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Research Report

En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder

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ABSTRACT

Autism spectrum disorder (ASD) is a prevalent and inheritable neurodevelopmental disorder. Recent human genetic studies are consistent with the homeobox transcription factor, *ENGRAILED 2* (*EN2*), being an ASD susceptibility gene. *En2* knockout mice ($En2^{-/-}$) display subtle cerebellar neuropathological changes similar to what has been observed in the ASD brain. To investigate whether $En2^{-/-}$ mice displayed abnormal behavior relevant to ASD, they were monitored in tasks designed to assess social maturation as well as learning and memory. Deficits in social behavior were detected in $En2^{-/-}$ mice across maturation that included decreased play, reduced social sniffing and allogrooming, and less aggressive behavior. Deficits in two spatial learning and memory tasks were also observed. Because locomotor activity was a component of many of the behavioral tasks, this was measured at various stages of development. Locomotor activity was not compromised in the knockout. However, a more thorough analysis of motor behavior in $En2^{-/-}$ mice revealed deficits in specific motor tasks. To determine whether neurochemical changes were associated with these behavioral phenotypes, monoamine levels in specific brain regions were assessed. A cerebellar-specific increase in serotonin and its metabolite was observed. Interestingly, several reports have suggested that the serotonin pathway is affected in ASD. We conclude that $En2^{-/-}$ mice display behavioral and neurochemical changes, in addition to genetic and neuropathological changes, relevant to ASD. Therefore, these mice may be useful as an animal model of autism.

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1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by limited or absent verbal communication, deficiencies in reciprocal social interactions as well as restricted and repetitive interests and behaviors. Twin, family, and disease modeling studies support a polygenic multifactorial basis in the etiology of ASD (Folstein and Rutter, 1977; Ritvo et al., 1985; Risch et al., 1999; Folstein and Rosen-Sheidley, 2001; Liu et al., 2001; Alarcon et al., 2002; Auranen et al., 2002). Since the etiology of autism remains unknown, the development of animal models reflecting behavioral, neuropathological, and genetic aspects of this disorder is important.

Recent data have demonstrated that two allelic variants in the human *EN2* gene are consistently inherited more frequently in individuals with ASD than unaffected siblings. This was initially reported for 167 pedigrees and has now been replicated in two additional data sets (Gharani et al., 2004; Benayed et al., 2005). When all three data sets (518 families) were combined and analyzed, a $P=0.00000427$ value was obtained, providing statistically significant evidence that these *EN2* polymorphisms were associated with ASD. Population Attributable Risk (PAR) calculations using the entire sample of 518 families determined that the *EN2* risk allele contributes to as much as 40% of ASD cases in the general population. These data represent one of the more significant associations of any gene with ASD. Furthermore, *EN2* is one of the few ASD associated genes in which a statistically significant association was observed for the same genetic alleles in the replication data sets. These genetic data are consistent with *EN2* encoding an ASD susceptibility locus (ASD [MIM 608636]; EN2 [MIM 131310]), and being one of an unknown number of genes that contribute to increased risk for ASD.

Studies in the mouse have revealed that *En2* is expressed transiently in the pons, periaqueductal gray, and colliculi but is primarily restricted to the cerebellum during nervous system development. *En2* is expressed throughout cerebellar development, starting at the neural plate stage (E8.5) and continuing in at least a subset of cerebellar neurons throughout embryogenesis and postnatal development. Given that *En2* is a transcription factor, it is likely to regulate gene expression necessary for normal cerebellar development. Consistent with this, $En2^{-/-}$ mice display cerebellar phenotypes, including hypoplasia, a reduction in the number of Purkinje cells, and foliation defects (Millen et al., 1994, 1995; Kuemerle et al., 1997).

Post-mortem and MRI studies have identified a number of neural structures that are anatomically abnormal in autism. These include the cerebellum, limbic system, cerebral cortex, and brainstem (for review see Bauman and Kemper, 2005). Interestingly, two consistent cerebellar phenotypes have been reported in the ASD brain: cerebellar hypoplasia and a decrease in the number of cerebellar Purkinje cells (Bauman and Kemper, 1985, 1986; Ritvo et al., 1986; Gaffney et al., 1987; Murakami et al., 1989; Hashimoto et al., 1995; Courchesne, 1997; Bailey et al., 1998; Courchesne et al., 2001, 2003; Palmen et al., 2004, Bauman and Kemper, 2005). This is similar to the cerebellar phenotypes observed in *En2* knockout mice. However, to our knowledge, no human post-mortem study has reported abnormal cerebellar foliation patterns in individuals with autism.

It has been hypothesized that the cerebellar anatomical defects observed in the ASD brain may contribute to some of the behavioral phenotypes. Functional MRI studies indicate that the cerebellum is active in tasks such as language generation, attention, and problems solving. Therefore, the cerebellum may anticipate these tasks and allow them to be performed in a coordinated fashion (Kim et al., 1994; Raichle et al., 1994; Gao et al., 1996; Akshoomoff et al., 1997; Allen et al., 1997; Courchesne and Allen, 1997; Allen and Courchesne, 2003; Corina et al., 2003; McDermott et al., 2003). Similarly, the neuropathological changes observed in the knockout mouse may lead to neurobehavioral alterations. Thus, we examined whether *En2* knockout mice would display ASD-like neurobehavioral phenotypes, using a strategy previously used in our lab to detect autistic-like behavior in mice (Wagner et al., 2006).

In addition to behavioral changes, we also examined neurochemistry in $En2^{-/-}$ mice. Neurochemical alterations have been reported in a number of transmitter systems in ASD, including serotonergic, dopaminergic, noradrenergic, and others (for review see Lam et al., 2006). However, alterations in serotonin are one of the more consistently reported neurochemical phenotypes, supported by physiological, pharmacological, and genetic studies (Chugani et al., 1999; Anderson et al., 1990; Cook et al., 1997; Posey and McDougle, 2000; Yirmiya et al., 2001, Lam et al., 2006). In addition, *Engrailed* genes are involved in the survival of serotonergic neurons of the raphe nucleus (Simon et al., 2005) as well as midbrain dopaminergic neurons (Simon et al., 2001) during development. Since many areas of the brain receive projections from these nuclei, we assessed serotonin and dopamine levels in $En2^{-/-}$ mice in cerebellum, hippocampus, striatum, and frontal cortex.

