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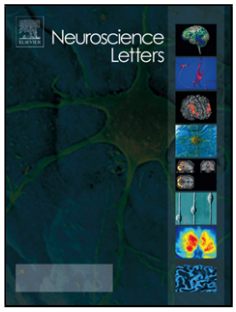

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Effect of cGMP-activated aquaporin 1 on TRPV4 in rats with allodynia induced by chronic compression of the dorsal root ganglion

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[1] **List of abbreviations**

DRG: dorsal root ganglion; CCD: chronic compression of the dorsal root ganglion; AQP: aquaporin; TRP: transient receptor potential; TRPV4: TRP vanilloid receptor 4; TRPV1: TRP vanilloid receptor 1; cGMP: cyclic guanosine monophosphate; ODQ: 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one; PWMT: paw withdrawal mechanical threshold; shRNA: short hairpin RNA; ANOVA: analysis of variance; SEM: standard error of the mean.

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Graphical abstract

Highlights

- AQP1 might play a role in chronic compression of dorsal root ganglion causing pain.
- Lentivirus-mediated interference of AQP1 reduced CCD-induced pain.
- Lentivirus-mediated interference of AQP1 reduced TRPV4 expression in CCD rats.
- A cGMP inhibitor attenuated allodynia in CCD rats via the AQP1-TRPV4 pathway.
- There is a cause–effect link between AQP1 and neuropathic pain.

Abstract

Background: The aim of this study was to investigate the effects of aquaporin 1 (AQP1) knockdown on allodynia in rats with chronic compression of the dorsal root ganglia (DRG) and the role of TRPV4 in these effects.

Methods:

Adult male Wistar rats were subjected to chronic compression of the dorsal root ganglia (CCD) via surgery. Behavioral tests were performed to calculate the paw withdrawal mechanical threshold (PWMT). Gene silence was induced by injecting rats with lentivirus expressing AQP1 short hairpin RNA (shRNA, Lv-shAQP1). Western blot analyses were performed to examine AQP1 and TRPV4 protein expression. The concentration of cyclic guanosine monophosphate (cGMP) was determined via enzyme-linked immunosorbent assay.

Results: AQP1 protein levels in DRG neurons were significantly increased in CCD rats and were accompanied by a decrease in the PWMT. Lentivirus-mediated RNA interference of AQP1 decreased AQP1 protein expression in CCD rats and normalized their PWMT, but not in rats infected with lentivirus-expressing negative control short hairpin RNA. Furthermore, AQP1 was identified as a cGMP-gated channel. cGMP concentration was upregulated in CCD rats. This effect was attenuated by treatment with a cGMP inhibitor. Additionally, the cGMP inhibitor decreased the mechanical allodynia and AQP1 protein expression in CCD rats. Finally, levels of TRPV4 expression were upregulated in DRG neurons and the L4/L5 spinal cord following surgery, and these effects were reversed by treatment with Lv-shAQP1 or a cGMP inhibitor.

Conclusion: AQP1 plays a vital role in CCD-induced allodynia as Lv-shAQP1 significantly

reduced the allodynia in CCD rats by inhibiting TRPV4 expression.

Keywords: cyclic GMP; AQP1; gene therapy; CCD; low back pain

Introduction

After tissue injury and inflammation, sensory signals from the primary sensory neurons to the spinal dorsal horn change significantly, ultimately leading to the development of chronic pain [1]. Low-back pain is among the most common forms of chronic pain. Radicular neuralgia is a typical type of low-back pain that occurs when the radix spinalis or dorsal root ganglia (DRG) are stimulated by harmful factors [2]. Consequently, as they are excited, they create and transmit neuropathic pain signals. Chronic compression of the dorsal root ganglion (CCD) is a typical example of radicular neuralgia, which may lead to spontaneous pain and allodynia. Currently, treatments for radicular neuralgia are inadequate, as the mechanisms underlying the condition remain unclear.

