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# Genomics and variation of ionotropic glutamate receptors: implications for neuroplasticity

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**Summary.** We used two approaches to identify sequence variants in ionotropic glutamate receptor (IGR) genes: high-throughput screening and resequencing techniques, and "information mining" of public (e.g. dbSNP, ENSEMBL) and private (i.e. Celera Discovery System) sequence databases. Each of the 16 known IGRs is represented in these databases, their positions on a canonical physical map are established. Comparisons of mouse, rat, and human sequences revealed substantial conservation among these genes, which are located on different chromosomes but found within syntenic groups of genes. The IGRs are members of a phylogenetically ancient gene family, sharing similarities with glutamate-like receptors in plants. Parsimony analysis of amino acid sequences groups the IGRs into three distinct clades based on ligand-binding specificity and structural features, such as the channel pore and membrane spanning domains. A collection of 38 variants with amino acid changes was obtained by combining screening, resequencing, and informatics approaches for several of the IGR genes. This represents only a fraction of the sequence variation across these genes, but in fact these may constitute a large fraction of the common polymorphisms at these genes and these polymorphisms are a starting point for understanding the role of these variants in function.

Genetically influenced human neurobehavioral phenotypes are likely to be linked to IGR genetic variants. Because ionotropic glutamate receptor activation leads to calcium entry, which is fundamental in brain development and in forms of synaptic plasticity essential for learning and memory and is essential for neuronal survival, it is likely that sequence variants in IGR genes may have profound functional roles in neuronal activation and survival mechanisms.

**Keywords:** Ionotropic glutamate receptor genes – Sequence variant – Genomics – Polymorphism – Neuroplasticity

## Introduction

Ionotropic glutamate receptors (IGR) play the largest role in excitatory neurotransmission in the brain and play central roles in a variety of behaviors such as learning that are fundamental to survival. Glutamatergic pathways are directly responsible for the bulk of long-range interconnectivity in the human brain. Therefore, it is not surprising that this is a phylogenetically ancient family of receptors. Glutamate receptors are found in both animals and plants. The size of the glutamate receptor gene family is also consistent with the critical role of these receptors in connectivity. There are at least 16 IGR genes in humans.

Glutamate receptor activation leads to calcium entry, which is fundamental in brain development and in forms of synaptic plasticity essential for learning and memory. In addition, earlier work has supported an essential role in neuronal survival, suggesting a role for glutamate receptors in neurological disorders such as epilepsy, brain injury from trauma or focal ischemia, and perhaps in disorders such as schizophrenia, Parkinson's disease, or Huntington's chorea, and amyotrophic lateral sclerosis (ALS). Although IGRs are largely expressed in the brain, they also have important roles in the peripheral nervous system, acting in other tissues to modulate secretion of insulin by the pancreas, regulating bone resorption, and in the perception of pain. Originally defined on the basis of their agonist binding and electrophysiological properties, N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA), or kainate (KA), molecular cloning of cDNAs encoding IGR subunits during the late 1980s and early 1990s made it clear that these receptors were members of related gene families. The recent availability of largely complete genomic sequence data for rodent and human IGR genes has enabled a comprehensive informatics approach focused on understanding the functional diversity of this class of receptors.

## Materials and methods

*Amplification of samples*

PCR conditions for the various primer pairs were as follows. Each reaction was performed in a total volume of 20 $\mu$l and contained 1 $\mu$l DNA 10 ng/$\mu$l, 2.0 $\mu$l 10× Buffer II, 5 mM $MgCl_2$, 0.8 mM dNTPs, 1 $\mu$l 10 nM forward primer and 1 $\mu$l 10 nM reverse primer. The temperature program consisted of a twelve minute hotstart at 95°C, followed by thirty cycles of 15 seconds of denaturing at 95°C, 20 seconds at the optimal annealing temperature, and 30 seconds at 72°C for product elongation. There was a final seven minute elongation step at 72°C and then the reaction was stored at 4°C. For variant screening with dHPLC, the screening panel consisted of five panels of 96 individual genomic DNA samples for a total of 480 individuals, which constituted both a clinically and ethnically diverse screening panel. All DNA samples were obtained from cultured white blood cells originally obtained either from repositories or blood samples.

