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Genetic analysis of neuronal ionotropic glutamate receptor subunits

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Abstract In the brain, fast, excitatory synaptic transmission occurs primarily through AMPA- and NMDA-type ionotropic glutamate receptors. These receptors are composed of subunit proteins that determine their biophysical properties and trafficking behaviour. Therefore, determining the function of these subunits and receptor subunit composition is essential for understanding the physiological properties of synaptic transmission. Here, we discuss and evaluate various genetic approaches that have been used to study AMPA and NMDA receptor subunits. These approaches have demonstrated that the GluA1 AMPA receptor subunit is required for activity-dependent trafficking and contributes to basal synaptic transmission, while the GluA2 subunit regulates Ca^{2+} permeability, homeostasis and trafficking to the synapse under basal conditions. In contrast, the GluN2A and GluN2B NMDA receptor subunits regulate synaptic AMPA receptor content, both during synaptic development and plasticity. Ongoing research in this field is focusing on the molecular interactions and mechanisms that control these functions. To accomplish this, molecular replacement techniques are being used, where native subunits are replaced with receptors containing targeted mutations. In this review, we discuss a single-cell molecular replacement approach which should arguably advance our physiological understanding of ionotropic glutamate receptor subunits, but is generally applicable to study of any neuronal protein.

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Abbreviations ACh, acetylcholine; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; iGluR, ionotropic glutamate receptor; LTD, long-term depression; LTP, long-term potentiation; nAChR, nicotinic acetylcholine receptor; NMDAR, *N*-methyl *D*-aspartate receptor; PSD, postsynaptic density; RNAi, RNA interference.

Introduction

Understanding the molecular mechanisms underlying fast, excitatory synaptic transmission is a daunting task given that hundreds of proteins have been identified in the postsynaptic density (PSD). In approaching this problem, it is important to realize that the primary role of the vast web of proteins in the PSD is to position ionotropic neurotransmitter receptors across from a presynaptic active zone. Thus, the ionotropic receptors present an appropriate starting point in understanding postsynaptic physiology. Much of our early understanding of fast synaptic transmission comes from work on nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction, initiated by the pioneering studies of Bernard Katz. These studies were the first to suggest that acetylcholine (ACh) might 'short circuit' the membrane to 'create aqueous channels through which small ions can pass without distinction' (Fatt & Katz, 1951; Del Castillo & Katz, 1956). Later biochemical and cloning studies revealed that nAChRs are transmembrane proteins composed of five subunits, which form an ion pore upon binding to ACh. However, it was soon realized that ACh is not involved in the vast majority of fast synaptic responses

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in the brain, but that glutamate and its analogues potently excite virtually all central nervous system (CNS) neurons. Subsequent pharmacological and cloning studies identified three classes of ionotropic glutamate receptors (iGluRs): AMPA, NMDA and kainate receptors. These receptors are each composed of different subunit proteins with distinct biophysical and trafficking properties. Depending on the particular subunit composition of its ionotropic receptors, a synapse's specific ion permeability, transmission kinetics and plasticity could vary considerably. Proper understanding of synaptic physiology therefore requires identifying the distinct biophysical properties and trafficking behaviour of iGluR subunits. However, pharmacology alone cannot distinguish these properties (Beique & Huganir, 2009). What are the molecular biological and genetic approaches available for such an analysis? On the one hand, receptors can be overexpressed in heterologous cells or in wild-type neurons to positively identify subunit properties. On the other hand, we can study loss of function through specific subunit deletion, either by germline or conditional knock-out, or by RNA interference (RNAi). The ultimate goal is to determine the mechanisms and molecular interactions that explain the physiological role of a particular subunit. To accomplish this, we propose the use of molecular replacement, either through germline knock-ins or single cell re-expression on a 'null' background. This review will discuss the advantages and disadvantages of these strategies in the study of AMPA receptors (AMPARs) and NMDA receptors (NMDARs), but the approaches discussed apply equally well to the study of any protein.

