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Behavioral characterization of P311 knockout mice

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Abstract

P311 is an 8-kDa protein that is expressed in many brain regions, particularly the hippocampus, cerebellum and olfactory lobes, and is under stringent regulation by developmental, mitogenic and other physiological stimuli. P311 is thought to be involved in the transformation and motility of neural cells; however, its role in normal brain physiology is undefined. To address this point, P311-deficient mice were developed through gene targeting and their behaviors were characterized. Mutants displayed no overt abnormalities, bred normally and had normal survival rates. Additionally, no deficiencies were noted in motor co-ordination, balance, hearing or olfactory discrimination. Nevertheless, P311-deficient mice showed altered behavioral responses in learning and memory. These included impaired responses in social transmission of food preference, Morris water maze and contextual fear conditioning. Additionally, mutants displayed altered emotional responses as indicated by decreased freezing in contextual and cued fear conditioning and reduced fear-potentiated startle. Together, these data establish P311 as playing an important role in learning and memory processes and emotional responses.

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Keywords

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P311 was first identified as a complementary DNA (cDNA) whose expression was increased dramatically during late embryogenesis in mouse brain and was maintained at high levels in adult mouse hippocampus, cerebellum and olfactory bulb (Studler *et al.* 1993; www.brain-atlas.com). The amino acid sequence for P311 is highly conserved in humans, nonhuman primates, mice, rats and chickens (Studler *et al.* 1993). In mouse, P311 contains 68 amino acids, and it has a short half-life of about 5 min because of rapid degradation by the ubiquitin/proteasome system and an unidentified metalloproteinase (Taylor *et al.* 2000). Despite its transient nature, P311 expression is modulated by various physiological stimuli. For instance, P311 expression increases in rat cerebral granular cells that have been induced to undergo apoptosis (Roschier *et al.* 1998). Conversely, expression is decreased in mouse cerebral cortex following pentylenetetrazol-induced seizures (Kajiwara *et al.* 1995, 1996) and in cultured neuronal precursor cells that have been induced to differentiate by neural growth factor (Taylor *et al.* 2000). Furthermore, P311 expression decreases in both neural and smooth muscle cells that have been transformed by co-expression of the tyrosine kinase receptor, c-*Met* and its ligand, hepatocyte growth factor (Taylor *et al.* 2000), suggesting a possible role for P311 in tumor formation. A role in tumorigenesis is further implied by laser capture microdissection studies that indicate high expression of P311 along the invading edge of glioblastomas with substantially lower expression within the tumor core (Mariani *et al.* 2001). The association of modulated P311 expression with cellular differentiation appears to be more than coincidental, as ectopic expression of P311 *in vitro* can promote neural and muscle differentiation (Fujitani *et al.* 2004), induce differentiation of fibroblasts into myofibroblasts (Pan *et al.* 2002) and augment motility of gliomas (McDonough *et al.* 2005). Considered together, these results suggest important roles for P311 in neural function and cellular differentiation; however, the mechanisms through which P311 acts as well as its importance in physiological processes remain to be explored.

Although P311 is expressed in different brain regions (Studler *et al.* 1993; www.brain-atlas.com), including the cerebellum, hippocampus, and amygdala, no data exist concerning its normal physiological and behavioral functions in neural systems. For this purpose, we developed P311 knockout (KO) mice by targeting the murine *P311* gene for disruption and assessed general neurophysiological responses, as well as, responses in behavioral tests that were sensitive to abnormalities in cerebellar, hippocampal and amygdala functions.

Materials and methods

Animals and P311 gene targeting

A vector was constructed from a 12-kb *XhoI–NdeI* fragment of the mouse *P311* gene. A central 4-kb *Sac*I–*Nde*I fragment that contained the entire P311 coding region was deleted and replaced with pGKneoBpA (Bonin *et al.* 2001; Tessarollo 2001), which left intact 4 kb of the noncoding upstream and downstream regions of *P311* (Fig. 1a). This plasmid was electroporated into CJ7 embryonic stem (ES) cells (derived from 129S1/SvImJ mice), and homologous recombinants were selected and confirmed by Southern blotting of EcoRV-digested DNA with a 3′ external P311 probe (1.8-kb *NdeI–SalI* fragment). Chimeras for the targeted P311 allele were generated by injecting the targeted ES cells into C57BL/6NCr1Br mouse blastocysts. These chimeric animals were crossed with C57BL/6NCr1Br mice, producing heterozygous C57BL/6NCr1Br × 129S1/SvImJ mice. Non-brother or non-sister intercrosses were used to generate P311 wild-type (WT) and homozygous mutant (KO) animals; no sublines were generated. Mice were genotyped for P311 by polymerase chain

reaction (PCR). DNA was prepared from tail biopsies using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction was performed using a standard protocol with 95°C denaturing, 58°C annealing and 72°C extension steps with iTaq Supermix (Bio-Rad, Hercules, CA, USA). Primers were used that distinguished the WT from the mutant P311 allele (P311 sense for the WT oligo: 5′-GTG AAT TCA CCT CTC CAG CT-3′; the Neo oligo: 5′-CAT TTG TCA CGT CCT GCA-3′; P311 common antisense oligo: 5′-GGG GTT GTA AAC GTG GAT-3′). This PCR procedure was validated by Southern blotting (data not shown).

