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Review article

A chemogenetic technology using insect Ionotropic Receptors to stimulate target cell populations in the mammalian brain

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ABSTRACT

Chemogenetics uses artificially-engineered proteins to modify the activity of cells, notably neurons, in response to small molecules. Although a common set of chemogenetic tools are the G protein-coupled receptor-based DREADDs, there has been great hope for ligand-gated, ion channel-type chemogenetic tools that directly impact neuronal excitability. We have devised such a technology by exploiting insect Ionotropic Receptors (IRs), a highly divergent subfamily of ionotropic glutamate receptors that evolved to detect diverse environmental chemicals. Here, we review a series of studies developing and applying this "IR-mediated neuronal activation" (IRNA) technology with the *Drosophila melanogaster* IR84a/IR8a complex, which detects phenyl-containing ligands. We also discuss how variants of IRNA could be produced by modifying the composition of the IR complex, using natural or engineered subunits, which would enable artificial activation of different cell populations in the brain in response to distinct chemicals.

1. Introduction

A central question in neuroscience is how sensation, movement, emotion, and cognition emerge from the activity of complex neural circuits formed by different cell populations in the nervous system. Elucidating the principles of brain function is not only of fundamental interest: in humans, this knowledge might help understand the pathophysiology of – and promote development of treatments for – psychiatric/neurological disorders. The identification and characterization of brain cell types is highly advanced, thanks to extensive anatomical labelling studies, molecular profiling using single-cell transcriptomics (Marx, 2021; Ofengeim et al., 2017; Zeng and Sanes, 2017), and electrophysiological and optical imaging analysis of their activities. However, to understand the functions of defined cell populations and the circuits in which they are embedded, it also important to examine their necessity and sufficiency through loss- and gain-of-function perturbations, respectively (Sternson and Bleakman, 2020; Tonegawa et al., 2015).

To analyze a causal relationship between neural activity and behavior, optogenetic (Deisseroth, 2015, 2021; Kim et al., 2017) and chemogenetic (Atasoy and Sternson, 2018; Roth, 2016; Sternson and Roth, 2014) approaches have been instrumental. Optogenetic tools can reversibly perturb the activity of a target cell population under spatiotemporally restricted conditions by providing light stimulation of channelopsins. However, this approach is often highly invasive, especially in mammals, since an optical fiber must be implanted in the brain (Matsubara and Yamashita, 2021; Poth et al., 2021; Pouliopoulos et al., 2022). Chemogenetic tools induce a reversible perturbation in the target cell population triggered by the binding of specific ligands to the designated exogenous receptor. After the ligand is introduced into the body, the animal can be left to move freely, allowing remote manipulation of the target neuron activity. However, the onset and offset of the effect are difficult to control tightly (Poth et al., 2021; Sternson and Roth, 2014).

To help circumvent some of the disadvantages of current chemogenetic tools, we have recently developed a chemogenetic technology that uses Ionotropic Receptors (IRs) from the chemosensory system of the fruit fly *Drosophila melanogaster* (Fukabori et al., 2020; Iguchi et al., 2024). Here, we describe this system and compare to other chemogenetic tools, then present its applications for analyzing the neural mechanisms controlling emotional memory (Fukabori et al., 2020) and drug-induced behavior (Iguchi et al., 2024).

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2. Use of ligand-gated ion channels in chemogenetics

The most common class of chemogenetic tool are DREADDs (designer receptor exclusively activated by designer drugs) (Armbruster et al., 2007; Roth, 2016). These comprise a wide assortment of engineered G protein-coupled receptors (GPCRs) activated by distinct ligands (Nakajima and Wess, 2012; Vardy et al., 2015) that have high affinity and low off-target effects (Chen et al., 2015; Nagai et al., 2020). However, DREADDs work by manipulating endogenous G protein-signaling in the target cells, potentially leading to cellular responses beyond experimenters' expectations (Goossens et al., 2021; Pati et al., 2019; Van Steenbergen and Bareyre, 2021). Thus, there has long been a desire to develop chemogenetic approaches employing ligand-gated ion channels (LGICs), which do not require intracellular signaling pathways to manipulate neuronal activity (Atasoy and Sternson, 2018; Iguchi et al., 2024).

