ARCHIVAL REPORT

Neuregulin-3 in the Mouse Medial Prefrontal Cortex Regulates Impulsive Action

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Background: A deficit in impulse control is a prominent, heritable symptom in several psychiatric disorders, such as addiction, attention-deficit/hyperactivity disorder, and schizophrenia. Here, we aimed to identify genes regulating impulsivity, specifically of impulsive action, in mice.

Methods: Using the widely used 5-choice serial reaction time task, we measured impulsive action in 1) a panel of 41 BXD recombinant inbred strains of mice ($n = 13.7 \pm .8$ per strain; $n = 654$ total) to detect underlying genetic loci; 2) congenic mice ($n = 23$) to replicate the identified locus; 3) mice overexpressing the *Nrg3* candidate gene in the medial prefrontal cortex ($n = 21$); and 4) a *Nrg3* loss-of-function mutant ($n = 59$) to functionally implicate the *Nrg3* candidate gene in impulsivity.

Results: Genetic mapping of impulsive action in the BXD panel identified a locus on chromosome 14 (34.5–41.4 Mb), syntenic with the human 10q22-q23 schizophrenia-susceptibility locus. Congenic mice carrying the impulsivity locus (*Impu1*) confirmed its influence on impulsive action. Increased impulsivity was associated with increased *Nrg3* gene expression in the medial prefrontal cortex (mPFC). Viral overexpression of *Nrg3* in the mPFC increased impulsivity, whereas a constitutive *Nrg3* loss-of-function mutation decreased it.

Conclusions: The causal relation between *Nrg3* expression in the mPFC and level of impulsive action shown here provides a mechanism by which polymorphism in *NRG3* in humans contributes to a specific cognitive deficit seen in several psychiatric diseases, such as addiction, attention-deficit/hyperactivity disorder, and schizophrenia.

Key Words: Behavior, Erbb4, impulsivity, inhibitory control, neuregulin, 5CSRTT

Poor impulse control is a prominent symptom in several psychiatric diseases affecting prefrontal cognitive functioning, such as addiction, attention-deficit/hyperactivity disorder (ADHD), and schizophrenia. Deficits in impulse control manifest in a variety of impulsive behaviors and can specifically be studied in computerized response tasks. These tasks are broadly categorized into those that measure impulsive action (i.e., the inability to withhold from making a response) and impulsive decision making (i.e., the inability to wait for a larger reward) (1). In tasks measuring impulsive action, ADHD and schizophrenia patients consistently show enhanced impulsive responding (2–7). In humans, impulsive action is affected by genetic factors, with heritability estimates in the range of 18% to 50% (8,9), suggesting that genetic variants affecting impulsive action contribute to the heritability of the associated diseases. Some genes involved in monoamine neurotransmission have been implicated in tasks of impulsive action [*TPH2* (10), *DAT* (11), *HTR2B* (12), *SLC6A2* (13), and *DRD2* (14)].

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Tasks of impulse control in humans have excellent rodent counterparts that measure impulsive action and impulsive decision making (1). In particular, the 5-choice serial reaction time task (5CSRTT) (15), which is the most widely used task measuring impulsive action and attention performance, has been instrumental in defining the underlying neuroanatomy and neurochemistry (16,17) and genes pertaining to these cognitive functions (18–23). For instance, using the 5CSRTT as a readout, anatomical substrates contributing to impulsive action, including the medial prefrontal cortex (mPFC), have been documented (24,25).

Using a genetically diverse reference panel of BXD recombinant inbred strains, which are derived from an intercross of C57BL/6J and DBA/2J mice (26), we previously measured a substantial contribution of genetic factors to impulsive responding with a heritability of 34% (27). This indicated that forward genetic studies in this reference panel could potentially be used to identify novel genes contributing to impulsive action. The C57BL/6J and DBA/2J founders of the BXD panel show quantitative differences in several types and aspects of impulsive behavior (27–31), and alleles influencing various forms of executive control are known to segregate in this BXD panel (32,33).

Here, we report the detection, and an independent confirmation, of a locus on chromosome 14 influencing impulsive action in mice. Increased impulsive responding was related to increased *Nrg3* gene expression in the mPFC. The causal relationship between *Nrg3* expression in the mPFC and impulsive action was shown by viral overexpression and genetic loss of function of the *Nrg3* gene.

Methods and Materials

Mice and Genotyping

Male mice were single housed on sawdust in standard Makrolon type II cages (26.5 cm long, 20.5 cm wide, and 14.5 cm high; Tecniplast, Milan, Italy) enriched with cardboard nesting

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material (7 AM lights on, 7 PM lights off; tested during the light phase). Water and food were available ad libitum, except during food restriction in weeks of 5CSRTT training.

