Over-expression of a human chromosome 22q11.2 segment including \textit{TXNRD2}, \textit{COMT}, and \textit{ARVCF} developmentally affects incentive learning and working memory in mice

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Abstract

Duplication of human chromosome 22q11.2 is associated with elevated rates of mental retardation, autism, and many other behavioral phenotypes. However, because duplications cover 1.5 to 6 Mb, the precise manner in which segments of 22q11.2 causally affect behavior is not known in humans. We have now determined the developmental impact of over-expression of an approximately 190-kb segment of human 22q11.2, which includes the genes TXNRD2, COMT, and ARVCF, on behaviors in bacterial artificial chromosome (BAC) transgenic (TG) mice. BAC TG mice and wild-type (WT) mice were tested for their cognitive capacities, affect- and stress-related behaviors, and motor activity at 1 and 2 months of age. An enzymatic assay determined the impact of BAC over-expression on the activity level of COMT. BAC TG mice approached a rewarded goal faster (i.e., incentive learning), but were impaired in delayed rewarded alternation during development. In contrast, BAC TG and WT mice were indistinguishable in rewarded alternation without delays, spontaneous alternation, prepulse inhibition, social interaction, anxiety-, stress-, and fear-related behaviors, and motor activity. Compared with WT mice, BAC TG mice had an approximately 2-fold higher level of COMT activity in the prefrontal cortex, striatum, and hippocampus. These data suggest that over-expression of this 22q11.2 segment enhances incentive learning and impairs the prolonged maintenance of working memory, but has no apparent effect on working memory per se, affect- and stress-related behaviors, or motor capacity. High copy numbers of this 22q11.2 segment might contribute to a highly selective set of phenotypes in learning and cognition during development.
INTRODUCTION

The human genome contains many types of variation, ranging from single nucleotide polymorphisms (SNPs) in individual genes to duplication or deletion of full chromosomes. A surprisingly large number of intermediate structural variations in the human genome, including kilo- to mega-base copy number variations (CNVs), also exist and are associated with autism spectrum disorders and schizophrenia (1-5). However, the precise manner in which these CNVs cause or remain silent in phenotypic variation is poorly understood.

Human chromosome 22q11.2 is considered a hotspot of CNVs (6). Children and adolescents with 22q11.2 duplications consistently exhibit cognitive and intellectual impairments during development, and they are often diagnosed with mental retardation and autism (7-20). Moreover, CNV screenings in individuals with autism (2,3,21-23) and mental retardation (24,25) have identified duplications in 22q11.2. Unlike human association studies of SNPs, the association between 22q11.2 duplications and developmental cognitive impairments is remarkably consistent and replicable (18,20). Published studies, excluding meeting abstracts, collectively show that 97% of individuals with 22q11.2 duplications exhibit cognitive deficits (18). However, because the diagnosis of mental retardation and autism include variations in diverse cognitive and intellectual capacities, the precise nature of cognitive impairments caused by 22q11.2 duplication remains unclear.

Duplications of 22q11.2 encompass 1.5 Mb or larger regions, making it impossible to determine whether segments or single genes are responsible for phenotypes in humans. Although association studies with SNPs have suggested that single gene alleles in 22q11.2 might contribute to behavioral phenotypes, conflicting evidence has been reported. COMT is one of
the most extensively studied 22q11.2 genes related to cognitive function. Many studies have implicated a high-activity allele of \textit{COMT} in cognitive defects in humans (26). Moreover, haplotype analyses have indicated that a set of SNPs of a 22q11.2 region that includes thioredoxin reductase 2 (\textit{TXNRD2}), catechol-O-methyltransferase (\textit{COMT}), and armadillo repeat gene deletes in velocardiofacial syndrome (\textit{ARVCF}) are over-transmitted in individuals with schizophrenia, a disorder characterized by specific cognitive impairments (27,28). However, other reports have failed to confirm these associations (29-32).

To determine the impact of copy number variation of a specific 22q11.2 region on precise behavioral phenotypes, we tested mice that over-express an approximately 190-kb human chromosomal segment, including \textit{TXNRD2}, \textit{COMT}, and \textit{ARVCF}, in a battery of assays that evaluate cognitive functions, affect- and stress-related behaviors, and motor function. Because individuals with 22q11.2 duplications begin to exhibit many behavioral phenotypes early during development, our behavioral analysis primarily focused on the developmental period from 1 to 2 months. When no phenotype was seen during this time, we then tested the mice at 5 months of age. These ages correspond to developmental milestones in mice. Although strain differences exist, mice exhibit signs of sexual maturation at 1 month of age, but the development of many biological processes continues until they reach mature adulthood at 3 months. The period from 3 to 6 months of age is considered mature adulthood for C57BL/6J mice (33,34). Our data show that when this chromosomal segment is over-expressed, phenotypes related to a specific type of learning and memory are observed during development.
RESULTS

Mice were tested in a battery of tasks that are designed to evaluate cognition, learning, memory, affect- and stress-related behaviors, and motor activity, as described earlier (35). Cognition, learning, and memory were evaluated using rewarded approach, rewarded alternation, spontaneous alternation, and prepulse inhibition (PPI). The affect-related traits were examined using social interaction, anxiety-related behaviors in the elevated plus maze and in an inescapable open field, a fear response in an acoustic startle task, and an affect- and stress-related behavior in a tail suspension task. Motor function was examined using horizontal spontaneous locomotor activity in an inescapable open field.

