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Genome-wide gene expression profiling in GluR1 knockout mice: Key role of the calcium signaling pathway in glutamatergically-mediated hippocampal transmission

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Abstract

α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors convey fast synaptic transmission in the central nervous system (CNS) and mediate various forms of hippocampal plasticity. Disruption of glutamate receptor type 1 (GluR1), a member of the AMPA receptor family, causes synaptic alterations and learning/memory deficits in mice. To gain mechanistic insight into the synaptic and behavioral changes associated with GluR1 deletion, hippocampal genome-wide expression profiling was conducted using groups of GluR1 knockout (KO) mice and their wild-type littermates. Regulation of 38 genes was found to be altered more than 30% ($p < 0.01$, $n=8$) and seven of these genes were studied with additional quantitative experiments. A large portion of the altered genes encoded molecules involved in calcium signaling, including calcium channel components, calcium binding proteins, and calcium-calmodulin-dependent protein kinase II subunit. At the protein level, we further evaluated some genes in the calcium pathway that were altered in GluR1 KO mice. Protein levels of two key molecules in the calcium pathway—glutamate receptor, ionotropic, N-methyl-D-aspartate (NMDAR)-1 and calcium/calmodulin-dependent protein kinase II alpha (CAMK2A), showed similar changes to those observed in mRNA levels. These findings raise the possibility that calcium signaling and other plasticity molecules may contribute to the hippocampal plasticity and behavioral deficits observed in GluR1 KO mice.

Keywords

AMPA; receptor; microarray; neuron; synaptic

Introduction

Ionotropic glutamate receptors mediate most excitatory synaptic transmission in the mammalian central nervous system (CNS). Three major classes of these receptors are

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expressed throughout the CNS. They are categorized on the basis of pharmacological criteria and primary sequence similarity as α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors. These receptors are critically involved in activity-dependent, long-term changes in synaptic strength and, therefore, are central to processes thought to underlie a variety of long-term plasticity events in the hippocampus.

AMPA receptors (AMPA receptors) comprise four subunits—GluR1, GluR2, GluR3, and GluR4 (alternatively called GluRA-D)—that combine to form tetramers. The subunit composition of AMPARs varies with cell type, development, and brain region (Seeburg *et al.*, 2001). A number of studies indicate that the synaptic delivery of different AMPAR subunit combinations is governed by molecular rules encoded in the intracellular C-terminals of the receptor subunits (Malinow, 2003). Phosphorylation of serines 831 and 845 (S831 and S845) in the GluR1 subunit by calcium-calmodulin-dependent protein kinase II (CaMKII) and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), respectively, regulates the ion channel properties and synaptic trafficking of GluR1-containing AMPARs during hippocampal long-term potentiation (LTP) (Barria *et al.*, 1997; Derkach *et al.*, 1999; Esteban, 2003). Dephosphorylation of the GluR1 subunit at its PKA site by protein phosphatases (e.g., calcineurin and PP1) targets GluR1 for recycling endosomes during long-term depression (LTD), while re-phosphorylation by PKA may target the receptor for reinsertion into the membrane (Banke *et al.*, 2000).

In addition, mitochondria are also able to modulate synaptic plasticity through cleavage of GluR1 mediated by activation of caspase-3. Exposure of radio-labeled GluR1 to recombinant caspase-3 results in GluR1 cleavage, demonstrating that this glutamate receptor protein is a direct substrate of caspase-3, which can be up-regulated by cytochrome C release from mitochondria (Lu *et al.*, 2002). In GluR1 knockout (KO) mice, initiation or completion of synaptic AMPAR exchange is compromised (McCormack *et al.*, 2006). Gene-targeted mice lacking the AMPAR subunit GluR1 have deficits in hippocampal CA3-CA1 LTP (Zamanillo *et al.*, 1999), suggesting that GluR1 is essential to several forms of hippocampal plasticity.

Several studies have evaluated the role of GluR1 in learning, memory, and behavioral plasticity using mutant mice. Furthermore, a variety of behavioral tests have shown that GluR1-deficient mice display abnormalities in several behaviors, notably those involving learning/memory (Reisel *et al.*, 2002; Mead & Stephens, 2003; Bannerman *et al.*, 2004; Schmitt *et al.*, 2005; Wiedholz *et al.*, 2008). Although these findings point to a critical role for GluR1 in hippocampal behavioral plasticity, the question of the precise downstream gene expression pathways that may underlie these deficits remains. In an effort to identify additional, and perhaps novel, targets underlying these synaptic and behavioral changes in GluR1 KO mice, we conducted the present series of studies to clarify changes associated with GluR1 ablation at the whole genome transcriptional level.

