Mice carrying a human GLUD2 gene recapitulate aspects of human transcriptome and metabolome development

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Whereas all mammals have one glutamate dehydrogenase gene (GLUD1), humans and apes carry an additional gene (GLUD2), which encodes an enzyme with distinct biochemical properties. We inserted a bacterial artificial chromosome containing the human GLUD2 gene into mice and analyzed the resulting changes in the transcriptome and metabolome during postnatal brain development. Effects were most pronounced early postnatally, and predominantly gene expression in neuronal development were affected. Remarkably, the effects of the GLUD2 gene partially recapitulate changes in the transcriptome and metabolome differennces seen between humans and macaques analyzed. Notably, the introduction of GLUD2 did not affect glutatione levels in mice, consistent with observations in the primates. Instead, the metabolic effects of GLUD2 center on the tricarboxylic acid cycle, suggesting that GLUD2 affects carbon flux during early brain development, possibly supporting lipid biosynthesis.

Significance

A novel version of the glutamate dehydrogenase gene, GLUD2, evolved in the common ancestors of humans and apes. Based on sequence and expression pattern, GLUD2 has been suggested to play a role in glutamate metabolism in human and ape brains. We have generated transgenic mice carrying a human GLUD2 gene. Analysis of transcriptome and metabolome changes induced by GLUD2 in the cerebral cortex revealed no changes in glutamate concentration but instead changes to metabolic pathways centering on the TCA cycle during early postnatal development. These changes mirrored differences seen between human and macaque during cortex development, suggesting that GLUD2 may play a role during brain development in apes and humans, possibly by providing precursors for the biosynthesis of lipids.

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genome, we constructed two independent transgenic lines (a and b). Anatomical, neurophysiological, and behavioral analyses of adult mice did not reveal any overt effects of the GLUD2 genotype in the two lines (13).

We assessed the effects of GLUD2 on gene expression and metabolite concentrations in the developing frontal cortex of hemizygous (GLUD2+) mice from both lines as well as control littersmates, using RNA sequencing (RNA-seq) and capillary electrophoresis coupled with mass spectrometry (CE-MS). RNA-seq data were collected from 30 GLUD2+ and 29 control individuals. Metabolite concentrations were measured in 56 GLUD2+ and 81 control individuals. The mice varied in age from 3 d to 18 mo, with ages and sexes matched between transgenic and control groups (Fig. 1 A and B and SI Appendix, Table S1).

To assess whether molecular changes induced by GLUD2 in the mice might recapitulate differences between hominoids (apes and humans) and other primates, we measured metabolite concentrations in prefrontal cortex of 35 humans and 31 rhesus macaques using CE-MS. For both species, samples covered the respective lifespans approximately corresponding to the one sampled in the mice: from 16 d postnatal to 90 y in humans, and from 18.4 wk to lifespans approximately corresponding to the one sampled in the mouse brain did not show any increased divergence between transgenic and control mice during early development (Fig. 2C).

Even of the 13 genes differentially expressed between transgenic and control mice fell into the same coexpressed module in an unsupervised hierarchical clustering analysis (Fig. 3A and SI Appendix, Table S5 and Fig. S7), an observation not expected by chance (permutations, P < 0.001) (SI Appendix, Fig. S8). The genes affected by GLUD2 are thus coexpressed during mouse development. The expression of these 11 genes decreased rapidly during early postnatal development in both transgenic and control mice, but in the transgenic mice, the decrease in expression was shifted to earlier stages of development (Fig. 3 B and C and SI Appendix, Fig. S9).

Based on analysis of Gene Ontology (GO) terms (15), the 11 coexpressed genes were significantly enriched in several biological processes, many of them related to neural development and transcriptional regulation (hypergeometric test, FDR corrected P < 0.05) (Fig. 5D and SI Appendix, Table S6), a result robust to the use of different background gene distributions. The genes affected by GLUD2 may thus to some extent be functionally related.

GLUD2 Effects in the Mice Mirror Differences Between Primates. To assess whether the effect of GLUD2 in the mice recapitulated differences between primate species that lack and that have GLUD2, we reanalyzed 579 million, 100-nt-long RNA-seq reads from the prefrontal cortex of rhesus macaques and humans. We detected the expression of 23,115 protein-coding and noncoding development in humans (Pearson correlation, r = 0.86, P < 0.0001; SI Appendix, Fig. S4). We also detected expression of a long noncoding RNA (lncRNA) originating from the opposite strand of the transcribed human genomic region upstream of the GLUD2 transcription start site (SI Appendix, Fig. S5). Expression profile of this lncRNA closely resembled the expression profile of GLUD2 (SI Appendix, Fig. S6), suggesting that it is expressed from the same bidirectional promoter as GLUD2 (SI Appendix, Table S4).