BXD Lines. Breeding pairs of BXD lines and their parental lines (C57BL/6J and DBA/2J) were received from The Jackson Laboratory (Bar Harbor, Maine) or from Oak Ridge National Laboratory (Oak Ridge, Tennessee) in case they were not available from The Jackson Laboratory at the time (BXD43, BXD51, BXD61, BXD62, BXD65, BXD68, BXD69, BXD73, BXD75, BXD87, BXD90) and were bred in the facility of the Neuro-Bsik consortium of the VU University Amsterdam (Amsterdam, The Netherlands). The breeding colonies of the different BXD strains were maintained largely in parallel, producing mice of each strain for this study every month, which were sampled from these colonies between June 2007 and August 2008. The cohort of BXD mice used in this study was subjected to one test of prepulse inhibition before 5CSRTT training, as reported earlier (33).

Generation of Congenic Mice. BXD96 mice were backcrossed for five generations to C57BL/6J mice [Charles River Laboratories, L'Arbresle, France; European supplier of C57BL/6J mice, genetically indistinguishable from those obtained from The Jackson Laboratory (34)]. The presence of the D2-derived impulsivity locus (*Impu1*) on chromosome 14 in the offspring was detected using high-resolution melting curve genotyping (Supplement 1) with primers surrounding the single nucleotide polymorphism (SNP) rs30199961 (Chr14:39.895,979 base pair [bp]), which is located in proximity of the genetic marker linked to impulsive action (~.75 Mb upstream).

Generation of *Nrg3* Mutant Mice. To generate mice with a mutant *Nrg3* allele, a neomycin cassette replaced exon 2 of *Nrg3*, which encodes the N-terminal part of the epidermal growth factor-like domain in *Nrg3*, thereby introducing a frame shift mutation in the truncated transcript (Figure S1 in Supplement 1). Subsequently, the line was backcrossed for more than 10 generations to the C57BL/6 background before it was used for the first 5CSRTT experiment. Marker-assisted backcrossing was performed using high-resolution melting curve genotyping on SNPs identified through the online SNP browser of the WebQTL database (www.genenetwork.org) to reduce the amount of hitchhiking genome of the embryonic stem cell donor strain (129/OlaHsd) around the mutant *Nrg3* allele (Supplement 1).

Behavioral Testing

5CSRTT. Mice were food-restricted to gradually decrease their body weight to 90% to 95% of their initial body weight before daily training in operant cages commenced (5 days each week). Water was available ad libitum throughout the experiment. Mice were trained to respond into the food magazine, to wait for a 5-second inter trial interval (ITI), and subsequently to respond to a 1-second stimulus presentation in one of five response holes to obtain a food reward. Premature responses into one of the five response holes before stimulus presentation were defined as impulsive responses, measuring impulsive action. Mice were trained to perform this 5CSRTT on an individually paced schedule (using criteria mentioned in Supplement 1), to ensure each individual BXD mouse reached a particular preset performance before commencing to the next training phase (27,29).

Behavioral Test Battery. For evaluation of differences in anxiety-related behavior, motor performance, and spatial memory, $Nrg3^{-/-}$, $Nrg3^{+/-}$, and $Nrg3^{+/+}$ littermates were tested in a battery of tests, most of which were reported previously [Loos *et al.* 2009 (27); Loos *et al.* 2012 (33)], entailing maximal one test per day in the following order: body weight, grip strength, novel cage induced hypophagia, elevated plus maze, open field, dark-light box, accelerating rotarod, and the Barnes maze (Supplement 1).

Viral Overexpression Experiments

Adeno-associated virus type 2 pseudotyped viral particles overexpressing *Nrg3* were generated using a two-plasmid cross-packaging system as described previously (35). Full-length *Nrg3* complementary DNA was synthesized (Eurofins, Ebersberg, Germany). This synthetic full-length *Nrg3* sequence was cloned between a cytomegalovirus promoter and an IRES2-eGFP cassette into the plasmid pTRCGW (36), yielding an *Nrg3*-IRES-eGFP virus (Nrg3 virus). An identical bicistronic vector lacking the *Nrg3* sequence was used for control experiment (control virus). Viral particles were produced and isolated according to previously described methods (37) (Supplement 1) and were stereotactically injected into the mPFC (anteroposterior +1.9 mm; lateral, ±.5 mm from Bregma and −2.5 mm from the surface of the skull) (38) under isoflurane anesthesia (1 μL per side, 11.7×10^{11} genomic copies per mL, at flow rate of .1 μL/min).

Molecular and Cellular Analyses

Real-Time Quantitative Polymerase Chain Reaction. Dissection of dorsal mPFC brain tissue, RNA isolation, and measurement of gene expression levels using gene-specific primers using real-time quantitative polymerase chain reaction was described in detail previously (27,39) (Supplement 1).

Fluorescence in situ Hybridization. Our protocol was adopted from previously published ones (40,41) (Supplement 1) using C57BL/6J mice.

Statistical Analyses

For evaluation of strain/mutant differences, analysis of variance or analysis of variance with repeated measures were used and upon significance ($p < .05$) followed by post hoc tests (Fisher's least significant difference). Estimates of the heritability (narrow sense) were calculated according to an adapted Hegmann and Possidente method (42), as described previously (27,43). Interval mapping analysis was performed in GeneNetwork (www.genenetwork.org) that uses the embedded MapManager software (44) to perform Haley–Knott regression (http://www.mapmanager.org). Empirical p values, derived from 1000 permutations, were used to assess whether the peak of a quantitative trait locus (QTL) was statistically significant (p value $< .05$) or suggestive (45) (on average one false positive per genome scan; genome-wide p value $< .63$). For the analysis of nonsynonymous mutations in genes under a QTL peak, GeneNetwork's comprehensive SNP browser was used (date: June 2010). Locations of genes and markers are based on the National Center for Biotechnology Information genome Build 37 (mm9). Data are expressed as mean ± SEM.