$En2^{-/-}$ mice were found to display neurobehavioral deficits in social as well as learning and memory tasks. Motor alterations were also observed in a subset of tasks. In addition, a cerebellar-specific increase in the level of serotonin and its metabolite was observed in the knockout. These data demonstrate that $En2^{-/-}$ mice display behavioral and neurochemical phenotypes similar to ASD and suggest that this genetically relevant mouse model may be useful in further studying its etiology.

2. Results

2.1. *Motor tests*

It has been reported that $En2^{-/-}$ mice do not display differences in locomotor activity in an open-field (Gerlai et al., 1996). We also monitored activity levels, using experimental conditions that we employed in the social and learning/memory tasks for juvenile (P30–40) and adult mice (P90–160). No difference in activity was observed between genotypes at either age when assessed individually (see Table 1). Therefore, it was appropriate to assess other behaviors under these experimental conditions.

Table 1 – Motor activity counts between genotypes under baseline (individual) conditions in juveniles and adults and for pairs of mice in the juvenile and adult social tests

Test condition	Mean ± SEM
Juvenile individual, 30 min session	$En2^{+/+}$ 3483 ± 473
	$En2^{-/-}$ 3373 ± 460
Adult individual, 15 min session	$En2^{+/+}$ 2671 ± 673
	$En2^{-/-}$ 2751 ± 627
Juvenile social pair, 30 min session	$En2^{+/+}$ 6396 ± 394
	$En2^{-/-}$ 3477 ± 433 *
Adult social pair, 15 min session	$En2^{+/+}$ 8022 ± 488
	$En2^{-/-}$ 8457 ± 457

* Indicates $p<0.05$.

We also examined the development of locomotor activity in young mice daily from P21 through P27. As shown in Fig. 1, $En2^{-/-}$ mice were hyperactive during this stage of development ($F(1,46)=9.606$, $p<0.003$), with a significant genotype by day interaction ($F(6,276)=3.067$, $p<0.006$). Post hoc analysis revealed significant effects on P21 ($p<0.01$), P22 ($p<0.01$), and P26 ($p<0.05$). The hyperactivity we observed was restricted to this developmental stage.

A thorough examination of motor maturation was also conducted in these mice. Five motor tests were conducted at developmentally appropriate time points: surface righting (P5–10), mid-air righting (P13–19), negative geotaxis (P13–19), hanging wire grip-strength (P13–19), and rotorod (adults). No significant differences were detected in the development of surface righting or negative geotaxis (data not shown). However, as shown in Fig. 1, significant differences were observed between genotypes in mid-air righting (P15: $\chi^2=4.691$, $p<0.03$; P16: $\chi^2=8.851$, $p<0.003$; P17: $\chi^2=10.991$, $p<0.001$; P18: $\chi^2=7.782$, $p<0.005$), hanging-wire grip strength ($F(1,65)=42.964$, $p<0.0001$), and rotorod ($F(1,28)=4.537$, $p<0.042$).

2.2. *Social tests*

Key social behaviors were examined in pairs of genotype-matched mice. Juveniles, both male and female, were observed in a neutral cage. Adult males were observed in both a neutral cage and a home cage. As shown in Fig. 2, juvenile $En2^{-/-}$ mice exhibited a striking reduction in social play, which consisted of chasing, pushing under, or crawling over the partner mouse ($F(1,40)=13.141$, $p<0.001$). They also engaged in significantly less social sniffing ($F(1,40)=9.489$, $p<0.004$) and allogrooming ($F(1,40)=13.020$, $p<0.001$) than controls. No sex differences were observed. Motor activity was monitored during this task and revealed that $En2^{-/-}$ mice were hypoactive ($F(1,40)=24.832$, $p<0.0001$; see Table 1). This hypoactivity was likely a reflection of decreased social interaction. Consistent with this, activity levels were positively correlated with the amount of social play ($r=.0441$, $p<0.003$), sniffing ($r=.0597$, $p<0.0001$), and allogrooming ($r=.0391$, $p<0.011$). In addition, each of these social behaviors was positively correlated with the others, including play and

