Genes, Brain and Behavior (2007) **6:** 141–154

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Complex, multimodal behavioral profile of the *Homer1* knockout mouse

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Proteins of the *Homer1* immediate early gene family have been associated with synaptogenesis and synaptic plasticity suggesting broad behavioral consequences of loss of function. This study examined the behavior of male *Homer1* knockout (KO) mice compared with wild-type (WT) and heterozygous mice using a battery of 10 behavioral tests probing sensory, motor, social, emotional and learning/memory functions. KO mice showed mild somatic growth retardation, poor motor coordination, enhanced sensory reactivity and learning deficits. Heterozygous mice showed increased aggression in social interactions with conspecifics. The distribution of mGluR5 and *N*-methyl-D-aspartate receptors (NMDA) receptors appeared to be unaltered in the hippocampus (HIP) of *Homer1* KO mice. The results indicate an extensive range of disrupted behaviors that should contribute to the understanding of the *Homer1* gene in brain development and behavior.

Keywords: Behavior, Homer1, knockout, learning, social interaction

Received 1 September 2005, revised 21 March 2006, accepted for publication 22 March 2006

Homer proteins are part of a complex scaffolding of proteins that comprise the postsynaptic density (PSD). This scaffolding links cell surface receptors to other PSD proteins, as well as to receptor proteins that regulate intracellular calcium homeostasis (Ango *et al*. 2002; Shin *et al*. 2003; Yuan *et al*. 2003). Homer proteins also play important roles in synaptogenesis, synapse function, dendritic spine development, receptor trafficking and axonal pathfinding (Foa *et al*. 2001; Xiao *et al*. 2000). Efficient synaptic transmission depends on these scaffolding proteins controlling the functional state of glutamatergic receptors at excitatory synapses (Brakeman *et al*. 1997).

In mammalian brain, Homer proteins are encoded by a family of genes, consisting of *Homer1, 2* and *3,* each with several transcriptional variants. The *Homer1* gene generates four variants named *Homer1a, 1b, 1c* and Ania 3, which are prominent components of the glutamatergic PSD complex (Brakeman *et al*. 1997; Tu *et al*. 1998). In brain, they are preferentially expressed in the hippocampus (HIP), striatum and cortex (CTX) (Sun *et al*. 1998; Tu *et al*. 1998). All members of the Homer protein family share a conserved N-terminal EVH1 [enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) homology 1] domain (Beneken *et al*. 2000; Kato *et al*. 1997). This domain can bind to the poly-proline-rich sequence (PPxxFR) found in metabotropic glutamatergic receptors type I (mGluRI), Shank, and inositol-triphosphate receptors (IP_3R) and ryanodine receptors (RyR). A coiled-coil (CC) domain at the C-terminal position allows multimerization of Homer proteins (Sun *et al*. 1998). Thus, Homer proteins appear to be positioned physically and functionally to link mGluRs, IP_3R, RyR and *N*-methyl-D-aspartate receptors (NMDA) through their ability to self-associate.

Of the Homer proteins, the Homer1a isoform is unique. It is a product of an immediate early gene (Bottai *et al*. 2002; Brakeman *et al*. 1997; Lanahan & Worley 1998). This protein lacks the CC domain, and, as a short isoform of Homer1 cannot dimerize. Homer1a is therefore thought to interfere with the ability of Homer1b and 1c long isoforms to form multimers and thus functions as a dominant negative of the long Homer1b and 1c scaffolding functions. Expression of Homer1a is induced during neuronal excitation (Ageta *et al*. 2001; Bottai *et al*. 2002), including convulsive seizures (Kato *et al*. 1997; Potschka *et al*. 2002) and long-term potentiation (French *et al*. 2001; Hennou *et al*. 2003; Kato *et al*. 1997).

Owing to their involvement in multiple signal transduction pathways, Homer proteins have been linked to synaptic plasticity, to neuronal development and to pathological conditions (Szumlinski *et al*. 2005; Wu *et al*. 2001). Homer1 proteins have been shown *in vitro* to regulate trafficking of mGluRI (Abe *et al*. 2003; Ango *et al*. 2000, 2002; Ciruela *et al*. 1999) and to participate in the modulation of glutamate-activated calcium and potassium signaling (Kammermeier *et al*. 2000; Tu *et al*. 1998; Westhoff *et al*. 2003). Specifically,

doi: 10.1111/j.1601-183X.2006.00240.x

Homer1b/c proteins regulate clustering of the mGluRs at dendritic synaptic sites by linking together and anchoring individual mGluRs in the plasma membrane (Ciruela *et al.* 2000). Homer1a expression in response to neuronal activity can promote additional receptor expression in both axons and dendrites (Ango *et al.* 2000; Kato *et al.* 1998; Roche *et al.* 1999). Nevertheless, Homer1a over-expression attenuates mGluR-evoked intracellular calcium release (Tu *et al.* 1998).

Homer proteins, through their interactions with Shank proteins at the PSD, are also involved in the development of dendritic spines (Sala *et al.* 2001, 2003; Tu *et al.* 1999) and differentiation of dendrites and synapses (Okabe *et al.* 2001;Shiraishi *et al.* 2003a; Tu *et al.* 1999). By disrupting Homer–Shank complexes, Homer1a reduces the density and the size of dendritic spines, thus regulating dendritic and synaptic function, differentiation and morphology. Finally, it has been demonstrated that Homer1 proteins have a function in axon guidance and target recognition, and that expression of Homer1a can cause axonal pathfinding errors by interfering with the ability of Homer1b and 1c to form protein–protein interactions (Foa *et al.* 2001).

These data suggested an important role for Homer1 proteins in the development of the nervous system as well as synaptic and behavioral plasticity, but their possible functions at the behavioral level have been little studied. Szumlinski *et al.* recently reported behavioral and neurochemical effects in *Homer1* knockout (KO) mice that were suggested to be consistent with a possible role for *Homer1* in the pathophysiology of schizophrenia (Szumlinski *et al.* 2005). In addition, deletion of the *Homer1* and *Homer2*, but not *Homer3* genes, also enhanced sensitivity to cocaine-conditioned reward (Szumlinski *et al.* 2004, 2005).

Analysis of the behavioral characteristics of mice with targeted *Homer1* gene disruption in combination with physiological and molecular data should contribute to a further understanding of the role of *Homer1* in brain function. Therefore, this study was carried out to systematically characterize the behavior of *Homer1* KO mice compared with wild-type (WT) and heterozygous *Homer1* (HET) animals. We predicted that *Homer1* KOs would show deficits in memory, poor motor learning and performance and that the localization and distribution of mGluR5 receptors would also be abnormal. We based these predictions on previous data showing that (i) brain glutamate levels are abnormal in *Homer1* KOs (Lominac *et al.* 2005; Szumlinski *et al.* 2005), (ii) Homer1 proteins are involved in the trafficking, retention and function of group 1 metabotropic glutamate receptors (mGluR1 and mGluR5) at the synapse (Ango *et al.* 2000, 2002; Ciruela *et al.* 2000; Kammermeier *et al.* 2000; Roche *et al.* 1999) and (iii) activation of mGluRI in the HIP and cerebellum is required for induction of protein synthesis-dependent LTD, a major form of synaptic plasticity (Aiba *et al.* 1994; Huber *et al.* 2001; Shigemoto *et al.* 1994).