Male and female WT and KO littermates were derived from the F_{10-17} generations of intercrossing. The same mice were used in some parts of the neurophysiological screen and a pilot fear-conditioning experiment and were up to 6 months of age at the end of testing. Naïve mice (2–4 months of age) were used to replicate these findings, and naïve mice were evaluated in additional experiments designed to evaluate electroshock-induced seizures, as well as performance in the social transmission of food preference (STFP), Morris water maze and fear-potentiated startle (FPS) tests. As no age, generation of breeding or sex differences were observed, the data were combined and analyzed by genotype. All mice were housed in a humidity-controlled and temperature-controlled specific-pathogen-free barrier facility with a 14–10 h light–dark cycle (lights on 0700 h) at 3–5 mice/cage with food and water provided *ad libitum.* All experiments were conducted to minimize potential pain or discomfort with approved protocols from the Veterans Administration and Duke University Institutional Animal Care and Use Committees.

Northern blotting and reverse transcriptase-polymerase chain reaction

RNA blotting was performed using procedures and probes as described (Taylor *et al.* 2000). Briefly, total cellular RNA was isolated from mouse tissues using a standard acid–phenol extraction procedure. Fifteen micrograms of RNA were separated on 1.2% agarose/formaldehyde gels for Northern blot analysis using mouse P311 and human glyceraldehydes-3-phosphate dehydrogenase probes.

The hippocampus and cerebellum were manually dissected and snap-frozen in liquid nitrogen. RNA was prepared from the tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Complementary DNA was generated from RNA using a Superscript First Strand Synthesis kit (Invitrogen, Carlsbad, Ca, USA), following the manufacturer's instructions and using random hexamers as primers. The cDNA was then subjected to a PCR procedure using a standard protocol (see above). Primers were used that recognized a P311-cDNA fragment (P311 sense: 5′-GGG AGA ATA GAA GAA GCC CA-3′; P311 antisense: 5′-CAG TGT GAA ATT CTG AGC G-3′) and a GAPDH fragment (GAPDH sense: 5′-ATC ACC ATC TTC CAG GAG CGA-3′; GAPDH antisense: 5′-GCC AGT GAG CTT CCC GTT CA-3′). The P311 primers were designed to span two intron splice sites, so that any product resulting from amplified cDNA could be easily distinguished from a product resulting from any contaminating genomic DNA.

Neurophysiological status

A neurophysiological screen was conducted to assess orienting and reflexive behavior, postural and righting reflexes, paw strength and motor co-ordination and balance (Ribar *et al.* 2000; Rodriguiz &Wetsel 2006; Rodriguiz et *al.* 2008). The ability of mice to discriminate olfactants from pooled urine samples was examined as described (Rodriguiz *et al.* 2004). Hearing was assessed as outlined (Carlson & Willott 1996) with an acoustic startle chamber (Med-Associates, St Albans, VT, USA). Following 10 min of acclimatization, 45 trials of pure tone startle stimuli were administered for 40 milliseconds in a pseudorandom

order. Nine trials each were administered at 4, 8, 12, 16 and 20 kHz [intertrial interval (ITI) = 30–90 seconds]. At each frequency, three stimuli were delivered at each of the following intensities: 100, 110 and 120 dB. For each animal, the mean acoustic startle response was calculated for each of the five frequencies at each of the three intensities. Spontaneous locomotor and rearing activities were evaluated over 30 min in an automated Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, OH, USA) as noted (Pogorelov et *al.* 2005). Motor co-ordination and balance were studied using a rotorod (Med-Associates) with accelerating (4–40 r.p.m. over 5 min) and constant speed (21 r.p.m.) protocols as described (Ribar *etal.* 2000). Anxiety-like behaviors were evaluated in the elevated zero maze illuminated at ~60 lux (Pogorelov et *al.* 2005; Rodriguiz & Wetsel 2006; Welch et *al.* 2007) and in the light–dark emergence test (Welch *etal.* 2007). Behavior was videotaped for 5 min and subsequently scored by trained observers using observer (version 5.0; Noldus Information Technology, Leesburg, VA, USA). The behaviors included percent time spent in the open areas, tota number of transitions between the two open areas, stretch-attend postures into the open areas and head-dipping over the side of the open area. Tail suspension (immobility time over 6 min) was used to examine depressive-like behaviors (Fukui et *al.* 2007). Prepulse inhibition (PPI) was examined as outlined (Pillai-Nair et *al.* 2005; Rodriguiz *et al.* 2008). The startle response was measured using maximum platform displacement recorded in mAmp following presentation of the startle stimulus. Prepulse inhibition was calculated as the ratio of prepulse trials to startle-only trials subtracted from 1 and expressed as a percentage [1 − (prepulse + pulse trials/pulse-alone trials)]100.

Electroshock-induced seizures

One or two drops of local anesthetic (1% lidocaine) were placed in each conjunctiva. Approximately 30–60 seconds later, the animal was held gently, a cup electrode was placed over each cornea and current was administered (Wahlquist Instrument Co., Salt Lake City, UT, USA). To test the initial seizure threshold, a stimulus of 6 mAmp at 60 Hz was administered for 200 milliseconds. The stimulus was repeated daily, and the intensity was increased by 2 mAmp each day until the stimulus evoked a tonic-clonic seizure. The animal's response to the stimulus was immediate. The initial phase observed in all animals consisted of a stun response. Later responses consisted of flexion and nodding of the head, facial clonus, clonus of the forelimbs and finally tonic-clonic seizures. The investigator evaluating the seizure pattern and duration was blinded to the genotype.

