Reversible Overexpression of *Bace1*-Cleaved Neuregulin-1 *N*-Terminal Fragment Induces Schizophrenia-Like Phenotypes in Mice

Xiaoyang Luo, Wanxia He, Xiangyou Hu, and Riqiang Yan

Background: Neuregulin-1 (Nrg1) is a pleiotropic signaling molecule that regulates neural development, and mutation of Nrg1 is a risk factor for schizophrenia. Cleavage of type I β1 Nrg1 isoform by *Bace1* releases a secreted *N*-terminal fragment (Nrg1-ntf$_\beta$), which can bind to a cognate ErbB receptor to activate the specific signaling cascade. This study aimed to determine whether increased expression of Nrg1 is beneficial for brain development and functions.

Methods: We generated transgenic mice overexpressing this fragment under the control of a tetracycline-inducible promoter and examined functional and behavioral changes in mice upon reversible expression of the transgene.

Results: Increased expression of full-length Nrg1 in mouse neurons has been previously shown to enhance myelination in the central nervous system. Overexpressing Nrg1-ntf$_\beta$ enhanced the expression of myelin proteins, consistent with the expected activation of the Nrg1 signaling pathway by Nrg1-ntf$_\beta$. Contrary to expectations, overexpressing Nrg1-ntf$_\beta$ transgene caused schizophrenia-like behaviors in transgenic mice, and these abnormal behaviors were reversible if the expression of the Nrg1-ntf$_\beta$ transgene was turned off. Our molecular assay suggests that protein levels of *N*-methyl-D-aspartate receptors are reduced in this transgenic mouse model, which might underlie the observed social and cognitive behavioral impairments.

Conclusions: Our results indicate that overexpressing the secreted form of Nrg1 is sufficient to cause schizophrenia-like behaviors in a mouse model, meaning the effect is independent of the transmembrane and C-terminal domains of Nrg1. Hence, genetic gain-of-function mutations of Nrg1 are also risk factors for schizophrenia.

Key Words: *Bace1*, neuregulin, NMDA receptor, schizophrenia, tetracycline control expression, transgenic mice

Mouse genetic studies have shown that Neuregulin-1 (Nrg1), a pleiotropic signaling molecule, controls developmental myelination (1) and cardiac development (2). Human genetic studies have also identified the *NRG1* gene as a risk factor for schizophrenia (3). Thus the mechanism(s) by which Nrg1 exerts its functional roles in development and disease pathogenesis has attracted great attention. The *Nrg1* gene encodes 33 spliced isoforms in six topology types due to alternative splicing (4,5). Types I and II transmembrane Nrg1 have *N*-terminal Ig- and epidermal growth factor (EGF)-like domains, whereas type III Nrg1 has the same transmembrane domain and another hydrophobic Cys-rich domain. All Nrg1 isoforms share an EGF-like domain, which is indispensable for initiating Nrg1 signaling via binding to its cognate ErbB receptors (6).

To exert signaling function, transmembrane Nrg1 undergoes proteolytic cleavage to allow binding of the *N*-terminal domain to an ErbB receptor. Enzymatic mapping shows that this cleavage of Nrg1 is mediated by *Bace1* (between Glu-Phe and Met-Glu) or ADAM10/ADAM17 (multiple adjacent sites) at the juxtamembrane region (7–9). After this ectodomain shedding, type I Nrg1 releases its *N*-terminal fragment (Nrg1-ntf) to the extracellular space, where it binds to ErbB receptors on nearby cells in a paracrine fashion, whereas type III Nrg1-ntf—which remains tethered on the lipid bilayer by the hydrophobic Cys-rich domain—signals to adjacent cells in a juxtacrine fashion. The distinct modes of signaling via isoform-specific Nrg1-ntfs seem to target specific in vivo functions (10).

In *Bace1*-null mice, full-length Nrg1 is increased, because cleavage of Nrg1 by *Bace1* is abolished. Due to a reduction in the availability of Nrg1 signaling fragments, *Bace1*-null mice exhibit hypomyelination during early development (11,12) and delayed remyelination in adulthood (7), consistent with an important role of Nrg1 in the control of myelination (1). Haploinsufficient Nrg1 in mice also causes schizophrenia-like behaviors (3). Indeed, *Bace1*-null mice exhibit schizophrenia-like phenotypes (13), further suggesting Nrg1 hypo-function upon *Bace1* deletion.