Materials and methods

Mice

The generation of the *Homer1* KO mice has already been described (Yuan *et al.* 2003). Male mice of each genotypic group for the *Homer1* gene – WT, HET and KO – were produced by the laboratory of Paul Worley, Department of Neuroscience, Johns Hopkins University School of Medicine. The experiments did not use a litter-based design. The 46 mice used in this study were derived from a total of 24 litters, with no more than three from any one genotype taken from any one litter. They were generated on a 129 × 1/SvJ × C57BL/6J background with between five and seven backcrosses with C57BL/6J. All mice were weaned, tagged and genotyped at 3 weeks of age and housed in same sex groups of mixed genotype until they were shipped to the Murine Behavioral Assessment Laboratory at The University of California, Davis, at an average age of 40 (±15) days. Animals were shipped in two cohorts 3 months apart but were tested at the same ages. Cohort 1 contained eight WT, eight HET and seven KO mice, and Cohort 2 contained eight WT, seven HET and nine KO mice. Final group sizes were thus WT = 16, HET = 15 and KO = 16. An additional 20 male control WT mice were used in the social dyad testing procedures.

All mice passed a general health examination before admission to the UC Davis Center for Laboratory Animal Science. Upon arrival, animals were individually housed under 12 h/12 h light–dark cycle, with water and mouse chow (Purina 5001) supplied ad libitum unless otherwise specified. Animals were acclimated for at least 3 weeks before the start of the experiments. Protocols were approved by the UC Davis Animal Care and Use Administrative Advisory Committee.

Western blotting

Detergent lysates (1% Triton-X-100) of cerebral CTX and HIP were prepared from *Homer1* WT, HET and KO mice. Thirty micrograms of protein per lane was loaded onto 4–12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and electroblotted to Immobilion P+ membrane (Millipore, Billerica, MA, USA). Blots were incubated with rabbit anti-Homer-1b/c-specific antibodies (1:2500) for 1 h at room temperature. HRP-conjugated anti-rabbit IgG (1:10 000; Amersham, Piscataway, NJ, USA) secondary antibodies were applied, after which blots were stripped and re-probed with specific rabbit antibodies to Homer-2a/b (1:2500) or Homer-3 (1:4000). The generation and use of the Homer antibodies were described earlier (Xiao *et al.* 2000). Blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA), and the relative amount of Homer1 expression was analyzed based on band intensity using NIH Image J software.

Behavioral test battery

Behavioral tests are described in the order that they were carried out, along with the approximate ages of the mice in postnatal days (PND). All tests were conducted during the light phase of the light/dark cycle.

Functional observational battery (PND 75)

The functional observation battery (FOB) is a series of observational ratings of sensory and motor development conducted during a 3-min observation of mice. The details of this battery have been published previously (Golub *et al*. 2004; Moser 2000; Sills *et al*. 2000). Righting and negative geotaxis were also tested at the end of the FOB.

Social dyadic interaction (PND 76)

Social interactions were characterized using a behavioral ethogram (Table 1) based on the work of Grant and Mackintosh, as modified by Calamandrei *et al*. (1999) (Grant & Mackintosh 1963). Briefly, an experimental mouse from one of the three genotypes was paired with an unfamiliar WT mouse for 10 min in a 10 × 10 × 10 cm Plexiglas chamber. The dyads were video recorded for later scoring using the Noldus Observe 4.1 software programmed with codes reflecting the behaviors specified by the ethogram. The duration and frequency of social, non-social and aggressive behaviors were measured using the ethogram in Table 1. The animal initiating each behavioral event was also determined. Video recording was preceded by a 5-min acclimation period alone in a similar box for each mouse. The unfamiliar WT mice were adult males of similar age to the experimental mice.

Elevated plus maze (PND 77)

The plus maze was used to examine fear and anxiety in *Homer1* mice (File 2001; Lister 1987). The maze was made of black Plexiglas, with two open (30 × 5 × 0.25 cm) and two closed (30 × 5 × 6 cm) arms emanating from a central platform (5 × 5 cm) and elevated 60 cm above the floor. Each mouse was placed onto the central platform and allowed 2 min of free exploration in the apparatus. Distance traveled, number of entries into each arm and into the central platform, time in open vs. closed arms and latency to first arm entry were recorded by a video-tracking system (San Diego Instruments, San Diego, CA, USA). Fearful anxious animals spend more time in the closed arms of the maze.

Auditory startle and prepulse inhibition (PND 78)

Prepulse inhibition (PPI) of the auditory startle response was measured to assess sensorimotor gating (Dulawa & Geyer 2000; Graham 1975). Mice were placed individually into the auditory startle apparatus (SR-LAB, San Diego Instruments, San Diego, CA, USA) and allowed to acclimate for 5 min. This was followed by a 20-min PPI session consisting of 50 test trials, 10 each for five conditions presented in a pseudorandom order with variable intertrial intervals of 5–20 ms. The test conditions were 120 dB auditory stimulus alone, 120 dB stimulus with a 74, 82 or 90 dB prepulse stimulus and background white noise only (70 dB). All prepulses were 20 ms long and were presented 100 ms before the 120 dB stimulus.

Water maze learning and cue use (PND 111)

A water maze task with simplified, well-defined cues was used to assess spatial learning and memory. The maze and procedures were designed by Lamberty and Gower (1991) and modified in our laboratory (Golub *et al*. 2000). The water maze (90 cm diameter) was enclosed by a gray PVC cylinder. Two distinct visual cues were provided; a salient cue (black strip of plastic just above the waterline) and a non-salient cue (a round opening in the cylinder). Four covered circular doors (7.5 cm diameter, bottom edge located 7 cm above the top of the basin) were equally spaced in the PVC cylinder at the quadrant borders and served as entry points for the mice. Distance, latency and swim pattern were recorded by an automated tracking system (Polytrack, San Diego Instruments).

Nine sessions of four trials (120 s each) were carried out over 11 days. Intertrial intervals were 5 min during which the animal waited in a warming cage. The entry door was pseudorandomized for each mouse on each training day. Daily training sessions 1–4 (days 1–4) were spaced 24 h apart, whereas session 5 was conducted 72 h later (i.e. day 7) to test for long-term memory retention. Sessions 6–8 (days 8–10) were also spaced 24 h apart. For these sessions, the entire maze (platform and cues) was rotated 180° such that the relative position of the cues to the platform remained unchanged. Probe trials (100 s, no platform) were conducted following the last training trial in sessions 4–8. An additional test was conducted in cohort 2 on PND 264 to determine whether the thermoregulatory response to the water immersion was altered. Body temperature was measured by rectal probe immediately before and immediately after a 120-s immersion in the water maze tank.

Nest building (PND 145)

Nest building was assessed over three consecutive days by providing individually housed mice with two pieces of cotton for 24 h and rating the nest quality as 1, no nest; 2, flat nest; 3, nest with raised side with an opening shape; 4, nest with higher raised sides with an enclosing shape or fully closed (Bult & Lynch 1997). Nest heights on four sides and the largest width were measured, and location of nest within the cage was noted.