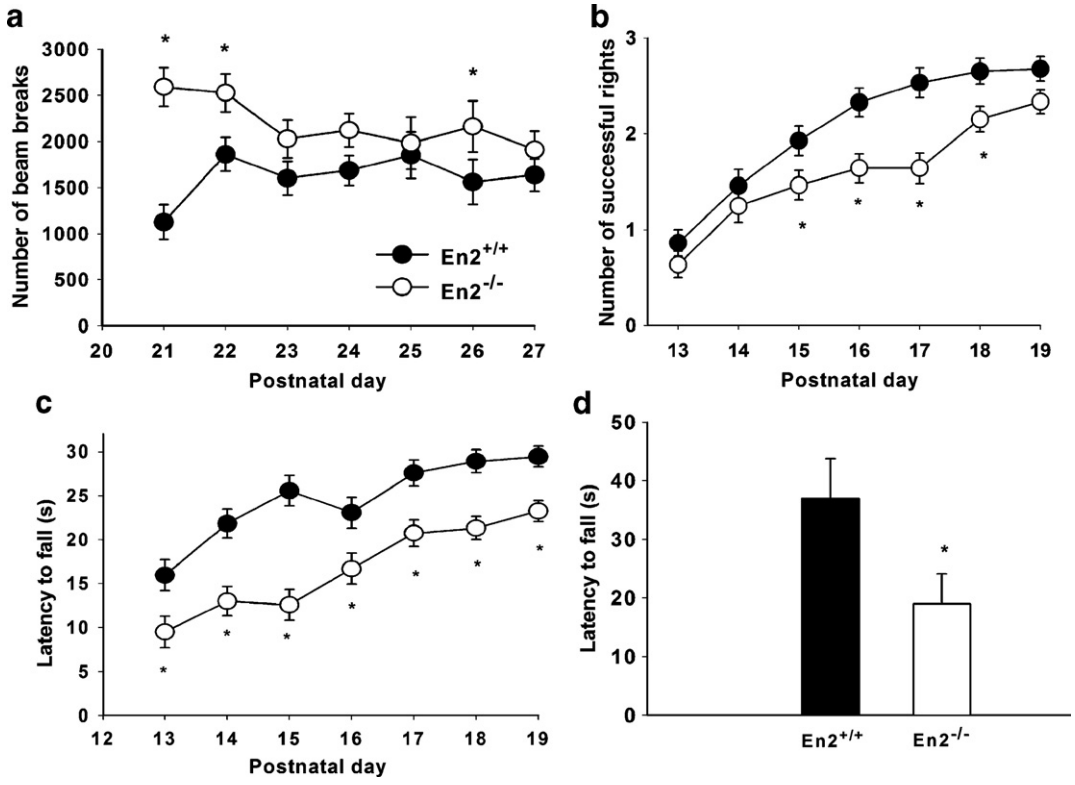

Fig. 1 – Motor maturation. (a) Activity counts from daily 15-min sessions across P21–27, (b) number of times mice were successfully able to right in mid-air out of 3 trial per day across P13–19, (c) latency for mice to fall when suspended from a wire across P13–19, and (d) latency for adult mice to fall from a rotating rod at 9 RPM is shown. * denotes $p<0.05$; error bars indicate SEM.

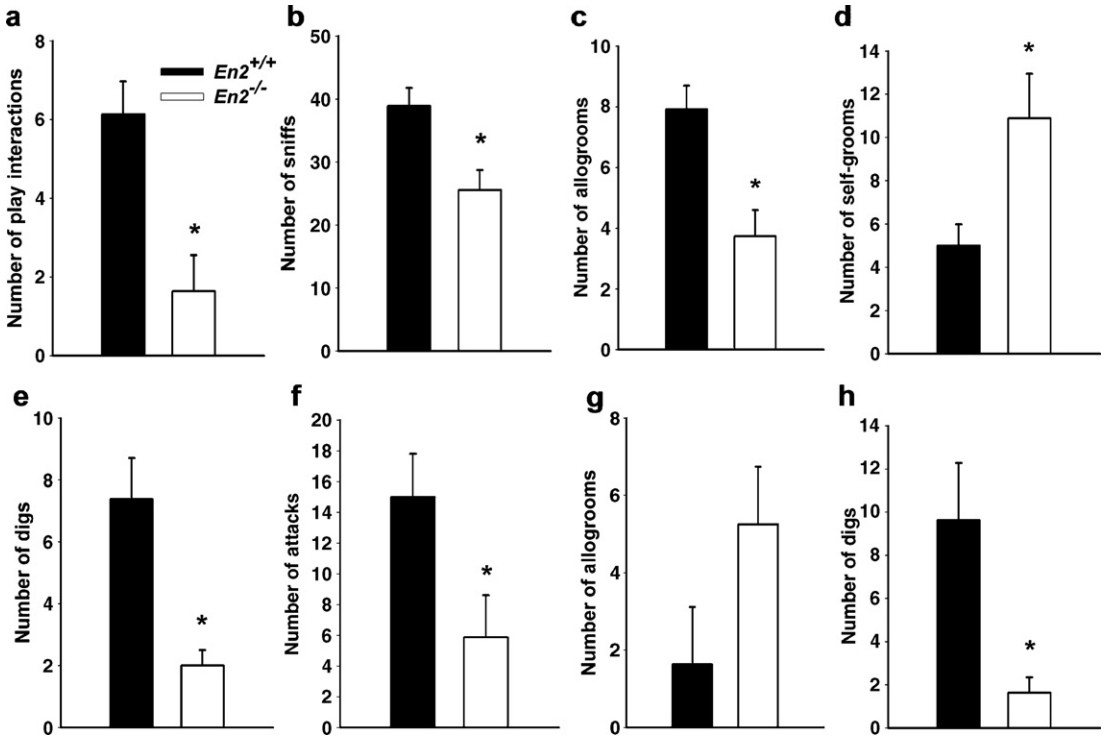

Fig. 2 – Social behavior. (a) Number of play interactions, (b) social sniffs, and (c) allogrooms displayed by pairs of juvenile mice in a neutral cage is illustrated. (d) Number of self-grooms and (e) woodchip digging episodes recorded for pairs of adult mice in a neutral cage. (f) Number of attacks and (g) allogrooms made by resident mice to an intruder during resident–intruder paradigm. (h) Number of times intruder mice engaged in woodchip digging during resident–intruder paradigm. * denotes $p<0.05$; error bars indicate SEM.

sniffing ($r=0.485$, $p<0.001$), play and allogrooming ($r=0.621$, $p<0.0001$), and sniffing and allogrooming ($r=0.491$, $p<0.001$).

Unlike juveniles, social assessment of adult males under neutral cage conditions revealed no difference between genotypes in social sniffing, crawl under/over, allogrooming, or motor activity. However, other behavioral alterations were noted in the knockout, including decreased digging in woodchips ($F(1,14)=13.962$, $p<0.002$), considered a form of exploratory behavior (Olivier, 1984), and increased self-grooming ($F(1,14)=6.467$, $p<0.023$), a self-maintenance behavior (Terranova et al., 1993) (see Fig. 2). Correlation analysis was performed and revealed no significant relationship between digging, self-grooming, or motor activity during this task. Of note, aggressive behavior appeared to be reduced in the knockout, such that none of the knockout males attacked one another in the neutral environment, whereas, 25% of the control mice fought.

Therefore, to elicit aggressive behavior, we assessed genotype-matched adult males in the resident-intruder paradigm. Knockout residents engaged in less aggressive behavior than controls, displaying a significant decrease in the number of attacks to an intruder mouse ($F(1,14)=5.379$, $p<0.036$) (Fig. 2). No significant difference in the latency to attack the intruder or number of tail rattles was observed (data not shown). There was also a trend toward an increase in resident $En2^{-/-}$ mice allogrooming the intruder (see Fig. 2). Therefore, the knockout mice engaged in more allogrooming but did not sustain species-appropriate attack behavior. We also monitored episodes of the intruder digging in the woodchips of the residents' home cage. Similar to what was observed in the neutral cage environment, intruder $En2^{-/-}$ mice exhibited reduced digging compared to controls ($F(1,14)=8.485$, $p<0.011$) (Fig. 2). Finally, none of the behaviors observed in the resident–intruder paradigm were significantly correlated with one another.