Aquaporins (AQP0-12) are a large family of proteins expressed commonly in plants as well as humans [3, 4], playing an important role in water transport and osmoregulation. Oshio et al. demonstrated that AQP1 protein was localized in the nerve fibers of small neurons, DRG, and spinal cord, and its deletion reduced responsiveness to thermal stimuli and capsaicin [5]. Additionally, Zhang and Verkman observed significantly reduced behavioral responses to inflammatory thermal and cold pain in AQP1$^{-/-}$ mice [6]. Therefore, we theorized that AQP1 might have a role in CCD-induced allodynia.

Transient receptor potential (TRP) channels, which have an important role in sensory

function, are Ca^{2+}-permeable cation channels. TRP vanilloid receptor 4 (TRPV4) plays a significant role in the mechanisms associated with high neuronal excitability, hyperalgesia, and allodynia after CCD surgery [7]. Capsaicin receptor TRP vanilloid receptor 1 (TRPV1) is a homologous tetramer of TRPV4 and co-localizes with AQP1 [5]. Therefore, we hypothesized that TRPV4 had a role in the effect of AQP1 on allodynia in CCD rats.

AQP1 also regulates non-selective cation channels when activated by intracellular cyclic guanosine monophosphate (cGMP) [8, 9]. This cGMP-dependent gating is localized in the intracellular loop between the fourth and fifth transmembrane domains [10]. Therefore, we theorized that the cGMP-dependent gating of AQP1 might have an important role in the regulatory effects of AQP1 on TRPV4.

Materials and Methods

Animals and surgical procedure

The study was conducted in compliance with the Chinese Institutional Animal Care Committee and performed following the Directive 86/609/EEC on the protection of animals used for scientific purposes. The total number of rats used in this study was 192. All efforts were made to minimize their suffering. The effect of Lv-shAQP1 on behavioral testing and the effect on expression of AQP1 and TRPV4 were investigated in the same rats to minimize the number used. Adult male Wistar rats weighing 180–220g were housed in a controlled environment with free access to food and water in Shandong University Lab Animal Center. Sodium pentobarbital (Nembutal, 50 mg/kg i.p.) was used to anesthetize the rats, following which two stainless steel rods were implanted unilaterally into the intervertebral foramen

between L4 and L5[11, 12]. Sham operations were not considered necessary because no differences were observed between sham and control rats in a previous study [7].

Behavioral testing

Gait patterns were assessed as an index of motor function. Rats that developed autophagy, sensory deficiency, or disability were excluded from the analysis [13].

The paw withdrawal mechanical threshold (PWMT) was measured using a von Frey hair monofilament (BME-404, Biomedical Engineering Institute of Chinese Academy of Medical Sciences) [14]. Each filament was pressed against the center of the hind paw for approximately 3 seconds. The filaments were used according to the "up-down" method, and immediate withdrawal of the paw was noted as a positive response.[14]. The procedure was repeated five times at intervals of at least 5 minutes, and the average value was recorded.

Behavioral testing of the ipsilateral hind paw was performed before surgery and on postoperative days 4, 6, 7, 14, and 28. The effects of chemicals and reagents on CCD-induced allodynia were assessed 2 hours after injection [11]. AQP1 expression was inhibited 24 hours after the injection of lentivirus [15]. To inhibit the expression of AQP1, the AQP1 lentivirus was injected for 3 days consecutively, and its effect on CCD-induced allodynia was tested 72 hours after the last injection [14].

Real-time quantitative PCR

Rats received intrathecal injections of lentivirus for 3 consecutive days. L4 and L5 ganglia from the operated side were harvested from CCD rats. Fragments of AQP1 were amplified

using the following primers: AQP1 (forward, 5′-CTGCTGGCCATTGACTACACT-3′; reverse, 5′-TCCCAATGAATGGTCCCACC-3′). Instrument control, automated data collection, and data analysis were performed using using the LightCycler software program, version 4.0. The $2^{-\Delta\Delta CT}$ method was used to analyze the data [16].