LGICs are generally classified into the Cys-loop, P2X, and ionotropic glutamate receptor (iGluR) families. Of these, Cys-loop receptor-dependent approaches have been well-studied, including both a neuronal silencing technology employing glutamate/ivermectin-gated chloride channels derived from *C. elegans* (Lerchner et al., 2007; Lin et al., 2011) and a neuronal activation technology employing an ivermectin-sensitive glycine receptor mutant endowed with cation permeability (Islam et al., 2016). Moreover, chimeric receptors have been developed in which the ligand-binding domain of Cys-loop LGICs (pharmacologically-selective actuator modules) transplanted to a transmembrane ion pore domain of other members of Cys-loop LGICs are activated by synthetic ligands (pharmacologically-selective effector molecules) (Atasoy and Sternson, 2018; Magnus et al., 2011, 2019). Although an ATP-sensitive P2X2-based technology has been reported in invertebrates (Lima and Miesenböck, 2005), P2X2 knockout background is required for application in mammals (Lima and Miesenböck, 2005; Zemelman et al., 2003). Until recently, chemogenetic tools using the iGluR family were unknown, and our interest in exploiting this family of receptors emerged through the discovery of a variant class of iGluR, as described in the next section.

3. Insect IRs: a functionally-diverse chemosensory family of iGluRs

As in all animals, insects detect chemical cues to extract information from the environment through olfaction and gustation (Benton, 2022; Montell, 2021; Shrestha and Lee, 2023). One important family of insect chemosensory receptors are the related Odorant Receptors (ORs) and Gustatory Receptors (GRs). Unlike most mammalian chemosensory receptors, which are GPCRs (Fulton et al., 2024; Spehr and Munger, 2009), insect ORs and GRs are ligand-gated ion channels (Benton, 2015; Joseph and Carlson, 2015), and represent the founder members of the seven transmembrane domain ion channel superfamily (7TMICs) (Benton and Himmel, 2023; Himmel et al., 2023).

Insects have a second family of chemosensory receptors to detect odors and tastants, the Ionotropic Receptors (IRs), best-characterized in *Drosophila melanogaster* (Benton et al., 2009; Koh et al., 2014; Montell, 2021; Sánchez-Alcañiz et al., 2018). IRs are derived from iGluRs, which are well-known for roles in mediating excitatory neurotransmission at synapses. However, IRs are distinguished by the large size of the repertoire in many insect species (for example, ~60 in *D. melanogaster*) and their high divergence in sequence, particularly within the extracellular ligand-binding domain (Croset et al., 2010; Rytz et al., 2013). Analogous to iGluRs, IRs are thought to assemble into heterotetrameric complexes to form cation channels gated by chemical ligands (Abuin et al., 2011, 2019). The vast majority of IRs are expressed in specific populations of peripheral sensory neurons, where they localize to ciliated sensory dendrites to detect specific environmental chemicals, mainly (but not only) acids, aldehydes and amines (Silbering et al., 2011; Yao et al., 2005). IR complexes are composed of tuning receptor subunits, which define ligand specificity (Abuin et al., 2011; Ni, 2020; van Giesen and Garrity, 2017) together with 1–2 co-receptors, which appear to provide structural stability to the complex as well as contributing to forming the channel pore (Abuin et al., 2011, 2019). One of the best-characterized *D. melanogaster* IR complexes is that containing the IR8a co-receptor and the IR84a tuning receptor, which recognizes the food-derived odors phenylacetic acid and phenylacetaldehyde, and controls male courtship behavior (Grosjean et al., 2011). The IR84a/IR8a complex can be functionally reconstituted both in other sensory neuron types in *D. melanogaster* and in *Xenopus* oocytes; in the latter, it is permeable mainly to monovalent cations (Na^+/K^+), with only low Ca^{2+} permeability (Abuin et al., 2011).

4. IR-mediated neuronal activation (IRNA) of mammalian neurons

The autonomous function of IR84a/IR8a in heterologous cells led us to investigate whether these IRs could be used as a novel type of chemogenetic tool for ligand-evoked activation of specific neuron types in the mammalian brain (Fukabori et al., 2020). To this end, we generated a transgenic mouse line expressing IR84a/IR8a in the catecholamine-containing cells. Patch clamp recording performed on acute brain slices of the locus coeruleus of these mice revealed excitatory responses of the IR84a/IR8a-expressing locus coeruleus neurons to phenylacetic acid. Furthermore, through a multifaceted analyses, including *in vivo* extracellular recording of the locus coeruleus neurons, *in vivo* microdialysis for norepinephrine release in the terminal region (the anterior cingulate cortex and basolateral amygdala), and measurement of norepinephrine-related behavioral effects, we demonstrated that the IR84a/IR8a-expressing locus coeruleus neurons exhibit a phenylacetic acid-dependent activation (Fig. 1).

