

GENETIC DISRUPTION OF THE AUTISM SPECTRUM DISORDER RISK GENE *PLAUR* INDUCES GABA_A RECEPTOR SUBUNIT CHANGES

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Abstract—Disruption of the GABAergic system has been implicated in multiple developmental disorders, including epilepsy, autism spectrum disorder and schizophrenia. The human gene encoding *uPAR* (*PLAUR*) has been shown recently to be associated with the risk of autism. The *uPAR*^{−/−} mouse exhibits a regionally-selective reduction in GABAergic interneurons in frontal and parietal regions of the cerebral cortex as well as in the CA1 and dentate gyrus subfields of the hippocampus. Behaviorally, these mice exhibit increased sensitivity to pharmacologically-induced seizures, heightened anxiety, and atypical social behavior. Here, we explore potential alterations in GABAergic circuitry that may occur in the context of altered interneuron development. Analysis of gene expression for 13 GABA_A receptor subunits using quantitative real-time polymerase chain reaction (PCR) indicates seven subunit mRNAs (α_1 , α_2 , α_3 , β_2 , β_3 , γ_{2S} and γ_{2L}) of interest. Semi-quantitative *in situ* hybridization analysis focusing on these subunit mRNAs reveals a complex pattern of potential gene regulatory adaptations. The levels of α_2 subunit mRNAs increase in frontal cortex, CA1 and CA3, while those of α_3 decrease in frontal cortex and CA1. In contrast, α_1 subunit mRNAs are unaltered in any region examined. β_2 subunit mRNAs are increased in frontal cortex whereas β_3 subunit mRNAs are decreased in parietal cortex. Finally, γ_{2S} subunit mRNAs are increased in parietal cortex while γ_{2L} subunit mRNAs are increased in the dentate gyrus, potentially altering the $\gamma_{2S}:\gamma_{2L}$ ratio in these two regions. For all subunits, no changes were observed in forebrain regions where GABAergic interneuron numbers are normal. We propose that disrupted differentiation of GABAergic neurons specifically in frontal and parietal cortices leads to regionally-selective alterations in local circuitry and subsequent adaptive changes in receptor subunit composition. Future electrophysiological studies will be useful in determining how alterations in network activity in the cortex and hippocampus relate to the observed behavioral phenotype. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neocortex, hippocampus, interneurons, neurodevelopmental disorders.

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Abbreviations: ASD, autism spectrum disorder; CA, cornu ammonis; P, postnatal day; PCR, polymerase chain reaction; PKC, protein kinase C; PLAUR, plasminogen activator, urokinase receptor; uPAR, urokinase-type plasminogen activator receptor.

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The formation of appropriate inhibitory circuits during telencephalic development underlies the fine-tuning of sensory maps—through the regulation of the timing of the critical period—and the expression of normal behavior (Fagioli et al., 2004; Hensch and Stryker, 2004). This requires a functional interaction during development between presynaptic elements, comprised of GABAergic interneurons, and postsynaptic elements, comprised of GABA_A receptor subunits. Subtle disruptions in the differentiation of GABAergic interneurons, receptor subunit combinations, and the maturation of inhibitory innervation can result in significant functional alterations, such as occurs in epilepsy, schizophrenia and autism spectrum disorders (ASD; Levitt et al., 2004; Guidotti et al., 2005; Lewis et al., 2005; Eagleson et al., 2010). Recently, the plasminogen activator, urokinase receptor (*PLAUR*) gene was identified as an autism risk gene in a large genetic association study (Campbell et al., 2008) and we have previously described GABAergic interneuron deficits in the cerebral cortex of adult mice with a genetic deletion of the mouse homolog, the urokinase-type plasminogen activator receptor (*uPAR*) gene (Powell et al., 2003; Eagleson et al., 2005). From a mechanistic standpoint, upon binding of urokinase plasminogen activator, uPAR interacts with other proteins, including integrins and certain G-protein coupled receptors, to modulate cell migration, adhesion, proliferation and differentiation (reviewed in Binder et al., 2007). There are significant reductions in GABAergic interneurons in the adult *uPAR*^{−/−} mouse, which are restricted to specific regions of neocortex (frontal and parietal). These reductions can be observed as early as postnatal day (P) 21 (Eagleson et al., 2005). The parvalbumin subpopulation is affected preferentially, with the other interneuron subclasses remaining intact. Of the other telencephalic regions examined (all amygdala subnuclei and striatum), only the adult hippocampus shows a loss of GABAergic interneurons, although the somatostatin, rather than the parvalbumin, subpopulation contributes to this loss. Behavioral analyses demonstrate that adult *uPAR*^{−/−} mice exhibit alterations in anxiety and social behavior, as well as a high susceptibility to pharmacologically-induced convulsions (Powell et al., 2003; Levitt, 2005). Specifically, the performance of *uPAR*^{−/−} mice in three behavioral paradigms is consistent with increased anxiety; thus, these mice spend significantly less time on the light side in a light–dark avoidance task, very little time in the open arms of an elevated plus maze and decreased time in the center of an open arena. In a modified version of the resident intruder task, *uPAR*^{−/−} mice spend half the time interacting with a conspecific (assessed as time spent sniffing and

in body contact) as do their wild type counterparts. Finally, when challenged with a single threshold dose of pentylenetetrazol (PTZ), all *uPAR*^{-/-} mice tested exhibit motor convulsions, with the majority displaying full tonic extension. In contrast, only half of the wild type mice tested exhibit signs of motor seizure, with none progressing to tonic extension.

In the telencephalon, as in the rest of the central nervous system, fast inhibitory neurotransmission is mediated principally by GABA_A receptors, which are chloride-selective ion channels composed of five subunits. The specific subunit composition of each receptor is the main determinant of its physiological and pharmacological properties. Nineteen subunits encoded by distinct genes have been identified in mammals, although those including $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$, or $\alpha_3\beta_3\gamma_2$ are the most prevalent in the brain (reviewed in Mohler, 2006). In addition, the diversity of GABA_A receptors is increased by alternative splicing of some subunits, including the γ_2 subunit (Whiting et al., 1990). From a clinical standpoint, GABA_A receptors are the targets of multiple classes of therapeutic agents, such as benzodiazepines, that mediate their anxiolytic, muscle relaxant, sedative-hypnotic, amnesic, and anticonvulsant properties. Changes in GABA_A receptor subunit expression have been observed under a variety of experimental and pathological conditions, including chronic ethanol exposure, temporal lobe epilepsy and schizophrenia (Akbarian et al., 1995; Brooks-Kayal et al., 1998; Bouillere et al., 2000; Sanna et al., 2003; Nishimura et al., 2005; Evans et al., 2006; Hashimoto et al., 2007). While there are now a large number of animal models in which genes responsible for interneuron development have been studied (Roy et al., 2002; Carretta et al., 2004; Cobos et al., 2005; Carmona et al., 2006; Yabut et al., 2007; Andrews et al., 2008; Muller Smith et al., 2008; Wallis et al., 2008; Barber et al., 2009; Batista-Brito et al., 2009; Bond et al., 2009; Canty et al., 2009; Ibi et al., 2010; Mao et al., 2009; Price et al., 2009; Sakata et al., 2009), the impact of altered interneuron development on the long-term expression of GABA_A receptor subunits remains to be elucidated. Here, we assess the possibility of GABA_A receptor subunit adaptations in the adult male *uPAR*^{-/-} mouse telencephalon, in the context of altered interneuron development through genetic manipulation.

EXPERIMENTAL PROCEDURES

Animals

C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The *uPAR*^{-/-} mouse line was obtained originally from P. Carmeliet (Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium). The line arrived in our colony on a mixed 129/Sv:C57Bl/6 background and was subsequently back-crossed on the C57Bl/6 strain for >10 generations. Animals were provided free access to food and water and housed in a 12 h light:dark cycle. All research procedures using mice were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and conformed to NIH guidelines. All wild type and null mice used in this study were generated using a heterozygous by heterozygous breeding strategy. Only male mice were analyzed as alterations in GABA_A receptor subunit expression have been noted at different stages of

the estrous cycle (Diaz-Veliz et al., 2000; Jorge et al., 2002; Maguire et al., 2005). Genotyping was performed by polymerase chain reaction (PCR) as described previously (Eagleson et al., 2005), using mouse tail genomic DNA as a template.

