

Chemical Genetics: A Small Molecule Approach to Neurobiology

Minireview

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Chemical genetics, or the specific modulation of cellular systems by small molecules, has complemented classical genetic analysis throughout the history of neurobiology. We outline several of its contributions to the understanding of ion channel biology, heat and cold signal transduction, sleep and diurnal rhythm regulation, effects of immunophilin ligands, and cell surface oligosaccharides with respect to neurobiology.

The contemporary neurological and psychiatric pharmacopoeia employs a staggering array of small molecules of both natural and synthetic origin to modulate pathological processes. It is therefore only fitting that few areas of science have benefited as much as neurobiology from the use of small molecules to explore cellular processes.

The specific and discrete perturbation of the cellular milieu by small molecules has been formalized as chemical genetics (Crews and Splittgerber, 1999; Mitchison, 1994). The aims and strengths of this approach lie not in the recapitulation of classical reverse genetics and traditional genetic manipulation. Rather, chemical genetics complements genetic analysis by affording access to novel and previously hindered biological space. Among the foibles of classical genetics are limitations in the fine control of spatial and temporal dosimetry, as well as its awkwardness in dissecting cases of functional redundancy or the tangled skeins of complex signaling pathways.

Ion Channel Biology and Small Molecules

This strategy hardly constitutes a novel or uncommon approach in neurobiological research. One familiar example is MacKinnon's use of scorpion charybdotoxin as a specific and reversible inhibitor that enabled the mechanistic elucidation of cellular ionic currents in potassium channels.

Using electrical methods, MacKinnon and colleagues demonstrated that scorpion toxin occluded potassium channels without altering their gating properties. Since this initial observation, scorpion toxin has been used liberally in the physical characterization of potassium channels. The *Drosophila* gene *Shaker*, the first potassium channel to be cloned, was extensively mutagenized to identify the residues essential for toxin binding. These data provided not only a primary sequence potassium channel signature, but also clues to probable secondary and tertiary structural features. This in turn led to inferences regarding the physical size of the channel

pore by careful analysis of the electrostatic interactions between the toxin and the residues constituting the binding motif. Furthermore, the effort to crystallize the *Streptomyces lividans* potassium channel was validated by its interaction with scorpion toxin, suggesting that it was appropriately analogous to its eukaryotic cousins at a deep level of evolutionary conservation (Hille et al., 1999).

With this precedent, neurobiologists have dissected the biology of ion channels by avidly exploiting the remarkable specificity of a diverse array of small molecule toxins and venoms, many of which are considered the defining ligand for their respective targets (Bailey and Wilce, 2001; Lewis, 2000).

Probing Heat and Cold Sensation with Small Molecules

The power of a small molecule-based approach to receptor biology has been elegantly illustrated in recent years through the identification of the receptors responsible for heat and cold sensation. It had been known for some time that capsaicin, the cardinal irritant and pungent component present in chili peppers (Figure 1), was a highly selective compound unique among sensory neuron agonists as it elicited a refractory period of desensitization following the initial stimulatory phase (Buck and Burks, 1986). In addition, Szallasi and Blumberg identified resiniferatoxin (RTX) as an especially potent but structurally divergent analog of capsaicin that shares a homovanillyl moiety critical for biological activity (Szallasi and Blumberg, 1990). The characterization of structure-activity relationships and the discovery of the capsaicin antagonist capsazepine (Walpole et al., 1994) bolstered the notion of a vanilloid receptor, and its existence was finally confirmed by autoradiographic visualization of a tritiated resiniferatoxin probe in tissues of various species (Szallasi, 1995).

Later, capsaicin was used as a molecular probe in the context of an expression cloning strategy to isolate the first nociceptive receptor, vanilloid receptor 1 (VR1). Characterization of VR1 revealed it to be a relative of the TRP ion channel and a nonselective cation channel activated by capsaicin or elevated temperatures (Caterina et al., 1997). Subsequently, other investigators identified the capsaicin-like substance *N*-arachidonoyl-dopamine (NADA) as a putative endogenous ligand (Huang et al., 2002). Intriguingly, a recent report identified another TRP-related ion channel based on its ability to transduce menthol or cold stimulation. The discovery of this new receptor family member, CMR1 (cold- and menthol-sensitive receptor), suggests that TRP channels play a general role in the signal transduction of thermal stimuli (McKemy et al., 2002).