Generation of lentivirus-expressing AQP1 short hairpin RNA (shRNA)

AQP1-specific target sequences were chosen based on online shRNA tools provided by Invitrogen (www.invitrogen.com/rnai) using an AQP1 reference sequence. The following target sequences were used for AQP1:

5′-CAGGGTGGAGATGAAGCCCAA-3′ (AQP1-shRNA-#1),

5′-ATCAGCATCGGTTCTGCCCTA-3′ (AQP1-shRNA-#2), and a

5′-CAGGGCCTGGGCATTGAGATC-3′ (AQP1-shRNA-#3) followed by chemically synthesized shRNA (GenePharma Co, Ltd, Shanghai, China) and lentiviral vector constructed per the Invitrogen lentiviral vector protocol. A scrambled sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as a negative control shRNA. Vectors expressing specific shRNA were confirmed via sequencing.

Western blot analysis

CCD rats were intrathecally injected with chemicals or AQP1 lentivirus, following which the L4/L5 ganglia and spinal cord were harvested from the operated side 7 days after surgery. After incubation overnight at 4 °C in a polyclonal anti-TRPV4 preparation (1:1000, Abcam, Cambridge, UK) or anti-AQP1 preparation (1:500, Waleibiology, Shenyang, China), the

polyvinyl fluoride membranes were incubated with an anti-rabbit peroxidase (HRP)-conjugated secondary antibody (1:10,000, Zhongshan Goldenbridge, Beijing, China) for 1 hour. Binding was detected using the chemiluminescent HRP method (Millipore, Billerica, MA, USA). Protein levels were expressed as a ratio of density of the detected band relative to β-actin (1:1000, Cell Signaling Technology Inc.) [17].

Enzyme-linked immunosorbent assay (ELISA)

The L4 and L5 ganglia of the operated side, as well as the L4/L5 spinal cord, were harvested from CCD rats following the injection of chemicals. The concentration of cGMP was measured via ELISA using a cGMP Enzyme Immunoassay kit (USCN, Wuhan, China), following the manufacturer's instructions.

Chemicals and reagents

The following chemicals were used in this study: GSK1016790A (TRPV4 synthetic agonist, Sigma, 0.5μM, 10μl), acetazolamide (inhibitor of AQPs, SELLECK, 10nM, 10μl), and 1H-[1, 2, 4] oxadiazole [4, 3-a] quinoxaline-1-one (ODQ, inhibitor of soluble guanylate cyclase [sGC], APEXBIO, 100mM, 10μl). All chemicals were dissolved in ethanol or dimethyl sulfoxide, and the final experimental dilutions were prepared on the day of the experiment. The dosing and administration schedules were selected based on the results of pilot experiments, and all efforts were made to minimize the side effects of chemicals and reagents.

Data analysis

Data were analyzed using Sigmaplot and SPSS 21.0 software. A one-way repeated-measures analysis of variance (ANOVA) was used to analyze differences in the PWMTs, AQP1 and TRPV4 protein levels, and cGMP concentrations. A Mann-Whitney rank-sum test was used to investigate differences in the levels of AQP1 gene expression. Values in the text and figures were expressed as the mean ± the standard error of the mean (SEM). The level of statistical significance was set at $P < 0.05$.

Results

Identification of small interfering RNA targeting AQP1 mRNA

As shown in Figure 1C, levels of AQP1 gene expression were significantly decreased by Lv-shRNA-ERK-#1 and Lv-shRNA-ERK-#3, but significantly upregulated by Lv-shRNA-ERK-#2, when compared with Lv-shRNA-NC. Therefore, we chose Lv-shRNA-ERK-#1 and Lv-shRNA-ERK-#3 to silence AQP1 gene expression in all subsequent experiments.

Identification of the optimal titration of Lv-shRNA-AQP1 in vivo

To reduce animals' pain, we investigated whether the titration of 10^4 or 10^5 TU/mL could suppress the expression of AQP1 significantly as Lv-shAQP1 in 10^6 TU/mL. As shown in Figure 1D, the titer of lentivirus expressing AQP1-shRNA at 10^6 TU/mL, but not at 10^4 or 10^5 TU/mL, significantly downregulated AQP1 protein expression, as measured by RT-qPCR analysis. This finding indicated that 10^6 TU/mL of lentivirus was optimal for silencing the

targeted gene expression *in vivo*.

Role of AQP1 in CCD-induced allodynia

All rats walked normally after CCD surgery, indicating that CCD surgery did not impair motor behavior. However, these rats exhibited mechanical allodynia (Figure 2A).