Heterologous expression of receptor subunits

Initial studies in this field involved the expression of various cloned subunit proteins in non-neuronal cells to compare their biophysical properties to native receptors. These studies led to a number of key discoveries. For instance, it was shown that a functional AMPAR is a tetramer composed of various combinations of four different subunit proteins, GluA1–4 (Fig. 1*A*) (Keinanen *et al.* 1990; Rosenmund *et al.* 1998). Also, the GluA2 subunit was found to limit the permeability of AMPARs to monovalent cations, blocking the flow of calcium ions (Burnashev *et al.* 1992). Using this information, researchers could demonstrate that the GluA2 content of AMPARs varied with neuronal type, synaptic plasticity and disease (Cull-Candy *et al.* 2006; Isaac *et al.* 2007). NMDARs, also tetrameric non-selective cation channels, were found to be unique in that they only pass current during depolarization due to extracellular Mg^{2+} blocking the receptor pore at resting membrane potentials. A functional NMDAR requires co-expression of a GluN1 subunit protein with any one of four GluN2 subunits, GluN2A–D (Monyer *et al.* 1994), which confer distinct functional properties to the NMDAR (Fig. 1*A*). Specifically, GluN2B-containing NMDARs have dramatically slower deactivation kinetics as compared with GluN2A, allowing much greater charge transfer and Ca^{2+} signalling (Vicini *et al.* 1998). This could result in dramatically different effects of the total contribution to synaptic transmission of NMDARs and the types of Ca^{2+}-dependent plasticity supported by synaptic NMDARs (Cull-Candy & Leszkiewicz, 2004).

The information gained from *in vitro* overexpression, however, is limited for at least two reasons. First, in some instances the expressed receptors do not match the biophysical properties of the neuronal receptors. For example, heterologously expressed GluA4 subunits showed significantly reduced conductance in response to kainate as compared with native GluA4 receptors in cerebellar granule cells (Wyllie *et al.* 1993; Swanson *et al.* 1997). This discrepancy was later solved by the discovery of a family of transmembrane AMPA receptor regulatory proteins (TARPS), which increase kainate efficacy and play crucial roles in trafficking and kinetics of neuronal AMPARs (Jackson & Nicoll, 2011). Second, the synaptic trafficking of different receptor subunits and their role in synapse development and function cannot be determined in heterologous cells. Therefore, a more complete understanding of iGluR function also requires study in neuronal systems.

Overexpression of subunits in wild-type neurons

To study subunit function in a more natural setting, subunit proteins can be overexpressed in wild-type neurons. This approach has been used with considerable success to study primarily AMPAR function (Hayashi *et al.* 2000; Shi *et al.* 2001). It was found that overexpressed green fluorescent protein (GFP)-tagged GluA1 did not traffic to synapses in hippocampal pyramidal neurons under basal conditions, whereas similarly tagged GluA2 subunits did. However, delivery of GluA1 to the synapse could be induced by long-term potentiation (LTP) or co-expression with an active form of Ca^{2+}–calmodulin-dependent kinase II (CaMKII). Based on these overexpression studies, a model was developed in which GluA2/3 heteromers constitutively traffic to and from the synapse, with GluA1/2 heteromers inserted following activity. This differential trafficking role for GluA1 *versus* GluA2 resides in the strikingly different cytoplasmic C-terminal domains, as swapping the C-termini swaps their trafficking behaviour. Similar overexpression of GluA1 has since been used in several contexts to study activity-dependent AMPA receptor trafficking *in vivo* (Takahashi *et al.* 2003; Jitsuki *et al.* 2011).

The main limitation of overexpression in wild-type neurons is the presence of a full complement of

endogenous receptor subunits that may compensate for expressed mutant or truncated subunits. Also, determining the native subunit composition and contribution of individual subunits to synaptic function is not feasible through overexpression. Instead, it is useful to study the loss of function when subunits are absent or altered, as discussed below.