Real-time PCR assays

As an initial evaluation of potential alterations in the repertoire of GABA_A receptor subunits in discrete areas of the *uPAR*^{-/-} telencephalon, the relative levels of 13 GABA_A receptor subunit mRNAs (α_1 , α_2 , α_3 , α_4 , α_5 , β_1 , β_2 , β_3 , γ_1 , γ_2S , γ_2L , γ_3 , δ) were analyzed using real-time PCR. Adult (>P90) male wild type ($n=3$) and *uPAR*^{-/-} ($n=3$) mice were anesthetized with isoflurane (Western Medical Supply, Arcadia, CA, USA) vapor, decapitated, and the brains removed. Following a midsagittal cut, the diencephalon was removed. Five subregions of the telencephalon were dissected and immediately frozen on dry ice. Specifically, the regions comprised: (1) the entire striatum, (2) the entire hippocampus including all CA subfields and the dentate gyrus, (3) frontal cortex (predominantly frontal association and primary and secondary motor regions), (4) parietal cortex (predominantly primary and secondary somatosensory regions), and (5) occipital cortex (predominantly primary and secondary visual regions). Each animal was considered an independent sample. Total RNA was isolated using the Trizol method according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Following the initial extraction, RNA aliquots were further purified using RNeasy Mini spin columns (Qiagen, Valencia, CA, USA). The concentration and purity of each sample was determined by spectrophotometry (NanoDrop, Wilmington, DE, USA).

Primers (Oligos Etc, Wilsonville, OR, USA) and probes (TaqMan probes, Applied Biosystems, Foster City, CA, USA) were designed using Primer Express software (Applied Biosystems). Ribosomal RNA (18 S) probe and primers were purchased from Applied Biosystems. The sequence of primers and probes were: α_1 primers, 5'-CCCCGGCTTGGCAACTA-3' and 5'-TGGTTTTGTCTCAGGCTTGAC-3', α_1 probe, 5'-TGCTAAAGTGCGACCATAGAACCAGAAAGA-3'; α_2 primers, 5'-GTAT-TACTGAAGTCTTCACTAACATT-3' and 5'-CGAAAGAAACA-TCTATTGTATACTCCATATC-3', α_2 probe, 5'-ACGTGACCA-GTTTTGGCCCTGTCTCAGA-3'; α_3 primers, 5'-CGCGACGGC-CATGG-3' and 5'-CAAATTCATCAGTGCAGAAAATACAA-3', α_3 probe, 5'-CTGGTTCATGGCCGTCTGTTATGCC-3'; α_4 primers, 5'-TGAAATCTTGAGGCTGAATTAATAG-3' and 5'-AGACA-GATTTCTTTCCATTCTGAAG-3', α_4 probe, 5'-TGGTCACCAA-AGTTTGGACCCCTGAT-3'; α_5 primers, 5'-GACTCTTGATG-GCTATGACA-3' and 5'-CACCTGCGTGATTCGCTCT-3', α_5 probe, 5'-CAGACTGCGGCTGGGCTGG-3'; β_1 primers, 5'-CCGGCAAGGGGCGCA-3' and 5'-TCAGTCAAGTCGGGGA-TCTTCACT-3', β_1 probe, 5'-CCGCAGGCGCGCCTCGCAG-CTCA-3'; β_2 primers, 5'-ACCCAGGAGCACAATGC-3' and 5'-AGGCAGCCAGCTTTTCG-3', β_2 probe, 5'-TGCCTATGATGC-CTCCAGCATCCAGTA-3'; β_3 primers, 5'-AGAGCATGCCAAG-GAAGG-3' and 5'-AGGTGGGTCTTCTGTGCG-3', β_3 probe, 5'-CTTCTGTCTCCCATGTACCGCCCATG-3'; γ_1 primers, 5'-CCCAGGTCTCCATGCTGG-3' and 5'-TTCCCTTGTTGGCAAA-GAAATG-3', γ_1 probe, 5'-CACTCTCATTTCCCATGAACA-3'; γ_2S primers, 5'-AAACCCTGCCCTACCATTG-3' and 5'-GGCATT-GTTCATTTGAATGGT-3', γ_2S probe, 5'-TTCGTCCAGATCAG-3'; γ_2L primers, 5'-TTTCCTTCAAGCCCTACC-3' and 5'-TGTGTGGCATTGTTCAATTTG-3', γ_2L probe, 5'-TGATATTTCG-TCCCAGATCAGCAACCA-3'; γ_3 primers, 5'-TCGTTATTACATC-CAGATTCCACAAGA-3' and 5'-GTCCAGTAGAGATGAATTA-GAGGGTG-3', γ_3 probe, 5'-CTGATCGAATAAGCCTTCAA-3'; δ primers, 5'-AGGGCAGAGATGGATGTGAG-3' and 5'-TGACCCC-AGCAGCTGAGAG-3', δ probe, 5'-CCATCGTCCTTTTCTC-3'.

Quantitative one-step real-time PCR assays were performed in an ABI prism Applied Biosystems 7900HT Sequence Detection System using QuantiTect™ Probe RT-PCR kit (Qiagen). Stan-

dard curves for relative quantification were generated with 1–500 ng of total RNA from wild type mouse cortex. Total RNA (50 ng) from wild type and *uPAR*^{-/-} mice was tested. Reactions were performed in triplicate in a total volume of 50 μ l containing QuantiTect Probe RT-PCR master mixture, 250 μ M receptor probe, 900 nM receptor primers, 1 μ M 18 S rRNA probe, and 250 μ M 18 S rRNA primers. Two aliquots of 20 μ l/reaction were loaded in a 384-well plate and incubated as follows: 48 °C for 30 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. The relative amount of each GABA_A receptor subunit RNA was normalized to the relative amount of 18 S rRNA (internal control).

In situ hybridization

Based on the real time PCR data, select GABA_A receptor subunits (α_1 , α_2 , α_3 , β_2 , β_3 , γ_{2L} and γ_{2S}) were analyzed using *in situ* hybridization. This method permits semi-quantitative analysis of transcript expression in more circumscribed anatomical subregions for the telencephalon. The tissue processing, probe synthesis and hybridization protocols were as described previously (Middleton et al., 2002; Campbell and Levitt, 2003). Briefly, adult (>P90) male wild type ($n=6$) and *uPAR*^{-/-} ($n=6$) mice were anesthetized with isoflurane vapor and decapitated. Brains were removed and flash frozen using cold 2-methylbutane and stored at -80 °C. Twenty-five micrometres sections were cut through the entire telencephalon with a cryostat. Three to four serial sections were collected on Superfrost Plus glass slides (VWR, West Chester, PA, USA) and stored at -80 °C until the day of hybridization. For each region analyzed (frontal, parietal and occipital cortices, striatum, and the CA1, CA3 and dentate gyrus subregions of the hippocampus), two slides from each mouse, processed in two independent hybridization runs, were used to examine the expression of each transcript. An additional two slides were used for sense controls. In each hybridization run, sections from all 12 brains were represented, with two slides from each animal (one antisense and one sense control) processed in parallel. Slides were hybridized with 3–5 ng of [³⁵S]-labeled sense or antisense probe at 55 °C overnight, followed by a series of buffer washes. The probes for α_2 , α_3 , γ_{2L} and γ_{2S} have been used previously in Northern blot and RNase protection assays (Steiger et al., 2002, 2003). Plasmid clones for α_1 (IMAGE 5360723), β_2 (IMAGE 6847737) and β_3 (IMAGE 4503196) were obtained from Open Biosystems (Huntsville, AL, USA). After air drying, the slides were exposed to BioMax MR film (Eastman Kodak, Rochester, NY, USA) for 1 day (α_1 , β_2), 3–5 days (α_3 , β_3 , γ_{2L} and γ_{2S}) or 10–12 days (α_2).