Social transmission of food preference

Testing was divided into three sessions: familiarization, a 20-min short-term memory test, and a 24-h long-term memory test (Rodriguiz *et al.* 2004, 2008). On the day before testing, group-housed mice were placed on food restriction for 18 h. For familiarization, a single mouse (i.e., demonstrator) was removed from the home cage and placed into a clean arena and allowed to eat from a single bowl of chocolate-flavored or vanilla-flavored diet. The choice of chocolate or vanilla designator diets was randomized across cages. After 30 min, the demonstrator was returned to its home cage and allowed to interact with cage mates (i.e., tester) for 20 min. Tester mice were subsequently placed into the arenas and presented with two bowls for 30 min, one containing the familiar diet (fed to demonstrator) and the other containing the novel flavor. The amounts of the diets consumed in each bowl were recorded. Tester mice were examined for long-term memory 24 h later using the same familiar–novel diet comparison. Preference for a given diet was calculated as the difference between the two flavored diets consumed relative to the total amount eaten. Positive scores indicated preference for the diet fed to the demonstrator, whereas negative scores signified preference for the novel diet.

Morris water maze

Learning and memory were assessed in the Morris water maze as outlined (Lipp & Wolfer 1998; Rodriguiz & Wetsel 2006; Rodriguiz *et al.* 2008) where performance was assessed as the total swim distance and swim velocity from tracking profiles created by Ethovision (version 3.1; Noldus Information Technology). For all trials, mice were released into the maze and given 60 seconds to find the platform. Acquisition testing was conducted over 8 consecutive days with the hidden platform placed in the north-east (NE) quadrant; release points at seven different locations around the perimeter of the pool were randomized across test trials. Animals were given four trials per day and run in blocks of two trials (ITI = 1 min) with blocks separated by 40–45 min. Probe tests (platform removed) were administered 1 h after completion of acquisition training on days 2, 5 and 8. For visible platform trials, a separate group of mice was run over 2 days.

Sensitivity to foot-shock

The sensitivity to scrambled foot-shock was examined as described (Grove *et al.* 2004; Rodriguiz & Wetsel 2006).

Fear conditioning

Animals were examined in a 3-day paradigm, consisting of conditioning on day 1, context testing on day 2 and a cued testing on day 3. Context testing was conducted in the conditioning chamber in the absence of the conditioned stimulus (CS) and foot-shock (UCS). Cued testing was conducted in a novel chamber that had a different level of illumination and different floor, walls, dimensions and shape than the conditioning chamber. All training and testing were conducted in a mouse fear-conditioning apparatus (Med-Associates) as noted (Pillai-Nair *et al.* 2005; Rodriguiz & Wetsel 2006; Rodriguiz *et al.* 2008). In a second set of studies to examine short-term retention of the conditioned response, mice were tested in the context or cued test 1 h after conditioning. Finally, a third set of mice were ‘doubled trained’ where they were given the conditioning procedure on day 1, it was repeated on day 2 after 24 h, and animals were tested 24 h later in either the context or the cued test (Ferguson *et al.* 2000). For all experiments, behavior was videotaped during conditioning and testing, and the time spent in freezing was scored with the Observer program (version 5.0; Noldus Information Technology) by trained observers ‘blind’ to the genotypes of the mice.

Fear-potentiated startle

Fear-potentiated startle testing was conducted in a Med-Associates potentiated startle apparatus as described (Falls *et al.* 1997; Rodriguiz & Wetsel 2006). Testing occurred over 4 days. On day 1, baseline startle responses were assessed over 18 trials with 40 millisecond bursts of white noise at 100, 105 and 110 dB. Each stimulus was presented six times in a pseudorandom order with an ITI of 30–90 seconds. On day 2, mice were given 9 trials of baseline startle stimuli (three presentations/intensity) followed by 18 test trials. On one-half of the trials, startle stimuli were administered immediately following the CS; on the remainder, the CS was presented alone. Conditioned stimulus + startle trials were interspersed in a pseudorandom order with the CS-only trials (ITI of 30–90 seconds). Twenty-four hours later, animals were given 10 pairings of the CS with 0.25 seconds scrambled shock (UCS) separated by an ITI of 90–180 seconds. One-half of the mice were conditioned with 0.2 mAmp foot-shock, while the remainder was conditioned with a 0.4 mAmp foot-shock. On the final day of testing, animals were examined for FPS under the same procedures described above for day 2. Potentiation to the CS was defined as the magnitude of response to the CS + startle trials relative to the startle-alone trails on both pre-conditioning (day 2) and post-conditioning (day 4) days and expressed as a percentage (CS

+ startle trials/startle-only trials)*100. The magnitude of FPS was calculated and presented for each of the startle intensities at 100, 105 and 110 dB.

Statistics

Inter-observer and intra-observer reliabilities for all behavioral scoring were assessed with Cohen's κ (Bakeman & Gottman 1997). In al cases, κ exceeded 0.90. Behavioral data are presented as means ± SEM, and statistical analyses were performed with the SPSS-1 1 statistical programs (SPSS Inc., Chicago, IL, USA). Student's t-test was used for WT and KO comparisons in the neurophysiologica screen, elevated plus maze, light–dark emergence test, spontaneous activity in the open field, null activity in the PPI and FPS tests and baseline startle responses for PPI. The percent mice exhibiting barbering in the neurophysiological screen was analyzed with chi-square analyses. Analysis of variance (ANOVA) was used to examine genotype differences in freezing behavior for the different training procedures and test times. Repeated measures ANOVA (RMANOVA) was used to evaluate startle responses to different frequencies and intensities in hearing tests, activity in the open field (5-min blocks), prepulse intensity (4, 8 and 12 dB) for PPI, olfactory preferences for multiple two-way comparisons, preference scores for STFP at 20 min and 24 h, sensitivity to different foot-shock intensities, freezing during conditioning sessions for fear conditioning and stimulus intensity in FPS as the within-subjects effects. In all cases, genotype was the between-subjects effect. Bonferroni corrected pair-wise comparisons were used for all *post hoc* analyses. In all cases, a P value of < 0.05 was considered significant.

