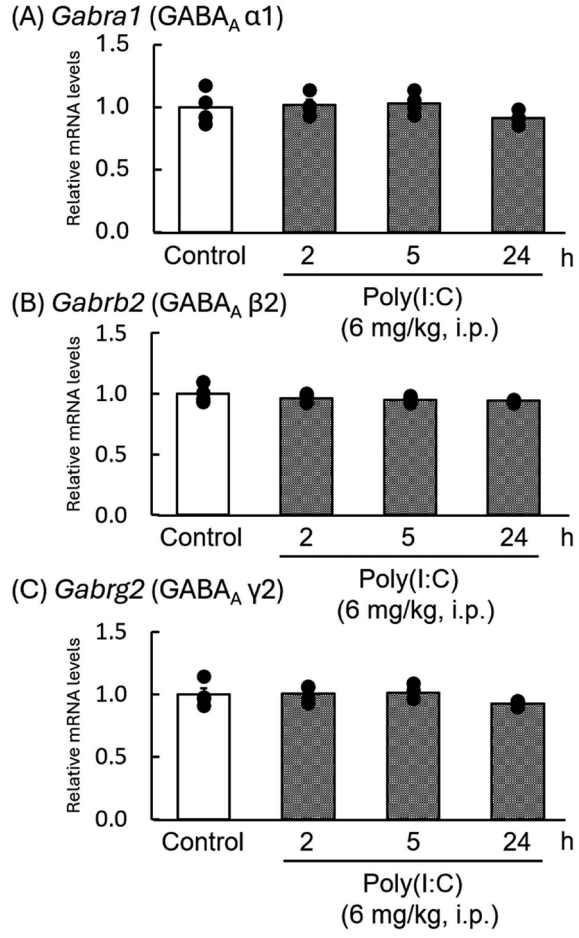

Fig. 4. Effect of Poly(I:C) on the mRNA Expression of GABA$_A$ Receptor Subunits (A: *Gabra1*, B: *Gabrb2*, and C: *Gabrg2*) in the Hippocampus of Mice.

Mice were injected with poly(I:C) (6 mg/kg, i.p.) 1 day before decapitation. Data are expressed as mean ± standard error of mean (SEM; n = 4 for each group). Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparisons test.

ronal transporters in zolpidem-induced LORR, mice were pretreated with bumetanide, an NKCC1 blocker, before poly(I:C) and zolpidem administration. Bumetanide alone did not significantly affect zolpidem-induced LORR under control conditions. In contrast, bumetanide pretreatment significantly attenuated poly(I:C)-induced prolongation of zolpidem-induced LORR ($F[3,20] = 6.07$, $p < 0.01$; Fig. 5).

Effects of Poly(I:C) on *Slc12a2* and *Slc12a5* mRNA Expression in the Hippocampus To determine whether mRNA expression of *Slc12a2* and *Slc12a5,* which encode NKCC1 and KCC2, respectively, was altered during inflammation, we examined their expression levels at 2, 5, and 24 h after poly(I:C) injection. Although *Slc12a2* mRNA expression increased significantly 5 h after poly(I:C) administration ($F[3,16] = 3.31$, $p < 0.05$; Fig. 6A), *Slc12a5* mRNA expression remained unchanged following poly(I:C)-induced inflammation ($F[3,20] = 1.45$, $p = 0.27$; Fig. 6B).

DISCUSSION

A previous study showed that poly(I:C) administration increased serum levels of the proinflammatory cytokines IL-6 and TNF-α.[8] In the present study, poly(I:C) administration significantly increased *Il6* and *Tnf* mRNA expression in the hippocampus, indicating the induction of systemic and neuroinflammatory responses. Overall, these findings underscore the importance of inflammation induced by both bacterial and viral stimuli in enhancing the sensitivity of the central nervous system to GABAergic sedatives. Poly(I:C) and LPS activate innate immune responses, leading to increased production of proinflammatory cytokines, including IL-6 and TNF-α, in both peripheral tissues and the central nervous system.[10, 16, 17] Poly(I:C) activates TLR3 and initiates downstream signaling pathways, including the activation of nuclear factor-kappa B (NF-κB), thereby promoting cytokine production.[6]