Results

BXD Strain Differences in Impulsive Responding

To map QTL underlying impulsive action in mice, we measured 5CSRTT performance of 41 BXD strains, with substantial numbers per strain, to accurately assess strain mean performance (average $n = 13.7 \pm .8$ per strain, Table S1 in Supplement 1). In the 5CSRTT, impulsive responding is defined as inappropriate premature responses to a food-predictive stimulus. BXD strains differed significantly in impulsive responding (Figure 1A), and their performance transgressed beyond that of the C57BL/6J and DBA/2J founders,

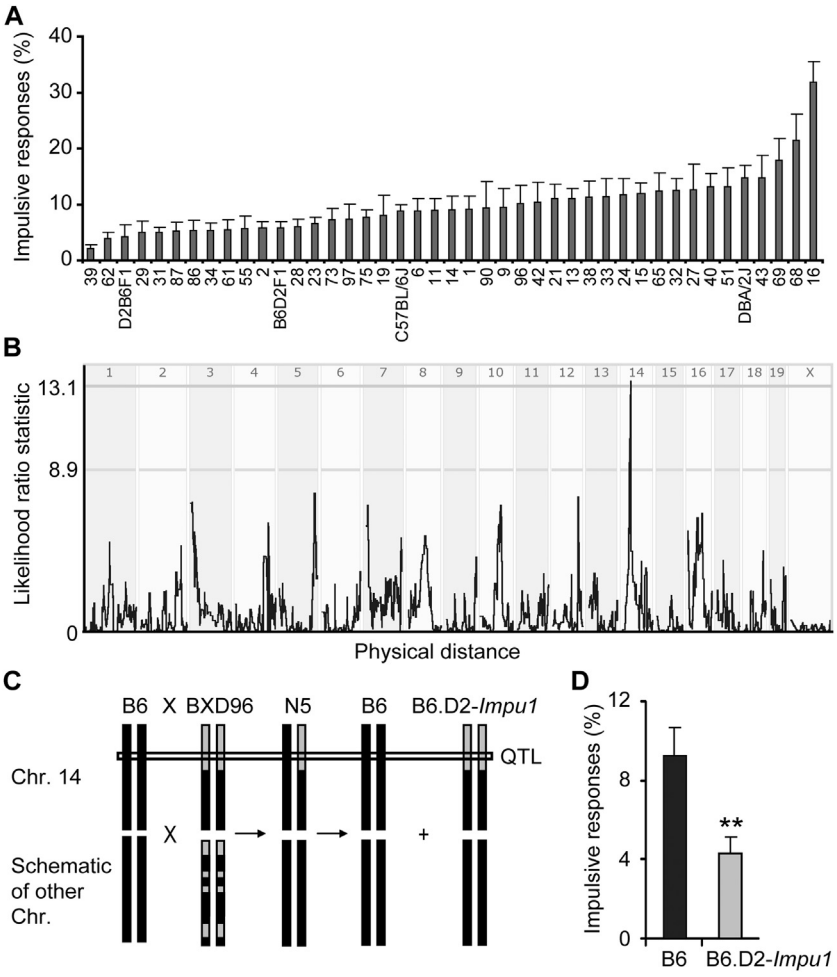

Figure 1. Significant quantitative trait locus (QTL) for impulsive action (*Impu1*). **(A)** Significant strain differences in impulsive responding in the 5-choice serial reaction time task (5CSRTT) ($F_{42,545} = 5.00$; $p < .001$). **(B)** The likelihood ratio statistic (LRS) score (y-axis) quantifies the relation between genomic markers (x-axis) and impulsive action in the 5CSRTT. The threshold for significance (LRS = 13.1; $p = .05$) and suggestive significance (LRS = 8.9; $p = .63$) are indicated. Genome-wide significance was reached at the *Impu1* locus on chromosome 14. Also, after Arcsine transformation of premature responding [appropriate for percentage data (61)], the QTL was significant. **(C)** The BXD96 strain, carrying a DBA/2J derived region on chromosome (Chr.) 14 (0–55.7 Mb), including the *Impu1* locus, was backcrossed to C57BL/6J mice (B6) for five generations (N5) generating a congenic C57BL/6J strain carrying the DBA/2J allele (B6.D2-*Impu1*), as well as littermate control carrying the recipient C57BL/6J allele (B6). Black is indicating C57BL/6J origin, gray is indicating DBA origin. **(D)** Congenic mice carrying the DBA/2J-derived *Impu1* locus at chromosome 14 (B6.D2-*Impu1*, $n = 13$) show reduced impulsive responding in the 5CSRTT compared with C57BL/6J ($n = 10$). $^{**}p < .01$. Error bars represent SEM.