A key factor for any chemogenetic system is minimizing activation of the designated exogenous receptor when a cognate ligand is not externally applied. Previous studies have shown that a low level of phenylacetic acid is present in rodent brain and human cerebrospinal fluid (Durden and Boulton, 1982; Sandler et al., 1982). However, we found no significant differences in the basal firing frequency of the locus coeruleus neurons and basal extracellular norepinephrine concentration in the terminal region between the IR84a/IR8a-expressing mice and wild-type littermate controls (Fukabori et al., 2020). These observations indicate that endogenous phenylacetic acid has negligible effect on IR84a/IR8a activation.

The mammalian brain is protected from exposure to harmful substances circulating in the blood by the blood-brain barrier (Daneman and Prat, 2015), making it challenging to transfer efficiently phenylacetic acid from the peripheral bloodstream into the brain. In the above-mentioned *in vivo* experiments, phenylacetic acid was administered directly into the brains of mice through a surgically implanted

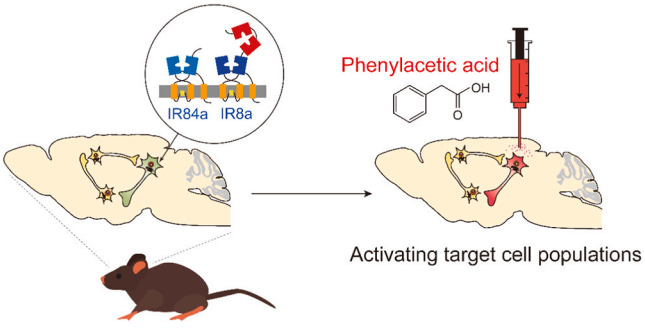

Fig. 1. Activation of mammalian neurons expressing IR84a/IR8a in the brain. Transgenic expression of IR84a/IR8a in a target neuronal population in the mammalian brain confers novel activation upon microinjection of the cognate ligand, phenylacetic acid.

cannula. To overcome this highly invasive delivery method, we sought to establish a prodrug strategy in which a chemically modified inert form of phenylacetic acid is peripherally injected into the bloodstream from which it could be transferred into the brain (Iguchi et al., 2024). We hypothesized that a methyl ester form of phenylacetic acid might readily crosses the blood-brain barrier through passive diffusion thanks to its lipophilic properties and then be converted to active phenylacetic acid by esterase activity in the brain (Shukuri et al., 2011; Suzuki et al., 2004; Takashima-Hirano et al., 2010, Fig. 2). Indeed, phenylacetic acid methyl ester administered intravenously into the lateral tail vein increases central noradrenergic activity in IR84a/IR8a-expressing mice, as measured by an increase in the firing frequency of the locus coeruleus neurons and an elevation in norepinephrine release in the anterior cingulate cortex. These results indicate that the IR84a/IR8a-expressing cells in the mammalian brain can be remotely activated by peripheral administration of phenylacetic acid methyl ester and support a prodrug strategy for chemogenetic approaches.

Finally, we employed the striatum of rats as a model to examine whether IRNA could be applied to non-catecholamine-containing cells in other mammalian species (Iguchi et al., 2024). The striatum controls behavior through the activity distributed across two subpopulations of GABAergic spiny projection neurons (SPNs), which express distinct dopamine receptor subtypes that have opposing responses to dopamine: direct SPNs (dSPNs) express type 1 receptors projecting to the substantia nigra pars reticulata and entopeduncular nucleus; indirect SPN (iSPNs) express type 2 receptors projecting to the external segment of the globus pallidus (Alexander and Crutcher, 1990; Gerfen and Bolam, 2016). We employed *Drd2*-Cre transgenic rats that express the Cre recombinase predominantly in iSPNs (Nonomura et al., 2018). A lentiviral vector pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G, Kato et al., 2007) was constructed to express IR84a and IR8a (gene sizes of 1851 bp and 2760 bp, respectively) in a Cre-dependent manner (the flip-excision switch system: FLEX, Kato et al., 2007; Matsushita et al., 2023) and then microinjected into the dorsal striatum of the *Drd2*-Cre rats. Immunohistochemistry found an efficient and specific expression of IR84a and IR8a in the striatal iSPNs, and patch-clamp recordings performed on acute brain slices of the vector-treated striatum revealed excitatory responses of the IR84a/IR8a-expressing iSPNs to phenylacetic acid.

5. Application of IRNA to the analysis of the neural mechanisms of behavior

We successfully developed IRNA as a novel chemogenetic approach to stimulate neuronal types of interest, laying the foundation to study open questions in mammalian neuroscience. Here, we present two examples in which IRNA-based gain-of-function perturbation was applied to the central noradrenergic system and emotional memory processing (Fukabori et al., 2020), and a specific type of striatal projection neurons and drug-induced movement (Iguchi et al., 2024).

