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# *Comt1* Genotype and Expression Predicts Anxiety and Nociceptive Sensitivity in Inbred Strains of Mice

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## Abstract

Catechol-O-methyltransferase (COMT) is an ubiquitously expressed enzyme that maintains basic biologic functions by inactivating catechol substrates. In humans, polymorphic variance at the *COMT* locus has been associated with modulation of pain sensitivity (Andersen & Skorpen, 2009) and risk for developing psychiatric disorders (Harrison & Tunbridge, 2008). A functional haplotype associated with increased pain sensitivity was shown to result in decreased COMT activity by altering mRNA secondary structure-dependent protein translation (Nackley *et al.*, 2006). However, the exact mechanisms whereby COMT modulates pain sensitivity and behavior remain unclear and can be further studied in animal models. We have assessed *Comt1* gene expression levels in multiple brain regions in inbred strains of mice and have discovered that Comt1 is differentially expressed among the strains, and this differential expression is cis-regulated. A B2 Short Interspersed Element (SINE) was inserted in the 3′UTR of *Comt1* in 14 strains generating a common haplotype that correlates with gene expression. Experiments using mammalian expression vectors of full-length cDNA clones with and without the SINE element demonstrate that strains with the SINE haplotype (+SINE) have greater Comt1 enzymatic activity. +SINE mice also exhibit behavioral differences in anxiety assays and decreased pain sensitivity. These results suggest that a haplotype, defined by a 3′ UTR B2 SINE element, regulates *Comt1* expression and some mouse behaviors.

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## Introduction

Dysregulation of catecholamine signaling has been linked to a variety of neuropsychiatric disorders (reviewed by Harrison & Tunbridge, 2008, Lachman, 2008). The *COMT* gene encodes an enzyme of the same name and functions to eliminate catecholamines. The COMT enzyme catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to one of the hydroxyls on a catechol structure (Axelrod & Tomchick, 1958) leading to catecholamine inactivation. Catecholamines such as adrenaline and noradrenaline prepare an organism for immediate action in response to a perceived threat (the fight-or-flight response) and both physiological and psychological stressors induce the release of catecholamines (Guyton, 1991). Altered COMT enzymatic activity has been linked to disproportionate anxiety responses (Domschke *et al.*, 2007, Evans *et al.*, 2009, Pooley *et al.*, 2007), differences in pain perception (Diatchenko *et al.*, 2005, Emin Erdal *et al.*, 2001, Zubieta *et al.*, 2003) and Attention Deficit Hyperactivity Disorder (ADHD), (Deyoung *et al.*, 2010, Palmason *et al.*, 2009, Qian *et al.*, 2003) in humans.

*COMT* is highly conserved between mouse and human, with nearly 80% amino acid homology. Several *Comt1* mouse models have been developed. A knockout of the mouse gene, Comt1 (Gogos *et al.*, 1998) and a transgenic line expressing human *COMT* (Papaleo *et al.*, 2008) were engineered to provide a model for understanding catecholamine processing. Experiments with the knockout and transgenic lines demonstrated that COMT activity affects stress and pain responses in a manner consistent with human studies. Comt1 null and heterozygous mice have increased sensitivity to thermal nociception and increased stress reactivity (Papaleo *et al.*, 2008). Transgenic mice over-expressing the human gene have higher COMT enzymatic activity and a blunted thermal pain response (Papaleo *et al.*, 2008). Differences in catecholamine metabolism between C57BL/6J and DBA/2J mice has also been reported (Eleftheriou, 1975). This study found that COMT enzymatic activity varies with age and brain region and also with stress.

In the present study, we measured behavioral phenotypes and brain gene expression in 29 common inbred strains of mice. We use a method called Haplotype Association Mapping (HAM) (McClurg *et al.*, 2007, McClurg *et al.*, 2006, Pletcher *et al.*, 2004) to correlate genotypic differences with gene expression in an effort to identify regions of the genome, expression quantitative trait loci (eQTL), that contribute to inbred strain variation. This genome-wide analysis revealed Comt1 to be differentially expressed among strains and cis-regulated. At least four haplotypes exist at the Comt1 locus but the defining polymorphic feature is the presence of an insertion of a SINE element in the 3′UTR of the gene. The presence of this SINE element correlates with differential gene expression and an increase in enzymatic activity. Thus, the natural allelic variations that alter COMT1 protein levels or enzyme function, in common inbred strains of mice are of potential interest for behavioral studies.

## Materials and Methods

### Animal Husbandry

All experiments conformed to the guidelines in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Behavioral measurements and gene expression data were collected at the Genomics Institute of the Novartis Research Foundation (GNF) and all procedures were approved by the GNF Institutional Animal Care and Use Committee. Animals were housed in an SPF barrier colony and were maintained on a 12-h light: 12-h dark cycle, housed in groups of 2–4 in standard high efficiency particulate air-filtered polycarbonate mouse cages containing a layer of Bed-o-cob bedding and one cotton nestlet. Food (Pico rodent chow 20; Purina, St Louis, MO, USA) and water were

made available ad libitum. Animals used for q-PCR and ELISA experiments were housed under similar conditions as those described above and sacrificed according to guidelines set by the Institutional Animal Care and Use Committee (IACUC) at UNC Chapel Hill.

## Inbred Strains

The 29 strains tested were: 129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBR T+ tf/J, BUB/BnJ, C3H/HeJ, C57BL/6J, C57BR/cdJ, C58/J, CBA/J, CE/J, DBA/2J, FVB/NJ, I/LnJ, KK/HIJ, MA/MyJ, MRL/MpJ, NOD/LtJ, NON/LtJ, NZO/HILTJ, NZW/LacJ, P/J, PL/J, RIIIS/J, SJL/J, SM/J, SWR/J, and WSB/EiJ.

## Behavioral Testing

**Animals—**Six to eight week old male and female mice of each strain were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were habituated in quarantine for approximately 6 weeks until they were transferred to the main barrier colony. Mice were habituated to the main colony holding room for at least one week prior to behavioral testing. All behavioral testing was conducted during the light part of the light/dark cycle and occurred between the hours of 8:00AM – 12:00PM. On the day of testing, mice were transported to a quiet anteroom adjacent to the testing room and acclimated for at least one to two hours prior to the start of testing. All testing equipment was cleaned with a light bleach solution (0.1%) in between each animal. Mice were tested in two behavioral cohorts that each went through a separate series of tests. One group of mice were tested in the open field and a second group were tested on the elevated plus maze and light/dark test. Mice were experimentally naïve for both open field and elevated plus maze testing. Animals tested in the light/dark assay had previously been tested in the elevated plus maze.

**Open Field Assay (OF)—**The open field is a 17″ × 17″ × 13″ arena with a white Plexiglas floor and clear Plexiglas walls (ENV-515-16; Med Associates, St Albans, VT, USA), which is surrounded by infrared detection beams on the x-, y- and z-axes that track the animals' position and activity over the course of the experiment. The apparatus is isolated within a 73.5 × 59 × 59 cm testing chamber fitted with overhead fluorescent lighting (lux level 14). Animals were removed from their home cage, immediately placed in the corner of the open field arena and allowed to freely explore the apparatus for a test interval of 10 min. Animals were scored for a number of behaviors in the open field, including total distance traveled (in cm), ambulatory episodes (number of times animal breaks user-defined number of beams before coming to rest), percent time resting, average velocity (in cm per second), number of rearings and percent time spent in the center of arena (defined as nine square-inch central part of arena). These data were recorded during testing and scored in post-session analyses using commercially available software (Activity Monitor 5.1, Med Associates). We have found in previous studies (Bailey *et al.*, 2008, Eisener-Dorman *et al.*, 2010) that factor analysis can reduce the data from the OF assay to two distinct classes of behaviors - activity-related behaviors and anxiety-related behaviors and rearing behavior represents a separate behavior. For subsequent analyses, we included only one representative behavior from each class in our analysis; total distance (activity), percent time in the center (anxiety) and rearing behavior.

**Elevated Plus Maze (EPM)—**The elevated plus maze (7001-0336; San Diego Instruments, San Diego, CA, USA) consists of two open arms and two closed arms [26.5″ × 2.5″] that are directly opposing each other. The walls of the enclosed arms completely surround the end of the runway and are 6″ high. The top of the enclosed arms is open to the testing room. The entire apparatus is 15″ high and is placed on the floor for testing. A video camera above the maze captures the animal's location in the maze. Data is collected and analyzed with Actimetrics LimeLight software (Actimetrics, Wilmette, IL, USA). The

animals are placed in the center of the maze and allowed to investigate the maze for 5 minutes. Data recorded include distance traveled in each region and percent time spent in the open arms of the maze.

**Light/Dark Assay (LD)—**The light/dark enclosure (ENV-511; Med Associates) inserts into the Med-Associates open field apparatus and is a light-impermeable box that covers one third of the area of the open field. The dark box has a classic “mouse hole” entry for the animal to enter and exit the box. The animals were placed in the center of the open field directly in front of and facing the dark enclosure and allowed to explore the arena for ten minutes. Transitions between light and dark quadrants and time spent in each quadrant were assessed.