Table 1: Behavioral ethogram for scoring social dyadic interaction

Behaviors/categories	Description
Behavior State (measured as duration in state)	
Social	
Extended groom	Licking or picking partner >2 s after groom event has been coded
Social	At least one animal pays attention to partner, within proximity or in contact with partner >2 s after approach event has been coded
Non-social	
Extended freeze	Sudden startle-like immobility in response to partner > 2 s after freeze event has been coded
Extended non-social inactivity	Motionless, out of proximity >2 s after inactivity event has been coded
Extended self-groom	Licking or picking self >2 s after self-groom event has been coded
Extended Stereotypy	Consecutive repetitions of a particular behavioral pattern >2 times after stereotypy event has been coded
Non-social	Not paying attention to partner and out of proximity >2 s after withdrawal event has been coded
Aggressive	
Extended aggression	Bite, hit and other aggressive moves >2 s after aggression event has been coded
Behavioral events (measured as frequencies of behavior)	
Social	
Approach	Coming into proximity (i.e. within an animal's body width)
Crawl on/under	Crawling on top/ under/ around partner
Follow	Moving alongside partner for at least 2/3 of arena's width
Groom	Licking or picking partner
Mounting	Positioning body on top of partner's rear end and thrusting
Non-social	
Escape	Attempt or committed escape from the arena, mainly by jumping
Freeze	Sudden startle-like immobility in response to partner >2 s
Inactivity	Motionless, out of proximity >2 s
Locomotion	Moving independently for at least 2/3 of arena's width
Mince	Pacing in circles, zigzag or figure-8 patterns after aggression episode >3 times
Rearing	Stands on hindlimbs with both front paws off ground
Self-groom	Licking or picking self
Stereotypy	Consecutive repetitions of a particular behavioral pattern >3 times
Withdrawal	Coming out of proximity (i.e. more than an animal's body width apart)
Aggressive	
Aggression	Bite, hit and other aggressive moves
Offensive/defensive rearing	Stands on hindlimbs, with front paws in air, upper body hunched forward, prepared to initiate or receive attacks
Push/pull	Aggressive pushing or pulling partner
Submissive crouch	Crouching near floor or at corner in response to partner
Rattling	Snake-like movement of the tail directed at partner

Social transmission of food preference (PND 152)

This procedure was adapted from Wrenn *et al*. (2003). 'Demonstrator' and experimental mice were maintained on a 20 h/day food restriction schedule for 1 week and shaped to eat ground mouse diet from a plastic cup for 30 min 'Demonstrator' WT mice were then given 5 g of either 1% (w/w) cinnamon-flavored ground chow (McCormick ground cinnamon) or 2% (w/w) cocoa-flavored ground chow (Hershey's cocoa) for 2 h with water available. Immediately thereafter, each demonstrator was paired with an experimental mouse for 30 min. Twenty-four hours later, the experimental mice were given a choice between 5 g of each flavored chow in plastic cups for 2 h (position randomized). The amount of each flavored food eaten was measured to determine preference. Transfer of food preference was evident as a preference for the same flavored food given to the demonstrator mouse.

Activity/metabolism (PND 255, cohort 2 only)

General activity and activity rhythms of the mice were measured for 48 h as previously described (Golub *et al*. 2004). The automated apparatus (Integra, Accuscan, Columbus, OH, USA) was only available for cohort two, and sample

sizes are thus smaller ($N = 7$–9/group). Vertical and horizontal activity counts were summarized in 3-min intervals. Localized non-ambulatory movement, defined as repeated breaking of the same beam or set of beams, was also measured. The first 2 h of activity in the arena were used to examine movement in a novel environment.

Rotarod (PND 259)

A rotarod (Rota-rod 7600, Ugo Basile Biological Research Apparatus, Malvern, PA, USA) was used to measure balance and motor coordination as described previously (Golub *et al.* 2004). Testing consisted of two 2-min training sessions 2 h apart on 1 day using a speed of 16 r.p.m. On the following day, a single 2-min test trial using 24 r.p.m. was conducted. During training, the mouse was replaced on the rod when it fell. During testing the trial terminated when the mouse fell. Rotating with the rod ('passive rotation' or 'flipping') was also recorded.

Home cage stereotypy (PND 300–330, cohort 2 only)

Mice were videotaped in their home cage during the first 2 h of the dark cycle. Videotapes were then scored for the frequency of occurrence of common stereotypy patterns, including bar mouthing, digging, jumping, route-tracing and twirling.

Histology

Mice were anesthetized and perfused transcardially with 0.1 M phosphate buffer, pH 7.4, followed by 4% (w/v) paraformaldehyde. Brains were dissected, postfixed 2 h and cryoprotected in 30% (w/v) sucrose in 0.01 M phosphate buffer, pH 7.4, at 4 °C for 24 h. Coronal sections were cut at 20 μ on a cryostat. Free-floating sections were initially treated with hydrogen peroxide (3%, v/v) in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 30 min at room temperature and then blocked with bovine serum (10%, v/v) and bovine serum albumin (3%) in PBS/Triton-X-100 (0.3%, v/v), pH 7.4, for 1 h at room temperature. Sections were then incubated overnight at 37 °C with rabbit polyclonal antimetabotropic glutamate receptor 5 (1:6000 in PBS, 0.3% triton and 3% bovine serum) or rabbit polyclonal anti-NMDA/NR2A subunit (1:1000, Novus Biologicals, CO, USA). Sections were washed in 0.01 M PBS and incubated with a biotinylated bovine anti-rabbit antibody (1:2000, Santa Cruz Biotech, CA, USA) for 2 h at 37 °C. Sections were subsequently stained by the avidin/biotinylated peroxidase complex method using the Vectastain ABC Elite kit and 3′,3-diaminobenzidine tetrahydrochloride as the hydrogen peroxidase substrate (Vector Laboratory, Burlingame, CA, USA). Absence of non-specific staining was verified by incubating without the primary antibody. Additional sections were stained with cresyl violet and examined for evidence of gross morphological brain differences between the genotypes. Photomicrographs of coronal sections were taken at the same anterior–posterior position from five animals in each genotype. Images were converted to grayscale, and relative density in stratum pyramidale and oriens of hippocampal subregions CA1 & CA3, the dentate gyrus and hilus were analyzed using NIH Image J software.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between genotypes were analyzed with analysis of variance (ANOVA) using Fisher *post hoc* comparisons (Statview, SAS Institute, Cary, NJ, USA). Maze data were analyzed by repeated measures (RMANOVA) across sessions. The activity/metabolic data were analyzed using the Bonferroni *post hoc* test (SAS, SAS Institute). Contingency analysis was used for categorical variables. Body weight was used as a covariate for statistical analysis at later ages when genotype effects on body weight were statistically significant. Cohort was initially used as a factor in the ANOVA for each endpoint and was retained if significant cohort effects were noted. Frequencies of behavioral events in the social dyad were analyzed by chi square tests and are presented as percent occurrence and frequency.

Results

Western blotting

Western blots for Homer1 proteins are shown in Fig. 1 for CTX and HIP. An absence of immunostaining for Homer1b/c proteins in the KO animals confirms the gene KO. As expected, Homer1b/c protein levels in the HETs were reduced by approximately one-half in CTX and HIP, with levels in the HIP averaging 45.7 ± 1.8% of WT ($n = 3$). The expression of Homer proteins 2a/b and 3 was largely unaffected by the *Homer1* gene targeting in the tested brain areas. Homer3 protein was not detected in the cerebral CTX as previously reported (Xiao *et al.* 1998).

General observations

At the beginning of the behavioral assessments (75 days of age), the WT, HET and KO groups did not differ in weight or length (Table 2). However, the ponderal index (weight/length) indicated that the KO mice were slightly underweight relative to their body length ($F_{2,44} = 3.4$, $P < 0.05$; WT vs. KO, $P < 0.01$). By the end of the experiment (260 days of age), the KO and HET mice weighed less than the WT mice ($F_{2,43} = 6.3$, $P < 0.005$; WT vs. KO, $P = 0.001$; WT vs. HET, $P < 0.05$). One WT mouse died on PND259 before the conclusion of the study.

