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Resistance to change and vulnerability to stress: Autistic-like features of GAP43 deficient mice

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Abstract

There is an urgent need for animal models of autism spectrum disorder (ASD) to understand the underlying pathology and facilitate development and testing of new treatments. The synaptic growth-associated protein-43 (GAP43) has recently been identified as an autism candidate gene of interest. Our previous studies show many brain abnormalities in mice lacking one allele for GAP43 (GAP43 (+/-)) that are consistent with the disordered connectivity theory of ASD. Thus, we hypothesized that GAP43 (+/-) mice would demonstrate at least some autistic-like behaviors.

We found that GAP43 (+/-) mice, relative to wild-type (+/+) littermates, displayed resistance to change, consistent with one of the diagnostic criteria for ASD. GAP43 (+/-) mice also displayed stress-induced behavioral withdrawal and anxiety, as seen in many autistic individuals. In addition, both GAP43 (+/-) mice and (+/+) littermates demonstrated low social approach and lack of preference for social novelty, consistent with another diagnostic criterion for ASD. This low sociability is likely due to the mixed C57BL/6J 129S3/SvImJ background.

We conclude that GAP43 deficiency leads to the development of a subset of autistic-like behaviors. Since these behaviors occur in a mouse that displays disordered connectivity, we propose that future anatomical and functional studies in this mouse may help uncover underlying mechanisms for these specific behaviors. Strain-specific low sociability may be advantageous in these studies, creating a more autistic-like environment for study of the GAP43-mediated deficits of resistance to change and vulnerability to stress.

Keywords

Autistic Disorder; Disease models, animal; Behavior, animal; GAP43; Mice, mutant strains; Maze learning; Reversal learning; Anxiety behavior; Social Behavior

INTRODUCTION

Autism spectrum disorder (ASD) involves impaired social interactions, communication deficiencies, and repetitive and stereotyped patterns of behavior (DSM IV). There is no single cause for ASD. Rather, it is an epigenetic phenomenon with numerous paths to a common outcome (Herbert, 2005, Minshew & Williams, 2007, Veenstra-Vanderweele *et al.*, 2004).

One hypothesized path to ASD is abnormal development and function of cortical circuits (Belmonte *et al.*, 2004, Rippon *et al.*, 2007). A central concept of this “disordered connectivity theory” is decreased connections *between* specialized brain areas, with increased or maintained connections *within* areas (Just *et al.*, 2004, Rippon *et al.*, 2007). This over connection of local circuit axons could produce excessive excitability, a proposed neurological state in ASD (Rubenstein & Merzenich, 2003). A major contributor to disordered connectivity may be transient brain overgrowth, seen in many autistic children (Courchesne *et al.*, 2001, Herbert, 2005).

Recently, growth-associated protein-43 (GAP43) has been identified as an autism candidate gene in the human gene region 3q13.2-q13.31 (Allen-Brady *et al.*, 2009). Findings from this study, and others (Schellenberg *et al.*, 2006, Szatmari *et al.*, 2007, Trikalinos *et al.*, 2006), suggest that genetic variants in this region may predispose males to broad spectrum ASD. GAP43 is found in growth cones of extending axons in the central nervous system (Meiri *et al.*, 1986, Skene *et al.*, 1986). Its many functions include growth cone navigation, neurite outgrowth, stabilization of axonal branches, neurotransmission and synaptic plasticity ((Denny, 2006).

Mice lacking one allele for GAP43 (GAP43 (+/-) mice) show multiple failures to establish or maintain long-distance cortical connections. These include poorly arborized thalamocortical axons with aberrant pathfinding (Mcilvain *et al.*, 2003) and decreased corpus callosum and hippocampal commissure volumes (Shen *et al.*, 2002). GAP43 (+/-) mice also display transient neonatal enlargement of barrel fields, which is normalized by adulthood (Mcilvain & McCasland, 2006, Mcilvain *et al.*, 2003). Despite this anatomical recovery, adult GAP43 (+/-) mice show enlarged excitatory receptive fields (Dubroff *et al.*, 2006). Taken together, these defects are consistent with both early brain overgrowth and disordered connectivity found in some individuals with ASD.

Mice lacking GAP43 (GAP43 (-/-) mice) also show interesting anatomical and functional deficits (Albright *et al.*, 2007, Donovan *et al.*, 2002, Donovan & Mccasland, 2008, Dubroff *et al.*, 2006, Maier *et al.*, 1999). However, the autistic-like findings of early overgrowth and disordered connectivity in GAP43 (+/-) mice cannot be confirmed in (-/-) mice due to high mortality rates (Maier *et al.*, 1999). GAP43 (-/-) mice also display multiple sensorimotor deficits that would confound tests for autism-like behavior, none of which are observed in GAP43 (+/-) mice (Metz & Schwab, 2004). For these reasons, we focused this study on GAP43 (+/-) mice.

Taken together, neurological abnormalities and human genetic findings suggest that GAP43 (+/-) mice may model at least some aspects of the ASD behavioral phenotype. To determine the utility of this animal model, we performed comprehensive testing for autistic-like behaviors in GAP43 deficient mice.