An effect of the GLUD2 genotype, albeit small, was detectable: using analysis of covariance (ANCOVA) with linear, quadratic, and cubic models, we identified 12 protein-coding genes and one lncRNA showing significant expression differences between the two transgenic lines and control littersmates (F-test, BH corrected P < 0.05, permutations P < 0.05, FDR < 0.01, SI Appendix, Table S5). Strikingly, the effect of the GLUD2 genotype was observed only during early postnatal development and disappeared at approximately 2 wk of age (Fig. 2B). By contrast, other genes expressed in the mouse brain did not show any increased divergence between transgenic and control mice during early development (Fig. 2C).

Eleven of the 13 genes differentially expressed between transgenic and control mice fell into the same coexpressed module in an unsupervised hierarchical clustering analysis (Fig. 3A and SI Appendix, Table S5 and Fig. S7), an observation not expected by chance (permutations, P < 0.001) (SI Appendix, Fig. S8). The genes affected by GLUD2 are thus coexpressed during mouse development. The expression of these 11 genes decreased rapidly during early postnatal development in both transgenic and control mice, but in the transgenic mice, the decrease in expression was shifted to earlier stages of development (Fig. 3 B and C and SI Appendix, Fig. S9).

Based on analysis of Gene Ontology (GO) terms (15), the 11 coexpressed genes were significantly enriched in several biological processes, many of them related to neural development and transcriptional regulation (hypergeometric test, FDR corrected P < 0.05) (Fig. 5D and SI Appendix, Table S6), a result robust to the use of different background gene distributions. The genes affected by GLUD2 may thus to some extent be functionally related.

GLUD2 Affects Gene Expression During Early Postnatal Development. Based on 613 million, 100-nt-long sequence reads collected from the mouse polyA-plus RNA (SI Appendix, Table S3), we detected expression of 18,610 protein-coding and noncoding genes. Global expression variation among the samples showed a clear effect of age when analyzed by principal component analysis (PCA), whereas neither genotype nor sex affected gene expression substantially (Fig. 2A and SI Appendix, Fig. S2). Consistently, as many as 15,663 of the 18,610 detected genes, including the GLUD2 transgene, showed significant age-dependent expression changes in the mouse cortex (F-test, Benjamini–Hochberg (BH) corrected P < 0.05, false discovery rate (FDR) < 0.01).

Both transgenic mouse lines showed the same developmental expression profile of GLUD2, which was distinct from the expression profile of GLUD1 (SI Appendix, Fig. S3). Notably, the GLUD2 expression trajectory in the developing mouse brain strongly resembled the trajectory of GLUD2 expression during prefrontal cortex
genes. PCA analysis of the expression of these genes demonstrated substantial species-dependent as well as age-dependent divergence (Fig. 4A).

Among the 13 genes differentially expressed between transgenic and control mice, 9 were expressed in human and macaque prefrontal cortex (SI Appendix, Table S5). Remarkably, the expression of these 9 genes showed a large divergence between humans and macaques at the earliest stages of postnatal development after which the divergence rapidly decreased. Thus, the expression differences between humans and macaques resembled those seen between mice carrying a GLUD2 gene and control littermates (Fig. 4B). By contrast, other genes expressed in human and macaque prefrontal cortex showed no such divergence patterns (permutation P = 0.05, Fig. 4C).

Furthermore, seven of the nine genes showed a rapid expression level decrease during human and macaque early postnatal development, i.e., the same developmental expression trajectory as the genes affected by GLUD2 in the transgenic mice (Pearson correlation, r > 0.9, permutation P < 0.001, SI Appendix, Figs. S10 and S11). After adjusting for the differences in lifespan between humans and macaques, we found a shift in the timing of the expression to earlier developmental stages in humans relative to macaques (Fig. 4D and SI Appendix, Fig. S10), similar to what is seen in the GLUD2 mice. This was not caused by any general mismatching of human and macaque developmental timing, as other genes did not show such a coordinated shift to earlier developmental stages (Pearson correlation, r > 0.9, permutation P = 0.002, SI Appendix, Fig. S12). Thus, gene expression changes in GLUD2 mice indeed recapitulate gene expression differences between human and macaque expression profiles during early postnatal development.

