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Cadherin 13: Human *cis*-Regulation and Selectively Altered Addiction Phenotypes and Cerebral Cortical Dopamine in Knockout Mice

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The cadherin 13 (*CDH13*) gene encodes a cell adhesion molecule likely to influence development and connections of brain circuits that modulate addiction, locomotion and cognition, including those that involve midbrain dopamine neurons. Human *CDH13* mRNA expression differs by more than 80% in postmortem cerebral cortical samples from individuals with different *CDH13* genotypes, supporting examination of mice with altered CDH13 expression as models for common human variation at this locus. Constitutive *CDH13* knockout mice display evidence for changed cocaine reward: shifted dose response relationship in tests of cocaine-conditioned place preference using doses that do not alter cocaine-conditioned taste aversion. Reduced adult *CDH13* expression in conditional knockouts also alters cocaine reward in ways that correlate with individual differences in cortical *CDH13* mRNA levels. In control and comparison behavioral assessments, knockout mice display modestly quicker acquisition of rotarod and water maze tasks, with a trend toward faster acquisition of 5-choice serial reaction time tasks that otherwise displayed no genotype-related differences. They display significant differences in locomotion in some settings, with larger effects in males. In assessments of brain changes that might contribute to these behavioral differences, there are selective alterations of dopamine levels, dopamine/metabolite ratios, dopaminergic fiber densities and mRNA encoding the activity dependent transcription factor *npas4* in cerebral cortex of knockout mice. These novel data and previously reported human associations of *CDH13* variants with addiction, individual differences in responses to stimulant administration and attention deficit hyperactivity disorder (ADHD) phenotypes suggest that levels of *CDH13* expression, through mechanisms likely to include effects on mesocortical dopamine, influence stimulant reward and may contribute modestly to cognitive and locomotor phenotypes relevant to ADHD.

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INTRODUCTION

Cadherin 13 (*CDH13*) encodes a glycosylphosphatidyl inositol-anchored cell adhesion molecule whose variation is likely to alter connections between neurons in which it is expressed (1,2). *CDH13* mRNA is prominently expressed by ventral tegmental area and substantia nigra pars compacta neurons that are implicated in reward, locomotor control and cognitive modulation (3,4). This localization increases interest in effects of *CDH13* variation on dopaminergic brain systems and dopamine-associated behaviors.

Experiments that test the consequences of modifying *CDH13* expression are also motivated by human molecular genetic studies. For addiction vulnerability and attention deficit hyperactivity disorder (ADHD), the *CDH13* locus is marked by groups of clustered, nearby single nucleatide polymorphisms (SNPs) that display associations with $10^{-2} > p > 10^{-8}$ statistical significance in numerous independent case versus control genome wide association (GWAS) studies (5–15). *CDH13* associations with the numbers of cigarettes smoked per day, reward reported after

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oral modest amphetamine doses and effects of individuals' first five uses of alcohol are also described (7,16,17).

PubMed searches for cadherin 13 identify nine publications relating to normal brain, most of which relate to abovementioned CDH13 associations with substance dependence or ADHD. Two reports describe different *CDH13* knockout mice, one with altered hippocampal function and modest influences on freezing and reversal learning during fear conditioning and Barnes maze tests (19,20). We now report the correlation of human *CDH13* genotypes with individual differences in levels of *CDH13* mRNAs in postmortem cerebral cortical samples. This finding, and failure to identify common disease-associated missense *CDH13* variants, indicate that altered levels of *CDH13* expression may represent a major genetic contribution to human interindividual differences at this locus. These human results help to motivate a variety of studies in mice with altered *CDH13* expression.

In this paper, we study mice with constitutively altered and with adult (*via* conditional knockout) reductions in levels of *CDH13* mRNA. We test these mice using conditioned place preference (CPP), a model of rewarding influences from drugs that has been highly validated in pharmacologic studies (18). Results support influences of *CDH13* variation on addiction-related phenotypes and the possibility that drugs that modify activities of *CDH13* might be useful when given to adults. Conclusions from CPP tests are buttressed by results of evaluations of other phenotypes, including tests of motor abilities (reductions might confound CPP results), learning/ memory (reductions might confound CPP results), aversive features caused by cocaine (which might provide an alternative explanation for CPP results to "altered reward"), impulsivity (which might fit with ADHD influences but confound CPP results) and anxiety (which might confound CPP results). We do report more subtle influences of CDH13 knockout on ADHD-related tests of locomotion in male mice and on performance during early acquisition trials for complex tasks.

When we test levels of monoamines in brains of the knockout mice as possible contributors to observed behavioral differences, we identify selectively altered cerebral cortical dopamine/ metabolite ratios and differences in dopaminergic fiber densities. These novel data, and recent work by others, provide substantial *a posteriori* likelihood that common human *CDH13* "level of expression" variation contributes to differences in brain connections that are reflected in phenotypes relevant to addiction, stimulant actions, locomotor control and learning of complex tasks.

MATERIALS AND METHODS

Human brain samples for studies of *CDH13* mRNAs in individuals with different *CDH13* SNP alleles were obtained under protocols overseen by the Johns Hopkins (19% of samples) and University of Maryland (81% of samples) Institutional Review Boards. Mouse breeding and all experiments were performed under protocols approved by the NIDA-IRP Institutional Animal Care and Use Committee that complied with the Guide for the Care and Use of Laboratory Animals (8th edition, NRC 2011).

