Expression of the G72/G30 gene in transgenic mice induces behavioral changes

Lijun Cheng (M.D., Ph.D.), Eiji Hattori (M.D., Ph.D.), Akira Nakajima (Ph.D.), Nancy Woehrle (Ph.D.), Mark D. Opal (B.A.), Chunling Zhang (M.S.), Kay Grennan (Ph.D.), Stephanie C. Dulawa (Ph.D.), Ya-Ping Tang (M.D., Ph.D.), Elliot S. Gershon (M.D.) and Chunyu Liu (Ph.D.)

From the Department of Psychiatry, Institute of Human Genetics, The University of Illinois at Chicago, Chicago, IL 60607 (LC, CZ and CL), the Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama 351-0198, and Sangenjaya Station Mental Health Clinic, Tokyo 154-0024, Japan (EH), the Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, 466-8560 Japan (AN), the Department of Psychiatry and Behavioral Neuroscience, The University of Chicago, Chicago, IL 60637 (NAS, MDO, KG, SCD, ESG), and the Department of Cell Biology and Anatomy, Louisiana State University Health Sciences Center, New Orleans, LA 70112 (YPT).

Corresponding author:
Chunyu Liu, Ph.D.
900 S. Ashland Ave. Room 1006
Chicago, IL 60607
Office Phone: 312-413-2599
Fax: 312-355-1741

E-mail: liucy@uic.edu

This work was supported by 1R21MH083521 and 4R33MH083521 (to CL)**.** The views expressed in this manuscript are not necessarily those of NIH.

Abstract

The G72/G30 gene complex is a candidate gene for schizophrenia and bipolar disorder. However, G72 and G30 mRNAs are expressed at very low levels in human brain, with only rare splicing forms observed. We report here G72/G30 expression profiles and behavioral changes in a G72/G30 transgenic mouse model. A human BAC clone containing the G72/G30 genomic region was used to establish the transgenic mouse model, on which gene expression studies, Western blot and behavioral tests were performed. Relative to their minimal expression in humans, G72 and G30 mRNAs were highly expressed in the transgenic mice, and had a more complex splicing pattern. The highest G72 transcript levels were found in testis, followed by cerebral cortex, with very low or undetectable levels in other tissues. No LG72 (the long putative isoform of G72) protein was detected in the transgenic mice. Whole-genome expression profiling identified 361 genes differentially-expressed in transgenic mice compared to wild-type, including genes previously implicated in neurological and psychological disorders. Relative to wild-type mice, the transgenic mice exhibited fewer stereotypic movements in the open field test, higher baseline startle responses in the course of the prepulse inhibition test, and lower hedonic responses in the sucrose preference test. The transcriptome profile changes and multiple mouse behavioral effects suggest that the G72 gene may play a role in modulating behaviors relevant to psychiatric disorders.

Key words: G72/G30; transgenic mouse model; gene expression; stereotypic behavior; hedonic response; psychiatric disorders

Introduction

Several linkage studies and meta-analyses support a broad region on chromosome 13q32-q34 as genetically linked and associated with schizophrenia (SZ) and bipolar disorder (BD) (1-4). Chumakov *et al.* interrogated a 5 Mb region on 13q33 and identified a 65 Kb region that harbored significant SZ association signals in two sample sets (5). Within the associated region, a pair of sense and anti-sense genes, G72 and G30, were identified by reverse transcription-PCR (RT-PCR) (5). This region's association with SZ and BD has been replicated in multiple studies, although negative results exist as well (6-9). Our meta-analysis supported the association with SZ in Chinese samples but not in Europeans (10). The G72/G30 gene complex is regarded as an important candidate for SZ (11;12), BD (13;14), or both (11;15-18). G72 genotypes have also been associated with brain function in humans during verbal fluency tests (19;20), and with brain structure changes in bipolar patients (21).

No gene homologous to G72 has been found in any non-primate species, including mouse (5). Furthermore, no conserved functional domains are found in any predicted G72 protein sequences. G72 is expressed at very low levels in human brain. Six different splicing forms have been previously identified in various parts of the human brain, spinal cord, and testis (5;22).

To date, two hypotheses regarding the function of G72 have been proposed: the G72–D-amino acid oxidase-*N*-methyl-D-aspartate (G72–DAAO-NMDA) receptor hypothesis and the G72-mitochondrial hypothesis. The original G72–DAAO-NMDA receptor hypothesis was based on *in vitro* experiments suggesting that LG72 (a long putative isoform of G72 protein) binds to and activates DAAO, which oxidizes D-serine, an endogenous ligand of the NMDA receptor. These observations linked G72 to the glutamate signaling hypofunction theory of schizophrenia (23) and indicated a possible role for LG72 in the regulation of NMDA-type glutamate receptors in the human brain (5).

However, the activator function was challenged in a later study, which found an inhibition rather than an activation of DAAO activity by LG72 (24). In contrast to these two studies, Kvajo *et al*. failed to confirm any functional interaction at all between G72 and DAAO in two different cell lines (25). Instead, their transfection experiments in multiple mammalian cell lines and in rat primary neurons indicated that the LG72 isoform encodes a mitochondrial protein that can promote mitochondrial fragmentation and dendritic arborization. Therefore, they proposed a connection between the LG72 gene and mitochondrial functionality. These inconsistent *in vitro* results suggest that the actual biological mechanisms of G72 remain to be further explored and fully clarified.

To address the *in vivo* expression and behavioral effects of G72, Otte *et al*. established a G72/G30 BAC (bacterial artificial chromosome) transgenic mouse model. They reported that G72 expression was highest in the granular cells layer of the cerebellum, dentate gyrus, and olfactory bulb. They also reported phenotypic changes that included reduced mitochondrial complex I activity and synaptic defects, impairments in spatial memory and prepulse inhibition, a higher sensitivity to phencyclidine, increased compulsive behaviors, and deficits in motor coordination and smell identification (26;27).