MATERIALS AND METHODS

Animals

This study investigated 54 GAP43 (+/-) adult mice (3–9 months, male and female) in a mixed C57BL/6J 129S3/SvImJ (B6129S3) background, and 50 (+/+) littermate controls. Mice were generated as previously described (Maier *et al.*, 1999). The initial mutation was bred in two strains - a 129S3/SvImJ strain and the progeny of a seventh-generation backcross into C57BL/6J. Since initial anatomical studies revealed no differences between the two strains (Maier *et al.*, 1999), the lines were subsequently interbred for efficient colony maintenance. The current mixed background is a result of approximately five generations of interbreeding between these two lines. For the social behavior testing, six adult male

C57BL/6J mice (B6, 2 months) were purchased from Jackson Laboratory (Bar Harbor, ME) and used as additional controls. Based on the availability of testing equipment, mice were tested at either State University of New York Upstate Medical University (SUNY Upstate) or the University of Texas Southwestern Medical Center (UT Southwestern), as detailed in Table 1.

All animals were handled in accordance with guidelines set forth by the Department of Laboratory Animal Resources at SUNY Upstate. Mice tested at SUNY Upstate were on a 14-hour light cycle (light on at 6 a.m.), and behavior tests were done between 8 a.m. and 4 p.m. after a minimum 30-minute period of habituation in the test room. Mice that were tested at UT Southwestern were shipped from SUNY Upstate at 3–5 months of age, and underwent 5 weeks of quarantine before testing. These mice were on a 12-hour light cycle (light on at 7 a.m.), and behavior tests were done between 8 a.m. and 5 p.m. after a minimum 30-minute period of habituation in the test room. All behavioral testing and scoring was done while blinded to animal genotype.

Behavioral Tasks

Learning and Memory

T-Maze: The T-maze test was performed to evaluate spatial learning and reversal learning, as previously described (Moy *et al.*, 2007). Mice were habituated to the apparatus (15 cm entrance tube, 5 cm in diameter, split into two 35 cm tubes at right angles) during 10 minute trials for 6 days with a food reward (sweetened condensed milk – Wegmans, Rochester, NY) in both arms. Mice were then trained for 10 consecutive trials per day to receive the food reward from one arm of the T-maze (learning phase), with a brief pause between trials to replenish the food, if needed. Once subjects met the learning criteria of 80% correct arm choice for 3 consecutive training days, the food reward was moved into the opposite arm and the test was repeated (reversal phase). Reversal criteria were also 80% correct arm choice for 3 consecutive training days. Both the number of correct arm choices per day and days to attain learning and reversal criteria were measured. If a mouse did not meet learning criteria after 9 training days, it was excluded from the reversal phase. If a mouse did not achieve reversal criteria by day 9, it was assigned a 10 for “days to reach reversal criteria”.

Morris Water Maze (MWM): The MWM task was performed to evaluate spatial learning and reversal learning, similar to previously described protocols (Crawley, 2004, Moy *et al.*, 2007). A pool of 122 cm diameter, 91.5 cm height was filled with 20 cm of 26±2°C water. Four geometric spatial cues were placed around the walls of the pool in each quadrant (named NE, NW, SE, and SW).

For all mice, an initial probe trial was carried out in the open pool to habituate them to the apparatus, and ensure they were capable of swimming. These sessions were videotaped. Two litters (N=19) were randomly selected for *post hoc* analysis of swimming behavior to evaluate thigmotaxis (the tendency to stay near the edge of the pool). Traces of swimming were manually prepared, and time spent in the outer 25% of the tank was hand scored.

After habituation, mice were trained to locate a translucent platform of 12 cm diameter, covered by 1 cm of water, at a fixed location (SE quadrant). There were four trials per day, with 10–20 minutes between trials. The latency to find the platform and the number of days to reach learning criteria (average latency to find platform <15 seconds for 3 consecutive training days) were measured. After mice met learning criteria, a probe trial in an open pool was performed. If mice demonstrated spatial bias (≥40% of time in SE quadrant, and at least 10% more time than in any other quadrant), they moved onto the reversal phase. If a mouse did not exhibit bias, training continued. In the reversal phase, the platform was moved to the

NW quadrant, and the latency to find the platform and number of days to reach learning criteria were measured. Probe trials for spatial bias were repeated. Each phase lasted for up to 9 days.

Fear Conditioning: Both context- and cue-dependent fear conditioning were completed to assess learning and memory using a contextual conditioning system (Med Associates, St. Albans, VT), as previously described (Powell *et al.*, 2004). The chamber was 9.5in wide × 12in long × 5¾in tall, with clear plastic walls and ceilings and a floor with standard 4.8mm grid rods. The chamber was equipped with a light and a fan. For Trial 1 (training), mice were placed in the chamber with both the light and fan on. After a 2-minute habituation, a 30 second 90-dB tone that coterminated with a 2 second 0.5mA shock was delivered twice, with a 1-min interstimulus interval. Mice remained in the context for 2 minutes prior to return to home cage overnight. After 24 hours, Trial 2 assessed context learning by placing mice into the same context as training but in the absence of any shock. After 3 hours, Trial 3 assessed cue determined learning by placing mice in a novel context for a 3 minute baseline assessment, followed by 3 minute presentation of the 90-dB tone. To create a novel context the box size was reduced by 50%, made from a different type of plastic, scented with vanilla, and the box light and fan were removed. In each of these sessions, freezing behavior was scored manually by two independent observers (within 80% agreement) at 10 second intervals.