2.3. *Learning and memory tests*

In order to test whether $En2^{-/-}$ mice displayed deficiencies in learning and memory, $En2^{+/+}$ and $En2^{-/-}$ adult male mice were examined in two tasks: the water maze and modified open-field with objects. Mice were first trained in the hidden-platform water maze for 7 days followed by a probe trial. A significant effect of training was observed, such that the latency to escape improved over testing for all mice ($F(6,72)=18.151$, $p<0.001$). A significant effect of genotype revealed that $En2^{-/-}$ mice displayed a deficit in performance of the hidden platform task ($F(1,12)=8.395$, $p<0.013$). As shown in Fig. 3, $En2^{-/-}$ mice had longer escape latencies on all test days except for the first day of testing. Probe trial data revealed that control mice spent a significantly greater proportion of time in the target quadrant, as well as significantly less time, on average, in the non-target quadrants compared to $En2^{-/-}$ mice ($F(1,12)=16.622$, $p<0.002$; see Fig. 3). Furthermore, probe trial data were negatively correlated with the sum of the average daily latencies during training of the hidden-platform task ($r=-0.538$, $p<0.047$). These

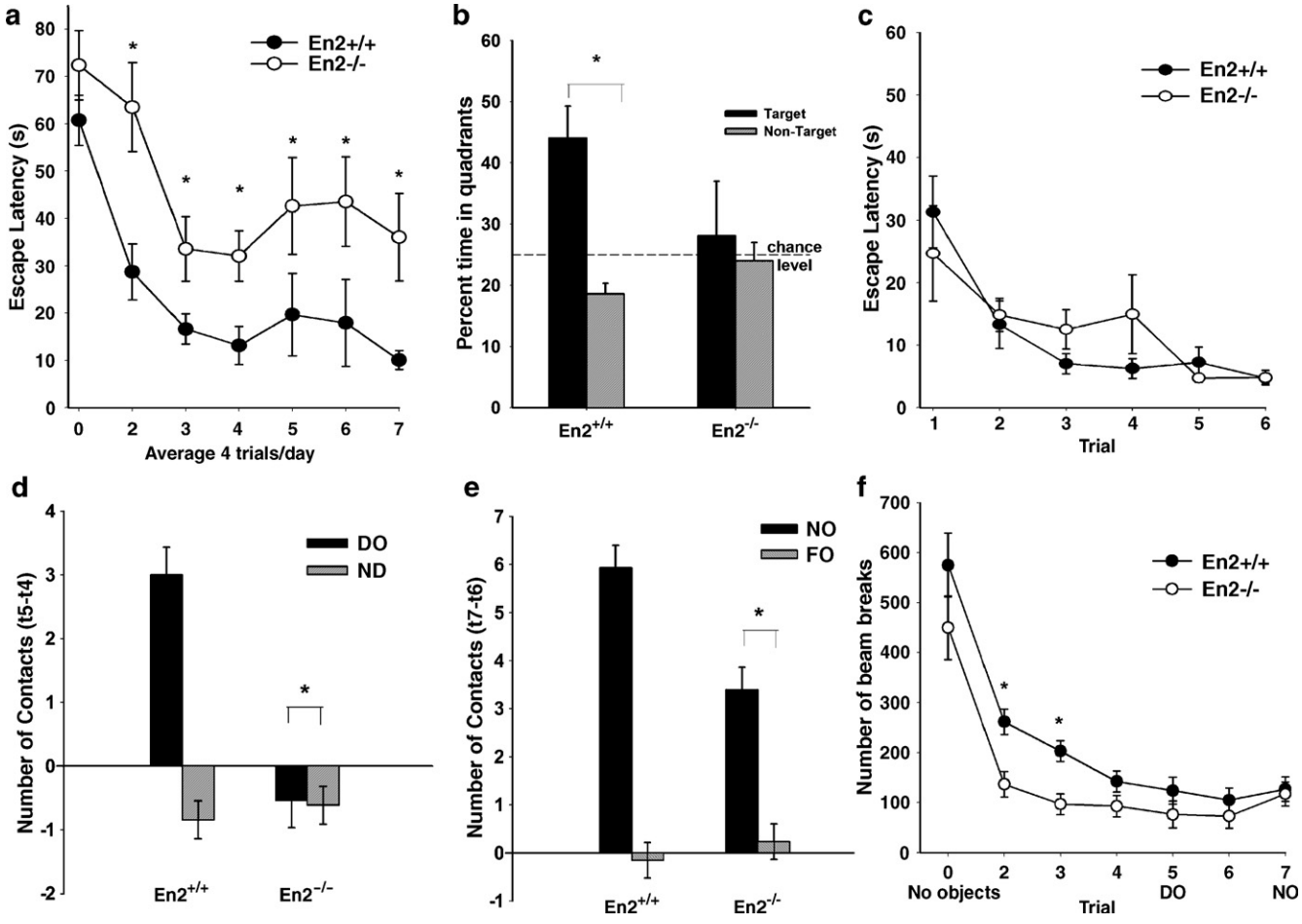

Fig. 3 – Learning and memory. (a) Latency for adult mice to escape to a hidden platform in the Morris water maze across seven days of testing is shown. (b) Percent time spent in the target and non-target quadrants of the Morris water maze during a probe trial is shown. (c) Latency to escape to a visible platform across six trials is depicted. (d) To determine exploration of the displaced object relative to non-displaced objects in the arena in the modified open-field with object task, the value of DO minus ND was analyzed between genotypes. DO = contacts to the displaced object minus contacts to the object in the same position on the previous trial. ND = average contacts to the non-displaced objects on the test trial minus average contacts to the objects in the same position on the previous trial. (e) To determine exploration of the novel object relative to familiar objects in the arena in the same task, the value of NO minus FO was analyzed between genotypes. NO = contacts to the novel object minus contacts to the object in the same position on the previous trial. FO = average contacts to the familiar objects on that trial minus average contacts to the objects in the same position on the previous trial. (f) Activity counts in each 4-min trial are shown; t1: no objects, t2–t4: 3 objects stably positioned, t5: object displaced, t6: no change, t7: novel object introduced. * denotes $p < 0.05$; error bars indicate SEM.

data indicate that the control mice, and not the knockout mice, learned to escape to the hidden-platform using spatial cues.