Among the 7 genes showing similar expression and developmental shifts in the transgenic mice and in human cortex, 3 are transcription factors (TFs) implicated in neural development: SRY (sex determining region Y)-box 4 (SOX4), SRY (sex determining region Y)-box 11 (SOX11), and Distal-less homeobox 2 (DLX2) (16-22). A total of 15 known neural-related target genes of these TFs (16-29) were expressed in the mice and 14 in the primates (SI Appendix, Table S7). The expression divergence profiles of these target genes and the corresponding TFs between the transgenic and control mice and between humans and macaques at different ages were significantly correlated (Pearson correlation, r > 0.5, permutation P = 0.008 for mice and P = 0.001 for primates, SI Appendix, Fig. S13), suggesting that the effects of GLUD2 may go beyond the 13 genes showing differences between the transgenic and control mice.

As the effects of GLUD2 in the mice were strongest at the earliest stages of postnatal development, it is possible that GLUD2 plays a role mainly in prenatal development. To test if this may be the case in humans, we analyzed the expression of the 13 genes affected by GLUD2 expression in the mice using RNA-seq data from prenatal prefrontal cortex development of humans (30) and macaques. Of the 13 genes, 9 had detectable expression in our human and macaque data, as well as in public fetal human cortex data. Strikingly, expression of the 9 genes was substantially more divergent between humans and macaques before than after birth (Fig. 5 A and B and SI Appendix, Fig. S14), whereas other
expressed genes showed no obvious increase in expression differences before birth (Fig. 5C).

Brain Metabolism in GLUD2 Mice and Primates. We assessed the effects of GLUD2 on metabolism in the brains of the GLUD2 mice using CE-MS. For comparisons, we similarly analyzed human and macaque brains. We detected and quantified 110 and 88 metabolites in the mouse and in humans and macaques, respectively (SI Appendix, Tables S8 and S9). PCA analysis based on concentration levels of these metabolites revealed a substantial effect of age in both mice and primates (Fig. 6 A and B).

We first focused on the concentration levels of glutamate, the direct substrate of the GDH2 enzyme encoded by GLUD2. We detected no effect of GLUD2 on glutamate concentration in the mouse cortex (Fig. 6C). By contrast, glutamate concentrations in the prefrontal cortex of the humans are substantially lower than in the macaques (Fig. 6C). Previous work using gas chromatography coupled with mass spectrometry (GC-MS) has shown that compared with humans, chimpanzees as well as macaques have higher glutamate levels in the brain (31), something that reexamination of the published glutamate data confirms (Fig. 6C). Because both humans and chimpanzees carry GLUD2 genes, a mechanism other than the mere presence of GLUD2 must be responsible for the lower glutamate concentrations in the human brain.

Despite the absence of a GLUD2 effect on glutamate levels in the mice, we detected that the overall differences between the metabolomes of the transgenic and the control mice were about threefold larger shortly after birth relative to 2 mo after birth (SI Appendix, Fig. S15). To test if this may be at least partially connected to gene expression differences, we compared the concentration profiles of 24 metabolites associated with the metabolic pathways where the 13 genes differentially expressed between transgenic and control mice are located (SI Appendix, Table S8). Concentration differences of the 24 metabolites were substantially larger than the differences for the 86 remaining compounds analyzed (Fig. 7 A). Similar results were obtained using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation and the Small Molecule Pathway Database (32) to identify metabolites and pathways associated with the 13 genes (SI Appendix, Fig. S16).

The 13 genes and 24 metabolites showing increased divergence between the transgenic and control mice converged within seven KEGG pathways (SI Appendix, Table S10). For three of these pathways, the HIF-1 signaling pathway, the pentose phosphate pathway, and carbon metabolism, metabolite concentration differences were significantly greater than expected by chance (permutations, P < 0.05) (Fig. 8A). The three pathways are closely related as the “carbon metabolism” pathway includes the pentose phosphate pathway and the TCA cycle, which is in turn linked to the HIF-1 signaling pathway (Fig. 8B).

We next analyzed metabolic differences between human and macaque brains. As the human samples were frozen after substantial postmortem delay (PMD), we first analyzed the effects of PMD by comparing three macaque samples that were intentionally collected with substantial PMD with macaque samples that were not (SI Appendix, Table S11). Of the 88 metabolites detected in the macaque and human brains, 21 were affected by PMD in at least one of the three samples analyzed (SI Appendix, Fig. S17 and Table S9) and were therefore excluded from further analyses.

For the remaining 67 metabolites, the differences between humans and macaques explained 26% of the total variation in metabolite concentrations. By comparison, the GLUD2 genotype explains 2% of the metabolic variation in the mice (SI Appendix, Fig. S18). Despite the much greater overall metabolic differences between macaques and humans than between the transgenic and control mice, it is notable that in both cases, the greatest metabolic differences are seen in early development (SI Appendix, Figs. S15 and S19).

