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Use of multicomponent reactions in developing small-molecule tools to study GABA_A receptor mechanism and function

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Abstract

We discuss the potential use of multicomponent reactions in developing small-molecule probes of GABA_A receptor function. Two examples that illustrate this approach are presented: the synthesis of a class of compounds that specifically modulate the function of GABA_A receptors containing the δ -subunit, and also ‘caged’ GABA derivatives. A caged GABA is a photolabile precursor of GABA that releases GABA upon photolysis.

GABA_A receptor-mediated processes play a central role in regulating signal transmission within the CNS [1]. Understanding the cellular and molecular processes of **GABA_A receptors** is not only important for determining how these receptors regulate signal transmission but also critical for understanding how malfunctioning GABA_A receptors relate to neurological diseases [2,3], such as epilepsy [4]. Small molecules, such as inhibitors, modulators and caged compounds, continue to be used to understand the mechanism of GABA_A receptors, as well as their role in the CNS. Here we present two examples that demonstrate how careful consideration of synthetic approaches using combinatorial chemistry can minimize difficulties in creating small libraries of compounds in the search for small-molecule tools to study GABA_A receptors.

Multicomponent reactions

(MCRs) can generate structural complexity in a single step from three or more reactants [5–7]. These selective, atom-economical, and synthetically convergent processes represent a cornerstone of combinatorial chemistry and generate libraries of closely related compounds for structure–activity studies. Several MCRs, such as the Hantzsch (1882), Biginelli (1891), Mannich (1912), Passerini (1921), Strecker (1850) and Ugi (1959) reactions, are well established and have been utilized by researchers and in industry over the years. However, only minimal development of novel or improved MCRs has taken place. Recently, interest in MCRs has been revived in academia and industry as researchers recognize the potential of improving known MCRs and creating new MCR synthetic routes [5–9]. Such developments will lead to the more economical and efficient synthesis of new compound libraries. Combinatorial chemistry and diversity-oriented small-molecule synthesis will likely prove

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advantageous in preparing new agonists, modulators and photolabile precursors of molecular probes for the investigation of many biological targets, including ligand-gated ion channels such as the GABA_A receptor. We believe opportunities abound for combinatorial chemistry and diversity-oriented small-molecule synthesis to contribute significant advances on this front.

GABA_A receptors are transmembrane ligand-gated ion channels that when activated by GABA can repress neuronal activity. These receptors are composed of five subunits from the 19 subunits in the human genome (α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , θ , π and ρ_{1-3}). Most GABA_A receptors in the CNS are thought to contain at least two α - and two β -subunits. The other subunits, except for ρ , can be incorporated as the fifth component of the receptor, modulating receptor function [1,2]. The ρ -subunits are primarily found in the retina and form functional receptors without α - and β -subunits [10]. The subunit composition of a receptor subtype dictates its pharmacology, cellular localization and kinetic properties [10,11]. For example, γ -subunit-containing receptors localize in neuronal synapses and contribute to phasic inhibition, whereas δ -subunit-containing receptors localize in extrasynaptic areas on neurons and have a tonic response to environmental GABA concentrations [12,13]. The expression profile of receptor subunits in the CNS has indicated that a few receptor subtypes are expressed ubiquitously in the CNS (e.g., $\alpha_1\beta_2\gamma_2$), while others are expressed regionally or in specific neurons (e.g., $\alpha_1\beta_2\delta$), suggesting that different subtypes have unique roles [14–16]. While attributes of various GABA_A receptor subtypes have been examined in detail, there is only a cursory understanding of how the various subtypes contribute to signal transmission within the CNS.

Many tools and techniques have been employed to elucidate GABA_A receptor function at the cellular and molecular level. Site-directed mutagenesis has been instrumental in identifying amino acids and subunit domains contributing to the receptor mechanism [17], the binding of GABA [18], the binding of modulatory compounds [19–21] and the selectivity of the ion channel for anions [22]. Investigations of GABA_A receptors containing genetically heritable point mutations, found in individuals with diseases such as epilepsy [23–25], have contributed to understanding the receptor mechanism [26], subunit interactions [27] and receptor trafficking within neurons [2,28]. Molecular tagging and immunological techniques have been instrumental in elucidating the regulation of synthesis, transport and localization of GABA_A receptors in the CNS [2,29]. However, the most widely used techniques for examining ligand-gated ion channels involve the use of small molecules to probe, identify, activate and modulate GABA_A receptors.

