Published in final edited form as:

Synapse. 2015 September ; 69(9): 434–445. doi:10.1002/syn.21830.

Deletion of $G_{\alpha q}$ in the telencephalon alters specific neurobehavioral outcomes

Devon L. Graham¹, Matthew A. Buendia², Michelle A. Chapman², Heather H. Durai², and Gregg D. Stanwood¹

¹Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL 32303

²Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232, United States

Abstract

$G_{\alpha q}$ -coupled receptors are ubiquitously expressed throughout the brain and body, and it has been shown that these receptors and associated signaling cascades are involved in a number of functional outputs, including motor function and learning and memory. Genetic alterations to $G_{\alpha q}$ have been implicated in neurodevelopmental disorders such as Sturge-Weber syndrome. Some of these associated disease outcomes have been modeled in laboratory animals, but as $G_{\alpha q}$ is expressed in all cell types, it is difficult to differentiate the underlying circuitry or causative neuronal population. To begin to address neuronal cell type diversity in $G_{\alpha q}$ function, we utilized a conditional knockout mouse whereby $G_{\alpha q}$ was eliminated from telencephalic glutamatergic neurons. Unlike the global $G_{\alpha q}$ knockout mouse, we found that these conditional knockout mice were not physically different from control mice, nor did they exhibit any gross motor abnormalities. However, similarly to the constitutive knockout animal, $G_{\alpha q}$ conditional knockout mice demonstrated apparent deficits in spatial working memory. Loss of $G_{\alpha q}$ from glutamatergic neurons also produced enhanced sensitivity to cocaine-induced locomotion, suggesting that cortical $G_{\alpha q}$ signaling may limit behavioral responses to psychostimulants. Screening for a variety of markers of forebrain neuronal architecture revealed no obvious differences in the conditional knockouts, suggesting that the loss of $G_{\alpha q}$ in telencephalic excitatory neurons does not result in major alterations in brain structure or neuronal differentiation. Taken together, our results define specific modulation of spatial working memory and psychostimulant responses through disruptions in $G_{\alpha q}$ signaling within cerebral cortical glutamatergic neurons.

Graphical Abstract

Correspondence to: Gregg D. Stanwood, Ph.D., Florida State University College of Medicine, Department of Biomedical Sciences, 1115 W. Call St. Tallahassee, FL 32306-4300, Tel: +1-850-644-2271, Fax: +1-850-644-5781, gregg.stanwood@med.fsu.edu.

The authors have no conflicts of interest to declare.

Keywords

Gnaq; conditional knockout; locomotor activity; spatial working memory; Emx1; anxiety; glutamate

Introduction

G-protein coupled receptors (GPCRs) signal intracellularly largely through heterotrimeric G protein complexes, consisting of α , β , and δ subunits. Following ligand binding to the receptor, the G protein complex is activated, and GDP, previously bound to the G_α subunit, is exchanged for GTP, allowing the α subunit to undergo a conformational change and dissociate from the G_γ complex. The identity of the recruited second messenger system is dependent upon the subunit, which can be subdivided into four primary families: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$ (Baltoumas *et al.*, 2013). Genetic deletion of $G_{\alpha q}$ is not fatal but results in a number of behavioral impairments such as movement dysfunction, memory deficits, and seizures (Frederick *et al.*, 2012, Offermanns *et al.*, 1997, Wettschureck *et al.*, 2006), while loss of $G_{\alpha 11}$ does not appear to produce any significant phenotype (Offermanns *et al.*, 1998). However, it would appear that these two highly homologous isoforms are functionally redundant, as loss of both $G_{\alpha q}$ and $G_{\alpha 11}$ is fatal (Offermanns *et al.*, 1998, Strathmann & Simon, 1990).

The $G_{\alpha q}$ isoform exerts its downstream effects by activation of phospholipase C β (PLC β), which in turn hydrolyzes phosphatidylinositol biphosphate (PIP $_2$), activating protein kinase C (PKC) via either diacylglycerol (DAG) or inositol triphosphate (IP $_3$)-induced Ca $^{+2}$ release (Dickson *et al.*, 2013, Exton, 1996, Falkenburger *et al.*, 2013, Hubbard & Hepler, 2006). $G_{\alpha q}$ serves as the transducer for several neurotransmitter receptor subtypes, including group I metabotropic glutamate receptors (mGluR1 and mGluR5), muscarinic acetylcholine receptors 1 and 3, α -1 adrenergic receptors, serotonin-2 $A/B/C$ receptors, and perhaps dopamine D1 receptors (Alexander *et al.*, 2013, Brown, 2010, Raymond *et al.*, 2001, Valenti *et al.*, 2002, Wang *et al.*, 1995, Zhong & Minneman, 1999). Deficiencies in $G_{\alpha q}$ contribute to a number of neurological disorders, including Sturge-Weber Syndrome, a developmental disorder characterized by seizures, stroke-type events, visual deficits, skin and vascular conditions, brain tumors, and intellectual deficits (Shirley *et al.*, 2013).