## Gene Expression Studies

**Tissue Collection—**Eight to ten week old male and female mice of each strain were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were habituated for one week prior to tissue collection. Mice were sacrificed by cervical dislocation without anesthesia to avoid gene expression differences in response to anesthetic. All dissections were conducted between the hours of 9:00AM to 11:30AM. Prefrontal cortex, amygdala, hypothalamus, hippocampus, nucleus accumbens, striatum and pituitary were dissected as follows: Immediately following euthanasia, mice were decapitated and the whole brain was removed from the skull. The hypothalamus was lifted from the ventral surface of the brain using curved forceps. The pituitary was lifted from the sella tursica in the base of the skull using microforceps. The brain was then transferred, ventral side up, to an ice-cold brain matrix with 0.5mm spacing (505C Braintree Scientific, Braintree, MA, USA). A single razor blade was placed into the first space on the brain matrix and the rostral surface of the brain was placed in the matrix and touching this blade. Thin, double-edged razor blades were placed in the twelve most anterior spaces. Following removal from the matrix, the 0.5 mm brain slices were placed flat onto an ice-cold dissection stage and specific regions dissected using anatomical landmarks as described below. The prefrontal cortex was taken from the slice corresponding to approximately 2.5 mm to 2.0 mm anterior to Bregma. To do so, a “V-shaped” wedge was made just medial to the corpus callosum with the apex terminating at about the lateral ventricle (viewed from the caudal side of the slice). Nucleus accumbens and striatum were taken from the adjacent slice approximately 2.0mm to 1.5mm anterior to Bregma. To isolate nucleus accumbens, 1mm diameter punches were taken just ventromedial to the anterior commissure (AC). For striatum, 1mm diameter punches were taken dorsolateral to the AC, midway between the AC and corpus collosum, and lateral ventricle and corpus collosum. Amygdala was dissected from the slices corresponding to approximately 0.5mm to 1.5mm posterior to Bregma, ventromedial to the ventral boundary of the external capsule and directly below the caudate putamen. Hippocampus was dissected from slices at approximately 1.0mm to 2.0mm posterior to Bregma. The dissected regions included the following - Prefrontal cortex consists of the piriform cortex, secondary motor cortex, cingulate cortex area 1 and medial orbital cortex; nucleus accumbens consists of the anterior portion of the nucleus accumbens core and shell; striatum is a one mm diameter punch of striatum only; amygdala contains all amygdaloid nuclei plus 50% of the piriform cortex and the entire ventral endopiriform nucleus; hippocampus contains hippocampus only as it is easily peeled away from the surrounding tissue.

**Gene expression analysis—**After dissection tissues were immediately frozen on dry ice and stored in a -80 freezer until RNA preparation. Tissue was pulverized to a fine powder while frozen to obtain a mixture of homogeneous tissue. A small (~10mg) aliquot was homogenized using a rotor-stator (Omni TH polytron, Omni International, USA) homogenizer in Trizol (Invitrogen, Carlsbad, CA, USA) and total RNA purified with

RNeasy columns (Qiagen, Valencia, CA, USA). RNA quality was tested using an Agilent Bioanalyzer (Santa Clara, CA, USA) and high quality RNA was pooled from three animals per strain to run one microarray. Male and female samples were pooled separately and processed on separate microarrays. Gene expression analysis was performed according to standard procedures (Su *et al.*, 2004). RNA was amplified and labeled using the Affymetrix one-cycle target labeling kit. Samples were hybridized to Affymetrix GeneChip 430v2 whole-genome mouse arrays and data were processed using the gcRMA algorithm (Wu *et al.*, 2008). Raw data were deposited in GEO (http://ncbi.nih.gov/geo) under series accession GSE20160. In this study, data were filtered to remove probe sets whose expression was either undetectable (maximum expression across strains <200) or invariant across strains (ratio of maximum expression to minimum expression across strains). Although summarization algorithms are designed to be robust to single-probe outliers, the presence of SNPs in the probe sequence could theoretically lead to spurious detection of cis-eQTL. An analysis performed after removing all probes overlapping a SNP in dbSNP from the CDF file resulted in qualitatively similar results.

### qPCR Validation of Affymetrix GeneChip array

Microarray data was validated by q-PCR in cDNA prepared from pituitary and cortex RNA from male animals. The first round of q-PCR used RNA remaining from the original samples prepared for the microarray. The sixteen strains (from 48 male animals) used in the first round of q-PCR validation were: BTBR T+ tf/J, C3H/HeJ, C57BL/6J, C57BR/cdJ, CBA/J, CE/J, KK/HIJ, MRL/MpJ, NOD/LtJ, NON/LtJ, NZO/HILTJ, NZW/LacJ, P/J, PL/J, RIIIS/J and SM/J. The most significant change in gene expression was validated by an ABI custom designed Taqman assay in the 3′UTR interval (data not shown). The primer and probe sequences are: Forward primer $^{5\prime}$AAACCCCTCACGGTGAATCC$^{3\prime}$, Probe $^{5\prime}$TCTGCACCCAAGAACA$^{3\prime}$, Reverse primer $^{5\prime}$CATCTCACCAGTCCCCCTTTTT$^{3\prime}$, (Applied Biosystems Incorporated, Foster City, CA, USA). The second round of validation used cortex from four 10 week old males from strains AKR/J, BALB/cByJ, C3H/HeJ and SJL/J. The material was not pooled in the second q-PCR assay. Data was collected from sixteen individual animals, from each of four strains. Animals were housed and sacrificed as previously described. ABI TaqMAN probe Mm00514377_m1, which measures transcript in the coding region, was used for the cortex q-PCR.

### Identification of B2 SINE element

BAC clone sequence for 129S6/SvEvTac (AC012399) and C57BL6/J (AC133487) was aligned with Sequencher software. A 234 bp insert was discovered in the 3′UTR. This sequence was run through Repeat Masker (http://www.repeatmasker.org), which identified it as a B2 SINE element. PCR primers flanking the region (forward primer $^{5\prime}$TTTCCTCAGGGCCTGTGGCT$^{3\prime}$ and reverse primer $^{5\prime}$GAGGCCATCAGGATGACACC$^{3\prime}$) were designed using publically available Primer3 software (http://frodo.wi.mit.edu/primer3/). PCR was performed on both DNA and cDNA. Liver and whole brain cDNA was sequenced and referenced against Comt1 cDNA transcript NM_001111063.1 in 129S1/SvImJ, BALB/cByJ, C57BL/6J, CBA/J and WSB/EiJ.

### Development of COMT ELISA assay

COMT1 protein was measured from three pooled brain regions (two male animals), determined by proximity: region HHA (hypothalamus, hippocampus and amygdala), region AS (nucleus accumbens and striatum) and region C (cortex). To detect the levels of COMT1 in brain lysates, the Meso Scale discovery (MSD) electrochemiluminescence (ECL) assay was performed using a MSD Sector Imager 2400 according to the manufacturer's protocol

(Meso Scale Discovery, Gaithersburg, MD, USA). The MSD assay is based on a sandwich immunoassay that utilizes ECL to measure protein levels. Frozen brain regions were pulverized to a powder in liquid nitrogen. The pulverized tissue was mixed with RIPA buffer (Pierce, Thermo Fisher Scientific, Rockford, IL, USA, Cat. # PIH9901) and protease inhibitor cocktail (Pierce, Cat. # PI-78430) and were then centrifuged at 13,000 RPM for 30 min at 4 °C to obtain the supernatant. Coomassie Plus (Pierce, Cat. # PI-23200) assays were performed according to manufacturer's protocol to determine the protein concentrations in the lysates. 10 μl of primary antibody at a 1:100 concentration (Abcam, Cambridge, CA, USA Cat. #36144) was spotted in each well of a 96-well plate (MSD, Cat. # MA2400), and allowed to dry at room temperature overnight. The plate was incubated for 1 h with blocking buffer containing 3% bovine serum albumin at room temperature, then 25 mg/ml protein lysates in 50 μl lysis buffer were added to the ELISA plates. The plate was incubated at 4 °C overnight, and washed three times with 150 μl of the MSD wash buffer (50 mM Tris pH 7.5, 1.5 M NaCl, 0.2% Tween-20). 25 μl of the secondary antibody (Abcam, Cat. #51984), diluted 1:10 and labeled with the MSD SULFO-TAG detection antibody solution was added to the wells and the plates were incubated for 2 h at room temperature, while rocking. The plates were again washed three times with the wash buffer and 150 μl of Buffer T from MSD was added to each well. The plates were analyzed with the SECTOR Imager 2400. ECL intensities obtained from the assay were normalized by the blank wells of the secondary antibody to the bovine serum albumin-coated spot with no lysate.

### Enzymatic analysis of brain lysates

**Sample preparation—**Whole brains from two 10 week old males from strains 129S1/SvImJ, C3H/HeJ, C57BL/6J, C57BLKS/J, NZB/BlNJ, NZW/LacJ, and PL/J were purchased from Jax Laboratories. Animals were killed by cervical dislocation, brains removed, frozen on dry ice, shipped, and thawed for our dissection of the frontal cortex. Tissue was pooled by strain, refrozen on dry ice, pulverized, and homogenized in 0.1mM CDTA (Sigma Chemical Company, St. Louis, MO, USA) with a 16 gauge syringe. A second round of experiments used non-pooled material from four 10 week old males from strains AKR/J, BALB/cByJ C3H/HeJ, and SJL/J. Animals were housed and sacrificed as previously described. Cortex was dissected from fresh tissue, and frozen on dry ice. Samples were then pulverized and homogenized in 0.1mM CDTA as for the first experiment. In both experiments, homogenized brain lysate was centrifuged at 2000g for 10 minutes and filtrate quantified with the Pierce BCA assay (Thermo Fisher Scientific, Rockford, IL, USA) in accordance with the manufacture's recommendation.

**Enzymatic assay—**COMT1 activity was assessed with the Normetanephrine ELISA kit (RE59171, IBL, Hamburg, Germany as described (Nackley & Diatchenko 2010, Nackley *et al.*, 2006). In this method, a known amount of substrate, L-norepinephrine, is added to a biological lysate. The measure of COMT1 enzymatic activity is the amount of product, normetanephrine (NMN), produced in the reaction of lysate and added substrate. Briefly: after lysates were normalized to equal protein concentrations, 8 μl was incubated with 200μM S-adenosyl-L-methionine (SAMe; ICN Chemical, Aurora OH, USA), 7.5 mM L-norepinephrine (NE; Sigma Chemical Co.) and 2mM $MgCL_2$ in 50mM phosphate buffered saline for 1 hr at 37 C°, final volume 21μl. The reaction was terminated using 20μl of 0.4M hydrochloric acid and 1μl of 330 mM EDTA. 10μl of the halted reaction mixture was then used in the Normetanephrine ELISA kit in accordance with the manufacture's recommendation. Lysates from cortex regions of brains in four strains of the -SINE haplotype and 6 strains of the +SINE haplotype were assayed for COMT1 enzymatic activity. Four technical replicates were conducted per animal or per strain in two separate experiments. C3H/HeJ was run in both experiments and data were normalized to C3H/HeJ COMT1 activity.