Results

Generation of mice lacking P311

Although a role for P311 in neural function is obscure, high levels of P311 messenger RNA have been identified in several brain areas of adult rodents (Studler *et al.* 1993). We used gene targeting to generate mice that lack expression of this gene, and the genotypes of the mice were routinely identified by PCR (Fig. 1b). Northern blot (Fig. 1c) and reverse transcriptase-polymerase chain reaction analyses (Fig. 1d) confirmed that P311 transcripts were absent in skeletal muscle and brain samples from the homozygous mutants. Knockout mice were produced at Mendelian frequencies, displayed no apparent gross abnormalities, and reproductive and mortality rates of adult mutants were similar to WT controls. Furthermore, a thorough histological analysis of all major tissues including brain showed nothing remarkable (data not shown).

Neurophysiological status

Examination of WT and KO mice for general appearance, visual orientation and reflexive behaviors, paw strength, balance and co-ordination, olfaction, hearing, spontaneous activity, anxiety-like responses, PPI and susceptibility to seizure showed no remarkable genotype differences (Table S1). The only exceptions were analyses of barbering behavior, horizontal pole-walking behavior and immobility in the tail suspension test.

Social transmission of food preference

After interacting with demonstrator mice for 20 min, WT and KO animals were tested immediately at 20 min and 24 h later (Table 1). Wild-type mice showed a strong preference for the familiar diet at 20 min and remembered this preference 24 h later. By contrast, KO animals failed to develop a preference for either diet at 20 min or 24 h. Parenthetically, no genotype differences emerged with regard to latency to contact the bowls, latency to eat or the frequency of bowl contacts during testing – although the total amount eaten by the KO mice was reduced at 20 min but not at 24 h relative to WT controls (Table S2). These data

suggest that the ability to develop stimulus–stimulus associations and retention of this relationship are deficient in KO mice.

Morris water maze

The impairments of KO mice in the STFP test suggest a loss of associative learning and memory. We investigated further these processes in the Morris water maze. In the visible platform test, no genotype differences were observed for swim time (Fig. 2a) or swim distance or velocity (Figs S1a and S2a), suggesting that sensory and motor functions were normal for WT and KO mice. Although swim velocity for the mutants during the first four test trials was slower than WT controls, no genotype differences were observed over the next four trials (Fig. S2b). Parenthetically, it is unclear why the swim velocities of the KO mice were reduced only over the first four trials of visible platform testing; however, it may be related to their increased depressive-like behavior displayed in tail suspension (Table S1). In acquisition testing, the mutants showed a marked impairment in learning the location of the hidden platform, with little change across testing in swim times (Fig. 2b), swim distances (Fig. S1b) and swim velocities (Fig. S2b) relative to WT controls. Analyses of swim time for probe trials conducted at the end of test days 2, 5 and 8 confirmed that WT animals had developed a clear preference for the NE quadrant on each probe trial (Fig. 2c). By comparison, P311 mutants were impaired on the probe tests (Fig. 2d). Analyses of swim distances for the probe trials confirmed these results (Fig. S1c–d) as did the representative tracings from the acquisition and probe trials for the mice (Fig. 2e,f). Together, these data support the idea that learning and memory are abnormal in the KO mice.

Conditioned emotional memory

Conditioned emotional responses were examined in WT and KO mice using fear conditioning and FPS. As both tests use electric shock, animals were tested for sensitivities to foot-shock, and no genotype differences were observed (Table S3). For fear conditioning, no genotype differences were noted for the percent time spent freezing during training on day 1 for WT and KO mice in the different experiments (Table S4). Under the different conditioning sessions on day 1, freezing was relatively low before and during presentation of the tone or CS and foot-shock or UCS, but it increased in all animals during the post-CS/UCS period. Following a single exposure to the UCS, freezing in contextual testing was reduced for KO mice relative to WT mice at 1 and 24 h (Fig. 3a, left and middle). By comparison in cued fear conditioning at the 1-h test, WT and KO animals spent approximately the same percent time freezing during presentation of the CS (Fig. 3b, left). Although WT mice maintained this response, mutants failed to show freezing at 24 h (Fig. 3b, middle). To determine whether additional training could improve KO responses, animals were given two sessions of CS–UCS pairings and tested 24 h later in contextual or cued fear conditioning. Freezing increased for both genotypes during the second conditioning session in double training, and WT mice showed a greater increase in freezing relative to KO animals (Table S4). Under contextual testing, levels of freezing were high and similar for WT mice given single or double training (Fig. 3a). By comparison, freezing at 24 h was reduced for KO mice under single training and it was not further augmented by double training. Under cued testing, freezing behavior was augmented for WT mice with double training compared with single training (Fig. 3b). Freezing for KO animals was significantly enhanced with double training; however, the percent time spent freezing was lower than that for double-trained WT animals. These data show that KO mice are deficient in short-term and long-term contextual fear conditioning. Cued-induced freezing behavior of KO animals is intact in the short-term but not in the long-term test; however, repeated training can increase their freezing responses.

