Adrenergic modulation with photochromic ligands

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Abstract: Adrenoceptors are ubiquitous and regulate heart and respiratory rate, digestion, metabolism, and vascular tone. They can be activated or blocked with adrenergic drugs, but systemic administration causes broad adverse effects. We have developed photochromic ligands (adrenoswitches) to switch on and off adrenoceptor activity on demand at selected locations. Their pharmacology, photochromism, bioavailability and lack of toxicity allow photomodulating adrenergic signalling, as demonstrated by controlling locomotion in zebrafish and pupillary responses in blind mice.

Adrenergic neurotransmission plays an essential role over our body's unconscious regulation of vital functions such as heart and respiratory rate, digestion, smooth-muscle contraction, gland secretion and pupil diameter. More generally, it is part of that branch of the autonomic nervous system known as sympathetic nervous system (SNS), which mobilizes a rapid response whenever a situation of acute stress and/or danger is presented. Because of the almost ubiquitous innervation of the sympathetic fibres, adrenergic receptors (ARs), both α and β subtypes, are extensively expressed in the human body. Over the years, this has made them extremely attractive and fruitful targets for pharmaceutical research and industry. Adrenergic modulators are used to effectively treat a variety of conditions such as hypertension, asthma, glaucoma, rhinitis, depression, heart failure, several types of cardiac arrhythmias, anaphylaxis, and migraine. However, the list of potential side effects is even longer, which hampers the use of the aforementioned treatments or lowers their tolerability[1]. Limitations of classical pharmacotherapy are mainly ascribable to poor drug selectivity and are the most restrictive when it comes to agents characterized by low therapeutic indices (i.e. high acute toxicity) and/or by the necessity of chronic and systemic administration (i.e. protracted exposure to off-target effects, low compliance). In this regard, photopharmacology allows to precisely deliver drug activity in space and time[2,3]. Photochromic groups that respond to specific light stimulation (e.g. azobenzene, which reversibly photoisomerizes between *cis* and *trans* configurations) are introduced into the structure of bioactive compounds thus allowing for non-invasive, on-demand control over a variety of biological targets. Successful examples of photoswitchable drugs include small molecule ligands of ion channels, receptors and enzymes, peptides, lipids and nucleic acids[4]. However,

despite the expanding applications of photopharmacology, drugs to photoswitch adrenergic neurotransmission are not available. Considering the physiological importance of the sympathetic system, we aimed our efforts at developing a small library of novel adrenergic ligands that could be remotely and dynamically controlled with light. Among the large database of sympatholytic and sympathomimetic agents built over decades of investigation, we focused our attention on a class of cyclic amidines structurally related to clonidine and to other widely commercialized drugs targeting α-ARs. (**Figure 1A**).

All adrenergic agents can be simplified to a common pharmacophoric motif, which consists in a primary or secondary aliphatic amine, protonated at physiological pH, and separated by a short linker (1-3 atoms) from a substituted benzene ring. We postulated that α-adrenergic ligands were suitable candidates for "azologization"[5,6] as the structural changes required to obtain photo-responsive derivatives could be introduced without altering essential pharmacophoric elements. In addition, the prospect of obtaining dark-inactive adrenergic azologs was supported by the lack of biological activity of *trans*-like epoxydic analogues compared to *cis*-like compounds[7]. We thus designed a set of putative photoswitchable adrenergic ligands, named "adrenoswitches", by replacing the two-atom linker with an azo group while constraining the cyclic amidine moiety to the closest structurally related aromatic derivatives. Since small structural changes can drastically alter the pharmacological profile of the molecules[8–11], we opted for a classical medicinal chemistry approach maintaining unvaried the substituted benzene moiety while exploring different heterocycles in order to afford both adrenergic activity and photochromism. The *ortho*-dichlorobenzene system common to clonidine and lofexidine seemed a feature worth maintaining for both purposes. First, halogen substitution increases the lipophilicity of a molecule, thus improving its absorption and its permeability to the blood brain barrier or the blood ocular barrier[12]. This property is correlated with the potency of hypotensive agents acting on the central nervous system (CNS)[13,14]. Moreover, lipophilic substitutions at the positions 2 and 6 of the phenyl ring are well tolerated in terms of pharmacological activity both by α_1- and α_2-ARs[15]. Secondly, from a photochromic point of view, *ortho*-halogenated azobenzenes benefit of enhanced thermal stability, longer photoisomerization wavelengths, and higher isomerization ratios when compared to their parent compounds[16–18].