**Cell Construct—**A *Comt1* cDNA clone in expression vector pCMV-SPORT6 was purchased from the I.M.A.G.E. Consortium (ATCC, Manassas, VA, USA, clone ID 4210097). The clone contained the full length 5′UTR and aligned to the Comt1 NM_001111063 transcript. The full length 3′UTR was not present in the clone. To construct the +SINE and -SINE expression vectors, the truncated 3′UTR was excised by a double digestion of *Not1* and *Bsu36I*. Primers to genomic DNA were designed flanking the 3′UTR genomic region to be ligated into the double digested expression construct. The 5′ primer was 5′ of the *Bsu36I* cut site, and the 3′ primer had a *Not1* linker 3′ of the sequence aligning to genomic sequence. C57BL6/J and WSB/J genomic DNA was PCR amplified for the 3′UTR, resulting in +SINE and -SINE 3′UTR fragment. The amplimer was double digested with *Not1* and *Bsu36I*, gel purified, and ligated into the double digested expression vector. Clones were verified by sequencing the entire insert in both directions.

**Transient transfection of Comt1 cDNA clones—**This transfection assay has been previously described (Nackley *et al.*, 2007). A rat pheochromocytoma cell line (PC12) was transiently transfected in 35-mm six well plates using FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, USA) according to manufacturer's recommendations. The amount of +SINE or -SINE construct was at a concentration of 0.9 μg/ml of media. To account for transfection efficiency, pSV-βGalactosidase vector (Promega, Madison, WI, USA) was co-transfected at 0.1 μg/ml of media. Transfection with empty vector was done for each experiment. Cell lysates were collected approximately 24 hours post-transfection.

## Enzymatic analysis of cell constructs

**Sample preparation and assay—**After removing media, cells were washed once with ice cold 0.9% saline solution (1ml/35 mm well), and collected by scraping the wells (on ice) with 150μl/well of ice cold 0.1mM CDTA. The lysate was collected in 1.8 ml tubes and freeze/thawed (-80 C°/RT) twice. The tubes were centrifuged at 2000g for 10 min and filtrate removed. Filtrate was quantified with the Pierce BCA assay in accordance with the manufacture's recommendation and lysates normalized. The Normetanephrine ELISA kit was also used for the cell constructs, in the same manner as for brain lysates. COMT1 activity was determined after subtracting the amount of NMN produced by endogenous enzymatic assay (transfection with empty vector). COMT1 activity was then normalized for transfection efficiency by measuring the β-galactosidase activity for each lysate. β-galactosidase activity was determined by incubating 50μg of normalized lysate with 2× β-galactosidase buffer (39.3 mM $NaH_2PO_4$ •$H_2O$, 154.84 mM $Na_2HPO_4$, 4.3 mM $MgCl_2$, 4.45 mM O-Nitrophenyl β-D-galactopyranoside, 12.mM β-mercaptoethanol) in a 100μl reaction for 30 minutes at 37 C°. The lysates were then read with a luminometer at 405nM filter. The read for blank, 50μl 0.1mM CDTA and 50μl 2× β-galactosidase buffer, was subtracted from each lysate.

## Statistical Analysis

**Genome-wide eQTL mapping—**The detailed algorithm underlying the Haplotype Association Mapping (HAM) method has been previously described (McClurg *et al.*, 2007, McClurg *et al.*, 2006, Pletcher *et al.*, 2004). Briefly, HAM uses ANOVA to calculate the strength of genetic associations between an input phenotype and the ancestral haplotype structure (as inferred using a local window of three adjacent SNP alleles across the genome). A weighted bootstrap method was introduced to detect association peaks conditional on the population structure in the mouse diversity panel. At each genetic locus, the association score was represented as the negative log10-transformed P value. A score of −LogP=6 is a maximal score resultant from $10^6$ permutations performed at each locus. This score is not corrected for genome-wide significance. HAM analysis was performed for *Comt1*, across 29 strains using the web-based analysis SNPster (http://snpster.gnf.org/cgi-bin/snpster_ext.cgi)

with the expression phenotype transformed to log scale. The genomic mapping of all genes and SNPs was based on Mouse Genome NCBI Build 35 (mm7).

**Analysis of B2 SINE element and Comt1 mRNA expression and enzymatic activity—***Comt1* mRNA expression data (Affymetrix 430v2 probe set 1449183_at) was analyzed in seven brain regions using analysis of variance (ANOVA) (SPSS, v.16 for Mac, Chicago, IL USA) with sex and SINE status as independent variables.

Replicate enzymatic assays were performed and C3H/HeJ was included in all replicates. COMT1 enzymatic data were normalized to C3H/HeJ. An independent t-test (SPSS) was performed to determine differences in enzymatic activity between +SINE and -SINE strains.

**Analysis of behavioral data—**A total of 744 mice (355 females from 32 strains and 389 males from 31 strains) were tested in the OF assay and 223 mice (113 female and 110 males from 24 strains each) were tested in both the EPM and the LD assays as described above. Fifty-one percent of the mice tested in the OF were -SINE and forty-nine percent were +SINE. Thirty-nine percent of the mice tested in the EPM and LD assays were −SINE and sixty-one percent were +SINE. The complete data set is available on (http://www.jax.org/phenome; Project: MPD: 214). The analyses described below were conducted on the behavioral data from individual mice. However, for those results that showed significance with individual test scores we also conducted the analysis using inbred strain means to avoid potential strain bias of uneven numbers of animals between strains. Only results that support significance by strain means as well as with individual behavioral scores were considered to have significant genotype-phenotype associations. Strain mean data and numbers of animals tested for each phenotype is listed in Supplemental Table 1.

We used multivariate analysis to account for large numbers of data vectors (Marron, 2007). This methodology allows us to test the single hypothesis that mice with the +SINE haplotype are behaviorally different from -SINE mice. All of the behavioral data from both cohorts of mice were used in single analyses in both male and female mice. This technique does not assign a *P-value* to individual behavioral assays, and contributions from each behavioral assay are visualized by distance weighted discrimination (DWD) plots. After the data is plotted, hypothesis testing then confirms the impressions gained from the visualization of the data. This can be done without the loss of information entailed by classical dimensionality reduction, using a Direction Projection Permutation hypothesis test (DiProPerm) (Benito *et al.*, 2004, Hu *et al.*, 2006, Marron, 2007).

DWD focuses on two-class discrimination in multi-dimensional space formed by the data. The objective is to find a direction, a loading vector, which best separates the two classes. The variables are the 11 behavioral test scores for all of the individual animals. The length of a bar corresponds to the importance in separating the two classes and a positive loading value indicates that the -SINE haplotype tends to have a higher level of the corresponding variable than the +SINE haplotype, while the negative loading value means the opposite.

The second step projects all behavioral test scores from the +SINE and -SINE haplotypes in a DWD direction to obtain a pair-wise t-statistic. The data projection plots a cloud of data points from each of the two classes, with the Gaussian distribution of both classes under the data points. The Gaussian distribution of all data points is represented above the two curves of +SINE and -SINE Gaussian distribution. A pair-wise t-statistic was then obtained.

The DiProPerm test is used to assess the significance of the t-statistic. All of the data from each behavioral test and each mouse was randomly re-labeled into two classes. This permutation was performed 100 times, and each time there was a new t-statistic. The

empirical p-value corresponds to the proportion of the t-statistics of the permutated data at or above the t-statistic (http://www.stat.colostate.edu/~chihoon/FDA_ratPMdata.pdf).

**Genetic Correlation Analysis with Nociception Assays—**To determine if the absence of the SINE element is genetically related to sensitivity in pain models of several fundamental nociceptive modalities, *Comt1* SINE status was compared to sensitivity in 22 nociception and hypersensitivity assays previously collected in twelve inbred mouse strains. Six of the strains had the SINE element: A/J, AKR/J, BALB/cJ, C57BL/6J, C57BL/10J and SM/J. Six of the strains did not: 129P3/J, C3H/HeJ, C58/J, CBA/J, DBA/2J and RIIIS/J. Brief descriptions of the assays are found in Table 3, with greater detail available on The Jackson Laboratory's Mouse Phenome Database website (http://www.jax.org/phenome) and in the original reports (Lariviere *et al.*, 2002,Mogil *et al.*, 1999a,Mogil *et al.*, 1999b). Multivariate statistical analyses were used as previously described (Lariviere *et al.*, 2002) to simultaneously assess the genetic correlations between absence of the SINE element in standard inbred strains and their sensitivity in: six inflammatory, six thermal and one mechanical nociception assay; one mechanical sensitivity assay; and eight mechanical, thermal and afferent-dependent hypersensitivity assays (see Table 3). Pearson product-moment correlation coefficients were calculated between the strain means for each assay and the SINE status of the strain, with 1 = absence and 0 = presence due to the expectation of a negative correlation with pain sensitivity, and with strain means corrected by multiplication by -1 so that higher numbers indicated greater sensitivity. As such, a positive correlation indicates that absence of the SINE element is observed in strains more sensitive in the particular assay. Multidimensional scaling (MDS) and principal components analysis (PCA) were used to visualize all pairwise correlations simultaneously (Systat 13, Chicago, IL, USA). Briefly, in MDS, coordinates in two-dimensional space are reiteratively computed for a set of points representing SINE status and the assays to fit as closely as possible the measured similarities of Pearson correlations using a Kruskal loss function with monotonic regression. High positive correlations are represented as small distances between points, and high negative correlations are represented as large distances between points. Uncorrelated points have intermediate distances between them. In PCA, two linear combinations of the points are constructed, and the weights of the linear combinations are plotted in a two-dimensional space to produce a vector for each point. Highly positively correlated points are represented with vectors with angles close to 0° between them, and high negative correlations as angles close to 180° between vectors. The results can also be viewed in three dimensions to determine if the same groupings are observed as in the two-dimensional representation.