Benzodiazepines, barbiturates and many other compounds act as modulators of GABA_A receptor function [30] and have been used both as tools for investigating the role of these receptors in the CNS and as therapeutic agents for the treatment of anxiety, insomnia, seizures and other afflictions [31,32]. While a few of these compounds, if used carefully, may be used to differentiate GABA_A receptor subtypes, most of these modulators act on a large number of GABA_A receptor subtypes. This indiscriminate activity of drugs greatly impedes elucidation of the role of specific GABA_A receptor subtypes in the CNS and results in therapeutics that have sedative, ataxic and other side effects [32,33]. Evidence has shown that modulation or activation of different receptor subtypes contributes to the various effects and side effects of drugs. A clear example of this phenomenon comes from studies with diazepam administration to knock-in mice containing a mutant α_1 -subunit, which renders GABA_A receptors containing this subunit benzodiazepine insensitive [34]. The benzodiazepine diazepam typically causes sedation, amnesia, antianxiety, muscle relaxation and motor impairment in animals. Administration of diazepam to knock-in mice with the mutant α_1 GABA_A subunit did not cause sedation or amnesia but did show other diazepam

phenotypes [34], indicating α_1 -containing GABA_A receptors account for only sedative and amnesic effects.

Receptor subtype-selective compounds

Subtype-selective modulators and inhibitors of ion channels are of great interest as both tools and lead compounds for the development of therapeutics, but only a few are presently known for GABA_A receptors. One such compound is TPA-023 [35], a selective partial agonist for α_2 - and α_3 -containing receptors at an allosteric site distinct from the GABA-binding site. TPA-023 was identified in a set of molecular derivatives of 3-phenyl-6-(2-pyridyl)methoxy-1,2,4-triazolo[3,4-a]phthalazines compounds [36]. These compounds were found in a Merck sample collection and contain structural features similar to those of the class of GABA_A **receptor modulators** known as benzodiazepines. TPA-023 exemplifies how the development of subtype- or subunit-specific compounds help determine subunit contributions to the function of the CNS and circumvent complications and drug-induced side effects. In rodents and primates, TPA-023 demonstrates nonsedating anticonvulsant and anxiolytic activities, thus implicating these subunits in anxiolytic responses [37]. NS-11394 is a molecule with selectivity for α_5 - and α_3 -subunits and demonstrates anxiolytic effects in rats with minimal sedation, ataxia and ethanol interactions [38]. Likewise, indiplon acts selectively on receptors containing the α_1 -subunit [39]. Recent reports also describe certain **dihydropyrimidinones** (DHPMs) and benzamides that selectively modulate δ -subunit-containing receptors [40–41]. In contrast to selective modulation, several compounds have been identified that modulate most GABA_A receptors, but exclude subtypes containing specific subunits. These include the diazepam derivative SH-053-S-CH₃-2'F, which selectively modulates α_2 - and α_3 -containing receptors to a greater extent than α_1 - and α_5 -containing receptors [33], and a compound designated 2–261, which lacks activity on β_1 -subunit-containing receptors [42].

In the search for other subtype-selective molecules for modulation of the GABA_A receptor, it will likely be necessary to generate families of related compounds that contain various active groups and occupy different molecular space. MCRs offer a great opportunity for developing these groups of related compounds. As an example, the Biginelli reaction (Figure 1) was used to prepare a small library of DHPM heterocycles 1 (Table 1) in a one-step three-component reaction [40]. First reported in 1891 [43], the Biginelli process involves the acid-catalyzed cyclocondensation of a 1,3-dicarbonyl compound (or equivalent), an aldehyde and a urea or thiourea. Of the various DHPM heterocycles generated (Table 1), several were found to preferentially potentiate peak currents of δ -subunit-containing GABA_A receptors expressed in HEK293T cells; 1a had the largest effect (Figure 2) [40]. While neither the precise binding site nor the mechanism for how these compounds selectively potentiate currents from δ -subunit-containing GABA_A receptors is known, it is particularly interesting that these compounds increase the maximal current observed. This finding contrasts with many other allosteric modulators, as these compounds often (but not always) potentiate receptor currents only at submaximal GABA concentrations, due to an increasing sensitivity of the receptor to GABA.