Our previous biochemical studies show that expression of type I Nrg1-ntf$_\beta$ in ErbB-expressing MCF-7 cells activates the Nrg1-ErbB pathway by enhancing phosphorylation of the downstream signaling molecules Akt and Erk (8). In this study, we used mouse models to investigate whether an increase in the expression of *Bace1*-cleaved Nrg1-ntf (termed as Nrg1-ntf$_\beta$) would have beneficial effects on brain development and functions. For this purpose, we generated transgenic mice overexpressing Nrg1-ntf$_\beta$ under the control of tetracycline (Tet) responsive element (Tet-Off promoter). We found that increased expression of the Nrg1-ntf$_\beta$ transgene in mouse forebrain is sufficient to increase expression of myelin proteins, consistent with activation of the Nrg1-ErbB pathway. Unexpectedly, these mice also developed schizophrenia-like behaviors, which were reversed if transgene expression was turned off. Hence, our results suggest that Nrg1 levels should be finely balanced and that sustained high levels of soluble Nrg1 can cause schizophrenia-like behaviors.

Methods and Materials

Generation of Human N1β Transgenic Mice

The *BACE1*-cleaved *N*-terminal fragment of human *NRG1* β1a (N1β) was subcloned into the BamHI and NotI sites of pTRE2hyg

From the Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio.

Address correspondence to Riqiang Yan, Ph.D., Department of Neurosciences, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195; E-mail: yanr@ccf.org.

Received May 20, 2013; revised Aug 30, 2013; accepted Sep 26, 2013.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.09.026

vector (Clontech Laboratories, Mountain View, California). A linearized NheI fragment containing the transgene was used for transgenic mouse production. Five TRE-N1β founders in the C57BL/6-CBA(J) background were identified by polymerase chain reaction with primers (forward CATCGTGGAATCAAACGAGA; reverse TTTGCCCCCTCCATATAACA) and further confirmed by Southern blotting. The Tg-N1β mice were backcrossed with C57BL/6J mice for six generations before crossing with CaMK2α-tTA mice (Jackson Laboratories, Bar Harbor, Maine; stock number 007004). Mice were housed in designated animal rooms at 23°C on a 12-hour light/dark cycle with food and water available ad libitum. For doxycycline (Dox) (Sigma-Aldrich, St. Louis, Missouri) treatment, the drug was added to drinking water at .5 mg/mL, supplemented with 2% sucrose. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Lerner Research Institute in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory Animals.

Western Blotting and Antibodies

Mouse tissues were freshly dissected, and proteins were extracted with modified radio immunoprecipitation assay buffer (50 mmol/L Tris-hydrochloride, pH 7.4, 1% NP-40, .25% sodium-deoxycholate, 150 mmol/L sodium chloride, 1 mmol/L ethylenediamine tetraacetate, 1 mmol/L sodium vanadate, protease inhibitors). At least two mice from each group were used for western blot analysis. Equal amounts of protein (40 μg) were resolved on a NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, California) and transferred onto a nitrocellulose membrane (Invitrogen). After protein transfer, blots were incubated with the specified antibodies: Nrg1 (H210), SNAP25, Complexin, and VAMP (Santa Cruz Biotechnology, Santa Cruz, California); p-ErbB4 (sc-33040, Santa Cruz), Akt and p–Akt (S473) (Cell Signaling Technology, Danvers, Massachusetts); myelin basic protein (MBP) (Sternberger Monoclonal, Lutherville, Maryland); Actin (Sigma-Aldrich); ErbB4, NR1, NR2A/B, γ-aminobutyric acid receptor (GABAR)α1, and Parvalbumin (EMD Millipore, Billerica, Massachusetts); and Synaptophysin and GAP43 (Sigma-Aldrich). The antibody to proteolipid protein (PLP) was previously obtained as a gift from Dr. Pfeiffer at the University of Connecticut.