## Results

### Expression QTL (eQTL) analysis of microarray data reveals cis-regulation of Comt1

The use of gene expression as a quantitative phenotype for SNP haplotype mapping allows for identification of genomic regions that control gene expression (eQTL). The correlation of differential gene expression for a specific gene with the haplotype pattern at its own chromosomal location indicates that the gene is cis-regulated - meaning that a difference at or near the gene influences its expression. *Comt1* gene expression (intensity scores) for all strains was used as a quantitative phenotype for haplotype association mapping analysis. A genome-wide association plot for *Comt1* gene expression from pituitary (Affymetrix probeset 1418701_at) using the SNPster algorithm shows an eQTL peak with a -LogP score of 6 on chromosome 16 (Figure 1a) near the physical location of the Comt1 gene, indicating that *Comt1* is cis-regulated. Analyses of Comt1 expression for other brain regions, (nucleus accumbens, cortex, hippocampus, amygdala and striatum) also demonstrate cis-regulation (data not shown).

More detailed investigation of the Chr 16 region identified only 6 SNPs that define the 2.5MB haplotype interval immediately surrounding Comt1 (Figure 1b). The other SNPs in the interval that show no association are non-informative for the strains included here. The genomic location of each SNP is indicated with its association score on the Y-axis. The SNP with the maximal -LogP score of 6, rs4165252, is at genomic location 20650976. We sequenced coding regions of Comt1 in brain and liver cDNA in several +SINE and -SINE strains and did not discover new SNPs, confirming earlier reports of identity by descent (IBD) in this region (Yang *et al.*, 2007). HAM analysis uses a 3 SNP window to define haplotypes and for the surveyed strains, there were three distinct haplotypes in this SNP position: AGT, AAC, and AAT (Figure 1c). We did not include the wild-derived strains CZECHII/EiJ, CAST/EiJ, MOLF/EiJ or PWD/PhJ in the original survey, but note: these strains exhibit an additional haplotype at this position, GGC.

## A B2 SINE element defines haplotype

We discovered the SINE insertion by aligning BAC clone sequence from 129/SvEvTac with C57BL6/J. C57BL6/J had an insertion of roughly 240 bp in the 3′UTR. RepeatMasker identified the inserted sequence as a B2 SINE element (http://www.repeatmasker.org). Although we found no other polymorphisms within *Comt1*, the HAM analysis pointed us toward this insertion as the cis-element contributing to regulation of *Comt1*.

A PCR to detect the presence or absence of the SINE element was developed to assess the status of the insertion among mouse strains (Figure 1d and e). Almost all of the -SINE strains are haplotype AGT, and almost all of the +SINE strains are haplotype AAT, except for A/J, PL/J, and SM/J. We included a single 129 strain, 129S1/SvImJ in our analysis, but included 129×1/SvJ in our SINE element PCR assay. Although both these 129 sub-strains have the haplotype that exhibit -SINE, these two strains are +SINE. We examined a third 129 strain, 129P3/J, and found this 129 parental strain to be a -SINE strain; 129/SvEvTac, is also a -SINE strain (data not shown).

## Expression QTL (eQTL) analysis of microarray data reveals significant effect of SINE haplotype on Comt1 expression

ANOVA identified a significant main effect of both SINE element status and sex for all brain regions examined. Overall, +SINE strains had higher *Comt1* expression and males showed higher expression levels than females (see Table 1). A significant SINE by sex interaction effect was also observed for expression levels in the nucleus accumbens ($F_{(1,55)}$=24.4;P<0.0001), prefrontal cortex ($F_{(1,57)}$=8.2;P<0.01), amygdala ($F_{(1,57)}$=18.0;P<0.0001) and striatum ($F_{(1,51)}$=9.9;P<0.01). Posthoc analyses indicate that female mice show increased *Comt1* expression in the striatum and +SINE females have slightly lower *Comt1* expression in the nucleus accumbens in contrast to males (Table 1).

## Validation of array data

Typical microarray data is shown for cortex and nucleus accumbens (Figure 2). To validate results from the microarray data we developed TaqMAN assays. qPCR of cortex cDNA replicated the array findings (Figure 3a, P<0.01). In 10-week old male mice, the average increase in Comt1 mRNA is approximately 20% across the surveyed strains. Resultant from increased mRNA presence we expected to find an increase in COMT1 protein levels and developed an ELISA assay to ascertain protein levels. However, we could not show differences observed in COMT1 protein levels in prefrontal cortex were significant for the strains surveyed (Figure 3b).

### Comt1 Enzymatic Activity is higher in brain lysates and cell constructs

In the absence of determining robust differences in COMT1 protein levels among the strains we investigated whether there was any change in enzymatic activity. We examined this in both *in-vivo* and *in-vitro* assays. The +SINE strains had an average of 20% more activity, mirroring gene expression profiles ($t(8)=-4.4$; $P<0.01$; Figure 3c). To validate the hypothesis that the presence of the SINE element was driving the increase in activity, we constructed full-length (cDNA) Comt1 clones of the NM_001111063 transcript with and without the B2 SINE element. Rat adrenal (PC-12) cells were transiently transfected with each construct. COMT1 enzymatic activity was measured and found to be 5-fold greater in +SINE transcripts (Figure 3d).

### SINE haplotype has an effect on anxiety and exploratory phenotypes

The DiProPerm analysis showed that +SINE strains have a significantly different behavioral profile than the -SINE strains. Four behaviors had a significant effect in separating the two classes. These were open field rearing and total distance, percent time in the light side of the light/dark arena and total distance in the elevated plus maze (Figure 4a). The results were similar for both male and female mice. When viewed in the distance-weighted discrimination (DWD) loading plot, the length of the bar corresponds to its contribution in discriminating between the two haplotypes. A positive loading value indicates that the -SINE mice have an increased measure of the behavior than the +SINE mice and a negative loading value means the opposite. Based on the DWD results, -SINE mice rear more in the open field assay and spend more time in the lighted area of the LD assay and +SINE mice exhibit greater locomotion in the elevated plus maze and open field. All behavioral data from the DWD loading plot for all strains were plotted on a data projection plot (Figure 4b) and this clearly shows discrimination between the absence (black) or presence (grey) of the SINE insertion. The DiProPerm analysis assessed the significance of the t-statistic by a permutation test and found it to be highly significant in both female and male mice (Figure 4c and Table 2). When individual behavioral tests are analyzed the elevated plus maze contributes a significant effect to the DWD loading plot for males only.

When strain means, rather than individual scores, for combined tests were analyzed, distance in the elevated plus maze was no longer significantly different in +SINE vs −SINE mice. Open field distance and rearing were significant in females ($t=2.6$;$P<0.05$) but only exhibited borderline significance in males ($t=2.3$; $P=0.07$). Percent time spent in the lighted area of the LD assay was significant in both males ($t=2.2$;$P<0.05$) and females ($t=2.2$;$P<0.05$).

**Strains without the SINE insertion are more sensitive to spontaneous inflammatory nociception—**Strains without the SINE insertion are more sensitive to spontaneous inflammatory nociception. Absence of the SINE insertion is positively and significantly correlated with increased pain sensitivity of inbred strains of mice in spontaneous inflammatory nociception assays. These include assays of subcutaneous injection of the inflammatory irritants bee venom, capsaicin or formalin to evoke paw licking, and intraperitoneal injection of acetic acid or magnesium sulfate to evoke abdominal constrictions or writhes (see Table 3 for statistical significance; (Lariviere *et al.*, 2002 for more details of the assays) for more details of the assays). Thermal nociception was also positively and significantly correlated with absence of the SINE haplotype in two of six assays, with the majority of assays showing no relation to SINE haplotype (Table 3). There was no consistent relationship of SINE haplotype with mechanical sensation or nociception in the von Frey and tail clip tests, respectively, or with a range of hypersensitivity assays tested with thermal or mechanical stimuli including hypersensitivity evoked by inflammatory irritants or nerve injury (Table 3). Multivariate analyses and the MDS and

PCA plots in Figure 5 confirm these individual findings and demonstrate graphically that absence of SINE haplotype is most genetically related to increased sensitivity to spontaneous inflammatory nociception. Note that the clustering of SINE status with spontaneous inflammatory nociception assays in the PCA plot was preserved when viewed in three dimensions (with 0.65 of the total variation accounted for), and more closely matched the correlations reported in Table 3 (e.g. strong correlation of absence of SINE element with Hargreaves' test of thermal nociception).

## Discussion

Commonly used inbred strains of mice exhibit broad phenotypic and genotypic variation. We measured behavioral phenotypes and gene expression variation across 29 inbred mouse strains and identified *Comt1* as being one of a number of genes that exhibit differential expression among inbred strains of mice. A cis-regulated pattern of gene expression was observed for all brain regions we examined. We have linked this gene expression difference to the insertion of a B2 SINE element in the 3′UTR of the gene such that mice with an insertion of the SINE element show increased expression and increased COMT1 enzymatic activity. Using a distance-weighted discrimination technique, we identified four behavioral phenotypes that contributed most strongly to discrimination of the status of the Comt1 haplotype. Our results indicate that mouse strains with the SINE element display increased locomotor activity, decreased rearing behavior and exhibit an increased anxiety response in the light/dark assay.

### The SINE element insertion is a recent event in inbred strains

The use of gene expression data as a quantitative trait has been successful for identification of eQTL (Chen *et al.*, 2008, Wu *et al.*, 2008). The actual polymorphism responsible for cis-regulation of gene expression, however, can often be difficult to identify. In mouse, Comt1 is located in a genomic region for which most strains share common haplotypes. We identified very few polymorphisms in the *Comt1* region within the common inbred strains (6 SNPs within 2.5 Mb). More extensive resequencing data (Frazer *et al.*, 2007) shows polymorphic variance only for wild-derived inbred strains and a similar pattern of two major haplotypes for laboratory inbred strains. Comt1 itself exhibits no coding sequence polymorphisms. In an attempt to identify non-coding regulatory polymorphisms we aligned sequence from BACs of two strains that were in different inferred haplotypes (C57BL/6J, 129/SvEvTac). A SINE element was discovered in the 3′UTR of Comt1. The SINE element insertion is likely to be of recent origin within the inbred strains as it is not found in wild-derived strains and it is not present in all C57 or 129-derived strains.