The deficiencies in contextual and cued fear conditioning suggest that learning and memory processes associated with emotional responses may be abnormal in KO mice. To examine this prospect, mice were tested in FPS. No genotype differences were discerned in null activity, baseline startle responses to 100, 105 and 110 dB stimuli and preconditioning potentiation of the startle response by the CS (Table S5). Pairing of the CS with the UCS on day 4 showed that 0.2 mAmp did not illicit potentiated startle in WT or KO animals (Fig. 3c); however, 0.4 mAmp produced fear potentiation in WT mice but not in the mutants (Fig. 3d). Taken together, these FPS and fear-conditioning results show that emotional responses are perturbed in P311 mutants.

Discussion

Previous work has suggested that P311 may be an important factor in the transformation or morphogenesis of neural cells, as well as for other cell types including muscle and fibroblasts (McDonough *et al.* 2005; Pan *et al.* 2002; Taylor *et al.* 2000). Additionally, P311 expression in adult cerebellum, hippocampus and amygdala suggests a function for the protein in these brain areas (Studler *et al.* 1993; www.brain-atlas.com). To date, the physiological role of P311 in normal brain function has not been elucidated. To address this point, P311-deficient mice were developed using gene-targeting procedures. These mutants have normal reproductive function, life span and gross behavioral function. Despite pronounced expression of P311 in cerebellum (Studler *et al.* 1993), an area of brain known to regulate motor co-ordination, balance and motor learning (Houk & Wise 1995), results from our neurophysiological screen indicate that these responses are normal in KO mice. Although the seizure-inducing agent PTZ has been shown to affect P311 expression in brain (Kajiwara *et al.* 1995, 1996), the mutants did not display a differential sensitivity to electroshock-induced seizures.

P311 mice were evaluated in the STFP, Morris water maze and contextual fear-conditioning tests – tasks that are sensitive to hippocampal dysfunction (Alvarez *et al.* 2002; Fendt & Fanselow 1999; Morris *et al.* 1986; Phillips & LeDoux 1992) but are known also to involve other brain areas. In STFP, KO mice failed to show preferences for the demonstrator or novel diet. This deficiency could be due to several factors that include olfaction, motivation and learning and memory. It should be recalled that P311 is expressed in the olfactory bulb and hippocampus (Studler *et al.* 1993). Because discrimination between olfactants is not different by genotype in the olfactory test, this sensation appears to be intact in the mutants. Similarly, possible motivational differences do not seem to underlie the genotype effect because the latencies to approach the bowls to eat the different flavored diets and the frequencies of bowl contacts were similar for the WT and KO mice. Nevertheless, the deficiency at 20 min and at 24 h in STFP suggests that nonspatial hippocampally mediated short-term and long-term learning and memory processes may be impaired in the P311 KO mice.

The deficits of KO mice in the Morris water maze are concordant with impairments in STFP. In the maze, mutants had difficulty in acquiring the task, and responses on probe trials were markedly deficient. These abnormalities may be attributed to perturbations in spatial learning and memory processes because performance over much of the visible platform version of the test was intact – suggesting that sensory and motor functions in KO mice are normal. This contention is further supported by results from the neurophysiological screen where sensory and motor functions are similar for WT and KO animals.

Besides STFP and the Morris water maze, P311 animals were examined also in fear conditioning. In tests of contextual fear conditioning at 1 and 24 h, KO mice were deficient compared with WT controls. Notably, double training did not improve contextual learning

and memory in the KO animals when they were tested at 24 h. Examination of responses following cued fear conditioning indicated that the KO mice were not deficient at 1 h but were impaired at 24 h. While double training improved cued fear conditioning at 24 h, the percent time freezing of KO animals was still below that of the double-trained WT controls. These differential responses in contextual and cued fear conditioning could be because of several different factors. Contextual fear conditioning is hippocampally dependent, while cued fear conditioning is not (Fendt & Fanselow 1999; Phillips & LeDoux 1992). P311 is expressed at relatively high levels in adult mouse hippocampus, whereas expression is much lower in amygdala (Studler *et al.* 1993; www.brain-atlas.com). Thus, P311 may have a greater opportunity to affect processes related to hippo-campal than amygdala function. In cell culture, P311 can modulate the numbers of neurons through apoptosis and other mechanisms (Roschier *et al.* 1998; Taylor *et al.* 2000). One population of stem cells is located in the subgranular zone of the dentate gyrus (Kempermann *et al.* 2004), and these hippocampal cells give rise to new neurons. Irradiation destroys hippocampal stem cells (Monje & Palmer 2003) and disrupts contextual fear conditioning (Winocur *et al.* 2006). Interestingly, P311 mice show a similar behavioral deficiency. P311 can affect cell migration and this protein colocalizes with Filamen A (McDonough *et al.* 2005), which can bind many other molecules at the leading edge of migrating cells. Rac1 is important for cell migration, and P311 has been shown to activate this small RhoGTPase (McDonough *et al.* 2005). Rac1 regulates dendritic spine formation and it can recruit AMPA receptors to the synapse (Wiens *et al.* 2005). As this process is important at least for long-term memory, it is not surprising that responses in contextual and cued fear conditioning at 24 h are disturbed in P311 KO mice. Double training can partially rescue cued fear conditioning at 24 h, whereas contextual fear conditioning is still impaired. These findings suggest that P311 controls different cellular processes within the hippocampus and amygdala and that plasticity in these brain regions is differentially affected by loss of this gene.