*Variant screening using dHPLC*

To screen for single nucleotide polymorphisms (SNPs) we used a ''resequencing'' strategy based on high performance liquid chromatography (dHPLC) and direct sequence analysis. We have described an approach for SNP discovery using dHPLC (Rudolph et al., 2002). Briefly, PCR samples were denatured at 95°C for 5 min and then cooled to 65°C over a period of 30 minutes to enhance formation of DNA heteroduplexes. Samples were then analyzed on a Transgenomic HPLC system (Omaha, NE) with the DNASep™ HPLC column. The HPLC system consisted of a cooled 96-well autosampler, column oven, pumps, degasser, variable wavelength UV detector, 20 $\mu$l sample loop and a PC-based data collection system. Buffers used for were as follows: Buffer A; 10 mM TEAA (tri-ethyl ammonium acetate), pH 7.4 and Buffer B; 10 mM TEAA and 25% acetonitrile. Loading buffer consisted of 10% acetonitrile for the DNASep column. Melting temperatures and buffer gradients were determined using the Transgenomic WAVEMAKER™ melting temperature prediction software.

*DNA sequence analysis*

PCR samples with differential dHPLC elution profiles were selected for direct sequence analsyis. Typically, the 10 $\mu$l sequencing reaction mixture contained 4 $\mu$l BigDye Terminator RR Mix (ABI, Foster City, CA); 2.84 $\mu$l of $dH_2O$; 1.6 pmol of forward or reverse primer; and 3 $\mu$l of purified HTTLPR PCR amplicon. Cycle conditions for sequencing were 25 cycles consisting of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. Sequencing reaction products were purified using Performa DTR (Edge BioSystems, Gaithersburg, MD) columns, dried, diluted with 25% formamide (v/v), denatured at 95°C for 5 min and analyzed on a 3100 Genetic Analyzer (ABI, Foster City, CA).

*Parsimony analysis of amino acid sequences*

Analyses were performed using Informax life science software (Frederick, MD). Phylogenetic tree calculation was based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm of Saitou and Nei (1987). The ClustalW program allows progressive multiple alignments by performing pairwise comparisons of sequences and sequence alignments with sequence weighting and gap penalties (Thompson et al., 1994).

*Genome informatics*

Non-synonymous substitutions at IGRs were also identified by a genome informatics approach. These data were compiled from translated exons from different IGR genes. Nucleotide sequence variants were detected by comparing available sequences via multiple sequence alignments and from entries in sequence variant databases. IGR genes were examined using dbSNP BUILD 113 (indexed April 14, 2003), Ensembl, and Celera RefSNP Release R4.0 (indexed February 21, 2003).

*Mutagenesis, transfections, and cell survival assays*

Site-directed mutagenesis of plasmids with NR1 subunit cDNAs (D. R. Lynch, University of Pennsylvania) was performed using the QuickChange protocol (Stratagene, La Jolla, CA) and mutations confirmed by direct DNA sequencing. HEK 293 cells were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Cell survival assays were based on the assay described by Marini et al. (1989), but used GFP-positive cells as the indicator in cotransfection experiments with the NR2B-GFP fusion cDNA.

## Results

### *Phylogenetically-ancient gene families*

Ionotropic glutamate receptors are encoded by at least six gene families based on nucleotide and amino acid sequence identity (Table 1). There is a single family of AMPA receptors, two families of KA receptors, and three families of NMDA receptors. Sequence similarity, including partial conservation of exon-intron structure, suggests a common but ancient evolutionary origin for all IGR gene families. In humans, amino acid sequence similarities among the gene family members ranges from 16 to 62%. Thus, the individual genes within gene families are themselves ancient. The primate brain has undergone a large expansion in size and capacity. However, the IGR genes involved in long-range connectivity in primate brain are probably orthologous to genes found in other vertebrates.