### Insertion of a B2 SINE element increases COMT1 enzymatic activity

Since Comt1 mRNA expression correlated to the presence or absence of the SINE insertion we predicted that we would detect concomitant changes in COMT1 protein levels. Our data did not reveal that protein levels correlated in a robust and consistent manner with SINE element status and although this could be due to secondary compensatory mechanisms it is more likely resultant from assay variability. However, our cell-based studies established that the presence of the SINE element does impact enzyme activity levels. It is not clear how a SINE element insertion can functionally cause a variation in the amount of measurable (mRNA) Comt1 and how this translates to variable enzyme activity and changes in protein levels. It is plausible that insertion of this SINE element affects mRNA secondary structure leading to alteration in mRNA degradation rate and protein folding. Protein folding may in turn affect protein stability, enzymatic activity or post-translational modification efficiency. Recently, the COMT enzyme has been shown to be phosphorylated and N-acetylated in rats (Overbye & Seglen, 2009) indicating that post-translational alterations of the protein are also

important and might be more mechanistically relevant considering the requirement for rapid action of COMT1 for catecholamine modulation. The exact mechanism by which the SINE element alters COMT1 enzymatic function will require further investigation.

## Behavioral effects of COMT enzymatic activity in inbred strains

The COMT protein is involved in catecholamine regulation, a pathway that has been implicated in behavioral function in both humans and animal models. Human COMT has been implicated in phenotypes related to pain, stress, anxiety and ADHD (reviewed by (Andersen & Skorpen, 2009, Harrison & Tunbridge, 2008, Lachman, 2008). In humans, a non-synonymous G→A base pair substitution results in a valine→methionine substitution at position 158. The $Met^{158}$ amino acid substitution results in a less active form of the enzyme (Chen *et al.*, 2004) thereby resulting in higher brain concentrations of dopamine. Presence of the $Met^{158}$ allele of *COMT* has been associated with poor emotional regulation for anxiety-related traits (Domschke *et al.*, 2004, Enoch *et al.*, 2003) and low sensation seeking (Stein *et al.*, 2005). However, it should be noted that the role of COMT in these behaviors in humans has not been consistent across all studies.

Animal models have also been useful for studying the role of COMT on behavior. Examination of *Comt1* knockout (KO) and transgenic (Tg) mice has addressed anxiety-related behaviors. Female *Comt1* KO mice show increased anxiety as measured by latency to emerge from the dark quadrant in the light/dark assay (Gogos *et al.*, 1998) and Tg mice carrying the human $Val^{158}$ allele exhibit decreased anxiety in the elevated plus maze (Papaleo *et al.*, 2008). The results from these animal studies concur with human studies linking increased enzymatic activity to decreased anxiety.

Based on the data observed in humans and animal models, one would expect that +SINE mice that have increased enzymatic activity, would exhibit decreased anxiety rather than increased anxiety as indicated by the observation that +SINE mice spend less time in the lighted quadrant of the light/dark arena in comparison with -SINE mice. However, +SINE mice also exhibit increased locomotor activation in a novel environment (the open field) and increased locomotion in response to novelty is believed to reflect decreased anxiety or emotionality (Fujita *et al.*, 1994, Kabbaj *et al.*, 2000). This theory is supported by the observation that some anxiolytics, particularly benzodiazepines like diazepam and chlordiazepoxide at moderate doses, increase locomotor activity in anxiety-related assays in mice (Choleris *et al.*, 2001, Vlainic & Pericic, 2009). Interestingly, female *Comt1* KO mice also display significantly less ambulatory activity than wild-type animals in the lighted quadrant of the light/dark arena (Gogos *et al.*, 1998) although we did not observe this difference among the inbred strains. The observation of increased locomotor response to novelty in +SINE mouse strains does seem to concur with the human observation that increased COMT enzymatic activity is associated with decreased anxiety. The seemingly disparate results from two behavioral measures of anxiety may not be surprising since different anxiety assays often do not correlate and may measure different components of anxiety-related behavior (Lister, 1990).

Exploratory activity in novel environments has also been described as an animal model for novelty seeking (Piazza *et al.*, 1989). Novelty and sensation seeking traits are correlated in human studies (McCourt *et al.*, 1993). A decrease in novelty-induced locomotion in mice with decreased COMT1 enzymatic activity corresponds with human studies that link the $Met^{158}$ allele of COMT with a decrease in sensation-seeking behavior (Lang *et al.*, 2007, Stein *et al.*, 2005).

+SINE mice also exhibit decreased rearing behavior in the open field. Rearing behavior in a novel environment has been described as both a measure of exploratory behavior and an

indicator of anxiety (Crusio *et al.*, 1989a, Crusio *et al.*, 1989b). However, previous studies in our laboratory and by others indicate that rearing behavior might also represent a distinct class of movements based on the lack of correlation between rearing behavior and anxiety- and locomotor-related behavior in common rodent tests for anxiety (Bailey *et al.*, 2008, Fernandez, 1997, Henderson *et al.*, 2004). Various studies have reported that anxiolytics cause a decrease (Crabbe *et al.*, 1998, Fahey *et al.*, 2001, Gray & McNaughton, 2000, Hughes, 1993, McNaughton, 1985), an increase (Crabbe *et al.*, 1998) or have no effect on rearing (Choleris *et al.*, 2001, Czech *et al.*, 2003). Some of these differences may reflect differences in the animal model (rat vs. mice), anxiolytic, dose or behavioral assay. Based on studies that have shown a decrease in rearing behavior in response to anxiolytics, one might hypothesize that the decrease in rearing behavior observed in the +SINE mice is also a reflection of decreased anxiety. However, until the neurobiological and neurochemical mechanisms that govern rearing behavior and anxiety are more fully understood, such an interpretation is conjecture at this point.

### Relevance of the SINE element to pain sensitivity

In humans, lower levels of COMT activity have been linked to heightened pain perception (Diatchenko *et al.*, 2006a, Diatchenko *et al.*, 2005). *Comt1* KO mice also exhibit increased pain sensitivity (Papaleo *et al.*, 2008) and *Tg* mice carrying the human $Val^{158}$ allele are more resistant to pain. The results of our study are consistent with decreased pain sensitivity in inbred strains with increased COMT activity. Previous reports in both rodents and humans show a stronger relationship of *COMT* genotype with noxious thermal stimuli over pressure stimuli (Diatchenko *et al.*, 2006b) which is consistent with our results indicating a stronger positive association with sensitivity in several thermal nociception assays but not with mechanical nociception. However, in the current study the strongest relationship between *Comt1* genotype was with inflammatory nociception – an assay that has not been assessed with regard to COMT activity in humans. This novel finding suggests specific relationship of *Comt1* genotype with the immediate effects of and spontaneous responses to nociceptive inflammatory insults, but not with the more prolonged consequences of inflammation- or nerve injury-induced hypersensitivity (Table 3). It is currently unknown how this specificity arises, but because *COMT* genotype differences are likely to be mediated via beta-2 and beta-3 adrenergic receptors, a stronger positive association with sensitivity in several thermal nociception assays but not with mechanical nociception (Nackley *et al.*, 2007) is consistent with our results. Specific hypotheses regarding heritable differences in the effects of modulation of adrenergic receptor activity and relative specificity for inflammatory pain over other pain types should be tested in both humans and animal models.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

Andersen S, Skorpen F. Variation in the COMT gene: implications for pain perception and pain treatment. Pharmacogenomics. 2009; 10:669–684. [PubMed: 19374521]

Axelrod J, Tomchick R. Enzymatic O-methylation of epinephrine and other catechols. J Biol Chem. 1958; 233:702–705. [PubMed: 13575440]

Bailey JS, Grabowski-Boase L, Steffy BM, Wiltshire T, Churchill GA, Tarantino LM. Identification of QTL for locomotor activation and anxiety using closely-related inbred strains. Genes Brain Behav. 2008

Benito M, Parker J, Du Q, Wu J, Xiang D, Perou CM, Marron JS. Adjustment of systematic microarray data biases. Bioinformatics. 2004; 20:105–114. [PubMed: 14693816]

Chen J, Lipska BK, Halim N, Ma QD, Matsumoto M, Melhem S, Kolachana BS, Hyde TM, Herman MM, Apud J, Egan MF, Kleinman JE, Weinberger DR. Functional analysis of genetic variation in catechol-O-methyltransferase (COMT): effects on mRNA, protein, and enzyme activity in postmortem human brain. Am J Hum Genet. 2004; 75:807–821. [PubMed: 15457404]

Chen Y, Zhu J, Lum PY, Yang X, Pinto S, MacNeil DJ, Zhang C, Lamb J, Edwards S, Sieberts SK, Leonardson A, Castellini LW, Wang S, Champy MF, Zhang B, Emilsson V, Doss S, Ghazalpour A, Horvath S, Drake TA, Lusis AJ, Schadt EE. Variations in DNA elucidate molecular networks that cause disease. Nature. 2008; 452:429–435. [PubMed: 18344982]

Choleris E, Thomas AW, Kavaliers M, Prato FS. A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. Neurosci Biobehav Rev. 2001; 25:235–260. [PubMed: 11378179]

Crabbe JC, Gallaher EJ, Cross SJ, Belknap JK. Genetic determinants of sensitivity to diazepam in inbred mice. Behav Neurosci. 1998; 112:668–677. [PubMed: 9676982]

Crusio WE, Schwegler H, Brust I, Van Abeelen JH. Genetic selection for novelty-induced rearing behavior in mice produces changes in hippocampal mossy fiber distributions. J Neurogenet. 1989a; 5:87–93. [PubMed: 2703943]

Crusio WE, Schwegler H, van Abeelen JH. Behavioral responses to novelty and structural variation of the hippocampus in mice. I. Quantitative-genetic analysis of behavior in the open-field. Behav Brain Res. 1989b; 32:75–80. [PubMed: 2930636]