Materials and Methods

Animal study

The breeding of male C57BL/6 GluR1 (GRIA1 or GluR-A) KO mice (GluR1^{-/-}) has been previously described (Zamanillo *et al.*, 1999). The founding knockout (KO) mice were provided by Dr. Rolf Sprengel (Max Planck Institute for Medical Research, Heidelberg, Germany). Homozygous (GluR1^{-/-}), heterozygous (GluR1^{+/-}), and wild-type (WT) (GluR1^{+/+}) mice were maintained in the animal facility of the National Institute of Alcohol Abuse and Alcoholism (NIAAA-NIH). Mice were housed three to four per cage in a 12-hour light/dark cycle and had free access to water and food. All animal treatments, procedures,

and care were approved by the NIAAA Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals* (ISBN 0-309-05377-3). At sacrifice, the average weights of the mice (in grams, mean $\pm$ SD) were: GluR1 $^{-/-}$: 28.16 $\pm$ 3.39; GluR1 $+/-$: 27.04 $\pm$ 3.92; and GluR1 $+/+$: 27.21 $\pm$ 3.91.

RNA extraction and microarray slide hybridization

RNA extraction from the hippocampus was conducted using RNeasy Midi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA concentrations were determined using NanoDrop spectrophotometer. The quality of RNA was evaluated through visual inspection of electrophoresis results plus densitometry analysis of the rRNA 28S/18S (optical density ratio of 28S and 18S rRNA bands was set to levels greater than 1.5 in our study). Mouse 70mer oligonucleotide microarray slides were provided by the National Human Genome Research Institute (NHGRI)/National Institute of Mental Health (NIMH)/National Institute of Neurological Disorders and Stroke (NINDS) Microarray Core Facility of the National Institutes of Health (NIH). Printed on the slide are 32,600 oligos that represent 25,152 unique Ensembl genes (<http://www.ensembl.org/>) or 24,729 RefSeq genes (<http://www.ncbi.nlm.nih.gov/RefSeq/>).

An indirect labeling system was adopted to generate a cDNA probe pool for microarray slide hybridization. There are two steps in the labeling process: the first is aminoallyl dUTP (aa-dUTP) incorporation in the reverse-transcribed cDNA synthesis, and the second is conjugating Cy dyes through the amine group of aa-dUTP. Briefly, 10 μ g total RNA were denatured together with 2 μ g anchor oligo-dT (the formula of anchor oligo-dT used in our study was 5'-T₂₀VN-3', V=G+A+C, N=A+C+G+T) at 70°C for five minutes. First strand cDNA synthesizing was catalyzed by 10U/ μ l SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with different reagents added as follows: 1XFirst Strand Buffer, which was diluted from the 5X stock, 0.1M DTT to a final concentration of 0.01M, dATP, dCTP, and dGTP to the final concentrations of 500 μ M, dTTP to a final concentration of 200 μ M, and aa dUTP to a final concentration of 300 μ M (all reagents obtained from (Sigma, St. Louis, MO, USA)). The total reaction volume was 30 μ l. After incubation at 42°C for two hours, the first strand cDNA was purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Purified and vacuum-spin dried cDNA was re-suspended in 5.0 μ l nuclease-free water, and mixed with 5.0 μ l Cy3 or Cy5 reactive dye (GE Healthcare/Amersham Biosciences, Piscataway, NJ, USA) dimethyl sulfoxide (DMSO) solution for coupling reaction. The mixture was incubated at room temperature for one hour and then purified with Qiaquick PCR purification kit as described for cDNA. Recovered Cy dye coupled cDNA was diluted to 220 μ l with 10 mM Tris-HCl (pH 8.5) solution, supplemented with 220 μ l 2Xhybridization buffer (Agilent Technologies, Santa Clara, CA, USA), mixed thoroughly, denatured at 95°C for five minutes and then put on ice. The prepared hybridization mixture was applied on microarray slide surface with Agilent gasket slide and then mounted in an Agilent hybridization chamber (Agilent Technologies, Santa Clara, CA, USA). Microarray slide hybridization was carried out at 60°C for 16~24 hours and rotated at about three cycles/minute.

Analyses based on both mathematical models and actual experiments support dye-swap as an effective method for quality control (Yang *et al.*, 2002). In addition, dye-swap also substantially affects the specificity of the results (Yao *et al.*, 2004). Therefore, in order to counterbalance the possible bias caused by two different Cy dyes, we alternately labeled the control and treatment samples with Cy3 and Cy5. In order to achieve better quality control, the procedure was replicated eight times for all three genotypes.

Image and statistical analysis

Microarray slides were scanned in ScanArray 5000 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Images were analyzed using IPLab with microarray extensions (Scanalytics, Fairfax, VA, USA). The mathematical core of the program was based on research at NIH regarding ratio-based quantitative microarray image analysis (Chen *et al.*, 2002).

Because it has been demonstrated that normalization is slide-dependent (Tseng *et al.*, 2001), we normalized the data for each slide separately. For biological studies investigating neuropharmacologic effects, we believe that consistency of effect is as important (if not more important) than simple fold changes. Indeed, a bioinformatics study demonstrated that error probabilities (as measured using *t*-test) can be used as important indicators of consistency (Bilke *et al.*, 2003). Furthermore, there is no general cut-off level of fold change as “positive”, as shown by some representative studies that used rodent brain tissues, and whose criteria were proven effective in the corresponding verification procedure and follow-up studies (McClung & Nestler, 2003; Newton *et al.*, 2003). Thus, we chose as a primary criterion a *t*-test value of $p < 0.01$. Furthermore, we believe that a $\pm 30\%$ -fold change is tenable as a selection standard. Thus, our criterion for picking out positive targets—combining the fold change with the P value originating from eight tests—is relatively stringent. This may cause the results to bias toward true positives at the cost of pseudonegatives.