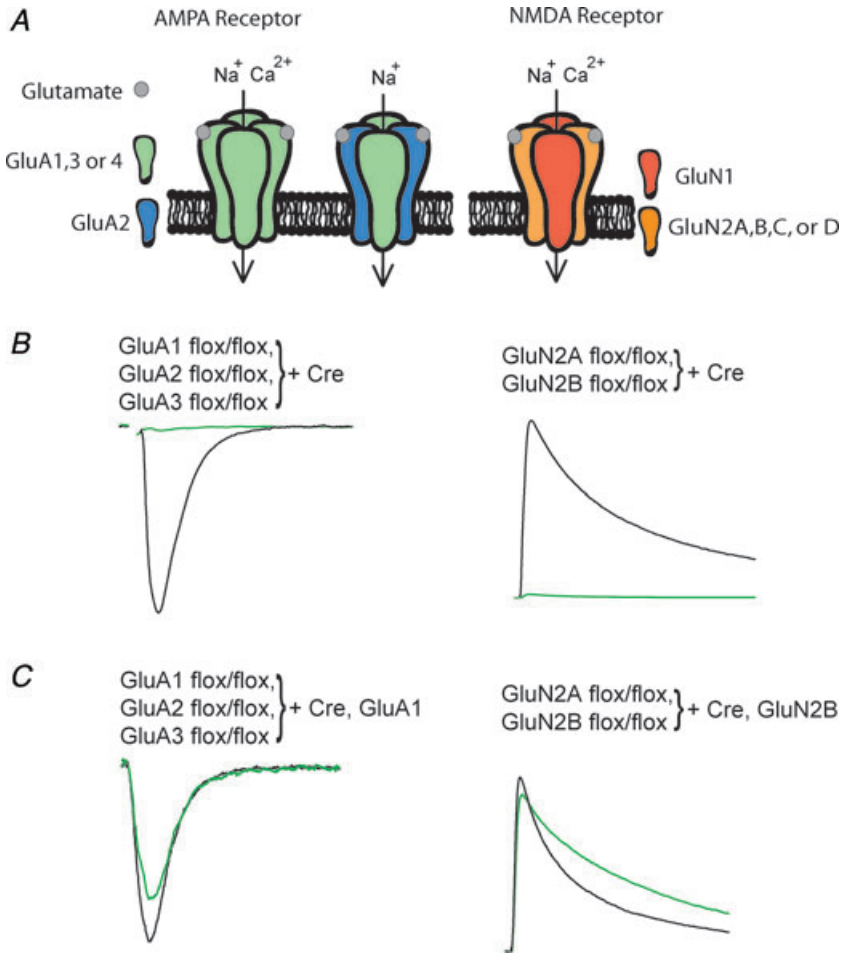

Figure 1. Conditional deletion and replacement of AMPA and NMDA receptor subunits
A, AMPA and NMDA receptors are transmembrane complexes each composed of four subunits. AMPA receptors are made of combinations of four different subunit proteins, GluA1, GluA2, GluA3 or GluA4. They can exist as homomeric complexes of a single subunit which act as non-selective cation channels following glutamate binding, allowing the flow of Na^+, K^+ and Ca^{2+} ions. Alternatively, they can exist as heteromers containing GluA2, which renders the ion pore impermeable to Ca^{2+}. The GluA2 subunit alone does not readily form homomeric receptors. In contrast, NMDA receptors must contain two GluN1 subunits and two GluN2 subunits, of which there are four isoforms, GluN2A, GluN2B, GluN2C and GluN2D. Glutamate-associated NMDA receptors also act as cation channels, allowing the flow of Na^+, K^+ and Ca^{2+}. However, they only pass current at depolarized membrane potentials, as a Mg^{2+} ion blocks the pore at resting membrane potentials. *B*, example traces of synaptic currents from AMPA (left) and NMDA (right) receptors in hippocampal CA1 pyramidal cells. AMPA receptor-mediated synaptic currents (left) can be isolated by recording at –70 mV, a potential where NMDA receptors are blocked by extracellular Mg^{2+}. NMDA receptor currents (right traces) have much slower decay kinetics than AMPA receptors, and can be isolated by recording at +40 mV in the presence of the AMPA receptor antagonist NBQX. The black traces show the current evoked in a wild-type control neuron, while the green traces are recorded simultaneously from a neighbouring Cre-expressing neuron. Expression of Cre in mice with floxed GluA1, GluA2 and GluA3 genes is sufficient to completely eliminate the AMPA-mediated current (left), indicating that all native AMPA subunits are deleted. Likewise, in mice with floxed GluN2A and GluN2B genes, Cre expression removes all NMDA receptor current. *C*, co-expression of a replacement subunit on these backgrounds can rescue AMPA or NMDA receptor current. Co-expression of Cre with the GluA1 subunit rescues synaptic AMPA receptor current to approximately 75% that of controls (left). Likewise, co-expression of Cre with a GluN2B subunit rescues NMDA receptor current (right). Note the slower decay kinetics in the NMDA receptor replacement cell, typical of purely GluN2B-containing receptors.