Czech DA, Jacobson EB, LeSueur-Reed KT, Kazel MR. Putative anxiety-linked effects of the nitric oxide synthase inhibitor L-NAME in three murine exploratory behavior models. Pharmacol Biochem Behav. 2003; 75:741–748. [PubMed: 12957214]

DeYoung CG, Getchell M, Koposov RA, Yrigollen CM, Haeffel GJ, af Klinteberg B, Oreland L, Ruchkin VV, Pakstis AJ, Grigorenko EL. Variation in the catechol-O-methyltransferase Val 158 Met polymorphism associated with conduct disorder and ADHD symptoms, among adolescent male delinquents. Psychiatr Genet. 2010; 20:20–24. [PubMed: 19997043]

Diatchenko L, Nackley AG, Slade GD, Bhalang K, Belfer I, Max MB, Goldman D, Maixner W. Catechol-O-methyltransferase gene polymorphisms are associated with multiple pain-evoking stimuli. Pain. 2006a; 125:216–224. [PubMed: 16837133]

Diatchenko L, Nackley AG, Slade GD, Fillingim RB, Maixner W. Idiopathic pain disorders--pathways of vulnerability. Pain. 2006b; 123:226–230. [PubMed: 16777329]

Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, Goldman D, Xu K, Shabalina SA, Shagin D, Max MB, Makarov SS, Maixner W. Genetic basis for individual variations in pain perception and the development of a chronic pain condition. Hum Mol Genet. 2005; 14:135–143. [PubMed: 15537663]

Domschke K, Deckert J, O'Donovan M C, Glatt SJ. Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. Am J Med Genet B Neuropsychiatr Genet. 2007; 144B:667–673. [PubMed: 17357147]

Domschke K, Freitag CM, Kuhlenbaumer G, Schirmacher A, Sand P, Nyhuis P, Jacob C, Fritze J, Franke P, Rietschel M, Garritsen HS, Fimmers R, Nothen MM, Lesch KP, Stogbauer F, Deckert J. Association of the functional V158M catechol-O-methyl-transferase polymorphism with panic disorder in women. Int J Neuropsychopharmacol. 2004; 7:183–188. [PubMed: 15009906]

Eisener-Dorman AF, Grabowski-Boase L, Steffy BM, Wiltshire T, Tarantino LM. Quantitative trait locus and haplotype mapping in closely related inbred strains identifies a locus for open field behavior. Mamm Genome.

Eleftheriou BE. Regional brain catechol-O-methyl transferase: age related differences in the mouse. Exp Aging Res. 1975; 1:99–103. [PubMed: 1053209]

Emin Erdal M, Herken H, Yilmaz M, Bayazit YA. Significance of the catechol-O-methyltransferase gene polymorphism in migraine. Brain Res Mol Brain Res. 2001; 94:193–196. [PubMed: 11597779]

Enoch MA, Xu K, Ferro E, Harris CR, Goldman D. Genetic origins of anxiety in women: a role for a functional catechol-O-methyltransferase polymorphism. Psychiatr Genet. 2003; 13:33–41. [PubMed: 12605099]

Evans J, Xu K, Heron J, Enoch MA, Araya R, Lewis G, Timpson N, Davies S, Nutt D, Goldman D. Emotional symptoms in children: The effect of maternal depression, life events, and COMT genotype. Am J Med Genet B Neuropsychiatr Genet. 2009; 150B:209–218. [PubMed: 18535998]

Fahey JM, Pritchard GA, Grassi JM, Pratt JS, Shader RI, Greenblatt DJ. Pharmacodynamic and receptor binding changes during chronic lorazepam administration. Pharmacol Biochem Behav. 2001; 69:1–8. [PubMed: 11420062]

Fernandez EE. Structure of the mouse behaviour on the elevated plus-maze test of anxiety. Behav Brain Res. 1997; 86:105–112. [PubMed: 9105588]

Frazer KA, Eskin E, Kang HM, Bogue MA, Hinds DA, Beilharz EJ, Gupta RV, Montgomery J, Morenzoni MM, Nilsen GB, Pethiyagoda CL, Stuve LL, Johnson FM, Daly MJ, Wade CM, Cox DR. A sequence-based variation map of 8.27 million SNPs in inbred mouse strains. Nature. 2007; 448:1050–1053. [PubMed: 17660834]

Fujita O, Annen Y, Kitaoka A. Tsukuba high- and low-emotional strains of rats (Rattus norvegicus): an overview. Behav Genet. 1994; 24:389–415. [PubMed: 7993317]

Gogos JA, Morgan M, Luine V, Santha M, Ogawa S, Pfaff D, Karayiorgou M. Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. Proc Natl Acad Sci U S A. 1998; 95:9991–9996. [PubMed: 9707588]

Gray, J.; McNaughton, N. The neuropsychology of anxiety. Oxford University Press; Oxford: 2000.

Guyton, AC. Textbook of Medical Physiology. Wonsiewicz, MJ., editor. W.B. Saunders Company; Philidelphia: 1991. p. 676

Harrison PJ, Tunbridge EM. Catechol-O-methyltransferase (COMT): a gene contributing to sex differences in brain function, and to sexual dimorphism in the predisposition to psychiatric disorders. Neuropsychopharmacology. 2008; 33:3037–3045. [PubMed: 17805313]

Henderson ND, Turri MG, DeFries JC, Flint J. QTL analysis of multiple behavioral measures of anxiety in mice. Behav Genet. 2004; 34:267–293. [PubMed: 14990867]

Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, Nobel A, Parker J, Ewend MG, Sawyer LR, Wu J, Liu Y, Nanda R, Tretiakova M, Ruiz Orrico A, Dreher D, Palazzo JP, Perreard L, Nelson E, Mone M, Hansen H, Mullins M, Quackenbush JF, Ellis MJ, Olopade OI, Bernard PS, Perou CM. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics. 2006; 7:96. [PubMed: 16643655]

Hughes RN. Effects on open-field behavior of diazepam and buspirone alone and in combination with chronic caffeine. Life Sci. 1993; 53:1217–1225. [PubMed: 8412479]

Kabbaj M, Devine DP, Savage VR, Akil H. Neurobiological correlates of individual differences in novelty-seeking behavior in the rat: differential expression of stress-related molecules. J Neurosci. 2000; 20:6983–6988. [PubMed: 10995843]

Lachman HM. Does COMT val158met affect behavioral phenotypes: yes, no, maybe? Neuropsychopharmacology. 2008; 33:3027–3029. [PubMed: 18923401]

Lang UE, Bajbouj M, Sander T, Gallinat J. Gender-dependent association of the functional catechol-O-methyltransferase Val158Met genotype with sensation seeking personality trait. Neuropsychopharmacology. 2007; 32:1950–1955. [PubMed: 17299513]

Lariviere WR, Wilson SG, Laughlin TM, Kokayeff A, West EE, Adhikari SM, Wan Y, Mogil JS. Heritability of nociception. III. Genetic relationships among commonly used assays of nociception and hypersensitivity. Pain. 2002; 97:75–86. [PubMed: 12031781]

Lister RG. Ethologically-based animal models of anxiety disorders. Pharmacol Ther. 1990; 46:321–340. [PubMed: 2188266]

Marron JS, Todd MJ, Ahn J. Distance-Weighted Discrimination. Journal of the American Statistical Association. 2007; 102:1267–1271.

McClurg P, Janes J, Wu C, Delano DL, Walker JR, Batalov S, Takahashi JS, Shimomura K, Kohsaka A, Bass J, Wiltshire T, Su AI. Genomewide association analysis in diverse inbred mice: power and population structure. Genetics. 2007; 176:675–683. [PubMed: 17409088]

McClurg P, Pletcher MT, Wiltshire T, Su AI. Comparative analysis of haplotype association mapping algorithms. BMC Bioinformatics. 2006; 7:61. [PubMed: 16466585]

McCourt WF, Gurrera RJ, Cutter HS. Sensation seeking and novelty seeking. Are they the same? J Nerv Ment Dis. 1993; 181:309–312. [PubMed: 8501447]

McNaughton N. The effects of systemic and intraseptal injections of sodium amylobarbitone on rearing and ambulation in rats. Australian Journal of Psychology. 1985; 37:15–27.

Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. Pain. 1999a; 80:67–82. [PubMed: 10204719]

Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. Heritability of nociception II. 'Types' of nociception revealed by genetic correlation analysis. Pain. 1999b; 80:83–93. [PubMed: 10204720]

Nackley AG, Diatchenko L. Assessing potential functionality of catechol-O-methyltransferase (COMT) polymorphisms associated with pain sensitivity and temporomandibular joint disorders. Methods Mol Biol. 2010; 617:375–393. [PubMed: 20336436]

Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchynskyi O, Makarov SS, Maixner W, Diatchenko L. Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. Science. 2006; 314:1930–1933. [PubMed: 17185601]

Nackley AG, Tan KS, Fecho K, Flood P, Diatchenko L, Maixner W. Catechol-O-methyltransferase inhibition increases pain sensitivity through activation of both beta2- and beta3-adrenergic receptors. Pain. 2007; 128:199–208. [PubMed: 17084978]

Overbye A, Seglen PO. Phosphorylated and non-phosphorylated forms of catechol O-methyltransferase in rat liver, brain and other tissues. Biochem J. 2009; 417:535–545. [PubMed: 18831714]

Palmason H, Moser D, Sigmund J, Vogler C, Hanig S, Schneider A, Seitz C, Marcus A, Meyer J, Freitag CM. Attention-deficit/hyperactivity disorder phenotype is influenced by a functional catechol-O-methyltransferase variant. J Neural Transm. 2009

Papaleo F, Crawley JN, Song J, Lipska BK, Pickel J, Weinberger DR, Chen J. Genetic dissection of the role of catechol-O-methyltransferase in cognition and stress reactivity in mice. J Neurosci. 2008; 28:8709–8723. [PubMed: 18753372]

Piazza PV, Deminiere JM, Le Moal M, Simon H. Factors that predict individual vulnerability to amphetamine self-administration. Science. 1989; 245:1511–1513. [PubMed: 2781295]