Repetitive Behavior

Marble Burying: Marble burying was performed to evaluate repetitive behaviors, as previously described (Thomas *et al.*, 2009). Briefly, clean cages were filled with 4 cm of Sani-chip bedding (P.J. Murphy Forest Products, Montville, NJ). Eighteen 30mm blue marbles were placed in a 3×6 grid on top of the bedding. The number of marbles buried at least 50% by bedding were counted at the end of a 30-minute trial.

Social Behavior

Social Interaction: GAP43 (+/+) and (+/−) mice were evaluated for social interaction with a caged unfamiliar adult mouse, as previously described (Tabuchi *et al.*, 2007). The apparatus was built to the following specifications – an open field (48cm × 48cm) containing a wire mesh cage (6.0cm × 9.5cm), with a defined “interaction zone” (15cm × 30cm) surrounding the wire cage. For trial 1, the wire cage was empty. For trial 2, the wire cage housed a novel adult B6 mouse. For both trials, mice were allowed to freely explore the open field for 5 minutes. Time spent in an “interaction zone” during each trial was quantified using Ethovision 2.3.19 videotracking software (Noldus, Leesburg, VA). The same protocol was followed for B6 controls, except that each session lasted 2.5 minutes (Lagace *et al.*, 2010).

3-Chambered Social Interaction Test: The 3-chambered task was performed, as previously described (Crawley, 2004, Tabuchi *et al.*, 2007). The apparatus was built to the following specifications – three 15 × 29 cm chambers, with empty wire cages in the 2 end chambers. During the habituation trial (10 minutes), mice were allowed to freely explore all three chambers. Next, during the sociability trial (10 minutes), mice were allowed to interact with a same-sex, unfamiliar B6 mouse in the wire cage in one end chamber, versus the still-empty wire cage in the other end chamber. Lastly, during the social novelty trial (10 minutes), the now familiar mouse remained in its cage, while a novel same-sex B6 stranger mouse was placed in the wire cage in the other end chamber. Locations of empty cages and target mouse, as well as novel vs. familiar mouse, were counterbalanced across subjects. Time spent in all three chambers was calculated for each trial, using Ethovision 2.3.19 videotracking software from (Noldus, Leesburg, VA).

Juvenile Interaction: A juvenile interaction paradigm was performed to evaluate social interaction and social memory, as previously described (Tabuchi *et al.*, 2007). All mice were habituated for 15 minutes to dark with red light in testing room in home cage. Test mice were placed in a clean unfamiliar mouse cage with no bedding, with an unfamiliar 4 week old B6 mouse of the same sex for 2 minutes. Three days later, mice were again tested in the same condition, in a novel cage with the same juvenile mouse for a 2-minute trial. Time spent interacting (following, sniffing etc.) with the juvenile was measured.

Social Communication

Social Transmission of Food Preference (STFP): The STFP test was performed to evaluate social communication, as previously described (McFarlane *et al.*, 2008). Mice were habituated to feeding cup (4cm diameter, 3 cm high, glued in a Petri dish to collect spillage) filled with unflavored ground chow (PMI Nutrition, Brentwood, MO) for 3 days prior to the start of the test. After overnight food deprivation, sex-matched (+/+) adult demonstrator mice were given free access to ground chow, flavored either 1% cinnamon (McCormick, Hunt Valley, MD) or 2% cocoa (Hersey's, Hersey PA) (split 50/50), for 1 hour. Demonstrator mice were then placed in a fresh cage with observer mice for a 30-minute interaction, and returned to their home cage. Observer mice were then food-deprived overnight. The next day they were placed in a clean cage and given free choice between cued food (flavor consumed by demonstrator) and non-cued food for 1 hour. To control for innate flavor preference, ~50% of the observers were cued to each flavor. After the trial, mice were returned to their homecage and the amount of each food consumed was measured. To control for appetite, a ratio of "cued food eaten/total food eaten" was calculated.

To ensure that we were actually measuring *social* transmission of food preference, a non-social transmission of food preference (NSTFP) control was performed. The same protocol was followed, with the exception that demonstrator mice were replaced with a novel object (cube built with children's interlocking blocks – Mega Brands, Montreal QC, Canada). The cued flavored chow was packed between layers of blocks so that the scent could be detected, but the chow would not be accessible for consumption. Again, ~50% of the mice were cued to each flavor to control for innate flavor preference. This control was done to test if exposure to the odorant alone, without the social component, was adequate to convey flavor preference.

Anxiety and Stress-Induced Behavioral Withdrawal

Elevated Plus Maze (EPM): The EPM was performed to evaluate anxiety, as previously described (Mulder & Pritchett, 2004). The apparatus was built to the following specifications - elevated 32 cm high, 2 open and 2 closed arms each 30 cm long, 2 cm wide, walls on closed arms 17 cm high. Mice were placed in the center of the apparatus and allowed to freely explore for 5 minutes. All sessions were videotaped. Time spent in each arm was measured. Data are presented as ratio of "time spent in open arm/(time spent in open arm + time spent in closed arm)" to control for any locomotor differences.

Light Dark Test (LDT): The LDT was performed to evaluate anxiety, as previously described (Liu *et al.*, 2007). The apparatus consisted of a light and a dark chamber, each 10 inches by 10 inches, separated by a wall with a small vestibule. The chambers were constructed and automated as described by Gershenfeld and Paul (1997). Briefly, the light chamber was open, transparent, and illuminated at 40 watts. The dark chamber was closed, painted black, and dark. Movements between the two chambers were detected by photocells. Mice were placed into the dark box for a 2-minute habituation. They were then allowed 10 minutes of access to the light box. The latency to scan the light box (head, but <4 paws, in

light box), latency to completely emerge from the dark box, and time spent in light box were measured.