We and others have demonstrated that $G_{\alpha q}$ plays an important role in locomotor and spatial working memory functions (Frederick *et al.*, 2012, Offermanns *et al.*, 1997). These findings

could be due to loss of G_{aq} in the cerebellum and basal ganglia, regions important in motor output (Graybiel, 2000, Manto *et al.*, 2012), and the frontal cortex, which plays a role in working memory. These regions are highly enriched in G_{aq} (Milligan, 1993). However, it is difficult to differentiate between these two outputs when G_{aq} is globally eliminated. Thus, we used the *Emx1-cre* line to drive conditional knockout of G_{aq} , whereby G_{aq} is not expressed in glutamatergic cells (and glia) (Gorski *et al.*, 2002, Guo *et al.*, 2000). We hypothesized that loss of G_{aq} in telencephalic glutamatergic neurons would result in altered working memory function while having little effect on motor output.

Materials and Methods

Animals

Homozygous G_{aq} -floxed ($G_{aq}^{fl/fl}$) mice on a C57Bl/6J background (Wettschureck *et al.*, 2001) and homozygous *Emx1-cre* knock-in mice (*Emx1-cre*^{+/+}; C57Bl/6J background) (Gorski *et al.*, 2002), obtained from Jackson Laboratory (Stock #022762; Bar Harbor, ME), were used for this study. $G_{aq}^{fl/fl}$ mice were bred with *Emx1-cre*^{+/+} mice to obtain $G_{aq}^{fl/wt}$, *cre*^{+/-} offspring. These double heterozygotic offspring were bred to homozygotic floxed mice ($G_{aq}^{fl/fl}$, *cre*^{-/-}) to obtain littermate $G_{aq}^{fl/fl}$, *cre*^{+/-} (G_{aq} conditional knockout; cKO) and $G_{aq}^{fl/fl}$, *cre*^{-/-} (G_{aq} homozygous flox; flox control); heterozygous flox, *cre*-negative offspring ($G_{aq}^{fl/wt}$, *cre*^{-/-}) were discarded and offspring that were heterozygous at both loci ($G_{aq}^{fl/wt}$, *cre*^{+/-}) were used as additional breeders. As an additional control, the *cre* knock-in mice ($G_{aq}^{wt/wt}$, *Emx1-cre*^{+/+}) were bred to wild-type C57Bl/6J mice, and offspring were used for testing ($G_{aq}^{wt/wt}$, *Emx1-cre*^{+/-}; *cre* control). Offspring were kept with the dam and sire until weaning (postnatal day (P)21), when they were separated into same sex cages. Adult mice were housed 3–5/cage and were provided with rodent chow and tap water *ad libitum*. Mice were housed in a temperature- and humidity-controlled AAALAC-approved facility that is maintained on a 12:12 h light:dark cycle (lights on 0600–1800 h). All protocols were approved by the VU Institutional Animal Care and Use Committee and performed in accordance with the NIH's Guide for the Care and Use of Laboratory Animals.

Genotypes were confirmed by polymerase chain reaction analysis of tail tissue obtained at weaning and then reconfirmed at death. The forward and reverse primers were used to identify the presence of the floxed allele(s) as previously described (Dettlaff-Swiercz *et al.*, 2005) (WT band at ~600 bp; flox band at ~750 bp, with single flox animals exhibiting both bands). Primers (0.5 μ M final concentration) against the *cre* allele were the following sequences: 5'-GCG GTC TGG CAG TAA AAA CTA TC-3' (forward) and 5'-GTG AAA CAG CAT TGC TGT CAC TT-3' (reverse). An additional set of forward and reverse primers were added to establish the presence of the wild-type allele: 5'-AAG GTG TGG TTC CAG AAT CG-3' and 5'-CTC TCC ACC AGA AGG CTG AG-3', respectively (http://jaxmice.jax.org/protocolsdb/f?p=116:2:0::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:5682,005628).

Behavioral Analyses

Mice from each of the genotypes (cKO, flox control, and *cre* control) were used in behavioral studies. Male mice (P70–140; N=5–8/genotype) were tested in a behavioral

battery consisting of elevated zero maze, inverted screen test, Y-maze, rotarod, and locomotor activity with and without a cocaine challenge; all testing occurred during the light and followed the order of tests listed below. Mice were age-matched for each behavioral test, were weighed at the start of each test, and only one test was performed per day with at least 24 h between each test. Mice were extensively handled for at least 3 consecutive days prior to the initiation of the testing battery.