Pletcher MT, McClurg P, Batalov S, Su AI, Barnes SW, Lagler E, Korstanje R, Wang X, Nusskern D, Bogue MA, Mural RJ, Paigen B, Wiltshire T. Use of a dense single nucleotide polymorphism map for in silico mapping in the mouse. PLoS Biol. 2004; 2:e393. [PubMed: 15534693]

Pooley EC, Fineberg N, Harrison PJ. The met(158) allele of catechol-O-methyltransferase (COMT) is associated with obsessive-compulsive disorder in men: case-control study and meta-analysis. Mol Psychiatry. 2007; 12:556–561. [PubMed: 17264842]

Qian Q, Wang Y, Zhou R, Li J, Wang B, Glatt S, Faraone SV. Family-based and case-control association studies of catechol-O-methyltransferase in attention deficit hyperactivity disorder suggest genetic sexual dimorphism. Am J Med Genet B Neuropsychiatr Genet. 2003; 118B:103–109. [PubMed: 12627475]

Stein MB, Fallin MD, Schork NJ, Gelernter J. COMT polymorphisms and anxiety-related personality traits. Neuropsychopharmacology. 2005; 30:2092–2102. [PubMed: 15956988]

Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A. 2004; 101:6062–6067. [PubMed: 15075390]

Vlainic J, Pericic D. Effects of acute and repeated zolpidem treatment on pentylenetetrazole-induced seizure threshold and on locomotor activity: comparison with diazepam. Neuropharmacology. 2009; 56:1124–1130. [PubMed: 19345234]

Wu C, Delano DL, Mitro N, Su SV, Janes J, McClurg P, Batalov S, Welch GL, Zhang J, Orth AP, Walker JR, Glynne RJ, Cooke MP, Takahashi JS, Shimomura K, Kohsaka A, Bass J, Saez E, Wiltshire T, Su AI. Gene set enrichment in eQTL data identifies novel annotations and pathway regulators. PLoS Genet. 2008; 4:e1000070. [PubMed: 18464898]

Yang H, Bell TA, Churchill GA, Pardo-Manuel de Villena F. On the subspecific origin of the laboratory mouse. Nat Genet. 2007; 39:1100–1107. [PubMed: 17660819]

Zubieta JK, Heitzeg MM, Smith YR, Bueller JA, Xu K, Xu Y, Koeppe RA, Stohler CS, Goldman D. COMT val158met genotype affects mu-opioid neurotransmitter responses to a pain stressor. Science. 2003; 299:1240–1243. [PubMed: 12595695]

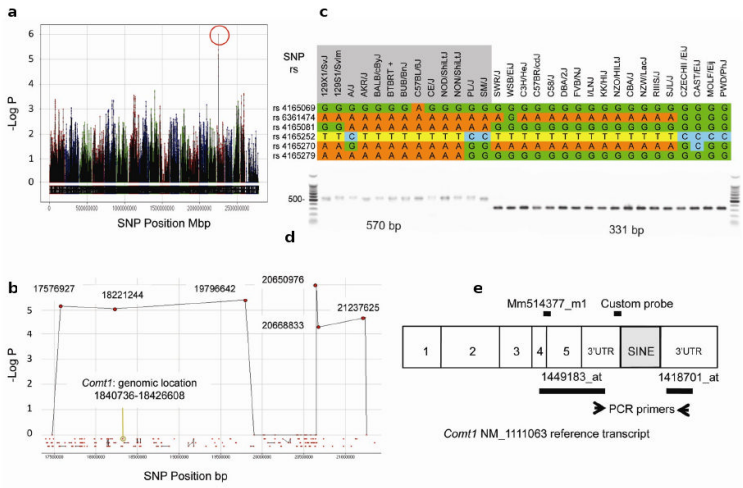


**Figure 1.**

Identification of *Comt1* as a cis-regulated gene. **a.** Genome wide association analysis (HAM) of *Comt1* RNA expression levels for probe set 1418701_at in male pituitary across 29 strains. The genomic position of SNPs is displayed on the X axis in cumulative position in the genome. Chromosomes are sequentially colored (1-19 and X). The Y axis displays the association score (-LogP) for association of between strain gene expression patterns with each inferred haplotype. The highest association on Chr. 16 has a maximal score, -LogP = 6. **b.** Expanded view of the 4 MB region surrounding the *Comt1* locus. Eight SNPs with −LogP=0 are non-informative for these strains, thus producing a break in the QTL locus. The red markers below zero on the Y axis define all Affymetrix 430v2 probe sets in their correct genomic location. **c.** SNP allele calls for 29 inbred mouse strains at this locus define at least four distinct haplotypes. However, there are two major haplotype groups and all strains in this interval are also characterized by the presence (grey) or absence of a SINE element in the 3′UTR of *Comt1*. **d.** The presence of the B2 SINE element is demonstrated by PCR. Strains are aligned in the same order. **e.** cDNA structure of *Comt1* illustrating exons, position of the SINE insertion, Affymetrix and TaqMAN probe sets, diagnostic PCR primers and coding exons.

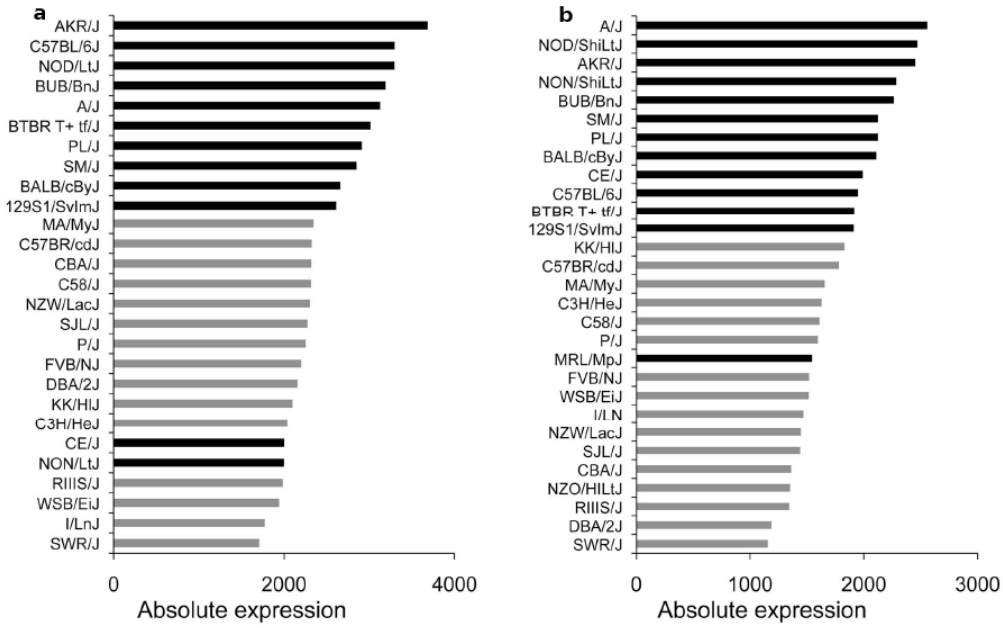


**Figure 2.**

Gene expression values for *Comt1* (Affymetrix probe 1449183_at) in: **a.** prefrontal cortex (27 strains) and **b.** nucleus accumbens (29 strains). Three male animals were pooled from each strain. Each result is also characterized by the presence or absence (grey) of a SINE element in the 3′UTR of *Comt1*.

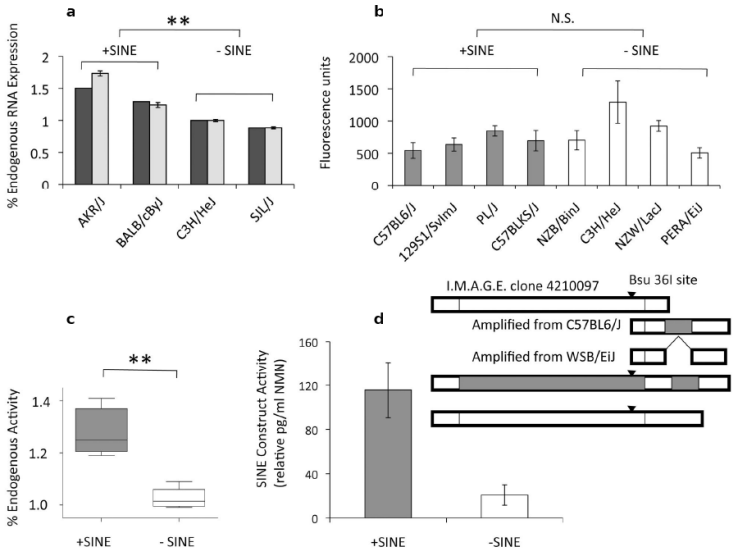


**Figure 3.**

Functional analysis of *Comt1* variants. Microarray gene expression results were validated using TaqMan assays. **a.** Array data for Affymetrix probe 1449183_at and qPCR (grey) from prefrontal cortex RNA: 4 strains, 4 male animals per strain demonstrate reproducible values between q-PCR and array data. Data normalized to C3H/HeJ; error bars are S.E. **P<0.01, 1-tailed paired t-test. **b.** An ELISA assay was developed to assess COMT1 protein levels. COMT1 protein levels in prefrontal cortex were measured for male animals in 8 strains, (+SINE grey) error bars are S.E. **c.** Enzymatic activity of COMT1 protein was measured using a Normetanephrine ELISA assay. Prefrontal cortex lysates from 2 to 4 male animals per strain were collected. Ten strains were assayed: 6 +SINE (grey), and 4 −SINE. Data normalized to C3H/HeJ and then analyzed by 1-tailed Mann Whitney test, **P < 0.01 **d.** Full length cDNA constructs of both +SINE and -SINE haplotypes were prepared and transiently transfected into PC12 cells. Cell lysates were tested for enzymatic activity in the same manner as brain tissue samples.