To investigate the gene's effect on phenotypes, including whole genome expression profile and behaviors, we independently developed a G72/G30 BAC transgenic mouse model using a different BAC clone and different mouse strain than Otte *et al*. First, we performed a thorough search for G72/G30 transcripts by RT-PCR, cloning and sequencing, then measured G72/G30 levels in multiple tissues and brain regions using TaqMan quantitative PCR (qPCR). We used Illumina's Sentrix Mouse-6 Expression BeadChip to profile whole genome expression in the mouse cortex to study the response of the mouse transcriptome to the introduced gene. To assess to what extent the transgenic mouse reflects G72/G30 expression in human, we also assayed G72/G30 transcripts in multiple human tissues and brain regions (*Supplemental*

Information). We also tested for LG72 protein expression in the transgenic mice by Western blot. Finally, we examined the behavioral phenotypes of these mice using a battery of behavioral tests.

Materials and Methods

Generation of a G72/G30 transgenic (Tg) mouse model

We used a human BAC clone, RP11-166E2 (GenBank: AL359751), from Children's Hospital Oakland Research Institute (CHORI, Oakland, CA) to make the transgene. This clone contained 117,068 bp of genomic region encompassing the entire G72/G30 gene complex *(Supp. Figure 1).* The BAC clone was microinjected into the fertilized ova of embryos from a female B6CBAF1/J mouse (The Jackson Laboratory, Bar Harbor, ME). Microinjection and generation of transgenic mice were performed by the Transgenics/ES Cell Technology Mouse Core Facility at The University of Chicago. Mouse tail DNA extraction, genotyping, and animal care are described in *Supplemental Information*.

RNA isolation, RT-PCR and T-A cloning

Transgenic mice were sacrificed at five different postnatal stages: one day, two weeks, one month, two months and five months. They were decapitated for immediate dissection of multiple tissues and brain regions (cerebral cortex, striatum, hippocampus and cerebellum) on dry ice. Total RNA was extracted using the RiboPure Kit *per* the manual instructions (Ambion, Austin, TX).

G72/G30 specific primers, RT-PCR, T-A cloning and sequencing are described in *Supplemental Information*.

G72/G30 TaqMan qPCR

Pre-designed TaqMan gene expression assays for G72 and G30 were obtained from Applied Biosystems (Foster City, CA). Expression levels of G72 and G30 were normalized to 18s ribosomal RNA. Details of qPCR and analysis procedures are described in *Supplemental Information.*

Mouse whole genome expression profile analysis

The Sentrix Mouse-6 Expression BeadChip (Illumina, San Diego, CA) was used to screen for differential expression in the cerebral cortex between G72 Tg and wild-type (WT) mice. These BeadChips have genome-wide coverage of 34,492 genes. Total RNA extracted from the cerebral cortex of 18 adult (2–3 months old) mice (13 Tg, 5 WT) was reverse transcribed to synthesize complementary DNA (cDNA). After *in vitro* transcription to biotin-labeled complementary RNA (cRNA) and hybridization, BeadChips were scanned with the BeadArray Reader (Illumina). The experiments were performed at the Genomics Core facility of Northwestern University. The average signal of each probe was log2 transformed. Batch effects were removed with ComBat (www.bu.edu/jlab/wp-assets/ComBat/Abstract.html) (28). After array-level quantile normalization (Partek software, http://www.partek.com), ANOVA was used to test for differential expression between Tg and WT mice. Functions and Canonical Pathways analysis was performed for differentially expressed genes with Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, Redwood City, CA). SYBR Green qPCR was used to validate the differential expression between Tg and WT mice for several mouse genes including *Dlg4* (Discs, large homolog 4 (Drosophila)), *Kcne1* (Potassium voltage-gated channel, Isk-related subfamily, member 1), *Pcdh8* (Protocadherin 8, transcript variant 2) and *Psap* (Prosaposin, transcript variant 2). Details of SYBR Green qPCR are described in *Supplemental Information.*

Mitochondrial isolation, whole cell protein preparation, and Western blot

We looked for LG72 protein in both mitochondrial fractions and whole cell lysates from Tg mouse tissues with Western blot assay.

Mitochondria were isolated from mouse tissues using the Mitochondrial Isolation Kit for Tissue from Pierce (Rockford, IL). Mitochondrial pellets ranging from 40-50 µg were solubilized in 1% *n*-dodecyl β-maltoside (Sigma-Aldrich, St. Louis, MO, USA) in PBS and run on 4-12% gradient NuPage gels (Invitrogen, Carlsbad, CA, USA). Mitochondrial proteins extracted from G72, G72-myc, and empty vector (mock) transfected Cos-7 cells were used as controls, and were gifts from Dr. Mirna Kvajo and Dr. Joseph Gogos (25). Western blot was performed as previously described (25). After incubation with the second antibody and washing, the blot was developed using highly sensitive Western Lightning Plus-ECL reagent (PerkinElmer, Shelton, CT) and exposed to film. Antibodies used were rabbit anti-prohibitin (1: 2,000, Abcam, Cambridge, MA, USA) and purified rabbit polyclonal-anti G72 antibody (1:100), a gift from Kvajo and Gogos (25). This antibody was raised against amino acids (aa) 51-69 of the LG72 protein and its specificity was determined as described in Kvajo *et al.*'s study (25).

For cell total lysate preparation, mouse tissues were homogenized in cold RIPA (radio-immunoprecipitation assay) buffer with PMSF (phenylmethylsulfonyl fluoride) and protease inhibitor on ice using a Kotes homogenizer. After sonication and centrifugation, 80-90 µg of supernatant was loaded into a NuPage gel. Rabbit polyclonal-anti G72 (1:100) (gift from Kvajo and Gogos), and mouse anti-GAPDH (1:10000, Abcam) were used. Other anti-G72 antibodies used included LG72 N-terminal and C-terminal antibodies (chicken anti LG72 4-16 aa and 114-125 aa, respectively; a gift from Dr. Husseini Manji, NIMH), and a purified goat polyclonal antibody against N-terminal of LG72 (sc-46118, Santa Cruz Biotech, CA).

Behavioral Testing

We used five behavioral tests to assess the effect of G72/G30 on behavioral traits with potential relevance to SZ.