Porsolt Forced Swim Test (FST): The FST was performed to evaluate behavioral withdrawal induced by stress, as previously described (Krishnan *et al.*, 2008, Nakasato *et al.*, 2008). Mice were placed in a 1000 ml beaker (Corning Inc. Life Sciences, Lowell, MA), filled with 700 ml of $26\pm 2^{\circ}\text{C}$ water. After a 2-minute habituation, latency to immobility, time spent immobile (behavioral withdrawal), and time spent swimming/climbing (escape-oriented behavior) were measured for the next 4 minutes. The entire 6-minute session was videotaped.

Tail Suspension Test (TST): The TST was performed to evaluate stress-induced behavioral withdrawal, as previously described (Liu *et al.*, 2003). During a 6-minute trial, mice were suspended by their tail on an automated TST device (Med Associates Inc, St. Albans, VT). The device's force transducer used pre-set thresholds to determine time spent immobile (behavioral withdrawal) and time spent struggling (escape-oriented behavior) for each mouse. These measurements were detected and calculated by the computer.

Baseline Activity

Locomotor Testing: Locomotor activity was assessed using photo beams linked to computer data acquisition software (San Diego Instruments, San Diego, CA), as previously described (Tabuchi *et al.*, 2007). Mice were placed for 2 hours in a fresh home cage with minimal bedding. After allowing the mice to habituate for the first hour, horizontal activity and total distance moved was measured for the second hour and averaged into 5-minute bins.

Statistics

All measures are reported as mean $\pm$ SEM. The percent attaining reversal data from T-Maze was analyzed using *Fisher's exact probability test*. For the FST, EPM, and LDT, data were analyzed using a two-way analysis of variance (ANOVA) with factors of sex and genotype, followed by Tukey *post hoc* comparisons if a significant *F*-value was determined. For all other tests, differences between genotypes were analyzed using two-tailed *Student's t-test*. All GAP43 (+/+) vs. (+/-) comparisons assumed unequal variances. Paired t-tests were used for within genotype comparisons (e.g. trial 1 vs. trial 2) for social paradigms.

RESULTS

General Appearance

There were no obvious differences in physical appearance between (+/+) and (+/-) littermates, consistent with previous reports (Metz & Schwab, 2004). There was no significant difference in weights between (+/+) and (+/-) mice (Groups B,C) - $t_{69}=0.65$, $p=0.52$. Therefore, weight-matched controls were unnecessary. The average weight for (+/+) mice was $34.56\pm 1.49\text{g}$, and the average weight for (+/-) mice was $33.10\pm 1.66\text{g}$.

Behavior

Behavioral results are summarized in Table 1, and presented in detail below.

Learning and Memory—In the learning phase of the T-Maze test, there were no significant differences between GAP43 (+/+) and (+/-) mice in the 1) number of mice that met learning criteria (Figure 1a); 2) latency to reach learning criteria ((+/+) 5.43 ± 0.84 days, (+/-) 5.00 ± 0.72 days); or 3) average number of correct arm choices ((+/+) 8.10 ± 0.70 , (+/-)

8.32±0.56). Both genotypes had a ~50% failure in initial task acquisition, with no sex differences. Similar acquisition rates have been reported in other mouse strains (Moy *et al.*, 2007). Only the 14 mice that met criteria in the learning phase were assessed for reversal learning.

In the reversal phase of the T-Maze test, GAP43 (+/-) mice displayed significantly impaired performance compared to (+/+) mice for 1) number of mice that met reversal criteria (Figure 1b) – Fisher exact probability test: $df=1$, $p\leq 0.05$; 2) latency to reach reversal criteria ((+/+) 5.29±0.57 days, (+/-) 8.29±1.11 days) – $t_{12}=2.31$, $p\leq 0.05$; and 3) average number of correct arm choices ((+/+) 8.10±0.039, (+/-) 4.43±1.37) – $t_{12}=2.35$, $p\leq 0.05$.

As an additional assessment for spatial and reversal learning, GAP43 (+/+) and (+/-) mice also completed the Morris water maze (MWM). There was no apparent difference in swimming capabilities between genotypes in the initial probe trial in the open pool with no escape platform. However, there was a difference in swimming behavior. GAP43 (+/-) mice displayed increased thigmotaxis (Figure 2a), spending significantly more time than (+/+) mice in the outer 25% of the tank's diameter (Figure 2b) – $t_{17}=4.83$, $p\leq 0.001$. Considering this behavior, it was not surprising that GAP43 (+/-) mice had significantly increased latencies to find the hidden platform (located toward the center of the pool) during early days of training (Figure 3a) – day 1 $t_{38}=3.02$, $p\leq 0.01$, day 2 $t_{38}=2.56$, $p\leq 0.05$, day 4 $t_{38}=2.72$, $p\leq 0.01$, day 5 $t_{38}=2.69$, $p\leq 0.01$. This increase in latency resulted in GAP43 (+/-) mice taking significantly more days to attain learning criteria than (+/+) mice (Figure 3b) – $t_{38}=3.00$, $p\leq 0.01$.