Sleep and Diurnal Rhythm Regulation by Small Molecules

Chemical genetic approaches have proven equally facile with lipid-based compounds. For example, Cravatt and colleagues (1995) isolated the lipid oleamide, or *cis*-9,10-octadecenoamide (cOA) (Figure 2), from the cerebrospinal fluid of sleep-deprived cats, and demon-

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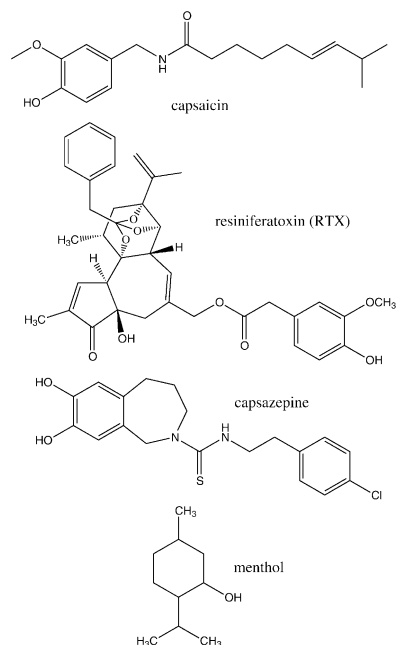


Figure 1. Structures of Capsaicin, Resiniferatoxin, Capsazepine, and Menthol

strated that exogenous synthetic oleamide induced sleep in rats.

Believing this to represent a novel signaling pathway, the investigators focused on elucidating an inactivating mechanism, and noted an enzymatic activity that hydrolyzed oleamide and the related fatty acid endocannabinoid anandamide into oleic and arachidonic acids, respectively. Mechanism-based isolation of this activity led to the purification and cloning of fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). This line of inquiry was supported by the observation that the human cerebrospinal fluid isolate 2-octyl γ -bromoacetoacetate, which had been shown to lengthen REM-associated sleep in cats, also inhibited FAAH (Patricelli et al., 1998).

Although oleamide and anandamide provoke ethological responses consistent with the action of cannabinoids, only anandamide has been demonstrated to bind the cannabinoid receptor CB1. A recent study conducted with FAAH-deficient mice established that both compounds are subject to catabolic regulation by FAAH, but that only anandamide's behavioral effects could be abolished by a deficiency of CB1 engineered by either genetic means or pharmacological inactivation with the small molecule antagonist SR141716A (Lichtman et al., 2002). Hence, it appears that oleamide and anandamide exert their effects virtually orthogonally in vivo. Exploration of the chemical space surrounding oleamide and anandamide has yielded a number of potent inhibitor analogs, which would be of utility in further characterization of these effects (Boger et al., 2000).

Various groups have shown interactions between oleamide and GABA(A) receptors, and ablation of the β 3 subunit of GABA(A) receptors in a murine deficiency system abrogates oleamide's effects (Laposky et al., 2001). There are even suggestions that oleamide may

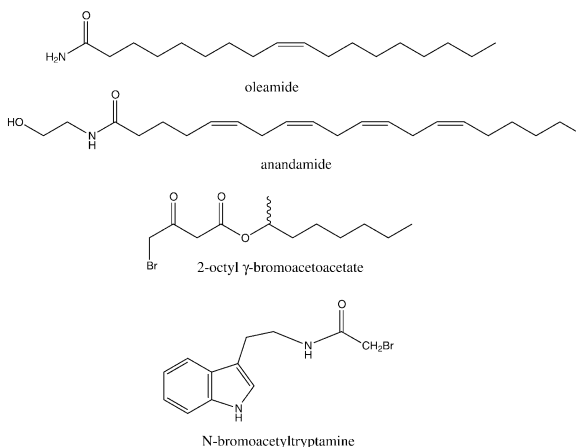


Figure 2. Structures of Diurnal Rhythm Regulators (Oleamide, Anandamide, 2-octyl γ -bromoacetoacetate, and N-bromoacetyltryptamine)

possess additional properties, as it appears to interact with mammalian voltage-gated sodium channels in a manner reminiscent of many anaesthetics (Nicholson et al., 2001).