Human *CDH13* mRNA correlation with *CDH13* genomic markers was sought in frontal cortical autopsy samples (Johns Hopkins and University of Maryland brain banks) from European American individuals who lacked gross neuropathology. We focused on this genetic background and this brain region since this focus allow us to assemble a large a group of brain samples not possible using material from other brain regions or individuals with different racial/ ethnic backgrounds. Most common causes of death were accidents/multiple trauma, cardiovascular disease and pulmonary embolisms. Average time to freezing was14 h. Brains came from individuals who were 50.5% female and with an average age 42. RNAs were prepared with the RNeasy lipid tissue mini kits (Qiagen), cDNA synthesized with SuperScript III first strand synthesis supermix (Invitrogen) and levels of mRNAs assessed by quantitative real-time polymerase chain reaction (RT-PCR) using SybrGreen master mix (Applied Biosystems) according to the manufacturer's protocol using oligonucleotide primers (sequences available from authors on request) that targeted the dominant long *CDH13* mRNA isoform 1 (www.ncbi.nlm.nih.gov/IEB/Research/Acembly) and the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and ubiquitin C (UBC).

CDH13 SNP genotyping was performed in DNA extracted from brain samples using Qiagen kits genotyped in multiplexed Sequenom panels (see Supplementary Materials).

Constitutive *CDH13* knockout mice were produced by standard methods using embryonic stem cells from 129/sv mice and backcrossed for more than 15 generations to C57BL/6J mice (19). Mice from heterozygote × heterozygote matings were genotyped as described (19) and tested at 90 ± 43 d of age.

Conditional *CDH13* knockout mice. $CDH13_{loxP}$ mice were produced as described (20) and bred with UBC-Cre/ERT2 mice (Jackson Laboratory; #008085) to obtain homozygous $CDH13_{loxP/loxP}$; UBC-Cre/ERT2 ± and $CDH13_{loxP/loxP}$; $UBC\text{-}Cre/ERT2_{-/-}$ mice.

CDH13 deletion was induced by treating adult mice (average 89-d-old) with tamoxifen (Sigma; 200 mg/kg/day intraperitoneal (*i.p.*)) for five days approximately two weeks prior to testing.

Cocaine-conditioned place preference was assessed as described (21,22). Briefly, preferences for one side of the apparatus were assessed, four 20 min conditioning trials conducted (two cocaine, two saline) and preferences were assessed again 24 h after the last conditioning session. Differences between times spent on the drug-paired side during the post- versus pre-tests were noted.

Cocaine-conditioned taste aversion consisted of habituation, conditioning

and two-bottle testing, as described (23). After acclimation, handling and 20 min/d access to water, mice were given four series of 20-min conditioning sessions during which they were provided access to 1 g/L saccharin solution, then injected within 20 min with 0, 5, 10 or 20 mg/kg cocaine i.p., then 2 d of water recovery. Saccharin solution consumed when mice were given access to both saccharin and water for 20 min in a two-bottle-conditioned taste avoidance test was assessed.

Locomotion was recorded for 60 min in 42 × 42 cm dark, sound-attenuated boxes to which the mice had not been previously exposed and during conditioned place preference sessions of 20 min pretest (access to both halves of the 20 × 40 conditioning apparatus), conditioning (access to only 20 × 20 cm half of the apparatus) and test (access to 20 × 40 cm) sessions. Distances traveled were calculated from infrared beam breaks (Optovarimax ATS System) (22).

Motor coordination and learning were tested once per day over three consecutive days on a rotarod that accelerated from 4 to 40 rpm over 5 min.

Memory and learning were evaluated in a Morris water maze (24). A black 90-cm diameter pool filled with opaque 22°C water contained a 9-cm platform in the center of one quadrant that was visible for the first six trials and hidden 0.5 cm below the water level for subsequent trials. Mice received two trials, each lasting a maximum of 60 s, separated by a 15 s rest period on the platform. They were returned to home cages for 4 h, then given an additional 2-trial session. After acquisition, defined by an average latency to reach the platform of less than 10 s, the platform was removed and a 60 s probe trial conducted. Probe trial data including the path of the subject in the pool and the time spent in each quadrant were analyzed with Ethovision software (Noldus). After probe trials, the platform was placed in the opposite quadrant to assess reversal learning using the same procedure.

Five choice serial reaction time/learning was tested in light-proof, ventilated sound attenuated operant chambers (MED-NP5L; Med-Associates) with masking white noise, five wall holes that could be illuminated and an opposite-wall food tray that received 45-mg food pellets. Mice initially received a pellet if they poked the appropriate hole during the 60-s period during which it was illuminated (FR1 schedule). Any response during the response period ended the trial, which was followed by a 5-s intertrial interval. The 60-s stimulus duration used for the first block of trials was decreased to 30, 15, 7.5 and 4 s in successive weekly blocks of trials. Sessions ended after 50 food presentations or 40 min.

Anxiety was evaluated by testing time for emergence from a dark box (18 × 18 cm) through a 5-cm opening into a brightly illuminated 18 × 18 cm field, and thigmotaxis, time spent in near walls versus near the center of the 42 × 42 cm open field apparatus under red light conditions.