Image analysis

For all analyses, the material was coded so that the individuals performing the analyses were blind to genotype. In each run, [¹⁴C]-labeled standards were included to cross calibrate signal intensity. The films containing the autoradiographic images were scanned at high resolution (2400dpi) and the average signal intensity across each region of interest quantified using the public domain program Image J, version 1.33. For the frontal, parietal and occipital cortices, the region of interest spanned all cortical layers. For the hippocampus, a line was drawn through the pyramidal (CA1 and CA3) or granule (dentate gyrus) cell layers, while the entire striatum was outlined. Sections were analyzed between the following Bregma levels using stereotaxic coordinates: striatum (+1.4 and +0.1 mm), hippocampus (-1.7 and -2.5 mm), frontal cortex (+2.5 and +1.7 mm), parietal cortex (+0.2 and -1.5 mm), and occipital cortex (-2.5 and -3.4 mm). For the cortical regions, it was not possible to define precisely the boundaries between cytoarchitectonic regions. Therefore, we placed the region of interest in the same position laterally such that the region of interest in frontal cortex comprised primarily secondary motor cortex, in parietal cortex comprised primarily primary somatosen-

sory cortex and in occipital cortex comprised primarily primary visual cortex and lateral secondary visual cortex (Fig. S1). Signal for each of the GABA_A receptor subunits examined was virtually absent in the white matter. Thus, quantification was performed by subtracting the signal in the white matter from the signal in the area of interest. For each brain region in each animal, a single value was obtained by averaging the signal across six to eight sections.

Statistical analyses

Data are expressed as means \pm SEM. For each receptor subunit, a two-tailed *t*-test was used to determine significance, with the alpha level set at 0.05 for significance. All analyses were implemented in Statview, Version 5.0.1 (SAS Institute, Inc., Cary, NC, USA).

Figures were prepared digitally in SigmaPlot 7.0 (SPSS Incorporated, Chicago, IL, USA) and Adobe Photoshop 6.01 (Adobe Systems Incorporated, San Jose, CA, USA).

RESULTS

Regional-selective alterations in GABA_A receptor subunit mRNA in *uPAR*^{-/-} mice

An alteration in GABA_A receptor number and/or subunit composition has been implicated in multiple behavioral states. The repertoire of behaviors displayed by adult *uPAR*^{-/-} mice, namely increased anxiety, atypical social behavior and a higher susceptibility to pharmacologically-induced convulsions, prompted us to explore whether there are changes in GABA_A receptor subunits in these mice. We first compared the expression level of 13 GABA_A receptor subunit mRNAs (α_1 , α_2 , α_3 , α_4 , α_5 , β_1 , β_2 , β_3 , γ_1 , γ_{2S} , γ_{2L} , γ_3 , δ) in select regions of the telencephalon in wild type and *uPAR*^{-/-} adult male mice using quantitative real-time PCR. We focused on these subunits because they are the most prevalent in the telencephalon; the remaining subunits are more prominent in more caudal brain structures, such as the hypothalamus and cerebellum, and in the periphery. Significant changes in expression were observed in select regions for two subunits. There was increased expression of the γ_{2S} subunit in parietal cortex (wild type: 1.491 ± 0.119 , *uPAR*^{-/-}: 2.851 ± 0.371 , $P=0.0252$) and of the β_2 subunit in frontal cortex (wild type: 1.044 ± 0.017 , *uPAR*^{-/-}: 1.393 ± 0.099 , $P=0.0254$) and the hippocampus (wild type: 0.771 ± 0.030 , *uPAR*^{-/-}: 0.901 ± 0.035 , $P=0.0469$). We also noted potential alterations in the α_2 (frontal, wild type: 0.140 ± 0.010 , *uPAR*^{-/-}: 0.236 ± 0.110 ; hippocampus, wild type: 0.246 ± 0.019 , *uPAR*^{-/-}: 0.470 ± 0.237), α_3 (frontal, wild type: 1.226 ± 0.210 , *uPAR*^{-/-}: 0.841 ± 0.122) and β_3 (frontal, wild type: 0.784 ± 0.392 , *uPAR*^{-/-}: 0.482 ± 0.042) subunits, but an increased degree of variability in either the null or wild type samples precluded this from reaching significance.

Based on the real-time PCR data, we focused on the expression levels of five GABA_A receptor subunits (α_2 , α_3 , β_2 , β_3 and γ_{2S}) in our next level of analysis, semi-quantitative *in situ* hybridization. In addition, for reasons noted below, we included analysis of the α_1 and γ_{2L} subunits. In adult wild type mice, each subunit mRNA displayed a

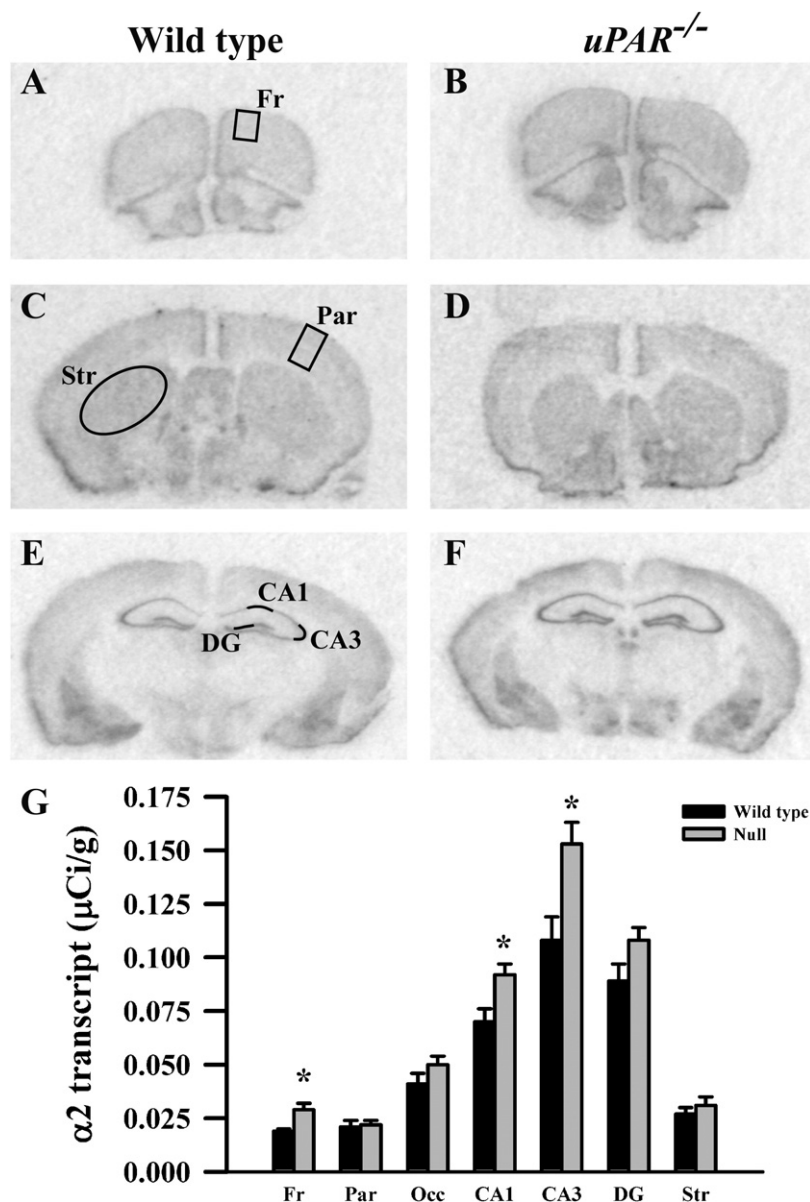


Fig. 1. Film autoradiographs from coronal sections depict expression of the GABA_A receptor α_2 subunit in adult wild type (A, C, E) and *uPAR*^{-/-} (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the different subfields of the hippocampus (E) are indicated. Quantitative analysis (G) reveals a significant increase in α_2 message in frontal cortex, and in the CA1 and CA3 subfields of the hippocampus. Asterisks, significantly different than wild type ($P < 0.05$). Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