Rewarded approach

Mice gradually learned to reach a rewarded arm faster on an L-maze over time (Day, F_{5,3280} = 421.52, P < 0.01) and performed faster at the age of 1 month than at 2 months (Age, F_{1,656} = 89.14, P < 0.01) (Fig 1). At both 1 and 2 months of age, BAC TG mice exhibited a shorter latency to reach the goal than WT mice on early learning days than later learning days (Genotype, F_{1,656} = 21.99, P < 0.01; Genotype x Day, F_{5,3280} = 2.37, P < 0.05; Genotype x Age, F_{1,656} = 1.42, n.s.; Genotype x Age x Day, F_{5,3280} = 0.31, n.s.). Newman-Keuls post-hoc comparisons showed that BAC TG mice reached the goal faster on the first 4 days compared to WT mice.

Rewarded alternation

Given a choice between two arms following a visit to one of them during a preceding run, mice tend to visit a previously unvisited arm (i.e., rewarded alternation). Mice achieved better rewarded alternation at the age of 2 months than at 1 month (Age, F_{1,54} = 21.98, P < 0.01), and
performed with higher percentages of alternation on earlier rather than later days (Day, \( F_{4,216} = 4.05, P < 0.01 \)) (Fig. 2). WT and BAC TG mice performed rewarded alternation equally well at both 1 and 2 months of age (Genotype, \( F_{1,54} = 1.06, \) n.s.; Genotype x Age, \( F_{1,54} = 0.97, \) n.s.; Genotype x Age x Day, \( F_{4,216} = 1.53, \) n.s.).

**Delayed rewarded alternation**

When delay intervals were imposed between the sampling run and the choice run in rewarded alternation, mice performed more poorly following longer delays (Delay, \( F_{2,304} = 87.87, P < 0.01 \)) (Fig 3). Two-month old mice performed better than 1-month-old mice (Age, \( F_{1,152} = 6.55, P < 0.05 \)). BAC TG mice performed more poorly than WT mice (Genotype \( F_{1,152} = 13.69, P < 0.01 \)).

Exploratory ANOVAs showed that BAC TG mice were impaired at 2 months (Genotype, \( F_{1,76} = 10.89, P < 0.01 \); Genotype x Delay, \( F_{2,152} = 0.69, \) n.s.), but not at 1 month of age (Genotype, \( F_{1,76} = 3.87, \) n.s.; Genotype x Delay, \( F_{2,152} = 1.12, \) n.s.). This impairment was due to a lack of developmental maturation of rewarded alternation; WT mice showed better performance at 2 months than at 1 month of age (Age, \( F_{1,82} = 6.39, P < 0.05 \)), but such developmental maturation was absent in BAC TG mice (Age, \( F_{1,70} = 1.33, \) n.s.).

**Spontaneous alternation**

Spontaneous alternation is the innate tendency to visit a previously unvisited, rather than visited arm, even without reward, in a T-maze. Genotype had no effect on choice accuracy (Genotype, \( F_{1,145} = 0.08, \) n.s.), and spontaneous alternation did not change during the developmental stages tested (Age, \( F_{2,145} = 0.29, \) n.s.) (Fig. 4).
PPI

Mice showed higher levels of PPI at higher prepulse intensities ($F_{3,474} = 46.40, P < 0.01$), but genotype had no effect on PPI ($F_{1,158} = 0.32, \text{n.s.}$) (Fig. 5). Five-month-old mice exhibited considerably higher levels of PPI, compared to the other two age groups ($\text{Age}, F_{2,158} = 7.39, P < 0.01; \text{Age} \times \text{Prepulse}, F_{6,474} = 3.95, P < 0.01$). No other effect was significant.

Social Behavior

When a pair of non-littermate mice are placed into a homecage-like environment where neither is a resident, they engage in non-aggressive, affiliative social behaviors (35). Active affiliative social behaviors differed depending on age and session ($\text{Age} \times \text{Session}, F_{2,46} = 3.55, P < 0.01$) (Fig. 6). Although the genotype alone did not affect active social interaction ($F_{1,46} = 0.01, \text{n.s.}$), it had a significant effect on this behavior together with the session ($\text{Genotype} \times \text{Session}, F_{1,46} = 6.64, P < 0.05$). However, Newman-Keuls post-hoc comparisons failed to detect a significant difference between WT and BAC TG mice at any session in any age group. No other single or interaction effect was significant.

Mice exhibited very low levels of passive affiliative social interaction behaviors under our experimental conditions (data not shown). Neither genotype nor age had any effect on these behaviors ($\text{Genotype}, F_{1,46} = 0.72, \text{n.s.}; \text{Age}, F_{2,46} = 0.50, \text{n.s.}$). No other effect was significant.