suggesting the contribution of multiple genetic loci. Both F1 lines were significantly less impulsive than the DBA/2J parental line ($p < .01$) but did not significantly differ from the C57BL/6J parental line, suggesting dominance of the C57BL/6J allele(s). Impulsive responding was not confounded by differences in motivation between strains, since measures related to motivation did not correlate with impulsive responding (e.g., magazine latency, relative body weight; Table S2 in Supplement 1). Increasing the demand for impulse control by randomly providing trials with ITIs longer than the standard 5 seconds (i.e., 7.5 and 12.5 sec) enhanced impulsivity (ITI: $F_{1,539} = 884.26$, $p < .001$; ITI × strain: $F_{42,539} = 2.39$, $p < .001$). Comparing the performance at an ITI of 5 seconds and 12.5 seconds showed an increase in premature responding at longer ITIs in all strains, which reached significance in all strains except for BXD9 and BXD27 (Figure S2 in Supplement 1), indicating that all strains used a similar strategy to solve the task. Thus, the 5CSRTT showed a specific difference in impulsive action among BXD strains. From within-strain and between-strain variances, we estimated heritability of 12% in this BXD panel.

QTL Analysis of Impulsivity and Identification of *Nrg3* as Candidate Gene

Interval mapping identified a significant QTL for impulsivity around marker rs6197032 on chromosome 14 (Figure 1B; a one logarithm of odds support interval 34.5–41.4 Mb [build 37, mm9]). At this locus, named *Impu1*, the C57BL/6J allele that contributed to higher impulsivity. To independently replicate the contribution of the *Impu1* locus to impulsivity, we generated a congenic line by backcrossing BXD96 mice to C57BL/6J mice (Figure 1C). The genome of BXD96 provided an efficient start for the backcross, since it carried a Chr14 that is inherited from C57BL/6J (55.7–125 Mb), except a region encompassing the *Impu1* region derived from DBA/2J (0–55.7 Mb). C57BL/6J mice carrying a Chr14 with this DBA/2J derived region, which includes the *Impu1* locus (B6.D2-*Impu1*), were significantly less impulsive than their B6 littermate control mice (Figure 1D). No other behavioral differences in the 5CSRTT were detected between the B6 and B6.D2-*Impu1* strains, except an increase in incorrect responses in the impulsive B6 strain (Table S3 in Supplement 1), in line with the correlation between impulsive and incorrect responding as observed in the BXD panel (see above and in Table S2 in Supplement 1).

The location of the most significant genetic marker (40,646,888 bp) of the *Impu1* locus was 1.46 Mb downstream of the start of the *Nrg3* gene (39,183,612 bp complementary strand). This region is syntenic with the human 10q22-q23 region, a susceptibility locus for schizophrenia (46,47), and known for its complex rearrangements in several families with cognitive and behavioral abnormalities (48). Human genetic studies have associated the *NRG3* gene with ADHD (49), the categorical diagnosis of schizophrenia (50), and delusionary (51) and cognitive (52,53) symptoms of this disease.

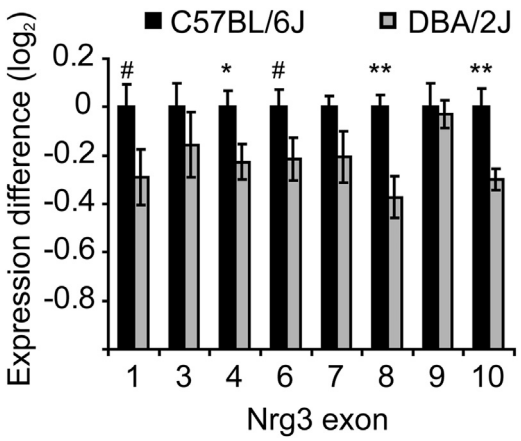

Figure 2. *Nrg3* transcript levels in C57BL/6J and DBA/2J mice. Significantly higher *Nrg3* transcript levels were detected in the medial prefrontal cortex of C57BL/6J ($n = 6$) compared with DBA/2J ($n = 6$) mice using multiple primer sets targeting different exons. The expression level of all individually measured exons was higher in C57BL/6J mice, reaching significance in several exons. $^{\#}p < .1$, $^{*}p < .05$, $^{**}p < .01$. Error bars represent SEM.

No nonsynonymous mutations are present in the *Nrg3* gene between the C57BL/6J and DBA/2J parental strains, indicating no differences in Nrg3 protein function between parental strains. However, there are 930 SNPs in the genomic region of *Nrg3*, including in the promoter sequence and the first intron, which may affect the expression level of the gene. In the mPFC, a brain area known to be involved in impulsive responding in humans (54,55) and rodents (24,25), we detected a significantly higher level of *Nrg3* messenger RNA in C57BL/6J than in DBA/2J mice (Figure 2; $F_{1,10} = 7.139$, $p = .023$). As yet, there is no clear evidence to conclude whether the expression difference of *Nrg3* between BXD parental strains in mPFC tissue was caused by genetic variation inside (acting in cis) or outside (acting in trans) the QTL region. Other genes in the *Impu1* locus contain relatively few SNPs and appear to reside in regions that are identical by descent (for SNPs in other genes, see Table S4 in Supplement 1). Given the involvement of the neuregulin gene family in the central nervous system function and neuropsychiatric disease (56) and the mPFC expression difference of *Nrg3* between BXD parental strains, we suspected *Nrg3* to be the causal gene in the *Impu1* locus regulating impulsivity, and additional experiments were designed to test the possible implication of Nrg3 in impulsive action.