Germline knock-out of ionotropic receptor subunits

One means to study iGluR loss of function is through germline genetic deletion. Studies on germline knock-out mice for individual AMPAR subunits have provided important insights into the function of neuronal AMPARs. Indeed, germline deletion of the GluA1 subunit essentially abolished hippocampal LTP in adult mice and eliminated somatic extrasynaptic receptors, the latter proposed to act as a reserve pool for potentiation of synaptic responses (Zamanillo *et al.* 1999; Jensen *et al.* 2003). In contrast, LTP was normal in GluA3 knock-out (Meng *et al.* 2003) or even enhanced in GluA2 knock-out mice (Jia *et al.* 1996; but see Panicker *et al.* 2008), further supporting the unique role for GluA1 in activity-dependent trafficking. This approach was less successful in determining the subunit composition of AMPARs during basal synaptic transmission. While knock-out of GluA2 caused a strong reduction of synaptic transmission in the hippocampus (Jia *et al.* 1996; Meng *et al.* 2003), knock-out of either GluA1 or GluA3 had little or no effect (Zamanillo *et al.* 1999; Andrasfalvy *et al.* 2003; Jensen *et al.* 2003; Meng *et al.* 2003). These results appear incompatible, because GluA2 alone does not readily form homomers and cannot fully account for basal synaptic transmission (Burnashev *et al.* 1992; Hollmann & Heinemann, 1994; Lu *et al.* 2009). One potential drawback to germline knock-outs is the possibility for developmental compensation, which may account for the modest phenotypes on basal synaptic transmission observed in GluA1 or GluA3 knock-outs. Also, in severe cases, germline deletion can result in embryonic or perinatal lethality, providing little insight into their role in synaptic function. Such is the case with GluN1 knock-out mice (Forrest *et al.* 1994), which suffer from respiratory abnormalities, and GluN2B knock-out mice, which do not suckle appropriately (Kutsuwada *et al.* 1996).

Conditional knock-out of ionotropic receptor subunits

To circumvent the problems of developmental compensation and neonatal lethality associated with germline knock-outs, one can use conditional knock-out mice. Here, mice engineered to have the subunit of interest flanked by locus-of-crossover (flox) sites can be bred with available reporter mice that express Cre recombinase in targeted tissues and at specific time points. Homologous recombination will then excise the subunit only in those cells that express Cre. This technique has been used with particular success to study the contribution of glutamate receptor subunits to behaviour. For example, deletion of GluN1 in adult hippocampal CA1 cells caused a specific impairment in a spatial memory task, but not in non-spatial memory, with an accompanying loss of LTP (Tsien *et al.* 1996). A similar impairment of LTP, long-term depression (LTD), and spatial memory was seen with CA1-specific GluN2B deletion (Brigman *et al.* 2010). These experiments convincingly demonstrate the importance of NMDARs to synaptic plasticity and support the link between synaptic plasticity and learning. However, LTP was normal in postnatal forebrain knock-out of GluA2, even though spatial memory was impaired, presumably due to the profound decrease seen in overall excitatory transmission (Shimshek *et al.* 2006). Conditional knock-outs can also be used to restrict subunit deletion to specific circuits, such as all dopaminergic neurons. Engblom *et al.* (2008) showed that the removal of GluA1 and GluN1 in dopamine neurons inhibited the extinction and reinstatement, respectively, of cocaine-induced conditioned place preference.