Monoamine/metabolite levels were examined in extracts of ventral midbrain, hippocampus, striatum and cerebral cortical specimens that were dissected on ice, frozen and stored at –80°C. Tissue samples were weighed, homogenized in 4°C 0.1 N $HClO_4$ and concentrations of dopamine (DA), serotonin (5-HT) and their respective metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) quantified using high-pressure liquid chromatography with a C_{18} column, mobile phase 50 mmol/L sodium phosphate monobasic, 250 ⌈M Na_2 ethylenediamine tetraacetic acid (EDTA), 0.03% sodium octanesulfonic acid/25% methanol (final pH 2.75), Coulochem III detector (Thermo Scientific Dionex) and Empower 2 software Waters Corp.) as described (25). Peak heights of unknowns were compared to those of standards; lower limits of detection were 1 pg/20 μL.

Cortical dopamine fiber densities were examined in 50-μm coronal vibratome sections cut through six levels of frontal cortices (bregma +1.38 to +0.68 mm) of pentobarbital-anesthetized mice perfused through their left ventricles with 4% depolymerized paraformaldehyde in phosphate-buffered saline (PBS). Brains were postfixed for 4 h at 4°C, sectioned, and sections washed 3 times for 10 min in Tris buffered saline (TBS), then for 1 h at 4°C in TBS/3% normal donkey serum/0.1% Triton, incubated overnight at 4°C with rat antibodies recognizing dopamine transporter N-terminal sequences (MAB369, Millipore) diluted 1:1,000 in TBS/1% normal donkey serum/0.1% Triton, washed three times for 10 min in TBS, incubated for 2 h at 22°C with Alexa Fluora 488-conjugated donkey anti-rat IgG (A-21208, Life Technologies) diluted 1:2,400 in TBS/1% normal donkey serum/0.1% Triton, washed 3 times for 10 min in TBS and mounted onto slides using Prolong Gold (P-36931, Life Technologies). Tiled confocal immunofluorescence projection images from 11-level z stack images (1 μm total) were produced from each section using a Zeiss 710 microscope (40× objective, excitation 488 and emission 519 nm). The number of pixels that were members of groups of at least 100 contiguous pixels in which dopamine transporter (DAT) immunofluorescence was greater than two standard deviations above the mean value for each section was quantitated using ImageJ (version 1.48, NIH) by an observer unaware of genotype.

Mouse mRNAs were prepared and quantitated as noted for the human specimens from regional brain samples rapidly dissected from mouse brains. Expression of *CDH13* mRNA in conditional knockouts was examined one week after CPP testing using oligonucleotides flCDH13f - flCDH13r anchored in the exon framed by the loxP sequences (exon 3) and CDH13af - CDH13ar, anchored in exons 7 and 8 (sequences available on request). Levels of mRNA corresponding to most mouse genes were compared using Affymetrix mouse gene 2.0 ST arrays, reagents recommended by the manufacturer (http://www.affymetrix.com/estore/catalog/131477/AFFY/Mouse+Genome+430+2.0+ Array#1_3),

a 3000 7G scanner and software AGCC v3.0, expression console v1.3 and transcriptome analysis v2.0.

Statistical analyses from mouse behavioral data used analyses of covariance (ANCOVA) with "PASW" statistics 18 (SPSS). We use age as a cofactor and genotype, dose and gender as between subjects factors and report influences of age and gender as well as interactions, below which they reach significance. Secondary analyses used Scheffe's *post hoc* and Student *t* tests (Excel). Saccharin preferences were analyzed using factorial ANOVA with between-subjects factors of genotype, sex and cocaine dose. Neurochemical data were expressed as pg/mg wet weight, including data for monoamines, metabolites and metabolite-to-parent ratios: [DOPC+HVA]/dopamine and 5-HIAA/5-HT. Data were analyzed using two-way ANOVA (genotype and sex). Averaged densities of dopamine transporter immunoreactive elements in projected images of six sections from five mice of each genotype were compared using *t* tests. Mouse expression array data was analyzed for triplicate arrays for each sample, three samples per genotype, using Gene Chip Operating Software v1.1.1 and a threshold of > two-fold difference in expression. Correlations between SNP genotypes and levels of expression in human postmortem samples used the association analysis toolset PLINK (26). Bonferroni corrections for multiple testing with observed, average 0.4 correlations (*r*) between CDH13 SNPs tested were performed using software available at (http://www.quantitativeskills.com/sisa/calculations /bonfer.php).

All supplementary materials are available online at www.molmed.org.

RESULTS

We report results from mice with constitutively altered and with adult (via conditional knockout) reductions in levels of *CDH13* mRNA. We test these mice using CPP, a model of rewarding influences from drugs that has been highly validated in pharmacological studies (18) and supported by results of several molecular genetic studies (see below). We identify evidence for shifted dose response relationships for cocaine reward in constitutive *CDH13* knockouts and in mice with reduced adult expression. These data fit with human observations that support influences of *CDH13* variation on addiction-related phenotypes, and with the idea that drugs that modify activities of *CDH13,* might be useful when given to adults.

We contrast these data with results of evaluations of other phenotypes that are of interest for several reasons. These include tests of motor abilities (reductions might confound CPP results), learning/memory (reductions might confound CPP results), aversive features caused by cocaine (which might provide an alternative explanation for CPP results to "altered reward"), impulsivity (which might fit with ADHD influences but confound CPP results) and anxiety (which might confound CPP results). Results of these tests identify no obvious alternative or confounding behavioral explanation for these CPP differences. We report more subtle influences of *CDH13* knockout on ADHD-related tests of locomotion in male mice and on performance during early acquisition trials for complex tasks.

