Optical control of insect behavior via ionotropic GABA receptors

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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Author contribution statement

X.S. conceived the research, supervised the study and wrote the manuscript. Q.H. and C. Z. synthesized and characterized the PCLs, and performed physiochemical analysis, *in vivo* bioassays and behavioral studies. Q.H. and C.Z performed TEVC experiments and prepare the manuscript. Q.X. and Y.D. analyzed data and helped prepare the figures. Z.L., Z.X and J.C. discussed data and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

BACKGROUND AND PURPOSE

Ionotropic GABA receptors (GABARs) in insects are the major inhibitory receptor and common targets in insecticides for pest control. Due to their high spatiotemporal resolution, the photopharmacological ligands have been developed in vertebrates, but only a few in insect yet.

EXPERIMENTAL APPOACH

In this study, two types of photoswitchable ligands (PCLs) by incorporating photoswitch azobenzene or dithienylethene into the antagonist, fipronil (FIP) generated the DTFIPs (DTFIP1 and DTFIP2) and ABFIPs (*p*-, *m*-, and *o*-ABFIP). Their photomodulation was measured by mosquito larval behavior, and the potential action mechanism of them was explored by two-electrode voltage clamp (TEVC) technique *in vitro*.

KEY RESULTS

DTFIP1 and *m*-ABFIP exhibited biggest difference of insecticidal activity between unirradiated and irradiated formation, and allowed for optical control of insect locomotors activity in swimming. The TEVC assay results indicted *m*-ABFIP and DTFIP1 enable optical control over the homomeric RDL-type GABAR, which is achieved by regulating the chloride channel of insect resistance to RDL-type GABAR by photoisomerization.

CONSLUSION AND IMPLICATIONS

Our results suggested that PCLs provide an alternative and precise tool for studying insect ionotropic GABARs and GABA-dependent behavior.

Keywords: azobenzene; dithienylethene; fipronil; ionotropic GABA receptor; photopharmacology;

Introduction

Ionotropic γ-aminobutyric acid receptors (GABARs) are inhibitory neurotransmitter activated ion channels in animal. They are associated with epilepsy, schizophrenia, insomnia, alcoholism and many other neurological diseases in human (Möhler, 2010; Braat *et al.*, 2015; Pin *et al.*, 2016), and with olfactory, visual, and mechanosensory processing in insect (Buckingham *et al.*, 2005). For example, the ionotropic GABAR subunit, RDL, is widely expressed in the *Drosophila melanogaster* central nervous system (Enell *et al.*, 2007) and it plays roles in sleep, memory and feeding (Liu *et al.*, 2007; Agosto *et al.*, 2008; Chung *et al.*, 2009; Cheung *et al.*, 2017). Ionotropic GABARs are formed by five subunits, usually as heteromer, surrounding a chloride channel, and depending on subunit composition the types of subunits and display differential sensitivities to pharmacological agents. Insect ionotropic GABARs respond to an array of natural or synthetic conventional ligands classified as agonists, antagonists, blockers or potentiators, which are critical for exploring the novel physiological function of ionotropic GABARs and pharmacological studies, e.g., synthesis of novel insecticides (Froestl, 2010; Olsen, 2018). However, the conventional ligands suffer from the limitation of low spatiotemporal resolution, due to this molecular complexity of ionotropic GABAR. Inspiringly, the photoresponsive ligands not only resolved this limitation, but also revolutionized the study of neuronal activity (Hüll *et al.*, 2018; Paoletti *et al.*, 2019).

Extensive efforts has been made to develop photoresponsive tools for targeting mammalian ionotropic GABARs only, such as photoaffinity ligands (PALs), photocaged ligands, photochromic tethered ligands (PTLs) and photochromic ligands (PCLs). PALs use gabazine-based photoreactive groups to inactivate ionotropic GABAR by covalently binding to receptor residues in the presence of light (Stewart *et al.*, 2011; Mortensen *et al.*, 2014). Photocaged ligands, such as caged GABA and diazepam (Matsuzaki *et al.*, 2010; Amatrudo *et al.*, 2014; Sansalone *et al.*, 2019), bear covalently-anchored photo-protecting groups and can irreversibly release the active form by one- or two-photon excitation. PTLs interact with mammalian ionotropic GABARs by covalent incorporation onto a mutant site (Lin *et al.*, 2014; Lin *et al.*, 2015).