Mice that attained learning criteria showed no genotype difference in percent time spent in correct quadrant during the test probe trial – (+/+) 39.2±2.1%, (+/-) 37.8±1.7%. Five mice – (+/+) 3 females, (+/-) 1 female, 1 male – did not display spatial bias after training, and were excluded from the reversal phase. In the reversal phase, there was no significant genotype difference in latency to find the platform on any day between genotypes (Figure 3a). There was also no difference between genotypes in the number of days to reach reversal criteria (Figure 3b). Mice that attained reversal criteria showed no genotype difference in percent time spent in correct quadrant during the test probe trial – (+/+) 41.3±2.5%, (+/-) 40.7±0.3%. Two male (+/-) mice did not display spatial bias after reversal training.

In addition to spatial learning, fear conditioning was tested to assess both context- and cue-dependent memory. Both genotypes demonstrated low levels of unconditioned freezing in Trial 1 (<5%) and spontaneous freezing (freezing before tone presentation) in Trial 3 (<15%). There was no difference between genotypes in either contextual or cued freezing percentage (Figure 4a). Both genotypes displayed a significant increase between spontaneous and cued freezing in Trial 3 (Figure 4b) – (+/+) $t_{32}=10.84$, $p\leq 0.001$, (+/-) $t_{32}=8.94$, $p\leq 0.001$.

Stress-Induced Behavioral Withdrawal—Significant sex × genotype differences in escape oriented (swimming/climbing) behavior were observed during the FST – $F_{1,36}=5.67$, $p\leq 0.05$. Tukey *post hoc* comparisons revealed two sources of significant difference. GAP43 (+/-) males, but not females, displayed a significant decrease in time spent swimming/climbing compared to (+/+) counterparts (Figure 5a) – $p\leq 0.05$. In addition, GAP43 (+/+) males exhibited significantly more swimming/climbing (escape oriented behavior) than (+/+) females (Figure 5a) – $p\leq 0.05$. This sex difference was not seen between (+/-) males and females – $p=.95$. These findings were not associated with any significant differences in latency to immobility (male: (+/+) 26.45±12.12s, (+/-) 18.33±4.58s; female: (+/+) 19.89±12.21s, (+/-) 14.63±5.87s) or time spent immobile (male: (+/+) 92.45±10.09s, (+/-) 119.33±8.17s; female: (+/+) 103.00±12.96s, (+/-) 86.50±14.91s).

In the tail-suspension test (TST), GAP43 (+/-) mice demonstrated a significant decrease in time engaged in struggling relative to (+/+) mice (Figure 5b) - $t_{31}=2.28$, $p\leq 0.05$. This occurred in the absence of any significant difference in time spent immobile ((+/+) 274.14±6.60s, (+/-) 286.21±5.12s). There were no sex-specific effects detected in any of the TST measures.

Anxiety—In the elevated plus maze (EPM) task, there appears to be a qualitative difference in performance between (+/+) males and females in the ratio of time in open arm/(time in open + closed arms) (Figure 6a). However, differences between groups were not statistically significant.

In the light dark test (LDT), there was a near-significant sex × genotype effect in time to emerge ($F_{1,33}=3.94$, $p=0.056$). GAP43 (+/+) males were much faster to emerge than any other group. There were no significant differences in time to scan or time spent fully emerged between groups (Figure 6b).

Social Behavior—In the social interaction task with adults, B6 controls showed the expected social behavior (Crawley, 2004) by spending significantly more time in the interaction zone with a social target present - $t_{10}=2.18$, $p\leq 0.05$. In contrast, both GAP43 (+/+) and (+/-) mice spent significantly more time in the interaction zone during the habituation phase (empty cage) than they did during the social approach phase (social target in cage) - (+/+) $t_{32}=2.37$, $p\leq 0.05$, (+/-) $t_{32}=2.00$, $p\leq 0.05$ - (Figure 7a). There was no genotype difference on this test in our mixed strain.

There was also no significant difference for interaction time with a juvenile between GAP43 (+/+) and (+/-) mice during either initial or subsequent exposure. Both genotypes, as well as B6 controls, displayed a significant decrease in social interaction on second exposure - (+/+) $t_{32}=5.82$, $p\leq 0.001$, (+/-) $t_{32}=4.31$, $p\leq 0.001$, B6 $t_{10}=5.44$, $p\leq 0.001$ (Figure 7b).

During the sociability trial of 3-chambered social interaction test, B6 controls displayed the expected behavior (Moy *et al.*, 2009) by spending significantly more time with a stranger mouse (labeled as stranger 1) than in chamber with an empty cage - $t_{10}=2.82$, $p\leq 0.05$. In contrast, neither GAP43 (+/+) nor (+/-) mice showed a significant preference for the stranger mouse rather than an inanimate object (Figure 8a). Similarly, in the social novelty trial of the 3-chambered test, B6 controls displayed the expected preference (Moy *et al.*, 2009) by spending significantly more time in the chamber with stranger 1 (now familiar mouse) than in the chamber with stranger 2 (novel mouse) - $t_{10}=4.27$, $p\leq 0.01$. In contrast, once again, neither GAP43 (+/+) nor (+/-) mice showed a significant preference for the novel mouse (Figure 8b). There was no genotype difference in any trial of the 3-chambered social interaction test in our mixed strain.

These effects occurred in the absence of any significant differences in the analysis of locomotion in GAP43 (+/+) and (+/-) mice, which revealed similar overall distance beam breaks after habituation to a novel home cage - (+/+) 42.94±4.39, (+/-) 57.35±11.73).