In the FOB tests, all mice were able to right themselves, displayed negative geotaxis, and had no indication of lacrimation or salivation abnormalities. There were no abnormalities in appearance of fur and eyes. None of the mice demonstrated hindlimb splaying or dragging or gait abnormalities.

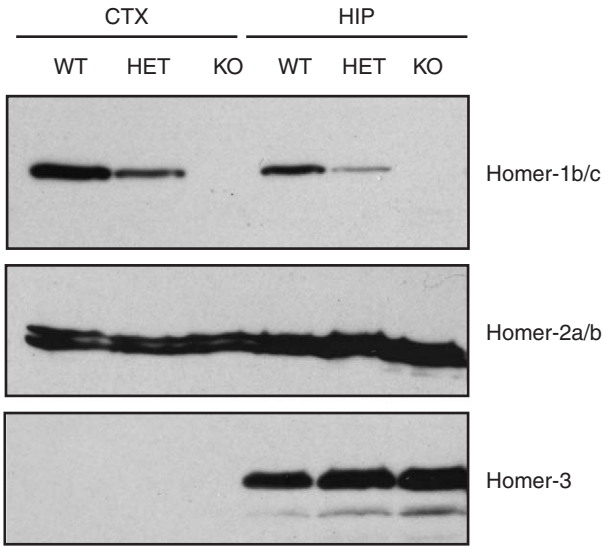

Figure 1: Western blot. Western blots of cerebral cortex (CTX) and hippocampus (HIP) showing presence of Homer1b/c protein in wild type (WT), reduced levels in heterozygous *Homer1* (HET) and absence in KO mice. Expression of Homer proteins 2a/b and 3 were largely unaffected by the gene targeting. Homer3 was not detected in the CTX.

However, KO mice showed a larger response than WT to approach of a probe to the face ($P < 0.001$), touch to the flank with the probe ($P < 0.001$) and to activation of a metal clicker overhead ($P < 0.001$). Analysis of stereotypy did not show any difference between groups in the frequency of home cage stereotypy, with bar mouthing the most common stereotypy for all mice.

Sensory function/emotionality (data not shown)

There were group differences in startle amplitude on the trials without a prepulse tone ($F_{2,44} = 3.7$, $P < 0.03$), with HET mice showing significantly lower startle amplitudes than WT mice ($P = 0.019$). There were no effects of genotype on sensory gating as measured by PPI. There were no differences in the elevated plus maze between groups in the total number of arm entries, closed arm entries or percent time spent in the open arms.

Table 2: Body weights and ponderal index at the beginning and end of the experiment

	Wild type	Heterozygous	Knockout
PND75			
Weight (g)	28.0 ± 1.0	28.4 ± 0.9	28.3 ± 0.7
Weight/length (g/cm)	3.17 ± 0.09	3.00 ± 0.06	2.92 ± 0.05*
PND 260			
Weight (g)	38.5 ± 1.9	36.7 ± 2.0†	37.2 ± 1.5‡

*$P < 0.01$ vs. wild type.
†$P < 0.05$ vs. wild type.
‡$P < 0.001$ vs. wild type.

Social behavior

Assessment of social interactions revealed a significant difference among genotypes for duration of time spent in social (vs. non-social) state during the dyadic interactions ($F_{2,43} = 8.2$, $P < 0.001$) (Fig. 2). As shown in Fig. 2(a), KO mice spent more time in the social state than WT ($P > 05$) or HET ($P < 0.01$), and HET spent less time in the social state than WT ($P < 0.05$). Duration in the non-social state also differed ($F_{2,43} = 6.6$, $P < 0.01$), with KO mice spending less time in the non-social state than WT ($P < 0.05$) or HET ($P < 0.01$). Finally, duration in the aggressive state also differed significantly ($F_{2,43} = 5.1$, $P < 0.01$), with HETs spending more time in the aggressive state than WT or KOs ($P < 0.05$).

The frequencies of specific behavioral events were also scored along with the identity of the initiator. Figure 2(b) shows that KOs had increased numbers of following and crawling (under/over) behaviors compared with WT ($P < 0.05$) and HETs ($P < 0.05$), consistent with more time spent in social interaction (i.e. social state). In contrast, a greater percentage of HETs initiated aggression (i.e. bite, attack, hit) than WT ($P < 0.01$), and also received ($P < 0.05$) more aggression than WT or KOs ($P < 0.05$), also consistent with the increase in the duration of aggression (Fig. 2c). HETs initiated and received more rattles than either WT or KOs ($P < 0.05$) (data not shown).

Finally, in the social transmission of food preference test (Fig. 2d), WT and HET, but not KOs, showed social transfer of food preference by consuming more of the familiar-flavored food than the novel food (paired *t*-test, familiar vs. novel flavor: WT, $P = 0.047$; HET, $P = 0.073$; KO, $P = 0.64$)

Motor function and nest building

Swim speed

Swim speed was measured as part of the water maze protocol (Fig. 3a). There was a significant difference among genotypes in swim speed ($F_{2,44} = 7.6$, $P < 0.001$), with the KO group swimming slower than WT and HET groups ($P < 0.001$). There was also a significant effect of session ($P < 0.0001$), with speed generally increasing across sessions. A significant interaction ($P = 0.02$) appeared to be due to the KO group failing to increase their swim speed across sessions. Nine of the 47 mice, seven in the KO group, demonstrated floating on at least one trial, but floating occurred on no more than five of the 36 trials for any mouse. Therefore, floating (no movement in the water) did not appear to be the major reason for the slower swim speeds in the KOs.