Elevated zero maze (EZM)

While it is associated with motor and cognitive deficits, Sturge-Weber Syndrome is also linked with psychiatric disorders and elevated levels of emotional distress (Chapieski *et al.*, 2000, Sujansky & Conradi, 1995). Used as a measure of anxiety, the EZM was performed as previously described (Frederick *et al.*, 2012). Briefly, mice were placed into an open area of an elevated circular platform (40 cm off ground, 50 cm in diameter with two open (5 cm wide) and two enclosed (5 cm wide with 15 cm high walls) areas opposite each other). Mice were allowed to explore the apparatus for 5 min, and activity was monitored via an overhead camera connected to a computer using ANY-maze software (Stoelting, Wood Dale, IL) in a separate room. Percent time spent in open or enclosed arenas, entries into open arenas, speed, and total distance traveled were output measures. As with all behavioral assays, the apparatus was cleaned with Vimoba spray between animals.

Inverted Screen

Inverted screen is a test of grip strength and was used as Sturge Weber syndrome is associated with hemiparesis (Suskauer *et al.*, 2010). Inverted screen was tested over two days with 3 trials per day (15 min intertrial interval (ITI)), 4 days following EZM. Using a metal grid screen (10 × 14 cm) subdivided into separate compartments, 2–3 littermates were placed on the screen and given time to grip the grid before the screen was suspended upside down approximately 60 cm over a cage filled with fresh bedding. Latency to fall was recorded, and data from the three daily trials were averaged together.

Y-Maze

The Y-maze, a test of spatial memory, was performed as previously described using a three-armed apparatus with distinct spatial cues at the end of each arm (Carpenter *et al.*, 2012, Coke-Murphy *et al.*, 2014, Frederick *et al.*, 2012). This test was performed one week after the completion of inverted screen and consisted of only one trial. A mouse was placed into one of the three arms of the apparatus, and its movements were recorded by an overhead camera over the course of 5 min. Arm entries were recorded by an observer located in another room, and these data were used to calculate total arm entries and spontaneous alternation, which was defined as the successive entry into each of the three arms (Gustin *et al.*, 2011, Lalonde, 2002, Thompson *et al.*, 2005). The number of possible alternations (# of total entries - 2) was used to calculate percent spontaneous alternations [(# of spontaneous alternations/# of possible alternations) × 100].

Rotarod

Rotarod was performed as previously described using 3 trials per day (15 min ITI) over the course of 3 consecutive days (Coke-Murphy *et al.*, 2014, Frederick *et al.*, 2012), 24 h after the completion of Y-maze. Mice were placed on an accelerating rotarod apparatus (Ugo Basile #7650; Collegeville, PA), subdivided into 6 cm compartments by plastic dividers. The rotating cylinder (3 cm diameter) increased in speed from 5 to 40 rpm over the course of the 5 min trial. The latency to fall was recorded, and the values for the 3 daily trials were averaged each day.

Locomotor activity

Locomotor activity measurements were performed over 3 days as previously described using commercially available chambers (29 × 29 × 20.5 cm; Med Associates; St. Albans, VT) (Frederick *et al.*, 2012, Frederick *et al.*, 2015), 4 days following the completion of rotarod. All movements and animal location were analyzed with the associated software, as measured by beam breaks on the x-y-z axes (16 infrared beams, 50 ms intervals). The first two days of testing allowed for habituation to the chamber, whereby the animal was placed in the open field for 30 min, removed and injected with 0.9% saline, and returned to the chamber for 60 more minutes. On the third day of testing, mice were again allowed to habituate to the apparatus for 30 min but instead were injected with 30 mg/kg cocaine prior to being placed back into the chamber for 60 minutes. Ambulatory distance and time, as well as time spent in the center or surround zones (i.e., thigmotaxis), were analyzed within 5 min blocks. Only the first 60 min of each trial are represented graphically (Fig. 7).

Immunoblotting

Tissue from the frontal cortex, striatum, hippocampus, amygdala, thalamus, and cerebellum (N=5–6/genotype) was prepared, and Western blots were performed, as previously described with minor modifications (Stanwood *et al.*, 2005). Equal concentrations of protein were loaded and probed for by antibodies against rabbit G_{αq/11} (1:500; Santa Cruz Biotechnology, Dallas, TX) and mouse GAPDH (1:125,000; Life Technologies, Grand Island, NY) as well as HRP-conjugated secondaries (1:10,000; Jackson ImmunoResearch). Blots were detected by ECL, and data were normalized to GAPDH, and expressed as normalized optical density (OD).