Having defined the hydrophobic moiety and the photoresponsive bridge of our derivatives, we moved on to identify suitable aromatic heterocycles. In our first analogue (adrenoswitch-1, **Figure 1B**) we substituted the imidazoline ring with an imidazole. As phenylazoimidazoles are known to undergo fast *cis-trans* thermal back-isomerization (i.e. fast-relaxing photoswitches), in adrenoswitch-2 we sought to reduce the rate of this process by employing an *N*-methyl imidazole[19]. In adrenoswitch-3, we introduced a permanent positive charge with a *N*,*N*-dimethyl imidazolium in order to better mimic the electronic properties of the original cyclic amidine in its physiologically protonated form. An alternative strategy for the same purpose was using 2-aminothiazole in adrenoswitch-4.

Our library of compounds was prepared via a divergent synthetic approach involving a standard azo coupling reaction (**Figure 1B**). Commercially available 2,6-dichloroaniline was converted into the corresponding diazonium salt and reacted under mild basic conditions either with imidazole to provide adrenoswitch-1 or with 2-aminothiazole to afford adrenoswitch-4. Adrenoswitch-2 and adrenoswitch-3 were obtained from adrenoswitch-1 through reactions of mono- or di-*N*-methylation, respectively.

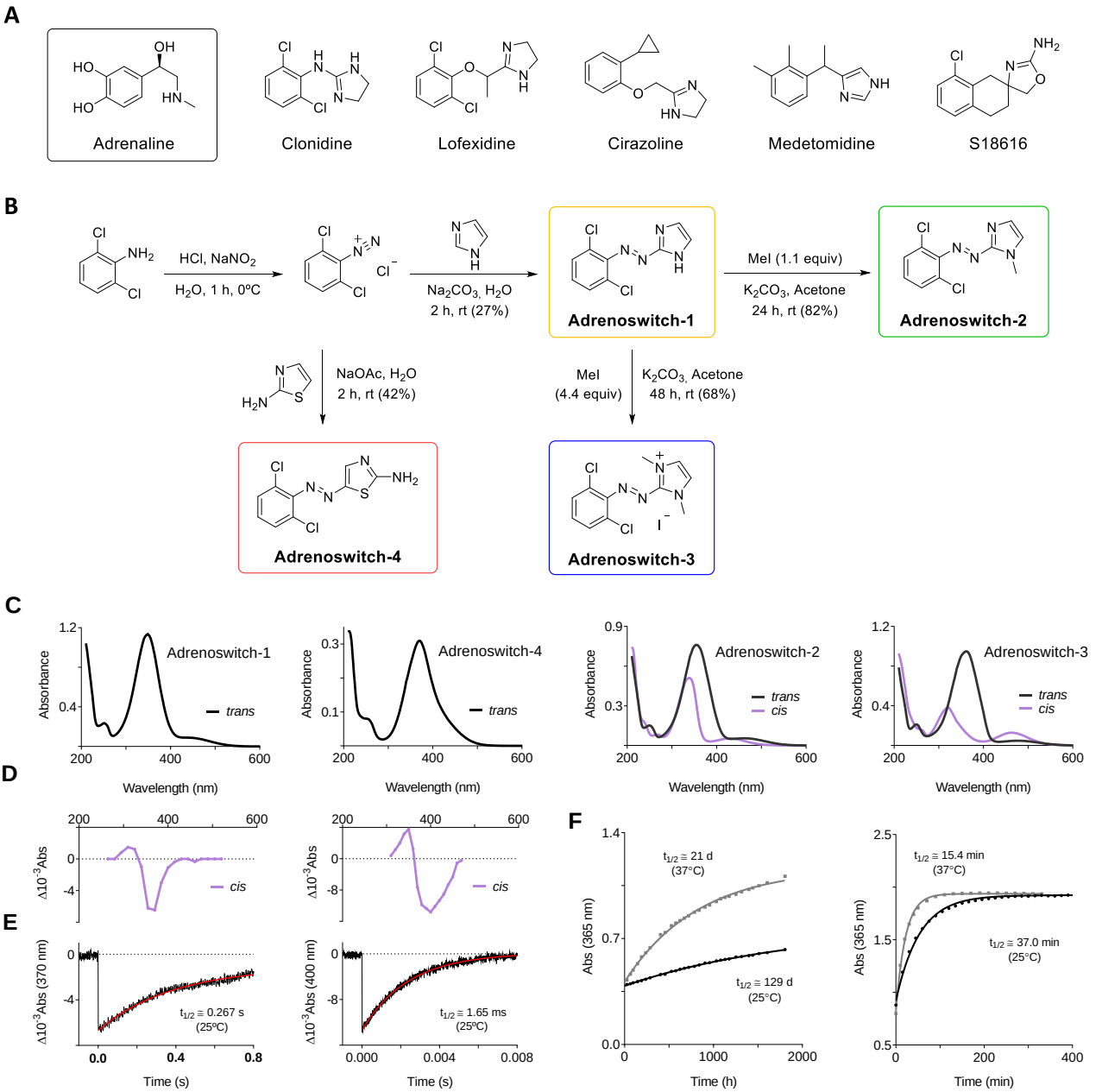

Figure 1. A) Chemical structures of adrenaline and some of the synthetic adrenergic ligands that inspired this work. **B)** Divergent chemical synthesis of adrenoswitches 1-4. **C)** UV-Vis absorption spectra of the *trans* isomers, for slow-relaxing adrenoswitches -2 and -3, UV-Vis absorption spectra of the *cis* isomers are also reported. Spectra were extracted from UPLC chromatograms after elution with a mixture of water and acetonitrile supplemented with trifluoroacetic acid. Spectra of *cis* and *trans* isomers were normalized at their isosbestic points (see SI – **Figures S3A-C**). **D)** Transient absorption spectra of adrenoswitch-1 and -4 at $t = 0$ upon pulsed excitation at λ_{exc} = 355 nm in physiological