**Table 1.** Human ionotropic glutamate receptors genes and their chromosomal locations

| Receptor | Family | Subunit | Gene name | Chromosome |
|---|---|---|---|---|
| AMPA | 1 | GluR1 | *GRIA1* | 5q33 |
| AMPA | 1 | GluR2 | *GRIA2* | 4q32-33 |
| AMPA | 1 | GluR3 | *GRIA3* | Xq25-26 |
| AMPA | 1 | GluR4 | *GRIA4* | 11q22-23 |
| KA | 2 | GluR5 | *GRIK1* | 21q21.1-22.1 |
| KA | 2 | GluR6 | *GRIK2* | 6q16.3-q21 |
| KA | 2 | GluR7 | *GRIK3* | 1p34-p33 |
| KA | 3 | KA1 | *GRIK4* | 11q22.3 |
| KA | 3 | KA-2 | *GRIK5* | 19q13.2 |
| NMDA | 4 | NR1 | *GRIN1* | 9q34.3 |
| NMDA | 5 | NR2A | *GRIN2A* | 16p13.2 |
| NMDA | 5 | NR2B | *GRIN2B* | 12p12 |
| NMDA | 5 | NR2C | *GRIN2C* | 17q24-q25 |
| NMDA | 5 | NR2D | *GRIN2D* | 19q13.1qter |
| NMDA | 6 | NR3A | *GRIN3A* | 9q34 |
| NMDA | 6 | NR3B | *GRIN3B* | 19p13.3 |

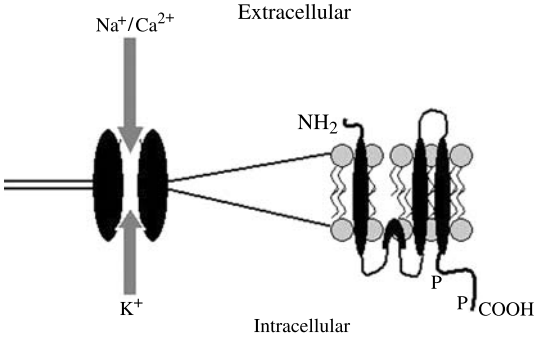


**Fig. 1.** Ionotropic glutamate receptor topology. The receptor (shown on left) is portrayed as a channel composed of heteromeric subunits. Each subunit (shown to the right) has four predicted transmembrane segments. However, only three actually span the membrane (TM1, TM3, and TM4). The second segment (TM2) constitutes a cytoplasmic-facing membrane reentrant loop involved in forming the channel pore of the functional receptor. The area of the intracellular domain that is phosphorylated is indicated (*P*). Not shown are post-transcriptional (editing) or post-translational features (glycosylation)

The predicted topology of mammalian IGRs is shared across the receptor families, and is illustrated for the NMDA receptor family in Fig. 1. Each subunit has four predicted transmembrane segments. However, only three actually span the membrane (TM1, TM3, and TM4). The second segment (TM2) constitutes a cytoplasmic-facing membrane reentrant loop involved in forming the channel pore of the functional receptor. These segments are highly conserved in humans (particularly TM3) and play critical roles in channel function.

Parsimony analysis of amino acid sequences groups the IGRs into three distinct clades, which correspond to the three defining ligands: AMPA, KA and NMDA (Fig. 2). Thus, ligand specificity parallels phylogeny.

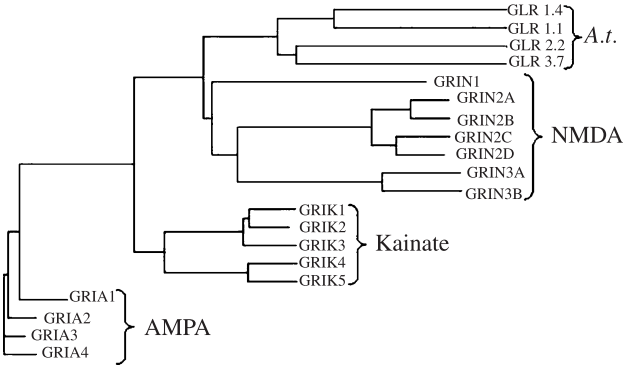


**Fig. 2.** Human and *Arabidopsis thaliana* clades. Phylogenetic tree calculation was based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm of Saitou and Nei (1987). The ClustalW program allows progressive multiple alignments by performing pairwise comparisons of sequences and sequence alignments with sequence weighting and gap penalties (Thompson et al., 1994)