Immunohistochemistry

A separate cohort of adult (P70–110, N=5–7/genotype) mice was anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Brains were removed and fixed overnight in paraformaldehyde at 4°C. Following cryoprotection in a sucrose gradient, brains were cut coronally into 40 μm sections on a freezing microtome and stored at –20°C in freezing solution until further experimentation. Staining was performed as previously described using the chromogen 3,3' diaminobenzidine (DAB) to visualize proteins (Stanwood *et al.*, 2009, Stanwood *et al.*, 2005). Antibodies against mouse GAD67 (1:2000; Millipore, Billerica, MA), mouse parvalbumin (PV; 1:500; Sigma-Aldrich, St. Louis, MO) or mouse microtubule-associated protein-2 (MAP2; 1:1500; Sigma-Aldrich) were used with biotinylated secondary antibodies (1:1000; Jackson ImmunoResearch, West

Grove, PA). Sections were visualized via a Zeiss AxioImager microscope with a Zeiss AxioCam HRc camera and corresponding AxioVision 4.1 software. Sections were selected from the medial frontal cortex (approximately +0.38–+1.98 mm from Bregma, focusing on the anterior cingulate cortex), with bilateral hemispheres from at least three sections analyzed by an observer blinded to genotype. Analysis of MAP2 staining was performed as previously described (Stanwood *et al.*, 2005) in order to evaluate dendritic appearance and development. Briefly, images of the ACC (10× magnification) were analyzed and scored based on the following rating system: 0 = very straight processes; can be traced in single focal plane from cell body to pial surface; no significant deviations in x-y coordinates as well; 1 = occasional fragmented or bent dendritic bundle; 2 = ~50% of dendritic processes show deviation in either the x-y or z coordinate systems; 3 = vast majority of processes appear “wavy” and take tortuous routes to the pial surface. For GAD67 and PV cell counts, sections were imaged at 20× as previously described (Graham *et al.*, 2015). Cells counts were corrected for profile size (Abercrombie, 1946).

To determine if cell type-specific loss of $G_{\alpha q}$ altered cellular number and distribution, additional sections were stained via fluorescent immunohistochemistry as previously described (Jacobs *et al.*, 2009). An antibody against mouse NeuN (1:100; Millipore, Billerica, MA), a neuronal marker, or rabbit S-100 (1:4000; Dako, Carpinteria, CA), a glial marker (due to the antibody’s high affinity for S-100B), followed by incubation with cyanine- or DyLight 488-tagged secondary antibodies (1:1000, Jackson ImmunoResearch). Qualitative analyses of sections was performed by blinded observers.

Statistical Analysis

GAD67 and PV histological data and behavioral data (except where noted) from the mice were analyzed via one-way analysis of variance (ANOVA) with a *post hoc* Tukey’s multiple comparison test using GraphPad Prism 5 (GraphPad Software; San Diego, CA), with genotype as the main factor. EZM, rotarod, and locomotor activity (collapsed data pertaining to the 35–65 min time points) data were analyzed via two-way ANOVA with a *post hoc* Bonferroni’s test using Genotype and Zone or Time as factors. Locomotor activity was analyzed via a repeated measures 2-way ANOVA with a *post hoc* Bonferroni’s test using Genotype and Time as factors. MAP2 data were analyzed via a non-parametric one-way ANOVA (Kruskal-Wallis test) with a *post hoc* Dunn’s Multiple Comparison test;. Significance was set at $p = 0.05$; data are presented as means $\pm$ SEMs. Individual data points were considered outliers if their value was greater than 2 standard deviations above or below the mean for each trial and were thus eliminated for that particular trial or test.

Results

Protein expression in the $G_{\alpha q}$ conditional mutant mouse

As a proof of concept, we assessed $G_{\alpha q}$ levels within various brains regions of the cKO and control mice. Immunoblot analyses revealed that there were no significant differences in protein levels of $G_{\alpha q}$ between control and cKO mice in the striatum, thalamus, and cerebellum (Fig. 1). However, significant differences were found in the hippocampus ($F(2,14)=33.78$, $p<0.001$) and the frontal cortex ($F(2,14)=19.99$, $p<0.001$), with the $G_{\alpha q}$ /

GAPDH values within these regions being significantly decreased in the cKO mice relative to either of the control groups. There was an overall significant effect of genotype in the amygdala as well ($F(2,14)=3.741$, $p<0.05$), with cKO values being significantly less than cre controls.

Glutamatergic $G_{\alpha q}$ -mediated behavior

Mice were used in a behavioral battery similar to that performed with the $G_{\alpha q}$ constitutive knockout mice (Frederick *et al.*, 2012). There was no difference in the weight between groups at the start of behavioral analysis (Fig. 2, $p=0.058$). Mice were initially tested in the EZM, a test of anxiety and exploratory behavior. There was no significant difference in distance traveled (Fig. 3a; $p=0.67$) or speed (Fig. 3b; $p=0.67$) between the genotypes. All genotypes spent significantly less time in the open arenas compared to the closed arenas (Fig. 3c; $F(1,36)=205.1$, $p<0.001$), and there was no interaction of zone and genotype. Moreover, there was no overall effect of genotype on entries into the open arenas (Fig. 3d, $p=0.90$), indicating that *Emx1*-induced conditional loss of $G_{\alpha q}$ does not affect anxiety behavior, as assessed by EZM.