We test levels of monoamines in brains of the knockout mice as one possible contributor to the observed behavioral differences, and also test mRNA expression differences. In *CDH13* cerebral cortices, we find selectively altered dopamine/metabolite ratios, differences in dopaminergic fiber densities and changes in an activity-dependent transcription factor mRNA. Each of these observations fits with *CDH13* expression by dopaminergic neurons and subsets of cerebral cortical neurons and with contributions of changed dopaminergic innervation to the observed behavioral data.

Human CDH13 Variation Versus Expression Levels

CDH13 mRNA was detected in RNA extracted from cerebral cortical specimens from each of 184 individuals. There was good ($r > 0.85$) agreement between results obtained with amplimers targeting different parts of this mRNA. Levels of *CDH13* expression were significantly associated with allele frequencies for SNPs in *CDH13* intron 2 (Table S4). Nearby intron 2 SNPs, rs8059696, rs4783277, rs12596958 and rs2199430, displayed minor allele frequencies *ca* 0.35, span about 8 kb and displayed nominally significant, $0.045 > p > 0.001$ association with levels of *CDH13* expression. Brains from individuals with rs2199430 = GG displayed 181% of the mean levels of *CDH13* mRNA found in AA homozygotes (Supplementary Figure S1; $p = 0.0007$ ANOVA; Bonferroni-corrected threshold $p = 0.003$).

Mouse CDH13 Expression

Heterozygote constitutive knockouts expressed approximately 50% of the wild-type levels of *CDH13* mRNA. Homozygote knockouts did not express detectable levels of this RNA (Supplement, Supplementary Figure S2A). Individual conditional knockouts sacrificed following CPP testing revealed varying levels of cerebral cortical expression of *CDH13* mRNA (Supplementary Figure S2B; see below).

Cocaine CPP in Constitutive CDH13 Knockout Mice

Knockouts displayed altered dose/response relationships for cocaine CPP (Figure 1A, $p = 0.002$ for genotype*dose interaction, ANCOVA with age as covariate). In wild-type mice, 5, 10 and 20 mg/kg doses of cocaine induced significant place preferences (Scheffe's $p = 0.035$, < 0.001 and < 0.001, respectively). In wild-type animals, significantly more robust preference was produced by 10 than by 5 mg/kg doses ($p = 0.006$). By contrast, homozygous *CDH13* knockout mice displayed significant preferences only for places paired with 5 mg/kg cocaine ($p = 0.002$). Preferences induced by pairing with 10 or 20 mg/kg doses did not differ significantly from values in control, saline-injected animals ($p = 0.38$ and 0.10). Heterozygous mice

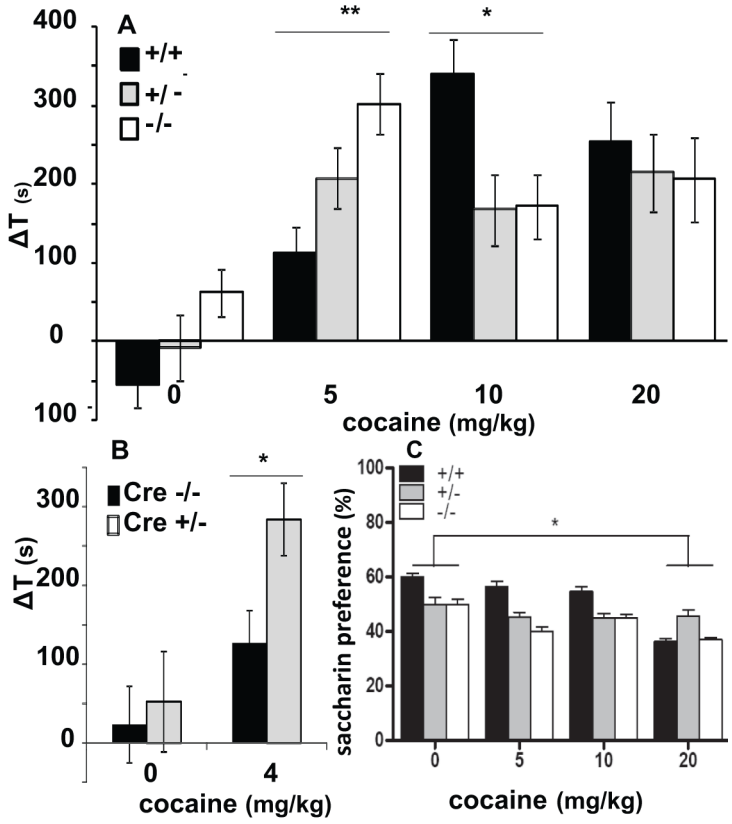

Figure 1. Constitutive (A, C) and adult (B) CDH13 knockout alters conditioned place preference (A, B) but not conditioned taste aversion (C) provided by modest to moderate cocaine doses. (A, B) Mean difference ± SEM in time spent on the cocaine-paired side before and after conditioning (n = 1 –12/group). There was no significant difference between genotypes in time spent on the drug-paired side during the pre-tests for constitutive (ANOVA, $p = 0.740$) or conditional ($p = 0.542$) knockouts. (C) Preference for cocaine-paired saccharin solution in two bottle testing after taste aversion conditioning. *$p < 0.05$, **$p < 0.01$.

provided intermediate preferences for the places paired with 5, 10 and 20 mg/kg cocaine doses that achieved statistical significance (Scheffe's $p = 0.006$, 0.029 and 0.007). Mice with reduced *CDH13* expression thus display reduced reward from a normally highly rewarding 10 mg/kg cocaine dose (ANOVA $p = 0.012$). They also display even more significant increases in preference for places paired with the 5 mg/kg dose (ANOVA $p = 0.003$). These data suggest a leftward shift of the initial limb of the inverted U dose-response relationship typically noted for cocaine reward and reinforcement. Dose–response relationships were similar in male and female mice (ANCOVA sex*genotype*dose, $p = 0.633$).