In the search for a new family of GABA_A receptor modulators, DHPMs were investigated because of their resemblance to barbiturates, a known class of such modulators. In many ways, the discovery of DHPM-selective modulation of δ -subunit-containing receptors was similar to the discovery of the effects of TPA-023, previously described. The subtle, but labor-saving, difference in the discovery of these two compounds is that MCRs enabled both the design and synthesis of individual pure DHPMs with a relatively simple one-step reaction rather than searching pre-existing compound libraries and using more elaborate synthetic routes. While MCRs will not expedite the process of screening, they do offer the

ability for customized and specific libraries to be generated relatively quickly by parallel synthesis. The Biginelli reaction and other combinatorial chemistry routes have been used to generate a variety of new bioactive modulators and inhibitors for various protein targets [44–46]. A specific example of MCRs used to develop other GABA_A receptor ligands is the use of isonitrile-based MCRs to generate benzodiazepine derivatives [47,48].

Light-activated probes

On another front, MCRs may provide the ability to generate small-molecule probes for structural and kinetic investigations of ligand-gated ion channels. Inhibitors and modulators of the GABA_A receptor modified with photoactivatable groups (e.g., azides and diazirines) can covalently react with amino acids composing the ligand-binding domains upon photolysis. These methods have identified residues of the GABA_A receptor involved with binding *t*-butylbicycloorthobenzoate [49], flunitrazepam [50], and recently the general anesthetic etomidate [51]. The development of another form of photolabile compound, known as caged compounds or caged neurotransmitters, has greatly aided the investigation of GABA_A receptor kinetics [52–54] and subcellular receptor localization [55–57]. Caged compounds are biologically inert, photolabile reagents that undergo a chemical reaction when illuminated to release a biologically active compound [58]. MCRs can permit further development of these types of light-sensitive compounds, whether it be new derivatives of previous compounds or novel photosensitive compounds. Our second example of MCR contribution to the study of GABA_A receptors demonstrates how an MCR was utilized to create a novel set of visible light-sensitive **caged GABAs**.

Caged neurotransmitters have two advantageous properties: they can release neurotransmitter with a high degree of spatial control and they permit rapid increases in neurotransmitter concentration. Certain caged neurotransmitters, when used with two-photon excitation techniques, have afforded precise 3D control over the release of neurotransmitters within neuronal tissue samples [55,56]. Neurotransmitter receptor-mediated reactions occur on a micro- to-millisecond time scale and must be studied with the protein embedded in a membrane. The rapid change in neurotransmitter concentration upon photolysis of the caged precursor occurs in the microsecond time region, making these molecules powerful tools in transient kinetic investigations of GABA_A and other neurotransmitter receptors [26,52,59]. The caged compound α -carboxynitrobenzyl-caged (α -CNB-caged) GABA [60] was used to determine changes that occur in the kinetic mechanism of GABA_A receptors when the epilepsy-associated K289M mutation is present in the γ 2L subunit [26]. Elucidation of the mechanism of GABA_A receptor modulation by phenobarbital and inhibition by picrotoxin was also possible using caged GABA [52,59].

While numerous caged neurotransmitters (including caged-GABA derivatives) have been reported [53,55,57,60–64], it should be noted that most compounds have limitations and no single caged neurotransmitter is appropriate for all assays. The requirement that a caged neurotransmitter and its photolysis byproducts (other than the released neurotransmitter) be inert towards the receptor is particularly important. Because these compounds are light sensitive and must satisfy several criteria for the experimental system used, the generation of small ensembles of caged compounds and their empirical screening are both typically slow processes. However, access to a panoply of different caging groups can usually uncover a suitable caged compound. Moreover, having a spectrum of different caging groups aids in finding a compound with a suitable rate of photolysis and quantum yield for investigating the receptor of interest.