buffer (pH=7.4 at 25°C). **E)** Absorption loss and recovery kinetics of adrenoswitch-1 and adrenoswitch-4, measured at λ = 370 and 400 nm respectively, upon irradiation with a single ns laser pulse (t=0) at λ_{exc} = 355 nm and 25 °C in physiological buffer (pH=7.4). Red lines correspond to monoexponential fitting of the experimental data. **F)** *Cis*-to-*trans* thermal relaxation of adrenoswitch-2 and -3 at 25 ˚C (in black) and 37 ˚C (in gray) under dark conditions. Data were fitted to a monoexponential decay model to estimate the half-lives of the *cis* isomers.

The photochromic behaviour of our compounds was then investigated. It is worth mentioning that, although several azoheteroaryl photoswitches have been already reported in the literature[20], the photoswitches contained in our compounds have never been described before. Slow-relaxing adrenoswitch-2 and -3 were characterized by steady-state UV-Vis absorption spectroscopy (**Figures 1C-F**), while transient UV-Vis absorption spectroscopy was used for fast-relaxing adrenoswitch-1 and -4 (**Figures 1D-E**). All our compounds

can be photoisomerized from *trans* to *cis* with (ultra)violet light (365–400 nm) and from *cis* to *trans* with blue or green light (450–500 nm). As intended by design, thermal relaxation rates vary considerably along the series. Measured half-lives spanned from milliseconds (adrenoswitch-4) to seconds (adrenoswitch-1), minutes (adrenoswitch-3), and months (adrenoswitch-2) (**Figures 1E-F**).

With all the information in hand to effectively photoswitch our ligands, we moved on to assess their biological activity as a function of illumination. The affinity of the library towards α_2-ARs was measured by competitive radioligand binding assay in pre-frontal cortex membranes obtained *post mortem* from human brains. All the adrenoswitches competed in binding to the receptor albeit at weaker affinities than clonidine. Most notably, the slow-relaxing adrenoswitch-2 and -3 showed a significant change in binding potency upon UV illumination (**Figure S4A**).