Verification of array results

Quantitative real-time polymerase chain reaction (Q-RT-PCR) was adopted to verify the microarray results for seven selected genes. This process included two steps: reverse transcription and SYBR green based quantitative real-time PCR. The reverse transcription reaction was carried out using Omniscript reverse transcription kit (Qiagen, Valencia, CA, USA) (50 μ l reaction volume including: 1x reaction buffer, 500 μ M each dNTP, 1 μ M oligo-dT primer, 0.5U/ μ l RNase Inhibitor, 0.2U/ μ l Omniscript reverse transcriptase and 2 μ g total RNA). The mixture was incubated at 37°C for 60 minutes. The reverse transcription products were then diluted accordingly for standard curve and sample runs. Real-time PCR was conducted with SYBR Green Supermix Kit (Bio-Rad Laboratories, Hercules, CA, USA) in a 25 μ l reaction volume containing 50mM KCl, 20mM Tris-HCl, pH 8.4, 0.2mM each dNTP, 20 U/ml iTaq DNA polymerase, 6mM MgCl₂, SYBR Green I, 10nM fluorescein, 500nM each probe, and 2 μ l diluted reverse transcription products. The 50 μ l cDNA products from reverse transcription were first diluted with 1:5 and used as a starting solution, then, for a standard curve run, the solution was further serially diluted by 1, 1/2, 1/4, 1/8, and 1/16; for the sample run, the solution was diluted by 1/5. Reactions were carried out using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA); the accompanying software was used for data collection according to the manufacturer's instructions.

The reaction mixture was initially denatured at 95°C for three minutes then cycled 40 times. In each cycle, the mixture was kept at 95°C for 10 seconds plus 55°C for 45 seconds. The PCR reaction was supplemented by the melting curve analysis process. For this, the mixture was denatured at 95°C for one minute, annealed at 55°C for one minute, and then incubated for 10 seconds at each of the 80 continuous steps, with the temperature being increased by 0.5°C at each step (i.e. from an initial 55°C to a final 95°C in this procedure).

Clustering of Genes

Genes were clustered according to either the functional groups they belong to or the pathways in which they are included. Experimental data were imported into Microsoft

Excel. Visual Basic for Applications (VBA, Microsoft Corporation) was adopted as a programming tool to create custom programs for conditional sorting and statistical testing. Gene Expression Profile Analysis Suite (GEPAS, version 3.1), developed by Vaquerizas and colleagues (Vaquerizas *et al.*, 2005), was used to functionally cluster the regulated genes. This functional annotation analysis method is based on Gene Ontology (GO), which is probably the most successful of the initiatives for standardizing the nomenclature of gene function. GO analysis has been widely used in gene profiling studies in the neurosciences (Kabbaj *et al.*, 2004; Ryan *et al.*, 2006), and represents biological knowledge as a tree (more precisely, as a directed acyclic graph).

As an example, the calcium channel, voltage-dependent, alpha 1G subunit (CACNA1G) gene was up-regulated in GluR1^{-/-} mice as showed in Table 1. This gene can be annotated within different functional groups at different levels. When categorized from higher to lower levels, it can be classified as: ion transporter activity (GO:0015075)→cation transporter activity (GO:0008324)→cation channel activity (GO:0005261)→calcium channel activity (GO:0005262), etc. In this manner, the upper nodes represent more general concepts, so more genes can be included in each category, but the information is less distinguishable and precise. When the functional annotation tree traverses towards deeper levels, the definitions are more and more precise but fewer genes will be left in any given category. Because most functional genes can be categorized at different levels and/or into more than one group, multiple-level analyses may provide more concisely differentiated information. FatiGO plus, a functional annotation tool included in GEPAS, was used for pathway analysis of significantly regulated genes. In pathway analysis, the KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg>) pathway database was also consulted, so that each gene that appeared through program-based analysis could be verified manually for membership in the corresponding pathway(s).