To assess the ability of these same mice to navigate the water maze, a visible platform version was employed. There was a significant effect of training for the visible platform task, such that all mice learned to escape more rapidly with repeated testing ($F(5,60) = 11.301$, $p < 0.001$). However, no significant effect of genotype was observed on latency to escape to the visible platform (see Fig. 3). There was also no correlation between performance in the hidden and visible platform versions of the task. These data indicate that *En2*$^{-/-}$ mice display a specific spatial learning deficit, which does not appear to be caused by a motor confound.

In the modified open-field with objects, mice were placed in an open-field apparatus and the number of contacts with each of three distinct objects was scored over a period of six 4-min trials, following an introductory trial with no objects present. As shown in Fig. 3, data revealed that *En2*$^{-/-}$ mice exhibited significantly less exploration to the displacement of an object than *En2*$^{+/+}$ mice, indicated by a decrease in the number of contacts with the displaced object minus contacts with non-displaced objects value (DO minus ND) ($F(1,24) = 7.561$, $p < 0.011$). *En2*$^{-/-}$ mice also exhibited significantly less exploration directed toward introduction of a novel object, indicated by a significant decrease in contacts with the novel object minus familiar object value (NO minus FO) ($F(1,24) = 5.895$, $p < 0.023$).

Table 2 – Mean±SEM neurotransmitter levels in μg/g wet tissue weight detected in cerebellum, hippocampus, striatum, and frontal cortex in wild-type and knockout mice

	$En2^{+/+}$	$En2^{-/-}$
A. Cerebellum		
5-HT	0.12±0.007	0.23±0.025*
5-HIAA	0.04±0.003	0.07±0.008*
B. Hippocampus		
5-HT	0.62±0.04	0.58±0.03
5-HIAA	0.20±0.004	0.24±0.04
C. Striatum		
DA	11.81±0.94	11.27±0.56
DOPAC	0.88±0.06	0.82±0.05
HVA	1.39±0.09	1.57±0.14
5-HT	0.66±0.03	0.71±0.04
5-HIAA	0.18±0.01	0.20±0.01
D. Frontal cortex		
DA	1.36±0.31	1.23±0.26
DOPAC	0.18±0.03	0.23±0.03
HVA	0.25±0.03	0.25±0.02
5-HT	0.65±0.04	0.70±0.03
5-HIAA	0.12±0.02	0.15±0.04

* Indicates $p<0.05$.

We noted that both genotypes made a similar number of contacts to the objects on all but one trial; therefore, it was appropriate for the data to be analyzed as the difference between contacts with the target and non-target objects. Only on trial 2, when the objects were first introduced to the open field, did $En2^{-/-}$ mice make significantly fewer total object contacts ($En2^{+/+}$ mice: 20.2±1.2; $En2^{-/-}$ mice: 14.7±1.8 ($F(1,24)=6.354$, $p<0.019$). Analysis of activity levels during this task showed that $En2^{-/-}$ mice were also significantly hypoactive on trial 2 ($F(1,24)=12.431$, $p<0.002$) and trial 3 ($F(1,24)=13.131$, $p<0.001$), as shown in Fig. 3. Activity levels were not different on other trials, including the test trials (t5 and t7).

2.4. *Neurochemistry*

To investigate whether altered levels of monoamines could contribute to some of the social, cognitive, and motor deficits observed in $En2^{-/-}$ mice, levels of serotonin and dopamine and their metabolites were measured using HPLC-electrochemical detection. As shown in Table 2, a significant increase in serotonin ($F(1,14)=16.95$, $p<0.001$) and its metabolite 5-hydroxyindolacetic acid ($F(1,14)=10.36$, $p<0.006$) was observed in the cerebellum of $En2^{-/-}$ mice compared to controls. Analysis of hippocampus, striatum, and frontal cortex revealed no significant alterations between genotypes in serotonin or dopamine levels (see Table 2).

3. Discussion

$En2^{-/-}$ mice were examined for behavioral and neurochemical changes associated with autism based on recent human genetic studies that indicate that *EN2* is involved in ASD susceptibility (Gharani et al., 2004; Benayed et al., 2005). *En2* knockout mice display cerebellar hypoplasia and a reduction in Purkinje cells. Similarly, a decrease in Purkinje cell number has been observed in 21 out of the 29 post-mortem studies of autistic brains (Palmen et al., 2004), and cerebellar hypoplasia has been consistently reported by a number of different investigators (Murakami et al., 1989; Hashimoto et al., 1995; Courchesne, 1997, Courchesne et al., 2001). ASD is a behaviorally defined disorder, therefore, it is important for an animal model to reflect the behavioral syndrome. The symptoms of ASD are inherently difficult to model in lower species because of the absence of verbal communication and higher cognitive behaviors, as well as the variability of associated symptoms reported (Crawley, 2004). We were, however, able to observe a number of social and learning alterations in *En2* knockout mice that are particularly relevant.

Most strikingly, we observed reduced social play, social sniffing, and allogrooming in pairs of juvenile $En2^{-/-}$ mice compared to controls. This reduction in social and play behavior was observed during the period when increased peer interaction and play become prevalent behavior in mice (Terranova et al., 1993). Similarly, in ASD social deficits become increasingly evident as peer interaction and play become prevalent in children at about 3–4 years of age (Wetherby et al., 2004).

$En2^{-/-}$ mice also displayed abnormal behavior in adult social assessments. We observed a trend toward decreased attack behavior in knockout mice in the neutral cage setting. When assessed in the resident–intruder paradigm to elicit aggressive behavior, significantly fewer attacks were made to the intruder. These data indicate significant disruption in territorial aggression in the knockout, an important component of social behavior in adult males. However, no difference in the latency to attack the intruder or in the number of tail rattles was observed. Both measures indicate that there was a similar level of arousal between genotypes upon the introduction of an intruder (Miczek et al., 2001). It is interesting to note that juvenile play behavior is considered a way to learn species-specific adult behavior, such as fighting (Panksepp, 1981). Therefore, the reduction in play behavior displayed by juvenile $En2^{-/-}$ mice may have influenced their behavioral development, leading to decreased adult aggression.

