Heterologous mammalian expression systems for investigating the properties of metabotropic glutamate receptors

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Introduction

The amino acid L-glutamate is the principal excitatory neurotransmitter in the central nervous system. Through its interaction with a broad variety of receptors present at the surface of responding cells, L-glutamate has been proposed to be involved in learning activity and memory acquisition, but also in neurotoxicity and diverse neurological disorders. Distinct families of glutamate receptors have been identified and these are either directly coupled to changes in cation conductance (ionotropic receptors) or to GTP-binding proteins (metabotropic receptors) (reviewed in [1]). Ionotropic glutamate receptors (iGlu receptors) are involved in triggering the immediate actions of released glutamate at the postsynaptic neuron. Metabotropic glutamate (mGlu) receptors are involved in the control of glutamatergic neurotransmission through the modulation of glutamate release at a presynaptic level and its actions at a postsynaptic level, and therefore have been proposed to have important regulatory roles in synaptic plasticity and neuronal excitability [1]. In the central nervous system, the nature of glutamate transmission is dependent on the combinations of these different subtypes of receptors at the synapse [2]. Although the physiological activities of mGlu receptors seem to be restricted to the central nervous system, a substantial proportion of the information currently accruing on mGlu receptor function is derived from experiments performed in heterologous expression systems, which often utilize non-neuronal mammalian cell models. This review provides an overview of the information derived from the use of such systems in the characterization of the properties of mGlu receptors, but we also assess critically the limitations and the possible risks of extrapolating data derived from studies in model cells to neuronal systems.

The structure of mGlu receptors

Molecular cloning studies have revealed the existence of a large family of mGlu receptors containing at least eight different subtypes, with most of these displaying alternative splicing at their C-terminus (reviewed in [3]). Although all these receptors possess the seven-transmembrane (7-TM) domain structure common to G-protein-coupled receptors, they are clearly distinguished from the vast majority of the other members of this receptor superfamily by their particularly long extracellular N-terminus and by their relatively short intracellular and extracellular connecting loops. This structure is shared by some other recently cloned G-protein-coupled receptors, including the type B gamma-aminobutyric acid receptors [4], the Ca2+ sensing receptor [5] and a subset of the pheromone receptor family [6]. These receptors do not present any significant sequence similarity to other G-protein-coupled receptors even within the 7-TM domains. Together, they constitute a subfamily of G-protein-coupled receptors in which the long extracellular N-terminus is proposed both to bind the agonist and then, after a conformational change, to act as an activator of the receptor [7,8]. The principal structural and biochemical characteristics of the mGlu receptors are summarized in Table 1.

On the basis of their amino acid sequence homologies, mGlu receptors can be subdivided into three groups [3]. Group I contains mGlu1 and mGlu5 receptors, group II contains mGlu2 and mGlu3 receptors, and group III contains mGlu4 and mGlu6 receptors. Structural homologies within each group coincide with common intracellular signalling pathways, indicative of the nature of the subsets of G-proteins activated. Group I mGlu receptors preferentially couple to the activation of phosphoinositide-specific

Abbreviations used: 1-AP4, 1,2-amino-4-phosphonobutyrate; BHK cells, baby hamster kidney cells; CHO cells, Chinese hamster ovary cells; HEK cells, human embryonic kidney cells; IPTG, isopropyl-beta-D-thiogalactoside; iGlu receptor, ionotropic glutamate receptor; MCPG, 2-methyl-4-carboxyphenylglycine; mGlu receptor, metabotropic glutamate receptor; 7-TM, seven-transmembrane.

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The extracellular N-terminus
- Particularly long, consisting of approx. 550 residues
- Similarity with bacterial periplasmic binding proteins involved in amino acid transport [52]
- Contains the glutamate-binding site [13,53] and determinants of ligand specificity
- Cysteine-rich region proximal to the membrane domains
- Contains Ca²⁺-sensing domain(s) [54]
- Involved in the dimerization of at least some mGlu receptor subtypes [55]

The seven TM domains and extracellular and intracellular loops
- Relatively short intracellular and extracellular loops
- Some regions are highly conserved between all mGlu receptors (i₁, i₃, TM6)
- Less conserved regions (the second intracellular (i₂) loop and the region of the C-terminus proximal to TM7) determine the nature of the coupling to G-proteins [10,11]. However, the other loops (i₁ and i₃) are also likely to contribute to G-protein discrimination and activation