In a related system, small molecules have been employed in the analysis of the melatonin-pineal gland diurnal rhythm axis. The photoregulated enzyme serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase [AANAT]), transfers acetyl from acetyl-coenzyme A (acetyl-CoASH) to serotonin in the first and rate-limiting step of *N*-acetylserotonin formation en route to the biosynthesis of melatonin (5-methoxy-*N*-acetyltryptamine). AANAT also possesses a secondary alkyltransferase activity, which is potently inhibited by the small molecule *N*-bromoacetyltryptamine and its cognate *N*-haloacetyltryptamines.

The acetyltransferase and alkyltransferase domains of AANAT are functionally distinct, and the mechanism of *N*-haloacetyltryptamine-mediated inactivation of AANAT is rather unusual. In brief, *N*-haloacetyltryptamines are prodrugs activated by transfer of CoASH at the alkyltransferase active site to generate species that subsequently bind and inhibit an AANAT acetyltransferase active site in either *cis* or *trans*.

A more subtle consequence is the potential amplification of the inhibitory signal, since the alkyltransferase site remains unaffected by the inhibition of the physiologically relevant acetyltransferase site. Hence, multiple *N*-haloacetyltryptamine molecules can be converted to their inhibitory analogs even after their host enzyme's primary acetyltransferase activity has been neutralized. Cole and colleagues have termed this type of inhibition "molecular fratricide," and as the *N*-haloacetyltryptamines are the only cell-permeable AANAT inhibitors described to date, these compounds hold much promise for the elucidation of melatonin's role in circadian rhythm biology (Zheng and Cole, 2002).

Neurobiological Activities of Immunophilin Ligands

Small molecules such as cyclosporin A (CsA), FK506, and rapamycin (Figure 3) are well characterized immunosuppressive compounds that form ligand-receptor

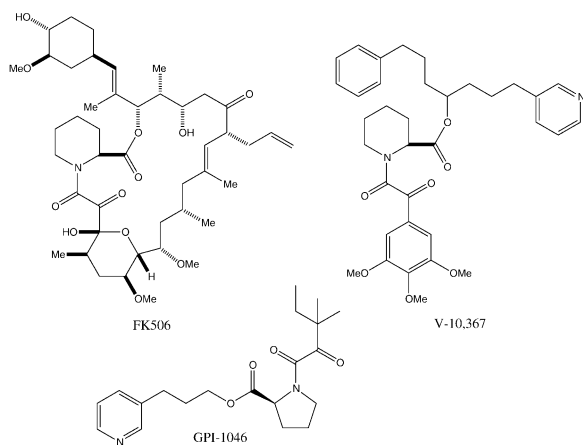


Figure 3. Structures of Immunophilin Ligands (FK506, GPI-1046, and V-10,367)

complexes with immunophilins, which in turn bind to and inhibit secondary targets such as calcineurin or FRAP/RAFT1 (FKBP and rapamycin-associated protein/rapamycin and FKBP12 target 1). These immunophilin ligands also exert a number of neurobiologically salient activities, including neuroprotective and neurotrophic effects for damaged neurons, modulation of neurotransmitter release secondary to NOS inhibition, and nerve regeneration.

In an effort to dissect the effects of calcineurin inhibition from these neurobiological effects, rational drug design of a nonimmunosuppressive ligand, which bound the immunophilin FKBP12, but not calcineurin, yielded 3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate (GPI-1046).

GPI-1046 acts as a neurotrophin that stimulates neurite outgrowth, and it has also been credited with spurring axonal regeneration following serious CNS injury to either the optic nerve or spinal cord. It also exhibits neuroprotective effects of varying degrees against lesions induced by 3-acetylpyridine, hydrogen peroxide, 6-hydroxydopamine, 1-methyl-4-phenylpyridinium, and parachloroamphetamine. There is some evidence for a protective elevation of antioxidative glutathione levels, although strong evidence for any direct target is lacking. Interestingly, insult by 1-methyl-4-phenylpyridinium to both wild-type neuronal cells treated with GPI-1046 and their FKBP12-deficient counterparts treated with FK506 result in similar neuroprotective profiles, which further supports a distinction between the immunosuppressive and neuroprotective effects of immunophilin ligands.