PWMTs and AQP1 protein levels were examined 2 hours after injection of the inhibitor and 72 hours after injection of AQP1 lentivirus. Figure 2B shows that treatment with either AQP1 lentivirus or the inhibitor attenuated CCD-induced mechanical allodynia. GSK1016790A, a TRPV4 agonist, was then injected to investigate whether AQP1 could modulate CCD-induced allodynia via TRPV4. Results showed that the decrease of allodynia was attenuated following the injection of GSK1016790A (Figure 2B).

Levels of AQP1 protein expression in the DRG and spinal cord were significantly higher in CCD rats than in control rats (Figure 3A and 3B). These CCD-induced increases in AQP1 protein expression levels were significantly attenuated following the administration of the lentivirus or AQP1 inhibitor ($P < 0.05$ and $P < 0.01$ compared to the CCD+Lv-shNC group).

Role of cGMP in CCD-induced allodynia

ODQ was intrathecally injected to investigate the effects of cGMP pathways on allodynia in CCD rats. Our *in vivo* experiments revealed that cGMP levels were significantly higher in CCD rats than in control rats (Figure 2D and 2E; $P < 0.01$), and that these effects were attenuated by treatment with ODQ ($P < 0.01$). Furthermore, CCD rats exhibited significant mechanical allodynia after surgery, which was attenuated by intrathecal administration of

ODQ ($P < 0.05$) (Figure 2C). Additionally, GSK1016790A injection markedly decreased PWMTs in CCD rats treated with Lv-shAQP1, acetazolamide, and ODQ (Figure 2B and 2C).

Expression of TRPV4 in CCD rats

As demonstrated in Figure 3, the level of TRPV4 protein expression was significantly increased in the operated side in CCD rats ($P < 0.01$ compared to the control group). TRPV4 protein expression decreased when animals were intrathecally injected with AQP1 lentivirus or an AQP1 inhibitor (Figure 3C; $P < 0.05$, and $P < 0.01$ compared to CCD + Lv-shNC group). Additionally, pretreatment with ODQ significantly decreased the expression of TRPV4 protein in the DRG after CCD surgery (Figure 3C; $P < 0.01$).

We also examined the effects of Lv-shAQP1, acetazolamide, and ODQ on TRPV4 protein expression in the L4/L5 spinal cord. As shown in Figure 3D, similar to findings were observed in DRGs, levels of TRPV4 protein expression were significantly increased in the L4/L5 spinal cord isolated from CCD rats, relative to those in control rats. As hypothesized, lentivirus-mediated AQP1 gene silencing reduced levels of TRPV4 protein expression, relative to those observed for negative control shRNA. Moreover, TRPV4 protein expression was also decreased following the injection of acetazolamide or ODQ.

These data further support the notion that TRPV4 is a downstream mediator of AQP1 in the spinal cord, where it contributes to neuropathic pain.

Discussion

In the present study, we aimed to characterize the role of AQP1 in CCD-induced allodynia, as well as the mechanisms underlying this effect. Our findings demonstrated that AQP1 is likely to have an essential role in the development of radicular neuralgia. In CCD rats, regulation of TRPV4 protein expression was associated with the effects of intrathecal injection of Lv-shAQP1 on behavioral hyperalgesia. To the best of our knowledge, our study is among the first to establish a cause-effect link between AQP1 and neuropathic pain *in vivo*.

Low-back pain is associated with high economic costs to the community. For many patients, pharmacotherapy for neuropathic pain remains challenging, and effective treatments are lacking. We developed a new approach for pain control using lentivirus-mediated shRNA infection technology to specifically silence AQP1 gene expression or injection of acetazolamide to depress AQP1 activation in CCD rats. Oshio et al. reported mild impairments in nociceptive responses to thermal and chemical stimuli in $AQP1^{-/-}$ mice, although no differences in responses to mechanical stimuli were noted [18]. Shields et al. confirmed that $AQP1^{-/-}$ animals exhibited no significant differences in behavioral pain tests [19]. In our study, we observed that Lv-shAQP1 attenuated CCD-induced mechanical allodynia, which is not consistent with the findings of previous studies. The first reason may be that AQP1 is not essential in pain signaling pathway, although it plays a role in neuropathic pain [19]. Furthermore, the role of AQP1 is distinctive in different neuropathic models. Many factors could explain these differences, such as surgical disease model, animal differences, etc. Our results showed that Lv-shAQP1 and AQP1 inhibitor could suppress the CCD-induced allodynia. The mechanisms of lentivirus and inhibitor are different. AQP1 lentivirus was injected before the formation of allodynia to inhibit the gene expression of