Immunoblotting (Western blot)

Immunoblotting was conducted as previously described (Chen *et al.*, 1998), with modifications. Briefly, mouse hippocampal samples were suspended in ice-cold lysing buffer, which contained 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 150 mM NaCl, and 1 mM β -glycerophosphate. Immediately before use, the buffer was supplemented to the final concentrations of 5 mM DTT: 1% Phosphatase Inhibitor Cocktail I, 1% Phosphatase Inhibitor Cocktail II, and 10% Phosphatase Inhibitor Cocktail (Sigma, St. Louis, MO, USA). Samples were homogenized by sonication (setting 2.5, 1 second $\times$ 10 times, VirSonic ultrasonic cell disrupter (Virtis, Gardiner, NY, USA)), and then centrifuged (15 seconds at 14000 $\times$ g) to remove undissolvable debris. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

For immunoblotting, equal amounts of protein were loaded to 8-16% SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) and separated by electrophoresis. Proteins were then electrophoretically transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose was blocked with Tris Buffered Saline plus Tween-20 (TBST), 5% nonfat dry milk. The antibodies for CACNA1G (Sigma, St. Louis, MO, USA), glutamate receptor, ionotropic, NMDA1 (zeta 1) (NMDAR1) (Cell Signaling, Danvers, MA, USA), myosin light chain kinase (MLCK) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and calcium/calmodulin-dependent protein kinase II alpha (CAMK2A) (GenWay Biotech, San Diego, CA, USA) were diluted according to the manufacturer's recommendations and, if necessary, further adjusted according to initial test results. The secondary antibodies were horseradish peroxidase (HRP) conjugated anti-rabbit antibody. Antibody for β -actin (Cell Signaling, Danvers, MA, USA) was applied for loading calibration, either simultaneously or after initial blotting. The former method was used if the membrane could be divided into two

parts, and each part covered the molecular range of the target protein or of β -actin separately with enough margins; the latter method was used if the molecular ranges of the target protein and β -actin were similar. In the latter situation, Re-Blot Plus Western Blot Recycling Kit (Chemicon, Billerica, MA, USA) was used according to the manufacturer's instructions. The immunocomplex was detected with an ECL plus kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of the immunoblots was performed by Syngene G:BOX Gel Documentation and Analysis System (Syngene, Cambridge, UK).

Results

Common and significant hippocampal gene expression changes in GluR1^{+/-} and GluR1^{-/-} mice: initial findings from microarray experiments

Genome-wide gene expression profiling was carried out to reveal differences in hippocampal gene expression between GluR1^{+/+}, GluR1^{+/-}, and GluR1^{-/-} mice. Microarray data showed that hippocampal expression of 21 genes was increased, and hippocampal expression of 17 genes was decreased in both GluR1^{+/-} and GluR1^{-/-} compared to GluR1^{+/+}, based on our criteria (30%-fold change plus *t*-test *p*-value <0.01) (Table 1). These changes appeared to be gene-dose dependent (Table 1). Expression of 28 additional genes occurred only in GluR1^{-/-} mice (Supporting Table S1).

The following quality control methods for the comparison were incorporated into the investigation: 1) the comparison was repeated eight times (i.e. one GluR1^{+/-} mouse or one GluR1^{-/-} mouse vs. one WT mouse separately without sample pooling; this method ensured higher specificity at the cost of more fluctuation); 2) Cy-3 and Cy-5 dyes were swapped when labeling KO and control mice in order to balance the possible bias caused by different dyes; and 3) because the comparisons were conducted through two-channel paired hybridization, Student's *t*-tests were used to test statistical significance. As noted previously, a 30%-fold change is high in such investigations, because it was frequently observed that very high gene expression level changes in the hippocampus happened in some cell groups or cell layers; thus, when the whole hippocampus was homogenized for analysis, the amplitude of change was drastically lowered (Newton *et al.*, 2003).

Validation of selected microarray findings with quantitative real-time PCR

Seven genes were selected for further validation using quantitative PCR evaluation (Supporting Table S2). These genes were chosen based on the following criteria: 1) they were altered in either an up- or down-direction; 2) they belonged to different categories; 3) the function of the gene was known to relate to synaptic function or behavioral plasticity; and 4) resource constraints.

With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control, hippocampal expression levels of GAPDH did not differ between GluR1^{+/+}, GluR1^{+/-}, and GluR1^{-/-} mice (data not shown). The samples first went through a gradient standard curve run; samples for comparison were then diluted to the same proper concentration (within the linear range of the standard curve) and went through the sample run together. Expression levels were calculated according to the standard curve (for representative data, see Supporting Figure S1). Because the accuracy of SYBR green-based Q-RT-PCR depends on the specificity of the PCR reaction, agarose gel electrophoresis was carried out to visually evaluate the primers; the results supported the specificity of the primers (Supporting Figure S2). Consistent with the microarray results (Table 1), hippocampal expression levels of CACNA1G, calcium binding protein 4 (CABP4), glutamate receptor, ionotropic, kainate (NMDAR1), and glutamate receptor, ionotropic, kainate 2 (GRIK2) were significantly higher in GluR1^{+/-} and GluR1^{-/-} mice, and the expression levels were gene-dose

dependent (Figure 1). Similarly consistent with the microarray results (Table 1), hippocampal expression levels of MLCK, CAMK2A, and S100 calcium binding protein A11 (calizzarin) (S100A11) were significantly lower in GluR1 +/- and GluR1 -/- mice, although the gene-dose effects were less clear (Figure 1).