The roles of the central noradrenergic system in the acquisition, consolidation, and reconsolidation of the memory conditioned to a cue that signals the occurrence of a biologically significant event (Pavlovian conditioned stimulus: CS) have been suggested by many studies (Ferry et al., 2015; LaLumiere et al., 2003; Villain et al., 2016). However, whether the noradrenergic system plays a role in the retrieval process was unclear (Miranda et al., 2007; Murchison et al., 2004). We sought to use IRNA to determine if the retrieval of the Pavlovian aversive memory conditioned to a flavor CS is affected by activating the noradrenergic system originating from the locus coeruleus. Mice were allowed to intake a sucrose solution as CS and then received an intraperitoneal injection of lithium chloride, which induced gastric illness (Garcia et al., 1955). Mice usually like sucrose, but they acquired an aversion to the CS solution through the conditioning, resulting in rejection responses to the CS presented forcibly and intraorally during testing (Yasoshima and Shimura, 2017). We found that IR84a/IR8a-expressing mice showed a reduced latency for the rejection responses to the CS when they were microinjected with phenylacetic acid into the bilateral locus coeruleus immediately before the CS test compared to control conditions (Fukabori et al., 2020). This finding suggests that activation of the norepinephrine cells in the locus coeruleus accelerated the retrieval of the aversive memory associated with the CS. Furthermore, adrenergic receptor antagonism in the basolateral amygdala blocked the facilitation of the taste aversion memory retrieval (Fukabori et al., 2020), suggesting that the locus coeruleus-basolateral amygdala noradrenergic pathway mediates the enhanced aversive memory retrieval.

Disorders of contraversive movement by the lesion in each brain hemisphere have been extensively studied in neurological patients (Heilman et al., 1985; Karnath et al., 2002). Unilateral lesions of the nigro-striatal dopaminergic system cause profound asymmetries in

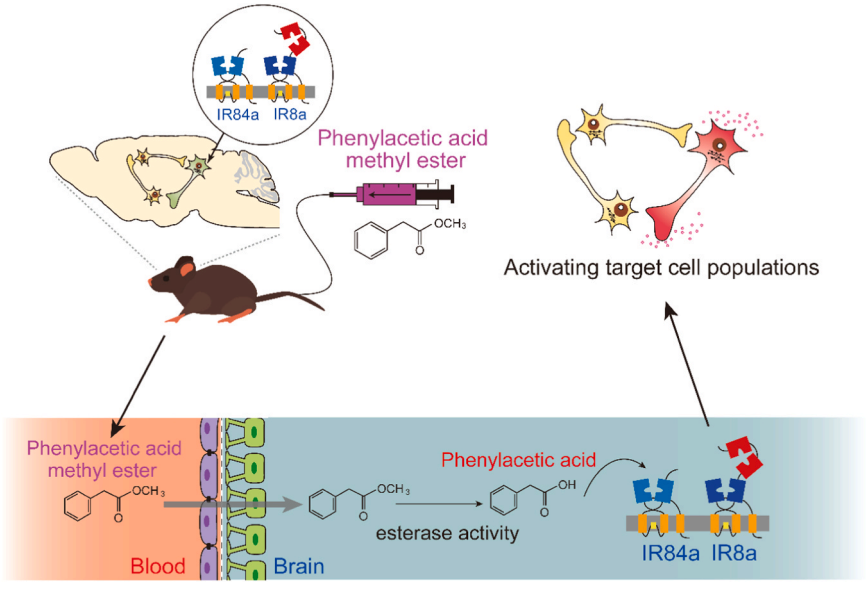

Fig. 2. A working model of the prodrug strategy for IRNA. The lipophilic phenylacetic acid methyl ester, a chemically-modified inert form of phenylacetic acid, is administered peripherally, readily crosses the blood-brain barrier through passive diffusion, and then converted to the active phenylacetic acid ligand by esterase activity in the brain.

