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Chemical genetics: receptor–ligand pairs for rapid manipulation of neuronal activity

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Toward the functional dissection of neuronal circuits, a number of new genetic tools have been developed that enable rapid and reversible manipulation of genetically defined neuronal subtypes in intact mammalian brain circuits. Alongside the breakthrough technology of optogenetics, receptor–ligand pairs provide complementary approaches to modulate neuronal activity using chemical genetics.

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Introduction

In neuroscience, electrical stimulation, lesions, and inactivation of brain areas have allowed functional mapping of discrete regions and nuclei [1–5]. However, to understand how these regions produce meaningful output, analysis has to zoom into the level of individual cell-types that constitute local circuits. To this end, clever transgenic methods employing cell type-specific promoters have been used for neuronal ablation [6,7], inactivation [8–11], or inhibition of transmitter release [12–15]. Although some of these methods permit regulation on a timescale of days to weeks, they are in principle chronic and preclude precise temporal deconstruction of complex biological processes. In addition, chronic interventions are susceptible to compensatory interference [16]. Alongside the exquisite temporal resolution afforded through optogenetics [17–19], complementary chemical-genetic approaches that permit rapid and reversible manipulation of neuronal function have now advanced from a proof of principle stage to physiological application.

Here, we review recent developments in chemical-genetic tools for manipulating neuronal activity via receptor–ligand pairs and discuss their application in mammalian brain circuits.

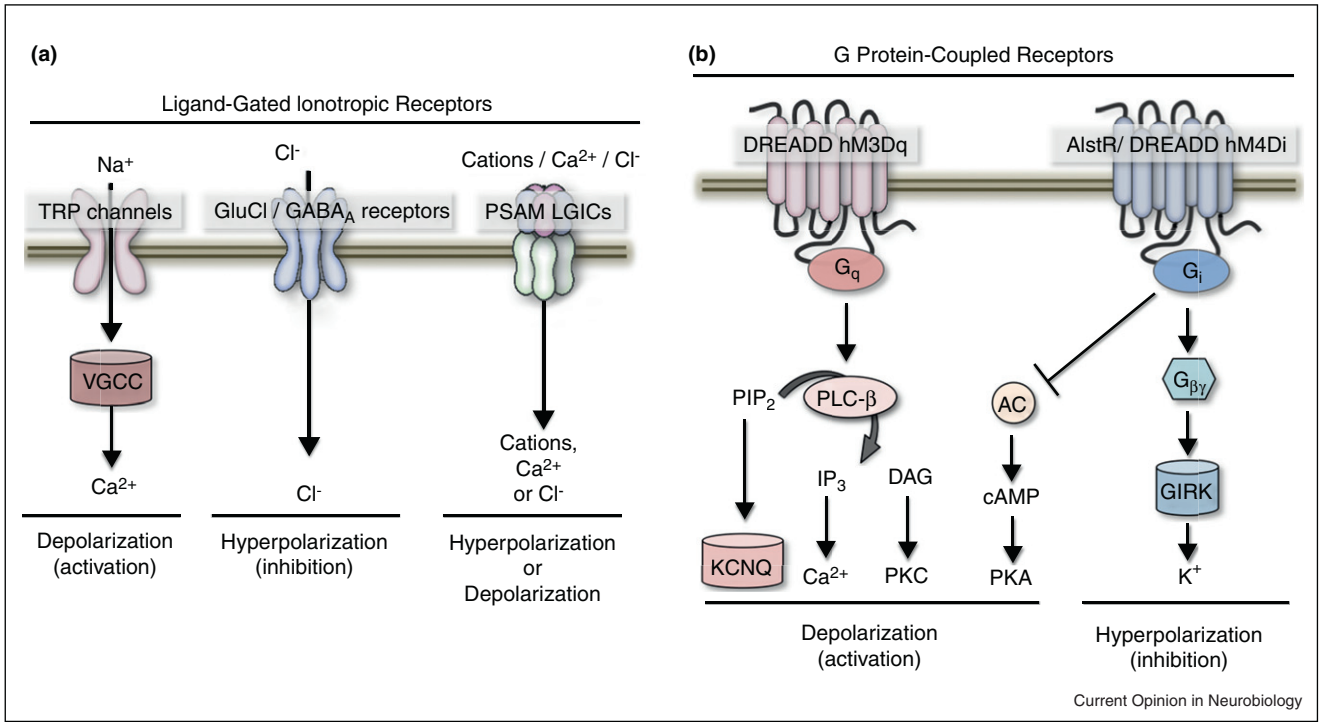
Manipulating neuronal activity with ionotropic receptors