Sequence conservation among the IGR gene has been used to discover new glutamate receptors in other eukaryotes, including plants. The availability of expressed sequence tag databases and the *Arabidopsis thaliana* genome project led to the discovery of three glutamate receptor-like gene families with 20 genes as we found by BLAST searches using known *Arabidopsis* glutamate receptor-like receptor (GLR) cDNAs as the query sequence. Comparison of the human sequences to *Arabidopsis* glutamate receptor sequences representative of three known *Arabidopsis* clades (*At*GLR1.4, *At*GLR2.2, and *At*GLR 3.7) enables ''rooting'' of the ionotropic glutamate receptor cladogram. This comparison revealed moderate similarity between the mammalian and plant sequences. Using this approach, we also showed that human NMDA receptor subunit (*GRIN1*) had the greatest overall similarity to *Arabidopsis* GLR compared to other IGR family members (Fig. 2). In order to test if phylogenetic tree estimation algorithms model protein structure, which in turn, mediates protein function, we performed individual sequence comparisons among IGR subtypes. By creating similarity groups (>50%, 41%–50%, 30%–40%, and <30%), we produced a visual representation of overall sequence similarity among individual IGR family members (Fig. 3, Panel A). These results suggested that IGRs encoded by *GRIA*, *GRIK*, and *GRIN3* (particularly *GRIN3B*) were more closely related to the three representative *Arabidopsis* GLRs than initially appreciated. In this analysis, *GRIN2*, which encodes NR2 subunits, was clearly distinquished from other IGR family members by having the least similarity with *Arabidopsis* sequences or any of the other IGR family members. We also produced a sequence comparison of IGR family members focusing on regions defining the proposed ligand binding, channel pore, and membrane spanning domains (Fig. 3, Panel B). Sequence differences between the three functional classes were enhanced, showing close similarity only among immediate family members along the diagonal (Fig. 3, Panel B), supporting the idea that these structural features are functionally constrained. The exception of the AMPA and kainate receptor subunits encoded by *GRIA* and *GRIK*, respectively, which showed higher sequence similarity for this important functional region of the receptor (Fig. 3, Panel B). Overall, the results suggest that while *GRIA* and *GRIK* showed the highest similarity to *Arabidopsis* GLR sequences (>50%), they also shared the highest level of similarity by function. NMDA receptors subclasses encoded by *GRIN1* and *GRIN3* still shared greater than 40% sequence similarity with *Arabidopsis* sequences, suggesting that plant GLRs