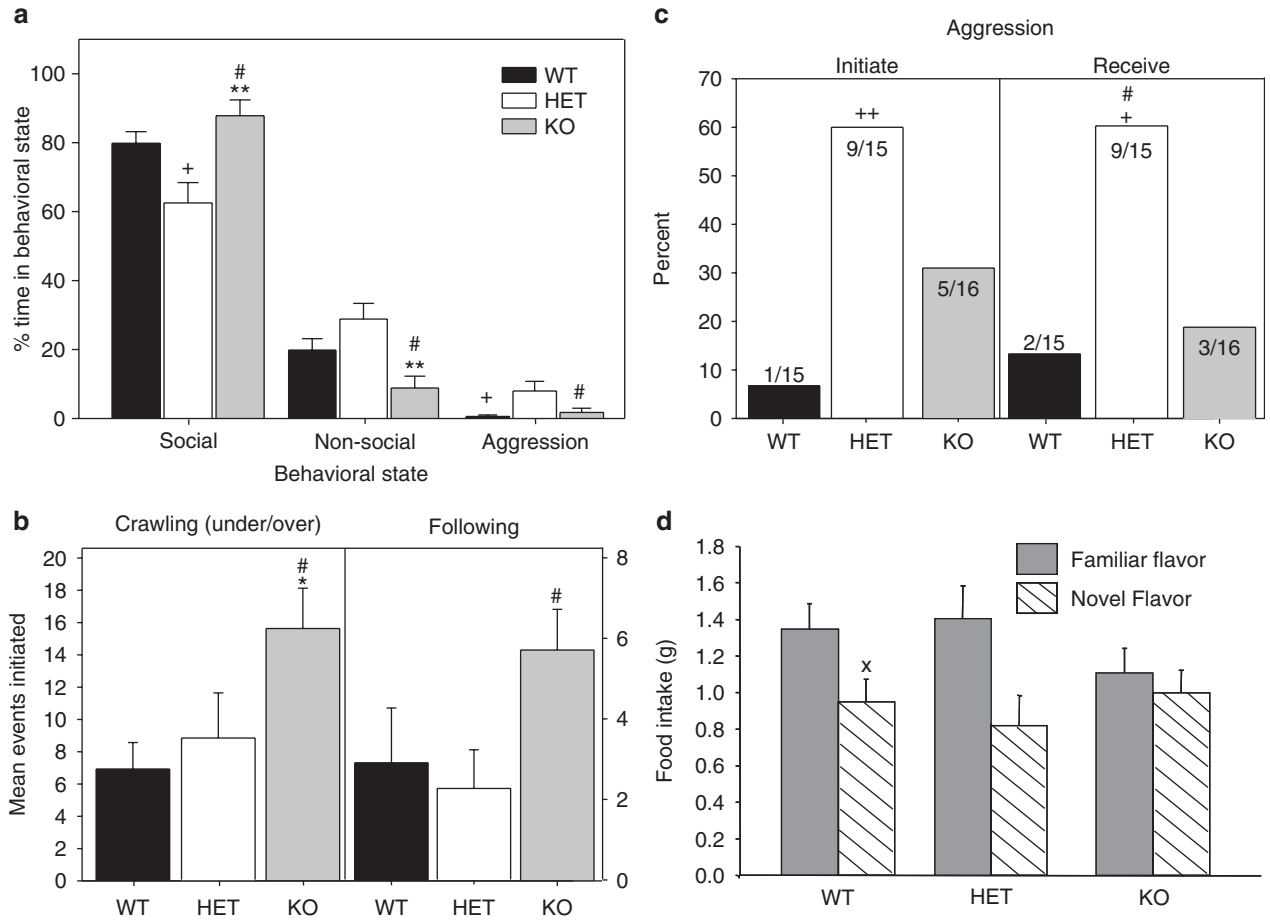

Figure 2: Social behavior in *Homer1* mutants. (a) In the social dyad, knockout (KO) mice showed more time in social interaction and heterozygous *Homer1* (HET) more time in aggression compared with wild type (WT). (b) KOs also showed more social behaviors (i.e. crawling and following) and (c) HETs more aggressive behaviors in the social dyad. (d) KOs also failed to show social transfer of food preference. $^{*}P = 0.05$, $^{**}P = 0.01$, KO vs. WT; $\#P = 0.05$, KO vs. HET; $^{+}P = 0.05$, $^{++}P = 0.01$ HET vs. WT; $^{X}P < 0.05$, novel vs. familiar food.

Forelimb grip strength

Forelimb grip strength was weaker in the KO mice than in the WT and HET mice ($F_{2,44} = 5.6$, $P < 0.01$; KO vs. WT, $P < 0.01$; KO vs. HET, $P = 0.005$) (Fig. 3b). The HET mice had significantly better hindlimb grip performance than WT and KO ($F_{2,44} = 4.7$, $P = 0.01$; KO vs. HET, $P < 0.005$; HET vs. WT, $P = 0.052$).

Rotarod performance

Rotarod performance differed among groups, with KO mice showing significantly more passive rotations during the two training sessions ($F_{2,43} = 4.6$; $P = 0.01$; KO vs. WT, $P < 0.05$; KO vs. HET, $P = 0.01$) as well as during the test session ($F_{2,43} = 3.2$, $P = 0.05$; KO vs. WT $P < 0.05$) (Fig. 3c). There were no significant differences between groups in the number of falls from the rotarod during the 120-s test trial.

Nest quality

Nest quality differed between genotypes on all three test days (ANOVA, $F_{2,43} = 8.5, 10.8, 4.4$; $P = 0.0002, 0.0001, 0.01$ for days 1, 2 and 3) (Fig. 3d). The KO had lower nesting scores than WT or HET on each of the 3 days ($P < 0.05$), indicating incomplete nest construction. Four KO, three HET and one WT failed to make a nest on at least 1 day. Although nests did not differ in width, depth was lower for the nests of the KO group ($F_{2,43} = 11.8$, $P < 0.0001$) compared with WT or HET ($P < 0.001$).

Water maze learning and cue use

A repeated measure ANOVA demonstrated a significant effect of genotype on swim distance (Fig. 4a, $F_{2,44} = 3.7$, $P < 0.05$), escape latency (Fig. 4b, $F_{2,44} = 5.7$, $P < 0.01$) and swim speed (Fig. 3a). For each of these measures, the KO group differed significantly from the WT and HET groups ($P < 0.05$). Group by session interactions were not

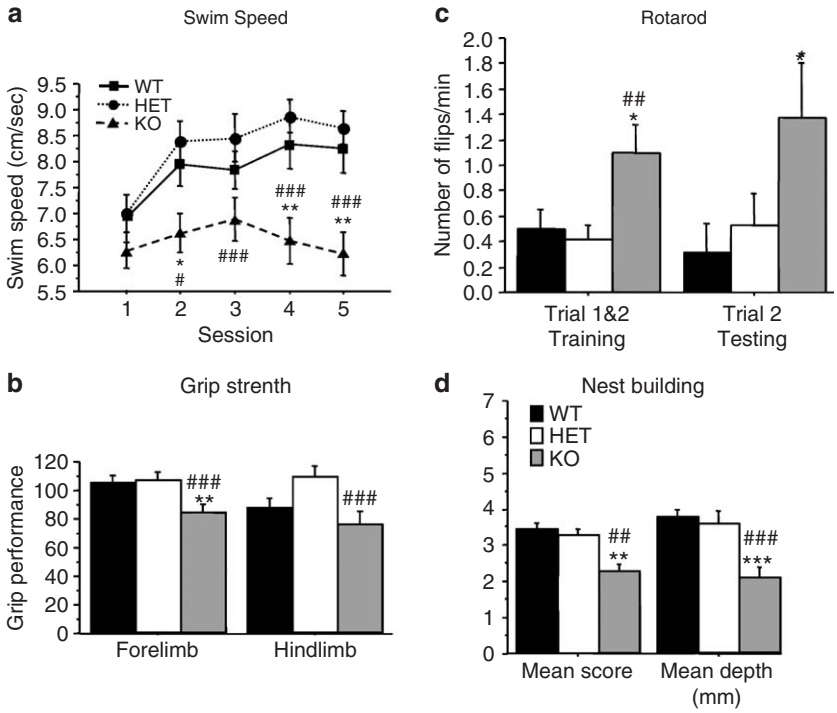

Figure 3: Motor deficits in *Homer1* mutants. The knockout (KO) group showed deficits relative to wild type (WT) on (a) swim speed in the water maze; (b) grip strength; (c) passive rotations on the rotarod; and (d) nest quality. $*P = 0.05$, $**P = 0.01$, $***P = 0.001$, KO vs. WT; $\#P = 0.05$, $\#\#P = 0.01$, $\#\#\#P = 0.001$, KO vs. heterozygous *Homer1* (HET).

significant, but planned comparisons on individual sessions demonstrated that KO mice had longer swim distances ($P < 0.01$) and longer escape latencies ($P < 0.001$) on session 2 than WT and HETs. In addition, the decreases in distance swum and escape latency between sessions 1 and 2 were only significant for WT and HET mice ($P < 0.05$), and not for KO mice, again demonstrating delayed improvement in this task. During the probe trials on sessions 5 and 6, all groups spent significantly more time in the escape platform quadrant than in any of the other quadrants ($P < 0.0001$) indicating memory retention of the platform location. Time in the platform quadrant and distance swum during the probe trials did not differ among groups (Fig. 4c). However, after relocation of the maze and cues on session 6 (Fig. 4d), KO mice spent less time ($F_{2,44} = 7.0$, $P < 0.005$; KO vs. HET and WT, $P < 0.01$) and swam a shorter distance ($F_{2,44} = 9.0$, $P < 0.0005$; KO vs. HET and WT, $P < 0.001$) in the escape platform quadrant than WT and HETs. Although KO mice spent less time in the escape quadrant, they did not show a significant preference for any of the remaining three quadrants. No effects of genotype on basal temperature before or temperature drop after water maze testing were found.