Even greater specificity can be achieved by expressing Cre into single neurons to study the cell-autonomous function of glutamate receptor subunits. Such an approach was used with GluN1 to demonstrate that NMDARs negatively regulate AMPAR trafficking to synapses during development (Adesnik *et al.* 2008; Lu *et al.* 2011), and with both GluN2A and GluN2B to show that NMDA subunit composition can regulate synaptic development (Gray *et al.* 2011) and growth of the dendritic arbor (Espinosa *et al.* 2009). The major study on single-cell knock-out of AMPARs used deletion of all possible combinations of GluA1, GluA2 and GluA3 to definitively demonstrate the receptor composition at synapses in CA1 pyramidal neurons (Lu *et al.* 2009). This showed a large role for GluA1/2 heteromers in basal synaptic transmission, with an approximate 15% contribution for GluA2/3 heteromers, neither of which was seen in the germline knock-outs.

RNA interference of ionotropic receptor subunits

A major disadvantage of conditional knock-outs is the great time and cost involved in engineering the necessary mouse lines. This need for genetically engineered mice can be bypassed using RNA interference (RNAi) to block expression of targeted proteins. RNAi also shortens protein turnover duration relative to conditional knock-outs, and can be used across multiple experimental organisms. For example, RNAi-mediated knockdown of GluA2 was used in rat dissociated neuronal cultures to demonstrate its requirement for homeostatic synaptic scaling (Gainey *et al.* 2009). For NMDA receptors, RNAi against GluN2A and GluN2B has suggested opposing roles in regulating AMPA receptor trafficking. While GluN2B knockdown increased synaptic AMPA receptors (Hall *et al.* 2007), GluA1 surface delivery was inhibited by knockdown of GluN2A (Kim *et al.* 2005*b*). Another advantage of RNAi is that knockdown of combinations of proteins is greatly simplified, as demonstrated by Tracy *et al.* (2011).

This study used a single RNAi construct to knockdown expression of all three – GluA1, 2 and 3 – ultimately showing that the GluA2 N-terminal domain can act as a retrograde signal for presynaptic maturation. This triple RNAi did not change spine density, a result consistent with conditional deletion of GluA1, 2 and 3 (Lu *et al.* 2009). However, several papers have shown that RNAi against GluN2B (Gambrill & Barria, 2011) or GluA2 (Passafaro *et al.* 2003) results in decreased dendritic spine density.

These discrepancies may be due to the several caveats associated with RNAi. First, knockdown by RNAi is typically incomplete, so some amount of native protein remains (Tracy *et al.* 2011). Also, RNAi expression can cause significant off-target effects independent of the decrease in target protein expression. These off-target effects may be caused by activation of the cell's innate interferon response, perturbation of endogenous microRNA machinery, or post-transcriptional silencing of proteins other than those targeted (Sioud, 2011). For example, Alvarez *et al.* (2006) showed that RNAi in rat neurons against luciferase, which has no sequence homology in the rat genome, can cause a robust decrease in dendritic branching, spine density and synaptic transmission. Controlling for this with a scrambled RNAi sequence alone is not sufficient, as these off-target effects are often sequence specific. Instead, a combination of controls should be used, such as direct assay of interferon activation, rescue with an RNAi-proof version of the target protein, validation with pharmacological block or dominant negative overexpression (Alvarez *et al.* 2006), or the lack of effect in a knock-out background for the protein in question.