Social Communication—There were no differences in the social transmission of food preference (STFP) task between genotypes. Both GAP43 (+/+) and (+/-) mice had a “cued food eaten/total food eaten” ratio greater than .50, indicating that they consumed more cued than non-cued food. However, both genotypes also had a “cued food eaten/total food eaten” ratio greater than .50 in the non-social transmission of food preference (NSTFP) control task. There was no difference in this ratio in either genotype between the STFP and NSTFP tasks (Figure 9) - (+/+) $t_{33}=0.21$ $p=0.83$, (+/-) $t_{30}=0.44$ $p=0.78$.

Repetitive Behavior—In the marble burying task, there was no difference between GAP43 (+/+) and (+/−) mice in the number of marbles buried ((+/+) 10.33±1.52, (+/−) 8.78±1.19).

DISCUSSION

Autism affects an estimated 1% of children in the United States (Kogan *et al.*, 2009, Mulvihill *et al.*, 2009). Animal models are needed to better understand its disease processes and develop new treatment strategies for this growing population. Here, we present findings that a specific subset of autistic-like behaviors is mediated by GAP43 deficiency in our mice.

GAP43 (+/−) mice display resistance to change

Individuals with ASD demonstrate resistance to change and/or repetitive behaviors (DSM-IV). In the T-maze, GAP43 (+/−) mice displayed clear deficits in reversal learning, indicating resistance to change (Crawley, 2004, Moy *et al.*, 2007). However, they did not show this deficit in the MWM. This difference may be due to task/reward variables that more strongly motivate reversal learning in the MWM. The T-maze rewarded correct choices, but did not punish incorrect ones. However, failure in the MWM task led to the aversive outcome of remaining in the water. This prolonged period of immersion was a strong motivation for mice to find the hidden platform, regardless of location. We propose that resistance to change in GAP43 (+/−) mice is overcome in the MWM due to the stronger aversive consequence of failure.

While GAP43 (+/−) mice demonstrated resistance to change, they did not exhibit repetitive behavior, at least as measured by marble burying. However, a diagnosis of autism does not require both behaviors, as they are grouped into one diagnostic domain (DSM-IV).

GAP43 (+/−) mice display increased stress-induced behavioral withdrawal and anxiety

Many autistic individuals show depression and social withdrawal in response to stress (Pearson *et al.*, 2006). Decreased struggling by GAP43 (+/−) mice in both the FST and TST indicate a lack of escape-oriented behavior, suggesting stress-induced behavioral withdrawal (Lucki, 1997, Nakasato *et al.*, 2008). Interestingly, in the FST, this decrease was only observed in GAP43 (+/−) males. These data are especially intriguing because autism is ~4× more common in males (Kogan *et al.*, 2009, Mulvihill *et al.*, 2009). Although GAP43 is an autosomal gene, this sex-specific finding is consistent with the finding that sex steroids modulate GAP43 mRNA levels in some postnatal brain regions (Shughrue & Dorsa, 1993, Shughrue & Dorsa, 1994).

There is no evidence for increased baseline anxiety in GAP43 (+/−) mice from the EPM and LDT tasks. However, when challenged with the stress of being placed in water (Nakasato *et al.*, 2008), increased thigmotaxis in the MWM suggests that GAP43 (+/−) have heightened stress-induced anxiety compared to (+/+) mice. This evidence, along with decreased escape-oriented behaviors in the FST and TST, indicate that GAP43 (+/−) mice, especially males, are less able to cope with stress than (+/+) mice. Increased vulnerability to stress has been shown in many individuals with autism, and may substantially contribute to autistic behaviors (Groden *et al.*, 1994).

Spatial learning is modulated by heightened anxiety in GAP43 (+/−) mice

We evaluated spatial learning in GAP43 (+/−) mice, since deficiencies have been reported in many autistic individuals (Steele *et al.*, 2007). GAP43 (+/−) mice did not display impaired learning compared to (+/+) in T-maze. However, GAP43 (+/−) mice displayed learning

delays in the MWM compared to (+/+) littermates. This is probably due to modification of learning by the observed stress-induced anxiety, as mentioned above. We hypothesize that longer search times for (+/-) mice to find the hidden platform on early days of training were caused by initial reluctance to leave the side of the pool due to heightened anxiety. This modulation of behavior by anxiety creates an apparent spatial learning deficit that is probably not due to a cognitive defect.

Both GAP43 (+/-) mice and (+/+) littermates display decreased social interaction

Individuals with autism demonstrate low social interaction and decreased social communication (DSM-IV). Social mice prefer interaction with other mice over inanimate objects, and novel mice over familiar mice (Bolivar *et al.*, 2007, Moy *et al.*, 2009, Moy *et al.*, 2007). While our B6 control group displayed the expected social behavior, neither GAP43 (+/-) nor (+/+) mice displayed high social approach to a stranger or preference for social novelty. This lack of normal social behavior is not due to impaired social memory, since both GAP43 (+/+) and (+/-) demonstrated memory for a juvenile mouse upon re-exposure.

Since both (+/-) and (+/+) demonstrated low sociability, we cannot determine GAP43's role in social behavior. The observed social deficits are likely due to strain-specific low sociability of the mixed B6129S3 background, as different strains exhibit varying levels of social behavior (Moy *et al.*, 2007). Future backcross studies into the highly social C57BL/6J background will determine whether GAP43 dependent social deficits are being masked by B6129S3 strain-specific low sociability.