Nrg3 Overexpression in mPFC Increases Impulsive Responding

To functionally test the effect of *Nrg3* expression level on impulsive responding, we created a bicistronic vector expressing both *Nrg3* and enhanced green fluorescent protein (Nrg3 vector) and a control vector lacking the Nrg3 sequence (control vector). To target this vector to the correct mPFC cell types, we first investigated the cellular expression of endogenous *Nrg3*. Double in situ hybridization using C57BL/6J mice showed that *Nrg3* co-localized with the calcium-calmodulin-dependent protein kinase type II alpha transcript, a marker of excitatory/pyramidal neurons (Figure 3). Furthermore, *Nrg3* was also expressed in *Gad67*-positive interneurons. Therefore, we chose an adeno-associated virus pseudotype with viral capsid serotype 2 as expression vector, which is known to transduce neurons in the frontal cortex (57). Bilateral mPFC injections of the Nrg3 virus, expressing both *Nrg3* and enhanced green fluorescent protein, provided transduction of cells throughout the mPFC (Figure 4A), i.e., encompassing the cingulate, prelimbic, and infralimbic areas in which *Nrg3* gene expression difference between parents was detected. Using sequence-specific polymerase chain reaction primers (Figure S3 in Supplement 1), viral expression of the synthetic *Nrg3* transcript was confirmed (Figure 4B). Synthetic *Nrg3* transcript levels were approximately eightfold higher than those of the endogenous *Nrg3* transcript. A significant increase in impulsive responding was detected in mice that had received Nrg3 virus injections before testing at 6 to 7 weeks of age compared with control-virus injected mice (Figure 4C; Table S5 in Supplement 1). Apart from impaired attentional performance, as indicated by the decrease in response accuracy and increase in response variability by *Nrg3* overexpression (Table S5 in Supplement 1), no difference in total locomotor activity was detected (Figure 4D), indicating that *Nrg3* overexpression in the mPFC had specifically deteriorated impulse control and attention. Thus, these data showed a causal relation between elevated expression of *Nrg3*, specifically in the mPFC, and decreased impulse control and attentional performance.

Constitutive Loss of *Nrg3* Decreases Impulsive Responding

Next, we generated *Nrg3*-null mice (Figure S1 in Supplement 1), expecting that absence of *Nrg3* would decrease impulsive responding. A first cohort of $Nrg3^{-/-}$ mice showed significantly decreased levels of impulsive responding in the 5CSRTT compared with $Nrg3^{+/+}$ littermates (Figure S4 in Supplement 1), without differences in other task parameters (Table S6 in Supplement 1). Despite more than 10 generations of unsupervised backcrossing, we detected SNPs in two genes upstream of the *Nrg3* gene (i.e., *Ppyr1* and *Mmrn2*) between $Nrg3^{-/-}$ and $Nrg3^{+/+}$ littermates of this first cohort, in line with the presence

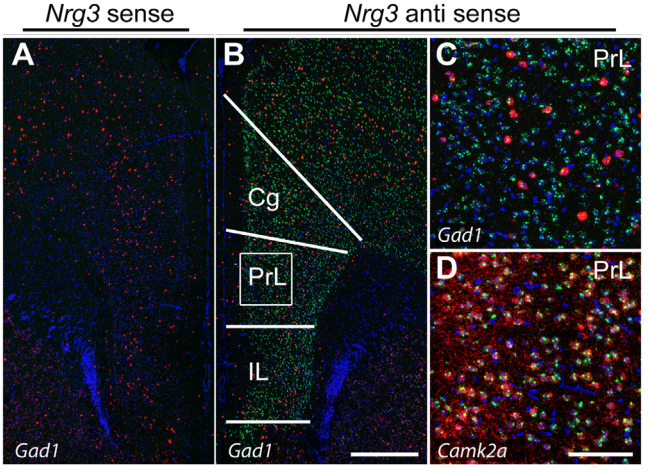

Figure 3. Cellular localization of *Nrg3* messenger RNA in the medial prefrontal cortex of C57BL/6J. **(A, B)** The *Nrg3* sense probe (green) did not show any staining **(A)**, indicating the specificity of the hybridization signal of the *Nrg3* antisense probe **(B)**. *Nrg3* is expressed in the cingulate (Cg), prelimbic (PrL), and infralimbic (IL) areas of the medial prefrontal cortex. Gene expression of *Gad* is indicated (red), and nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). **(C)** Enlargement of the boxed area of the PrL **(B)**: *Nrg3* transcripts (green) co-localize with *Gad1* messenger RNA (red) encoding Gad67 in inhibitory interneurons. **(D)** The *Nrg3* hybridization signal (green) also overlaps with *Camk2a* positive somata of excitatory pyramidal neurons (red). The majority of small glial nuclei is free of *Nrg3* signal **(C, D)**. Scale bars: 500 μm **(B)** and 100 μm **(D)**.