The most direct way to pharmacologically regulate the activity of neuronal cell-types is through targeted expression of ligand-gated ion channels (LGICs), followed by activation with exogenous ligands (Figure 1a). An example is the transgenic overexpression of high affinity acetylcholine receptors (nAChRs) in dopaminergic neurons, which resulted in hyperdopaminergic behavior upon low-dose administration of nicotine [20]. However, to allow versatile and precise manipulations of neuronal activity, receptor–ligand pairs must be orthogonal. That is, neither the receptor nor its ligand must have endogenous interaction partners. In addition, the receptor must not show activity in the absence of the ligand, which in turn must not be toxic. Common approaches have been to either hijack receptor–ligand pairs from other tissues and species, or to re-engineer endogenous ionotropic receptors.

Neuronal activation with transient receptor potential channels

One of the first successful methods to drive neuronal firing using targeted expression of ionotropic receptors was afforded through the identification of the transient receptor potential cation channel subfamily V member 1 (TRPV1), which is mainly expressed in nociceptive peripheral neurons [21]. When expressed in primary neuronal cultures, TRPV1 drives strong inward currents and membrane depolarization in the presence of vanilloid-like ligands, including the pungent molecule capsaicin [21–23]. Similarly, TRPM8 transduces electrochemical signals in the presence of menthol [22,24]. Although effective for ligand-dependent membrane depolarization, use of TRPV1 for circuit analysis is complicated by baseline effects in the absence of ligand gating, and excitotoxicity in the presence of high agonist concentrations [22,25•]. Nevertheless, in mice conditionally expressing TRPV1, moderate doses of capsaicin have been shown to reversibly induce dose-dependent neuronal firing on a timescale of seconds without overt agonist-independent baseline effects or excitotoxicity [26•]. Furthermore, unilateral activation of striatal neurons in these mice resulted in contra-lateral turning

Fig. 1



Receptor–ligand systems for rapid modulation of neuronal activity. **(a)** Left, Ligand-gated influx of free Na^+ activates voltage-gated calcium channels (VGCC), resulting in Ca^{2+} influx, depolarization, and increased firing. Middle, ligand-gated influx of Cl^- results in hyperpolarization and neuronal inhibition. Right, Pharmacologically selective effector molecule (PSEM)-gated influx of cations, calcium, or chloride through combination of pharmacologically selective actuator modules (PSAMs) with different ion-pore domains to manipulate neuronal activity or inhibition, respectively. **(b)** Second messenger cascades associated with G_q, and G_i signaling. Left, G_q signaling activates Phospholipase C beta (PLC-β), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). This leads to increased levels of free Ca^{2+} or protein kinase C (PKC) activation. Reductions in PIP_2 levels may also lead to closure of KCNQ channels causing depolarization and increased neuronal firing. Right, G_i signaling activates inward rectifying potassium channels (GIRKs), resulting in hyperpolarization and inhibition. Independently, activated G_i also inhibits adenylyl cyclase (AC), which promotes cAMP formation and protein kinase A (PKA) activation.

behavior starting within 5 min of capsaicin application and lasting for about 10 min [26•]. 'Caging' ligands for light controllable photo release [22] further enhances the temporal and spatial resolution of this technology. A disadvantage of the TRPV1 approach is that capsaicin activates peripheral pain receptors and does not readily cross the BBB. *In vivo* experiments will thus benefit from TRPV1 null backgrounds, and the use of other vanilloid-like molecules with better binding kinetics, and BBB permeability to allow systemic ligand administration.