In the STFP task, both GAP43 (+/+) and (+/-) mice consumed a greater ratio of cued:non-cued food, indicating intact social communication (Galef & Whiskin, 1995). However, this also occurred when the "demonstrator" was a novel object, as opposed to a stranger mouse. These results suggest that the olfactory cue can be transmitted from an inanimate object, and that the social component is not necessary. In both paradigms, it is likely that decreased consumption of the non-cued food was a result of neophobia. Future pup vocalization studies (Crawley, 2004) are planned to more directly measure if GAP43 deficiency affects social communication.

Altered synaptogenesis may contribute to abnormal GAP43 (+/-) mouse behavior

Taken together, our published studies provide strong evidence for disordered connectivity in GAP43 (+/-) mice (Dubroff *et al.*, 2006, Mcilvain & McCasland, 2006, Mcilvain *et al.*, 2003, Shen *et al.*, 2002). It has been proposed that disordered connectivity is the common aberrant neurological finding resulting from the various insults that lead to the development of ASD (Belmonte *et al.*, 2004, Just *et al.*, 2004, Rippon *et al.*, 2007). This theory proposes that observed early brain overgrowth in autistic individuals (Courchesne *et al.*, 2001, Herbert, 2005) coincides with the peak of synaptogenesis and myelination, preventing formation of proper connections (Rippon *et al.*, 2007). Thus, it is likely that abnormal synaptic development and elimination contribute to the development of autistic behavior (Pfeiffer & Huber, 2009, Zoghbi, 2003).

We propose that abnormal behavior in GAP43 (+/-) mice could be due in part to aberrant synaptogenesis. We have recently shown that these mice display early abnormalities in glutamate receptor composition and dendritic organization in the cortex subsequent to sparse thalamocortical input (Kelly *et al.*, 2010). Interestingly, both deficits are then normalized by compensatory changes. These findings emphasize the importance of complex interplay between pre- and postsynaptic elements for proper development of cortical networks (Kelly *et al.*, 2010).

Further study of altered GAP43 (+/–) synaptic development may reveal novel treatment strategies for the specific autistic-like behaviors observed. Planned experiments will test whether mGluR5 antagonists, which affect NMDA receptor function (Lea *et al.*, 2002), can reduce these abnormal behaviors. This drug treatment has been shown to be effective for reducing repetitive behaviors in the BTBR T+tf/J mouse model of autism (Silverman *et al.*, 2010).

Utilizing the B6129B3 GAP43 (+/–) mouse to study ASD

Since autism is a disorder of human behavior, there can never be a truly “autistic” mouse. Nevertheless, mice with abnormal behaviors that parallel human ASD can serve as valid animal models (Crawley, 2004). However, the behavioral profile of ASD is extremely complex, and likely has multiple underlying causes (Happé *et al.*, 2006). Therefore, modeling and studying all aspects in one mouse model is proving very difficult, and may not be advantageous.

We have isolated specific autistic-like behaviors that can be attributed to GAP43 deficiency, including resistance to change and stress-induced behavioral withdrawal and anxiety. Since these behaviors occur in a mouse that displays disordered connectivity, we propose that future anatomical and functional studies in this mouse may help uncover underlying mechanisms for these specific behaviors. In addition, our mice exhibit strain-specific low sociability, similar to the BTBR T+tf/J mouse model of autism (McFarlane *et al.*, 2008, Moy *et al.*, 2009, Moy *et al.*, 2007). This inherent deficit in sociability can be used to our advantage, creating a more autistic-like environment in which to study these GAP43-mediated behavioral deficits.

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Figure 1. Number of mice to reach passing criteria during (a) the learning phase and (b) the reversal phase of the T-maze task

There was no difference in the number of GAP43 (+/+) and (+/-) mice to attained learning criteria within 9 days of training. However, significantly fewer GAP43 (+/-) than (+/+) mice attained reversal criteria within 9 days of training. (* $p \leq 0.05$)

Figure 2. (a) Qualitative and (b) quantitative analysis of swimming behavior during initial probe trial of MWM

Composite traces of swimming behavior of all mice showed that (+/-) mice had a strong tendency to swim near the edge of the pool (thigmotaxis). Quantitative annulus analysis revealed that GAP43 (+/-) mice spent significantly more time in the outer 25% of the pool than (+/+) mice. (***) $p \leq 0.001$

Figure 3. (a) Average daily latency to find hidden platform and (b) days to reach passing criteria during the learning and reversal phases of MWM

GAP43 (+/-) mice take significantly longer to locate the hidden platform during early, but not late, training days during the learning phase. GAP43 (+/-) mice also take significantly longer than (+/+) mice to reach passing criteria during the learning phase. However, there is no difference between genotypes in daily latency to find hidden platform or in days to reach passing criteria during the reversal phase. (* $p \leq 0.05$, ** $p \leq 0.01$)

Figure 4. Freezing behavior during fear conditioning task

(a) There is no difference in either contextual (trial 2) or cued (trial 3) freezing between genotypes. **(b)** Both genotypes displayed significantly less spontaneous freezing than cued freezing in trial 3. (** $p \leq 0.001$)

Figure 5. Time spent engaged in escape-oriented behavior during (a) FST and (b) TST

In the FST, GAP43 (+/−) males displayed significantly decreased active swimming than (+/+) males. This difference was not seen between (+/−) and (+/+) females. GAP43 (+/+) mice displayed sex differences, with (+/+) males spending significantly more time engaged in active swimming than (+/+) females. In the TST, GAP43 (+/−) mice displayed significantly decreased struggling compared to (+/+) mice. There were no sex differences in the TST. (* $p \leq 0.05$, ** $p \leq 0.01$)

