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A Photochromic Agonist for μ-Opioid Receptors**

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Abstract: *Opioid receptors (ORs) are widely distributed in the brain, the spinal cord, and the digestive tract and play an important role in nociception. All known ORs are G-protein-coupled receptors (GPCRs) of family A. Another well-known member of this family, rhodopsin, is activated by light through the cis/trans isomerization of a covalently bound chromophore, retinal. We now show how an OR can be combined with a synthetic azobenzene photoswitch to gain light sensitivity. Our work extends the reach of photopharmacology and outlines a general strategy for converting Family A GPCRs, which account for the majority of drug targets, into photoreceptors.*

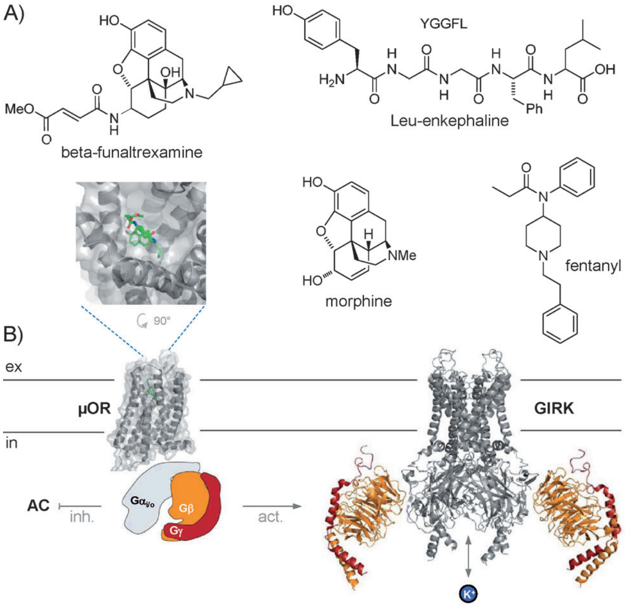

Figure 1. Pharmacology, structure, and function of the μ-opioid (MOR) receptor. A) MOR ligands. Beta funaltrexamine is a covalently bound agonist. Its binding mode is shown in the inset. Leu-enkephaline (LE) is an endogenous ligand, whereas morphine and fentanyl are naturally occurring and synthetic agonists, respectively. B) Functional features of MOR. When activated by agonists, MOR acts as a nucleotide exchange factor on heterotrimeric $G_{i/o}$ G-proteins, which results in dissociation of the G_{α} and $G_{\beta\gamma}$ subunits. While G_{α} has an inhibitory effect on the adenylyl cyclase (AC), the $G_{\beta\gamma}$ complex activates G-protein coupled inward rectifying potassium channels (GIRK channels). Both modes lead to decreased neuronal activity. ex = extracellular side, in = intracellular side.

Opioids have a long cultural and medicinal history and are among the most useful drugs available to humanity. Their best-known member, morphine, has been employed since antiquity to alleviate pain and induce euphoria (Figure 1 A).[1,2] With the advent of modern organic chemistry, many synthetic and semisynthetic opioids, such as fentanyl, became available, some of which facilitated the pharmacological characterization of receptor subtypes.[3,4] Subsequently, these transmembrane proteins were systematically explored through molecular cloning. Today, four human opioid receptor (OR) subtypes, namely, δ, μ, κ, and NOP, are known, all of which have been recently characterized by X-ray crystallography.[5–10] The X-ray structure of a μ-opioid receptor (MOR) in conjunction with the covalently bound antagonist β-funaltrexamine provided insights into how morphine and its derivatives bind and helped explain some of the structure–activity data accumulated over the decades (Figure 1).

Opioid receptors belong to Family A of the G-protein-coupled receptors (GPCRs), which includes many important drug targets.[11–13] GPCRs of this type are closely related to the opsin photoreceptors, which enable vision and shape the circadian rhythm in humans.[14] The MOR is the major target of morphine and is endogenously activated by small peptides, such as Leu-enkephaline (LE), β-endorphin, and the dynorphins.[15,16] Upon binding of an agonist, the receptor catalyzes the dissociation of a heterotrimeric $G_{i/o}$ protein into $G_{\alpha i}$ and $G_{\beta\gamma}$ subunits (Figure 1 B). The former inhibits adenylyl cyclase, resulting in decreased cAMP levels, whereas the latter activates G-protein-coupled inward-rectifying potassium (GIRK) channels.[17] Both actions translate to hyperpolarization and decreased neuronal excitability.[15,18]