Behavioral Testing

All testing except for prepulse inhibition (PPI) was performed at 23°–24°C in an isolated behavior room of the Rodent Behavioral Core at the Lerner Research Institute, Cleveland Clinic. Mice were taken to the testing room 1 hour before testing began to acclimate to the environment. All behavioral tasks were video-tracked and analyzed by the Ethovision XT software system (Noldus Information Technology, Leesburg, Virginia).

Y-Maze Test. The Y maze is composed of black plastic with three identical arms positioned 120 degrees apart. Mice were placed in the center of the maze and allowed to explore for 5 min, during which time a video camera was used to record the activity of the animal in the maze. The number of spontaneous alternations was used to assess spatial working memory. Mice were used for the Y-maze test first, and then half of the mice were used for the social behavioral test, and the other half were used for the contextual fear conditioning test.

Social Behavior Test. The sociability apparatus is a rectangular, three-chamber box (Stoelting, Wood Dale, Illinois). Each chamber measures 20 cm (length) × 40.5 cm (width) × 22 cm (height). Dividing walls are made from clear Plexiglas, with small openings (10-cm width × 5-cm height) that allow free access into each chamber. Photo beams are embedded across each doorway. An automated photo beam detector registers time spent in each chamber and the number of transitions. After mice were habituated in the center chamber for 5 min, their social interactions were assessed by evaluating the amount of time (during a 10-min period) the animal spent investigating an unfamiliar "stranger" mouse inside a wire cage located in one of the side chambers as compared with the time spent investigating an identical but empty wire cage contained in the other side chamber. In the second phase of testing, a second unfamiliar mouse was introduced into the previously empty chamber. The preference for social novelty was then tested for 10 min by measuring the amount of time the test mouse spent investigating the new, unfamiliar "stranger" mouse (also restricted in a wire cage) as compared with the time spent with the now-familiar mouse.

Open Field Test. The open field arena (41 cm × 41 cm) was equipped with a 16 × 16 grid of photo beam sensors 2.54 cm apart (San Diego Instruments, San Diego, California). An additional 16 photo beam sensors were used to measure rearing behaviors. In each trial, mice were placed into the center of the arena and allowed to explore freely. Beam breaks were recorded at 1-min intervals throughout the trial and converted to directionally specific movements. After 20-min initial habituation in the arena, mice received injection with MK-801 (.3 mg/kg in phosphate-buffered saline, IP; Sigma-Aldrich), and their locomotion was recorded for 90 min. Locomotor activity was measured as total distance travelled.

Contextual Fear Conditioning Test. This test consisted of three daily trials. On the first day, the conditioning period, the mouse was placed in the conditioning chamber (Med Associates, St. Albans, Vermont) for 3 min (phase A) before the onset of a sound at 2800 Hz and 85 dB for 30 sec (phase B, conditioning stimulus). The last 2 sec of the conditioning stimulus was coupled with a .7-mA continuous foot shock (phase C, unconditioned stimulus). After resting an additional 30 sec in the chamber, phases B and C were repeated once, and the mouse was returned to its home cage after resting in the chamber for another 30 sec. On the second day, mice were tested for their contextual memory in the same chamber for 3 min without either sound or foot shock. On the third day, mice were tested for their cue-induced memory in a different chamber environment with the sound but no foot shock. Fear learning ability of the mice was measured as the percentage of freezing, which was defined as the percentage of time completely lacking movement, except for respiration, in intervals of 5 sec.

Statistical Analysis

Statistical analysis was performed with Sigmastat 3.5 (Systat Software, Chicago, Illinois). All data are expressed as mean ± SEM. Social behavior tests were analyzed by two-way analysis of variance with Tukey's post hoc tests. Data from other experiments with 3 or more groups were analyzed by one-way analysis of variance with Tukey's post hoc tests. Two-tailed Student *t* tests were used to analyze data from experiments with two groups. Significant *p* values are denoted by the use of asterisks in the text and figures.

Results

Generation of Transgenic Mice Expressing Nrg1-ntf$_\beta$ Transgene

We have previously mapped *Bace1* cleavage of Nrg1 to the site between amino acids F237 and M238, which is located 10 amino