Although social behaviors were reduced in knockout mice as juveniles, we did not observe differences in the adults on measures of sniffing, crawl under/over, or allogrooming in the neutral environment. Housing conditions prior to testing may have influenced the motivation of the mice to engage in social interaction, since the juveniles were individually housed prior to testing but the adults remained group housed. Alternatively, social deficits could have been compensated for with development or overshadowed by behaviors that are more salient in adults, such as aggressive behavior. Taken together, these results indicate that the *En2* knockout mutation in mice leads to complex changes in social behavior that may be critically dependent on the developmental time period and the context of the behavioral test.

Learning and memory deficits are common in ASD, and up to 75% of individuals with autistic disorder may be diagnosed with co-morbid metal retardation (Bölte and Poustka, 2002).

Similarly, we observed learning and memory deficits in the knockouts in two different paradigms. First, deficits in the hidden-platform water maze were observed in the knockout across testing. Sensorimotor problems did not appear to be a contributing factor because the knockout mice performed similarly to controls when the platform was visible (D'Hooge and De Deyn, 2001). The knockout mice did show some improvement in their escape latency across water maze testing; however, unlike the control mice, they did not show a preference for the target quadrant on the probe trial. This suggests that they may have used a non-spatial strategy to improve their performance over testing, such as a learned sequence of movements. Second, in the modified open-field with objects task, knockout mice showed poor spatial memory, evidenced by less investigation an object that was displaced to a new position and less investigation a novel object introduced to the field than wild-type mice (Ricceri et al., 2000). These spatial learning and memory deficits may be related to reduced exploration, since *En2*$^{-/-}$ mice also displayed a reduction in total object contacts on their first exposure to the objects and decreased activity in the early training trials. Reduced exploration in an open-field, independent of activity levels, has been associated with poor learning in mice in a number of learning paradigms (Matzel et al., 2003). Likewise, our knockout mice engaged in reduced exploratory behavior (i.e. digging in woodchips) in both adult social tests. Interestingly, reduced exploration has been observed in autistic children placed in a novel environment and positively correlated with cerebellar hypoplasia (Pierce and Courschesne, 2001).

We also observed complex changes in motor behavior that, like the social assessment, were dependent on age and context of the behavioral test. Knockout mice exhibited poor performance in the mid-air righting and grip strength reflexes in the neonatal period. These tasks are partially dependent upon cerebellar development (Altman and Sudarshan, 1975). Yet, the cerebellar-dependent reflexes that develop earlier, surface righting and negative geotaxis, were unaffected. *En2*$^{-/-}$ mice are not ataxic cerebellar mutants (Joyner et al., 1991). However, consistent with a previous report (Gerlai et al., 1996), we found that adult knockout mice displayed poor performance on the rotorod. Therefore, motor disruptions can be detected in neonates and in adults when more complex coordination is required for the task. Evaluation of locomotor activity was more complex. Hyperactivity was observed in a specific period during postnatal life (P21–27), when locomotor activity should begin to decline to adult-like levels (Erinoff et al., 1979). This may reflect a developmental delay. No difference in activity was observed in either juveniles or adults under baseline conditions. However, juvenile mice showed reduced activity in the social assessment and adult mice exhibited reduced activity in the modified open-field test. These reductions likely reflect motivation or anxiety, and do not appear to be a motor deficit.

We acknowledge that the alterations in motor coordination and locomotion make the assessment of other behaviors more complex. However, they may be an important component of the neurobehavioral alterations observed in these mice. In addition, they may not be independent from the ASD phenotype, as a number of studies have reported gross and fine motor deficits as well as deficits in postural control (Teitelbaum et al., 1998; Noterdaeme et al., 2002; Mineshew et al., 2004).

In addition to our behavioral data, neurochemical analysis of the *En2* knockout mice revealed a specific increase in cerebellar serotonin levels. Therefore, projections from the raphe nuclei may be abnormal in these mice. Consistent with this, a recent report revealed that the *Engrailed* genes are necessary for the development of serotonergic neurons (Simon et al., 2005). Altered serotonin levels are relevant to ASD for a number of reasons. Elevated platelet serotonin has been consistently reported in 20–25% of individuals with autism and their first-degree relatives (Schain and Freedman, 1961; Cook et al., 1993). Selective serotonin reuptake inhibitors (SSRIs) have been effective in treating some of the maladaptive behaviors associated with ASD (Posey and McDougle, 2000). Abnormal serotonin synthesis capacity has been observed in the ASD brain (Chugani et al., 1999). Finally, several human genetic studies support the possibility that the serotonin transporter gene (encoded by *SLC6A4*) contributes to increased ASD risk (Cook et al., 1997; Yirmiya et al., 2001; Sutcliffe et al., 2005). Thus, the serotonin system may be affected in ASD, making our finding of increased cerebellar serotonin in the *En2* knockout important.

To summarize, deficits in social behavior were observed in *En2* knockout mice, with variable expression at different ages. Learning and memory were also affected, which may have been related to reduced exploratory behavior. Motor alterations, which are inextricably linked to the behaviors in our tests, were observed in the knockout. Motor problems have been associated with autism; however, this is not a defining feature. Finally, serotonin levels were increased in the cerebellum of the knockout mice; and alterations in serotonin are a commonly reported associated feature in ASD. Collectively, our data are consistent with the *En2* knockout mouse displaying behavioral and neurochemical changes that are relevant to ASD.

An important caveat is that the wild-type and knockout mice we tested were maintained on a hybrid genetic background and were not littermates. Therefore, the observed effects could have been influenced by indirect effects such as maternal behavior or through genetic modifiers from the BL6 or 129 backgrounds affecting the penetrance or expressivity of the assessed phenotypes. We conducted an analysis on a random sample of mice for markers spanning the genome that are polymorphic between BL6 and 129. We established that each *En2*$^{+/+}$ and *En2*$^{-/-}$ mouse sampled had a different contribution of BL6 and 129 alleles, demonstrating genetic heterogeneity for both the wild-type and knockout lines. While background effects may play a role, they did not appear to negatively impact our results. Considerable variation was not observed within genotype for any of our behavioral and neurochemical assessments.