distinct pattern of probe hybridization (Figs. 1–7) that is similar to that reported for adult C57Bl/6 mice (Heldt and Ressler, 2007). Hybridizations conducted with corresponding sense probes produced no specific labeling patterns. For each subunit, the anatomical patterns of expression were similar in the *uPAR*^{-/-} brain compared to wild type. However, there were highly significant, quantitative differences in subunit expression. There was a significant increase in the levels of α_2 subunit mRNAs in frontal cortex ($P = 0.005$) and in the CA1 ($P = 0.013$) and CA3 ($P = 0.009$) subregions of the hippocampus (Fig. 1G). During normal development, as well as in some pathological conditions, an alteration in α_2 subunit expression is accompanied by

a reciprocal alteration in expression of the α_1 subunit (Fritschy et al., 1994; Volk et al., 2002; Hashimoto et al., 2007, 2009). However, there was no significant change in α_1 subunit expression in any of the regions examined (Fig. 2G). In contrast, there were significantly reduced levels of α_3 subunit mRNAs in frontal cortex ($P = 0.007$), parietal cortex ($P = 0.039$), and CA1 ($P = 0.006$) (Fig. 3G). There was a significant increase in the levels of β_2 subunit mRNAs in frontal cortex ($P = 0.006$) (Fig. 4G), and significantly reduced levels of β_3 subunit mRNAs in parietal cortex ($P = 0.002$) (Fig. 5G). Messenger RNAs specific to the γ_{2S} subunit were significantly increased in parietal cortex ($P = 0.005$) and decreased in CA3 ($P = 0.016$) (Fig.

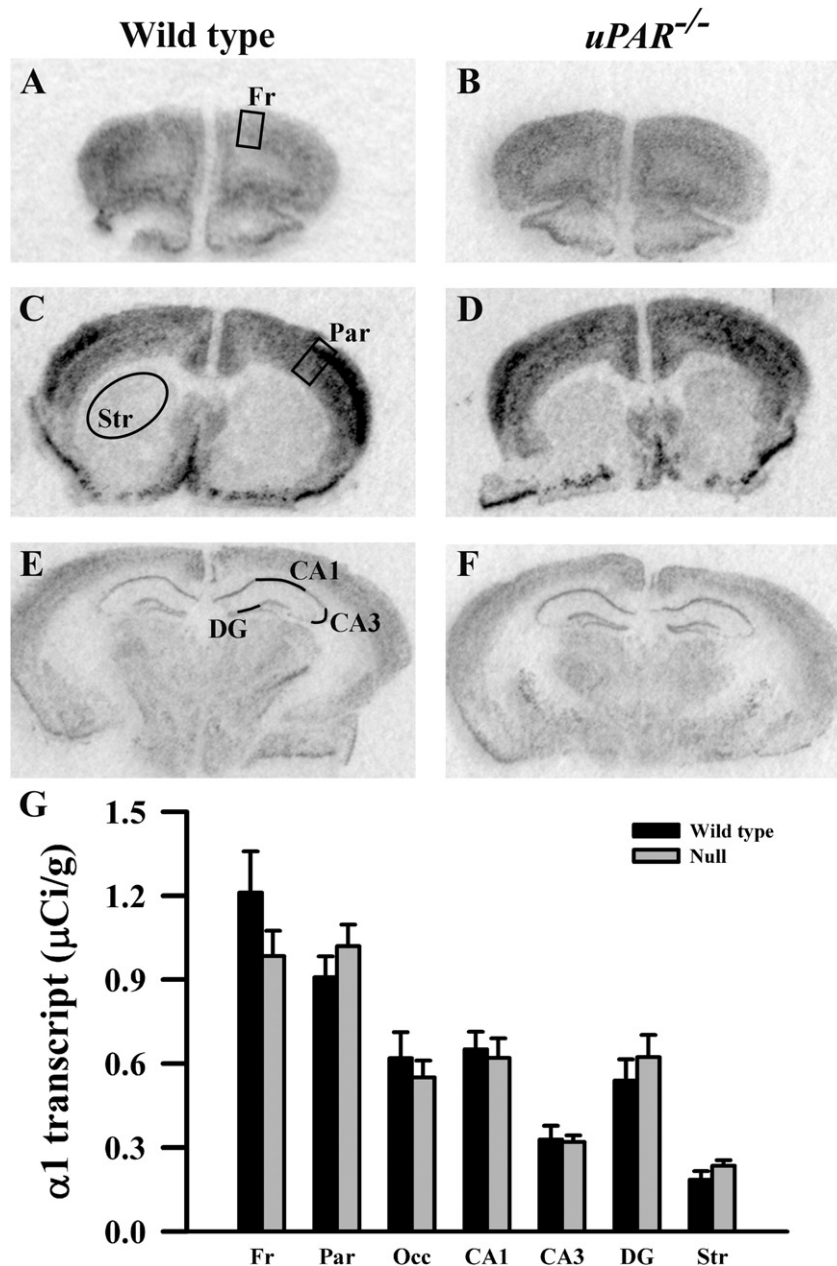


Fig. 2. Film autoradiographs from coronal sections depict expression of the GABA_A receptor α_1 subunit in adult wild type (A, C, E) and *uPAR*^{-/-} (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the different subfields of the hippocampus (E) are indicated. Quantitative analysis (G) reveals no significant difference in α_1 message in any region examined. Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

6G). Alterations in the ratio of γ_{2L} subunit to γ_{2S} subunit are thought to impact the function of the GABA_A receptor (Moss et al., 1992). We therefore included analysis of the expression levels of γ_{2L} subunit mRNAs and found this subunit transcript was increased significantly in the dentate gyrus ($P=0.004$) (Fig. 7G). The regional specificity of changes in gene expression was noteworthy, as it paralleled the identical telencephalic regions that exhibit interneuron disruption. Thus, for all subunits examined, no significant changes were observed in striatum and occipital cortex (Fig. 1G–7G), in which the num-

bers of GABAergic interneurons are unaltered in the *uPAR*^{-/-} mice.

DISCUSSION

The present study reveals a selective interaction between GABAergic interneuron development and adaptive changes in GABA_A receptor subunit expression. The relationship appears to be complex, as the data reveal distinct patterns of altered subunit expression that vary regionally. Thus, it is likely that unique features of input and local circuitry may