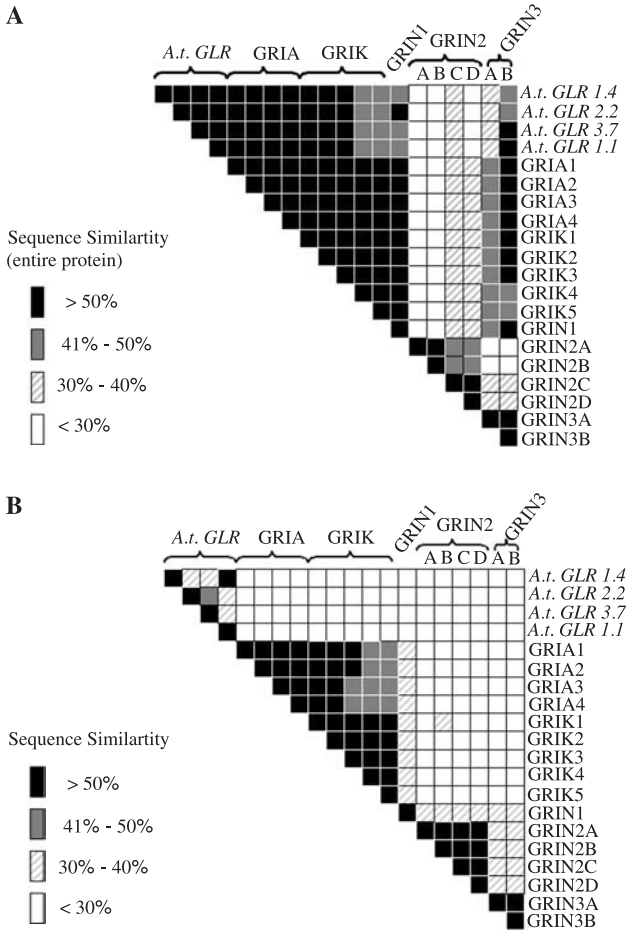


**Fig. 3.** Sequence similarities between human ionotropic glutamate receptors. Panel **A** shows the overall sequence similarity among individual IGRs. Panel **B** shows the similarity in sequences between IGRs based on the proposed ligand binding, channel pore, and membrane spanning domains

share some functional qualities with human NMDA receptors. In this context, it is useful to note that NR1 and NR3 differ from other NMDA receptor subunits (NR2) in that glycine is the primary ligand for NR1 (Kuryatov et al., 1994) that may act as an excitatory neurotransmitter when NR1 and NR3 subunits are co-expressed (Chatterton et al., 2002). In contrast, NR2 receptors have glutamate binding domains (Laube et al., 1997). This difference in functionality was mirrored by sequence divergence in the ligand binding, channel pore, and membrane spanning domains.

### *Chromosomal localization and conserved synteny*

The ionotopic glutamate receptor genes are dispersed over many chromosomes (Table 1). In humans, two pairs of genes are localized to the same chromosomal region. *GRIA4 and GRIK4* are located on the long arm of chromosome 11. However, they are separated by several million base pairs. Also, *GRIK5* and *GRIN2D* are located

**Table 2.** Comparison of human and murine glutamate receptor genes and their chromosomal locations

| Receptor | Subunit* | Gene* | Chromosome | |
|---|---|---|---|---|
| | | | Human | Murine |
| AMPA | GluR4 | *GRIA4* | 11q22-23 | 3F3 |
| KA | KA1 | *GRIK4* | 11q22.3 | 9A5.1 |
| KA | KA-2 | *GRIK5* | 19q13.2 | 7A3 |
| NMDA | NR2D | GRIN2D | 19q13.1qter | 7B27B3 |
| NMDA | NR1 | *GRIN1* | 9q34.3 | 2A3 |
| NMDA | NR3B | GRIN3B | 9p13.3 | 4B |

* Human gene name and subunit classifications are used in describing species comparisons

relatively close to each other on the long arm of chromosome 19. Syntenic regions have been identified in mouse. However, the synteny of these two pairs of human IGR genes is not conserved in the mouse (Table 2). Therefore, while functional significance of these chromosomal colocalizations in the human cannot be ruled out, it is unlikely that these lead to coordinate regulation of gene expression. Because of conserved synteny (as shown in Table 2), genes in the immediate neighborhood of human IGRs are also in the immediate neighborhood of mouse IGRs. This would enable detection in the mouse of *cis*-effects on expression of IGRs and neighboring genes, if such effects are present.

### *Sequence variant discovery using dHPLC and sequence analysis*

We have identified IGR sequence variation by resequencing technologies, including denaturing dHPLC for detection of new sequence variants and direct sequencing. We have also information-mined publicly available (dbSNP, Ensembl) and private (Celera Discovery System) databases. We initially selected three genes for this approach. We chose the NMDA receptor subunit genes *GRIN1*, *GRIN2B*, and *GRIN3* because of the central role of NMDA receptors in neurotransmission, neuronal plasticity, and neuronal viability, and because of the potential for linkage to clinically important phenotypes including addictions, anxiety/dysphoria, post-brain injury responses, pain perception, schizophrenia, and epilepsy. It has long been appreciated that alcohol acts directly on NMDA receptors and that the NMDA receptor antagonists such as ketamine and MK801 produce schizophrenia-like symptoms and exacerbate symptoms in schizophrenia patients. Here we report on *GRIN1* and *GRIN2B* data based on genome informatics plus sequence variation detection across the entire coding sequences of these genes by