In this study, we focused on the hippocampus because this brain region is highly responsive to systemic inflammatory stimuli and has been repeatedly implicated in inflamma-

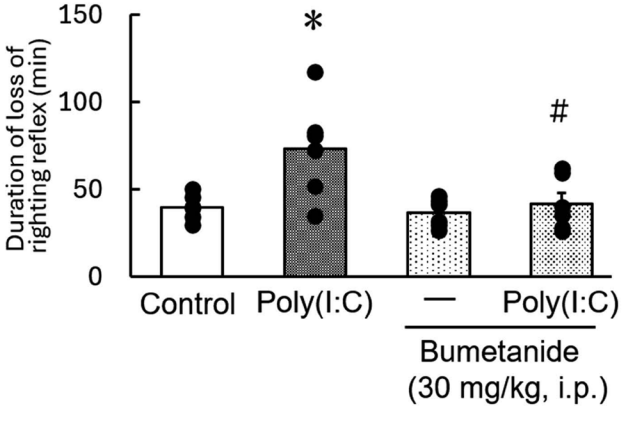

Fig. 5. Effects of Bumetanide on the Duration of Zolpidem-Induced Loss of Righting Reflex (LORR) in Poly(I:C)-Treated Mice.

Poly(I:C) (6 mg/kg, i.p.) was injected 1 day before the zolpidem-induced LORR test. Bumetanide (30 mg/kg, i.p.) was administered 30 min before poly(I:C) injection. Data are expressed as mean ± standard error of mean (SEM; n = 6 for each group). Data were analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test. *$p < 0.05$ indicates a significant difference from the control group, and #$p < 0.05$ indicates a significant difference from the poly(I:C) group.

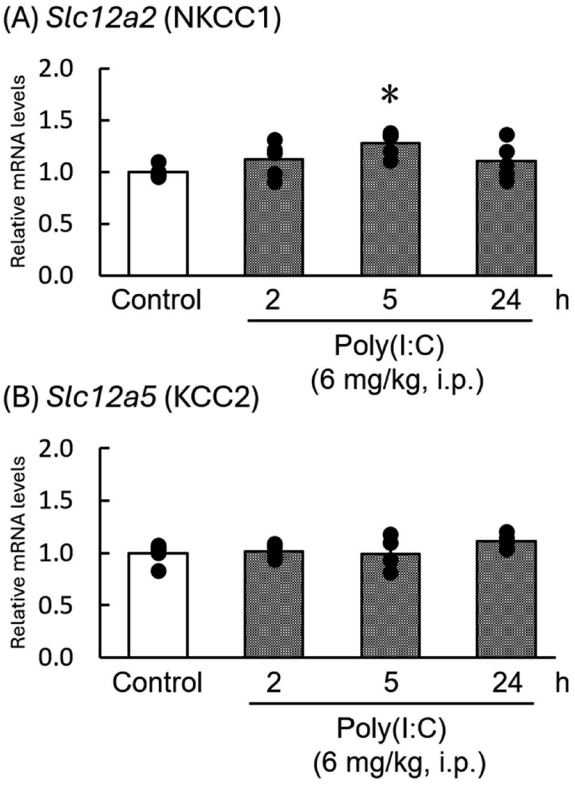

Fig. 6. Effects of Poly(I:C) on *Slc12a2* (A: NKCC1) and *Slc12a5* (B: KCC2) mRNA Expression in the Hippocampus of Mice.