Of the 24 metabolites linked to the genes differentially expressed between GLUD2 and control mice, 11 were among the

![Fig. 5. Prenatal gene expression. (A) Prenatal expression profiles of E2F7, SOX11, and SOX4 genes, which are among the 7 genes showing similar expression and divergence patterns in primates and the transgenic mice. Each point represents an individual. The colors indicate species information: red, humans; blue, macaques. Filled symbols indicate postnatal ages; empty points, prenatal ages (17 in humans and 4 in macaques). The vertical dashed line shows the human birth age; pcw, weeks postconception. (B) The mean normalized gene expression divergence between humans and macaques based on the 9 genes with expression affected by the GLUD2 genotype, calculated using a published fetal human dataset (red curve). The colored area shows variation of the divergence estimates obtained by bootstrapping the 9 genes 1,000 times. (C) The mean normalized gene expression divergence between humans and macaques, based on the remaining 15,897 detected genes in the public fetal human and our macaque time series data (light-red curve). The colored area shows variation of the divergence estimates obtained by subsampling 9 genes 1,000 times.](https://www.pnas.org/doi/10.1073/pnas.1519261113)
Discussion

GLUD2 originated as an evolutionary novel version of the glutamate dehydrogenase gene as a result of the retroposition of a GLUD1 transcript in the common ancestors of humans and apes. We investigated the function of GLUD2 by inserting the human GLUD2 gene and surrounding sequences carrying putative regulatory elements into the mouse genome. We isolated two independent transgenic lines to exclude artifacts caused by the insertion of the human gene in the mouse genome. When analyzed in the frontal cortex, both lines displayed similar developmental GLUD2 expression trajectories, closely resembling GLUD2 expression in human prefrontal cortex during development.

In both transgenic lines, GLUD2 affects the expression of 13 genes, 11 of which show similar ontogenetic expression profiles. These genes include several TFs, among which DLX2, SOX4, and SOX11 play important roles in neuronal differentiation and neurogenesis (16, 18, 19, 25). Some of the previously described targets of these TFs show changes in expression in the transgenic mice that are correlated with expression changes of the corresponding TFs. Nine primate orthologs of the 13 mouse genes affected by GLUD2, including DLX2, SOX4, and SOX11, differ in expression between human and macaque ontogenesis in ways that mirror the changes seen in the mice. Similarly, primate orthologs of target genes of DLX2, SOX4, and SOX11 show expression differences corresponding with those of these TFs. These results illustrate that the introduction of GLUD2 into the mouse genome induces effects paralleling evolutionary differences between primate species that carry a GLUD2 gene and those that do not. This adds to a mounting amount of evidence suggesting that human-specific variants of genes such as FOXP2, ASPM, EDAR, SRGAP2, and CMAH can be fruitfully studied in mouse models (33).

The gene expression changes induced by GLUD2 in the mice and the differences between humans and macaques were restricted to early development and were not observed past the first 2 wk postpartum in the mice or the first 2 y of life in humans.
This suggests that GLUD2 mainly functions during brain growth and early development, a notion that is in apparent contradiction to the idea that GLUD2 has a major role in the metabolism of the neurotransmitter glutamate.

The metabolic data lend support to the notion that GLUD2 mainly influences aspects of metabolism different from glutamate recycling. We detect no effects on glutamate concentrations in the mouse frontal cortex and our previous results show that glutamate concentrations do not differ between macaques and chimpanzees, are particularly prevalent at early developmental stages characterized by rapid brain growth. Given that lipids comprise more than 50% of the brain’s dry weight (34), we speculate that GLUD2 may support the rapid growth of the large ape and human brains by enhancing lipid biosynthesis.

Materials and Methods

A detailed description of materials and methods is provided in SI Appendix. Briefly, a human bacterial artificial chromosome containing the GLUD2 gene (RP11-610G22) was linearized by NotI and injected into the male pronucleus of C57BL/6 mice to construct GLUD2+ transgenic mice. Transgenic animals were identified by PCR targeting the 5′-end of the gene, the coding region, and the 3′-end of the gene. For metabolome analysis, CE-MS measurements were conducted in frontal cortex samples of 81 control and 56 transgenic mice and prefrontal cortex samples of 35 humans and 34 rhesus macaques. Transcriptome analysis was conducted in the frontal cortex of 29 control and 30 transgenic mice used for metabolite profiling on the Illumina platform, as well as in prefrontal cortex of 38 humans and 40 rhesus macaques (14).

Written consent for the use of human tissues for research was obtained from all donors or their next of kin. Use of human autopsy tissue is considered nonhuman subject research and is institutional review board exempt under NIH guidelines. Biomedical Research Ethics Committee of Shanghai Institutes for Biological Sciences completed the review of the use and care of the animals in the research project (approval ID: ER-SIBS-260802P).

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