**Table 3.** Ionotropic glutamate receptor missense variants and their frequencies

| Gene/variant | Minor allele frequency | Source |
|---|---|---|
| *GRIN1* | | |
| Ala211Val | 0.003 | LNG[†] |
| Cys744Tyr | 0.003 | LNG |
| Asp765Tyr | 0.003 | LNG |
| Ser864Gly | nd* | computational |
| *GRIN2A* | | |
| Asn3Asp | MONOMORPHIC | computational |
| Glu196Gln | [‡] | computational |
| Arg205Lys | [‡] | computational |
| Asn380Thr | [‡] | computational |
| *GRIN2B* | | |
| Ser116Thr | 0.003 | LNG |
| Leu120Ile | 0.003 | LNG |
| Thr275Ala | 0.02 | LNG, CONFIRMED |
| Gly603Ser | MONOMORPHIC | computational |
| Ile1167Val | MONOMORPHIC | computational |
| Gly1168Ser | MONOMORPHIC | computational |
| *GRIN2C* | | |
| Trp1180Arg | nd | computational |
| Ala1266Thr | MONOMORPHIC | computational |
| Ser1212Arg | 0.01 | LNG, CONFIRMED |
| *GRIN3A* | | |
| Met362Val | nd | computational |
| Arg487Gly | 0.18 | LNG, CONFIRMED |
| Asp835Asn | 0.13 | LNG, CONFIRMED |
| Arg1041Gln | 0.10 | LNG, CONFIRMED |
| *GRIN3B* | | |
| Thr157Met | 0.43 | LNG, CONFIRMED |
| Arg404Trp | nd | computational |
| Thr577Met | nd | computational |
| Ala845Thr | 0.19 | LNG, CONFIRMED |
| *GRIA2* | | |
| Glu241Gly | MONOMORPHIC | computational |
| *GRIA3* | | |
| Leu525Phe | MONOMORPHIC | computational |
| Asn786Ser | MONOMORPHIC | computational |
| Ile790Leu | MONOMORPHIC | computational |
| *GRIK1* | | |
| Val328Ile | nd | computational |
| Val445Ala | nd | computational |
| Ser475Leu | nd | computational |
| Val757Ile | nd | computational |
| Ala870Val | MONOMORPHIC | computational |
| Leu902Ser | 0.02 | LNG, CONFIRMED |
| *GRIK2* | | |
| Ile867Met | nd | computational |
| *GRIK4* | | |
| Ser700Gly | nd | computational |
| *GRIK5* | | |
| Pro140His | 0.07 | LNG, CONFIRMED |

* *nd*, not determined. [†] *LNG*, this study. [‡] *GRIN2A* variants are intronic, the codon change lies within IMP DH, transcribed from the complementary strand of GRIN2A, intron 3. *CONFIRMED* variants have a frequency of $\geq 0.01$ while *MONOMORPHIC* variants failed to replicate by direct sequencing of PCR products from 100 unrelated individuals (200 chromosomes)

dHPLC and DNA sequence analysis. *GRIN2B* expression is important developmentally and *GRIN1* must be co-expressed to produce fully functional receptors. Sequence variants in other IGRs were obtained using the genome informatics approach.