1. The open field test was used to measure general locomotor activity and stereotypic behaviors.

2. Prepulse inhibition (PPI) testing was used to evaluate the startle response and integrity of sensorimotor gating mechanisms.

3. The sucrose preference test was used as a measure of anhedonia.

4. The Morris water maze was used to test for spatial learning and memory deficits.

5. The modified Forced Swim Test (FST) was used to assess depression-like behavior.

A detailed description of these tests can be found in *Supplemental Information*.

Statistical Analyses

To determine the genotypic effects on G72 expression and behavioral responses, a two-way analysis of variance (ANOVA) was used, followed by a *post hoc* analysis or the Student's t-test where appropriate. Repeated measures ANOVA were used when data were collected in multiple trials. All data are expressed as the mean ± SEM (standard error of the mean). A p value less than 5% was considered a significant difference. For analyses specific to experiments, see *Supplemental Information*.

Results

G72 and G30 transcripts were highly expressed in the Tg mice, with multiple alternative splicing isoforms identified

We established the Tg mouse model carrying the human BAC clone RP11-

166E2. Pulse-field gel electrophoresis and PCR of fragments at both ends and in the middle of the BAC clone verified that the BAC had been incorporated into the founder mice (data not shown). The Tg mice were fertile and appeared healthy. Stable transgene transmission between generations was detected using qPCR (data not shown).

The primer pairs 1F2/R2, 1-2-3F/R and P1/P2 were used for RT-PCR, with Tg mouse brain cerebral cortex RNA used as template. The further cloning and sequencing results indicated that the six human G72 forms also existed in the Tg mouse cerebral cortex (data not shown). Furthermore, thirteen novel splicing forms were identified in the mouse cerebral cortex; four of these we also detected for the first time in the human testis and brain tissue (GenBank: DQ386869, DQ386870, DQ343761 and DQ357223) (Figure 1B, *Supp. Table 5*). The remaining nine novel isoforms were only observed in the Tg mouse cerebral cortex (G72-Tg1 to G72-Tg9, GenBank: JN413793 – JN413801, Figure 1B). They were identified through various combinations of primer pairs as described in *Supp. Table 1*.

The total nineteen splice variants defined ten exons (Figure 1B, C). Based on the inclusion level of each exon (29;30), transcripts generally contained major-form exons 3, 4, 6, 9, and 10, with the variable inclusion of minor-form exons 1, 2, 5, 7 and 8. In addition, exon 6 is included in all variants, while exon 5 is rare and only present in variant 3 (Figure 1B). Furthermore, on the basis of the variants we have seen, three modes of alternative splicing were identified for G72 transcripts: exon skipping, alternative 3' end, and intron retention (30) (Figure 1C).

TaqMan qPCR indicated that G72 mRNA was expressed the most in testis, followed by cerebral cortex , and expressed least in lung and heart; it was undetectable in other tissues (kidney, spleen, *etc*.) *(Supp. Table 2).*In brain, G72 was expressed at its highest level in cerebral cortex, followed by striatum and hippocampus, and was almost undetectable in cerebellum (Figure 2). In cerebral cortex, no significant differences in

G72 expression were observed among five postnatal time points (one day, two weeks, one month, two months and five months) (data not shown), indicating that G72 expression in the brain of Tg mice is not developmentally regulated. No significant difference in G72 expression was observed in cerebral cortex between male and female mice (data not shown).

RT-PCR and electrophoresis indicated strong G30 expression in the Tg mouse cerebral cortex with primer G30 F2/G30 R only. A novel long exon (3453 bp), created by the retention of the intron (1712 bp) between exons 6 and 7, was identified by sequencing. Similar to G72, qPCR detected a relatively high G30 expression level in the Tg mice cerebral cortex and testis, lower levels in striatum and hippocampus, and none in cerebellum *(Supp. Table 2)*.

Differentially-expressed genes were associated with genetic disorders and psychological disorder categories

After a one-way ANOVA, 6,472 probes showed differential expression in cerebral cortex between Tg (n=13) and WT (n=5) mice ($p \leq 0.05$); 371 probes survived multiple test correction with a false discovery rate (FDR) of $q \leq 0.05$. These 371 probes represented 361 individual genes (*Supp. Table 3*). Ingenuity Pathways Analysis indicated many of these differentially-expressed genes were associated with neurological disease, genetic disorders and psychological disorder categories (*Supp. Table 4*). These genes included several possible candidate loci such as DLG4 (synonym: postsynaptic density protein 95, PSD95) (31), PCDH8 (32), PSAP (33), CLDN5 (Claudin 5) (34;35), DPYSL2 (Dihydropyrimidinase-like 2) (36) for SZ, GRIN2B (glutamate receptor, ionotropic, NMDA2B (epsilon 2)) (37;38), HTR2C (5'-hydroxytryptamine (serotonin) receptor 2C) (39;40) for both SZ and BD, and ATP1A3 (ATPase, Na+/K+ transporting, alpha 3 polypeptide) (41), DUSP6 (Dual specificity phosphatase 6) (42),

KCNQ2 (potassium voltage-gated channel, subfamily Q, member 2) (43) for BD (*Supp. Table 3*). The differential expression levels of some genes between Tg and WT mice were validated with SYBR Green qPCR (*Supp. Figure 2*).

No native LG72 protein detected in Tg mice

We validated the anti-G72 antibody described by Kvajo *et al*. (25) for Western blot. This antibody readily detected specific LG72 protein signal in mitochondria of G72 and G72-myc transfected Cos-7 cells, and showed no specific signal in mitochondria of mock transfected cells (Figure 3A, left panel). The apparent molecular weight of G72 was ~18 kDa, while G72-myc was slightly heavier.

We then performed Western blot on mitochondrial extracts from cerebral cortex, testis and cerebellum of Tg mice and WT controls. To maximize the possibility of detecting even weak signals, we loaded 40-50 µg of mitochondria and 80-90 µg of total lysate on the gel, and developed the blot using the highly sensitive detection reagent Plus-ECL (benefits include low 1 - 10 picogram protein sensitivity level, and that signal is maintained for over two hours) in combination with long exposure time (>10 min). Even so, no specific signal for endogenous LG72 protein was observed in either mitochondrial fractions or whole cell total lysate at the expected molecular weight of ~18 kDa, even in cerebral cortex and testis (Figure 3A, right three panels; Figure 3B) where high expression of G72 mRNA was detected by qPCR.