Elevated Plus Maze

Mice naturally avoid open arms in the elevated plus maze, which is thought to reflect anxiety, since it is attenuated by anxiolytic drugs (35). Mice avoided open arms in the elevated
plus maze, as evidenced by less than 50% time spent in the open arms (Fig. 7A). Genotype had no effect on this anxiety-related behavior (Genotype, F_{1,113} = 2.17, n.s.), although this behavioral tendency was more pronounced at 2 and 5 months of age, compared to 1 month of age (Age, F_{2,113} = 29.62, P < 0.01).

Similarly, when analyzed for the frequency of visits to the open arms (Fig. 7B), genotype had no effect on this behavior (Genotype, F_{1,111} = 0.59, n.s.), but age was a significant factor (F_{2,111} = 23.90, P < 0.01).

**Thigmotaxis**

Thigmotaxis, a tendency to remain in the vicinity of a wall in an inescapable open field, is thought to reflect anxiety, stress, and fear (35,36). Mice tended to stay in the vicinity of the wall more during the first 5 min than at later time points (Time interval, F_{5,705} = 22.66, P < 0.01) (Fig. 7C-E). Thigmotaxis was not affected by genotype (F_{1,141} = 1.20, n.s.) and was more sustained at 1 and 2 months of age, compared to 5 months of age (Age, F_{2,141} = 6.55, P < 0.01; Age x Time interval, F_{10,705} = 2.12, P < 0.05).

**Tail Suspension**

Immobility during suspension by the tail is thought to reflect a basal affective state and a stress response (35,36). Genotype had no effect on immobility during this task (F_{1,83} = 0.85, n.s.), but mice tended to exhibit immobility longer at 2 and 5 months of age than at 1 month of age (F_{2,83} = 4.23, P < 0.05) (Fig. 8).
Startle

The sudden presentation of a loud acoustic stimulus causes a startle response. BAC TG and WT mice exhibited equally higher levels of startle in response to higher pulses (Genotype, $F_{1,158} = 0.13$, n.s.; Pulse, $F_{9,1422} = 250.41, P < 0.01$) (Fig. 9). One-month-old mice showed lower levels of startle at high levels of pulses than 2- and 5-month-old mice (Age, $F_{2,158} = 4.72, P < 0.05$; Age x Pulse, $F_{18,1422} = 3.91, P < 0.01$).

Spontaneous Motor Activity

BAC TG mice exhibited lower levels of motor activity compared to WT mice (Genotype, $F_{1,141} = 5.72, P < 0.05$) (Fig. 10). Although 1- and 2-month-old mice gradually exhibited less locomotor activity within a session, such habituation was absent in 5-month-old mice, primarily due to lack of hyperactivity at the beginning of a session (Time interval, $F_{5,705} = 35.30, P < 0.01$; Age x Time interval, $F_{10,705} = 9.61, P < 0.01$). Despite the significant genotype effect, further exploratory ANOVAs failed to reveal a significant genotype effect at 1 month (Genotype, $F_{1,48} = 2.65$, n.s.), 2 months (Genotype, $F_{1,54} = 3.68$, n.s.), or 5 months of age (Genotype, $F_{1,39} = 0.83$, n.s.).

COMT activity

One or two copies of the BAC expressed in the brain was reflected in higher levels of COMT activity in various brain regions examined (Table 1). COMT activities were higher in BAC TG mice compared to WT mice (Genotype, $F_{1,16} = 245.57, P < 0.01$). Basal levels of COMT were higher in the prefrontal cortex compared to the striatum and hippocampus (Region, $F_{2,32} = 187.54, P < 0.01$). For each brain region examined, BAC TG mice exhibited ~2-fold higher COMT activity than did WT mice.
DISCUSSION

Our results demonstrate that over-expression of the ~190-kb 22q11.2 human chromosomal region developmentally affects a select set of behaviors. BAC TG mice exhibited faster performance in rewarded approach, but were impaired in rewarded alternation with delay. These phenotypes are highly selective, since BAC TG mice did not exhibit any other phenotypes in rewarded alternation without delay, spontaneous alternation, PPI, social interaction, anxiety-, affect-, or stress-related behaviors, or motor activity. Over-expression of the BAC resulted in a functional consequence in the brain, as evidenced by ~2-fold elevated levels of COMT enzymatic activity in the prefrontal cortex, striatum, and hippocampus of BAC TG mice. We previously reported that over-expression of an adjacent ~200-kb region of human chromosome 22q11.2, including Septin 5 (SEPT5), glycoprotein Ib (platelet), beta polypeptide (GP1Bb), T-box 1 (TBX1), and guanine nucleotide binding protein, beta polypeptide 1-like (GNB1L), caused antipsychotic-responsive behavioral sensitization in mice (37) and that Sept5 deficiency caused deficits in social interaction and rewarded approach in mice (35). Our data extend these findings by demonstrating that the ~190kb region with a different set of genes contributes to a unique set of behavioral phenotypes.

Compared to WT mice, BAC TG mice showed a shorter latency to reach the rewarded goal during an early training phase in the rewarded approach. In this task, mice need to reach the baited goal arm in an L maze. One critical element of this task is that the goal arm is pseudorandomly determined by the experimenter for each trial. Because the spatial memory for a previously visited arm provides no information as to which arm is baited next, and since the same arm is not baited more than twice in a row, this task minimizes spatial working and reference memory and maximally evaluates an approach behavior guided by local cues,
represented by the only open arm regardless of its spatial location (i.e., incentive learning) (38). The faster approach in BAC TG mice does not seem to be due to improved motor capacity, because BAC TG mice were indistinguishable in motor activity from WT mice and even tended to exhibit less motor activity than WT mice in an open field (Fig. 10).