Calcium handling genes emerged as the most frequently differentially expressed genes in the hippocampus between GluR1 +/- and GluR1 -/- mice

Given the consistency between our microarray and Q-RT-PCR data (Table 1, Figure 1), we used the microarray data to further explore the gene expression changes associated with GluR1 ablation. The accession numbers of 38 altered genes (Table 1) were entered into GEPAS, and the annotations were carried out at all seven subcategory levels (level 3 to level 9). The annotation analysis showed that altered genes were much more frequent in the calcium handling gene group; these included calcium binding, calcium transporter, voltage-gated calcium channel activity, calcium channel activity, calcium transporter activity, voltage-gated calcium activity, calcium-activated potassium channel activity, and calcium-transporting ATPase activity (Supporting Figure S3).

FatiGO plus, a functional interpretation tool included in GEPAS, was used for pathway analysis. In this analysis, all pathways from the KEGG pathway database (<http://www.genome.jp/kegg/pathway.html>) and all differentially expressed genes (Table 1) were included. The program tests each of the input genes for membership in every node of the different pathways. Pathway hits are recorded with the corresponding gene. In our analysis, the 38 altered genes hit 11 pathways (Table 1). The most frequently hit pathway was the “calcium signaling pathway”, which was hit by six genes: 1) calcium channel, voltage-dependent, L type, alpha 1S subunit (M57976); 2) voltage-dependent alpha-1 E calcium channel (L29346); 3) calcium channel, voltage-dependent, T type, alpha 1G subunit (AJ012569); 4) glutamate receptor, ionotropic, NMDA1 (D10028); 5) sarco/endoplasmic reticulum calcium-ATPase, SERCA3c isoform (Y15736); and 6) calcium/calmodulin-dependent protein kinase II alpha (X87142) (Supporting Table S3).

Further checking of the pathway members yielded two more genes that could be manually added: MLCK (AF335470) and calcium channel, voltage-dependent, alpha 2/delta subunit 2 (AF247139). The former is a downstream target of calcium in the pathway, and the latter is an auxiliary subunit of all three subfamilies of voltage-gated calcium channels. Both were put into the calcium signaling pathway group. Among the 28 genes that were altered only in GluR1 -/- mice, two genes—neurotensin receptor (AB017027) and PTK2 protein tyrosine kinase 2 beta (NM_172498)—showed up under the calcium signaling pathway (Figure 2).

Similar to the mRNA results, NMDAR1 and CAMK2A showed significant protein level changes

Using Western blot analysis, protein levels of four genes were evaluated: CACNA1G, NMDAR1, MLCK, and CAMK2A. Levels were altered in four of them, although only two of these results were statistically significant. Antibodies for the remaining members of the pathway altered in GluR1 KO mice were either unavailable or did not work in our tests. Results showed that protein levels of NMDAR1 ($P=0.036$) were significantly up-regulated, and protein levels of CAMK2A ($P=0.020$) were significantly down-regulated. CACNA1G showed a trend towards increase and MLCK showed a trend towards decrease, but neither of these trends reached statistical significance (Figure 3).

Discussion

Genome-wide gene expression profiling in the hippocampus revealed that GluR1 ablation was associated with a range of gene expression changes, although only some of these changes were further confirmed by other methods. In addition, we found that a cluster of these changes occurred in the calcium handling and signaling group (Supporting Table S3, Supporting Figure S3). Intriguingly, the “on” reactions of calcium signaling appear to have been activated through up-regulation of cell membrane receptors and calcium channels; conversely, calcium is pumped back into endoplasmic/sarcoplasmic reticulum (ER/SR) through up-regulation of intracellular sarco/endoplasmic reticulum calcium-ATPase (SERCA), making it less effective. We also found that several effect molecules were down-regulated, thus suggesting that the “off” reactions of calcium signaling were also likely to be activated (Figure 2). This seemingly contradictory regulation may shed new light on the delicate manner through which AMPARs regulate cellular calcium signaling cascades.

One of the key features of the calcium signaling pathway is that the calcium transient has two functions. In addition to activating cellular responses, it also functions as part of the feedback mechanism that regulates the transcriptional events responsible for maintaining signal stability. This calcium-dependant self-stability regulation might play a central role in the compensatory mechanisms that enable cells to adapt to modifications of their calcium signaling system (Berridge *et al.*, 2003). In light of this major feature of the calcium signaling pathway, we propose the following hypothesis: in GluR1^{-/-} mice, cellular calcium signals are low, and membrane calcium permeable receptors and calcium channels increase to compensate for this state through feedback mechanisms. In addition, many downstream effectors of GluR1 are regulated through the calcium cascade, and a significant part of those downstream effectors are involved in neuroplasticity regulation. Thus, it appears that the calcium signaling pathway may play a central role in GluR1-induced neuroplasticity.

The complexity of the AMPA system and its wide range of regulatory roles complicate our understanding of its underlying molecular mechanisms and their role in regulating neuroplasticity. This study of GluR1 deficient rodents provides an important clue that the calcium signaling pathway may play a key role in the way that AMPA regulates neuroplasticity. The original study by Zamanillo and colleagues (Zamanillo *et al.*, 1999) showed that LTP was absent in the CA3 to CA1 synapses of GluR1^{-/-} mice, suggesting the importance of AMPARs for hippocampal synaptic plasticity. Furthermore, it appears that postsynaptic elevations in calcium and calcium-dependent protein kinases are required to establish LTP, and that AMPARs are a likely target of these kinases (Barria *et al.*, 1997).