We tested three other available anti-G72 antibodies with whole cell lysate of Tg mouse tissues and could not detect G72 protein expression in any of the samples (data not shown).

Tg mice exhibit fewer stereotypic movements in the open field test

We used open field test to assess general locomotor activity and stereotypic

behaviors of the Tg mice. We found Tg mice spent significantly less time in stereotypic behavior (small repetitive movements with no obvious goal) [$F(1, 79) = 5.27$; $p=0.02$] compared with controls (Figure 4A). There was no significant difference in total distance traveled [$F(1, 79) = 3.01$; $p = 0.09$] (Figure 4C) and ambulatory time [$F(1, 79) = 2.99$; $p = 0.09$] (Figure 4D) between groups, indicating that Tg mice had no deficit in general motor activity. No significant difference between groups was observed in time spent in central and peripheral areas [$F(1, 79) = 0.16$; $p = 0.69$] (Figure 4E) or in resting time [$F(1, 79) = 0.45$; $p = 0.5$] (Figure 4F).

To confirm the phenotype of fewer stereotypic behaviors observed, we used a new batch of Tg mice with controls in another activity tracking system. The tracking data collected from 15 and 30 minutes after the start of the experiment were used. The stereotypy count of Tg mice was significantly less than controls in the 15 minute period [$F(1, 27) = 4.46$; $p=0.04$] and even less in the 30 minute period [$F(1, 27) = 6.12$; $p=0.02$] (Figure 4B). As above, there was no significant difference between Tg and WT mice for other measurements like ambulatory distance, ambulatory time, *etc.* (data not shown).

Tg mice show higher baseline startle responses in the course of PPI test

Base startle reactivity in block 1 (six consecutive PULSE ALONE 120 dB bursts) was significantly higher in Tg mice compared to WT [$F(1, 26) = 6.69$; $p=0.02$] (Figure 5A). The lack of genotype x block interaction [$F(3, 78) = 0.48$; $p=0.70$] revealed that there were no differences between Tg and WT mice in terms of habituation, which is defined as the decrease of startle response from block 1 to 4 (the slope of the curve) (Figure 5B). No effect of genotype on PPI was found [$F(1, 26) = 0.29$; $p=0.60$] (Figure 5C).

Tg mice had lower hedonic responses in the sucrose preference test

Compared to WT, the Tg mice consumed significantly more water [$F(1, 39) = 8.00$; $p=0.007$] (Figure 6A) and marginally less sucrose [$F(1, 39) = 3.58$; $p=0.07$] (Figure

6B). The sucrose preference of the Tg mice was significantly lower than WT [$F(1, 39) = 7.03$; $p = 0.01$] (Figure 6C) across all sucrose concentrations. These data suggest that hedonic responses were reduced in G72/G30 Tg mice and that reward pathways may be affected.

Results for the Morris water maze and the modified Forced Swim Test were negative (see *Supplemental Information*).

Discussion

Our study indicated that the transgenic mice we established over-expressed G72/G30 relative to humans, where they are expressed at extremely low levels. It also demonstrated the altered expression of genes involved in neurological and psychological disorders, through a whole genome expression BeadChip study. No LG72 protein was detected in Tg mice. The behavioral changes in Tg mice suggested that the G72 gene does play a role in modulating behaviors.

G72 and G30 are very low abundance transcripts in human tissues (5;22), and the *in vivo* expression profiles and functions of G72/G30 are not well-delineated. To address the *in vivo* expression and behavioral effects of the G72/G30 gene complex, we independently generated G72/G30 BAC transgenic mice using a human BAC clone, RP11-166E2, which is available from a public library. This BAC clone retained the whole structure of G72/G30 gene (introns and exons) and upstream/downstream flanking regions, which may include native regulatory *cis* elements (*Supp. Figure 1*). We further assayed G72/G30 transcripts in both Tg mice and human, and compared expression levels and splicing forms between the two (*Supplemental Information* and Figure 1).

The transgenic mice over-expressed G72 and G30 transcripts relative to their extremely low level in humans. In Tg mice, we found G72 and G30 expressed at the highest levels in testis, followed by cerebral cortex, and very low or undetectable levels

in cerebellum and other tissues. To some extent, this pattern mimics the G72/G30 expression profile in human tissues (*Supp. Tables 2 and 5*). This expression pattern is contrary to the observation in Otte *et al.'s* transgenic mice, where G72 expression was highest in the cerebellum; however, in that study, a different BAC clone (RP11-111A8) and mouse strain (CD1) were used (26). In our Tg mouse cerebral cortex, the six known human G72 forms were detected, plus 13 novel splicing variants were identified. The total nineteen variants defined a total of ten exons and three modes of alternative splicing for G72 transcripts, which may indicate a more complex splicing profile and pattern in Tg mice than in human (Figure 1). However, these novel variants may have been detected because of the overall elevated G72 expression levels in Tg mice, *i.e.,* these forms may exist in humans, but at levels below the detection threshold.

Questions about the expression and physiological role of the predicted LG72 protein have not been resolved, and it is not clear that it actually exists in human brain. The original proposal of a direct G72 and DAAO interaction has not been confirmed in endogenous tissues, is inconsistent with later reports (24;25), and is challenged by an alternative G72-mitochondrial hypothesis.

Using Western blot, we investigated LG72 protein expression in the organs of our Tg mice. Using the same antibody as Kvajo *et al.*, plus other available antibodies, in combination with sensitive detection procedures, we were unable to detect native LG72 protein in either the mitochondrial fraction or whole cell protein lysates. These negative results are in line with Western blot results from Benzel *et al.*'s systematic search for LG72 protein in various human tissues. The antibody generated for Benzel *et al.*'s study, antibody #1411, is very similar to the antibody used by Kvajo *et al.* and us, because a very similar epitope was used to generate the two (25;44). Using Western blot with a range of sensitive detection methods, Benzel *et al.* failed to detect native G72 protein in a variety of human brain regions, including cerebellum and amygdala, or in spinal cord

and testis *in vivo* (44).