AQP1, which has a preventive effect. The inhibitor of AQP1 was injected at 7 days after surgery, which is therapeutic effect and nonspecific. The results of our study show that AQP1 is important in formation and maintenance of neuropathic pain, though the mechanism requires further investigation.

In our study, CCD rats exhibited increases in AQP1 and TRPV4 expression. Furthermore, TRPV4 expression and allodynia were attenuated by injection of Lv-shAQP1 or an AQP1 inhibitor, suggesting that AQP1-TRPV4 plays an important role in CCD-induced allodynia. ODQ, a specific inhibitor of sGC, blocks the classical sGC/cGMP signaling pathway and inhibits cGMP accumulation [20]. AQP1 is a cGMP-gated cation channel that can be blocked by ODQ. ODQ could significantly decrease the AQP1 and TRPV4 expression. Additionally, ODQ-treated CCD rats exhibited significant decreases in allodynia when compared with the non-ODQ-treated group, and these effects were partially attenuated by GSK1016790A. Therefore, these findings indicate that cGMP-gating plays an essential role in the effect of AQP1 on CCD-induced allodynia. However, further studies are required to elucidate the molecular mechanisms underlying these effects.

Central sensitization underlies some common clinical presentations of musculoskeletal pain, including chronic low back pain [21, 22]. In this context, central sensitization refers to the amplification of neural signaling within the central nervous system that elicits pain hypersensitivity. Sensory fibers from the spinal nerves to the spinal cord are responsible for the conduction of allodynia [23], and previous studies have indicated that spinal cord dysfunction is critical to neuropathic pain [24]. In the present study, protein levels of AQP1 and TRPV4 were significantly increased in the L4/L5 spinal cord, relative to levels observed

in control rats. Lentivirus-mediated AQP1 gene silencing reduced levels of TRPV4 protein expression in the L4/L5 spinal cord and neuropathic pain of CCD rats. These data support the crucial role of the AQP1-TRPV4 pathway in neuropathic pain in the spinal cord of CCD rats.

Conclusions

In conclusion, our findings demonstrated that effective lentivirus-mediated RNA interference of AQP1 significantly reduced CCD-induced allodynia in rats. Furthermore, our results indicated that cGMP regulated allodynia in CCD rats, at least in part, via blockade of the AQP1-TRPV4 pathway. Collectively, our data provide new insights into the molecular mechanisms underlying CCD-induced allodynia, which may aid in the development of novel antinociceptive agents that target the AQP1-TRPV4 pathway.

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Author contributions

All authors are listed, and they have all contributed substantially to the manuscript (H. Wei wrote the article, W.S. Gao, L. Jia, and L. Qi helped to write the article, Y.J. Qu provided language help, S.W. Yue and Y. Zhang were responsible for article conception). The content has been approved by all co-authors.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1 Efficacy of lentivirus expressing shRNA by targeting AQP1 mRNA *in vivo.* (A) Designs of shRNA targeting AQP1 mRNA. (B) The *in vivo* experimental protocols. (C) Effects of lentivirus-mediated AQP1 gene silencing in CCD rats. (D) qRT-PCR analysis of AQP1 gene expression in DRG injected with different titrations. n = 5 in each group for (C) and (D). ** P < 0.01, * P < 0.05 vs. CCD group. PCR, polymerase chain reaction; shRNA, short hairpin RNA; Lv-shNC, negative control shRNA; CCD, chronic compression of the dorsal root ganglia; DRG, dorsal root ganglia; AQP1, aquaporin 1.

