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,