The G72 antibody #1411 used in Benzel *et al.*'s study was also used by Otte *et al.* in their Tg mice. They reported detection of LG72 protein in cerebellar protein lysate separated on a two-dimensional polyacrylamide gel. Subsequently, they detected it in the mitochondrial fraction from *in vitro* transfected mouse neuroblastoma N2a cells. However, they did not present *in vivo* evidence of LG72 protein in their Tg mouse mitochondria (26;27). The discrepancy between the G72 Western blot results from Otte's and our mouse models could be resolved by a side-by-side comparisons of G72 protein expression in the two models under the same experimental conditions.

Taken together, the lack of demonstrable LG72 protein *in vivo* expression in our Tg mice and in human brain cannot support the existing G72 functional hypotheses.

High expression of G72/G30 in Tg mouse cerebral cortex was associated with changes in the transcriptome. Several genes previously implicated in SZ and BD showed significant differential expression between Tg and WT mice. Tg mice also exhibited some behavioral changes that may be related to clinical psychiatric phenotypes. For example, in open field explorations, Tg mice exhibited significantly fewer stereotypic behaviors than WT mice. Stereotypy, a common feature of SZ, is characterized by repetitive, unvarying and functionless behaviors which usually appear in the later stages of disease (45-47). Since diverse neurotransmitters, such as dopamine, serotonin and opioids, have been proposed to be involved in the stereotypies (48;49), it remains to be determined if the pathways of these neurotransmitters underlie the decreased stereotypic behaviors in Tg mice.

Compared with WT mice, Tg mice showed significantly higher baseline reactivity in the course of the PPI test. In addition, Tg mice showed decreased sucrose preference relative to WT in the sucrose preference test, indicating an anhedonia phenotype. Anhedonia, the reduced ability to experience pleasure, has always been regarded as a

core negative symptom of both SZ and depression (50). Thus, Tg mice exhibited some behavioral phenotypes that have relevance to SZ and mood disorders.

Some genes differentially expressed between Tg and WT mice were found to be correlated with some mouse behavioral phenotypes. For instance, *Dlg4* (synonym: PSD95) was overexpressed in Tg mouse model, which exhibited significantly fewer stereotypic behaviors than WT mice, while Feyder *et al.* reported that mice with *Dlg4* deletion ($Dlg4^{-/-}$) displayed significantly increased repetitive and stereotypical behaviors than $Dlg4^{+/+}$ WT mice did (51). In humans, DLG4 encodes the PSD95 protein, a member of the membrane-associated guanylate kinase family of synaptic scaffolding molecules that control the organization, composition, and function of synapses (52). DLG4 was associated with the susceptibility of SZ in Han Taiwanese SZ patients (31), and DLG4 mRNA expression was significantly decreased in Brodmann area 9 (B9) of the prefrontal cortex (PFC) of individuals with SZ compared with controls (53). Collectively, these findings indicated that the expression level of DLG4 may have a reverse correlation with the stereotypy in SZ, although the neural basis of DLG4 involvement in the abnormal stereotypic behaviors in Tg mice is still to be determined.

We also observed that the expression of the potassium channel gene KCNE1 was correlated with startle response phenotype. In humans, the copy number variants in the KCNE1 gene region identified this gene as a novel locus involved with SZ (54). Meanwhile, *Kcne1* knockout mice showed absence of reflex or even signs of startle response to loud noise (55;56). *Kcne1* was overexpressed in our Tg mouse model, which also had a significantly higher baseline startle response. The molecular interconnections between the *Kcne1* expression level and startle response need to be further clarified.

This G72/G30 mouse model presents us with multiple future research directions. We would like to determine the link between G72 expression and the transcriptome

changes in the Tg mice. To understand the molecular mechanisms underlying the behavioral changes, we could test the Tg mice's cerebral cortex for altered levels of neurotransmitters and other molecules involved in stereotypy, startle response, and reward pathways. With the lack of *in vivo* LG72 protein expression in our Tg mice and in human brain, we would be interested in testing whether G72 could play a regulatory role as a non-coding RNA gene.

Acknowledgment

*This work was supported by 1R21MH083521 and 4R33MH083521 (to CL)***.**

We are grateful to Dr. Mirna Kvajo and Dr. Joseph A. Gogos at Columbia University, and Dr. Guang Chen and Dr. Husseini K. Manji at NIMH for anti-G72 antibodies. We thank Dr. Cristianne R. M. Frazier, Dr. Jeff A. Beeler, and Dr. Xiaoxi Zhuang at The University of Chicago for technical assistance and data analysis of the mouse sucrose preference test.

Conflict of interest

The authors reported no biomedical financial interests or potential conflicts of interest.

The sequences of thirteen novel G72 splicing forms identified in human and transgenic mouse model have been accepted by NCBI GenBank (www.ncbi.nlm.nih.gov/genbank), with accession numbers as indicated in Figure 1B.

Supplementary information is available at *Molecular Psychiatry*'s website.

Table/Figure Legends

Figure 1. Summary of G72 alternative splicing forms and patterns of splicing in Tg mice and humans