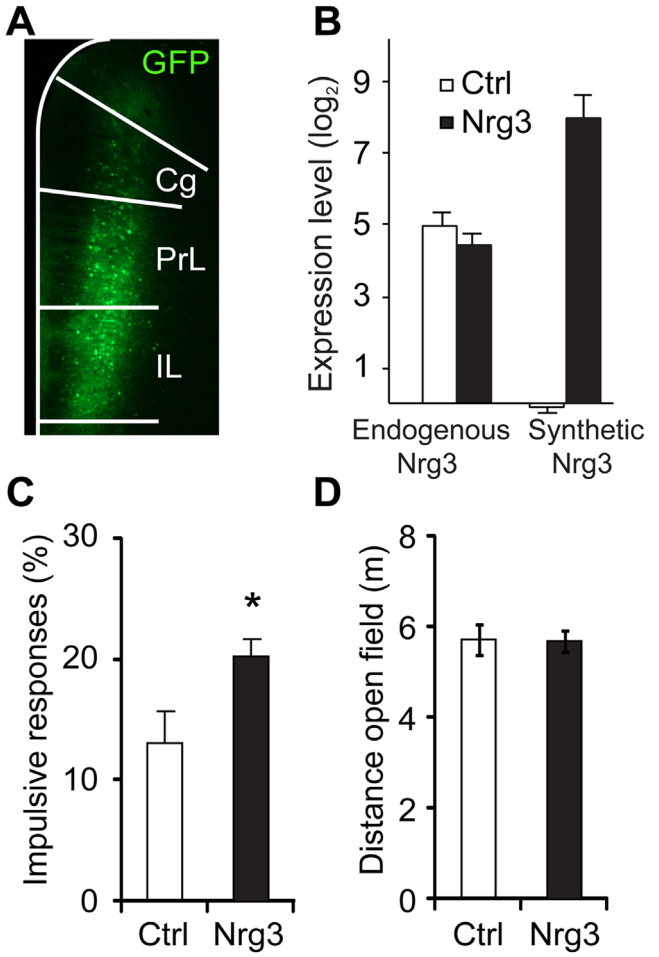

Figure 4. Increasing *Nrg3* expression in the medial prefrontal cortex (mPFC) of C57BL/6J decreases impulsive responding. **(A)** Transcranial injection of adeno-associated virus type 2 particles expressing the bicistronic cytomegalovirus-*Nrg3*-IRES-eGFP construct resulted in widespread expression of enhanced green fluorescent protein (green) in the cingulate (Cg), prelimbic (PrL), and infralimbic (IL) areas of the mPFC. **(B)** Levels of endogenous *Nrg3* transcripts were identical in mice injected with the control (Ctrl, white bars; cytomegalovirus-IRES-eGFP; $n = 3$) and the Nrg3 virus (Nrg3, black bars; $n = 3$). Exogenous *Nrg3* transcripts originating from the Nrg3 virus were only detected in Nrg3 virus injected brains (see Figure S3 in Supplement 1) and were about eightfold (three $\log_2$ units) higher than endogenous expression levels. **(C)** Overexpression of exogenous *Nrg3* in mPFC of C57BL/6J mice enhanced impulsive responding ($n = 12$) compared with control ($n = 9$), and **(D)** did not affect general activity in an open field in these mice. *$p < .05$. Error bars represent SEM. GFP, green fluorescent protein.

of hitchhiking genome of the embryonic stem cell donor strain (129/OlaHsd). After marker-assisted breeding, a second cohort of $Nrg3^{-/-}$ mice, backcrossed to C57BL/6J up to marker rs51174312 (35.2 Mb) and devoid of SNPs in Ppyr1 and Mmrn2, confirmed decreased levels of impulsive responding in *Nrg3* mutant mice (confirmatory one-sided test versus $Nrg3^{+/+}$ littermates, $p = .034$; Figure 5A; Table S7 in Supplement 1). Of note, the 5CSRTT training protocol of these $Nrg3^{-/-}$ and $Nrg3^{+/+}$ mice was slightly different (see Supplemental Methods and Materials in Supplement 1), explaining the higher impulsive responding compared with the previous experiment (e.g., Figure 1). $Nrg3^{-/-}$ and $Nrg3^{+/+}$ mice did not differ in 5CSRTT parameters indexing attentional performance (e.g., response accuracy and variability) or motivation to perform the task (e.g., magazine latency; Table S7 in Supplement 1). Moreover, we observed no differences in tests of anxiety-related behavior, motor function, and learning and memory (Table S8 and Figure S5 in Supplement 1). The decrease in impulsive responding in $Nrg3^{-/-}$ mice was not accompanied by a decrease in overall activity; in contrast, $Nrg3^{-/-}$ mice were significantly more active in a novel open field (genotype × time: $p < .001$; Figure 5B).

Together, these data indicate that the *Nrg3* loss-of-function mutation affected impulsive behavior selectively, without interfering with attentional performance, learning and memory functions, and the regulation of emotional behavior.