To our knowledge, only PCLs that primarily act on mammalian ionotropic GABAR have been

generated (Feliciano *et al.*, 2010; Bregestovski *et al.*, 2018), and no PCL was available for insects. Therefore, it is necessary to develop a host of subtype-selective PCLs to decipher the neurological roles of the different insect ionotropic GABAR subtypes.

To date, azobenzenes are most commonly used in photocontrol of biological events, although they display incomplete photoconversion and thermal reversibility (Crespi *et al.*, 2019). On the other hand, dithienylethenes provide the benefits of fast response rates, excellent thermal stability, and outstanding fatigue resistance, but are difficult to structurally modify and the space for molecular geometry change is relatively small (Komarov *et al.*, 2018). In this study, we first synthesized PCLs that act on insect ionotropic GABAR with fipronil, a well-known antagonist of insect ionotropic GABARs peripherally attaching azobenzene or dithienylethene (Casida *et al.*, 2015). Interestingly, the translation of photostimulation of PCLs is able to modulate the behavioral activity in insect larvae. Subsequently, insect ionotropic GABARs consisting of homomeric insect RDL-type GABAR heterologously expressed in African frog *Xenopus laevis* oocytes, PCLs could optical control over the homomeric RDL-type GABAR. Our results show that these PCLs provide a new photopharmacological toolkit for understanding the function of insect ionotropic GABARs and their roles in behavior.

Results

PCLs were prepared by linking fipronil to azobenzene or dithienylethene

In the present study, the structure-activity relationship was firstly investigated and the primary amine group of fipronil is receptive to chemical modification. Insertion of azobenzene or dithienylethene at this position produced the fipronil-deuterogenic PCLs and designated as ABFIPs and DTFIPs, respectively, which can acquire *trans*-to-*cis* transformation for ABFIPs and open-to-closed transformation for DTFIPs upon irradiation (Fig. 1) and supposedly lead to changes in PCL activity.

The ABFIPs and DTFIPs were synthesized according to the synthetic routes (Supporting information S1 and S2), and five PCLs were synthesized based on the fipronil. *p*-ABFIP and *m*-ABFIP were prepared by coupling 1-(4-(bromomethyl)phenyl)-2-phenyldiazene or 1-(3-(bromomethyl)phenyl)-2-phenyldiazene with one molecule of fipronil, while *o*-ABFIP was prepared by a five-step procedure involving Boc protection, chlorination, coupling with fipronil,

deprotection and azologization. DTFIP1 was synthesized by a seven-step procedure involving bromination, aldehyde protection, n-butyl lithium dehydrogenation, deprotection, reduction, chlorination and finally coupling with two molecules of fipronil. DTFIP2 was synthesized by a similar procedure starting from 2,5-dimethylthiophene. All compounds were structurally confirmed by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry (Supporting information S3).

PCLs have suitable photophysiochemical properties for biological studies

We firstly investigated the photophysiochemical properties to identify the maximum absorbance wavelength (λ_{max}), photoisomer ratios and half-life of thermal relaxation of each PCL (Supporting information S4). The photoconversion process was tracked by recording the absorbance and emission spectra changes of samples irradiated with UV light at regular intervals. *m*-ABFIP showed a typical absorption spectrum like that of azobenzene with a maximum absorption wavelength of π–π* transition ($\lambda_{trans\text{-}max\ \pi–\pi^*}$) at 321 nm and n–π* transition ($\lambda_{cis\text{-}max\ n–\pi^*}$) at 430 nm, respectively. Upon irradiation with ultraviolet light (365 nm), gradual intensity decreases at 321 nm and increases at 400-500 nm (Fig. 2a). Other ABFIPs exhibited similar spectral changes when illuminated (Supporting information S5). The reverse spectral changes were observed upon irradiation at 430 nm. *trans*-ABFIPs are the dominant isomers present prior to irradiation, ranging from 97% to 100% of the ABFIP population. After irradiation, more than 75% of the population consisted of *cis*-isomers. All *cis*-ABFIPs have slow thermal back isomerization ($t_{1/2}$ > 85.7 h) at 25 °C, guaranteeing stability during biological application. The isomerization processes can be switched back and forth more than 7 cycles (Fig. 2b).