(A) Schematic representation of the genomic location and structure of the human G72 gene. The G72 gene locus maps to the human chromosome band 13q33.2. The continuous line with arrows represents the genomic sequence, on which all ten splicing exons of G72 are shown as small rectangles and are numbered as the same as in panel (C). **(B)** Exon structure of G72 transcripts. The exons are shown as rectangles. Six known isoforms: LG72, SG72 and variants 1 to 4, were expressed in humans as reported in previous studies (5;22). By using RT-PCR and T-A cloning, we identified nine novel G72 splicing forms (G72-Tg1 to 9) in the cerebral cortex of Tg mice (JN413793 to JN413801, indicated by $^{\Delta}$). In these unique mouse forms, two were identified through G72 gene specific RT-PCR (G72-Tg3 and G72-Tg4,) and five (G72-Tg5 to 9) were identified through PCR detection of the longest transcript using G72 P2/G30 nest R primers. These five forms have a novel common 5' terminal 75 bp exon starting 3.5 kb earlier than the first exon previously reported. We also identified four novel G72 splicing forms (G72-7 to 10) in human testis (DQ386869, DQ386870, indicated by ^) and brain tissues (DQ343761, DQ357223, indicated by asterisk), as described in detailed in *Supplemental Information*. Taken together, nineteen G72 splicing forms defined a total of ten possible exons. GenBank accession numbers are indicated in brackets. **(C)** Patterns of alternative splicing of G72 gene. The splicing of 19 forms defined a total of ten possible exons, labeled as exon 1 ~10 and aligned with corresponding one in panel (B). The introns, shown as lines between exons, are not drawn to scale due to the size limit of the figure. The solid vertical lines in exon 2, 3, 4 and 9 indicate the variable length of each exon. Three basic modes of alternative splicing were identified for G72 mRNA and illustrated: 1) Exon skipping: exon 3, 5, 7, and 8. 2) Alternative 3' end: happened to exon 4 and 9. In this case, an alternative 3' splice junction (acceptor site) is used, changing the 5' boundary of the downstream exon. 3) Intron retention: happened to introns between exon 2 and 3, between exon 3 and 4, and, between exon 9 and 10.

Figure 2. Expression of G72 mRNA in Tg mice brain subregions

In four brain regions from Tg mice, qPCR indicated G72 was expressed at a relatively high level in cerebral cortex (0.87 ± 0.07), followed by the striatum (0.57 ± 0.27, $P_{\text{cortex/striatum}}$= 0.41) and hippocampus (0.34 ± 0.11, $P_{\text{cortex/hippocampus}}$= 0.02). G72

expression was almost undetectable in cerebellum (0.05 ± 0.05, $P_{cortex/cerebellum}$= 6.63E-05). Number of animals: *n*=5. Value represents mean ± SEM of the relative expression level.

Figure 3. LG72 protein was not detected in Tg mice

(A) Left panel: Western blot analysis of mitochondrial protein extracted from G72, G72-myc and mock transfected Cos-7 cells, all probed with anti-G72 antibody (gift from Kvajo). G72 was detected in the mitochondrial pellets of G72 and G72-myc, but not in mock. Right three panels: Western blot analysis of mitochondrial extracts from the cerebral cortex, testis and cerebellum of Tg mice and controls revealed no evidence of LG72 protein expression. All mitochondrial extracts were enriched for prohibitin, a 29.7 kDa protein located in the inner membrane of mitochondria, which was used to confirm that the membrane fraction was enriched for mitochondrial extracts. **(B)** Western blot analysis on whole cell total lysate from the cerebral cortex and testis of Tg mice and WT controls revealed no evidence of LG72 expression.

Figure 4. Tg mice exhibited fewer stereotypic movements in open field test

(A) Tg mice spent less time in stereotypic behaviors compared with controls [$F(1, 79) = 5.27$; $p=0.02$]. **(B)** To confirm the phenotype of fewer stereotypic behaviors observed, a new batch of Tg mice with controls were used in another activity tracking system. The stereotypy count of Tg mice was significantly less than controls within a 15 minute period [$F(1, 27) = 4.46$; $p=0.04$] and even less within a 30 minute period [$F(1, 27) = 6.12$; $p=0.02$]. **(C-F)** There was no significant difference in total distance traveled [$F(1, 79) = 3.01$; $p = 0.09$], ambulatory time [$F(1, 79) = 2.99$; $p = 0.09$], time spent in the central/peripheral area [$F(1, 79) = 0.16$; $p = 0.69$], or resting time [$F(1, 79) = 0.45$; $p = 0.5$] between the two groups. Number of animals used in **A, C-F**: WT, *n*=28; Tg, *n*=53. Number of animals used in **B**: WT, *n*=14; Tg, *n*=15.

Figure 5. Tg mice showed increased startle response in the course of PPI test

(A) Base startle reactivity in block 1 was significantly higher in Tg mice compared to WT [$F(1, 26) = 6.69$; $p=0.02$]. **(B)** ANOVA indicated that startle response decreased from block 1 to 4 in two groups (block 1 through 4, $F(3, 78) = 3.64$; $p=0.02$). There was no difference between Tg and WT mice in terms of habituation, which is defined as the decrease of startle response from block 1 to 4, *i.e.*, the slope of the curve [$F(3, 78) =$

0.48; p=0.70]. **(C)** No differences in PPI were found between Tg mice and WT mice [F(1, 26) = 0.29; p=0.60]. Prepulse intensities were 3 dB, 6 dB or 12 dB stimuli above a 65 dB background. Number of animals: WT, n=14; Tg, n=14.

Figure 6. Tg mice had abnormal hedonic responses

(A-B) Tg mice consumed significantly more water [F(1, 39) = 8.00; p=0.007] **(A)** and marginally less sucrose [F(1, 39) = 3.58; p=0.07] **(B)** than WT across all sucrose concentrations. All mice significantly decreased their water intake [F(5, 195) = 16.85; p< 0.0001] **(A)** and increased their absolute sucrose intake from the sucrose tube [F(5, 195) = 64.33; p< 0.0001] **(B)** from habituation to 15% sucrose concentration. **(C)** The sucrose preference of Tg mice was significantly lower than WT [F(1, 39) =7.03; p = 0.01] across all sucrose concentrations. As expected, all mice showed significant sucrose preferences when offered increasing sugar concentrations [F(5, 195) = 30.63; p< 0.0001]. Number of animals: WT, n=18; Tg, n=23.

Reference List

(1) Blouin JL, Dombroski BA, Nath SK, Lasseter VK, Wolyniec PS, Nestadt G *et al*. Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. Nat Genet 1998;20(1):70-73.

(2) Levinson DF, Holmans P, Straub RE, Owen MJ, Wildenauer DB, Gejman PV *et al*. Multicenter linkage study of schizophrenia candidate regions on chromosomes 5q, 6q, 10p, and 13q: schizophrenia linkage collaborative group III. Am J Hum Genet 2000;67(3):652-663.