Activity/metabolism

KO and HET mice showed less vertical activity over the 48-h monitoring period than WT mice ($F_{2,44} = 5.33$, $P = 0.01$; WT vs. HET, WT vs. KO, $P < 0.05$) (Fig. 5). This difference was also significant for the active phase of the cycle (night) when most of the vertical activity occurred ($F_{2,44} = 5.35$, $P < 0.01$; WT vs. HET, WT vs. KO, $P < 0.05$; data not shown). During the rest phase of the cycle, the KO group spent more time in locomotion than did the other two groups ($F_{2,44} = 4.94$; KO vs. WT, $P < 0.05$; KO vs. HET, $P = 0.05$), suggesting a disruption of the rest–activity cycle. Localized activity, including grooming and repeated movements without ambulation, formed the largest portion of the activity of all groups. HET mice spent less time in localized movement than KO mice and WT mice ($F_{2,44} = 4.43$, $P < 0.05$; WT vs. HET, $P < 0.05$; KO vs. HET, $P < 0.05$).

Because of the lower body weights of the KO mice by the end of the behavior testing series, additional statistical analyses were conducted to determine possible influences of body weight on the activity measures. Body weight was not correlated with vertical activity but showed an overall weak correlation with horizontal activity measures. This correlation was mainly attributable to the fact that KO mice with lower weights were more active (regression of weight on horizontal activity, KO group, $F_{2,44} = 14.94$, $P < 0.01$).

No genotype effects were seen in metabolic parameters, including oxygen consumption and respiratory exchange ratio, as measured over the 48-h period in the chambers.

Histology

Representative photomicrographs of coronal sections of HIP are presented in Fig. 6 showing mGluR5 (a–c) and NMDA/NR2A (d–f) immunoperoxidase staining for the three genotypes. Both receptor subtypes were expressed throughout the HIP, with no differences between groups in patterns or intensities of immunostaining for either receptor. In addition,

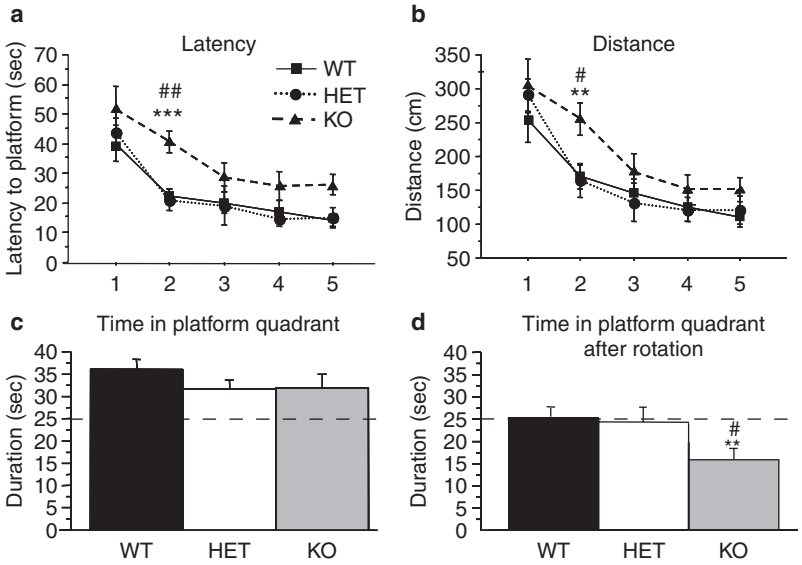

Figure 4: Water maze performance deficits in *Homer1* mutants. (a) Knockout (KO) mice had significantly longer swim distances and (b) longer latencies to locate the escape platform across sessions. Comparisons on individual days showed that KOs had longer swim distances and longer escape latencies on session 2 than wild type (WT) or heterozygous *Homer1* (HET) mice. (c) All groups showed increased time in the platform quadrant during the probe trial, but there were no significant group differences (- - - - - chance performance). (d) KO mice spend less time in the platform quadrant after maze rotation (- - - - - chance performance). $^{**}P = 0.01$, $^{***}P = 0.001$, KO vs. WT; $\#P = 0.01$, $\#\#P = 0.001$, KO vs. HET.

there were no striking morphological differences observed between groups with cresyl violet staining (g–h).

Discussion

The behavioral characteristics of the *Homer1* KO mice included altered performance in sensory, motor, social and learning/memory tests. Most variables showed a difference between the KO group and both the WT and HET groups. However, for some behaviors, including non-locomotor activity, auditory startle with no prepulse, hindlimb grip performance and aggression during dyadic interaction, the HET group differed from both WT and KO groups.

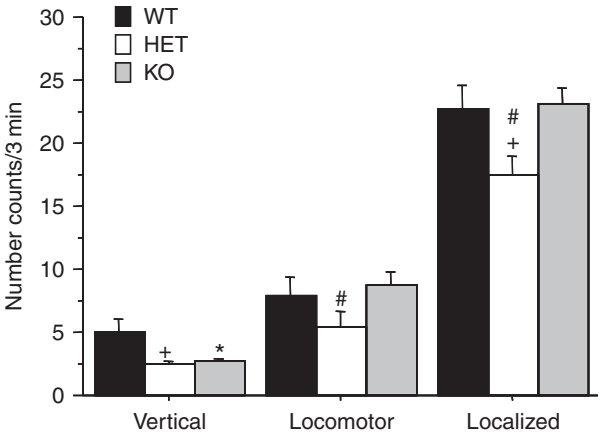

Figure 5: Reduced spontaneous activity measured in *Homer1* mutants. Vertical beam breaks, horizontal locomotion (distance) and localized non-ambulatory movement were totaled over 3-min periods and averaged over the 48-h period. The heterozygous *Homer1* (HET) group had lower activity than wild type (WT) group in all three categories, whereas the KO group had lower activity than WT only for the vertical category. $^{*}P = 0.05$, KO vs. WT; $\#P = 0.05$, KO vs. HET; $^{+}P = 0.05$, HET vs. WT.

Motor function and locomotor activity

Motor deficits were found in KO mice for forelimb grip strength and on the rotarod test. Motor impairment may have also contributed to the slower swim speeds in the water maze, less rearing in the activity assessment and poor nest quality by the KO mice. Homer1 proteins have been identified within skeletal and cardiac muscle (Ciruela *et al.* 2000; Sandona *et al.* 2000), where they physically associate with ryanodine receptor type 1 (RyR1) and modulate the gain of Ca^{2+} release (Feng *et al.* 2002). Therefore, deficiencies in Homer-RyR1 complexes in *Homer1* KO animals may contribute to decrease the efficiency of excitation–contraction coupling and could explain the motor dysfunction noted above.