Upon irradiation, the absorption peak of DTFIP1 at 271 nm decreased with enhanced absorption at 522 nm, due to the formation of a benzodithiophene conjugation system (Fig. 2c). Fluorescence intensity increased gradually at 600 nm with extended irradiation (Supporting information S6). The isomerization process was confirmed by ^{1}H NMR tracking (Supporting information S7). The closed-isomer accounts for 67% of the population in the photostationary state. DTFIP1 showed robust continuous ring-closed/ring-opening cycles, but experienced a 20% photodegradation after eight cycles were observed (Fig. 2d). In the dark, the closed-isomer was stable for several months. Meanwhile, similar photophysiochemical properties were observed on DTFIP2 (Supporting

information S5).

PCLs exhibit 'photoactivated' or 'photoinactivated' activities *in vivo*

To test whether the photoisomerization of PCLs would alter pharmacological activity *in vivo*, its activities were screened by monitoring lethality in mosquito *Aedes albopictus* larvae (Fig. 3a and Supporting information S8). All ABFIPs have high insecticidal activities with low LC_{50} values (0.027-0.301 μM), which have comparable toxicity level of fipronil. A maximum 4.6-fold lethality was decrease for *m*-ABFIP. The attachment position has little influence on efficacy levels and differences between isomers that indicate this location is able to accommodate a relatively large geometry change. The LC_{50} values of unirradiated and irradiated DTFIP1 were 29.8 μM and 0.33 μM, respectively, which indicates approximately 90-fold activity enhanced after light irradiation. Although both photoisomers of DTFIP2 have higher activity than DTFIP1, the difference in activities was relatively low (3.5-fold). The variation in light-dependent activity was not observed when solely irradiating fipronil, excluding the possibility that the change of activity was induced by photodegraded products of fipronil. Based on the above results, both *m*-ABFIP and DTFIP1, which had the largest difference of insecticidal activity between unirradiated and irradiated states, were selected for further investigations.

***m*-ABFIP and DTFIP1 enable optical control over the homomeric RDL-type GABAR**

To determine whether *m*-ABFIP or DTFIP1 enabled optical control over ionotropic GABAR of insects, we evaluated reversible antagonistic effects using two-electrode voltage clamp (TEVC) technique on homomeric RDL-type GABARs cloned from the small brown planthopper *Laodelphax striatellus* (Fallén) and heterologously expressed in *Xenopus laevis* oocytes. PCLs reduced the inward currents evoked by GABA in a dose-dependent pattern, indicating antagonistic action when acting on homomeric *Ls*RDL GABARs (Fig. 3b). A schematic illustrating reversible opening and closing of the chloride channel upon photoisomerization of DTFIP1 is depicted in Fig. 3c. The IC_{50} values of DTFIP1 and *m*-ABFIP were 18.5 and 7.8 nM, respectively before irradiation. In control experiments, no current elicited by UV light or green light was observed (Fig. 3d). The *X. laevis* oocytes were treated with 0.003 μM GABA and 0.001 μM closed-DTFIP1 to establish a steady baseline current, prior to being subjected to illumination. Repeated irradiation

at λ_{520nm} and λ_{365nm} resulted in profound cyclic current changes (n = 6 cells each), indicating that the expressed chloride channel can be reversibly closed and opened by photoisomerization. Oocytes treated with *m*-ABFIP showed a similar reversible photoswitching trend upon alternative irradiation by UV (λ = 365 nm) and blue (λ = 430 nm) light (Fig. 3e, 3f) (n = 6 cells each), indicating *trans*-configuration was more active.