The intracellular C-terminus
- Alternative splicing in this region results in highly variable length (20–350 residues)
- Involved in the determination of the functional coupling to G-proteins
- Proposed to determine the potency/coupling efficiency of some mGlu receptor agonists [12]
- Putative target for several protein kinases that regulate receptor activity [56–59]
- Involved in the regulation of agonist-induced receptor internalization [41]

Pharmacology of mGlu receptors
The pharmacological distinction between iGlu receptors and mGlu receptors was originally based on the specific recognition of the latter by the agonists 1-aminocyclopentane-1,3-dicarboxylic acid (groups I and II) and t.-2-amino-4-phosphonobutyrate (t.-AP4) (group III). The classification of mGlu receptors into three groups is also supported by pharmacological studies, as their distinction is facilitated by the use of a large number of agonists and antagonists. Provided that iGlu receptor interactions are excluded, t.-quisqualate, (S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine and t.-AP4 are specific agonists of group I, II and III mGlu receptors respectively, whereas 7-(hydroxyimino)cyclopropan[b]chromen-1a-carboxylic acidethyl ester, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine and (R,S)-α-methylserine- O-phosphate are relatively specific antagonists of groups I, II and III mGlu receptors respectively [9,13–15]. In contrast with the increasing range of agonists and antagonists that can distinguish between mGlu receptor groups I–III, there is only a small number of highly specific agonists or antagonists that exhibit pharmacological discrimination within mGlu receptor subgroups (e.g. between mGlu₄ and mGlu₅ receptors). The classification of the mGlu receptors, the transduction pathways that they activate and their respective pharmacologies are presented in Table 2.

Heterologous expression of mGlu receptors
The use of brain tissue preparations, or neurons in primary culture, for the study of the pharmacological and biochemical properties of...
mGlu receptors is limited by the wide and overlapping distributions of the various subtypes of mGlu receptors in the central nervous system and therefore the frequent co-existence of different mGlu receptor subtypes in any preparation. In addition, to our knowledge, no commonly used cell line has been found endogenously to express any characterized mGlu receptor, although the presence of group I mGlu receptors in a differentiated human teratocarcinoma (NTera2/c1.D1) cell line has been reported recently [16]. Another potentially interesting model is the use of astrocytes in culture, which have been found to express predominantly mGlu5 receptors [17].

An alternative approach has been made possible by the cloning of the mRNA sequences encoding the mGlu receptors from a variety of different species. This has allowed the heterologous expression of these receptors in eukaryotic cell models. As a consequence, most of the pharmacological studies concerning mGlu receptors, and the characterization of their signalling transduction pathways, have been performed in such expression systems. Although injected Xenopus oocytes also constitute a suitable model for electrophysiological studies, transfected cell lines are more convenient for extensive biochemical and pharmacological experiments. Transiently transfected human embryonic kidney (HEK) cells and pig kidney epithelial (LLC-PK1) cells expressing different mGlu receptors have been widely used as models for the study of these receptors [18–21]. However, quantitative measures of the receptor expression levels in such transfected cell models have proved problematic because of the lack of specific radio-ligands for each subtype. As a consequence, receptor density is usually assessed in immunoblotting experiments with receptor subtype-specific antisera. Less frequently, stable expression of mGlu receptor in HEK, Chinese hamster ovary (CHO), baby hamster kidney (BHK) and adenovirus-transformed Syrian hamster AV12 cells has also been reported [22–26]. However, it seems that an additional problem observed with these transfected cells is the difficulty of obtaining and maintaining the stable expression of functional mGlu receptors at a constant level.

Although these transfected cell lines constitute convenient models for the study of mGlu receptor properties, an analysis of the recent literature reveals important discrepancies between studies performed with different cell lines transfected with identical cDNA sequences. Significant variation in the efficacies and/or potencies of different mGlu receptor agonists has been reported [27,28]. Such variation is not restricted to agonist pharmacology: an interesting example of conflicting data on antagonists arises from studies reporting the activity of α-methyl-4-carboxyphenylglycine (MCPG) at the recombinant mGlu5 receptor. Thus MCPG has been reported to antagonize both l-quisqualate- and l-glutamate-stimulated responses in transfected L(tk−) mouse fibroblast cells [28]. However, Brabet et al. [19] have reported that antagonist activity is dependent on the nature of the agonist causing mGlu receptor activation, as only the response to l-quisqualate was inhibited by the antagonist, whereas that stimulated by l-glutamate was unaffected by MCPG [19]. In addition to such pharmacological discrepancies being reported, major discordances concerning differences in the biochemical coupling of mGlu receptors with intracellular transduction pathways are also commonplace. An example of the