Neuronal silencing with ivermectin-gated chloride channels

For many experimental applications that probe circuit function *in vivo*, inhibition rather than excitation of neuronal populations is desired. Toward this goal, Lechner *et al.* have taken advantage of a glutamate-gated chloride channel (GluCl) from *Caenorhabditis elegans*, which is activated by the anthelmintic drug ivermectin [27–29]. Mammalian neurons expressing the GluCl α and β subunits *in vitro* showed ivermectin-induced membrane potential hyperpolarization and action potential shunting within seconds, but delayed ligand unbinding and recovery [27]. *In vivo* intraperitoneal injection of ivermectin evoked turning behavior in mice with unilateral striatal GluCl expression, which peaked 12–48 hours after injection and lasted for days [29]. This silencing method has since been used in mice to investigate the roles of subtypes of GABAergic amygdala neurons in fear conditioning and hypothalamic neurons in aggression [30,31]. Challenges associated with variability in receptor expression, and the need for two subunits [29–31] might be overcome by implementing human α1 glycine receptor subunits engineered to have low glycine, but high ivermectin sensitivity [32]. For this system, however, potential interference with endogenous glycinergic transmission still has to be tested.

Chemically and genetically engineered ligand-gated ion channels

Magnus *et al.* have mutated the ligand-binding domain of the α7 nicotinic acetylcholine receptor to be un-responsive to the endogenous ligand acetylcholine, but highly sensitive to a variety of small molecule synthetic ligands which they coined pharmacologically selective effector

molecules (PSEMs) [33••]. Fusion of these modified nAChR ligand-binding domains, termed pharmacologically selective actuator modules (PSAMs), with the ion-pore domains of different Cys-loop receptors produced receptor channels with different ion selectivities (cations, calcium, or chloride). Chimeras of serotonin 5HT3, or glycine receptor ion-pore domains with PSAMs, showed potent activation or silencing in brain slices within seconds to minutes after addition of synthetic ligands. Proof of principle experiments *in vivo* showed that the chimeric glycine receptor efficiently suppressed Agouti-related-protein-expressing (AgRP) neuron-dependent feeding behavior tens of minutes after intraperitoneal ligand injection. The exact on/off-kinetics for each designer receptor will depend on the specific combination of ligand-binding and ion-pore domains, as well as the pharmacokinetic properties of the cognate ligand. Many of these response properties have yet to be determined. However, a notable advantage of this system is the potential to use different ligand and receptor channel combinations to manipulate separate ionic conductances in different neurons or circuits in the same animal [33••].

Allosteric modulation of GABAergic neurotransmission

While most neuronal manipulation techniques influence activity irrespective of network state, the 'zolpidem-method' modulates physiological $GABA_A$ receptor-mediated transmission via allosteric pharmacology. Mice engineered to harbor a point mutation in the 'floxed' $GABA_A$ receptor γ2 subunit show unaltered GABAergic transmission, but are insensitive to the allosteric ligands zolpidem and DMCM, which normally enhance and reduce GABA-induced chloride influx through α1–3βγ2 subunit containing receptors (~78% of all $GABA_A$ receptors in mammals), respectively [34–36]. Cell type-selective reintroduction of the wild-type γ2 subunit together with Cre recombinase allows cell type-selective subunit swap and reinstatement of drug sensitivity as shown for cerebellar Purkinje cells [37,38]. In these mice, zolpidem caused enhanced inhibitory postsynaptic currents in Purkinje cells *in vitro* and motor deficits within minutes after intraperitoneal injection *in vivo*. The half-life of zolpidem in rodents is about 20 min [39]. An advantage of this system is that the same animal may be used for bidirectional modulation with either zolpidem or the inverse agonist DMCM. Both drugs can be applied systemically and can be acutely antagonized with flumazenil [37,38]. A disadvantage is the requirement for a genetically engineered zolpidem-insensitive background, which limits its application to mice and rats [40].

Neuronal manipulation using G-protein-coupled receptors

The brain expresses a large family of G-protein-coupled receptors (GPCRs), which are activated by a variety of endogenous and pharmacological ligands. Depending on downstream signaling cascades, receptor activation can have a multitude of cellular effects [41]. These include the activation, or inactivation of potassium channels, which in turn lead to reduced or elevated neuronal firing (Figure 1b). The efficacy of GPCR-mediated neuronal silencing was nicely shown by transgenic expression of the $G\alpha_i$-coupled serotonin receptor (Htr1a) in the amygdala of $Htr1a^{-/-}$ knockout mice. Treatment with the selective agonist 8-hydroxy-2-(di-n-propylamino) tetralin resulted in qualitative changes in conditioned fear responses [42,43]. A clear caveat of this approach was the need for a knockout background. Engineered and heterologously expressed GPCRs address this issue.