Evaluation at the protein level provided further evidence highlighting the crucial importance of GluR1 in regulating synaptic plasticity. For those calcium inflow enhancing proteins on the cell membrane, NMDAR1 was up-regulated in GluR1 KO mice. NMDAR1 itself is a key regulator of synaptic plasticity, especially in the hippocampal CA1 region (Tonegawa *et al.*, 1996). CaMKII consists of four distinct but highly homologous chains (α , β , γ , and δ) (Si *et al.*, 2007). The α chain of CaMKII, also known as CAMK2A, plays a very important role in controlling neuronal excitability, either by regulating excitatory neuronal transmission (Liu & Jones, 1997), or by enhancing gamma-aminobutyric acid (GABA) synaptic response (Wang *et al.*, 1995). Notably, CAMK2A, like NMDAR1, also regulates synaptic plasticity in the hippocampal CA1 region (Hinds *et al.*, 1998); for instance, a deletion in the CAMK2A gene resulted in impaired LTP and LTD in the hippocampal CA1 region as well as spatial learning deficits (Silva *et al.*, 1992a; Silva *et al.*, 1992b). Interestingly, studies have shown that there are corresponding relationships between GluR1, NMDAR1, and CAMKII in regulating neuroplasticity (Zaitseva *et al.*, 2003; Zhao *et al.*, 2008). In this

study, we found that NMDAR1 levels were elevated, but that CAMK2A levels were decreased. This result reflected the changes that occurred when one member of a group of key regulators was absent. At the cell membrane level, NMDAR1 was up-regulated, probably to compensate for the lack of GluR1, but with the increasing calcium levels in cell plasma, CAMK2A levels decreased in order to balance the increased cell excitability. In this situation, calcium was the key factor linking upstream regulators (like NMDAR1) to downstream regulators (such as CAMK2A). With the knockout of GluR1—which is a key regulator of synaptic plasticity as well as calcium (Barria *et al.*, 1997; Derkach *et al.*, 1999; Ye *et al.*, 2006)—calcium-linked balance was damaged, suggesting that calcium may be the major molecular mechanism at play in GluR1-mediated neuroplasticity.

Taken together, these studies support the notion that GluR1 regulates neuroplasticity by regulating the calcium signaling pathway. While AMPARs were previously considered almost impermeable to calcium (Mayer & Westbrook, 1987), this view has since been challenged by the observation that non-NMDA glutamate receptors in a subset of cultured hippocampal neurons appear to be highly permeable to calcium (Iino *et al.*, 1990). These non-NMDARs were subsequently identified as AMPARs on the basis of their pharmacological profile (Ozawa & Iino, 1993), and several additional studies have since supported this key finding (Bochet *et al.*, 1994; Jonas & Burnashev, 1995). At excitatory synapses, the calcium influx through AMPARs is comparable to that through NMDARs at the resting membrane potential (Burnashev *et al.*, 1995; Koh *et al.*, 1995). The GluR1-containing complexes include GluR1/2, GluR1 homomers, and GluR1/3. GluR1/2 and GluR2/3 are the dominant forms of AMPAR channels and are calcium-impermeable; however, approximately 15-20% of GluR1 homo tetramers and a small percentage of the GluR1/3 expressed in the hippocampus are calcium permeable (Wenthold *et al.*, 1996). We would expect all the calcium-impermeable GluR1/2, calcium-permeable GluR1 homomers, and GluR1/3 to be reduced in GluR1 KO mice. In addition, because depolarization caused by activation of AMPARs is required to open NMDAR channels and other voltage dependent calcium channels, the reduction in calcium impermeable GluR1/2 receptors would eventually be expected to affect calcium influx in the neurons in response to various stimuli.

Another point of interest concerns voltage-dependent calcium channels (VDCCs). In the developing and mature CNS, VDCCs regulate the coupling of electrical excitation to gene expression, and modulate a wide variety of intracellular signaling pathways that, in turn, lead to neurite outgrowth, synaptogenesis, transmitter and hormone release, plasticity, and muscle contraction. Mammalian VDCCs are multimeric complexes of $\alpha 1$, β , $\alpha 2\delta$, and γ subunits; $\alpha 1$ is the only subunit that forms a calcium-conducting pore. The synaptic proteins syntaxin-1A, synaptotagmin, SNAP-25, and synaptobrevin interact with the calcium channel complex of the $\alpha 1$ subunit, coupling excitation to neurotransmitter release at nerve terminals (Burgess & Noebels, 1999). Our study showed that four calcium channel subunits were up-regulated in GluR1^{-/-} mice, although we could not show altered expression at the protein level. Notably, three of these four are $\alpha 1$ subunits. At least eight identified mammalian genes encode $\alpha 1$ subunits. These subunits are noticeable because, of the four kinds of VDCC subunits, they are the only ones that form a calcium conducting pore.