(3) Liu C, Badner JA, Christian SL, Guroff JJ, tera-Wadleigh SD, Gershon ES. Fine mapping supports previous linkage evidence for a bipolar disorder susceptibility locus on 13q32. Am J Med Genet 2001;105(4):375-380.

(4) Badner JA, Gershon ES. Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. Mol Psychiatry 2002;7(4):405-411.

(5) Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H *et al*. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc Natl Acad Sci U S A 2002;99(21):13675-13680.

(6) Mulle JG, Chowdari KV, Nimgaonkar V, Chakravarti A. No evidence for association to the G72/G30 locus in an independent sample of schizophrenia families. Mol Psychiatry 2005;10(5):431-433.

(7) Liu YL, Fann CS, Liu CM, Chang CC, Wu JY, Hung SI *et al*. No association of G72 and D-amino acid oxidase genes with schizophrenia. Schizophr Res

2006;87(1-3):15-20.

(8) Wood LS, Pickering EH, Dechairo BM. Significant support for DAO as a schizophrenia susceptibility locus: examination of five genes putatively associated with schizophrenia. Biol Psychiatry 2007;61(10):1195-1199.

(9) Vilella E, Costas J, Sanjuan J, Guitart M, De DY, Carracedo A *et al*. Association of schizophrenia with DTNBP1 but not with DAO, DAOA, NRG1 and RGS4 nor their genetic interaction. J Psychiatr Res 2008;42(4):278-288.

(10) Shi J, Badner JA, Gershon ES, Chunyu L, Willour VL, Potash JB. Further evidence for an association of G72/G30 with schizophrenia in Chinese. Schizophr Res 2009;107(2-3):324-326.

(11) Maier W, Hofgen B, Zobel A, Rietschel M. Genetic models of schizophrenia and bipolar disorder: overlapping inheritance or discrete genotypes? Eur Arch Psychiatry Clin Neurosci 2005;255(3):159-166.

(12) McGuffin P, Tandon K, Corsico A. Linkage and association studies of schizophrenia. Curr Psychiatry Rep 2003;5(2):121-127.

(13) Craddock N, Forty L. Genetics of affective (mood) disorders. Eur J Hum Genet 2006;14(6):660-668.

(14) DePaulo JR, Jr. Genetics of bipolar disorder: where do we stand? Am J Psychiatry 2004;161(4):595-597.

(15) Craddock N, O'Donovan MC, Owen MJ. The genetics of schizophrenia and bipolar disorder: dissecting psychosis. J Med Genet 2005;42(3):193-204.

(16) Craddock N, O'Donovan MC, Owen MJ. Genes for schizophrenia and bipolar disorder? Implications for psychiatric nosology. Schizophr Bull 2006;32(1):9-16.

(17) Bass NJ, Datta SR, McQuillin A, Puri V, Choudhury K, Thirumalai S *et al*. Evidence for the association of the DAOA (G72) gene with schizophrenia and bipolar disorder but not for the association of the DAO gene with schizophrenia. Behav Brain Funct 2009;5:28.

(18) Muller DJ, Zai CC, Shinkai T, Strauss J, Kennedy JL. Association between the DAOA/G72 gene and bipolar disorder and meta-analyses in bipolar disorder and schizophrenia. Bipolar Disord 2011;13(2):198-207.

(19) Krug A, Markov V, Krach S, Jansen A, Zerres K, Eggermann T *et al*. Genetic variation in G72 correlates with brain activation in the right middle temporal gyrus in a verbal fluency task in healthy individuals. Hum Brain Mapp 2011;32(1):118-126.

(20) Prata DP, Papagni SA, Mechelli A, Fu CH, Kambeitz J, Picchioni M *et al*. Effect of D-amino acid oxidase activator (DAOA; G72) on brain function during verbal fluency. Hum Brain Mapp 2012;33(1):143-153.

(21) Zuliani R, Moorhead TW, Job D, McKirdy J, Sussmann JE, Johnstone EC *et al*. Genetic variation in the G72 (DAOA) gene affects temporal lobe and amygdala structure in subjects affected by bipolar disorder. Bipolar Disord 2009;11(6):621-627.

(22) Hattori E, Liu C, Badner JA, Bonner TI, Christian SL, Maheshwari M *et al*. Polymorphisms at the G72/G30 gene locus, on 13q33, are associated with bipolar disorder in two independent pedigree series. Am J Hum Genet 2003;72(5):1131-1140.

(23) Konradi C, Heckers S. Molecular aspects of glutamate dysregulation: implications for schizophrenia and its treatment. Pharmacol Ther 2003;97(2):153-179.

(24) Sacchi S, Bernasconi M, Martineau M, Mothet JP, Ruzzene M, Pilone MS *et al*. pLG72 modulates intracellular D-serine levels through its interaction with D-amino acid oxidase: effect on schizophrenia susceptibility. J Biol Chem 2008;283(32):22244-22256.

(25) Kvajo M, Dhilla A, Swor DE, Karayiorgou M, Gogos JA. Evidence implicating the candidate schizophrenia/bipolar disorder susceptibility gene G72 in mitochondrial function. Mol Psychiatry 2008;13(7):685-696.

(26) Otte DM, Bilkei-Gorzo A, Filiou MD, Turck CW, Yilmaz O, Holst MI *et al*. Behavioral changes in G72/G30 transgenic mice. Eur Neuropsychopharmacol 2009;19(5):339-348.

(27) Otte DM, Sommersberg B, Kudin A, Guerrero C, Albayram O, Filiou MD *et al*. N-acetyl cysteine treatment rescues cognitive deficits induced by mitochondrial dysfunction in G72/G30 transgenic mice. Neuropsychopharmacology 2011;36(11):2233-2243.

(28) Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 2007;8(1):118-127.

(29) Modrek B, Lee CJ. Alternative splicing in the human, mouse and rat genomes is associated with an increased frequency of exon creation and/or loss. Nat Genet 2003;34(2):177-180.

(30) Xing Y, Lee C. Alternative splicing and RNA selection pressure--evolutionary consequences for eukaryotic genomes. Nat Rev Genet 2006;7(7):499-509.