Given the importance of opioids in medicine and their role in drug abuse, the development of new agonists and antagonists for ORs continues to be a very active field. One important goal, for instance, is to untangle the analgesic properties of opioids from their euphoric and addictive side effects. Another goal is to take advantage of the high precision light provides to elucidate the biological function of endogenous opioids and their receptors. Recently, photocaged versions of the enkephalins have been used to gain insight into the spatiotemporal scale at which neuropeptides act in the brain.[19] Additionally, a caged version of the antagonist naloxone has been employed to study opioid deactivation kinetics.[20] However, photoswitchable versions of opioids, that is, compounds that can be reversibly turned on

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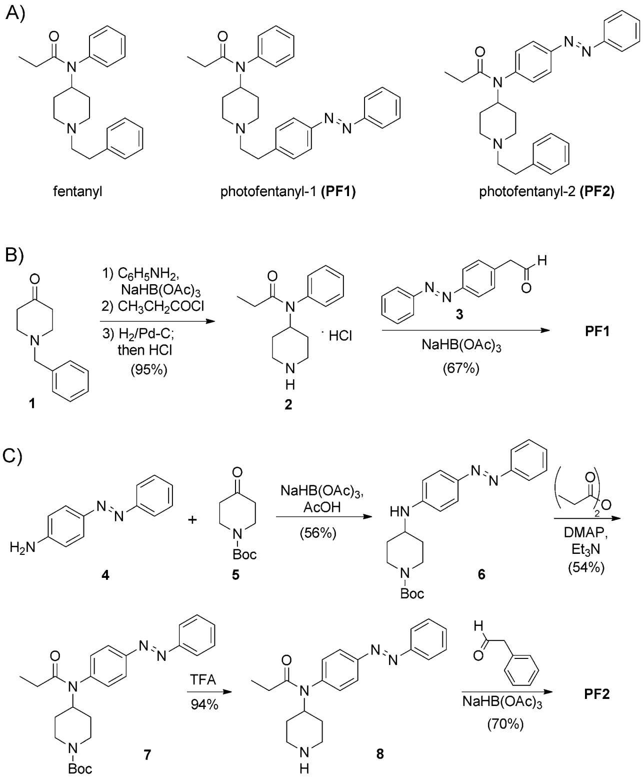
and off with light, have not been described to date. We now introduce an azobenzene derivative of the potent OR agonist fentanyl that can be used to optically control MOR in a reversible fashion.[21]

Although derivatives of morphine were initially considered,[22,23] fentanyl was ultimately chosen as a platform for the design of our photochromic ligands (PCLs). This was due to its potency, its relatively simple structure, and the presence of two aryl rings that could be extended to azobenzene photoswitches. As in the design of other PCLs, the photoswitch had to be installed in such a fashion that the biological activity of the ligand was not completely abrogated, whilst aiming for a maximum change of its efficacy upon photoisomerization.[21] Scheme 1 shows the structure of two candidates, photofentanyl-1 (**PF1**) and photofentanyl-2 (**PF2**), which we designed, synthesized, and evaluated for their biological activities.



Scheme 1. PCL design and synthesis. A) The known synthetic opiate fentanyl was used as a platform for photochromic MOR agonists **PF1** and **PF2**. B) Synthesis of **PF1** started from *N*-benzyl piperidone **1**, which was reductively aminated, acylated, deprotected, and reductively aminated with aldehyde **3**. C) Synthesis of **PF2** entails a reductive amination, followed by acylation, deprotection, and another reductive amination.

Our synthesis of **PF1** started from commercially available *N*-benzylpiperidone (**1**), which underwent reductive amination with aniline, followed by acylation and deprotection to yield piperidine **2** (see Scheme 1 and the Supporting Information). Reductive amination with azobenzene aldehyde **3**[24] then provided **PF1**. The synthesis of **PF2** started from commercially available *N*-Boc piperidone (**5**), which underwent reductive amination with 4-aminoazobenzene (**4**) to afford 4-aminopiperidine **6**. Acylation using propionic acid anhydride then gave access to propionamide **7**. Finally, deprotection under acidic conditions, followed by reductive amination of the resulting piperidine **8** with phenyl acetaldehyde gave **PF2**.