As an illustration, the UV-sensitive α -CNB-caged GABA has been successfully used for transient kinetic measurements of the $\alpha_1\beta_2\gamma_2$ L receptor subtype [26,52,60]; however, it acts

as a competitive inhibitor of the $\alpha 1\beta 2\delta$ receptor subtype [EAGEN, UNPUBLISHED DATA]. Similarly, the visible-light-sensitive RuBi-caged GABA, which uncages rapidly with an excellent quantum yield, has a concentration limitation of 20 μ M for some GABA_A receptor subtypes, because photolysis byproducts of the caging group alter receptor function at higher concentrations [57,63]. Traditional caged neurotransmitters (i.e., those activated by UV light) with fast photolysis rates also encounter certain biological limitations, since only two to three measurements are usually made before significant UV damage occurs to the receptors or cells containing the receptors. Of course, UV-sensitive caged neurotransmitters are also limited in use by the requirement for expensive lasers and special safety precautions.

Thus, the systematic development of a range of useful caged neurotransmitters is an important and challenging research undertaking for investigating receptor mechanisms in general and for GABA_A receptors in particular. Careful synthetic design can circumvent limitations; for example, the 7-(*N,N*-diethylamino)-4-(hydroxymethyl)-coumarin (DECM) chromophore serves as an alternative to the RuBi chromophore as a caging group that is photolyzed by visible light [64,65]. However, identifying and preparing a suitable caged neurotransmitter with the necessary physical properties may require the exploration of large areas of chemical structure space, a task that can often be lengthy, but may be shortened and simplified using combinatorial chemistry.

The well-known Passerini three-component condensation, which combines an aldehyde, a carboxylic acid and an isonitrile to afford an α -acyloxycarboxamide in a one-pot procedure (Figure 3) proved ideal for incorporating the DECM chromophore in aldehyde **2** into a collection of caged GABA compounds that released GABA efficiently at 400 nm [53]. Deprotection of Passerini product **3** afforded the amine salt **4**, which underwent photolytic decaging at 400 nm with a good quantum yield to afford GABA **5**.

Future perspective

One member of our group has developed a logic-based approach for the rational design (or improvement) of multiple-component reactions; the long-term goal being to expand the useful repertoire of such complexity-generating reactions [6]. Further development, improvement and discovery of MCRs will greatly contribute to finding molecules that possess the physical and biological properties needed for detailed investigations of GABA_A receptors.

As noted in this article, more selective ligands for channel-forming neurotransmitter receptors should yield better tools and therapeutics, because receptor subtypes are likely to correlate with observed pharmacological effects (e.g., sedative vs anxiolytic effects). Thus, there is a challenge to find more selective modulators of GABA_A receptors for specific receptor subtypes or subunits. While several such subtype/subunit-selective modulators have been identified for α -subunit-containing receptors, only a few modulators are currently known to selectively target other receptor subunits. Diversifying subunit-selective compounds away from primarily α -subunit-specific modulators will provide new insights into the function of other GABA_A receptor subunits in behavior and disease. The growing demand for improved specificity in agonists and modulatory compounds drives the ongoing search for compounds in molecular libraries and further encourages the development of faster and better methods to create new chemical libraries, areas in which MCRs may offer significant opportunities.

Synthetic schemes, such as MCRs, to develop diverse and alternative photolabile groups for photoreactive and caged compounds are also areas for future investigation. A larger and more ambitious goal beyond caged GABA derivatives is the development of other caged

agonists for GABA_A receptors, such as caged 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol (THIP; gaboxadol) or caged muscimol. The latter may permit the combination of both photolabeling and photoactivation of GABA_A receptors, an area that has not been examined. The additional development of photoreactive derivatives of inhibitors and modulators will likely continue to provide valuable insights into GABA_A receptor binding sites by reacting with residues of the receptor when photolyzed. One relatively unexplored area is the development of caged precursors of inhibitors and modulators for the GABA_A receptor. Such molecules would confer spatial and/or temporal control of inhibition and modulation of GABA_A receptors and would be excellent tools for examining receptor kinetics, as well as biological processes, such as synaptic plasticity, neural circuitry and receptor trafficking within a neuron. To achieve any of these objectives, however, the next generation of caged precursors must be fine-tuned to fit the appropriate biological and experimental criteria. While the time needed to test these individual candidate caging groups and resulting compounds is not likely to be expedited, the process of generating libraries of candidate photolabile compounds may be greatly aided by MCRs and other synthetic schemes that can readily generate structurally related compounds.