(31) Cheng MC, Lu CL, Luu SU, Tsai HM, Hsu SH, Chen TT *et al*. Genetic and functional analysis of the DLG4 gene encoding the post-synaptic density protein 95 in schizophrenia. PLoS One 2010;5(12):e15107.

(32) Bray NJ, Kirov G, Owen RJ, Jacobsen NJ, Georgieva L, Williams HJ *et al*. Screening the human protocadherin 8 (PCDH8) gene in schizophrenia. Genes Brain Behav 2002;1(3):187-191.

(33) Jungerius BJ, Hoogendoorn ML, Bakker SC, Van't SR, Bardoel AF, Ophoff RA *et*

al. An association screen of myelin-related genes implicates the chromosome 22q11 PIK4CA gene in schizophrenia. Mol Psychiatry 2008;13(11):1060-1068.

(34) Ye L, Sun Z, Xie L, Liu S, Ju G, Shi J *et al*. Further study of a genetic association between the CLDN5 locus and schizophrenia. Schizophr Res 2005;75(1):139-141.

(35) Wu N, Zhang X, Jin S, Liu S, Ju G, Wang Z *et al*. A weak association of the CLDN5 locus with schizophrenia in Chinese case-control samples. Psychiatry Res 2010;178(1):223.

(36) Hong LE, Wonodi I, Avila MT, Buchanan RW, McMahon RP, Mitchell BD *et al*. Dihydropyrimidinase-related protein 2 (DRP-2) gene and association to deficit and nondeficit schizophrenia. Am J Med Genet B Neuropsychiatr Genet 2005;136B(1):8-11.

(37) Ohtsuki T, Sakurai K, Dou H, Toru M, Yamakawa-Kobayashi K, Arinami T. Mutation analysis of the NMDAR2B (GRIN2B) gene in schizophrenia. Mol Psychiatry 2001;6(2):211-216.

(38) Fallin MD, Lasseter VK, Avramopoulos D, Nicodemus KK, Wolyniec PS, McGrath JA *et al*. Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. Am J Hum Genet 2005;77(6):918-936.

(39) Lerer B, Macciardi F, Segman RH, Adolfsson R, Blackwood D, Blairy S *et al*. Variability of 5-HT2C receptor cys23ser polymorphism among European populations and vulnerability to affective disorder. Mol Psychiatry 2001;6(5):579-585.

(40) Segman RH, Heresco-Levy U, Finkel B, Inbar R, Neeman T, Schlafman M *et al*. Association between the serotonin 2C receptor gene and tardive dyskinesia in chronic schizophrenia: additive contribution of 5-HT2Cser and DRD3gly alleles to susceptibility. Psychopharmacology (Berl) 2000;152(4):408-413.

(41) Goldstein I, Lerer E, Laiba E, Mallet J, Mujaheed M, Laurent C *et al*. Association between sodium- and potassium-activated adenosine triphosphatase alpha isoforms and bipolar disorders. Biol Psychiatry 2009;65(11):985-991.

(42) Lee KY, Ahn YM, Joo EJ, Chang JS, Kim YS. The association of DUSP6 gene with schizophrenia and bipolar disorder: its possible role in the development of bipolar disorder. Mol Psychiatry 2006;11(5):425-426.

(43) Borsotto M, Cavarec L, Bouillot M, Romey G, Macciardi F, Delaye A *et al*. PP2A-Bgamma subunit and KCNQ2 K+ channels in bipolar disorder. Pharmacogenomics J 2007;7(2):123-132.

(44) Benzel I, Kew JN, Viknaraja R, Kelly F, de BJ, Hirsch S *et al*. Investigation of G72 (DAOA) expression in the human brain. BMC Psychiatry 2008;8:94.

(45) Ridley RM. The psychology of perserverative and stereotyped behaviour. Prog

Neurobiol 1994;44(2):221-231.

(46) Garner JP, Meehan CL, Mench JA. Stereotypies in caged parrots, schizophrenia and autism: evidence for a common mechanism. Behav Brain Res 2003;145(1-2):125-134.

(47) Morrens M, Hulstijn W, Lewi PJ, De HM, Sabbe BG. Stereotypy in schizophrenia. Schizophr Res 2006;84(2-3):397-404.

(48) Kennes D, Odberg FO, Bouquet Y, De Rycke PH. Changes in naloxone and haloperidol effects during the development of captivity-induced jumping stereotypy in bank voles. Eur J Pharmacol 1988;153(1):19-24.

(49) Garner JP, Mason GJ. Evidence for a relationship between cage stereotypies and behavioural disinhibition in laboratory rodents. Behav Brain Res 2002;136(1):83-92.

(50) Andreasen NC, Olsen S. Negative v positive schizophrenia. Definition and validation. Arch Gen Psychiatry 1982;39(7):789-794.

(51) Feyder M, Karlsson RM, Mathur P, Lyman M, Bock R, Momenan R *et al*. Association of mouse Dlg4 (PSD-95) gene deletion and human DLG4 gene variation with phenotypes relevant to autism spectrum disorders and Williams' syndrome. Am J Psychiatry 2010;167(12):1508-1517.

(52) Feng W, Zhang M. Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. Nat Rev Neurosci 2009;10(2):87-99.

(53) Ohnuma T, Kato H, Arai H, Faull RL, McKenna PJ, Emson PC. Gene expression of PSD95 in prefrontal cortex and hippocampus in schizophrenia. Neuroreport 2000;11(14):3133-3137.

(54) Tam GW, van de Lagemaat LN, Redon R, Strathdee KE, Croning MD, Malloy MP *et al*. Confirmed rare copy number variants implicate novel genes in schizophrenia. Biochem Soc Trans 2010;38(2):445-451.

(55) Vetter DE, Mann JR, Wangemann P, Liu J, McLaughlin KJ, Lesage F *et al*. Inner ear defects induced by null mutation of the isk gene. Neuron 1996;17(6):1251-1264.

(56) Vidal PP, Degallaix L, Josset P, Gasc JP, Cullen KE. Postural and locomotor control in normal and vestibularly deficient mice. J Physiol 2004;559(Pt 2):625-638.