A total of 480 unrelated individuals were resequenced across *GRIN1* and *GRIN2B* coding exons using dHPLC as described previously (Rudolph et al., 2002). The screening sample was ethnically diverse, representing Caucasians, Asians, African Americans, Native Americans, and a clinically diverse group, representing schizophrenia, obsessive compulsive disorder, alcoholism, depression, anorexia nervosa, and normal controls. A total of 2.9 Mb of genomic sequence was screened for novel sequence variants in the sample, which amounted to approximately three kb per individual per gene. Individual samples having variants detected by dHPLC were amplfied by PCR using the same primers and the products subjected to direct DNA sequence analysis. Both strands were sequenced, identifying six non-synonymous *GRIN1* and *GRIN2B* substitutions (Table 3) plus one synonymous change. All were single nucleotide changes. The allele frequencies provided in Table 3 are based on the deliberately ethnically diverse screening sample of 480 individuals. Using a frequency of $\geq 1\%$, these single nucleotide variants are defined as polymorphic and thus can be classified as single nucleotide polymorphisms (SNPs). The exon 17 Cys744Tyr substitution was seen in only a single individual. All of the non-synonomous variants discovered in *GRIN1* and *GRIN2B* were novel, and not represented in public or commercially available databases. The number of variant sites normalized for the number of chromosomes and base pairs screened ($\theta$) for the sequence changes across the coding exons of *GRIN1* and *GRIN2B*, and based on non-synonymous substitutions, was $2.1 \times 10^{-5}$. The average heterozygosity per site ($\pi$) for non-synonymous substitutions was $1.0 \times 10^{-6}$.

Also listed in Table 3 are other non-synonymous substitutions at IGRs identified by a genome informatics approach. These data were compiled from translated exons from different IGR genes. Nucleotide sequence variants were detected by comparing available sequences via multiple sequence alignments and from entries in sequence variant databases. Of 16 IGRs examined using dbSNP BUILD 113 (indexed April 14, 2003) and Celera RefSNP Release R4.0 (indexed February 21, 2003), ten contained at least one non-synonymous SNP entry, but not G*RIN1* or *GRIN2B*. There were also no reported non-synonymous SNPs in *GRIA1*, *GRIA4*, *GRIK3*, and *GRIN2D*. As seen by the entries under the ''minor allele frequency'' column in Table 3, the vast majority of the

SNPs reported in the databases have not been validated by genotyping multiple ethnically-defined populations. Exceptions were *GRIK1* Leu902Ser (minor allele frequency 0.02), *GRIK5* Pro140His (minor allele frequency 0.07), *GRIN3B* Thr157Met (minor allele frequency 0.43), and *GRIN3B* Ala845Thr (minor allele frequency 0.19). Clearly, the validity of several of these sequence variants must be confirmed and their genotype frequencies determined in different human populations. Furthermore, it is clear from comparing our *GRIN1* and *GRIN2B* resequencing results to the genome informatics approach that many non-synonymous substitution polymorphisms at other IGRs are not represented in the databases or in publicly available sequence. It should also be noted that some ''computational'' SNPs, those sequence variants obtained through information mining of sequence databases, failed to replicate upon sequencing and were termed ''monomorphic'' (Table 3).

### *Functional analysis of* GRIN1 *Tyr744 variant*

The data in Fig. 4 show the effect of increasing NMDA concentrations on viability of human embryonic kidney (HEK 293) cells cotransfected with rat cDNAs for either NR1Cys744 and NR2B-GFP or NR1Tyr744 and NR2B-GFP. The effect of NMDA on cotransfected cells was normalized for transfection efficiency using an NR2B-Green Fluorescent Protein (GFP) fusion protein expressing plasmid. Because NR2B cDNAs were expressed as GFP fusion proteins, they also provided a means to monitor cell viability following exposure to increasing concentrations of NMDA. Untransfected HEK cell viability was not affected by NMDA because they do not produce functional NMDA receptors (data not shown). However, at higher concentrations of NMDA (1000 $\mu$M), NR1Tyr744-expressing cells showed increased survival over NR1Cys744-expressing cells, suggesting that the cells expressing the Tyr744 variant may be less sensitive to NMDA-mediated excitotoxicity.