Cocaine CPP in Conditional CDH13 Knockout Mice

Mice in which expression was reduced in adulthood also increased preference for places paired with modest doses of cocaine (Figure 1B, ANCOVA genotype*dose, $p = 0.017$; n = 11–12/group). Cre expressing mice showed significantly stronger preferences for places paired with 4 mg/kg cocaine than the control $Cre_{-/-}$ mice ($p = 0.018$). There was a $r = –0.42$; one-tailed $p = 0.035$ correlation between preference for the place paired with this modest cocaine dose and levels of *CDH13* mRNAs assessed in cortices of mice sacrificed following the conditioned place preference testing (Supplementary Figure S2B).

Cocaine-Conditioned Taste Aversion

There were no significant influences of genotype on acquisition of the taste aversion. In two-bottle tests (Figure 1C), there were significant effects of genotype and dose ($p = 0.029$ and 0.009, respectively) but no significant genotype*dose interaction (p = 0.30). Conditioning with 20 mg/kg of cocaine significantly reduced saccharin preference in mice of all genotypes ($p = 0.006$).

Locomotion

During the first exposure to the 49 × 49 cm arena, ANCOVA identified a $p = 0.023$ effect of genotype on locomotion. Genotype effects were significant when data from males were analyzed separately (Supplementary Figure S3A, $p = 0.034$) but not when data from females was analyzed separately (Supplementary Figure S3B, $p = 0.265$).

During the first preconditioning session in the CPP apparatus (Supplementary Figure S4A), there was a significant genotype*age interaction (ANCOVA, $p = 0.045$). Constitutive *CDH13* knockout mice older than 90 d displayed less locomotion than their wild type siblings (ANOVA, $p = 0.018$). In mice <90 d old, genotype effects on locomotion did not reach significance (ANOVA, $p = 0.095$). There was no significant effect of genotype on locomotion monitored when mice were confined to halves of the conditioned place preference apparatus after treatments with saline, 5, 10 or 20 mg/kg cocaine doses (Supplementary Figure S4B; ANCOVA, $p = 0.15$, 0.13 and 0.09, respectively; ANCOVA overall effect of genotype, $p = 0.31$, genotype*dose interaction, $p = 0.88$).

Rotarod Testing

Knockout mice displayed a significant overall influence of genotype on the arc of learning this task (repeated measures ANCOVA day of test*genotype interaction, $p = 0.040$) (Figure 2A).