### Table 2

**Biochemical and pharmacological classification of the mGlu receptors**

<table>
<thead>
<tr>
<th>Group</th>
<th>Subtypes</th>
<th>Intracellular effectors</th>
<th>Specific agonists</th>
<th>Specific antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>mGlu1, mGlu5</td>
<td>Phospholipase C (stimulation)</td>
<td>L-Quisqualate, (S)-DHPG</td>
<td>CPCCOEt</td>
</tr>
<tr>
<td>II</td>
<td>mGlu2, mGlu3</td>
<td>Adenylyl cyclase (inhibition)</td>
<td>DCG-IV, (2R,4R)-APDC</td>
<td>MCCG, LY341495</td>
</tr>
<tr>
<td>III</td>
<td>mGlu4, mGlu6, mGlu7, mGlu8</td>
<td>Adenylyl cyclase (inhibition)</td>
<td>L-AP4</td>
<td>MeSOP, MAP4, CPPG</td>
</tr>
</tbody>
</table>
latter is the existence of a positive functional coupling between mGlu receptors and adenylate cyclase activation in transfected LLC-PK1 cells [29], but not in CHO cells [30] or in astrocytes endogenously expressing this receptor [17]. Furthermore, constitutive activity of the mGlui receptor [20] is not observed in transfected CHO cells when medium and/or cell-derived L-glutamate is excluded from the incubation buffer (by incubation in the presence of glutamic-pyruvic transaminase and pyruvate) [31]. Analysis of the functional coupling of the mGlui receptor to G-proteins by using pertussis toxin has also produced conflicting results, as both positive and negative effects of toxin treatment have been observed with respect to effects on agonist-induced phosphoinositide hydrolysis [32,33].

Finally, desensitization of group I mGlu receptors by pretreatment with agonist or activators of protein kinase C has also been shown to be cell-type dependent. Although phorbol ester treatment of BHK cells expressing the mGlui receptor has been reported to desensitize further agonist-induced phosphoinositide hydrolysis [25], no similar effect was observed in transfected CHO cells [34].

One explanation that can account for some, but not all, of these discrepancies between studies performed in heterologous expression models could be based on the variations in receptor densities between model systems. As already mentioned, the question of receptor expression level is particularly difficult to assess for mGlu receptors because of the absence of satisfactory high-affinity, high-specific-activity radioligands. Differences in the density of functional (cell-surface) receptors can result in differences in the 'receptor reserve', which will affect the potency and efficacy of both positive and inverse agonists [35]. Although the coupling of some mGlu receptors is rather complex, possibly involving efficient coupling to multiple G-proteins [9], it has also been proposed that increasing the expression of G-protein-coupled receptors can lead to increasingly promiscuous coupling to multiple G-protein species [36]. Similarly, the constitutive activity of G-protein-coupled receptors might also depend on the density of receptors [37,38]. This has been shown for the mGlui receptor, in which constitutive activity has been shown to increase proportionately with receptor expression [20]. In addition, the nature of the cell type chosen for heterologous expression studies might also govern the properties of the expressed receptor. For example, the molecular processing of newly synthesized receptors might be different from one cell to another. This possibility has been highlighted by immunocytochemistry studies that have shown a widespread distribution of the mGlui receptor in transfected BHK cells [33], whereas the same receptor was found to be targeted principally to the plasma membrane in transfected HEK cells [12]. In the same way, differences in the nature of the functional coupling of the receptors might also reflect the nature of the endogenously expressed G-protein complement of a given cell.

**Glutamate-induced regulation of mGlu receptors**

In addition to these possible pitfalls of using transfected cells for the study of G-protein-coupled receptors, a further problem observed when studying glutamate receptors in cell culture is the presence of L-glutamate in the cell culture medium. Even when care is taken to avoid the addition of any exogenous L-glutamate, cultured cells can release this amino acid into the incubation medium. This has been observed for BHK cells [33,39], HEK cells [20] and adenovirus-transformed Syrian hamster AV12 cells [40]. The accumulation of this agonist of mGlu receptors in the incubation medium inevitably results in the tonic activation of mGlu receptors and the activation of regulatory mechanisms at the level of the receptor and/or downstream signalling cascade. Accordingly, L-glutamate-induced desensitization, internalization and down-regulation of mGlu receptors has been reported in some systems [17,41–44]. Evidence for a desensitization of mGlu receptors caused by cellular L-glutamate release into their incubation medium has been obtained from experiments in which cells were co-transfected with a glutamate transporter (which facilitates L-glutamate re-uptake from the medium) [40], or in which cells were preincubated in the constant presence of a mGlu receptor antagonist until immediately before the experiment [20]. These manipulations were both found to increase significantly the responsiveness of transfected cells to further stimulation by mGlu receptor agonists. This might provide an explanation of the difficulties reported by some groups in obtaining and maintaining the expression of functional mGlu receptors stably in transfected cells [28,44,45].
Inducible expression of mGlu receptors