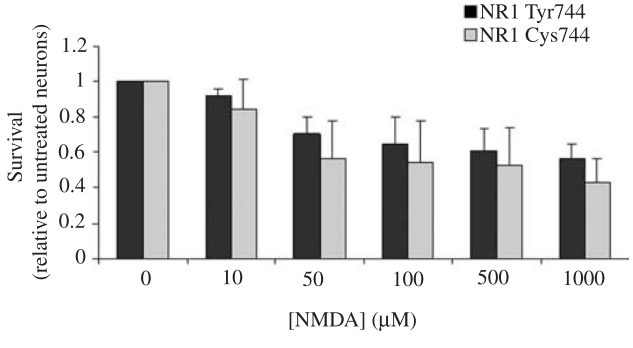


**Fig. 4.** Functional analysis of *GRIN1* Tyr744 variant. Human embryonic kidney (HEK 293) cells were cotransfected with rat cDNAs for either NR1Cys744 and NR2B-GFP or NR1Tyr744 and NR2B-GFP. Transfected cells were treated with increasing concentrations of NMDA. Cell viability was measured by counting GFP-positive cells and survival determined relative to untreated neurons as described in Materials and methods

## Discussion

The IGR genes are well conserved phylogenetically. There is a high degree of amino sequence identity among IGR gene family members within humans. Three clades were determined that corresponds to the three original groups defined by pharmacological and electrophysiological parameters. Conservation of ligand-receptor interactions and channel activity may be a major factor maintaining sequence conservation among the three clades.

Of the non-synonymous SNPs discovered in IGR genes, the vast majority of these candidate functional variants were rare. Others have screened IGR genes for variants and also found a low rate of non-synonymous substitutions (Cargill et al., 1999; Ohtsuki et al., 2001; Rice et al., 2001; Williams et al., 2002). Taken together, these data indicate that among the IGR genes, the *GRIN* family may be under the strongest selection. This is also seen in the high degree of amino acid sequence similarity between human family members and those of other species, at least for the ligand binding, channel pore, and membrane spanning domains. Because other factors influence IGR activity, such as receptor subunit composition and subunit stoichiometry, it is likely that sequence variants influencing transcription and messenger RNA stability may profoundly alter receptor function. These sequence variants may be more common. Thus, the contribution of a particular sequence variant may have a variable influence, depending on the neuronal population and the environmental conditions influencing the expression of the gene. However, our data showing that cells expressing the Tyr744 NR1 variant may be less sensitive to NMDA-mediated excitotoxicity, supports the idea that subtle changes in function can potentially have a large effect on phenotype. Thus, the task of relating the role of sequence variation to behavior, although potentially difficult, is not impossible.

There are examples of functional variants that have recently been linked both to behavioral differences and to intermediate phenotypes in the brain, suggesting a pathway by which functional variants at IGRs can be linked to an etiologically complex phenotype (Hariri et al., 2002; Egan et al., 2003; Zubieta et al., 2003). Identifying ''intermediate phenotypes'' in the CNS can provide greater power to

detect effect of the candidate gene on phenotype. A comprehensive discussion of this issue is beyond the scope of this report. However, one phenotype that can be used to measure ''plastic'' responses in human studies would be positron emission tomography (PET) during cognitive learning or acquisition of visuomoter skill. Measurements using $^{15}O$ PET could be performed on subjects where subjects have already suffered prior specific damage, a condition that may be applied to genetic association studies. Such a study population could be composed of traumatically brain-injured or stroke patients, which may have some injury-induced limitations in functional plasticity.

Developing methods to facilitate functional neuroplasticity in the injured human brain is ongoing (Levin and Scheller, 1997; Wishart et al., 2002; Newberg and Alavi, 2003; Levin, 2003). A study could take the form where subjects were selected based on confirmed lesions in structures that are required to perform the task (cerebellum, basal ganglia, motor cortex, parietal cortex and prefrontal cortex) as well as in uninjured controls, and in those with lesions in structures not viewed as relevant to the performance of either task (i.e.: temporal lobes). The role of IGR genotype could be evaluated in these subjects versus the degree and rate of cortical plasticity in patients, compared to matched controls. The influence of genotype on function in different brain regions could possibly be used to predict learning during the performance of the different tasks.

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