Deficiencies in both contextual and cued fear conditioning are indicative of emotional dysregulation (Fendt & Fanselow 1999; Kim & Davis 1993; Phillips & LeDoux 1992). Knockout mice were deficient in both versions of fear conditioning at 24 h. Although double training did not alter freezing responses in contextual fear conditioning, it enhanced freezing behaviors in cued testing – but time spent freezing was still significantly lower than that of the double-trained WT controls. P311 mutants also showed deficits in a second test of emotionality, FPS. Together, these findings suggest that P311 plays an important role in learning and memory processes and emotionality.

The P311 KO mice provide an interesting model to investigate potentially novel mechanisms underlying learning and memory and emotional responses. Notably, deficits in these behaviors are not confounded by impairments in sensory or motor function or anxiety-like behaviors. Currently, it is unknown whether the cognitive and emotional deficits in the KO mice can be attributed solely to expression of P311 in brain during late embryogenesis (Studler *et al.* 1993) or during adulthood in brain areas or circuits critical for these responses. It should be recalled that P311 is implicated in neuronal differentiation (Fujitani *et al.* 2004) and perhaps motility (McDonough *et al.* 2005). Hence, one may envision that deficiencies in P311 function could affect behaviors as diverse as those in Down's syndrome or contribute to cognitive decline in aged adults. Hence, further elucidation of a role for P311 in developing and adult brain may provide new insights into basic processes that contribute to cognitive dysfunction and emotional dysregulation in a variety of neurological and psychiatric conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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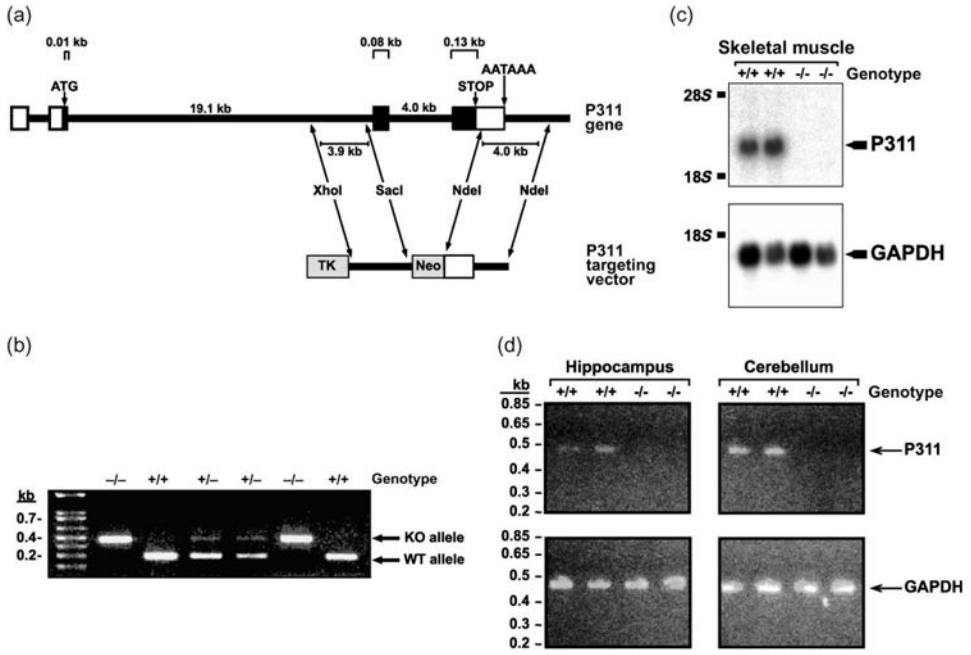

Figure 1. Development of P311-deficient mice

(a) AP311 gene targeting vector was created from the indicated *P311* gene fragments and used to generate P311-deficient mice. The third exon and part of the fourth exon of the *P311* gene, which contain the coding region for the protein, were deleted in targeted mice. Protein-coding exons are indicated by solid boxes, noncoding exons by open boxes and introns by solid lines. (b) Mice that carried the targeted P311 allele were identified by PCR and the products migrated on agarose gel at ~0.4 and ~0.28 kb for the respective WT and KO alleles. The positions of molecular weight markers are depicted on the left. (c) Total RNA was prepared from skeletal muscle isolated from WT and KO and then used for Northern blot where the P311 transcript migrated at ~2 kb for WT samples, and the GAPDH mRNA migrated at ~1.4 kb for both genotypes. Positions of the major ribosomal RNA species are shown on the left. (d) Total RNA was prepared from the hippocampus and cerebellum of WT and KO mice. Reverse transcriptase polymerase chain reaction identified a product for P311 of ~0.47 kb in WT samples; as a control the GAPDH product (~0.47 kb) can be seen in both WT and KO samples. Positions of the molecular weight markers are indicated at the left.