Designer GPCRs

As described for engineered ion channels above, one method to create orthogonal GPCR–ligand pairs is to render endogenous receptors insensitive to endogenous ligands, but sensitive to synthetic ones. Systematic mutations of the κ opioid receptor produced receptors activated solely by synthetic ligands (RASSLs) [44], which were sensitive only to synthetic agonists. RASSLs have since been used *in vivo* to investigate GPCR signaling in different tissues [45–47]. However, baseline receptor activities and off-target effects of the synthetic ligands precluded their use for precise brain circuit manipulation [48,49]. These teething problems were overcome in a second generation of RASSLs, so called designer receptors exclusively activated by designer drugs (DREADDs) [50].

Engineered from muscarinic acetylcholine receptors (mAChRs), Armbruster and colleagues generated DREADDs with little or no baseline activity that were insensitive to endogenous acetylcholine, but potently activated by the pharmacologically inert molecule clozapine-*N*-oxide (CNO). Introductions of $Y^{3.33}C$ and $A^{5.46}G$ mutations generated DREADDs that coupled to G_q, G_i, ($hM_{1-5}D$) or G_s (rM3/β1Ds) signaling pathways without obvious interference with endogenous GPCR signaling [50,51]. These receptors provided a genetic means for *in vivo* cell type-selective activation (G_q-coupled hM_3D_q) or inhibition (G_i-coupled hM_4D_i) of neuronal activity, respectively (Figure 1b). Experimentally, pyramidal cells in hippocampal slices of mice transgenically expressing HA-tagged hM_3D_q in forebrain showed robust phospholipase C-dependent depolarization and increased firing minutes after CNO application — presumably through closure of KCNQ channels. Apart from reduced locomotion, hM_3D_q-expessing mice showed no overt behavioral alterations in the absence of CNO. However, intraperitoneal injection of CNO caused dose-dependent and time-dependent increases in hippocampal network activity and locomotion, with seizures developing at high doses. Effects developed within 15 min, peaked approximately one hour post injection, and lasted for ~10 hours. Notably, comparable drug-induced phenotypes were

observed after re-injections [52••]. Since then, similar on/off-kinetics have been reported for mCherry-tagged hM_3D_q targeted to AgRP neurons of the mouse hypothalamus [53••]. In these animals CNO injections increased feeding behavior within minutes, which lasted for up to eight hours. Chronic CNO injections caused weight gain, which reversed after CNO withdrawal [53••]. The same authors also expressed the inhibitory DREADD hM_4D_i in AgRP neurons, where CNO caused hyperpolarization and reduced firing in slices (presumably via GIRK channels), as well as reduced food intake within two hours after intraperitoneal injection [50,53••]. *In vivo* silencing with $hM4D_i$ has also been successfully used to investigate the functions of the striatopallidal or striatonigral pathways in drug sensitization [54•], and the role of serotonergic neurons in respiratory control and thermo regulation [55].

Allatostatin receptor

Another method to drive neuronal hyperpolarization and inhibition of action potential firing has exploited the *Drosophila* allatostatin receptor (AlstR). Genetic transplantation of the AlstR into mammalian neurons induces G_i-coupled GIRK channel-mediated silencing in the presence of the insect peptide allatostatin (Figure 1b) [56,57]. In ferret cortical slices, allatostatin efficiently reduced membrane potential, input resistance, and action potential frequency within minutes. Similar silencing responses were reported for AlstR-expressing neurons in slices of mouse spinal cord, hippocampus, amygdala, and rat brainstem, as well as cortical neurons *in vivo* following surface superfusion with allatostatin [58–62]. *In vitro*, allatostatin-induced effects could be 'washed-out' within 15 min, but local allatostatin applications below the brain surface *in vivo* resulted in neuronal inactivation for minutes to hours [62]. Whereas Tan and colleagues found no decrease in silencing efficiency in the continued or repeated presence of allatostatin, Wehr *et al.* reported partial recovery of activity during allatostatin superfusion, and transient rebounds of hyperexcitability during washout [60,62]. As allatostatin does not cross the BBB, it has to be injected locally. Limited tissue diffusion might account for the reported variability in silencing efficiency and recovery times [62,63]. Nevertheless, this method has proven successful and has been widely used *in vitro* to study single neuron response properties, and *in vivo* to delineate the roles of neuronal subtypes in coordinating locomotor rhythms [61,63], respiration [58,64], and encoding fear memories [59].