motor performance (Björklund and Dunnett, 2019). In rodents, this manifests as an asymmetrical body posture, sensorimotor disorientation, and impaired use of the contralateral forelimb, which are widely used as an experimental model to mimic the loss of dopamine neurons seen in Parkinson's disease (Dunnett and Torres, 2011). More recently, the role of the two basal ganglia pathways originating in dSPNs and iSPNs in behavioral phenotypes of the rodent model has been investigated. For instance, several studies reported that unilateral activations of striatal iSPNs by optogenetics (Grimm et al., 2021; Kravitz et al., 2010; Lee et al., 2016) or DREADD-based chemogenetics (Alcacer et al., 2017) induce spontaneous ipsiversive rotations in mice, suggesting that iSPNs play an inhibitory role in spontaneous motor control. By contrast, in the dopaminergic drug-induced condition, selective ablation of the iSPNs in the unilateral striatum generates an ipsiversive rotation (Sano et al., 2003), implying that iSPNs facilitate the motor activation induced by drug administration. To further test the possibility, we induced IR84a/IR8a expression in the iSPNs of the unilateral striatum using the FLEX viral vector in combination with the *Drd2*-Cre rats described above. We then injected phenylacetic acid methyl ester intraperitoneally to induce unilateral activation of the iSPNs and examined whether a systemic cocaine administration would bias the rotation behavior in a specific direction (Iguchi et al., 2024). The results showed that animals exhibit more contraversive rotation to the unilateral striatum in which the iSPNs are activated, confirming the role of iSPNs in facilitating drug-induced motor activation.

6. Conclusions and perspectives

We have devised a new LGIC-based chemogenetic technology, IRNA, which uses iGluR-related IRs from insects (Benton et al., 2009) to induce ligand-evoked activation in target cell populations in the mammalian brain (Fukabori et al., 2020; Iguchi et al., 2024). We show how IRNA can be used to activate several types of neurons in mice and rats, emphasizing the likely general applicability of this approach. We also developed a prodrug system that allows remote, peripheral administration of a ligand precursor, which is transferred to the central nervous system to activate the IR-expressing target cells.

As with any new method, further technical improvements and developments are required. Phenylacetic acid activates the IR84a/IR8a complex expressed in the target neurons in the mammalian brain at a higher concentration range than the DREADDs ligands, leading to concern about non-specific effects. However, we found no significant change in the norepinephrine release when phenylacetic acid was microinjected into the locus coeruleus of the control animals at the concentrations that induced a significant behavioral effect in the IR84a/IR8a-expressing mice (Fukabori et al., 2020), suggesting that a single dose of phenylacetic acid does not impair normal cellular function, at least in the short-term. The possibility of cytotoxic effects of phenylacetic acid by repeated administration has not yet been examined. This chemical also has a unique odor, so that it is necessary to establish appropriate control conditions for behavioral experiments. Identifying a more efficient (and possibly less volatile) actuator for the IR84a/IR8a complex that overcomes these issues should enhance the specificity and usefulness of IRNA.

Despite these caveats, successful application of IRNA to the analysis of the neural mechanisms controlling emotional memory and drug-induced behavior provides new biological insights: first, that the locus coeruleus–basolateral amygdala noradrenergic pathway could be a therapeutic target in psychiatric disorders with abnormal control of aversive memory, and second, that the function of iSPNs in the behavioral control change between spontaneous and drug-induced conditions. Thus, IRNA can stimulate new research and clarifying previously unresolved issues. IRNA might also form the basis for future chemogenetic-based treatment of psychiatric/neurological disorders (Sternson and Bleakman, 2020; Urban and Roth, 2015; Walker and Kullmann, 2020).

We also envisage creating variations of IRNA beyond the IR84a/IR8a-phenylacetic acid receptor-ligand pairing. For example, a complex of IR75a and IR8a is activated by propionic acid, rather than phenylacetic acid (Abuin et al., 2011; Benton et al., 2009). Here, the potential is enormous: hundreds, if not thousands, of functionally-distinct IRs are encoded in insect genomes (Croset et al., 2010; Rytz et al., 2013), any of which are theoretically exploitable for artificial activation of mammalian neurons. Increased understanding of the molecular basis of ligand binding specificity of IRs (e.g., Prieto-Godino et al., 2021) could also enable custom-design of IRs with novel ligand-recognition properties. Similarly, insect OR and GR ligand-gated ion channels, which are arguably even better understood mechanistically than IRs (e.g. Butterwick et al., 2018; del Mármol et al., 2021; Frank et al., 2024; Gomes et al., 2024; Ma et al., 2024), could serve as the basis for other chemogenetic tools. By expressing different ion channels in distinct neuronal types under cell-type specific promoters – or by combining IRNA with DREADDs – it is conceivable to independently control the activity of two or more neuron populations with different ligands.

CRediT authorship contribution statement

Yoshio Iguchi: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Richard Benton:** Conceptualization, Funding acquisition, Writing – review & editing. **Kazuto Kobayashi:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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