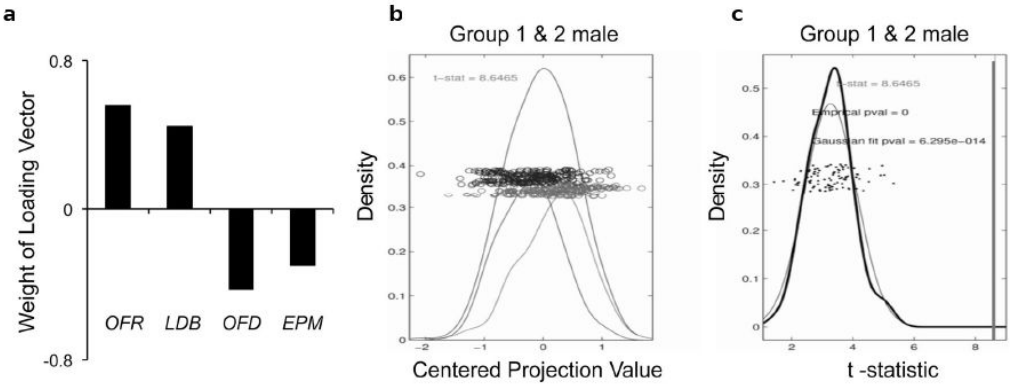


**Figure 4.**

Behavioral assays from individual animals are correlated with the presence or absence of the SINE element in *Comt1*. Males were tested in two separate groups depending on assay. **a.** The four most significant behaviors that discriminate for the presence or absence of the SINE element in Comt1 are shown on a DWD loading plot (male) *OFR* - open field, rearing, *LDB* - Light/Dark Box, % time in light, *OFD* - Open Field, total distance, *EPM* - Elevated Plus Maze, total distance. **b.** Data projection plot on DWD direction of contributing behavioral assays, grey points indicate scores from +SINE haplotype, black from -SINE. **c.** DiProPerm plot of t-statistics. The DiProPerm test plot depicts the 100 t-statistics as 100 black dots under a Gaussian peak. The t-statistic for the DWD direction plot is depicted as a black line. Similar scores are seen for female mice.

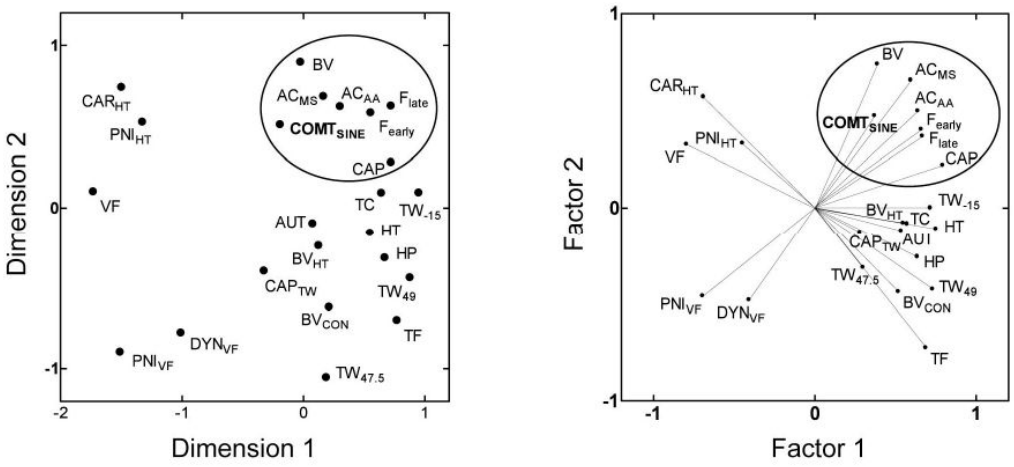


**Figure 5.**

Multivariate analyses of cross-correlations between negative SINE status of inbred mouse strains and strain means for 22 assays of sensation, nociception and hypersensitivity (corrected for sensitivity; see Table 2 for abbreviations). MDS (**a**) and PCA (**b**) plots show that strains without a SINE element have increased sensitivity to spontaneous inflammatory nociception assays. In the MDS plot, the Euclidean distances between the point for *Comt1* SINE status and the points for sensitivity in the pain models are representative of their Pearson product-moment correlations; points for traits with higher positive correlations are closer (see text). Using a Kruskal loss function with monotonic regression, the final stress was 0.21. The proportion of total variance accounted for is 0.81. In the PCA plot, the angles between rays projecting to the points are representative of the correlations between the two traits (see text). The proportion of total variance accounted for is 0.53. Circles indicate the set of all spontaneous inflammatory nociception assays and demonstrate the overall proximity and strong genetic correlation of negative SINE status with this type of nociception.

**Table 1**

Association of the presence or absence of the SINE element with gene expression of *Comt1* in seven brain regions.

| | Female | | | | | | | Male | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | - SINE Haplotype | | | + SINE Haplotype | | | | - SINE Haplotype | | | + SINE Haplotype | | | |
| **Brain Region** | **N** | **Mean** | **Std Err** | **N** | **Mean** | **Std Err** | **P value** | **N** | **Mean** | **Std Err** | **N** | **Mean** | **Std Err** | **P value** |
| *Z*-score | 16 | -5.86 | 0.15 | 13 | -2.47 | 0.36 | P<0.0001 | 16 | 2.24 | 0.37 | 13 | 6.94 | 0.52 | P<0.0001 |
| Nucleus Accumbens | 16 | 1141 | 72.9 | 13 | 1072 | 38.5 | P=0.437 | 15 | 2136 | 53.28 | 12 | 2887 | 146.84 | P<0.0001 |
| Prefrontal Cortex | 16 | 791.4 | 15.08 | 13 | 1134 | 57.98 | P<0.0001 | 16 | 1493 | 47.29 | 13 | 2131 | 77.2 | P<0.0001 |
| Amygdala | 16 | 320.8 | 7.78 | 13 | 458.6 | 13.03 | P<0.0001 | 16 | 1507 | 34.76 | 13 | 1888 | 44.39 | P<0.0001 |
| Hypothalamus | 16 | 789.8 | 13.89 | 13 | 1267 | 48.72 | P<0.0001 | 16 | 1310 | 104.5 | 13 | 1852 | 129.18 | P<0.01 |
| Hippocampus | 16 | 737.8 | 18.3 | 13 | 1027 | 28.13 | P<0.0001 | 15 | 2370 | 139.2 | 13 | 2690 | 96.5 | P<0.05 |
| Striatum | 16 | 893.1 | 25.15 | 13 | 1371 | 65.46 | P<0.0001 | 11 | 508.2 | 19.91 | 12 | 718.5 | 43.25 | P<0.0001 |
| Pituitary | 15 | 805.7 | 33.25 | 13 | 814 | 38.87 | P=0.872 | 16 | 2444 | 59.28 | 13 | 2679 | 89.93 | P<0.05 |

**Table 2**

Behavioral scores that best discriminate the +SINE/-SINE haplotypes

| | Female | Male |
|---|---|---|
| Behavioral phenotype | *OFR, LDB, OFD, EPM* | *OFR, LDB, OFD, EPM* |
| DiProPerm t-statistic | 10.501 | 8.6465 |
| P value | P<0.0001 | P<0.0001 |

## Table 3

Correlations between negative SINE status and strain means for 22 assays of sensation, nociception and hypersensitivity (corrected for sensitivity; positive correlation indicates absence of SINE haplotype associated with increased sensitivity in the assay).

| Abbreviation | Description of pain model | Pearson Correlation (Strains in Common) |
|---|---|---|
| *Spontaneous inflammatory nociception* | | |
| $AC_{AA}$ | Abdominal constriction (writhing) test – acetic acid | 0.46 (11) |
| $AC_{MS}$ | Abdominal constriction (writhing) test – magnesium sulfate | 0.61* (11) |
| BV | Bee venom-induced spontaneous pain behavior (licking) | 0.55* (12) |
| CAP | Capsaicin-induced spontaneous pain behavior (licking) | 0.27 (12) |
| $F_{early}$ | Early/acute phase of formalin test | 0.64* (11) |
| $F_{late}$ | Late/tonic phase of formalin test | 0.35 (11) |
| *Thermal nociception* | | |
| $TW_{-15}$ | Tail withdrawal from -15°C ethanol | 0.05 (12) |
| HP | Hot-plate test | 0.41 (11) |
| HT | Hargreaves et al.'s thermal paw-withdrawal test | 0.60* (11) |
| TF | Tail-flick from radiant heat source | -0.21 (10) |
| $TW_{47.5}$ | Tail withdrawal from 47.5°C water | -0.28 (12) |
| $TW_{49}$ | Tail withdrawal from 49°C water | -0.20 (11) |
| *Mechanical nociception* | | |
| TC | Tail-clip test | 0.33 (12) |
| *Mechanical sensitivity* | | |
| VF | von Frey monofilament test | -0.01 (11) |
| *Mechanical hypersensitivity* | | |
| $DYN_{VF}$ | Dynorphin-induced mechanical hypersensitivity assessed with von Frey monofilament test | 0.29 (7) |
| $PNI_{VF}$ | Peripheral nerve injury-induced mechanical hypersensitivity assessed with von Frey monofilament test | -0.23 (11) |
| *Thermal and afferent-dependent hypersensitivities* | | |
| AUT | Autotomy following sciatic and saphenous nerve transection | 0.00 (11) |
| $BV_{HT}$ | Bee venom-induced thermal hypersensitivity assessed with Hargreaves' test (ipsilateral) | 0.31 (12) |
| $BV_{CON}$ | Contralateral bee venom-induced thermal hypersensitivity assessed with Hargreaves' test | 0.14 (12) |
| $CAP_{TW}$ | Capsaicin-induced thermal hypersensitivity assessed with tail-withdrawal test, 47°C water | 0.20 (12) |
| $CAR_{HT}$ | Carrageenan-induced thermal hypersensitivity assessed with Hargreaves' test | -0.09 (11) |
| $PNI_{HT}$ | Peripheral nerve injury-induced thermal hypersensitivity assessed with Hargreaves' test | -0.08 (11) |

*Statistically significant ($P < 0.05$, one-tailed test, uncorrected).