Finally, because disease causing variants for common human diseases like ASD are likely to be functional polymorphisms that subtly alter the activity or expression of the associated gene, the *En2* knockout is expected to be a more severe allele than the *EN2* disease variant. Resequencing and RTPCR analysis on lymphoblastoid cell lines support this possibility (Benayed and Millonig, unpublished results).

However, with limited knowledge of the etiology of ASD, understanding the way in which absence of this gene affects the behavior of these mice is an important step in deciphering the contribution of this ASD susceptibility gene to behavior. Ongoing experiments are focused on identifying the disease allele(s) and generating a mouse model of the functional variant(s). It will be interesting in the future to compare the anatomical, behavioral, and neurochemical phenotypes between the two mutants.

4. Experimental procedures

4.1. Animals

The *En2* knockout mouse ($En2^{hd}$ allele) was obtained through The Jackson Laboratories (Bar Harbor, ME) by re-derivation of cyropreserved embryo stocks. $En2^{+/hd}$ mice on a mixed C57BL6J/129S2SV PAS inbred background were obtained and intercrossed to establish an $En2^{+/hd}$ mutant colony. Because the $En2^{hd/hd}$ mice are viable and fertile, two separate $En2^{+/+}$ and $En2^{hd/hd}$ colonies were established for our behavioral analysis. This was accomplished by genotyping progeny of $En2^{+/hd}$ intercross matings and identifying $En2^{+/+}$ and $En2^{hd/hd}$ by genotype, from which both colonies were generated. These colonies have been maintained by $En2^{+/+} \times En2^{+/+}$ and $En2^{hd/hd} \times En2^{hd/hd}$ matings. Mice were maintained under conditions meeting all AAALAC guidelines, with free access to food and water, and a 12:12 h light:dark cycle. The day of birth was recorded as postnatal day 0 (P0) and mice were weaned at approximately P25.

4.1.1. Genetic background

25 SSLP markers, representing each chromosome and spanning the genome, were typed for a random sample of at least 10 $En2^{+/+}$ and $En2^{-/-}$ mice after completion of behavioral assessments. Standard PCR and genotyping protocols were used. Control DNA (BL6J and 129 from Jackson stock), as well as their mixture, was run on an acrylamide gel with DNA from our sample of knockout and control mice for each SSLP marker. We visualized that individual mice across genotype had either the BL6 or 129 allele or their combination at each SSLP marker. This demonstrated that the genetic background was heterogeneous for all mice.

4.2. Motor tests

4.2.1. Motor activity

The activity chamber consisted of a standard large cage crossed by infrared beams (Opto-Varimex, Columbus, OH). All activity testing was conducted at the start of the dark cycle with the test room illuminated by an overhead red light. Motor activity was monitored daily in naive male and female mice from P21 through P27 ($En2^{+/+}$ $n=26$ and $En2^{-/-}$ $n=22$). Each day mice were placed individually in the same activity chamber for a 15-min session and total activity counts were recorded. Another group of naive male and female mice between P30 and 40 were individually placed in the motor activity chamber for one 30-min session and total counts were recorded ($n=14$ per genotype). A final group of naive adult male mice (90–120 days) were placed in the activity chamber for one 15-min session, and total activity counts were recorded ($n=8$ per genotype).

4.2.2. Motor development

For tests of early development, litters were culled to 6 pups or less prior to the start of testing. Pups were weighed daily from P5–19 and underwent behavioral testing appropriate to skill ontogeny. Data from surface righting, negative geotaxis, mid-air righting, and hanging wire grip-strength were recorded from the same male and female pups ($En2^{+/+}$ $n=34$ and $En2^{-/-}$ $n=33$) derived from six different litters per genotype. *Surface Righting:* Surface righting was evaluated on P5–10. Pups were placed dorsal side down with all four paws splayed outward and upward and the latency for the mouse to right to ventral side down with all four paws contacting the surface was measured with a maximum latency of 30 sec. *Mid-air Righting:* The ability to right in mid-air was evaluated on P13–19. Pups were held by their scruff, dorsal side down and feet upward 45 cm above a padded surface and dropped for 3 trials each test day. Ability to right in mid-air was determined if the pup landed ventral side down with all four paws on the surface. Data were recorded as the number of successful trials out of 3. *Grip Strength:* Grip strength was evaluated on P13–19. The front paws were placed on a wire mounted 45 cm above a padded surface. The latency for the pup to fall from the wire was measured with a 30-sec maximum. *Negative Geotaxis:* Negative geotaxis was tested on P13–19. Pups were placed with their head pointing downward on a metal grid positioned at a 45° incline. Latency to turn 180° from the incline was recorded with a 30-s maximum. *Rotorod:* Rotorod testing was conducted in adult males (90–120 days) ($En2^{+/+}$ $n=15$ per genotype). Mice were placed on a rod (13.5 cm circumference) for one 60-s trial to acquaint them with the task (if they fell the timer was stopped and the mouse was quickly returned to the rod). Mice were then placed on the rod while in motion at 9 RPM and tested for latency to fall (50 cm to a soft container) with a maximum latency of 60 s.

4.3. Social tests

4.3.1. Social and play behavior juveniles

Mice used in this study were naive male and female pups between P30 and 40. $En2^{+/+}$ $n=23$ sex-matched pairs derived from 10 litters and $En2^{-/-}$ $n=19$ sex-matched pairs derived from 9 litters. Mice were individually housed 4–5 days prior to the test session, conditions that have been shown to increase both play behavior (Panksepp, 1981; Terranova et al., 1993) and social interaction (File and Seth, 2003) in rodents. Time of housing was counterbalanced across groups and no pair was more than 5 days different in age. Testing was conducted at the start of the dark cycle and the test room was illuminated by an overhead red light. Two non-sibling mice of the same genotype and sex were paired together in a neutral large cage surrounded by a motor activity chamber. The mice were observed for a 30-min session and scored by two trained observers blind to genotype (with reliability at greater than 95%) for the number of times that a member of the pair engaged in social play (the sum of chase, push under, or crawl over the partner mouse), social sniffing, and allogrooming

(grooming the partner mouse). Motor activity of the pair was also recorded.