Hippocampal *Slc12a2* and *Slc12a5* mRNA expression was observed at 2, 5, and 24 h after poly(I:C) administration (6 mg/kg, i.p.). Data are expressed as mean ± standard error of mean (SEM; n = 5 for each group). Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison test. $*p < 0.05$ indicates significant differences from the control group.

tion-induced neurochemical and behavioral alterations. In our previous studies using an inflammatory mouse model, we observed hippocampal neuroinflammatory changes, including increased inflammatory cytokine expression, microglial activation, and decreased brain-derived neurotrophic factor (BDNF) expression.[8, 18] Similarly, we confirmed early inflammatory cytokine responses and alterations in hippocampal BDNF expression in the poly(I:C)-induced inflammatory model. Collectively, these findings suggest that the hippocampus is a relevant brain region for evaluating central responses to LPS- and poly(I:C)-induced inflammation. In addition, the hippocampus exhibits abundant $GABA_A$ receptor-mediated inhibitory neurotransmission, and alterations in GABAergic signaling or chloride ion homeostasis in this region may reflect, at least in part, changes in central nervous system sensitivity to GABAergic sedatives. Therefore, we examined the mRNA expression of inflammatory cytokines, $GABA_A$ receptor subunits, and chloride transporter-related genes in the hippocampus. However, zolpidem-induced LORR is not solely regulated by the hippocampus; rather, it is likely mediated by distributed neural circuits involved in arousal, sleep, consciousness, and postural control. Therefore, the findings of the present study should be interpreted as one aspect of inflammation-associated changes in GABAergic sensitivity rather than a complete explanation of the prolonged LORR.

Consistent with our previous observations after LPS administration, poly(I:C) treatment did not induce statistically detectable changes in the mRNA expression levels of the major $GABA_A$ receptor subunits in the hippocampus under the present experimental conditions. In particular, the individual values showed relatively small variability and no clear tendency toward poly(I:C)-induced change. Overall, these findings suggest that large transcriptional changes in the examined $GABA_A$ receptor subunits are unlikely to be the primary mechanism underlying the enhanced sensitivity to zolpidem. However, given the small sample size used in this analysis, the possibility of small-to-moderate effects on $GABA_A$ receptor subunit expression cannot be excluded. $GABA_A$ receptors are ligand-gated chloride channels, and their activation typically promotes chloride influx into mature neurons, which is a key mechanism underlying inhibitory neurotransmission. Intracellular chloride homeostasis is regulated by $GABA_A$ receptor-mediated chloride conductance and chloride transporters, such as NKCC1 and KCC2.

Although both LPS and poly(I:C) induced inflammatory cytokine responses in the hippocampus, the pattern of chloride transporter-related gene expression differed between the inflammatory models. Based on our previous findings in the LPS-induced inflammation model, we initially hypothesized that poly(I:C) would decrease *Slc12a5* mRNA expression without affecting *Slc12a2* mRNA expression. Contrary to this expectation, poly(I:C) transiently increased *Slc12a2* mRNA expression without significantly altering *Slc12a5* mRNA expression. The precise mechanism underlying this discrepancy remains unclear. However, this may be explained, at least in part, by differences in the innate immune signaling pathways activated by bacterial and viral mimetics. LPS primarily activates TLR4-dependent inflammatory signaling, whereas poly(I:C) activates TLR3-dependent signaling. Although both stimuli increase inflammatory cytokines, such as IL-6 and TNF-α, their downstream effects on neurons and glial cells may differ in terms of gene expression profiles and temporal patterns. Functionally, decreased KCC2 expression may reduce chloride extrusion, whereas increased NKCC1 expression may enhance chloride influx. Together, these changes could shift intracellular chloride homeostasis, thereby altering $GABA_A$ receptor-mediated responses. Consequently, LPS- and poly(I:C)-induced inflammation may alter GABAergic drug responsiveness through different chloride transporter-related mechanisms. However, this interpretation remains hypothetical because the present study did not directly compare these mechanisms or measure transporter protein expression or chloride transport function.

In the present study, poly(I:C) administration significantly increased *Slc12a2* mRNA expression at 5 h post-treatment. However, this increase was transient and was no longer significant at 24 h, when the zolpidem-induced LORR test was performed. Therefore, the present findings do not indicate that the sustained upregulation of *Slc12a2* mRNA expression at the time of the behavioral test directly accounts for the prolonged LORR. Rather, the transient increase in *Slc12a2* mRNA levels may reflect an early inflammatory response that contributes to subsequent changes in chloride homeostasis or GABAergic sensitivity. We previously reported that LPS-induced systemic inflammation prolonged zolpidem-induced LORR in mice.[10] In addition, poly(I:C) administration induced an early inflammatory cytokine response that subsequently returned to baseline.[8] Collectively, these findings suggest that early inflam-

matory responses or secondary neural alterations induced by these responses may contribute to the enhanced zolpidem responsiveness observed at 24 h. Consistent with this possibility, pretreatment with bumetanide attenuated the poly(I:C)-induced prolongation of zolpidem-induced LORR.