Molecular replacement

Ultimately, techniques that rely solely on removing selected subunits are limited in the types of questions that can be asked. To discover the specific molecular interactions that mediate subunit function, native receptors must be replaced with modified versions containing targeted mutations. Such molecular replacement can be achieved with knock-in mice, where a mutated version of a selected subunit is inserted into the endogenous gene locus. Several papers have used this technique with success to study the importance of the GluA1 C-terminus in synaptic plasticity. For example, knock-in mice with phosphonull mutations of S831 and S845 in the GluA1 C-terminus have no LTD and impaired LTP (Lee *et al.* 2003, 2010), whereas phosphomimetic mutations of the same sites show enhanced LTP (Makino *et al.* 2011). In addition, a knock-in mouse with a truncated GluA1 PDZ domain showed no change in synaptic transmission, despite previous overexpression experiments that suggested its requirement for synaptic targeting (Kim *et al.* 2005*a*). In contrast, similar knock-ins of GluN2A and GluN2B that inserted premature stop codons before their C-termini demonstrated the crucial importance of this region in receptor function, as these defects recapitulated the effects of the full knock-outs – perinatal lethality for GluN2B, impaired synaptic plasticity and contextual memory for GluN2A (Sprengel *et al.* 1998).

This technique is not without its disadvantages. First, considerable time and cost is involved in engineering knock-in mouse lines. Given this, careful consideration needs to be taken to select a mutation that will provide the most mechanistic insight. This also limits the number of mutations that can be feasibly studied. Second, as a global manipulation, the mouse may be affected by network-wide compensation or impaired survival due to defects other than those being studied, such as the GluN1 and GluN2B knock-out mice.

Single-cell molecular replacement, however, allows for screening of multiple mutations in a cell-autonomous fashion. In this technique, over-expression and conditional knock-outs are combined, permitting the study of mutated subunits without competition from native wild-type protein. In the case of hippocampal CA1 cells, Cre expression in mice with floxed GluA1, GluA2 and GluA3 genes is sufficient to produce a complete null background lacking any endogenous AMPARs (Lu *et al.* 2009), and floxed GluN1 or GluN2A/2B mice can be used to create a null background for NMDARs (Fig. 1*B*) (Gray *et al.* 2011). A replacement subunit protein can then be co-expressed with Cre, rescuing synaptic transmission (Fig. 1*C*). This technique has been used to discover a novel CaMKII phosphorylation site in the first intracellular loop of GluA1 which is necessary for normal synaptic targeting of surface AMPARs (Lu *et al.* 2010). A similar approach using RNAi instead of conditional knock-outs has also been used to demonstrate the requirement of the GluN2B C-terminus for induction of LTP (Foster *et al.* 2010). This technique still has potential drawbacks. Total protein turnover may take several weeks following Cre expression, and the replaced subunits may be expressed at unnaturally high levels. Nonetheless, single-cell molecular replacement represents the next step in studying iGluR function, and is the best currently available technique to find the crucial molecular interactions underlying synaptic transmission and plasticity.

Conclusion

In this review, we outlined various genetic approaches available to determine the physiological role of neuronal ionotropic glutamate receptors. As a result of these approaches, we now appreciate the unique role of individual iGluRs for neuronal function. In general, AMPARs are responsible for fast excitatory synaptic

transmission at resting membrane potentials. GluA1 in particular is required for basal synaptic transmission and activity-dependent trafficking, while GluA2 is important for regulating Ca^{2+} permeability, basal trafficking to synapses and homeostasis. NMDARs, in contrast, are only active at depolarized potential and appear to play a major role in synaptic plasticity and development, with GluN2A and GluN2B having complex and differential roles in regulating synaptic AMPARs. Future research should focus on restricted domains and molecular interactions that carry out the functions of these subunits. To accomplish this, molecular replacement with mutated subunits will be essential to identify physiologically important regions. Multiple mutations can be screened relatively quickly with the use of single-cell molecular replacement, and the most crucial mutations then tested in germline knock-in mice. This approach can be easily generalized to any important protein of interest and will permit the systematic uncovering of the mechanisms that regulate synaptic transmission and plasticity.

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