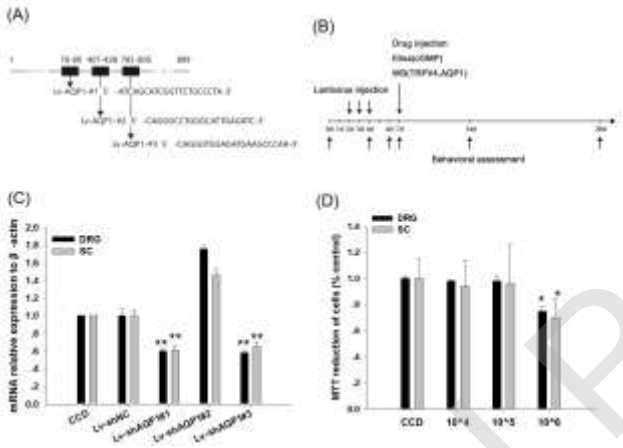

Fig. 2 Mechanical allodynia in CCD rats and concentrations of cGMP. (A) Mechanical allodynia of the ipsilateral hind paws induced by CCD. (B) Effects of lentivirus-mediated AQP1 gene knockdown on PWMT in CCD rats. (C) Effects of ODQ on PWMT in CCD rats. n = 9 in each group for (A), (B), and (C). (D) ELISA results for cGMP protein in the DRG of CCD rats. (E) ELISA results for cGMP protein in the spinal cord of CCD rats. n = 6 in each group for (D) and (E). ## P <0.01, # P < 0.05 vs. Control (CON) group; ** P < 0.01, * P < 0.05 vs. CCD group; & P < 0.01, P < 0.05 vs. CCD + LvshNC group; a P < 0.05 vs. CCD + Lv-shAQP1 group; b P < 0.05 vs. CCD + ACE group; c P < 0.05 vs. CCD + ODQ group.. shRNA, short hairpin RNA; Lv-shNC, negative control shRNA; PMWT, paw mechanical

withdrawal threshold; cGMP, cyclic guanosine monophosphate; CCD, chronic compression of the dorsal root ganglia; DRG, dorsal root ganglia; AQP1, aquaporin 1; ACE, acetazolamide; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one.

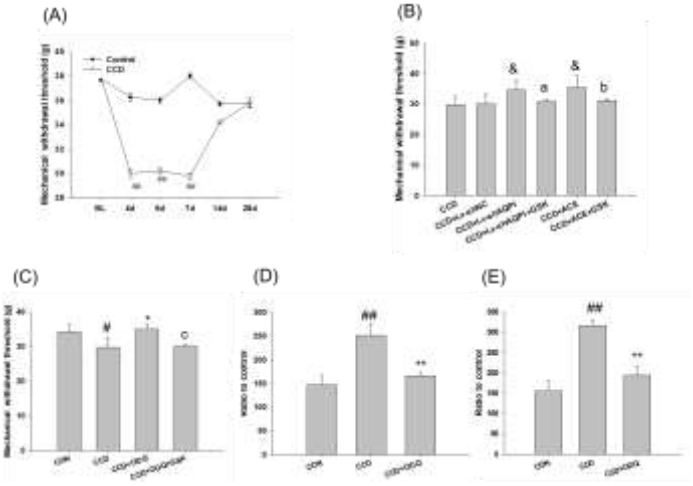

Fig. 3. Levels of protein expression. (A) Western blotting bands for AQP1 protein in the DRG of CCD rats. (B) Western blotting bands for AQP1 protein in the spinal cord of CCD rats. (C) Western blotting bands for TRPV4 protein in the DRG of CCD rats. (D) Western blotting bands for TRPV4 protein in the spinal cord of CCD rats. n = 6 in each group. β-actin served as an internal control. ## P <0.01, # P < 0.05 vs. control (CON) group; && P < 0.01, & P < 0.05 vs. CCD + Lv-shNC group. AQP1, aquaporin 1; TRPV4, transient receptor potential vanilloid receptor 4; CCD, chronic compression of the dorsal root ganglia; DRG, dorsal root ganglia; shRNA, short hairpin RNA; Lv-shNC, negative control shRNA,; ODQ, 1H-[1,2,4] oxadiazole [4,3-a] quinoxaline-1-one.