Because enhanced incentive learning was limited to an early training phase, some processes unique to the early phase of this learning might have been enhanced by overexpression of this 22q11.2 segment. Since COMT, a catalytic enzyme of dopamine, is encoded in the ~190 kb BAC, increased COMT activity may have altered the early phase of dopamine transmission during rewarded behavior. In fact, how dopamine is released in the rodent brain depends on the amount of training. In rodents, when the delivery of reward is not fully expected, dopamine release occurs. When training progresses and the reward is predictable, such a response subsides (39,40). One potential explanation for the enhanced incentive learning is the postulated differential role of COMT on tonic and phasic dopamine release. It has been speculated that tonic and phasic dopamine release are terminated by COMT and the dopamine transporter, respectively, in the nucleus accumbens. According to this hypothesis, increased COMT activity is likely to amplify signaling initiated by reward-triggered phasic dopamine by removing the inhibitory action of tonically released dopamine on dopaminergic neurons (41).

Our findings provide insight into how the over-expressed chromosomal segment contributes to memory and cognitive functions. The absence of developmental improvement in delayed rewarded alternation is in contrast to normal rewarded alternation without delay and normal spontaneous alternation in BAC TG mice. Rewarded alternation and spontaneous alteration in the T-maze require working memory in which the animal temporarily retains
information about a previously visited arm and subsequently chooses to visit the arm that does not match this arm. Our data suggest that BAC TG mice were selectively impaired in their cognitive capacity to retain working memory during delay.

The execution of rewarded alternation depends on multiple brain structures, including the prefrontal cortex, hippocampus, and diencephalon, but the precise way each of the different structures contributes to elements of this task may not be identical (42). Over-expression of this 22q11.2 segment in the hippocampal system could influence the phenotype in delayed rewarded alternation. Damage to the hippocampal formation selectively impairs the delayed component of rewarded alternation and other working memory tasks in rodents, which is not seen with damage to the prefrontal cortex (43-48). Consistent with studies reporting that COMT mRNA is abundantly expressed in the human and rodent hippocampus (49-51), our data showed that COMT enzymatic activity also was present in the hippocampus. Although an ample body of evidence supports the role of COMT in the prefrontal cortex in cognition (26), our findings suggest the need for more studies to explore a possible link between COMT levels and hippocampal functions in prolonged retention of working memory.

There was no phenotype in short-term retention of working memory (Fig. 2), spontaneous alternation (Fig. 4), PPI (Fig. 5), social interaction (Fig. 6), anxiety-related behaviors in the elevated plus maze or thigmotaxis (Fig. 7), immobility in tail suspension (Fig. 8), startle (Fig. 9), or motor activity in an open field (Fig. 10). The absence of phenotypes in these tasks is unlikely to reflect a ceiling effect or a floor effect; performance showed further changes over days (Fig. 2), sessions (Figs. 6, 7C,D, and 10), age (Figs. 7A,B, and 8), and a range of prepulse (Fig. 5) and pulse (Fig. 9) intensities. Moreover, mice generally are capable of exhibiting much higher or
lower levels of performance in all of these tasks (35). Although humans with 22q11.2 duplications variably exhibit affect-related phenotypes and delays in motor development (12,13,18), the chromosomal region we studied may not be responsible for these phenotypes. Alternatively, these phenotypes seen in humans with 22q11.2 duplication may not be well represented by the rodent tasks used in this study. It should be noted, however, that although not statistically significant, there was an overall trend of lower motor activity in BAC TG mice. The three genes encoded in the BAC and other 22q11.2 genes may additively contribute to delays in motor development.

Our BAC TG mice over-express a human chromosomal segment that includes TXNRD2, COMT, and ARVCF. Because these three genes are endogenously expressed in the developing mouse brain (51,52), behavioral phenotypes could reflect the impact of over-expression of any single human gene, more than one of the three human genes, or an epistatic action among some or all of the three genes. Thus, our BAC TG mice cannot be directly compared to mice that over-express COMT alone (53). Moreover, our BAC TG mice exhibited an approximately 2-fold increase in COMT activity in the brain, whereas COMT TG mice in the study by Papaleo et al. showed an approximately 16% increase in the levels of enzymatic activity in the frontal cortex, compared to WT mice (53). Duplication cases are expected to be associated with much higher levels of COMT activity compared to the approximately 38% increase in enzymatic activity associated with a COMT Val allele vs. Met allele in the human brain (54). In addition, adult COMT TG mice were tested at 3 to 7 months of age in the Papaleo et al. study (53), whereas our BAC TG mice were tested during development at 1 and 2 months of age, as well as during adulthood at 5 months of age.
Nevertheless, the differences and similarities between the phenotypes exhibited by our BAC TG mice and the COMT TG mice are interesting. Our BAC TG mice and the COMT TG mice were similarly impaired in the percentage of correct choices in discrete-trial rewarded alternation with delay. Moreover, COMT TG mice were impaired in only one version of an attentional shift task where the task difficulty is highest (53). High activity levels of COMT may exert a detrimental effect on the cognitive capacity when demand is high. More studies are needed to test whether COMT is involved in such a difficulty-specific deficit in many cognitive tasks.