Figure 6. Results from (a) EPM and (b) LDT

EPM data is presented as a ratio of time spent in the open arm to total time spent in all arms to correct for differences in locomotion. There were no significant differences between groups. LDT testing showed a near-significant trend with (+/+) males fully emerging more quickly than other groups. There were no significant differences in the other measures. (n.s. $p=0.056$)

Figure 7. Behavioral measures of (a) social approach and (b) social memory

Both GAP43 (+/+) and (+/-) mice spent a significantly greater percent of time in the interaction zone when the target mouse was absent than when the target mouse was present during the social approach paradigm. In contrast, B6 controls spent a significantly greater percent of time in the interaction zone when the target mouse is present. All mice decreased interaction time upon re-exposure to a juvenile mouse. (* $p \leq 0.05$, *** $p \leq 0.001$)

Figure 8. Time spent in outer chambers during the (a) sociability trial and (b) social novelty trial of the 3-chambered apparatus test

During the sociability trial, neither GAP43 (+/+) nor (+/-) mice spent more time in the chamber containing stranger 1 than the chamber without a target mouse. In contrast, B6 controls spent significantly more time in the chamber containing stranger 1. Similarly, in the social novelty trial, neither GAP43 (+/+) nor (+/-) mice spent more time in the chamber containing stranger 1 (familiar mouse) than the chamber containing stranger 2 (novel mouse). In contrast, B6 controls spent significantly more time in the chamber containing stranger 2 (novel mouse). (* $p \leq 0.05$, ** $p \leq 0.01$)

Figure 9. Ratio of cued/total food eaten during transmission of food preference tasks
There was no difference in the ratio of cued food eaten between genotypes in either the social transmission (STFP) or non-social transmission (NSTFP) tasks.

Table 1

Overview of behavioral tests performed and significant results. Tests for Group A/B were performed on a rotating schedule based on litter, with only one test being performed at a time. Tests for Group C/D were performed in order presented in table.

Test	Evaluates	Animal Group	Significant Results
T-maze	Spatial learning Resistance to change	Group A SUNY Upstate Total N=26 Male (+/+) N = 6; (+/-) N = 5 Female (+/+) N = 6; (+/-) N = 9	<u>Learning Phase</u> No differences between (+/-) and (+/+) mice <u>Reversal phase</u> Decreased number to attain reversal criteria in (+/-) mice Increased latency to attain reversal criteria in (+/-) mice Increased arm choice errors in (+/-) mice
Morris Water Maze (MWM)	Spatial learning Resistance to change	Group B SUNY Upstate (Group A plus 2 additional litters) Total N=39 Male (+/+) N = 11; (+/-) N = 10 Female (+/+) N = 9; (+/-) N = 9	<u>Habituation Phase</u> Increased time spent in outer 25% of pool by (+/-) mice (thigmotaxis) <u>Learning Phase</u> Increased latency to find hidden platform in (+/-) mice in early, but not late, days of training Increased latency to reach learning criteria in (+/-) compared to (+/+) mice <u>Reversal Phase</u> No differences between (+/-) and (+/+) mice
Elevated Plus Maze (EPM)	Anxiety	Group B	No differences between (+/-) and (+/+) mice
Marble Burying	Repetitive behavior	Group D Total N=33 Male (+/+) N = 10; (+/-) N = 12 Female (+/+) N = 5; (+/-) N = 6	No differences between (+/-) and (+/+) mice
Transmission of food preference Social (STFP) Non-social (NSTFP)	Social communication	Group B – STFP Group D – NSTFP	No difference between (+/+) and (+/-) mice No difference in either genotype in ratio of cued food/total food eaten between social and non-social transmission
Porsult Forced Swim Test (FST)	Stress-induced behavioral withdrawal	Group B	Decreased swimming/climbing in (+/-) compared to (+/+) mice Increased swimming/climbing in (+/+) males compared to (+/+) females
Social Approach	Social approach	Group C UT Southwestern N=32 Male (+/+) N = 7; (+/-) N = 9 Female (+/+) N = 8; (+/-) N = 8 B6 control group: N= 6 male	Both (+/+) and (+/-) spent more time in interaction zone with target absent than with target present B6 controls spent more time in interaction zone with target present
Juvenile Interaction	Reciprocal social interactions, social memory	Group C B6 control group	No significant difference between (+/+) and (+/-) All mice decreased interaction during subsequent interaction
3-Chambered Social Apparatus	Social approach, object novelty, social novelty	Group C B6 control group	Neither (+/+) nor (+/-) mice spent more time with mouse vs. object in sociability trial or with novel vs. familiar mouse in social novelty trial B6 controls spent more time with mouse in sociability trial and more time with novel mouse in social novelty trial
Tail Suspension Test (TST)	Stress-induced behavioral withdrawal	Group C	Decreased struggling in (+/-) compared to (+/+) mice
Locomotor Testing	Baseline activity	Group C	No differences between (+/-) and (+/+) mice
Light/Dark Test (LDT)	Anxiety	Group C	No differences between (+/-) and (+/+) mice

Test	Evaluates	Animal Group	Significant Results
Fear Conditioning	Fear, learning & memory	Group C	No differences between (+/-) and (+/+) mice