Another nonimmunosuppressive immunophilin ligand analog, V-10,367, also spurs neurite outgrowth in dopaminergic neuronal cell culture in addition to the acceleration of nerve regeneration in a rat model and neuroprotection in a murine traumatic brain injury (TBI) model. One group used the novel compound Lie120, a highly specific calcineurin inhibitor, to distinguish the neuroprotective effects of FK506 and V-10,367 from calcineurin inhibition. Their results suggested that calcineurin inhibition was strictly orthogonal to the neuroprotective effects of both FK506 and V-10,367 in cell culture (Snyder et al., 1998a, 1998b).

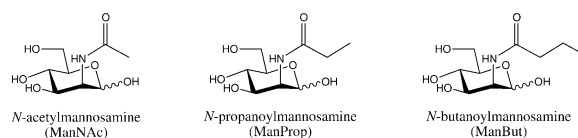


Figure 4. Structures of poly- α 2,8-sialic acid (PSA) Precursors (*N*-acetylmannosamine [ManNAc], *N*-propanoylmannosamine [ManProp], and *N*-butanoylmannosamine [ManBut])

Modulation of Cell Surface Chemistry Using Small Molecules

Finally, we wish to address a model study of the specific perturbation of a cellular system with small molecules to generate novel insights into neuronal biology that complements genetic analysis. Linear homopolymers of the saccharide α 2,8-sialic acid (poly- α 2,8-sialic acid [PSA]), are primarily concentrated on the cell surface upon neural cell adhesion molecules (NCAM) and play an important role in neuronal development and synaptic plasticity. Manipulation of PSA levels was previously limited to either enzymatic digestion or genetic means. The latter approach was inherently problematic, as multiple distinct enzymatic steps are responsible for the synthesis of oligosaccharides such as endogenous PSA.

Bertozzi and colleagues (Mahal et al., 2001) recently described the modulation of this oligosaccharide epitope by using the small molecule *N*-butanoylmannosamine (ManBut) (Figure 4) to inhibit PSA generation in a specific and reversible manner. ManBut is permitted in lieu of the natural substrate *N*-acetylmannosamine (ManNAc) in the cellular biosynthetic pathway leading to cytidine 5'-monophosphate-sialic acid (CMP-sialic acid), the quantum of PSA fabrication, and is metabolically transformed into an unnatural chain-terminating derivative when incorporated into α 2,8-sialic acid oligomers undergoing extension. The closely related compound *N*-propanoylmannosamine (ManProp) is also a suitable substrate for incorporation into unnatural sialic acid derivative, but does not interfere with the iterative catenation that leads to PSA and its analogs, rendering it an excellent matched control.

In this system, tunable inhibition and temporal potentiation of PSA expression is but a matter of adulterating endogenous sialic precursor pools with ManBut in a dose-dependent fashion. ManBut treatment at extremely low doses would alter the nature of population of PSA to include both full-length as well as truncated PSA molecules. Alternatively, increasing amounts of ManBut would attenuate both the population and mean length of PSA molecules by prematurely terminating PSA oligomers on average at increasingly earlier stages. This chemical genetic approach will enable finer resolution of PSA epitope function in neuronal processes through tractable methods (Mahal et al., 2001).

Conclusion

We have attempted to illustrate how chemical genetic analysis of various neurobiological processes offers additional analytical power in juxtaposition to traditional forward and reverse genetic analysis. The chemical genetic approach is limited theoretically only by the level of specificity and bioavailability of the small molecules marshaled in its employ. That said, precise target identi-

fication and validation remain significant challenges in the elucidation of chemical genetic pathways.

As evidenced by the continuous struggle to recapitulate the synthesis of natural products, the exploitation of chemical space tendered by small molecule-based scaffolds is not yet a mature science. The promise of this genetic paradigm is necessarily tempered by our collective chemical prowess and creativity.

Acknowledgments

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