COMT TG mice were reported to be impaired in rewarded alternation without delay (53), but our BAC TG mice were normal in this version of rewarded alternation. However, in the study of Palaleo et al., data were shown as the number of days needed to reach a correct choice 80% of the time, and COMT TG mice needed more days to reach this criterion than did WT mice (53). Our data are consistent with their observation in that WT mice reached this criterion, but BAC TG mice never did at one month of age (Fig. 2A). COMT levels may affect the amount of training needed to reach a certain criterion more than the choice accuracy of working memory during development.

The apparent absence of PPI deficits in BAC TG mice is consistent with mouse studies reporting that the COMT TG mice, as well as Comt KO mice, were normal in PPI at 3 to 7 months (53,55). Our findings extend these studies by demonstrating that elevated levels of the three 22q11.2 genes, including COMT, are not associated with any PPI phenotype during development, as well as in adulthood. No study to date has reported analysis of PPI in humans.
with 22q11.2 duplication. If humans with 22q11.2 duplications exhibited a PPI phenotype, then over-expression of other 22q11.2 genes are likely to contribute to such a phenotype.

Although COMT TG mice exhibit less acoustic startle than WT controls (53), our BAC TG mice were normal in this fear-related behavior. Likewise, COMT TG mice exhibit lower levels of anxiety-like behavior compared to WT mice, while our BAC TG mice were normal in this measure. Because individual genes encoded in 22q11.2 may produce conflicting phenotypic elements, and the final phenotype is likely determined by complex interactions among them in large deletion/duplication cases (35), such “hidden” phenotypic elements could account for the apparent inconsistency in phenotypes associated with large chromosomal deletions/duplications and the functional variation of individual genes. Another possibility is that the genetic background influenced phenotypic expression. Our BAC TG mice were a congenic line with the genetic background of C57BL/6J mice. The COMT TG and WT mice were on a mixed genetic background of C57BL/6J and CD-1 (53). The flanking loci of the COMT transgene are probably not balanced between COMT TG and WT mice: more C57BL/6J than CD-1 alleles are likely present in COMT TG mice and the opposite is likely to be true in WT controls. We recently reported that the phenotypic expression of mice deficient for Sept5, another 22q11.2 gene, depends on the genetic background (35). The phenotypic differences seen in the COMT TG mice and WT mice also might reflect this factor; the COMT TG mice may have shown a lower level of anxiety-related behavior than did WT mice (53) because C57BL/6 mice exhibit a lower level of anxiety compared to CD-1 mice (56). Consistent with this possibility, congenic Comt KO, HT, and WT mice are indistinguishable in the percentage of time spent on open arms of the elevated plus maze (53).
Individuals with 22q11.2 duplications exhibit developmental cognitive impairments, including mental retardation, learning disabilities, and autism (7-20). Moreover, when individuals with autism or mental retardation are screened for CNVs, 22q11.2 duplications encompassing the ~190 kb region are found (2,21,23). Although there is no rodent model to globally recapitulate these neuropsychiatric disorders, our findings provide a translational element of cognitive functions that can be objectively tested in individuals with 22q11.2 duplication. Humans with 22q11.2 duplication would be expected to exhibit heightened incentive learning but impairments in working memory with delays.

There are a number of chromosomal abnormalities that are associated with a set of symptomatic elements of mental retardation and autism spectrum disorders, but each chromosomal abnormality also causes a separate set of specific symptomatic elements (57). When viewed from the impact of genes on specific cognitive impairments, the boundary between DSM-defined neuropsychiatric disorders blurs. Our data show that over-expression of the ~190-kb segment of 22q11.2 affects prolonged retention of working memory, but working memory is non-specifically affected in many clinically defined neuropsychiatric disorders, including mental retardation, autism, and schizophrenia. A major future challenge will be to critically evaluate how diverse symptomatic elements constitute, or even causally relate to, the clinically-classified disease entities.

MATERIALS AND METHODS

Mice

The circular BAC DNA (BAC467.8), containing TXNRD2, COMT, and ARVCF, was injected into the pronucleus of fertilized FVB zygotes. The TG mice expressed one to two
copies of the BAC. The FVB inbred mice are homozygous for Pde6brd1, a recessive gene for retinal degeneration (58) that affects vision-guided behavior, learning, and memory (59-61). Moreover, FVB mice are impaired in motor performance and learning (62). To avoid these non-specific effects of the genetic background, BAC TG mice were backcrossed to C57BL/6J mice for 10 generations. The genotypes of the mice were determined using tail tissues at the age of 7-10 days. We used two sets of PCR primers for genotyping: (1) AGG AAG ACC ATG TCC AGT GTG and TGT ATG GGT GTG TAA AGA TGG C and (2) AAT GCT CAT CCG GAG TTC C and ACT GGT GAA ACT CAC CCA GG.