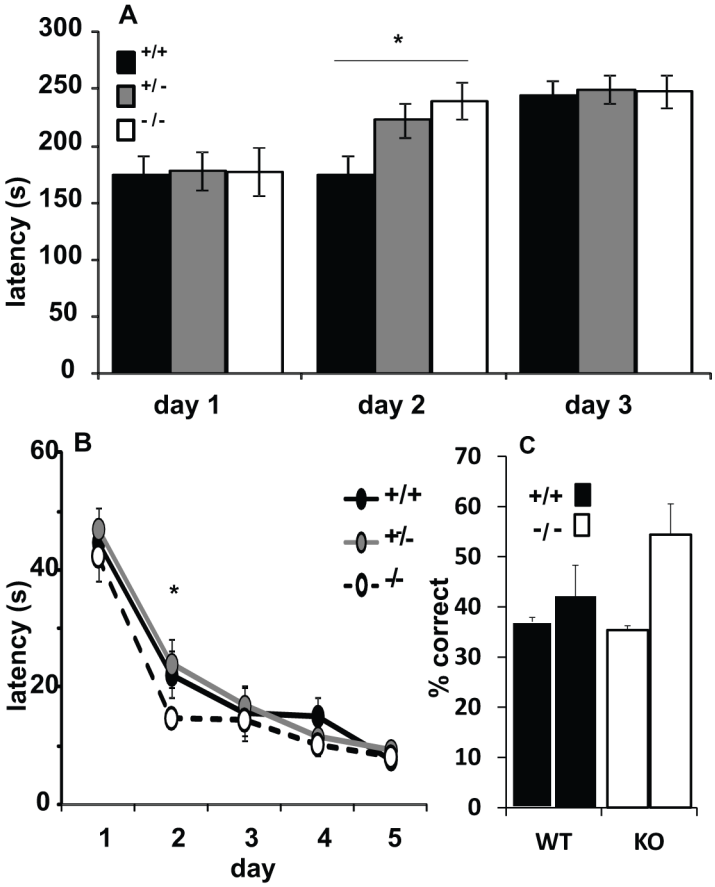

Figure 2. Acquisition and performance of the rotorod (A) Morris water maze (B) and 5-choice serial reaction time (C) tasks in constitutive *CDH13* knockouts and wild-type littermates. (A) Mean ± SEM of the latencies to fall from the accelerating rotorod in wild-type, heterozygous and homozygous constitutive *CDH13* knockouts. Knockouts showed faster acquisition of the task (repeated measures ANCOVA, $p = 0.040$). N = 24/genotype. *$p < 0.05$. (B) Mean ± SEM of the latencies to reach Morris water maze platform for wild-type, heterozygous and homozygous *CDH13* knockouts. Knockouts displayed no significant deficit in task acquisition (days 1–5, repeated measures ANOVA genotype*day effect, $p = 0.96$), and displayed better performance on d 2 of testing when compared with mice of both other genotypes *$p < 0.05$. (B) Mean ± SEM of the probe trial results. (C) Mean ± SEM of % correct responses in 5-choice serial reaction time testing in the first 2 trials of this task in male mice. Knockouts showed a trend toward faster acquisition of the task that did not achieve significance ($p = 0.1$). Females failed to display any trends (data not shown).

This influence was greatest during the second test day (ANVOVA, effect of genotype, $p = 0.017$), when knockouts performed better than wild-type littermates.

Morris Water Maze

Knockout mice displayed no significant deficit in Morris water maze tests of acquisition (Figure 2B; repeated measures ANCOVA genotype*day, $p = 0.96$), reversal learning (repeated measures ANCOVA genotype*day, $p = 0.81$), the first probe trial (Supplementary Figure S5B; including distance from the former platform location ANOVA, $p = 0.13$; time spent searching in the target quadrant $p = 0.12$; time spent searching in the opposite quadrant, $p = 0.32$; and swimming speed, $p = 0.45$) or the second probe trial after the platform had been moved to a new location. On the contrary, there was modest support for more rapid acquisition of the task in the knockouts, when compared to mice of both other genotypes (Figure 2B, $p = 0.039$; t test).

Five choice serial reaction time: Knockout mice displayed no significant deficit in 5-choice serial reaction time testing (Figure 2C, Supplementary Figure S6). During the first and second days of sessions in this apparatus, knockout male mice displayed 35 and 54% correct responses, while their wild-type littermates displayed 37 and 42% correct (Figure 2C), though these differences failed to achieve statistical significance ($p = 0.1$). Knockout mice made no more premature responses or omission errors than wild-type littermates (Supplementary Figure S6).

Anxiety

CDH13KO mice did not differ from their wild-type siblings in latencies to emerge from a dark compartment or time spent in the light (Supplementary Figure S7A; ANCOVA effect of genotype, $p = 0.2$). There were no significant differences in the amount of time that they spent in the center of an open field (Supplementary Figure S7B; ANCOVA effect of genotype, $p = 0.75$).

Mouse Brain Neurochemistry

In cortex, levels of dopamine were significantly reduced in constitutive CDH13 knockout mice (Figure 3A; ANOVA, $p = 0.006$). Ratios between dopamine and its metabolites also differed significantly (Figure 3D; ANOVA, $p = 0.007$). However, there were no significant differences in levels of dopamine, HVA or DOPAC in striatum, ventral midbrain or hippocampus (Figures 3B, C). There were no significant genotype-related differences in levels of norepinephrine, serotonin or its metabolite 5HIAA in cortex, striatum, hippocampus or ventral midbrain.

Cortical Dopamine Transporter (DAT) Immunohistochemistry

Infralimbic and frontal cortical regions displayed densities of DAT-immunoreactive elements in fiber/varicosity patterns