The KO group also showed a relatively high rate of passive rotations on the rotarod, suggesting impairment in motor learning. Homer1 proteins have been shown to be important for the localization of mGluR1 receptors to the cell surface of axons and dendrites (Ango *et al.* 2000; Minami *et al.* 2003; Roche *et al.* 1999; Xiao *et al.* 2000). A decrease in active mGluR1 receptors in *Homer1* KOs could therefore have interfered with the induction of long-term depression (LTD) in cerebellum which has been closely linked to cerebellar plasticity and motor learning (Anwyl 1999; Burguiere *et al.* 2005). For example, *mGluR1* KO mice fail to show cerebellar LTD in response to either parallel fiber or climbing fiber stimulation, show ataxia and gate anomalies and are impaired in acquisition of classical eyeblink conditioning (Aiba *et al.* 1994).

Homer1 KO mice were more active than other groups during the rest phase of the diurnal rest–activity cycle.

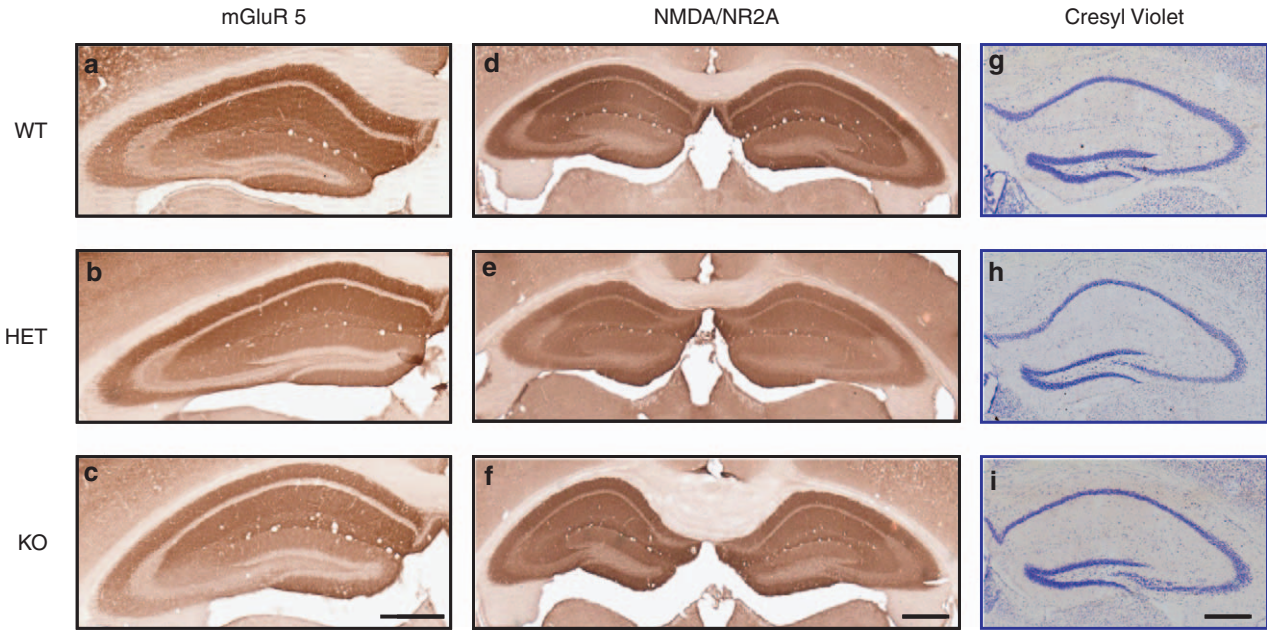

Figure 6: Photomicrographs showing immunostaining for mGluR5 (a–c) and NMDA/NR2A receptors (d–f) and cresyl violet staining (g–i) of hippocampus from representative mice in each genotype. Wild type (WT) (a,d,g), heterozygous *Homer1* (HET) (b,e,h) and KO (c,f,i). Bar, 500 μm.

Homer proteins may be involved in the photic entrainment of the circadian clock (Park *et al.* 1997). The retinohypothalamic tract transmitters pituitary adenylate cyclase activating polypeptide (PACAP) and glutamate are responsible for light-induced expression of Homer1a in the suprachiasmatic nucleus (SCN), and *Homer1a* seems to be differentially regulated by these two transmitters at early and late night (Nielsen *et al.* 2002). The absence of Homer1a expression in the mutant animals may therefore have prevented the phase shifting capacity of the two neurotransmitters on the clock, affecting circadian rhythms. In addition, mGluRI, whose functions are linked with Homer1 proteins, are also expressed in the region of the SCN that receives input from the retina and plays a role in regulating mammalian circadian rhythms (Mikkelsen *et al.* 1995). Greater activity of the KOs is also consistent with the increased activity and reduced habituation in a novel environment reported by Szuminski *et al.* (2004, 2005) in *Homer1* but not in *Homer2* KO mice.

Response to sensory stimulation

Greater reactivity to proximal sensory stimulation (i.e. approach, touch, auditory click) was noted in the KO mice during FOB screening. However, KO mice did not demonstrate greater auditory startle amplitudes later in the test battery. Rather, HET mice displayed reduced auditory startle amplitudes. The lower acoustic startle responses may be related to a hearing deficit in HET mice. Like their parental strain (C57Bl/6J), mutant mice may exhibit a genetically determined progressive cochlear degeneration that begins at about 1–2 months of age (Ison & Allen 2003). It is not clear why HETs and not KOs showed this effect, although it is possible that a partial loss of *Homer1* expression in HETs may amplify this phenomenon. PPI, which has been used as an operational measure of sensorimotor gating, was not affected by genotype. These results differ from those of Szumlinski *et al.* (2005) who report impaired PPI in young (5–6 weeks of age) *Homer1* KOs. Somewhat older mice were tested for PPI in this study (i.e. 11–12 weeks of age), and age of testing may underlie the differing results.

Water maze performance

Homer1 KOs were impaired in the water maze, showing longer swim distances, increased latencies and slower swim speeds to locate the escape platform. The longer swim distance to find the escape platform in KOs is consistent with a mild learning impairment in this task. However, the longer escape latencies of KOs are clearly related to their slower swim speeds, and this confounds interpretation of the latency data. These results are similar to those of Szumlinski *et al.* (2005) who reported that *Homer1* KOs show working memory deficits in the radial arm maze, and were slower to complete the maze compared with WTs, while *Homer2* KO were not impaired.

In this study, KOs were also impaired following maze rotation compared with WT and HETs. This suggests that KOs may have been attending to contextual cues in the general testing environment (e.g. sounds, odors, shadows) that were either irrelevant or distracting, leading to impaired

location of the escape platform following maze rotation. This observation may be related to the selective attention or sensory gating impairment in PPI demonstrated by Szumlinski *et al.* (2005) in *Homer1* KO mice though we did not find a similar impairment in PPI. KO mice were also impaired in social transmission of food preference, and learning in this test has been shown to be impaired by hippocampal lesions (Bunsey & Eichenbaum 1995).

Social interaction in the social dyad

This study found that KO mice spent more time in social interaction with a naïve WT stranger in the social dyad test than WT and HET. In contrast, HET mice were less social and more aggressive than WT or KO mice. Reduced aggression in the KOs was unexpected as they appear highly aggressive when colony housed (unpublished observations). However, the social dynamics of a colony and its attendant social hierarchy are clearly quite different from a dyadic encounter between strangers, such as that described in this study.