Animal handling and use followed a protocol approved by the Animal Care and Use Committee of Albert Einstein College of Medicine, in accordance with NIH guidelines. Mice were maintained under a 14 h light:10 h dark cycle and were tested during the light phase. Mice were maintained in a group of up to five in a cage and were given access to food and water ad libitum, unless otherwise specified. Male congenic BAC TG mice and their WT littermates were tested at the ages of 1 and 2 months, unless otherwise specified. If no phenotype was seen at these two age points, an additional group of 5-month-old mice was tested.

**Behavioral Analysis**

*Rewarded Approach.* Male BAC TG and WT littermates were tested at the ages of 1 or 2 months. Food deprivation started 2 days prior to testing. Food deprivation equally reduced body weights to 88-93% of baseline in WT and BAC TG mice during the rewarded approach and subsequent rewarded alternation (Day, $F_{12,648} = 55.20, P < 0.01$; Genotype, $F_{1,54} = 0.25$, n.s.; Age, $F_{1,54} = 0.11$, n.s., Genotype $\times$ Day, $F_{12,648} = 0.41$, n.s.; Genotype $\times$ Age $\times$ Day, $F_{12,648} = 1.62$, n.s.).
The apparatus and procedure have been previously reported (35). Briefly, following habituation to the apparatus, individual mice were allowed to run from the start compartment of the T-maze to one goal arm while the other goal arm was blocked by a door (i.e., L-maze). The experimenter pseudorandomly designated one of the two arms as the goal 50% of the cases for each day; the same arm was not chosen as the goal more than twice in a row. Because the previously visited arm provides no information, this procedure does not require spatial working or reference memory. Because the open goal arm served as a local cue that signaled the availability of reward, this task tests the animal’s ability to reach the cued goal (i.e., incentive learning) (38). Six trials of up to 10 min were given per day for 6 days, with the inter-trial interval set within 10 min. The reward was sweetened, condensed milk (diluted 50/50 with water) placed in the food well at the end of the arm. Immediately after consuming the reward, mice were picked up from the goal compartment. Latency to the first lick of milk was recorded and used for analysis.

Rewarded Alternation. After completion of the six day training in the rewarded approach, mice were trained for rewarded alternation on six trials per day for 5 days. We used the apparatus and the procedure described previously (35). Each discrete trial included a sampling run and a choice run, and six trials were given per day. The inter-trial interval was set within 10 min. During the sampling run for each mouse, the experimenter baited both goal arms, but pseudorandomly chose one arm as the only open arm and blocked the other arm with a closed door. The selection of the open arm differed for each mouse and day. Care was taken so that equal numbers of left and right goals were chosen as the goal for each day. Upon entering the goal arm, the mouse was left there for approximately 11 s to consume the milk; it was then
brought back to the start compartment. Both goal arms were opened 5 s later, and the choice run started. The previously unvisited arm was baited and the previously visited arm was not. If a mouse did not enter the goal during the sampling or choice run within 2 min, the mouse was returned to the home cage and such trials were not used for analysis. The number of total arm visits and correct and incorrect visits were recorded and analyzed.

Delayed Rewarded Alternation. Mice were tested in this task with no delay, a 1 min delay, and a 5 min delay imposed between the sampling run and the choice run. For each delay, mice were given six trials per day over a period of 3 days with the inter-trial interval set within 10 min. After completing testing with no delay, they were given testing with a 1 min delay and then a 5 min delay. Mice were returned to their home cages during delays. The delay was timed from the time the animal entered the goal arm and the door was lowered to the time when animals were picked up from the home cages and placed in the start compartment. Otherwise, the procedure was identical to that of the rewarded alternation without delay, as described above.

Throughout delayed rewarded alternation, food deprivation significantly and equally reduced and maintained body weights of WT and BAC TG mice at 85-94% of their free feeding body weights at the ages of 1 and 2 months (Day, $F_{16,768} = 54.49, P < 0.01$; Genotype, $F_{1,48} = 0.02$, n.s.; Age, $F_{1,48} = 0.04$, n.s.; Genotype × Day, $F_{16,768} = 0.85$, n.s.; Genotype × Age × Day, $F_{16,768} = 0.37$, n.s.).

Spontaneous Alternation. The apparatus used for rewarded alternation was used. Mice were placed in the start compartment facing away from the goal arms and confined there for 5 s before the door was opened. Both goal arms were open. Once mice chose and entered one of the goal
arms, a door was gently lowered to confine mice in that arm, and 30 s later, they were removed from the arm. The mice were brought back to the start compartment, and the door was opened 5 s later for the next trial. They were allowed to choose either the goal arm just visited or the opposite goal arm. A total of 10 trials were given. If a mouse did not enter the goal during the first trial within 2 min, the mouse was returned to the start compartment and left there for 30 s before resuming a new trial. If a mouse did not enter the goal arms within 2 min during the subsequent trials, it was placed in the correctly alternated arm and confined there for 30 s. Such trials were not used for analysis. The percentage of alternated choices was used for analysis.