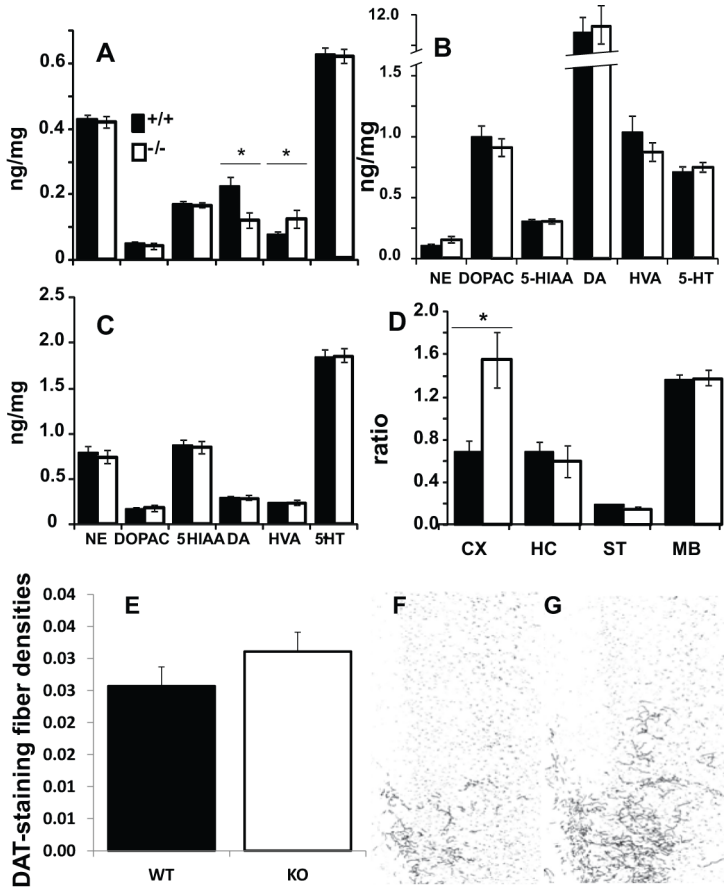

Figure 3. Altered dopamine levels (A–D) and dopamine fiber densities (E–G) in cortices of constitutive *CDH13* knockout mice. Regional concentrations of monoamines and metabolites in dopamine-associated brain regions. (A–C) Mean ± SEM of the concentrations of monoamines and metabolites in cerebral cortex (A), striatum (B) and ventral midbrain (C) samples dissected from brains of wild-type (black bars) and *CDH13* knockout (open bars) mice. (D) Ratios between dopamine metabolites and dopamine in: CX, cerebral cortex; HC, hippocampus; ST, striatum; MB, ventral midbrain. N = 10/ genotype. *$p < 0.05$. (E) Densities of dopamine transporter immumoreactive dopamine fibers in 50 µm sections through prefrontal cortex from constitutive *CDH13* knockouts and wild-type littermates. *$p < 0.05$. (F–G) Images of dopamine transporter immunoreactive elements > 2 SD above mean pixel density for each section from infralimbic/prefrontal cortical sections from wild-type (F) and *CDH13* knockout (G) mice. Midline is at the left in both images.

that were indistinguishable from those reported using several anti-DAT sera and material from several species (27,28). Knockout mice displayed 1.3× the density of dopamine transporter immunoreactivity displayed by wild-type littermates ($p = 0.005$, *t* test; Figures 3E, F).

DISCUSSION

CDH13 is a cell adhesion molecule expressed by neurons in brain circuits that include ventral midbrain substantia nigra and ventral tegmental area neurons, likely dopaminergic, whose activities are modulated by virtually every abused substance and by most ADHD pharmacotherapeutics (29–31). Homotypic interactions between CDH13 molecules on adjacent surfaces of expressing neurons are likely to alter the ways in which dopaminergic and other brain circuits that are implicated in addiction and motor modulation develop and are modified by pharmacological and physiological challenges (1,32–34).

The evidence for *cis*-regulation of *CDH13* expression in humans identified in the current work adds to the posterior probabilities that CDH13 variation does alter addiction and ADHD-related phenotypes. The robust, *ca*. 80% differences in cerebral cortical expression of *CDH13* mRNA that we identify between individuals with common 5′ *CDH13* genotypes provides a link between data from the knockouts and common human allelic functional variation at this gene locus. SNPs in this 8 kb *CDH13* intron 2 genomic region display nominally significant associations with addiction-related phenotypes; 9 of 17 and 8 of 20 tested SNPs display $0.05 > p > 2 \times 10^{-6}$ p_{min} associations with abilities to quit smoking or dependence on an addictive substance, repsectively (36). The intron 2 *CDH13* SNP that displays the strongest association with level of CDH13 mRNA expression lies within 2 kb of a cluster of *CDH13* SNPs that display the strongest associations with human temporal lobe volume (35). Mutually supporting positive and negative human and mouse data thus suggest that common *CDH13* alleles that alter levels of expression in ways that provide links between individual differences identified in humans and differences that we report here in mouse models.

Characterization of mice with lifelong alterations in *CDH13* expression supports a pattern of specific behavioral changes. Constitutive *CDH13* knockout mice display alterations in their preference for the places paired with several different doses of cocaine. There is no corresponding influence of reduced *CDH13* expression on the aversive features provided by these same cocaine doses, as assessed by conditioned taste aversion (36). There is no evidence for differences in anxiety phenotypes, which might otherwise interact with CPP testing (37). Constitutive knockout mice fail to display sizable alterations in locomotion or strength likely to confound CPP testing (38,39).

Effects on preference for places paired with a modest cocaine dose were also observed in conditional knockouts in which expression of CDH13 was changed only in adulthood. Developmental alterations in circuitry thus do not appear to be required to provide these most significant effects of altered CDH13 expression. We focused on influences of 4 mg/kg doses since these provided the largest differences in initial studies of these conditional knockouts. The significant correlations between mouse-to-mouse differences in levels of cortical *CDH13* mRNA and preferences for places paired with 4 mg/kg doses do provide evidence for a graded relationship between levels of adult *CDH13* expression and preference for this modest cocaine dose. It is conceivable that effects on reward from larger (e.g., 10 mg/kg) cocaine doses might require lifelong reductions in *CDH13* expression levels, however.

Subtle knockout influences on locomotion and cognitive performances reach significance in several interesting ways. Knockouts overall, and males analyzed separately, display significant differences in locomotion in a larger dark chamber. Knockouts of both genders display better performance during their second exposures to rotarod and Morris water maze testing. Each of these observations fit with reported modest human *CDH13* associations with ADHD, which is more frequently diagnosed in males (40). However, the sizes of these effects are small, as are the modest effects noted by others in some mnemonic parameters of the conditional knockout mice (20). There are also no effects of constitutive changes in *CDH13* on trained performance on the 5-choice serial reaction time task; the same result came from conditional *CDH13* knockouts (20). The impulsivity phenotype assessed by this test (41) is thus not changed with *CDH13* deletion.