4.3.2. *Social behavior adults*

Mice used in this study were naive adult males (90–120 days) that were housed in sex-matched groups of 2–3 per cage from time of weaning ($n=8$ pairs per genotype). Testing was conducted at the start of the dark cycle and the test room was illuminated by an overhead red light. Two unfamiliar mice of the same genotype were paired in a neutral large cage surrounded by an activity chamber. The mice were observed for a 15-min session and scored by two trained observers blind to genotype (with reliability at greater than 95%) for the number of times that a member of the pair engaged in social sniffing, crawl over/under, allogrooming, self-grooming, or digging in wood chips. Episodes of aggressive behavior were also monitored and motor activity of the pair was recorded. This shorter 15-min session was used in adults to prevent any prolonged episodes of aggressive behavior, in accordance with animal welfare guidelines.

4.3.3. *Resident–intruder adults*

Residents were naive adult male mice (120–160 days) that were individually housed 2 weeks prior to testing. Intruders were group-housed males previously used in the adult social interaction test, of the same genotype as the residents ($n=8$ pairs per genotype). Testing was conducted at the start of the dark cycle and the test room was illuminated by an overhead red light. An intruder was introduced to the home cage of the resident and latency for the resident to attack the intruder was recorded, beginning a 10-min session. Once the session began, the number of attacks, tail rattles, allogrooms, and episodes of the intruder digging in woodchips were recorded by two trained observers blind to genotype (with reliability at greater than 95%).

4.4. *Learning and memory tests*

4.4.1. *Water maze*

The water maze consisted of a circular galvanized steel tub 109 cm in diameter and 55 cm height. It was filled 3/4 of the way with water kept at approximately 23 °C and made opaque with non-toxic white latex paint. An 8×8 cm clear Plexiglas escape platform remained in a fixed position in the center of one quadrant of the maze 2 cm below the water surface. The spatial environment of the test room was held constant with geometric shapes on the walls to facilitate spatial mapping. Adult male mice ($n=7$ per genotype) were given a one-day pre-training session to habituate them to the platform and holding cages. Mice were given two trials in which they were placed on the platform with the water level below the platform surface, and remained there for 45 s; they were then placed in a holding cage for an 8 min inter-trial interval (ITI). The following day, hidden-platform testing began using a standard procedure (according to D'Hooge and De Deyn, 2001). Mice were given 4 trials per day for 7 days with a maximum latency of 90 s/trial to find the hidden platform. Each trial was started from a semi-randomly assigned quadrant of the maze. Mice that successfully found the platform remained there for 10 s, and were then placed in a holding cage for the 8 min ITI. If the trial was unsuccessful, the mouse was guided to the platform and held there for 15 s, then placed in the holding cage for the 8 min ITI. The data were analyzed by taking the average of 4 trials on the given test day. One hour following the last test trial, mice were given a 60-s probe trial, and time spent in the target quadrant was recorded. Probe trial data were converted to proportion of time spent in the target quadrant and average proportion of time spent in the non-target quadrants to determine whether or not there was a preference for the target quadrant. One week following the probe trial, a cued version of the water maze was conducted. Geometric shapes were removed from the walls and the platform was rendered visible by using a black 8×8 cm Plexiglas platform. The location of the visible platform varied across trials. Mice were given 6 trials, with an 8 min ITI during which they were placed in the holding cages. Data were analyzed as the average escape latency for each trial.

4.4.2. *Modified open-field with objects*

Adult male mice (90–120 days) were used in this test ($n=13$ per genotype). Testing was conducted with methods and statistical analysis performed similar to Ricceri et al. (2000) although only three objects were used. Mice were placed in a standard large cage surrounded by an activity monitor for the first trial (t1), followed by 3 trials (t2–t4) with 3 objects stably positioned in the cage to allow the mice time to habituate to the objects and their position. On the next trial (t5), one of the objects was moved to a different location in the cage and remained in that position for one additional trial (t6). Finally, on the last trial (t7), a novel object replaced a familiar object. The number of contacts to each object was recorded across each 4-min trial, with a 2-min ITI between trials, during which the mouse was returned to its home cage. Mice were observed on each trial for the number of exploratory contacts with each object (operationally defined as snout touching the object) and for general motor activity. Motor activity counts were expressed as activity counts per 4-min trial, not as the sum of all sessions, because the mice were returned to their home cage for the ITI. Data were analyzed using the number of contacts with the displaced object (DO) in its new location, expressed as a function of contacts with the non-displaced objects (ND), and for the number of contacts with the novel object (NO) expressed as a function of contacts with familiar objects (FO) (Ricceri et al., 2000). Specifically, for the displaced object, the value of DO minus ND was analyzed such that: DO = contacts to the displaced object minus contacts to the object in the same position on the previous trial and ND = average contacts to the non-displaced objects on the test trial minus average contacts to the objects in the same position on the previous trial. For the novel object, the value NO minus FO was analyzed such that: NO = contacts to the novel object minus contacts to the object in the same position on the previous trial and FO = average contacts to the familiar objects on that trial minus average contacts to the objects in the same position on the previous trial.

4.5. *Neurochemistry*

Male mice approximately 90 days old ($n=8$ mice per genotype) were sacrificed and cerebellum, hippocampus, striatum, and

frontal cortex were dissected, snap frozen in liquid nitrogen, and stored until homogenization. All tissue was homogenized in 0.3 ml of 0.4 N perchloric acid with 0.1 mM EDTA. Homogenized samples were centrifuged at 20,000×*g* for 20 min at 4 °C and the supernatant frozen in liquid nitrogen until analyzed. Supernatant was assayed for dopamine serotonin and their metabolites using HPLC-electrochemical detection (Bioanalytical System, West Lafayette, Indiana). The mobile phase consisted of 0.1375 M sodium phosphate (dibasic), 0.0625 M citric acid, 5.0 mg EDTA, and 14% methanol with a flow rate of 0.7 ml/min.

4.6. *Statistical analysis*

All data were analyzed using SPSS statistical package. No significant effect of sex was observed for the tests that used juvenile male and female pups; therefore, data for those tests are presented as the average of both sexes. Mid-air righting was analyzed using Kruskal–Wallis non-parametric test because data were scored as presence or absence of the skill. All other tests used ANOVA (repeated-measures ANOVA when the data was measured across days or trials) with Tukey's HSD as a post hoc test where appropriate.

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