Mice were then tested on the inverted screen, a test of forepaw and hindpaw strength (Deacon, 2013). We have previously shown that constitutive loss of $G_{\alpha q}$ results in significantly decreased strength, as latencies to fall were dramatically reduced in the null mice (Frederick *et al.*, 2012). There was no difference in the latency to fall between groups (Fig. 4; $p=0.52$ for average of both test days).

The Y-maze was used to test spatial working memory. The number of entries into arms (Fig. 5a; $F(2,16)=4.762$, $p<0.05$) was affected by genotype, with the cKO group significantly less than the flox control, but not the cre control, group. Most pertinently, there were significantly fewer percent spontaneous alternations in the cKO group relative to the control groups (Fig. 5b; $F(2,16)=12.07$, $p<0.001$). This reduction in alterations and entries cannot be attributed to distance or speed traveled, as neither of these outcomes were significantly altered based on genotype (data not shown).

The presence of motor deficits was tested via rotarod. Our previous study indicated that global deletion of $G_{\alpha q}$ resulted in poor performance of this task (Frederick *et al.*, 2012). In the present study, there was no significant effect of day ($p=0.17$). Following each daily session, the cKO group did not significantly differ from the control groups ($p=0.07$; Fig. 6). No significant Genotype $\times$ Time (Day) interaction was found ($p=0.98$).

Finally, mice were tested in a 3 day locomotor activity assay. The first two days of this test allowed the mice to habituate to the chambers and consisted of a 30 min baseline reading, removal from the chamber and injection with saline, and returned to the chamber for an additional 60 min (Fig. 7). During the habituation tests, there were significant effects of Genotype [Day 1: $F(2,285)=7.688$, $p<0.001$; Day 2: $F(2,271)=10.42$, $p<0.001$] and Time [Day 1: $F(17,285)=16.13$, $p<0.001$; Day 2: $F(17,271)=14.50$, $p<0.001$], but no significant interaction of Genotype $\times$ Time. Furthermore, there were no significant overall differences in total distance traveled during the 30–60 min period (i.e., time following injection) on the first two test days when total distance was collapsed over time, although cre and cKO mice

trended to slight hyperactivity (Fig. 7a ($p=0.059$) and 7b ($p=0.055$), insets). On day 3, mice were administered cocaine to test psychomotor stimulant response. Again, there was a significant difference between Genotype ($F(2,300)=9.911$, $p<0.001$) and Time ($F(17,300)=20.01$, $p<0.001$), as well as a significant Genotype $\times$ Time interaction ($F(34,300)=2.751$, $p<0.001$), whereby the cKO group was significantly different from control groups immediately after the cocaine injection. When data were analyzed at the 30–60 min block (i.e., immediately following cocaine injection), there was a significant difference between genotypes such that the cKO group was significantly hyperactive relative to the flox control group (Fig. 7c, inset; $F(2,18)=5.980$, $p<0.01$).

Histological alterations in the $G_{\alpha q}$ conditional mutant mouse

Coronal brain sections through the forebrain were immunostained for the somatodendritic marker MAP2 (Fig 8a–c), the inhibitory neuronal marker GAD67 (Fig 8d–f), and the Ca^{++} binding protein PV (Fig 8g–i). For the MAP2 analysis, we used a pseudo-quantitative scale (described above in Materials and Methods and in Stanwood *et al.*, 2005) to rate dendritic bundling and trajectories, and although a significant effect of Genotype was observed ($p<0.05$), *post hoc* analysis revealed no significant differences between any of the groups [flox control (mean $\pm$ SEM), 1.18 ± 0.15 ; cre control, 1.94 ± 0.24 ; cKO, 1.90 ± 0.11]. No differences between genotypes were found after cell counting for GAD67 ($p=0.64$; flox control, 301.9 ± 16.6 cells/mm²; cre control, 278.7 ± 16.6 ; cKO, 280.2 ± 21.0) and PV ($p=0.42$; flox control, 151.7 ± 7.6 cells/mm²; cre control, 146.6 ± 5.6 ; cKO, 140.8 ± 4.4) within the frontal cortex. To further monitor forebrain cytoarchitecture, we next stained for the neuronal and glial markers, NeuN and S-100. Although these immunostains did not undergo rigorous cell counting, two observers blinded to genotype found no obvious incongruities in neuronal or glial expression in the regions examined, including frontal cortex, striatum, hippocampus, amygdala, thalamus, and cerebellum (Fig. 9 and data not shown).