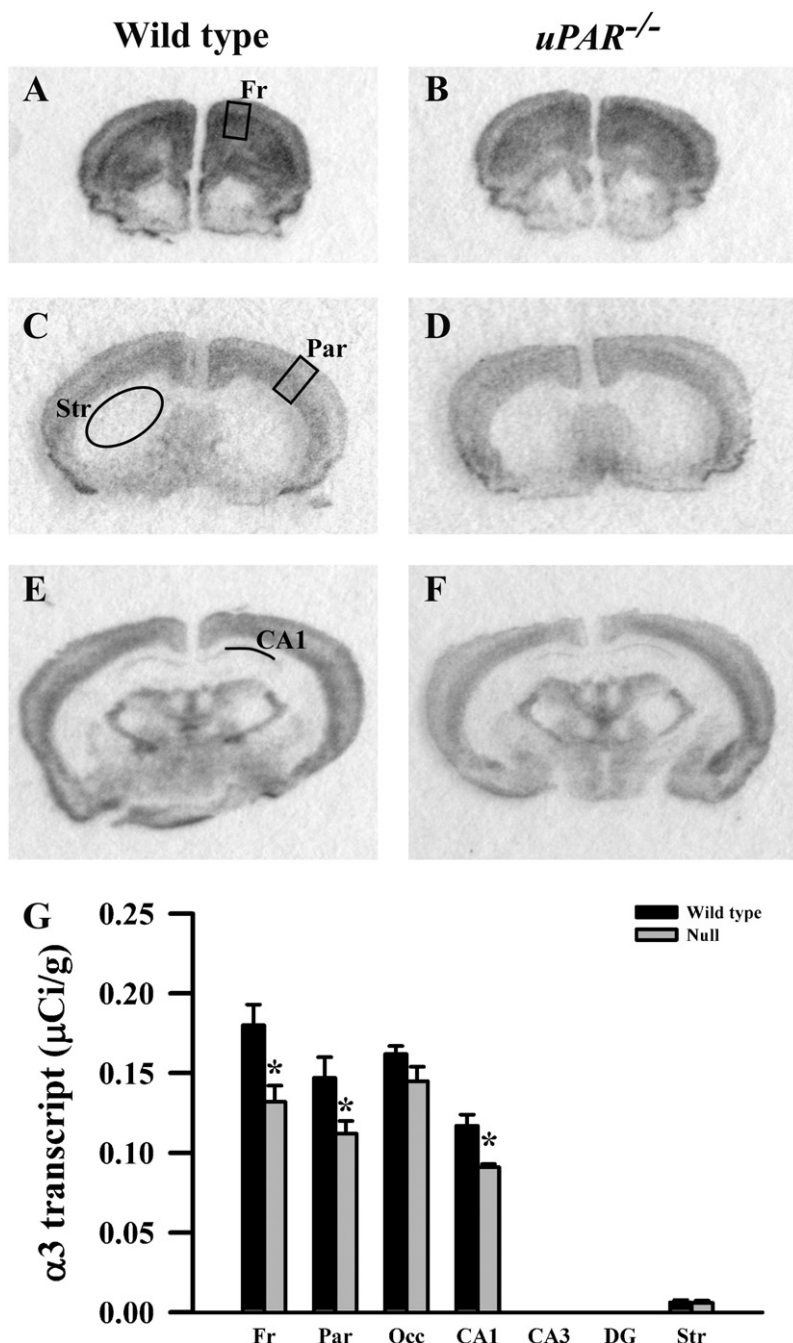


Fig. 3. Film autoradiographs from coronal sections depict expression of the GABA_A receptor α_3 subunit in adult wild type (A, C, E) and $uPAR^{-/-}$ (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the CA1 subfield of the hippocampus (E) are indicated. Quantitative analysis (G) reveals a significant decrease in α_3 message in frontal and parietal cortices, and in the CA1 subfield of the hippocampus. No signal was detected in the CA3 and dentate gyrus subfields in either genotype. Asterisks, significantly different than wild type ($P < 0.05$). Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

contribute to the differential adaptive changes in GABA_A receptor composition.

While a large number of studies have defined multiple mechanisms that control different aspects of interneuron development (Roy et al., 2002; Carretta et al., 2004; Cobos et al., 2005; Carmona et al., 2006; Yabut et al., 2007; Andrews et al., 2008; Muller Smith et al., 2008; Wallis et al., 2008; Barber et al., 2009; Batista-Brito et al., 2009;

Canty et al., 2009; Mao et al., 2009; Sakata et al., 2009), none have extended these findings to investigate directly potential adaptive changes to the receptors that ultimately are responsible for mediating physiological activity. In the present study, we identified a complex pattern of region-specific alterations in GABA_A receptor subunit gene expression in the adult $uPAR^{-/-}$ telencephalon (Fig. 8), which may contribute to the functional alterations exhibited

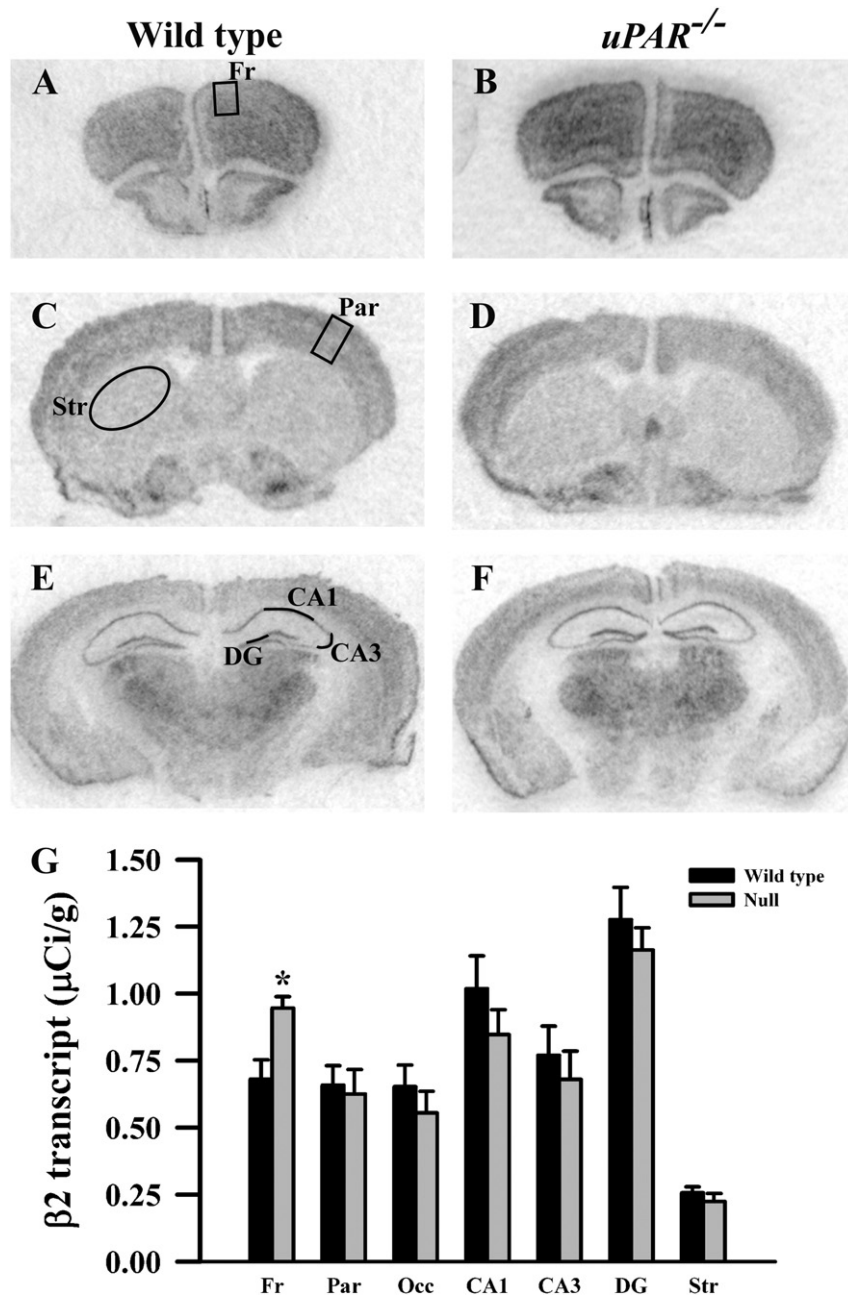


Fig. 4. Film autoradiographs from coronal sections depict expression of the GABA_A receptor β_2 subunit in adult wild type (A, C, E) and *uPAR*^{-/-} (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the different subfields of the hippocampus (E) are indicated. Quantitative analysis (G) reveals a significant increase in β_2 message in frontal cortex. Asterisks, significantly different than wild type ($P < 0.05$). Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

by this genetically altered mouse. The magnitude of these changes ranged from 25% to 55%, similar to the range that has been reported in mouse models of Rett syndrome (30%), Fragile X syndrome (35%–50%) and lissencephaly (50%) (Samaco et al., 2005; D'Hulst et al., 2006; Valdes-Sanchez et al., 2007), although these studies did not examine potential variability in receptor changes across different brain regions. Altered mRNA levels may reflect changes in the number of GABA_A receptors at the cell surface and/or in the subunit composition of GABA_A recep-

tors. We hypothesize that these changes in mRNA levels reflect alterations in network activity in the cortex and hippocampus. Although the precise molecular mechanisms are currently unknown, previous data suggest that uPAR-mediated signaling influences the differentiation and maturation of GABAergic neurons arising from the medial ganglionic eminence, leading to subtype specific deficits in select regions of the cortex and hippocampus during the period of cortical circuit formation. The subsequent disruption of inhibitory control in more rostral regions of the