Developing and using new synthetic approaches, such as MCRs, to aid in the discovery of compounds with greater specificity and fewer limitations will likely expand the repertoire of available tools for studying the mechanism and function of GABA_A receptors as well as their role in signal transduction throughout the CNS.

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Key Terms

GABA_A receptors	Pentameric proteins, which in the presence of GABA can form anion channels across a cell membrane. The resulting current flowing across the membrane can be recorded and analyzed.
Multicomponent reaction	Three or more starting materials react in a single step to yield a single product, dependent on a careful choice of reaction conditions.
Receptor modulators	Compounds that allosterically inhibit or potentiate the activity of receptors.
Dihydropyrimidinones	These heterocyclic compounds commonly display bioactivity.
Caged GABA	Photolabile precursor of GABA that can be equilibrated with GABA receptors and photolyzed <i>in situ</i> , releasing GABA and providing high temporal and/or spatial resolution.

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Executive summary

- Multicomponent reactions, a major tool in combinatorial chemistry, can generate structural complexity in a single step from three or more reactants, and can minimize difficulties in creating small libraries of small-molecule tools to study GABA_A receptors.
- Subtype-selective modulators and inhibitors of GABA_A receptors are of great interest as both tools and lead compounds for the development of therapeutics, but only a few are presently available. Some dihydropyrimidinone derivatives, generated with a Biginelli three-component reaction, are subtype selective.
- Using the Passerini three-component condensation reaction, 7-(*N,N*-diethylamino)-4-(hydroxymethyl)-coumarin was used to protect GABA. The resulting caged compound is sensitive to visible light and, upon photolysis at 400 nm, yields free GABA in sufficient quantum yield for use in studies of the GABA_A receptor.
- Multicomponent reactions have great potential for the further development of subtype-selective and light-activated tools for investigating reactions mediated by GABA_A receptors in the CNS.

Figure 1.
The Bignelli synthesis of dihydropyrimidinones.

Figure 2. Effect of dihydropyrimidinones in potentiating peak currents of δ -subunit-containing GABA receptors expressed in HEK293T cells

Whole-cell current-recording methods were used to examine the efficacy in potentiation of $\alpha_1\beta_2\delta$ GABA_A receptors of each dihydropyrimidinone in Table 1. The amount of current obtained is 100% when a saturating concentration (1 mM) of GABA was applied to $\alpha_1\beta_2\delta$ GABA_A receptors and is depicted by the control current bar (C). The relative amplitude of receptor currents evoked by co-application of 1 mM GABA with 1 mM of the compound tested (**1a–1m**) is shown. Compound **1g** was N.S. in the assay buffer used. N.S. Not soluble.

Figure 3. Synthesis of DECM-caged GABA derivatives using the Passerini reaction
(A) BOCNH(CH₂)₃CO₂H (1 equiv.), CNCH₂CO₂Et (1 equiv.), CH₂CL₂, rt, 12 h, dark (96%). **(B)** CF₃CO₂H (5 equiv.), CH₂CL₂, rt, 18 h, dark (90%). DECM: 7-(*N,N*-diethylamino)-4-(hydroxymethyl)-coumarin.

Table 1

Dihydropyrimidinone derivatives generated with a Biginelli three-component reaction.

	R ¹	R ²	R ³	R ⁴	X
1a	OMe	Me	H	Ph	O
1b	OEt	Me	H	Ph	O
1c	Me	Me	H	Ph	O
1d	OH	Me	H	Ph	O
1e	NH ₂	Me	H	Ph	O
1f	OMe	Me	Me	Ph	O
1g	OMePh	Me	H	Ph	O
1h	OMe	Me	H	4-Br-C ₆ H ₄	O
1i	OMe	Me	H	3-OMe-C ₆ H ₄	O
1j	OMe	Me	H	4-NO ₂ -C ₆ H ₄	O
1k	OMe	Me	H	3-OMe-4-OH-C ₆ H ₃	O
1l	OMe	Me	H	C ₃ H ₇	O
1m	OMe	Me	H	Ph	5