The current study is also associated with certain limitations. First, only some of the positive microarray findings in this study were validated. Second, the current data apply to gene expression, which has only been partially correlated with changes at the protein or functional level. Finally, although cluster analysis revealed that gene expression in the calcium signaling group was the most frequently affected, the altered expression of other functional groups cannot be fully ruled out.

Nevertheless, GluR1 receptor malfunction has recently been highlighted in studies of CNS disorders (Burgess & Noebels, 1999; Kwak & Weiss, 2006). To our knowledge, our study is the first to systematically evaluate genome-wide gene expression changes in GluR1 KO animals. Our findings have focused on calcium signaling pathways and the role they play in AMPAR-regulated hippocampal plasticity, especially the GluR1 receptors. Of those targets identified here, the roles of NMDAR1 and CAMK2A in synaptic and behavioral plasticity have been well-documented, while the roles of other calcium signaling/handling genes whose expression levels are altered in GluR1 $-/-$ mice are less clear. Further investigation of the molecules encoded by these genes may ultimately provide novel treatment targets for behavioral disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
CACNA1G	calcium channel, voltage-dependent, T type, alpha 1G subunit
CAMKII	calcium-calmodulin-dependent protein kinase II
CAMK2A	calcium/calmodulin-dependent protein kinase II alpha
CNS	central nervous system
GO	Gene Ontology
LTP	long-term potentiation
LTD	long-term depression
MLCK	myosin light chain kinase
NMDA	N-methyl-D-aspartate
PKA	protein kinase A

Figure 1.

Expression levels of seven genes of interest detected using SYBR green based quantitative real-time PCR. Hippocampal RNA samples were obtained from GluR1 +/+, GluR1 +/-, and GluR1 -/- mice. Q-RT-PCR analysis of seven genes was conducted. Data are mean±SEM from eight mice. *: P<0.05, **: P<0.01, ANOVA Tukey's HSD test. KO: knockout mice, WT: wild type.

Figure 2.

Pathway analysis of gene expression profile. Ten differentially expressed genes were found to be members of the calcium signaling pathway and are illustrated here according to KEGG's prototype. Rectangular boxes represent genes. Colored boxes represent one or more significantly changed genes; red boxes represent increased expression, and blue boxes represent decreased expression (GluR1 $-/-$ vs. WT). Additional outside rectangular boxes indicate that the result was also observed in GluR1 $+/-$ mice. Circled numbers are added beside highlighted boxes to index the corresponding genes that were significantly changed in the microarray analysis. These genes are: 1) calcium channel, voltage-dependent, L type, α 1S subunit; 2) voltage-dependent α -1 E calcium channel; 3) calcium channel, voltage-dependent, T type, α 1G subunit; 4) calcium channel, voltage-dependent, α 2/ δ subunit 2; 5) glutamate receptor, ionotropic, NMDA1; 6) neurotensin receptor; 7) sarco/endoplasmic reticulum calcium-ATPase, SERCA3c isoform; 8) myosin light chain kinase; 9) calcium/calmodulin-dependent protein kinase II α ; and 10) PTK2 protein tyrosine kinase 2 β .

Figure abbreviations (in alphabetical order): ADCY: adenylate cyclase; cADPR: cyclic ADP-ribose; ANT: adenine nucleotide translocator; CALM: calmodulin; CAMK: calcium/calmodulin-dependent protein kinase; cAMP: cyclic adenosine mono-phosphate; CD38: CD38 antigen; CaN: calcineurin; CaV: calcium channel, voltage-dependent; DAG: diglyceride; FAK: focal adhesion kinase; GPCR: G-protein coupled receptor; Gq: G-protein, Gq class; Gs: guanine nucleotide binding protein, stimulating; IP3: inositol 1,4,5-trisphosphate; IP3 3K: inositol 1,4,5-trisphosphate 3-kinase; IP3R: inositol 1,4,5-trisphosphate receptor; MLCK: myosin light chain kinase; NCX: Na $^{+}$ -calcium $^{+}$ exchanger; NOS: nitric oxide synthase; PDE: phosphodiesterase; PHK: phosphorylase kinase; PKA: protein kinase A; PKC: protein kinase C; PLC: phospholipase C; PLN: phospholamban; PMCA: plasma membrane calcium ATPase; PPID: peptidylprolyl isomerase D; PTK: protein tyrosine kinase; ROC: receptor-operated channel; RYR: ryanodine receptor; SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase; SOC: store-operated channel; TnC: troponin C; VDAC: voltage-dependent anion channel (*: not included in computer analysis result, manually added).

Figure 3.

Protein level evaluation of selected genes altered in GluR1 KO mice that are members of the calcium signaling pathway. Six GluR1 knockout mice and six WT mice were compared for hippocampal protein levels of a) NMDAR1; b) CACNA1G; c) CAMK2A; and d) MLCK. Protein levels were calibrated with the corresponding actin beta (ACTB) levels for loading amount. Bar graphs are in mean $\pm$ SEM format, *: $p < 0.05$, t -test, GluR1 KO vs. control.