Discussion

$G_{\alpha q}$ is a crucial component of GPCR signaling that contributes to the diverse heterogeneity in physiological function that this superfamily offers. The $G_{\alpha q}$ family itself consists of at least four known members: $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, and $G_{\alpha 15/16}$ (Hubbard & Hepler, 2006). $G_{\alpha q}$ and $G_{\alpha 11}$, in particular, share high sequence homology and are ubiquitously expressed in the body, particularly the brain, with $G_{\alpha q}$ more prevalent than the $G_{\alpha 11}$ isoform (Milligan, 1993, Strathmann & Simon, 1990); $G_{\alpha 14}$ and $G_{\alpha 15/16}$ are found predominantly in the periphery and stem cells, respectively (Hubbard & Hepler, 2006). Agonist binding to a $G_{\alpha q}$ -coupled receptor results in increased intracellular calcium mobilization via PKC and PLC-IP₃ second messenger pathways, which is in direct contrast to other G_{α} -subunits, such as $G_{\alpha s/olf}$ and $G_{\alpha i/o}$, which function by increasing or decreasing cAMP levels, respectively, via adenylyl cyclase. $G_{\alpha q}$ has been demonstrated to have significant effects on the development of several clinical disorders, such as Sturge-Weber Syndrome, a disorder characterized by seizures, intellectual deficits, paralysis and facial birthmarks (Shirley *et al.*, 2013). In preclinical models, loss of $G_{\alpha q}$ causes significant disruptions of central nervous system output such as motor function, memory processes, seizures, and maternal behavior (Frederick *et al.*, 2012, Offermanns *et al.*, 1997, Wettschureck *et al.*, 2004, Wettschureck *et*

al., 2006), as well as a number of peripheral processes, including cardiovascular effects (Offermanns *et al.*, 1998, Wettschureck *et al.*, 2001) and differentiation of stem-like chondrocytes (Chagin *et al.*, 2014). Many of these findings were found using a global knockout approach. However, $G_{\alpha q}$, while expressed in virtually all cell types and in all regions of the brain, is especially concentrated within the forebrain, specifically the frontal cortex and hippocampus (Milligan, 1993). Previous findings from our lab demonstrated that global loss of $G_{\alpha q}$ resulted in spatial working memory deficits (Frederick *et al.*, 2012), a test correlated with both the hippocampus and prefrontal cortex (PFC), among other regions (Lalonde, 2002). Furthermore, we and others have found deficits in motor coordination and activity (Frederick *et al.*, 2012, Offermanns *et al.*, 1997), a function correlated to basal ganglia and cerebellar output. It is thereby possible that some of the observed behavioral deficits could be complicated by gross motor deficits. We thus took a more focused approach using a conditional knockout mouse model, whereby $G_{\alpha q}$ was deleted from glutamatergic cells within the frontal cortex, hippocampus, and amygdala (Emx1-cre line). A comparison of the altered behavioral phenotype found in the Emx1-cKO model compared to that of the constitutive KO mouse can be found in Table 1.

Constitutive and brain-wide $G_{\alpha q}$ KO mice are significantly smaller than WT littermates (Frederick *et al.*, 2012, Wettschureck *et al.*, 2005). We noted no statistically significant differences in body weights in Emx1-driven cKO mice compared to controls ($p=0.06$), although the cKOs tended to be 1–2 g lighter. The cKO mice performed similarly to controls in all facets examined of the EZM, demonstrating that these mice have normal anxiety responses, similar to our previous findings (Frederick *et al.*, 2012). $G_{\alpha q}$ cKO mice also exhibited no significant motor deficits, as assessed by distance traveled in the EZM, inverted screen, rotarod, or basal locomotor activity. These data are in contrast to significant deficits in these measures in our previous study investigating the constitutive KO mice (Frederick *et al.*, 2012). The lack of motor deficits may be due to the fact that $G_{\alpha q}$ within the cerebellum and basal ganglia remained intact, as expected by Emx1 expression patterns and confirmed via immunoblotting. Thus, we were successfully able to tease out the motor impairments resulting from the global KO mouse.

On the contrary, cKO mice were significantly more responsive to the locomotor-stimulating effects of cocaine (Fig. 7). Locomotor activity is a highly complex behavior, with numerous neuronal circuits, neurotransmitters, and brain regions involved. Group I metabotropic glutamate receptors are $G_{\alpha q}$ -coupled, and these receptors, particularly mGluR5, are localized within the frontal cortex. It is thought that mGluR5 modulates locomotor activity through interactions with NMDA receptors, although mGluR5 ligands do not alter activity on their own (Henry *et al.*, 2002, Homayoun *et al.*, 2004). Interestingly, mGluR5 activation within the medial PFC regulates the expression and long-term maintenance of cocaine behavioral sensitization, presumably through glutamatergic projections to the ventral tegmental area (Timmer & Steketee, 2012), suggesting that cortical mGluR5 plays a role in cocaine-stimulated hyperactivity. However, other $G_{\alpha q}$ -coupled receptors may be involved. For instance, DA receptors—specifically D1 receptors—within the medial frontal cortex to some extent modulate locomotor activity, as loss of dopamine within this region results in increased activity levels (Tassin *et al.*, 1978), contrary to this receptor's role within the