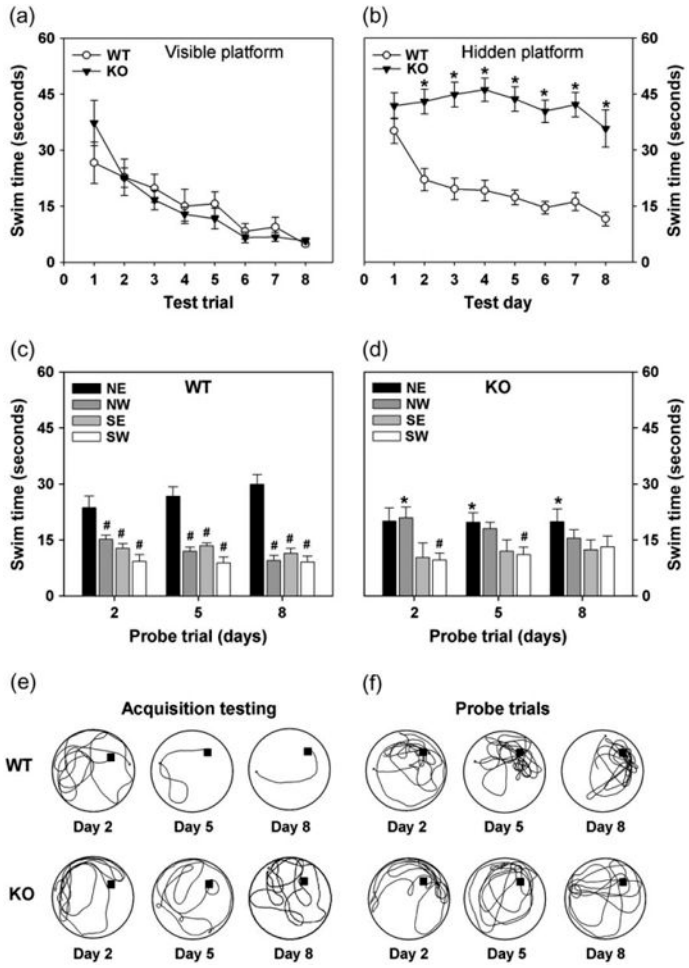

Figure 2. Swim times for P311 mice in the Morris water maze

(a) Performance of WT and KO mice are similar in the visible platform version of the water maze. Repeated measures ANOVA for swim time showed significant main effects of test trial ($F_{7,133} = 17.888$, $P < 0.001$), but no significant test trial by genotype interaction. Bonferroni comparisons showed that swim times for WT and KO mice were lower on trials 6, 7 and 8 than trial 1 ($Ps < 0.035$). $n = 10$ mice/genotype. (b) Wild-type mice rapidly locate the hidden platform as indicated by reductions in swim time across test days, whereas mutants are impaired. Repeated measures ANOVA found significant main effects of test day ($F_{7,133} = 12.907$, $P < 0.004$) and a significant test day by genotype interaction ($F_{7,133} = 3.921$, $P < 0.05$). Bonferroni comparisons confirmed that KO mice had longer swim times on test days 2–8 ($Ps < 0.001$). (c–d) Probe trials conducted after acquisition training on days 2, 5 and 8 for (c) WT and (d) KO mice. A RMANOVA for swim time showed significant main effects of test day ($F_{2,38} = 20.228$, $P < 0.001$) and quadrant ($F_{3,57} = 21.149$, $P < 0.001$), and a significant genotype by quadrant interaction ($F_{3,114} = 3.875$, $P < 0.044$); the between-subjects test was not significant. Bonferroni comparisons observed WT mice to spend more time swimming in the NE quadrant relative to the other quadrants on probe days 2 ($Ps < 0.045$), 5 ($Ps < 0.033$) and 8 ($Ps < 0.005$). By contrast, KO mice swim times between the NE and SW quadrant were different on days 2 ($P < 0.034$) and 5 ($P < 0.012$). Although swim times in the NE quadrant were similar for KO and WT mice on day 2 (P = 0.516), mutants spent considerably less time in the NE quadrant on test days 5 and 8 ($Ps < 0.048$). (e) Tracings of representative swim patterns for WT mice on test days 2, 5 and 8 when the hidden platform was located in the NE quadrant. (f) Tracings of swim patterns for

representative WT mice during probe trials on days 2, 5 and 8. The apex of the water maze in each tracing is north (N) and the platform location is designated by a filled square. $n = 10$ mice/genotype. $^{*}P < 0.05$, WT compared with KO mice; $^{\#}P < 0.05$, target NE quadrant compared with NW, SE or SW quadrants. NW, north-west; SE, south-east; SW, south-west.