Since the adrenergic system is a major regulator of the vascular tone, we further characterized the adrenergic effects of our compounds by measuring their vasoconstrictor/vasodilatory activity in *ex vivo* rat aortic rings where the endothelium had been preserved[21–25]. Compounds were first screened for their vasoconstrictor potencies by cumulative additions on plain quiescent vessels, on which they all proved unable to evoke contractions. Subsequently, we examined their vascular activity on rings pre-contracted with phenylephrine (PE). We observed that vascular smooth muscle relaxes when exposed to UV light alone, substantially abolishing the contraction evoked by PE[26]. These intrinsic photoresponses are due to nitric oxide (NO) release either from endogenous or exogenous donors or from storage vesicles[27,28]. To minimize unintended photorelaxation, we pre-incubated the vessels in a suitable nitric oxide synthase (NOS) inhibitor, namely N^G-methyl-L-arginine acetate (L-NMMA) which is reported to be unaffected by light[29]. Remarkably, all adrenoswitches displayed high vasodilatory efficacies (maximal evoked response E_{max}, **Figure 2A**) with adrenoswitch-1 coming through as the most potent compound and the only one displaying a light-dependent behavior in this assay (3x increase in potency upon UV illumination, **Figure S8A**). Although this assay was useful to identify and rank the adrenergic efficacy and potency of adrenoswitches, it had important shortcomings. Intrinsic photoresponses forced us to use a NOS inhibitor, thus partially blocking endothelium-dependent relaxation responses to α_2-AR agonists[30,31]. Moreover, the model proved complex to clarify adrenergic pharmacodynamics and it cannot exclude contribution of other receptors to the observed antihypertensive responses.

We next characterized the activity of adrenoswitches *in vivo*. Zebrafish (*Danio rerio*) is a well established animal model for neurobehavioural research[32,33] and toxicological studies,[34,35] and the transparency of their larvae makes them convenient for photopharmacological applications[36–39]. The expression and function of zebrafish ARs have been studied[40,41] and the effect of dexmedetomidine, a central-acting and structurally-related α_2-AR agonist, has also been described by Ruuskanen *et al.*[42].

In analogy to the sedative effect reported for dexmedetomidine, administration of clonidine (50 µM) caused locomotor inhibition when compared to untreated control animals (**Figure 2B**, fish trajectories shown in light blue and grey wells respectively). Clonidine effect was not significantly altered by UV illumination, although in vehicle-treated animals we observed an increase of the swimming distances as a result of their photomotor response to UV light.[33] In contrast, larvae treated with adrenoswitch-1 (50 µM) responded to UV light by progressively reducing their locomotion to levels comparable to clonidine-treated animals under UV (**Figures 2B-C and Figures S9-S10**). Therefore, adrenoswitch-1 displayed a clonidine-like behaviour upon activation with UV light, while its *trans* isomer was unable to evoke sedation. Although this effect is unequivocally light-induced, it did not revert after turning the light off despite the fast-relaxing nature of the compound (**Figure 1E and Figure S10**). Moreover, this behavioural set-up cannot exclude the involvement of other non-

adrenergic receptors in evoking locomotion responses. We thus continued studying adrenoswitch-1 in a different *in vivo* model.

Among the available experimental models for assessing autonomic responses *in vivo*, we sought one in which the adrenergic system played a major physiological role, and in which the target tissue was easily accessible to light. The quantification of mydriatic or miotic responses by pupillometry fulfilled both criteria.[43] In addition, this assay allows for a selective evaluation of α-adrenergic activity and it excludes the involvement of other targets, most notably imidazoline receptors[44–46]. As pupils naturally adapt their diameter in response to changes in light intensity (pupillary light reflex), we resorted to genetically engineered mice (*Opn4xRd10*). These animals do not express melanopsin and their rods and cones degenerate two months after their birth, thus becoming physiologically insensitive to any luminous stimuli. We tested if adrenoswitch-1 enabled photoregulation of pupil diameter after topical administration (1 mM, 0.02% w/v) to isoflurane-anesthetized blind mice. We observed that the compound exerted mydriasis only under concomitant UV illumination, and that the effects were reversed in the absence of light, which causes adrenoswitch-1 to rapidly relax to the *trans* configuration (**Figures 2D-E-F**). These pupillary responses were reproducible in at least two cycles of alternating UV light and darkness, and were neither elicited by application of the vehicle nor by exposure to UV light alone. The maximum photoresponses were consistently observed after approximately 20 min from its administration (**Figure 2F**), in agreement with the pharmacodynamics of adrenergic ligands with similar structure and lipophilicity to adrenoswitch-1[47].