ventral striatum (Delfs *et al.*, 1990, Dreher & Jackson, 1989, Swanson *et al.*, 1997, Xu *et al.*, 1994). This same group demonstrated that D1-like antagonist administered into the medial PFC enhances amphetamine-stimulated locomotor activity (Vezina *et al.*, 1991). Moreover, a subset of D1 receptors appear to couple to PLC β (i.e., G $_{\alpha q}$ -coupled receptors) (Friedman *et al.*, 1997, Undie & Friedman, 1990) and may mediate forward locomotion (Medvedev *et al.*, 2013). Loss of striatal G $_{\alpha q}$ -coupled D1 receptors, however, cannot explain the enhanced effects of cocaine locomotor activity exhibited by the cKO mice, as G $_{\alpha q}$ expression was unaffected within this region. Perhaps G $_{\alpha q}$ -coupled D1 or D5 receptors within the frontal cortex play a significant role in cocaine-induced hyperactivity, as the inhibition of motor activity normally induced by these receptors would be lost. Future studies will need to assess dose-response relationships and also examine cocaine-induced reward.

G $_{\alpha q}$ within the frontal cortex may also play an additional role in the apparent working memory deficits we found in the Y-maze (Khan & Muly, 2011). Deletion of G $_{\alpha q}$ in telencephalic glutamatergic neurons resulted in spatial working memory deficits similar to our previous study (Frederick *et al.*, 2012). Arnsten and colleagues noted that PKC overactivation via G $_{\alpha q}$ -coupled receptors within the PFC impaired working memory function (Birnbaum *et al.*, 2004). Other downstream effectors of the G $_{\alpha q}$ signaling cascade have also been implicated in working memory function, such as IP $_3$ within the PFC (Lopez-Tellez *et al.*, 2010). D1 receptors within the PFC also play a significant role in working memory (Sawaguchi & Goldman-Rakic, 1991, Williams & Goldman-Rakic, 1995). D1 receptors, possibly via the G $_{\alpha q}$ signaling cascade, mediated working memory function (Runyan *et al.*, 2005). Similarly, others have found that antagonism of the G $_{\alpha q}$ -coupled mGluR5 resulted in working memory impairments (Balschun & Wetzel, 2002, Homayoun *et al.*, 2004), implicating mGluR5 located within the frontal cortex and hippocampus. It is unlikely that the memory impairments in our study are due to altered cortical inhibitory circuitry (Lin *et al.*, 2014, Straub *et al.*, 2007, Wang *et al.*, 2004), as there were no significant differences in GAD67 or PV cell density in this region.

Due to the nature of the cre line utilized, we cannot rule out whether the loss of G $_{\alpha q}$ in the PFC, hippocampus, amygdala, or a combination of these regions contributed to this memory deficit. While studies suggest that the amygdala does not have a direct role in spatial working memory, its modulation of stress and stress hormones upon other regions, such as the PFC, alters memory function (Abush & Akirav, 2013, Aggleton *et al.*, 1989, Roozendaal *et al.*, 2004) (but see Barros *et al.*, 2002). The hippocampus is important in spatial memory tasks (Burgess *et al.*, 2002, Martin & Clark, 2007) and is involved in the neurocircuitry of spontaneous alternation, the key output of the Y-maze (Lalonde, 2002). However, others noted that within the hippocampus, aging—not G $_{\alpha q/11}$ signaling—was detrimental to spatial memory (Mcquail *et al.*, 2013); this study did not specifically implicate a particular subset of G $_{\alpha q}$ -coupled receptors. Regardless, these data indicate that the G $_{\alpha q}$ pathway is important for spatial working memory. Future studies using viral cre-based deletions could be used to define the exact brain regions responsible for this phenotype.

Based on previous observations that neither global nor neuron-specific deletion of G $_{\alpha q}$ resulted in neuroanatomical abnormalities (Offermanns *et al.*, 1997, Wettschureck *et al.*, 2005, Wettschureck *et al.*, 2006), we examined more detailed cellular and morphological

correlates of altered neuronal composition. We noted no changes in GAD67+ or PV+ cells, indicating that loss of $G_{\alpha q}$ did not alter GABAergic cell expression. Furthermore, we did not note any gross changes in NeuN or S-100 staining, or in dendritic morphology of pyramidal neurons via MAP2 labeling. Taken as a whole, loss of $G_{\alpha q}$ in telencephalic glutamatergic cells does not result in any gross alterations to cytoarchitecture or morphology, suggesting that the behavioral phenotypes observed are not necessarily due to changes in cellular differentiation or abnormal brain development.