_m_-ABFIP and DTFIP1 enable optical control of behavioral responses of insects

To examine the potential behavioral effects of *m*-ABFIP and DTFIP1 in living insects, the transparent larvae of mosquito were used to evaluate light-induced behavioral responses. We designed an experiment with three culture dishes containing two larvae each, treated with PCLs (by bath application) and/or light, individually and in combination and monitored their locomotor behavior (Fig. 4a). Initially, the optimal ligand concentration was screen to ensure insects would not die before the behavioral response appeared (Supporting information S8). Under dark conditions, DTFIP1-treated (5 μM) larvae performed as untreated larvae (Movie S1 and S2). Illuminated under UV light, mosquito larvae treated with DTFIP1 (5 μM) became excited and exhibited characteristic, increased motility (Movie S2). An obvious increase in movement distance was present with prolonged irradiation time (Fig. 4b). Although the activity difference of *m*-ABFIP was small, through careful selection of ligand concentration (Supporting information S9), we successfully managed to induce photostimulated behavioral responses in mosquito. After irradiation, a decrease in motility was observed in 0.6 μM *m*-ABFIP treated larvae (Fig. 4c and Movie S3), suggesting the ionotropic GABAR-mediated inactivation occurred in the larvae *in vivo*, which correlate with the electrophysiological data *in vitro*. Next, we investigated the reversibility of the behavioral modification using DTFIP1 (Supporting information S10). UV irradiation triggered locomotion in larvae (5 μM), whereas cessation of UV irradiation and bursts of green light quickly relaxed the larvae. We repeated these cycles of treatment by alternating irradiation with UV and green light and found that DTFIP1 worked well for reversible triggering excitement of mosquito larvae (Movie S4). The movement trajectory and distance are shown in the video clip (Fig. 4d), demonstrating significantly increased motility of DTFIP1-treated larvae in the presence of UV light.

Discussion

In the present study, we generated photoswitchable ligands that are able to control the ionotropic GABA receptors in insects. The PCLs, including *m*-ABFIP and DTFIP1, are versatile tools for modulating the ionotropic GABAR-mediated biological events. Fipronil, as a representative antagonist of insect ionotropic GABARs, is an ideal starting compound in PCLs due to its high affinity for homomeric insect RDL-type GABARs, its simple synthesis, and its tolerance to structural modification. A robust way to generate PCLs is by attaching a photoswitch to the exterior of existing, potent ligands (Velema *et al.*, 2015). Following this strategy, PCLs acting on insect ionotropic GABAR were generated by peripheral modification of fipronil with an azobenzene or dithienylethene as photoswitch. This also permits easy evaluation of different photoswitches to determine which one(s) fits best for the intended purpose. We demonstrate here the effectiveness of azologization for photochromic ligand modification. To our knowledge, dithienylethene is rarely used for drug modifications since it is recalcitrant to relatively small changes to its molecular geometry. However, in this study we found that its utility for activity switching, was even better than azobenzene.

We initially evaluated the ability of PCLs to induce differential activity *in vivo* via photoisomerization in insects, and identified two potent ligands, including DTFIP1 and *m*-ABFIP. As no activity was observed *in vivo* with azobenzene and dithienylethene, we presume that PCLs have the same mode of action as fipronil, and this was further verified via experiments with homomeric *Ls*RDL-type GABARs expressed in *X. laevis* oocytes. DTFIP1 works robustly in light-dependent reversible control over the opening and closing of ionotropic GABARs. However, UV light-initiated phenomena was not observed in the control groups, suggesting that insect homomeric RDL-type GABARs are not sensitive to UV light and we can exclude that UV or green light alone elicit inward currents.