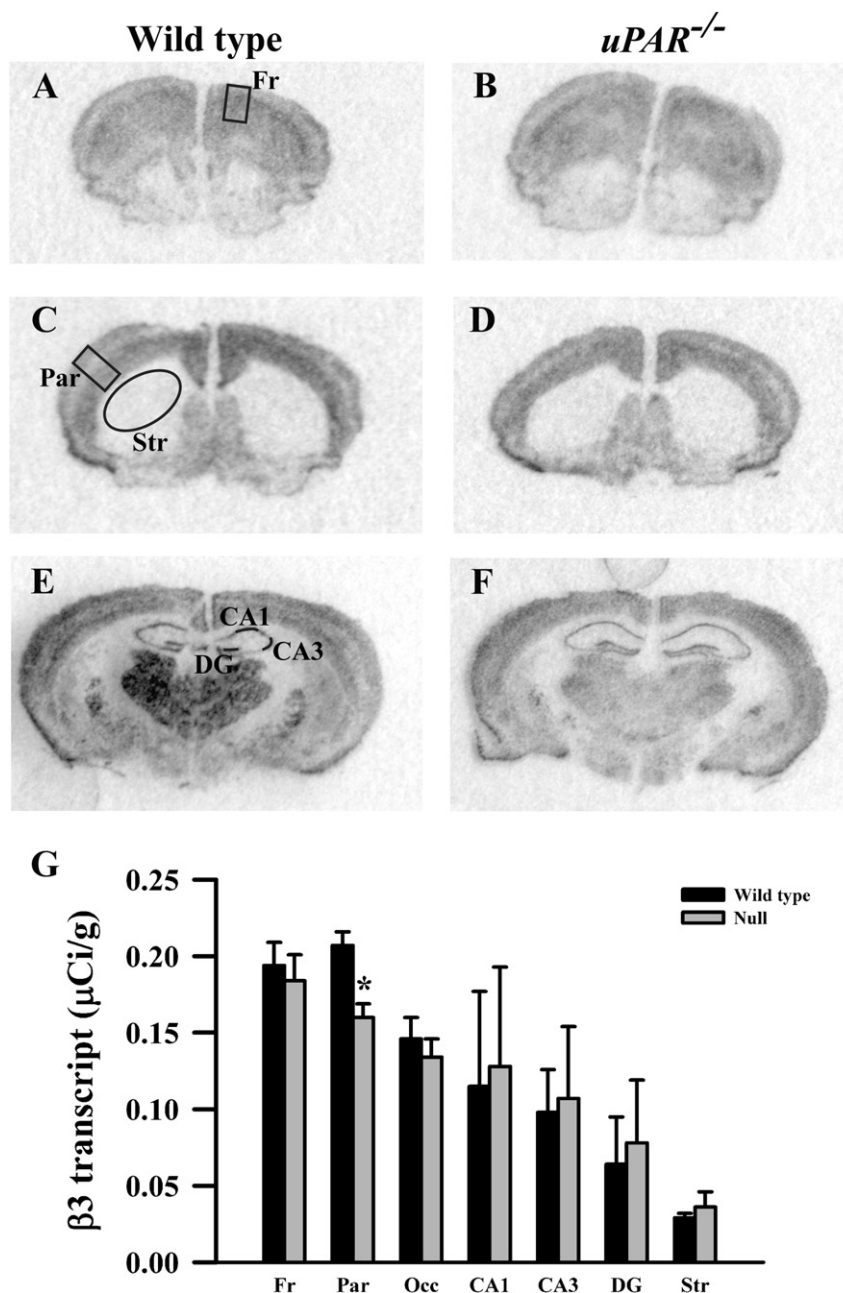


Fig. 5. Film autoradiographs from coronal sections depict expression of the GABA_A receptor β_3 subunit in adult wild type (A, C, E) and *uPAR*^{-/-} (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the different subfields of the hippocampus (E) are indicated. Quantitative analysis (G) reveals a significant decrease in β_3 message in parietal cortex. Asterisks, significantly different than wild type ($P < 0.05$). Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

cortex and the hippocampus likely impacts the development of local circuits in affected regions leading to a regionally-selective adaptive response in GABA_A receptor subunit expression.

Alterations in GABA_A receptor subunit composition have been observed following a variety of environmental challenges in model systems and, when examined, usually accompany changes in receptor pharmacology and physiology. Chronic ethanol or benzodiazepine exposure in rodents leads to region-specific alterations in the expres-

sion levels of discrete receptor subunits [for example; Tseng et al., 1994; Devaud et al., 1995; Holt et al., 1996; Pesold et al., 1997; Tyndale et al., 1997; Chen et al., 1999; Tietz et al., 1999; Raol et al., 2005]. Furthermore, following ethanol exposure *in vitro*, hippocampal neurons demonstrate an altered sensitivity to several benzodiazepine ligands, which is associated with changes in the expression levels of α and γ subunit mRNAs (Sanna et al., 2003). During development, prenatal malnutrition, stress and maternal behavior modulate GABA_A receptor subunit expres-