While these data indicate that particular populations of $G_{\alpha q}$ are involved in learning and memory and motor deficits, there are a number of drawbacks to this study. First, the behavioral studies were performed with relatively low sample sizes ($N=5-8$), which can contribute to Type II errors. The low sample size can be attributed to the difficulty in attaining the proper number of experimental mice given the complicated breeding scheme and additional control groups, with which we used multiple litters for analysis but also included littermates within groups when necessary. Also, while the y-maze is a test of short-term spatial working memory, it was the only assay used to test learning and memory in this study. Additional testing and increased sample sizes are necessary to further elucidate these data. For histological assays, we focused on inhibitory neuronal markers, despite the fact that $G_{\alpha q}$ was deleted from glutamatergic neurons. The balance between excitatory and inhibitory circuits is crucial for proper brain development (Marin, 2012, Yizhar *et al.*, 2011). It is of great interest that the inhibitory component (i.e., GABAergic interneurons) of this circuitry is unaffected by this genetic manipulation, indicating that the excitatory aspect is responsible for these alterations. Given that this is the component directly affected by the genetic manipulation, this is most likely. Future studies will examine these alterations directly.

Our data thus demonstrate that loss of $G_{\alpha q}$ in telencephalic glutamatergic neurons produces spatial working memory deficits and increased sensitivity to cocaine-induced locomotor activity but not basal motor function. These data suggest that activation of $G_{\alpha q}$ signaling in specific regions of the telencephalon could lead to new therapies to enhance learning and memory and to limit the effects of drugs of abuse.

Acknowledgements

We would like to thank Mary Akel for excellent technical support and Professor Stefan Offermanns (University of Heidelberg) for supplying the $G_{\alpha q}$ -floxed mice. Behavioral work was performed at the Vanderbilt Mouse Neurobehavioral Core, which is supported in part by NIH grant P30HD15052. This work was supported by NIH grant R01MH086629 (GDS).

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Figure 1.

Immunoblot analysis of G α q in various brain regions of the control and cKO mice. G α q levels were normalized against GAPDH. * p<0.05 vs. cre controls, *** p<0.001 vs. both cre and flox controls.

Figure 2.
Body weights of animals taken before behavioral testing.

Figure 3. Results of EZM as expressed in total distance traveled (a), average speed (b), percent time spent in the open and closed arenas (c), and number of entries into the open zones (d). *** $p < 0.001$ vs. respective closed arena

Figure 4. Latency to fall in the inverted screen test. No significant differences between groups were found.

Figure 5.

$G_{\alpha q}$ mutant animals were assessed in the Y-maze using the number of entries into the arms (a), and percent spontaneous alternations (b). * $p < 0.05$ vs. flox control; ** $p < 0.01$ vs. flox control; ++ $p < 0.01$ vs. cre control.

Figure 6.

No significant differences were found between any of the genotypes on the rotarod over the course of 3 days.

Figure 7.

Locomotor activity during habituation [Days 1 (a) and 2 (b)] and cocaine challenge (Day 3; c). The insets display total distance traveled across the 30–60 min bins (post-injection). Arrow indicates the SAL (Days 1 and 2) or cocaine (Day 3) injections. * $p<0.05$ flox control or cre control vs. cKO group; # $p<0.05$, ## $p<0.01$ flox control vs. cKO group.

Figure 8. Representative 20× images in the anterior cingulate cortex of MAP2 (a–c), GAD67 (d–f), and PV (g–i) staining in $G_{\alpha q}$ floxed control (a, d, g), cre control (b, e, h), and cKO (c, f, i) mice. Scale bar = 100 μ m

Figure 9.

Representative micrographs of $G_{\alpha q}$ floxed control (a, d, g, j), cre control (b, e, h, k), and cKO (c, f, i, l) mice stained with NeuN (a–f) or S-100 (g–l) at 10 $\times$ magnification in the ACC (a–c, g–i) or hippocampus (d–f, j–l). Scale bar = 100 μ m; ACC = anterior cingulate cortex; CA1 = CA1 hippocampal field; CC = corpus callosum; DG = dentate gyrus; SM = sensorimotor cortex

Comparison of behavioral data from current study utilizing the conditional $G_{\alpha q}$ knockout versus that of our previous study using constitutive knockouts.

Table 1

	Weights	EZM	Inverted Screen	Y-maze	Rotarod	Basal locomotor activity	Cocaine-stimulated locomotor activity
Constitutive KO (Frederick <i>et al</i> , 2012)	↓ weight	↓ speed ↔ anxiety	↓ latency to fall	↓ entries ↓ % spontaneous alternations	↓ latency to fall	↓ distance traveled (day 1)	↓ distance traveled
Conditional KO (Current study)	↔ body weights	↔ speed ↔ anxiety	↔ latency to fall	↓ entries ↓ % spontaneous alternations	↔ latency to fall	↔ distance traveled	↑ distance traveled