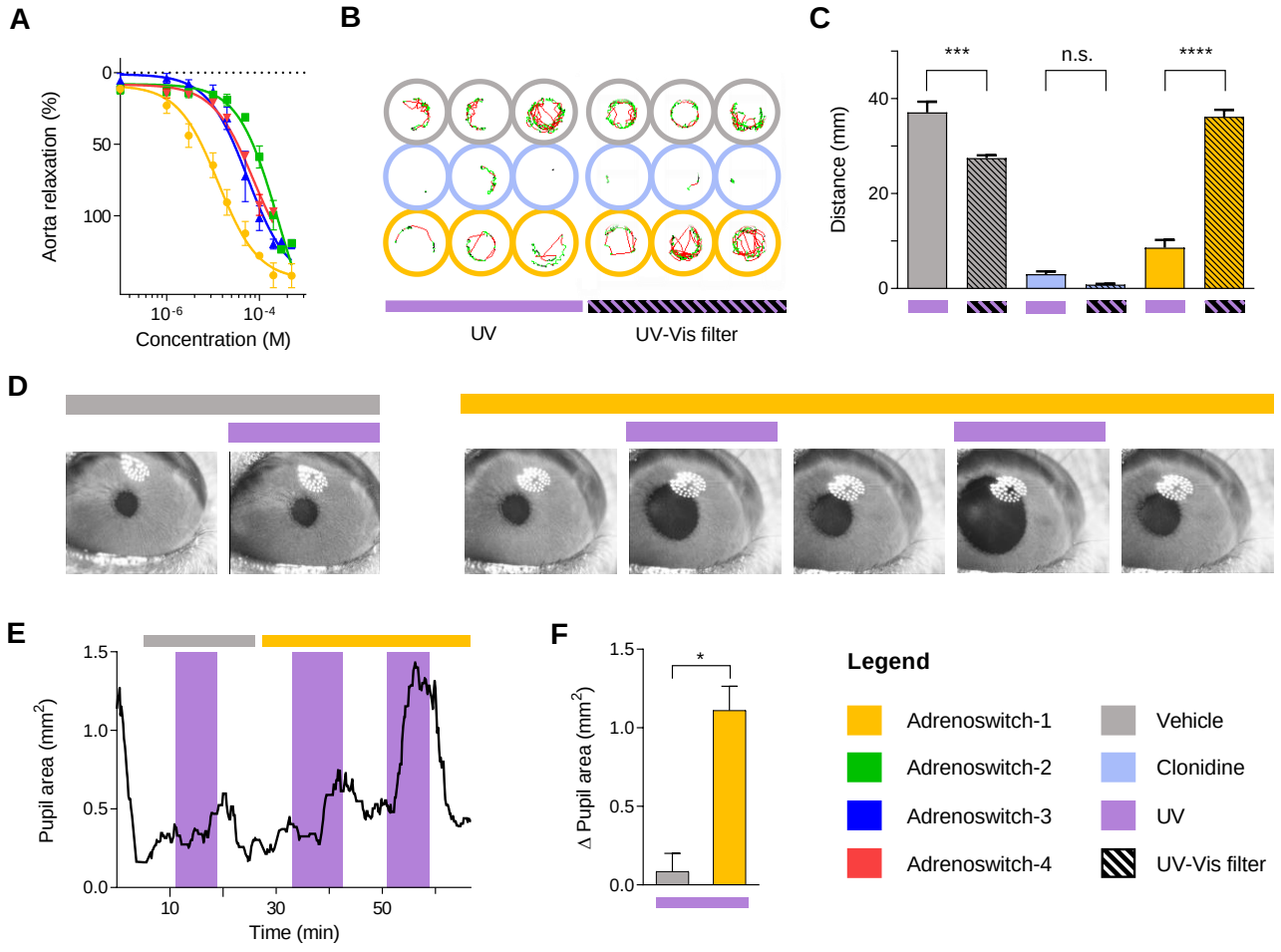

Figure 2. A) Dose-response curves comparing the vasodilatory potencies of *cis*-enriched adrenoswitches administered under UV illumination to rat aortic rings where the endothelium had been preserved. Vessels were pre-contracted with 10^{-6} M phenylephrine after treatment with 10^{-3} M N^{G}-methyl-L-arginine acetate (L-NMMA), a nitric oxide synthase inhibitor. Relaxation is expressed as

percentages of the reference contraction induced by PE. Data are means ± SEM (adrenoswitches 1-3, *n*=4; adrenoswitch-4, *n*=2). **B)** *Danio rerio* 7 days post-fertilisation (dpf) larvae swimming trajectories (movements with velocities over 6 mm·s^{-1}) after treatment with the vehicle (grey wells), 50 μM clonidine (light-blue wells) and 50 μM adrenoswitch-1 (yellow wells). Conditions were simultaneously analysed under 365 nm UV illumination (left panel, *cis*-enriched adrenoswitch-1) and under a UV-Vis filter (right panel, *trans* adrenoswitch-1) that only transmit infrared light for movement recording. **C)** Quantification of the swimming trajectories shown in **B)** after 20 minutes in the presence of the vehicle (grey), 50 μM clonidine (light blue) and 50 μM adrenoswitch-1 (yellow) under UV illumination or under a UV -Vis filter (shades bars). Data are means ± SEM (*n*=12 per treatment). Statistical differences between UV- and non-UV-exposed larvae were determined by two-way ANOVA with Tukey's multiple comparison test (n.s., not significant; ***, *p*-value<0.01; ****, *p*-value<0.001) **D-E)** Pupillary responses in an isoflurane-anestethized *Opn4xRd10* blind mouse first treated with the vehicle and then administered 1 mM adrenoswitch-1 in its right eye. Adrenoswitch-1 exerted mydriasis only under concomitant UV illumination. Nor the vehicle nor UV light alone elicited any pupillary responses in the animals. **F)** Change in area of vehicle vs. adrenoswitch-1 treated pupils of *Opn4xRd10* blind mice at 20 minutes from administration of the drug when under concomitant UV illumination. Data are means ± SEM (*n*=4 per condition). Statistical differences were determined determined by Student's *t* test for paired observations.