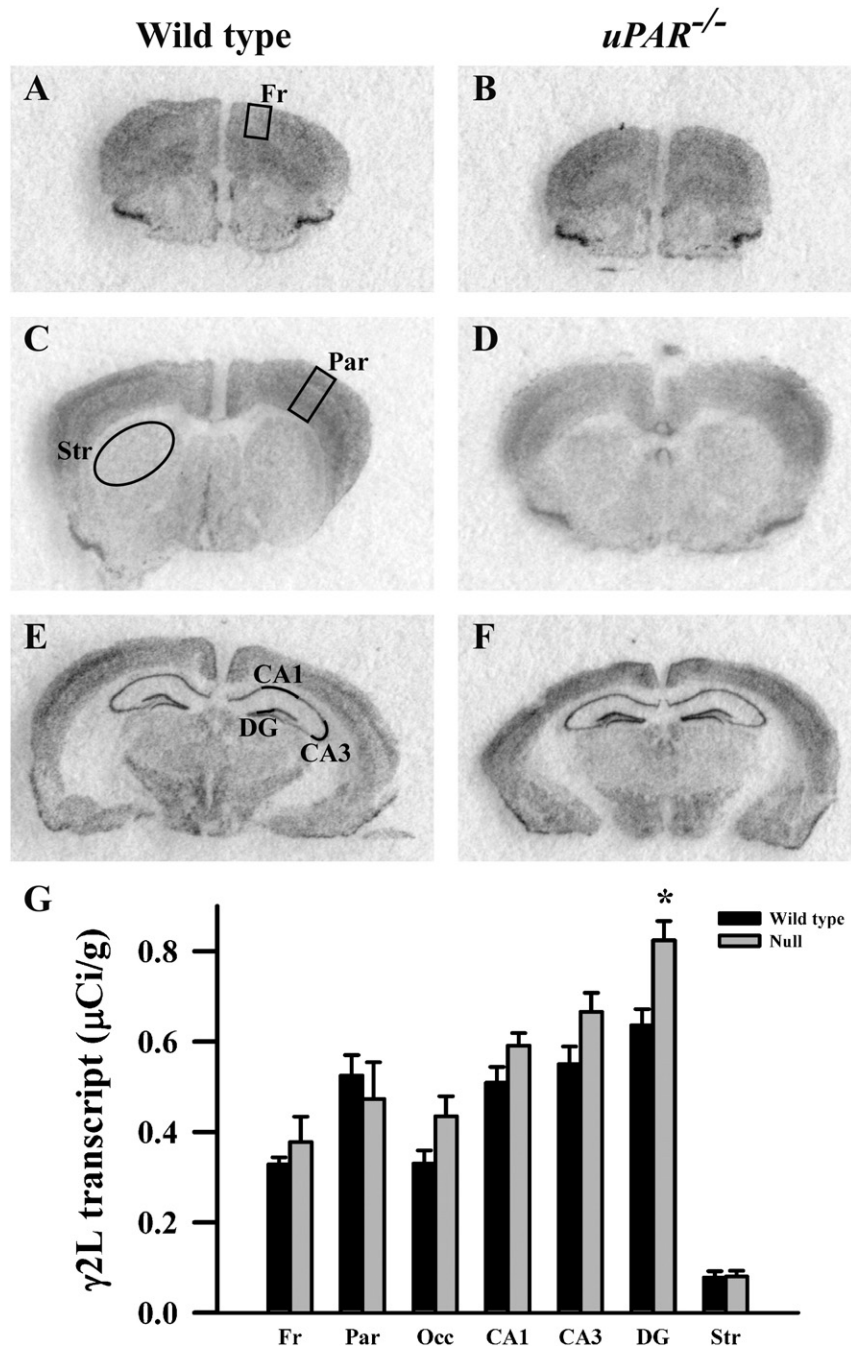


Fig. 6. Film autoradiographs from coronal sections depict expression of the GABA_A receptor $\gamma 2L$ subunit in adult wild type (A, C, E) and *uPAR*^{-/-} (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the different subfields of the hippocampus (E) are indicated. Quantitative analysis (G) reveals a significant increase in $\gamma 2L$ message in the dentate gyrus subfield of the hippocampus. Asterisks, significantly different than wild type ($P < 0.05$). Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

sion, as does prolonged social isolation in adult mice (Steiger et al., 2002, 2003; Caldi et al., 2003; Pinna et al., 2006; Jacobson-Pick et al., 2008). In patients with temporal lobe epilepsy and in animal models of this disorder, there is an increase in several key GABA_A receptor subunits in the dentate granule cell layer, which are thought to reflect axon sprouting and the formation of new circuits in response to increased excitability (Brooks-Kayal et al.,

1998; Nishimura et al., 2005). A reduction in $\alpha 3$ -containing receptors in the cortex and an increase in the $\alpha 2$ subunit in the CA regions also have been reported in this disorder (Loup et al., 2006). The subunit changes observed in temporal lobe epilepsy are reminiscent of, although not identical to, those observed in the *uPAR*^{-/-} telencephalon, in which there are altered levels of excitability. Given that the $\alpha 2$ subunit is localized primarily at the axon initial

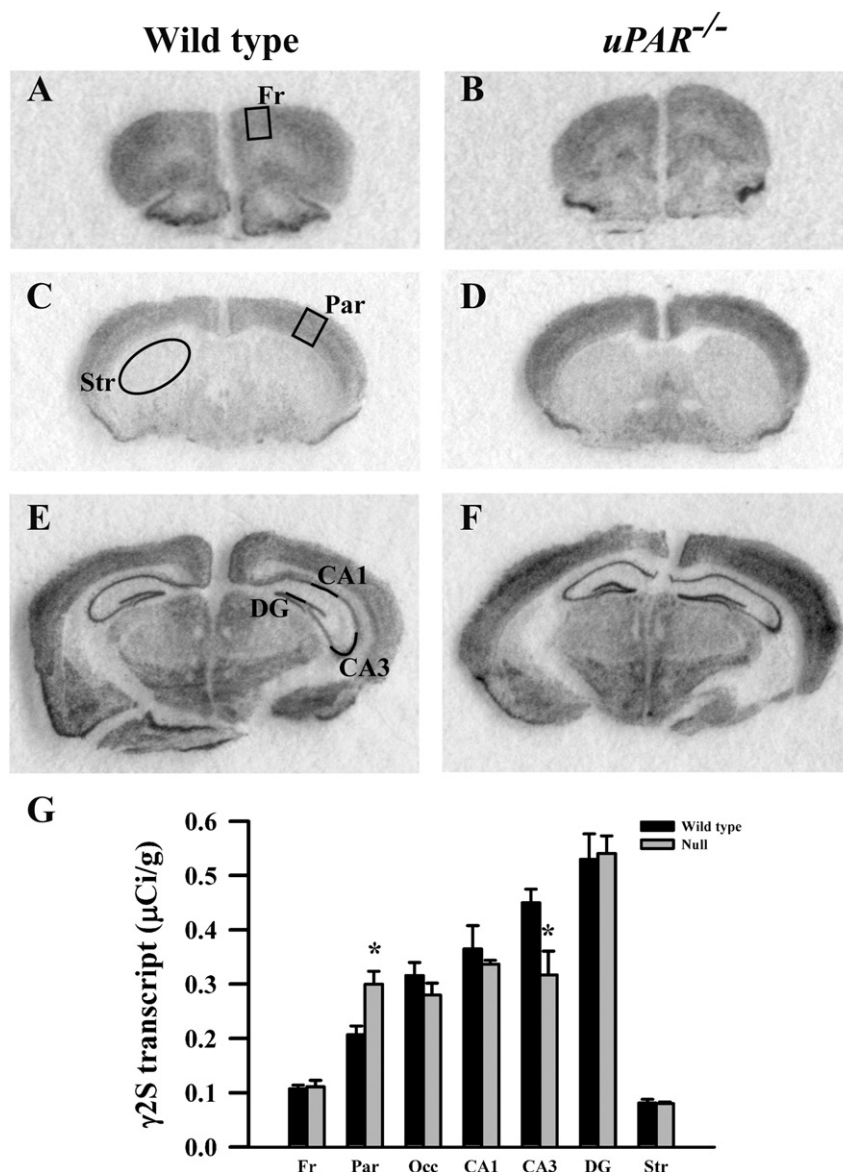


Fig. 7. Film autoradiographs from coronal sections depict expression of the GABA_A receptor γ_{2S} subunit in adult wild type (A, C, E) and *uPAR*^{-/-} (B, D, F) mice. The region of interest analyzed in frontal (A) and parietal (C) cortex, striatum (C), and the different subfields of the hippocampus (E) are indicated. Quantitative analysis (G) reveals a significant increase in γ_{2S} message in parietal cortex, and a decrease in the CA3 subfield of the hippocampus. Asterisks, significantly different than wild type ($P < 0.05$). Fr, frontal cortex; Par, parietal cortex; Occ, occipital cortex; DG, dentate gyrus; Str, striatum.

segment, the potential increase in α_2 containing receptors in the *uPAR*^{-/-} frontal cortex, CA1 and CA3 may reflect an attempt to dampen the output of pyramidal cells in these regions. In addition, the γ_2 subunit is important for targeting the GABA_A receptor to the synapse (Essrich et al., 1998; Schweizer et al., 2003). Thus, the increase in γ_2 subunit gene expression in the parietal cortex and dentate gyrus may, in effect, increase the number of receptors at the synapse in these regions.