In the present study, poly(I:C) administration significantly upregulated *Slc12a2* expression. NKCC1 is a Na^+-K^+-$2Cl^-$ cotransporter that mediates chloride influx into cells and contributes to the maintenance of intracellular chloride homeostasis. Therefore, NKCC1 upregulation under inflammatory conditions may increase intracellular chloride levels and modify $GABA_A$ receptor-mediated signaling. Consistent with this possibility, pretreatment with bumetanide attenuated poly(I:C)-induced prolongation of zolpidem-induced LORR. In the present study, bumetanide was administered 30 min before poly(I:C) injection, not immediately before the LORR test. Therefore, this experiment was not designed to evaluate the acute inhibitory effect of bumetanide on NKCC1 at the time of zolpidem administration. Rather, this dosing schedule was used to examine whether bumetanide-sensitive chloride transport mechanisms during the early phase of poly(I:C)-induced inflammatory responses contribute to the subsequent development of enhanced zolpidem responsiveness. In our previous study, we reported that bumetanide administration using a similar schedule attenuated LPS-induced prolongation of zolpidem-induced LORR.[10] Therefore, our findings suggest that altered chloride influx or chloride homeostasis during inflammation may contribute to increased zolpidem sensitivity.

Despite these promising findings, this study has some limitations. First, the expression of NKCC1, KCC2, and $GABA_A$ receptor subunits was assessed only at the mRNA level. We did not determine whether changes in *Slc12a2* or *Slc12a5* mRNA expression were accompanied by corresponding changes in NKCC1 or KCC2 protein expression. In addition, we did not examine the membrane localization, phosphorylation status, or functional chloride transport activity of these transporters. Given that NKCC1 and KCC2 are membrane transporters whose function is regulated not only by total protein expression but also by membrane localization and post-translational regulation, mRNA expression alone cannot establish functional changes in chloride transport. Moreover, we did not directly assess the effects of poly(I:C) administration on intraneuronal chloride levels. Therefore, NKCC1/KCC2-mediated chloride homeostasis should be interpreted as a mechanistic possibility based on mRNA expression and pharmacological findings, rather than as direct evidence of altered chloride transport function. Future studies should examine NKCC1 and KCC2 protein expression, membrane localization, phosphorylation status, and chloride transport activity to determine whether changes in *Slc12a2* or *Slc12a5* mRNA expression are associated with functional alterations in chloride homeostasis.

Clinically, the inflammatory conditions investigated in this study may provide insight into postoperative inflammatory states, in which benzodiazepine receptor agonists are sometimes used. Notably, the incidence of postoperative delirium is higher in patients treated with benzodiazepine receptor agonists.[3-5] Our findings do not establish a direct causal relationship between zolpidem use and postoperative delirium. However, they suggest that inflammatory states may influence the sensitivity of the central nervous system to GABAergic sedatives, at least under experimental conditions. In addition, these findings highlight the need for further studies to clarify the interaction between inflammation and GABAergic activity during infectious and inflammatory states.

In summary, poly(I:C)-induced viral-mimetic inflammation prolonged zolpidem-induced LORR in mice, mirroring observations from bacterial inflammation models. Importantly, this effect may be mediated by altered chloride homeostasis associated with NKCC1 dysregulation without transcriptional changes in the major $GABA_A$ receptor subunits in the hippocampus. Collectively, these findings emphasize the importance of chloride homeostasis in inflammation-mediated central nervous system drug responses and support the need for clinical vigilance and further mechanistic research to improve the management of vulnerable patient populations.

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Conflicts of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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