As mydriasis is mediated by postsynaptic α-adrenoceptors of the iris smooth muscle dilator, the results of **Figures 2D-E-F** unambiguously demonstrate that adrenoswich-1 modulates endogenous ARs with light. This opens the way to multiple applications in the SNS and CNS that were not previously accessible. For example on-demand adrenergic modulation at specific locations might allow to blunt hypertension, to treat glaucoma, or to single out individual adrenergic projections from the locus coeruleus. In addition to these novel applications, spatiotemporal modulation of adrenoceptors should improve the efficacy of treatments (including higher doses) and prevent side effects.

Optogenetic control of adrenoceptors has been shown but it requires overexpressing opsins in the target tissue using genetic manipulation, and thus several safety and regulatory hurdles should be overcome for therapeutic purposes[48–51]. Currently the only way to target endogenous receptors and physiological adrenergic pathways is by means of drugs. Uncaging of adrenergic ligands is irreversible and releases undesired by-products[52,53]. Here, we have rationally designed novel arylazoheteroarene reversible photoswitches and characterised their adrenergic action *in vitro* and *in vivo*. The drug-like properties of these adrenoswitches, the absence of acute toxicity in zebrafish larvae and most remarkably, the fact that adrenergic (photo)modulation was readily and reversibly achieved in mammals by topical application without formulation, all indicate that adrenergic photomodulation offers unique opportunities to understand physiological signaling and to develop safe and effective therapies.

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