Nrg3 Overexpression in *Nrg3* Mutant mPFC Does Not Rescue Impulsive Responding

In $Nrg3^{-/-}$ mice, the brain circuitry that finally gives rise to decreased impulsive responding has developed in the absence of *Nrg3*. To investigate whether *Nrg3* is dispensable during development and is only required during task performance, we rescued *Nrg3* expression in $Nrg3^{-/-}$ mice by injection of the Nrg3 virus at 6 weeks of age into the mPFC. Exogenous *Nrg3* expression did not alter impulsive responding significantly in comparison with *Nrg3* mutant mice injected with the control virus (Figure 6A). Also, attentional performance (Table S9 in Supplement 1) and open field behavior (Figure 6B) was not affected by *Nrg3* overexpression in $Nrg3^{-/-}$ animals. Together, these data suggest that constitutive loss of *Nrg3* during development results in adapted brain circuitry that has become insensitive to *Nrg3* expression at periadolescent and adult stages.

Discussion

We observed differences in a widely studied type of impulsivity, i.e., impulsive action, among BXD recombinant inbred strains, which mapped to a locus, *Impu1*, on chromosome 14. The contribution of the *Impu1* locus to impulsive action was replicated by a congenic strain carrying the D2-*Impu1* locus in a C57BL/6J background. The *Impu1* locus is syntenic with the human 10q22-q23 genomic region, linked to cognitive impairment (48) and known as susceptibility locus for schizophrenia (46,47). Human genetic studies have implicated *NRG3*, located in

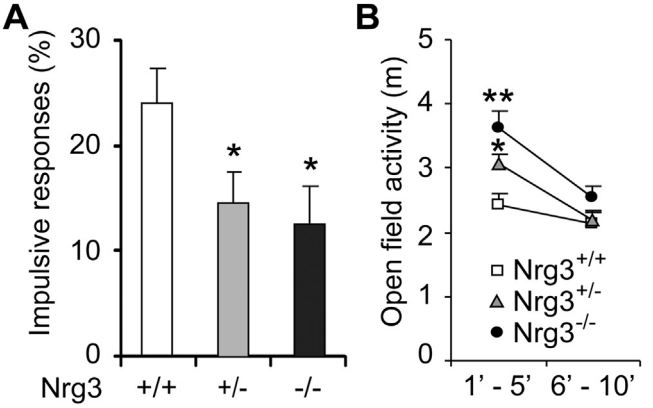

Figure 5. *Nrg3* loss of function decreases impulsive responding. **(A)** *Nrg3* genotype significantly affected the number of impulsive responses ($F_{2,37} = 3.29$, $p = .049$) with $Nrg3^{+/+}$ mice (white; $n = 18$) making more impulsive responses than $Nrg3^{+/-}$ (gray; $p = .045$; $n = 14$) and $Nrg3^{-/-}$ mice (black; $p = .034$; $n = 6$). **(B)** Activity of $Nrg3^{+/-}$ and $Nrg3^{-/-}$ mice was significantly higher during the first 5 minutes in a novel open field than of $Nrg3^{+/+}$ mice. *$p < .05$, **$p < .001$. Error bars represent SEM.

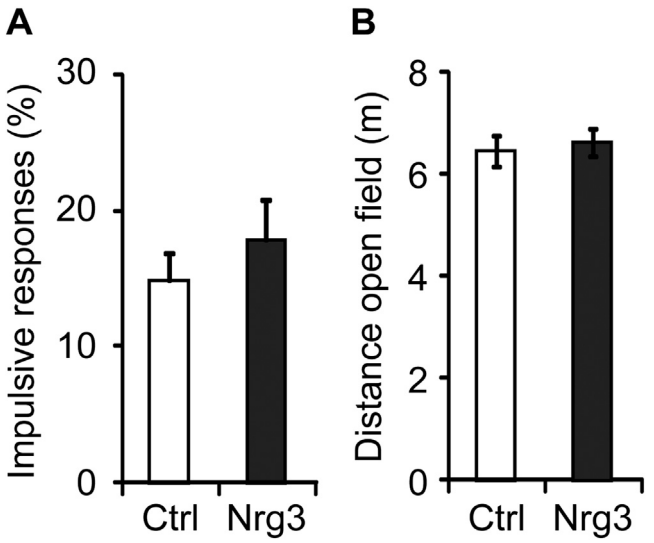

Figure 6. Viral *Nrg3* overexpression did not rescue the low impulsive phenotype of *Nrg3*$^{-/-}$ mice. Viral *Nrg3* overexpression (black bars) in medial prefrontal cortex did not affect **(A)** impulsive responding in the 5-choice serial reaction time task and **(B)** activity in a novel open field in comparison with *Nrg3*$^{-/-}$ mice injected with control (Ctrl) virus (open bars).

this genomic region, as candidate gene for schizophrenia and ADHD (49–53). In this study, we provide the first causal evidence for involvement of prefrontal *Nrg3* in impulsive action by studying the effects of viral *Nrg3* overexpression and *Nrg3* loss-of-function mutation. The direction and specificity of effects provide new insights into the neurodevelopmental role of *NRG3* in schizophrenia and ADHD.

