Behavioral Alterations in Mice Lacking the Gene for Tenascin-X

Kohei KAWAKAMI[a] and Ken-ichi MATSUMOTO*[,b]

[a] *Department of Experimental Animals, Center for Integrated Research in Science, Shimane University; and* [b] *Department of Biosignaling and Radioisotope Experiment, Center for Integrated Research in Science, Shimane University; Enya-cho, Izumo 693–8501, Japan.* Received November 13, 2010; accepted January 15, 2011; published online January 19, 2011

Tenascin-X (TNX) is the largest member in the tenascin family of large oligomeric glycoproteins of the extracellular matrix (ECM). TNX is expressed in the leptomeningeal trabecula and connective tissue of choroid plexus in the brain as well as in muscular tissues. Interestingly, single nucleotide polymorphism (SNP) analysis in human showed that TNX is significantly associated with schizophrenia. Previously we generated TNX-deficient (TNX−/−) mice by homologous recombination using embryonic stem (ES) cells. In the present study, we analyzed behaviors relevant to affect, learning and memory, and motor control in TNX−/− mice. TNX−/− mice showed increased anxiety in light–dark and open-field tests and superior memory retention in a passive avoidance test. Also, TNX−/− mice displayed higher sensorimotor coordination than did wild-type mice in a rotorod test. However, TNX−/− mice did not differ from wild-type mice in locomotor activity in a home-cage activity test using telemetric monitoring. These findings suggest that TNX has diverse roles including roles in behavioral functions such as anxiety, emotional learning and memory, and sensorimotor ability.

Key words tenascin-X; knockout mouse; behavioral analysis

The tenascin family constitutes a group of extracellular matrix (ECM) glycoproteins with a characteristic structure. Four members of this family [tenascin-C (TNC), tenascin-R (TNR), tenascin-X (TNX) (known as tenascin-Y in birds), and tenascin-W (TNW)] have so far been identified in vertebrates,[1] and they all have a cysteine-rich segment at the amino terminus followed by epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a fibrinogen-like domain at the carboxy terminus.

TNX is the largest member of the tenascin family with a size of about 450 kDa. Complete deficiency of TNX in humans leads to a rare recessive form of Ehlers-Danlos syndrome (EDS), and TNX haploinsufficiency is associated with hypermobility type of EDS.[2—4] There are several lines of evidence suggesting that TNX participates in collagen fibrillogenesis,[5,6] collagen deposition,[7] modulation of collagen stiffness,[8] and development and maintenance of elastic fibers.[9] TNX-deficient (TNX−/−) mice generated by TNX gene targeting in murine embryonic stem (ES) cells showed progressive skin hyperextensibility, similar to individuals with EDS. Biomechanical analyses indicated reduced tensile strength of their skin.[10] Furthermore, TNX−/− mice showed enhanced tumor invasion due to activation of matrix metalloproteinase (MMP)-2 and MMP-9.[11] TNX−/− mice also displayed increased amount of triglyceride and altered composition of triglyceride-associated fatty acids.[12]

TNX is expressed much more widely than other tenascins.[13] TNX is also present in the leptomeningeal trabecula and connective tissue of choroid plexus in the brain[14] as well as in the peripheral nerves[15] and in developing spinal cord meninges.[16] Interestingly, single nucleotide polymorphism (SNP) analysis has revealed a strong association between the *TNX* locus and schizophrenia.[17,18] Previously, we investigated the distribution of TNX in sciatic nerves by immunohistochemical staining.[19] TNX was found to be localized in the perineurium and the endoneurium of sciatic nerve fibers. These results suggested that TNX plays an important role in neural functions.

In the present study, we analyzed behaviors relevant to affect, cognition, and motor control in the TNX−/− mice to better elucidate the role of endogenous TNX.

MATERIALS AND METHODS

Animals TNX−/− mice were generated by TNX gene targeting in ES cells as described previously.[11] TNX−/− mice were further established by backcrossing original TNX−/− mice into a congenic line, C57BL/6J, for 10 generations. Male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used as wild-type (TNX+/+) mice. The animals were housed in the Department of Experimental Animals, Center for Integral Research in Science, Shimane University at room temperature of 23±2 °C, humidity of 55±10% and ventilation of 10—13 times per hour. The mice were kept on a 12:12 h light–dark schedule (lights on at 7:00 a.m.) with commercial chow (NMF, Oriental Yeast, Tokyo, Japan) and water given *ad libitum*. This study was approved by the Ethical Committee for Animal Research of Shimane University, and all of the experimental procedures were performed according to the institutional guidelines.

Behavioral Testing Male mice were tested at 8, 9, 10, 11 weeks of age for light–dark preference, open-field, passive avoidance, and rotorod tests and at 9, 13, 17 weeks of age for a home-cage activity test. All experiments were performed during the light period.