Preliminary pharmacological testing showed that **PF2** is an excellent μOR agonist. **PF1**, on the other hand, lacked MOR agonism at all concentrations. We therefore decided to focus on **PF2** for further physical and biological characterization.

Using UV/Vis spectroscopy, we first determined that **PF2** has all the hallmarks of a "regular" azobenzene (Figure 2). As shown in Figure 2 A, the $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions are well separated, allowing for clean photoswitching upon irradiation with 420 nm and 340 nm light, respectively (Figure S1). **PF2** in its *cis* form was also found to be thermally stable in Ringer solution (Figure 2B). After **PF2** had resided in the dark for 90 min, virtually no back-isomerization to its *trans* state was detected. This feature might be helpful in physiological experiments were prolonged illumination times are undesirable.

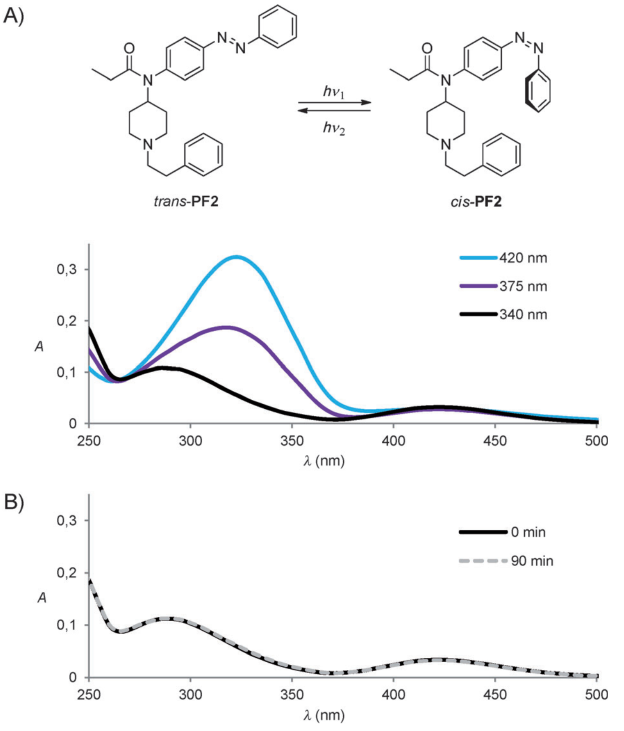


Figure 2. Photoswitching and thermal stability of **PF2**. A) Light-induced isomerization can be followed by UV/Vis spectroscopy. Illumination of a 50 μM **PF2** solution in Ringer buffer with different wavelengths leads to distinct absorption spectra. B) **PF2** that was previously switched to *cis* stays at this state for hours.

We next turned our attention to the functional characterization of **PF2**. Although GPCRs can be assayed using a variety of methods, we decided to use electrophysiology, due to its compatibility with optical stimulation protocols