To our knowledge, DTFIP1 is the first divalent ligand derived from fipronil to date. The 90-fold enhancement of light-induced insecticidal activity *in vivo* of DTFIP1 indicates that completed switching on and off of ligand activity was achieved. DTFIP1 has relatively low photoconversion efficiency with a photoisomer ratio of 67:33, implying that closed-DTFIP1 had higher activity than that tested in the photostationary state. We speculated that higher activity differences could be achieved with total conversion to the closed form. However, activity differences between the azobenzene modified fipronil and DTFIP2 were slim, although their activities were higher. This

finding indicates that the geometry change at this position cannot significantly influence the ligand's potency, suggesting that attaching large sized groups at the $-NH_2$ can preserve a substantial physiological activity (Kagabu *et al.*, 2010). The data of structure-activity relationship are consistent with previous findings that attaching a large-sized group at the amine ($-NH_2$) position does not quench activity. Therefore, geometry changes of photoswitches may not be the main cause for the light-induced activity turbulence of DTFIP1. The differences of activity might be caused by the pronounced N-N length change from 7.9 Å to 11.04 Å (Supporting information S11), which was previously determined vital for activity levels of divalent ligands (Chang *et al.*, 2001).

For testing in living organisms, the proper physiochemical properties and metabolic stability is necessary for the diffusible PCLs to reach the binding pocket and being photoisomerized after forming stable ligand-receptor complexes. Both DTFIP1 and *m*-ABFIP are useful in acute optical control of mosquito larvae. For behavioral control, the ligand concentration must be carefully optimized to avoid adverse effects on the mosquito larvae at high concentrations and yet trigger a detectable response on behavior. Upon UV irradiation, a critical amount of active closed-DFFIP1 was quickly achieved, which induced an excitatory behavioral response. The mosquito larvae rapidly recovered from the arousal when subjected to green light irradiation, indicating that DTFIP1 allows reversible, light-dependent control over mosquito larvae. When transitioning back from green to UV light irradiation, 20 s was required to resume the activation. This was probably due to the time required to accumulate sufficient amounts of effective ligand to exert observable behavioral response. Through careful titration of ligand concentration, a light-induced differential behavioral response of *m*-ABFIP was also achieved, albeit with smaller activity variations between photoisomers.

In this study, we firstly synthesized a library of PCLs acting on ionotropic GABARs, and the dithienylethene-derived PCLs demonstrate the utility of this photoswitch in GABAR photopharmacology. Fipronil displays weak photostability and a coumarin-caged fipronil was prepared to enhance stability (Gao *et al.*, 2017), but the photocontrolled release process is irreversible. Interestingly, the DTFIP1 may provide a lead for control of mosquito larvae. Furthermore, insect ionotropic GABARs have for a long time been targets of insecticides, and, the generation of PCLs could be helpful in studies of the mechanisms, timing and location of action of

ligands, as well as localizing the optimal neuronal target of the ligands. Appropriate PCLs will also facilitate our understanding of toxic mechanisms and selectivity between target and non-target organisms.

In conclusion, we developed azobenzene and dithienylethene-based PCLs, which target insect ionotropic GABARs and provide bidirectional optical control over activity of homomeric insect RDL-type GABAR *in vitro*, and behavior of insect. The photo responsivness of GABAR PCLs furthermore provides an invaluable tool for studying links between neuronal activity and behavior of insects and other invertebrates. Even in genetically tractable organisms such as *Drosophila,* PCLs will provide valuable complements in studies of GABA signaling.

Materials and Methods

Synthesis of PCLs

Information on chemicals, instrumentations, synthetic procedures and characterization of the synthesized compounds are provided in Supporting information S3.

Determination of photoswitching properties

PCL solution (0.02 μM in acetonitrile) was irradiated by hand-hold MQK-WFH-204B ultraviolet lamp (365 nm, 10 mW cm^{-2}, MQK, Shanghai) and blue light (430 nm, 5 mW cm^{-2}) or green light (520 nm, 5 mW cm^{-2}) alternatively. Absorption spectra was recorded on a Lambda 650 UV-Vis spectrophotometer. The fatigue resistance was determined after 12 cycles of alternative irradiation of ultraviolet or blue/green light. The ratio of closed- and open- isomer was determined by HPLC analysis before and after 365 nm light irradiation. The ratio of closed and open was calculated at the isosbestic points.

Calculation of N-N length

The length of N-N of DTFIP1 was calculated using PyMOL 1.7 (Schrödinger, LLC, 2015, New York, NY, USA).