In line with previous studies by others and us, we observed a higher level of impulsive responding in DBA/2J mice compared with C57BL/6J mice (27–29,58). The level of impulsive responding of BXD strains transgressed beyond the phenotypes of the founder strains C57BL/6J and DBA/2J, indicating the contribution of multiple genetic loci to impulsive action. The counterintuitive observation that the C57BL/6J allele at the *Impu1* locus on chromosome 14 was linked to higher impulsive responding is in line with the notion that other (undetected) alleles together with the *Impu1* locus determine the level of impulsive action in DBA/2J and C57BL/6J mice.

Numerous synonymous SNPs are present in *Nrg3* between C57BL/6J and DBA/2J. The majority are located in the proximal part of the gene (promoter and intron 1), potentially changing the expression of *Nrg3*. In the mPFC of C57BL/6J mice, a brain region involved in impulsive responding in the 5CSRTT (24,25), we detected elevated levels of *Nrg3* transcripts compared with DBA/2J mice, suggesting that an increase in *Nrg3* levels in the mPFC might cause an increase in impulsive behavior.

In line with these data in mice, a transcript profiling study in humans showed that the expression of *NRG3* transcript variants is increased in prefrontal cortex tissue of schizophrenia patients and that this elevation is associated with the presence of *NRG3* schizophrenia risk alleles (50). Control subjects carrying *NRG3* risk alleles showed worse performance in an attention task (52), providing some evidence for a relation between NRG3 expression and cognitive performance. Here, we functionally confirmed this relation by showing that viral overexpression of *Nrg3*, specifically targeted to neurons in the mPFC, deteriorated impulse control and impaired attentional performance in adult C57BL/6J mice.

Decreasing or abolishing *Nrg3* expression in *Nrg3*$^{+/-}$ and *Nrg3*$^{-/-}$ mutant mice decreased impulsive responding, without changes in attentional performance, motor coordination, spatial learning, and memory or anxiety-related behaviors. The decrease in impulsive responding in *Nrg3*$^{-/-}$ mice could not be explained by a decrease in general activity; in contrast, mutant mice showed hyperactivity in an open field test.

We note that the engineered loss-of-function mutation of *Nrg3*, as well as exogenous viral overexpression of *Nrg3*, likely have more potent effects on Nrg3 expression than the genetic polymorphisms segregating in the BXD strains. Moreover, despite the choice for a viral vector targeting exogenous Nrg3 to cells that endogenously express Nrg3, profound overexpression could have nonspecific effects. However, the effects of these interventions on mouse behavior both support the relation between Nrg3 expression and the level of impulsivity.

The present results do not exclude the presence of (other) causal polymorphisms in/around other genes in the *Impu1* QTL. Additional experiments are required to identify the causal polymorphism(s) in the *Impu1* locus influencing impulsivity in BXD strains, for instance, employing fine-mapping of the QTL region with additional BXD lines or a cross between different common inbred strains. Three genes, for instance, contained nonsynonymous SNPs (*Ppyr1*, *Mmrn2*, and *4930474N05Rik*). Nonetheless, the viral overexpression and the loss-of-function experiments in the present study clearly implicated *Nrg3* in impulsivity.

In contrast to the effect of viral overexpression of *Nrg3* in wild-type C57BL/6J mice, viral overexpression of Nrg3 in the mPFC of mice with a constitutive *Nrg3* loss-of-function mutation at the levels reached in the current experiment were insufficient to affect impulse control or attentional performance. Nrg3 interacts with the ErbB4 receptor (59), which is highly expressed in parvalbumin positive interneurons and localizes to their dendritic and somatic surface (60). In the absence of Nrg3-ErbB4 interaction, the brains of *Nrg3*$^{-/-}$ mice might not have developed the circuitry that is required for the effect of exogenous *Nrg3* overexpression on impulsive action. These results bear some resemblance to the observation that schizophrenia risk alleles associated with increased expression of *NRG3* variants were associated with relatively spared cognitive performance in schizophrenic patients, but not in control subjects (52), indicating that the effect of NRG3 expression level may depend on a specific brain circuitry. Thus, the particular neurodevelopment of mPFC circuitry of mice with constitutive *Nrg3* loss-of-function mutation and schizophrenic patients may prevent enhanced *Nrg3* expression to affect behavior.

In conclusion, this is the first study specifically implicating *Nrg3* in impulsive action. Notably, unlike previously investigated candidate genes [*TPH2* (10), *DAT* (11), *HTR2B* (12), *SLC6A2* (13), and *DRD2* (14)], this gene is not directly involved in monoamine neurotransmission, thereby providing new molecular insight into impulse control. Genetic intervention causally demonstrated that prefrontal expression of the *Nrg3* gene can influence impulse control in mice, depending on the neurodevelopmental state of the mPFC. These findings are relevant for a better understanding of functional consequences of polymorphisms in NRG3 in healthy subjects and in patients of psychiatric diseases characterized by impulse control deficits, such as addiction, schizophrenia, and ADHD.

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