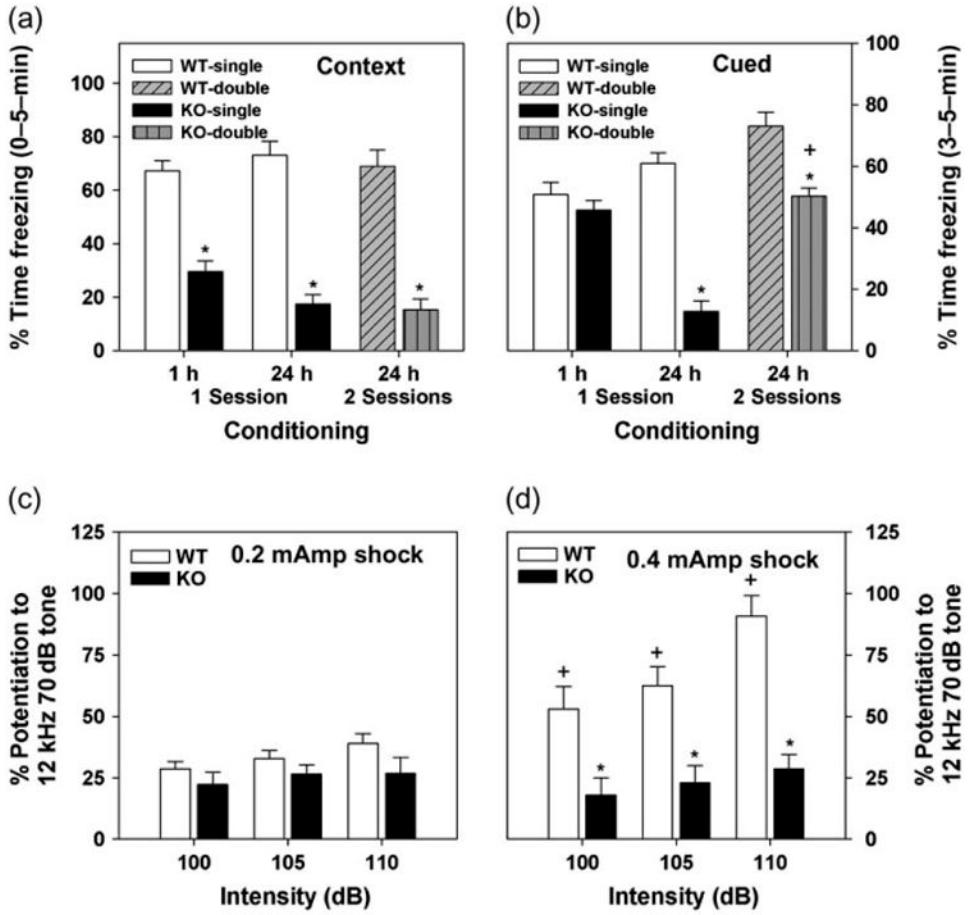

Figure 3. Fear conditioning and fear-potentiated startle responses of P311 mice

Wild-type and KO mice were tested in fear conditioning with a single training session and tested at 1 and 24 h or were given double training and tested at 24 h. (a) Knockout mice showed reduced freezing at testing in contextual fear conditioning at 1 and 24 h under single training and at 24 h under double training conditions. Analysis of variance showed significant main effects for genotype ($F_{1,32} = 159.662$, $P < 0.001$), but the experimental groups and the genotype by experimental group interaction were not significant. Bonferroni corrected pair-wise comparisons confirmed that WT mice spent more time freezing than KO animals under all conditions ($Ps < 0.001$) and that freezing for KO mice did not differ on any of the tests. (b) P311 mutants were also deficient in the cued test at 24 h but showed no deficits at 1 h with single training; they were deficient at 24 h following double training. Analysis of variance showed significant main effects for genotype ($F_{1,32} = 85.996$, $P < 0.001$) and experimental group ($F_{1,32} = 25.739$, $P < 0.001$), and a significant genotype by experimental group interaction ($F_{1,32} = 16.482$, $P < 0.001$). Bonferroni corrected pair-wise comparisons showed that WT mice spent more time freezing at the 24-h test following a single foot-shock exposure than KO animals ($Ps < 0.001$). No genotype differences were found at the 1-h test. However, double training increased freezing in KO mice at the 24-h test ($P < 0.001$) compared with mutants examined at 24 h with only a single training session. Nevertheless, freezing was still lower ($P < 0.001$) than that for WT animals. Wild-type animals had only a marginally significant increase in freezing at the 24-h test with double training ($P < 0.075$) compared with the single training episode. $n = 9–10$ mice/testing condition. $^{*}P < 0.05$, WT vs. KO mice, $^{+}P < 0.05$, single training vs. double training. (c) In FPS with 0.2 mAmp foot-shock, post-conditioning potentiation of the startle response to the

CS did not differ between genotypes. (d) For 0.4 mAmp foot-shock, post-conditioning potentiation of the startle response to the CS was significantly higher for WT mice than KO animals. Potentiation of the startle response to 0.4 mAmp foot-shock (d) was compared with mice tested with 0.2 mAmp foot-shock (c) with RMANOVA using startle intensity as the within-subject effect, and foot-shock intensity and genotype as the between-subject effects. Repeated measures ANOVA within-subjects tests yielded significant main effects of startle intensity ($F_{2,76} = 26.774$, $P < 0.001$) and a significant startle intensity by genotype ($F_{2,76} = 4.221$, $P < 0.018$) and a startle-intensity by foot-shock intensity by genotype interaction ($F_{2,76} = 5.909$, $P < 0.004$). Bonferroni corrected pair-wise comparisons confirmed no genotype differences when animals were tested with 0.2 mAmp foot-shock. For mice conditioned with 0.4 mAmp foot-shock, WT animals showed greater potentiation relative to mutants at each of the startle intensities ($Ps < 0.005$). Whereas WT mice had heightened potentiation of startle following conditioning with 0.4 mAmp foot-shock compared with conditioning with 0.2 mAmp foot-shock ($Ps < 0.001$), no such differences were observed for KO mice. $n = 10–12$ mice/genotype/test. $^{*}P < 0.05$, WT vs. KO mice; $^{+}P < 0.05$, 0.2 vs. 0.4 mAmp potentiation.

Table 1
Preference scores for STFP in WT and KO mice

Test	WT	KO
20 min	0.50 ± 0.073	−0.13 ± 0.134 *
24 h	0.44 ± 0.097	−0.03 ± 0.079 *

* Repeated measures ANOVA within-subjects test failed to show significant main effects of test–time or a significant genotype by test–time interaction. The between-subjects test found a significant genotype effect ($F_{1,17} = 20.759$, $P < 0.001$). Bonferroni corrected pair-wise comparisons showed that KO mice had significantly lower preference scores at 20 min and 24 h ($Ps < 0.001$) than WT litter-mates. $n = 10$ WT and 9 KO mice.