Conclusions

We have entered an era of experimental neurobiology where imaging, electrophysiological recording, and genetic manipulation technologies are merging [65]. New methods to manipulate neuronal activity through optogenetic and chemical genetic methods now allow interrogation of circuit function from the level of the synapse to behavior. Although all these methods provide the power to probe and map neuronal connectivity with unprecedented resolution, each has its own advantages and disadvantages. For example, optogenetics provides temporal control on a millisecond timescale, which in principle can be used to shape elaborate patterns of activity to investigate details of neuronal coding [66]. However, optogenetics relies on direct access of photons to brain tissue, a methodology that requires brain surgery and is difficult to achieve for prolonged periods of time, or in distributed neuronal populations. Chemical genetics

Table 1

Receptor–ligand systems applied *in vivo*.

System (receptor)	Ligand	Timescale induction	Timescale reversal	BBB permeability	Limitations	References
Activation						
TRPV1	Capsaicin	d.a. — s	d.a. — s	Unknown	Potential base-line effects, excitotoxicity with high ligand concentrations	[21–23,25•,26•]
hM_3D_q	CNO	d.a. — s/min sys — tens of min	sys — hours	Yes	Slow reversal, cellular effects may very with signaling pathways	[50,52••,53••]
PSAM-5HT3	PSEMs	d.a. — s	d.a. — s	Yes	Not yet tested *in vivo*	[33••]
Inhibition						
$GABA_A$	Zolpidem	d.a. — s/min sys — min	sys — tens of min	Yes	Requires zolpidem-insensitive background, no absolute silencing possible	[34–38]
GluCl	Ivermectin	d.a. — s/min sys — hours	d.a. — hours sys — days	Yes	Slow on-/off-kinetics, ligand may be toxic at higher concentrations	[27–31]
AlstR	Allatostatin	d.a. — min	d.a. — min/hours	No	Tissue diffusion of ligand might be limited, effects depend on signaling pathways	[57–64]
hM_4D_i	CNO	d.a. — s/min sys — hours	sys — hours	Yes	Slow reversal, cellular effects may vary with signaling pathways	[50,53••,54•,55]
PSAM-GlyR	PSEMs	d.a. — s/min sys — tens of min	d.a. — s/min	Yes	Requires further characterization *in vivo*	[33••]

d.a., direct tissue application *in vitro* or *in vivo*; sys, systemic application *in vivo*. Note that timescales for induction and reversal are only approximations and may vary with experimental conditions such as route of ligand application, target cell-type, and experimental read-out.

provide an alternative and complementary approach to modulate neuronal activity (see Refs. [53••,67•] for comparison of optogenetics and chemical genetics applied to the same cell type). However, chemical genetic methods have their own cast of drawbacks (Table 1). For example, ivermectin-based inhibition through expression of GluCl channels relies on the availability of multiple subunits, and is applicable with timescales of hours to days, whereas allosteric modulation of GABAergic transmission with zolpidem requires expression of wild-type γ2 subunits plus Cre, and is restricted to mice and rats. For GPCR-based methods, efficiency and time course of the manipulation may vary with the availability of downstream signaling pathways, and effector molecules in different cell types and developmental stages. In addition, G-protein-signaling will have multiple cellular effects, which may complicate data interpretation in some experimental settings. Regarding reversibility, it should be kept in mind that independent of the method a neuron might not be the same after a manipulation as before. A great advantage of chemical genetics over optogenetics is the potential for non-invasive or minimally invasive experimental design through systemic ligand application. This, however, requires transport of the ligand across the BBB, which has not yet been achieved for all systems.

Although neuro-technology is advancing at a breakneck pace, the challenge remains to further define, build upon, and optimize the evolving toolset for investigating brain circuit form and function. New frontiers include the development of synapse-specific manipulation strategies as well as the exploration of molecular receivers for physical signals with easy propagation in brain tissue to combine advantages of current optogenetic and chemical-genetic techniques. The functional deconstruction of neuronal circuits will help to understand human brain development and disease, and a hope for the future is to advance some of the creative genetic approaches used in the lab toward therapeutic design.

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