Comparison of qPCR and *in situ* analyses

Potential alterations in seven GABA_A receptor subunits were assessed by both qPCR and *in situ* hybridization. With one exception, all changes observed by qPCR were

recapitulated by the *in situ* analyses. In addition, significant changes were revealed by *in situ* hybridization that had not been appreciated by qPCR. This is particularly evident in the hippocampus, and most likely reflects the increased anatomical precision achieved with the *in situ* analysis, where each major subregion (CA1, CA3 and dentate gyrus) was considered independently, compared to the qPCR, where the hippocampus was considered a single entity. Similarly, the ability to detect changes in α_3 and β_3 subunit mRNA expression in parietal cortex by *in situ* hybridization that are not apparent by qPCR is likely a consequence of the ability to analyze a more circumscribed region of cortex in the former (primary somatosensory only) compared to the latter (primary and secondary

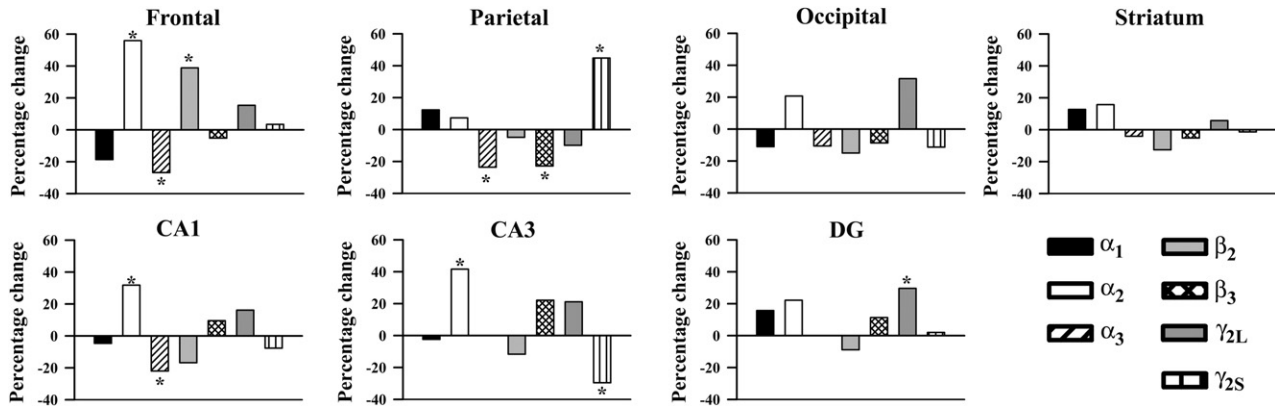


Fig. 8. Percentage change in the expression of the α_1 , α_2 , α_3 , β_2 , β_3 , γ_{2S} , and γ_{2L} subunits of the GABA_A receptor in *uPAR*^{-/-} mice compared to wild type in seven regions of the telencephalon (same data as in Fig. 1–7). Note that the pattern of change varies across different regions of the *uPAR*^{-/-} telencephalon. Asterisks, significant difference between wild type and null.

somatosensory). Further analyses will need to be done using both methods in new cohorts of mice to reconcile the apparent increase in α_3 subunit expression observed by qPCR in frontal cortex with the decrease in the same subunit seen by *in situ* hybridization.

Alterations in the $\gamma_{2L}:\gamma_{2S}$ subunit mRNA ratio

The γ_2 subunit is the predominant γ isoform expressed throughout the brain. The two alternatively spliced variants differ only by the addition of eight amino acids, which include a protein kinase C (PKC) phosphorylation consensus sequence, in the γ_{2L} form (Whiting et al., 1990). In addition, both of the splice variants can be phosphorylated by PKC on serine 327 (Krishek et al., 1994). Thus, because the two splice variants possess a differential capacity to be phosphorylated by PKC and PKC-induced phosphorylation negatively modulates GABA_A receptor function, it has been suggested that an alteration in the $\gamma_{2L}:\gamma_{2S}$ subunit ratio leads to a change in GABA_A receptor function (Moss et al., 1992). Consistent with this, mice in which the γ_{2L} subunit is deleted exhibit an increased level of anxiety and increased sensitivity to benzodiazepines (Quinlan et al., 2000). Region-specific increases in mRNA levels of the γ_{2S} , but not the γ_{2L} , subunit have been reported in animals following ethanol treatment (Devaud et al., 1995), while the $\gamma_{2L}:\gamma_{2S}$ mRNA ratio is altered in cultured cerebellar neurons after pentobarbital treatment (Tyndale et al., 1997). In the *uPAR*^{-/-} mouse telencephalon, there seems to be a differential adaptation in the gene expression of γ_{2S} and γ_{2L} subunits, such that there is an increase in mRNA levels of the γ_{2S} subunit in parietal cortex and in the γ_{2L} subunit in the dentate gyrus, with no compensatory change in the other variant.

GABA_A receptor expression and neurodevelopmental disorders

Genetic and postmortem studies have indicated altered GABAergic interneuron development and receptor expression in several neurodevelopmental disorders. Perhaps the most compelling evidence, coming from multiple stud-

ies, is for schizophrenia, with most attention focused on the prefrontal cortex. Here, as in the *uPAR*^{-/-} frontal cortex, there is a reduction in GAD67 expression, with the parvalbumin-expressing population particularly affected (Volk et al., 2000; Reynolds and Beasley, 2001; Reynolds et al., 2002; Hashimoto et al., 2003). In addition, there is an increase in α_2 subunit expression, detected at both the message and protein level, as well as a reduction in the levels α_1 , γ_2 and δ subunit mRNA, all of which are thought to occur in response to decreased GABA levels (Volk et al., 2002; Hashimoto et al., 2007). Furthermore, a marked decrease in γ_{2S} transcripts, with no significant change in γ_{2L} transcripts, has also been reported (Huntsman et al., 1998). Together, these changes are thought to reflect alterations in the underlying GABAergic circuitry in this brain region and to contribute to the cognitive deficits, in particular those in working memory, in patients with schizophrenia (Lewis and Hashimoto, 2007; Hashimoto et al., 2008).

We identified a functional common polymorphism in the human homologue of the *uPAR* gene, *PLAUR*, which increases the risk for ASD (Campbell et al., 2008). Several authors have postulated that alterations in the GABAergic system may contribute to the etiology of this disorder and the behavioral phenotypes expressed (Rubenstein and Merzenich, 2003; Levitt et al., 2004; Geschwind and Levitt, 2007; Eagleson et al., 2010). Consistent with this, allelic variants and chromosomal inversion in genes encoding GABA_A receptor subunits, including the β_3 subunit, have been associated with ASD (McCauley et al., 2004; Ma et al., 2005; Ashley-Koch et al., 2006; Vincent et al., 2006; Hogart et al., 2007). Furthermore, a reduced expression of the β_3 subunit in the frontal cortex, as well as a decreased density of GABA_A receptors in anterior cingulate cortex, has been reported in patients with ASD (Samaco et al., 2005; Oblak et al., 2009). The current study contributes additional evidence for a GABAergic contribution to ASD, providing an experimental link between an autism susceptibility gene and alterations in GABA_A receptor expression. Alterations in the expression of the GABA_A receptor have also been noted in rarer disorders, including Tuberous

Sclerosis, Prader-Willi, Angelman and Rett syndromes (White et al., 2001; Dan and Boyd, 2003; Lucignani et al., 2004; Samaco et al., 2005; Valencia et al., 2006). Indeed, there is a cluster of three GABA_A receptor subunits (α_5 , β_3 and γ_3) in the chromosomal regions 15q11–q13 that are deleted in most patients with Prader-Willi or Angelman syndromes, and it has been hypothesized that altered neurobehavioral function seen in patients with Prader-Willi could arise directly from an altered GABA_A receptor composition and expression (Lucignani et al., 2004). Similar to patients with ASD, reduced expression of the β_3 subunit of the GABA_A receptor has been reported in subjects with Angelman and Rett syndromes (Samaco et al., 2005). Finally, alterations in GABA_A receptor subunit expression have been observed in mouse models of Rett syndrome, Fragile X syndrome and lissencephaly (Samaco et al., 2005; D'Hulst et al., 2006; Valdes-Sanchez et al., 2007).

The present study demonstrates that in the telencephalon of *uPAR*^{−/−} mutant mice, the gene expression levels of six GABA_A receptor subunits are altered in a region-specific manner. The regional and subunit specificity that we report here will help focus examination of potential parallel changes in clinical samples. Moreover, the documentation of these changes lays the foundation for determining whether an alteration in the subunit composition of GABA_A receptors may underlie, at least in part, the abnormal behavioral repertoire of *uPAR*^{−/−} mutant mice. The role that adaptive gene regulation plays in GABA_A receptor subtype expression, and how this may contribute to genomic control over interneuron development, will be pursued in the future.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neuroscience.2010.03.066](https://doi.org/10.1016/j.neuroscience.2010.03.066).