In vivo assay

All experiments were conducted three times with three replicates in each case. Insect was considered as dead if it did not move when prodded with a syringe. Two groups of PCL solutions in distilled water were prepared and one group was irradiated by 365 nm UV light for 15 min. The 4^{th}-instar larvae of mosquito were obtained from National South Pesticide Initiative Center

(Shanghai, China). Ten fourth-instar mosquito larvae were transferred into each plastic container containing test solution. Larvae swim in the test solution and presumably PCLs are both ingested and taken up by permeation. The containers were covered with black cloth and positioned in the conditioned room (25 ± 1 ºC). The mortality rates were calculated after 24-hour treatment.

TEVC assay.

The insect RDL subunit was cloned from small brown planthopper *Laodelphax striatellus*, and expressed in *Xenopus laevis* oocyte as previously described (Sheng *et al.*, 2018). Electrophysiological experiments were carried out in room light at 18-20 ºC using a TEVC technique on Axoclamp 900A Microelectrode Amplifier plate (Molecular Devices, San Jose, CA, USA) under holding potential of -80 mV. The current signals were recorded by Axon Digidata 1440A Data Acquisition System (Molecular Devices). Oocytes were placed in a recording chamber perfused with a standard oocyte saline (SOS) [100 mM sodium chloride (NaCl), 2 mM potassium chloride (KCl), 1.8 mM calcium chloride ($CaCl_2$), 1 mM magnesium chloride ($MgCl_2$), and 5 mM HEPES, pH 7.6] by flowing at 8-10 mL min^{-1}. GABA dissolved in a SOS solution was applied to stimulate oocytes for 5 s, at intervals of 85 s. Dose-response curves and half maximal effective concentration (EC_{50}) were obtained by sequential applications of increasing concentrations of GABA to homomeric *Ls*RDL channels expressed in oocytes (Jiang *et al.*, 2021). Test compounds were firstly dissolved in dimethyl sulfoxide (DMSO), and subsequently diluted using a SOS solution to a final DMSO concentration less than 0.1% (v/v). Test compound solution was added to the perfusate after successive control applications of GABA, and then applied consecutively for remainder of experiments for 5 s at 85 s intervals during perfusion. Median inhibition concentration (IC_{50}) values were determined from the mean of six replications using standard probit analysis with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Light-regulated activation of homomeric *Ls*RDL-type GABAR

The experiment was carried out in a dark room. A UV light (λ_{max} = 365 nm), a green light (λ_{max} = 520 nm) or a blue light (λ_{max} = 430 nm) provided UV, green or blue stimulating light, respectively. In order to detect the regulatory effect of light on PCLs, 3 μM GABA was firstly applied for 5 s and SOS in 85 s. Subsequently, 3 μM GABA and 1 μM PCL continuously perfused together. Finally, multiple UV/blue or UV/green light flashes was applied during the perfusion of GABA and PCL. The light flash interval is 1 s. Electrophysiological data were obtained using Clampex

10.2 (Axon Instruments, Molecular Device) and analyzed using Clampfit 10.2 (Axon Instruments) and OriginPro 9.2 software (OriginLab, Northampton, MA, USA).

Photomodulation of mosquito larval behavior

The 4th-instar larvae of mosquito were transferred into a culture dish containing 4 mL tested solution and were kept at 25 ^{o}C for 6 h. Two 4th-instar mosquito larvae for each group and three sets of experiments were performed. The first group of larvae treated with 5% aqueous DMSO solution was irradiated with 365 nm UV light. The second group treated with PCL (5 μM DTFIP1 or 0.6 μM *m*-ABFIP) were kept in the dark. The third group treated with PCL (5 μM DTFIP1 or 0.6 μM *m*-ABFIP) was irradiated alternatively by 365 nm UV light and 520 nm green light (or 430 nm for *m*-ABFIP). Each treatment was performed in six replicates. Videos of mosquito larvae were recorded by camera. ImageJ software (Schneider *et al.*, 2012) was used for analysis of moving trajectory and distance.

Supplementary Information

Supplementary information accompanies this paper at…..

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