Molecular, neurochemical and anatomic findings from *CDH13* knockout mice provide evidence for specificity that complements behavioral results. Initial neurochemical studies highlighted specific changes in dopamine and its metabolites in cerebral cortex. These findings contrasted with lack of significant knockout versus wild-type differences in levels of other monoamines in any brain region sampled, or of dopamine in ventral midbrain or striatum. Neurochemical findings in cortex were then supported by changes in densities in dopamine-transporter immunoreactive fibers using a pixel based method; we obtained similar observations in initial studies using counts of labeled fibers by paired independent observers who were unaware of genotype. The greater fiber densities noted in knockouts and the elevated dopamine turnover (42) suggested by the altered ratios between dopamine and its major metabolites are each consistent with *in vitro* observations that *CDH13*-*CDH13* interactions can inhibit outgrowth of *CDH13*-expressing neuronal processes (34). Microarray results that point to substantial changes in cortical expression of only the activity-dependent transcription factor *Npas4* (43) comport with both subtly altered cortical circuitry in the *CDH13* knockouts and with the substantial specificity of the changes that *CDH13* deletion induces.

Evidence available prior to and following our CPP results also supports differences in dose response relationships for addictive substances in individuals with differences in *CDH13* genotypes (44). Prior to these mouse results, there were reports of associations between *CDH13* markers and smoking quantity/frequency (7) and dose-response relationships for symptoms of intoxication during individuals' first five exposures to alcohol (45). After we had obtained the initial CPP data from constitutive knockouts, Hart and colleagues reported associations between *CDH13* variants and human individual differences in the dose-response relationships for amphetamine effects (16). There was a robust *CDH13* association with individual differences in subjective positive responses to oral administration of 10 mg, but not 20 mg amphetamine doses.

Evidence from mouse studies that *CDH13* variation is associated with both cognitive and motor differences that could alter vulnerability to ADHD fits with human ADHD associations that were largely available as we performed the mouse experiments. The differences in locomotion that we identify here display interactions with gender that are reminiscent of the influences of gender on ADHD diagnoses and/or symptomatology. *CDH13* associations with cognitive function in children with ADHD or autism fit nicely with the influences of *CDH13* variation on task acquisition that we note here (46–48). We failed to identify any significant influences of *CDH13* variation on the sorts of impulsivity measured by 5-choice serial reaction time testing (49), although this "impulsivity" indicator may not assess all forms of impulsivity present in human disorders. Nevertheless, *CDH13* variation does appear to provide subtle influences on both cognitive and motor systems in ways that could (subtly) alter likelihood of obtaining an ADHD diagnosis in several ways.

Our observations of altered cerebral cortical dopamine/metabolite levels and ratios in the *CDH13* knockouts provide a plausible neurochemical correlate for at least some of the specific influences of the knockout on drug reward. Homomeric recognition of *CDH13* expressed by ventral tegmental area mesocortically projecting dopamine neurons and by many of the frontal cerebral cortical neurons that they target is likely to alter connections between these two important nodes of addiction-related circuitry.

Results from mice with altered *CDH13* expression are reinforced by data from human associations that were available both prior to and following availability of the initial mouse CPP results. Prior probabilities for influences of *CDH13* variation on addiction and ADHD phenotypes in mice were enhanced by findings, in several addiction and some ADHD association datasets, that clusters of nearby *CDH13* SNPs display nominally significant $10^{-2} > p > 10^{-8}$

case versus control allele frequency differences (5,7,9,10,14,16,45,50–58). Cautions in interpreting this human data, taken alone, included the lack of large effects of any single *CDH13* variant. Nevertheless, prior datasets, subsequent addiction-related studies and a meta-analysis of ADHD data (59) that do identify *CDH13* supported elevated prior probabilities that *CDH13* associations would be identified in the current work. In light of these prior probabilities, the robust influences of *CDH13* knockout on stimulant-conditioned place preference and modest influences on motor/learning phenotypes that are consistent with ADHD associations elevate posterior probabilities that *CDH13* variation does provide *bona fide* influences on addiction- and ADHD-related phenotypes.

CDH13 variation, implicated in addiction, in rewarding and/or aversive responses to stimulants and alcohol and in ADHD by human molecular genetic studies, provides a pattern of altered behaviors in a mouse model that accords remarkably with human data for these phenotypes. In future work, it will be interesting to see if mice with reduced *CDH13* expression display differences in responses to natural rewards and/or reward from other substances of abuse. Nevertheless, the present results support the important roles that detailed patterns of neuronal connections (6), including those modulated by *CDH13*, are likely to play in addiction and other complex brain phenotypes. These data add to support for *CDH13* as a candidate druggable target for therapies for addictions and/or ADHD (60).

CONCLUSION

Few of the likely polygenic influences on human vulnerabilities to addiction or attention deficit hyperactivity disorder (ADHD) have been elucidated in detail or in animal models. Mice with lifelong reductions in expression of cadherin 13 (*CDH13*) display sizable differences in tests of stimulant reward, modest/gender-specific influences on locomotor behaviors (though not 5-choice testing) of possible relevance to ADHD and altered cortical dopamine systems that could contribute to these behavioral observations. Mouse and human data are linked by differences in *CDH13* mRNA expression in brains of humans with different *CDH13* genotypes and by human associations of CDH13 variants with addiction, stimulant reward and ADHD phenotypes. These data and altered cocaine reward in mice with adult reductions in *CDH13* expression support *CDH13* as a novel therapeutic target.

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