Table 1

Genes with significantly altered expression levels in GluR1 KO mice

GeneBank#	Clone Title	Brief annotation	Ratio	t-test-P	Ratio	t-test-P
Up-regulated						
AJ012569	calcium channel, voltage-dependent, T type, alpha 1G subunit	Calcium channel	1.52	0.00183	1.34	0.00217
AF247139	calcium channel, voltage-dependent, alpha 2/delta subunit 2	Calcium channel	1.52	0.00774	1.41	0.00311
L29346	voltage-dependent alpha-1 E calcium channel	Calcium channel	2.27	0.00126	1.65	0.00160
M57976	calcium channel, voltage-dependent, L type, alpha 1S subunit	Calcium channel	1.48	0.00903	1.42	0.00115
AF022252	calcium-sensing receptor related protein 3	Calcium regulation	1.60	0.00354	1.51	0.00691
AY039218	calcium binding protein 4	Calcium regulation	1.62	0.00643	1.56	0.00918
AF419324	calcium binding protein 7	Calcium regulation	1.74	0.00145	1.42	0.00547
Y15736	sarco/endoplasmic reticulum Ca2+ - ATPase, SERCA3c isoform	Calcium regulation	1.52	0.00399	1.55	0.00515
AK036697	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 5	Calcium regulation	1.46	0.00815	1.44	0.00658
AB033123	C-terminal binding protein 2	cell signaling	1.70	0.00069	1.54	0.00245
AF094520	neuroepithelial cell transforming gene 1	cell signaling	1.79	0.00759	1.32	0.00375
Y08830	tumor-associated calcium signal transducer 2	cell signaling	1.61	0.00852	1.49	0.00108
AF448840	neuropilin (NRP) and tolloid (TLL)-like 1	cell signaling	1.74	0.00079	1.32	0.00129
BC023022	calcium binding atopy-related autoantigen 1	cell signaling	1.49	0.00287	1.45	0.00815
AK011949	poliovirus receptor-related 3	cytoskeleton	1.55	0.00831	1.47	0.00630
U31443	glutamate receptor subunit (GluR6)	Glutamate receptor	1.93	0.00609	1.70	0.00018
D10028	glutamate receptor, ionotropic, NMDA1 (zeta 1)	Glutamate receptor	1.65	0.00938	1.49	0.00487
AF357239	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1	Iron channel	1.52	0.00448	1.32	0.00868
AF357240	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	Iron channel	1.59	0.00387	1.35	0.00853
BC013651	serine (or cysteine) proteinase inhibitor, clade A, member 3N	metabolism	2.17	0.00291	1.76	0.00339
AK080718	synaptosomal-associated protein 25 binding protein	synapse	1.58	0.00303	1.45	0.00465
Down-regulated						
NM_02850 2	RIKEN cDNA 1700013N18 gene	metabolism	0.62	0.00094	0.69	0.00020
U41341	S100 calcium binding protein A11 (calizzarin)	Calcium regulation	0.61	0.00160	0.63	0.00002
AF169156	calcium binding protein 2	Calcium regulation	0.55	0.00084	0.57	4.51E-06
AF335470	myosin light chain kinase (fragment)	cytoskeleton	0.64	0.00142	0.66	0.00162
BC014711	serine/threonine kinase 6	cytoskeleton	0.57	0.00136	0.55	0.00104
AB045323	DNA segment, Chr 8, ERATO Doi 594, expressed	cytoskeleton	0.60	0.00130	0.68	0.00318

GeneBank#	Clone Title	Brief annotation	Ratio	t-test-P	Ratio	t-test-P
X57497	glutamate receptor, ionotropic, AMPA1 (alpha 1)	Glutamate receptor	0.18	2.23E-09	0.67	0.00011
AY008277	chloride channel calcium activated 4	Iron channel	0.63	0.00100	0.59	4.46E-06
J05663	aldo-keto reductase family 1, member B7	metabolism	0.54	0.00718	0.68	0.00850
AY073747	olfactory receptor MOR135-28	other	0.53	0.00156	0.56	0.00012
BC014763	far upstream element (FUSE) binding protein 1	other	0.56	0.00167	0.68	0.00006
AJ300716	Cx45 gene for connexin 45, exons 1-3	other	0.63	0.00032	0.70	0.00226
D50433	Mucosal vascular addressin cell adhesion molecule 1	other	0.58	0.00008	0.52	1.18E-06
X87142	calcium/calmodulin-dependent protein kinase II alpha	synapse	0.63	0.00033	0.70	0.00045
AK018680	ADP-ribosylation factor GTPase activating protein 3	synapse	0.53	0.00078	0.61	0.00014
BC014825	calcium/calmodulin-dependent protein kinase I	synapse	0.59	0.00231	0.61	0.00006
AK006538	RIKEN cDNA 1700030E15 gene	unclear	0.59	0.00040	0.59	0.00002