**PPI and Startle.** Mice were tested for startle response and PPI in a startle chamber (S-R LAB, San Diego Instruments, San Diego, CA). We used the procedure described previously (35). The amount of PPI was calculated as a percentage score for a prepulse trial: \(% \text{PPI} = 100 - \left[\frac{\text{startle response for PREPULSE+PULSE}}{\text{startle response for PULSE-ALONE}}\right] \times 100\). Startle was elicited by pulses ranging from 75 to 120 dB, and startle magnitude was calculated as the average response to all of the PULSE-ALONE trials, excluding the first and last blocks of five PULSE-ALONE trials. PPI\% and startle magnitude were analyzed.

**Social Interaction.** Our experimental protocol was designed to optimally elicit affiliative social interaction and minimize aggressive behaviors (35). Non-littermate male WT and BAC TG pairs were tested. Each mouse was individually placed in a new home cage in the experimental room for 30 min before a pair was placed in a new, third home cage in two 5-min sessions, with a 30-min interval. Social interaction included active forms (i.e., tail rattle, bites, kicks, sideway offense, boxing, wrestling, mounting, pursuit, olfactory investigation, and allogroom) and passive forms (i.e., escape, leap, side-by-side position, and submissive posture). Time spent in
active and passive social behaviors was rated by observers blinded to genotype (inter-rater correlation coefficient = 0.89, \( P < 0.0001 \)), and was analyzed separately.

*Elevated Plus Maze.* The apparatus and procedure were identical to those described in our previous studies (35,36). Mice were brought into a room adjacent to the testing room at least 1 hr before testing began. Individual mice were placed on the center platform facing one of the open arms, and tested for 5 min. Behavior was videotaped from an adjacent room through an observation window. An observer blinded to the genotype of the mice rated the behavior. The percentage of 1) time spent in and 2) entrances into the open arms with respect to the total time spent in and the number of entrances into the open and closed arms, respectively, was analyzed.

*Tail Suspension.* The apparatus and the procedure were described previously (35,36). Briefly, mice were suspended for 6 min by the tail from a metal bar 30 cm above the platform. Behaviors were recorded by a video camera, and a rater blinded to genotype recorded the length of time each mouse remained immobile.

*Locomotor Activity.* Spontaneous locomotor activity was tested in four sets of open field apparatus (Truscan, Coulbourn Instruments, PA, USA)(35,36) Horizontal activity was recorded for 30 min. Distance traveled (cm) was used as an index of horizontal locomotor activity. Time (s) spent in the marginal area along the walls was used as an index of thigmotaxis.

*COMT activity assay*

Two- to 3-month old mice were used for this analysis. COMT activity was determined by high-performance liquid chromatography with electrochemical detection using our standard
method with a slight modification (63,64). The enzyme preparation was incubated at 37 °C in 100 mM phosphate buffer (pH 7.4) containing 5 mM MgCl₂, 200 mM S-adenosyl-L-methionine, and 500 mM 3,4-dihydroxy benzoic acid (Sigma, St. Louis, MO, USA). The reaction products, vanillic and isovanillic acid, were analyzed with high-performance liquid chromatography with electrochemical detection. The system consisted of a sample autoinjector (Jasco AS-2057, Tokyo, Japan), a pump (Merck Hitachi LaChrom L-7100, Tokyo, Japan), an RP-18 column (3 mm, 4.6 × 150 mm; Waters Spherisorb, Milford, MA, USA), a coulometric detector (ESA Coulochem model 5100A detector and a model 5014B cell; ESA Inc., Chelmsford, MA, USA; detector potential -0.30 mV), and an integrator (Shimadzu C-R5A; Shimadzu Corporation, Kyoto, Japan). The mobile phase was 0.1 M Na₂HPO₄ (pH 3.3), 0.15 mM EDTA, and 25% methanol with a flow-rate of 0.8 ml/min. The protein concentration of the samples was determined with the Pierce protein assay kit, which is based on the bicinchoninic acid method (Pierce, Rockford, IL, USA). Specific activity of COMT is expressed as picomoles vanillic and isovanillic acid formed in one min per mg of protein in the sample.

**Statistical Analysis**

All data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined by analysis of variance (ANOVA) followed by Newman-Keuls post-hoc comparisons. The minimum level of significance was set at 5%.
FUNDING

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Conflicts of interest statement. NH served as a consultant for IntraCellular Therapies, Inc. in 2008. MAG holds an equity interest in San Diego Instruments, Inc.
Reference List


Legends to Figures

Figure 1. Rewarded approach at 1 month (A) and 2 months (B) of age. Time (means + SEM) spent to reach a rewarded goal in the L-maze is shown. Because the homogeneity of variance was violated (Hartley’s F-max = 3469.68, P < 0.01), data were transformed and are presented as square root values. Asterisks indicate statistically significant differences from WT mice at 5% (*) and 1% (**) levels, as determined by Newman–Keuls comparisons. One month group; WT, n = 30; TG, n = 30. Two month group; WT, n = 24, TG, n = 26.

Figure 2. Rewarded alternation without delays. The percentage of correct choices is shown as means + SEM. A) One-month-old mice, WT, n = 15; TG, n = 19; B) 2-month-old mice, WT, = 11; TG, n = 13. The dashed line indicates 50%, i.e., chance.

Figure 3. Rewarded alternation with delays. The percentage of correct choices is shown as means + SEM. A) One-month-old mice, WT, n = 15; TG, n = 11; B) 2-month-old mice, WT, = 13; TG, n = 13. The dashed line indicates 50%, i.e., chance.