Examples of gene mutations leading to increased social interaction are unusual and suggest that the present findings with the *Homer1* KO may be unique and could provide new insights into the neurobiology of social behaviors. Increased social interactions, impaired spatial learning, poor motor performance and increased auditory responsiveness seen in the *Homer1* KOs are reminiscent of Williams syndrome, a human disorder resulting from deletions in chromosome sub-band 7q11.23 (Francke 1999). Williams syndrome is associated with short stature, gait abnormalities, mild mental retardation with an abnormal positive bias toward strangers (Bellugi *et al.* 1999; Holinger *et al.* 2005). However, there is no direct evidence at the present time for any interactions between Homer1 proteins and genes or gene products associated with the Williams syndrome gene deletion region (e.g. elastin, syntaxin 1A, LIM kinase-1, Frizzled 1).

Histology

No gross morphological differences were observed in the brains of KO mice, and immunostaining for mGluR5 and NMDA NR2A receptor subunits appeared to be unaltered. This was unexpected as previous *in vitro* research had suggested that these receptors might show an abnormal distribution in *Homer* mutants (Ango *et al.* 2002; Roche *et al.* 1999; Xiao *et al.* 2000). It is possible that changes in hippocampal morphology or glutamate receptor distribution were present in the *Homer1* mutant but occurred at the level of the synapse (i.e. dendritic spines) and were therefore not apparent with the immunostaining procedures used in this study. Resolution of this issue will require a more detailed examination of the cytoarchitecture of *Homer1* mutants using procedures with greater resolution (e.g. electron or confocal microscopy).

Contribution of Homer isoforms to behavioral deficits

Homer1 KOs lack both long isoforms (Homer1b/c/d) and short isoforms (Homer1a and Ania 3) of Homer1 proteins (Berke *et al.* 1998; Brakeman *et al.* 1997; Kato *et al.* 1997). Therefore, it is not possible based on the present data to attribute specific behavioral differences among the three genotypes to loss (i.e. KOs) or reduction (i.e. HETs) in the levels of any specific Homer1 isoform. Homer long isoforms are constitutively expressed and play critical roles in membrane localization of glutamate receptors and their linkage to proteins in signaling pathways (Abe *et al.* 2003; Shiraishi *et al.* 2003a,b). Loss of these functions would be expected to disrupt excitatory signal transmission and could underlie the observed behavioral impairments in *Homer1* mutants. In contrast, Homer1 short isoforms are thought to function in a dominant negative fashion by competing with long isoform Homer proteins. In the absence of long isoform proteins, it could be argued that the loss of the short isoform proteins would not be expected to contribute additional independent effects on CNS function or behavior. However, recent findings provide evidence for independent behavioral effects of Homer1a when overexpressed in the prefrontal CTX of *Homer1* KO mice otherwise lacking all Homer1 proteins (Lominac *et al.* 2005). Specifically, overexpression of Homer1a, but not Homer1c, reversed the increased behavioral despair in the Porsolt swim test seen in *Homer1* KOs. In the same study, overexpression of Homer1c, but not 1a, reversed the deficits in memory and PPI as well as normalized prefrontal glutamate regulation. While these findings support the conclusion that the majority of behavioral deficits reported in *Homer1* mutant mice are due to the loss of the long Homer1 isoforms, they also suggest that Homer1a may have independent effects from those of Homer1c. Another consideration is the recent description of additional Homer1 isoforms (i.e. Homer1e,f,g,h) that would also be absent in *Homer1* KOs (Klugmann *et al.* 2005). Loss of these isoforms could contribute to the behavioral effects seen in *Homer1* KOs. For example, Homer1g lacks an EVH-1 domain, and it has been suggested that it could bind via its coiled-coiled domain with the Rho GTPase family to affect cytoskeleton assembly and possibly dendritic spine morphology (Klugmann *et al.* 2005).

Gene dosage

Western blot analysis in CTX and HIP showed Homer1 proteins to be absent in KOs and reduced by approximately one-half in HETs. However, the results do not support a simple gene–dosage relationship for behavioral differences among genotypes. Specifically, KOs differed significantly from WT and HETs in response to sensory stimulation (greater), forelimb grip strength (weaker), Morris water maze (longer latency, longer distance and slower swim speed to find escape platform), nest construction (less complete), social transmission of food preference (impaired) and rotarod

performance (impaired). In contrast, HET mice did not differ significantly from WTs in any of these tasks. If behavioral deficits in the HETs are related to abnormally low levels of Homer1 proteins, a pattern of behavioral performance intermediate between WT and KO would be predicted, which was not the case. It may be that only a relatively complete KO of Homer1 proteins results in gross behavioral deficits, and protein levels in HETs are sufficient to support behaviors comparable with WT for some tasks. Alternatively, compensatory mechanisms could ameliorate behavioral deficits in HETs but not in the KOs. Compensatory mechanisms could include genetic redundancy and alternative biochemical or cell signaling pathways (Crawley 1996). Another possibility is that gene dosage effects were present but were not linear and below the detection threshold for the HETs in this study. Indeed, if a particular behavioral trait is qualitative (i.e. present or not present), rather than quantitative, clear segregation between phenotypes may not be possible (Gerlai 1996). Such complex behavioral effects have been seen in other studies with single gene KOs. For example, Bozon *et al.* (2002) found that WT (+/+) and HET Z*if268* (+/–) were able to learn an object recognition task, whereas *Zif268* (–/–) KO were not. When the memory task was for spatial location, HET performance was impaired to a similar degree to that of the KOs. Again, no simple gene–dosage relationship was evident.

These interpretations are further complicated, however, for behavioral tests in which the HETs differed significantly from the WT and/or KO while WT and KO did not differ. These tests included aggression in the social dyad and auditory startle. Social behaviors represent a very high level of cognitive performance, and no simple gene dosage or compensatory explanation appears to explain these data for the HETs nor for auditory startle. A possible explanation may be that epistatic interactions due to lower levels of Homer1 proteins altered these behaviors while an absence of proteins did not. The possible, and even likely, influence of multigene interactions and flanking gene effects in determining phenotype in null and single gene mutations has been discussed extensively (Gerlai 2001; Routtenberg 2002; Wolfer *et al.* 2002). Resolution of these issues will clearly require additional experiments in *Homer1* mutants that directly relate gene expression, protein levels and behavioral or physiological phenotype, as well as use of converging techniques and lines of evidence to provide insight into gene function.

Conclusion

Deletion of the *Homer1* gene resulted in highly complex and multimodal behavioral outcomes. Sensory responsiveness, motor function, activity cycles, somatic growth and learning were all affected to some degree by deletion of *Homer1*. Because of the central role Homer1 proteins play in linking glutamatergic receptors to calcium-signaling pathways, and the importance of glutamate for synaptic plasticity (e.g. LTP and LTD), it was surprising that the deficits seen in the *Homer1* KO mice were relatively mild and that the animals appeared to be generally healthy and free of gross neurological symptoms (e.g. seizures, ataxia, etc.). This suggests that effective compensatory systems, possibly involving the other Homer isoforms, exist that enable KO mice to survive and function. Future research aimed at discovering how Homer proteins contribute to brain function, and behavior will undoubtedly require a combination of approaches including the use of conditional KOs targeting specific brain regions (e.g. HIP, CTX, cerebellum) and the use of animals overexpressing various Homer isoforms.

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Acknowledgments

This work was supported by NIEHS 1 P01 ES11269. We thank Melissa J. Marcucci for helping to setup the behavioral ethogram on the Noldus Observer.