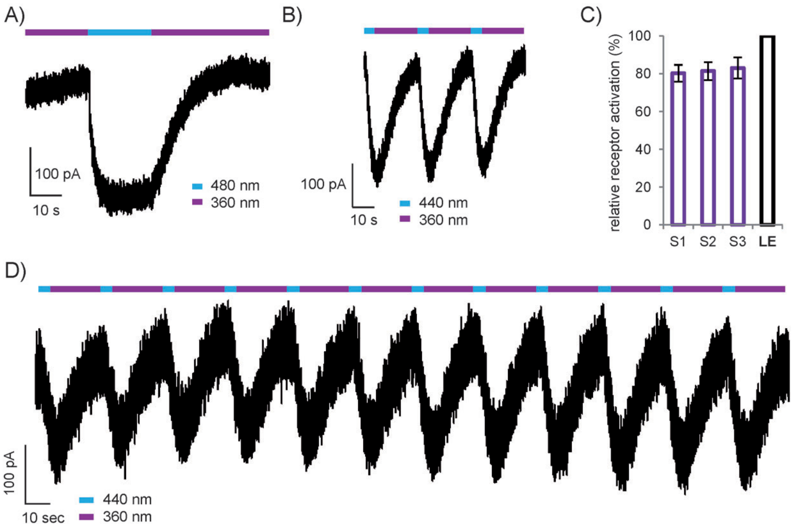


Figure 3. Light-dependent activation of MOR by **PF2**. HEK293t cells were transiently transfected with the human MOR and both GIRK1 and GIRK2 channels. A) Illuminating a 25 μM solution of **PF2** with 360 nm light keeps the system in an inactive state. Switching to 480 nm quickly activates the receptor. B) Rapid, repeated photoactivation of GIRK by action of MOR and **PF2**. C) Comparison of photocurrents (consecutive stimulation with a 10 μM solution of the native agonist LE ($n=11$, one error bar indicates ±SEM). D) Photoswitching can be repeated over many cycles without a noticeable decrease in photocurrent.

(Figure 3).[26] To this end, human MOR was transiently transfected into HEK293t cells together with the G-protein-coupled inward rectifier channels GIRK1 and GIRK2.[17] These channels are common effectors in $G_{i/o}$ signaling and native signaling partner of MORs in the locus coeruleus.[18,27] Upon testing several concentrations, we found that 25 μM **PF2** gave optimal results for photoswitching. As shown in Figure 3 A, *trans*-**PF2**, which predominates in the dark or under irradiation with 420–480 nm light, acts as an effective agonist of MOR. By contrast, *cis*-**PF2**, which predominates at 360 nm, is much less active. As a consequence, switching from 360 nm to blue light immediately triggers a potassium influx through the GIRK channels (high external potassium concentrations were applied). This process can be terminated by switching back to 360 nm light, which abrogates MOR activation. The action spectrum of **PF2** between 400 and 500 nm is shown in the Supporting Information (Figure S1). GIRK currents cease as βγ-G-proteins re-associate with inactive $G_{\alpha i/o}$ subunits bound to GDP. Interestingly, GIRK currents evoked through the action of *trans*-**PF2** are maintained at a high level. Desensitization of the system, as is the case of other GPCRs and agonists, is not observed.[17]

The repeated photoactivation of MOR with **PF2** is shown in Figure 3 B–D. Comparison between photocurrents of three consecutive switching cycles and subsequent activation of the same cell with a saturating concentration of LE revealed that **PF2** elicits, on average, 80 % of the current elicited by the native ligand (Figures 3 C,D and Figure S2). Photoactivation could be repeated a number of times, demonstrating the stability of the system (Figure 3 D). Since **PF2** acts as a *trans* agonist, the pharmacologically active isomer is also the thermodynamically more stable one. However, exposure to 360 nm light for deactivation can be kept at a minimum as *cis*-**PF2** remains stable under physiological conditions (Figure S3). In general, activation and deactivation occurred at timescales that can be expected for a metabotropic receptor.[26] As azobenzene photoswitching is known to occur on a sub-millisecond timescale[21] and MOR ligands dissociate rapidly,[19] it can be assumed that the rate of activation and deactivation is limited by the kinetic parameters of the signaling cascade that links the GPCR with the ion channel.

Recently, we described photochromic tethered ligands (PTLs) that are covalently attached to Family C GPCRs, namely, metabotropic glutamate receptors.[26] This system, termed LimGluR, was used to effectively control neuronal excitability with light. Our new results described herein should enable the extension of this concept to Family A GPCRs. These "LiGPCRs" could provide a valuable alternative to genetically encodable light-sensitive GPCRs (Opto-XRs), which are based on a fusion of rhodopsin with other Family A GPCRs.[25]

In conclusion, we have developed a method to optically control MORs with a photochromic ligand, **PF2**. In a sense, our PCL imparts the logic of rhodopsin onto a light-insensitive Family A GPCR. Rhodopsin is a member of this transmembrane protein class that uses retinal as a covalently bound photoswitchable inverse agonist. Our work outlines a potentially general chemical strategy to turn other members of Family A GPCRs, such as dopamine receptors or adrenergic receptors, into photoreceptors, laying the foundation for a photopharmacology of these targets. **PF2** itself could be used to investigate the role of ORs in various parts of the brain and the periphery in a precise spatiotemporal fashion. Its application in neuroscience as well as its potential use as a "photoanalgesic", perhaps as a variant with a red-shifted action spectrum, is currently under investigation and will be disclosed in due course.

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