During the past few years, different systems of controlled heterologous expression have been developed by using inducible or repressible transcription promoters. The use of such systems allows cell lines to be established in which the expression of any cloned gene can be maintained 'silent' and can be rapidly induced by the addition of a chemical inducer to the culture medium. Controlled expression of adrenoceptors has previously been achieved in transfected DDT,MF-2 cells by using an isopropyl-β-d-thiogalactoside (IPTG)-inducible system [46]. More recently, dexamethasone-controlled expression of the human mGlu₁ and mGlu₄ receptors has been reported in CHO cells [28]. By using the IPTG-inducible system, we have developed stably transfected CHO [31] and HEK cells (E. Hermans, S. R. Nahorski and R. A. J. Challis, unpublished work) expressing 'silently' the human mGlu₁ receptor. The maintenance of receptor expression at very low levels during the initial transfection and clonal selection, and during the normal passaging of cells, prevents any possibility of prolonged activation of the mGlu receptor by medium glutamate, and allows expression 'on demand' by simple addition of the inducing chemical. Furthermore the expression level can be tightly controlled as the amount of receptor protein synthesized is dependent on the concentration of inducer added (Figure 1). Such control of receptor expression levels allows the pharmacological and biochemical properties to be examined at different densities of cell-surface receptors [47].

Conclusion

The results summarized here highlight the contributions made to our understanding of the properties of single mGlu receptor subtypes by using heterologous expression systems. However, as discussed above, this approach is not without its own set of problems and limitations. Regarding the potential difficulties posed in determining the density of mGlu receptors in these model systems (but see [48,49]), as well as the possible receptor adaptations caused by glutamate in the incubation medium of cultured cells, the use of inducible mammalian cell models might prove to be extremely useful. In addition, heterologous expression systems provide a critical research tool for the discovery of new specific agonists and antagonists for each of the mGlu receptor subtypes, as they can provide sufficient cells 'in bulk' to allow high-throughput screening to be applied. Furthermore, inducible expression systems allow the 'titration' of physiologically relevant densities of mGlu receptors to be expressed at the cell surface ([31], and E. Hermans, R. A. J. Challis and S. R. Nahorski, unpublished work), providing an ideal system in which to assess the efficacy, as well as the potency, of novel mGlu receptor agonists, and to establish the preferred downstream G-protein coupling partners of the different mGlu receptors [49]. Ultimately, however, heterologous expression in neuronal cell backgrounds [51] will be required to address more functional aspects of mGlu receptor modulation of synaptic activities in physiological and pathophysiological circumstances.

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G-Protein-Coupled Receptor Signalling in the Central Nervous System


Evidence that a novel metabotropic glutamate receptor mediates the induction of long-term potentiation at CA1 synapses in the hippocampus

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Introduction
Long-term potentiation (LTP) at excitatory synapses in the CA1 region of the hippocampus is the most extensively studied model for understanding mechanisms of synaptic plasticity that are thought to be critically involved in learning and memory in vertebrates [1]. LTP is induced by the transient activation of one class of glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor. LTP is manifest as a persistent increase in the synaptic response that is mediated by the action of the neurotransmitter l-glutamate on a different subtype of glutamate receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (and on NMDA receptors, in conditions under which these contribute to the synaptic response).

There has been intense investigation into the possible role of a third type of glutamate receptor, the metabotropic glutamate (mGlu) receptor, in the induction of LTP at CA1 synapses (recent references are given in [2,3]). This receptor comprises a family of G-protein-coupled receptors of which eight have so far been identified by molecular cloning. These are subdivided by sequence homology, pharmacology and signal transduction mechanisms into three groups: group I (mGlu, and mGluS), group II (mGlu2 and mGlu3) and group III (mGlu6-8) [4]. However, the roles of mGlu receptors in hippocampal LTP is highly controversial and the identity of the subtypes that might be involved in not known.

Here we present pharmacological evidence that two distinct types of mGlu receptor are involved in the induction of LTP at CA1 synapses. One is involved in the setting of a 'molecular switch' [5] and the other is involved in the induction of LTP itself. The latter receptor has a pharmacology that suggests that it does not correspond to any of the cloned mGlu receptors.

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