Figure 4. Spontaneous alternation. The percentage of correct choices (means + SEM) is shown. One month, WT, n = 26; TG, n = 29; 2 months, WT, n = 27; TG, n = 28; 5 months, WT, n = 21, TG, n = 20. The dashed line indicates 50%, i.e., chance.

Figure 5. Prepulse inhibition (PPI). The percentage of PPI (means + SEM) is plotted against prepulse stimuli at 4, 8, 12, and 16 dB (69, 73, 77, and 81 dB presented with the 65 dB background noise. A) One month, WT, n = 36; TG, n = 32; B) 2 months, WT, n = 27; TG, n = 28; C) 5 months, WT, n = 21, TG, n = 20.
Figure 6. Active, affiliative social behaviors. Time (means + SEM) spent in active social behavior is shown in two successive 5-min sessions. One month, WT, n = 12; TG, n = 12; 2 months, WT, n = 8; TG, n = 8; C) 5 months, WT, n = 6, TG, n = 6.

Figure 7. The relative amounts (% means + SEM) of (A) time spent in and (B) frequency of visits to the open arms versus both the open and closed arms of the elevated plus maze are shown. One month, WT, n = 20; TG, n = 26; 2 months, WT, n = 21; TG, n = 23; 5 months, WT, n = 14, TG, n = 15. C-E) Thigmotaxis. Time (means + SEM) spent in the marginal area is shown over a period of 30 min. C) One month, WT, n = 25; TG, n = 25; D) 2 months, WT, n = 27; TG, n = 29; E) 5 months, WT, n = 21, TG, n = 21.

Figure 8. Immobility during the tail suspension test. Time (means + SEM) mice remained immobile is shown. One month, WT, n = 20; TG, n = 23; 2 months, WT, n = 14; TG, n = 15; 5 months, WT, n = 8, TG, n = 9.

Figure 9. Startle. A-C. The magnitude of a startle response (V average, means + SEM) is plotted against pulse stimuli (75-120 dB). A) One month, WT, n = 31; TG, n = 30; B) 2 months, WT, n = 27; TG, n = 28; C) 5 months, WT, n = 21, TG, n = 20.

Figure 10. Spontaneous locomotor activity in an inescapable open field. Horizontal distance traveled (means + SEM) was measured for 30 min. Each tick on the x-axis represents a 5-min bin. A) One month, WT, n = 25; TG, n = 25; B) 2 months, WT, n = 27; TG, n = 29; C) 5 months, WT, n = 21, TG, n = 21.
Table 1. COMT activity in various brain regions of WT and BAC TG mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal Cortex</td>
<td>39.32 (2.11)</td>
<td>83.45 (1.45)*</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
<td>n = 4</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>212.2</td>
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<tr>
<td>Striatum</td>
<td>24.66 (0.95)</td>
<td>49.26 (1.37)*</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
<td>n = 4</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>199.76</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>20.26 (0.58)</td>
<td>40.91 (1.08)*</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
<td>n = 4</td>
</tr>
<tr>
<td>%</td>
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<td>201.92</td>
</tr>
</tbody>
</table>

Means (SEM) of COMT activity (activity/pmol/min/mg protein) in three brain regions. The number of age-matched mice used is indicated. WT, wild-type mice; TG, BAC transgenic mice. * indicates a statistically significant difference from WT mice, as determined by Newman-Keuls comparisons.
Figure 1

A) 1 month

- ** WT
- ○ TG

B) 2 months

- ** WT
- ○ TG

Day 1 2 3 4 5 6

Latency (sec)
Figure 2

A) 1 month
   - ● WT
   - ○ TG

B) 2 months
   - ● WT
   - ○ TG
Figure 3
Figure 4

% Correct Choice

Month

WT
TG
Figure 5

A) 1 month

B) 2 months

C) 5 months
Figure 6

Time spent in active social behaviors (sec)

WT
TG

Session

1 month 2 months 5 months

0 20 40 60 80 100
Figure 7

A) % time in open arms

B) % entries to open arms

C) Time spent in marginal area (sec)

D) Time spent in marginal area (sec)

E) Time spent in marginal area (sec)

1 month

2 months

5 months
Figure 8
Figure 9

A) WT  
B) TG  
C) WT  
D) TG

1 month  
2 months  
5 months
Figure 10

A) 1 month
B) 2 months
C) 5 months
**List of Abbreviations:**

TXNRD2, thioredoxin reductase 2  
COMT, catechol-O-methyltransferase  
ARVCF, armadillo repeat gene deletes in velocardiofacial syndrome  
BAC, bacterial artificial chromosome  
WT, wild type  
TG, transgenic  
CNV, copy number variations  
SNP, single nucleotide polymorphism  
ANOVA, analysis of variance  
PPI, Prepulse inhibition  
SEPT5, septin 5  
GP1Bb, glycoprotein Ib (platelet), beta polypeptide  
TBX1, T-box 1  
GNB1L, guanine nucleotide binding protein (G protein), beta polypeptide 1-like  
DSM, Diagnostic and Statistical Manual of Mental Disorders