Light–Dark Preference Test The light–dark preference test measures the conflict between the tendency to explore a novel environment *versus* the aversive qualities of a lighted space. Longer time spent in the dark side of the apparatus is indicative of increase in anxiety-like behavior. Mice at 8 weeks of age were examined in a light–dark apparatus consisting of a light (illuminated) compartment (10 cm×25 cm×25 cm) with a 100 W bulb (luminescence: 2000 lux) connecting to a dark compartment (30 cm×30 cm×30 cm) separated by a board (Muromachi-Kikai Co., Ltd., Tokyo, Japan). Mice were placed in the center of the light box for 5 min to acclimate to the test environment. Then the separation board was removed and the connecting gate to the dark compartment

* To whom correspondence should be addressed. e-mail: matumoto@med.shimane-u.ac.jp

was opened. The trials were each 10 min, and the percentage of cumulative time spent in the light compartment and the total number of transitions between the two compartments were scored live by an experimenter.

Open-Field Test The open-field test is used to evaluate exploratory behavior and measures of anxiety by counting small movements such as grooming, large movements such as ambulation, and rearing. Intense anxiety causes mice to suppress grooming, ambulation, and rearing. Each mouse at 10 weeks of age was placed in a 56.5 cm×56.5 cm×6 cm brightly lit open arena (300 lux) equipped with a near-infrared sensor interfaced with a computer (SCANET-MV-10, Toyo-Sangyo Co., Ltd., Okayama, Japan) for 35 min. By the near-infrared sensor, small movements such as grooming were detected at a minimum distance of 6.0 mm (Grooming). A square of 15.0 cm was set, and when mice moved out of the square, the movement was counted as a large locomotion (Ambulation). In addition, the number of rearing movements was counted by detecting movement over a height of 4 cm (Rearing). The apparatus was cleaned with 70% ethanol between trials.

Rotorod Test To assess sensorimotor ability, mice at 9 weeks of age were tested using a rotorod apparatus (SN-445, Shinano Manufacturing Co., Ltd., Tokyo, Japan). Each mouse was habituated on the rotating rod (3.0 cm in diameter) for 1 min before testing began. The trials were conducted at turn speed of 15 rpm. The number of turns before the mouse fell from the rotating rod was counted. Mice were tested once per day on three consecutive days.

Passive Avoidance Test The passive avoidance test was used to examine emotional learning and memory. On day 1 of testing, each mouse at 11 weeks of age was placed in a light compartment connected to a dark compartment separated by a board, the same apparatus as that used in the light–dark preference test (Muromachi-Kikai Co., Ltd.). After 5 min of acclimation, the separation board was removed and the connecting gate to the dark compartment was opened. A mouse preferring the darkened side moves quickly through the gate to the dark compartment. Upon doing so, the mouse received a 0.3 mA electrical shock (3 s in duration) from the grid floor (Shock Generator SGS-002T, Muromachi-Kikai Co., Ltd.). On day 2, the same procedure was used except for removal of the shock. Cumulative time spent in the light compartment was measured.

Home-Cage Activity Test Pentobarbital anesthesia (50 mg/kg intraperitoneally (i.p.)) was used for all surgical procedures. A battery-operated free-floating transmitter (model TA10TA-F20, Data Sciences International, St. Paul, MN, U.S.A.) was inserted into the abdominal cavity of the mice at 9, 13, 17 weeks of age. The peritoneal muscle and skin layers were closed with sutures. Immediately after surgery, each mouse was returned to its home cage. Locomotor activity was continuously monitored using the Dataquest A.R.T. system (Data Sciences International). Locomotor activity was obtained by counting the number of impulses, detected by changes in signal strength at 10-min intervals. The signal was received by an antenna under each mouse's cage and transferred to a computer. After 24 h of acclimation, the data of cumulative locomotor activity (counts) during light and dark periods were collected separately every 12 h.

Statistics Data were analyzed for statistical significance by Student's *t*-test or ANOVA with *post hoc* by Scheffé's test in six to ten experiments, where $p<0.05$ was considered statistically significant. These analyses were performed using StatView version 4.0 (SAS Institute Inc., Cary, NC, U.S.A.). Results are expressed as means±S.E.

RESULTS

TNX−/− Mice Show Increased Anxiety-Like Behavior In the light–dark preference test, TNX−/− mice spent less time in the light compartment ($p<0.01$) (Fig. 1A) and moved less ($p<0.01$) (Fig. 1B). These results indicated that TNX−/− mice show more anxiety-like behavior than do wild-type mice.

Consistent with the increased anxiety-like behavior in the light–dark preference test, increased anxiety-like behavior in TNX−/− mice was also revealed in the open-field test. Small movements such as grooming ($p<0.01$) (Fig. 2A) and large movements such as ambulation ($p<0.05$) (Fig. 2B) as well as rearing ($p<0.01$) (Fig. 2C) in TNX−/− mice were significantly less frequent than those in wild-type mice.

TNX−/− Mice Display Superior Sensorimotor Coordination and Emotional Learning and Memory TNX−/− mice were superior to wild-type mice in the rotorod test as indicated by longer latency to fall from the rotorod. This tendency was strengthened as the number of trials increased ($p<0.05$) (Fig. 3A). The results indicated that TNX−/− mice have ability superior to sensorimotor coordination (Fig. 3B).

In the passive avoidance test of learning and memory, TNX−/− mice showed higher latency than did wild-type mice to re-enter the dark compartment ($p<0.01$) (Fig. 4).

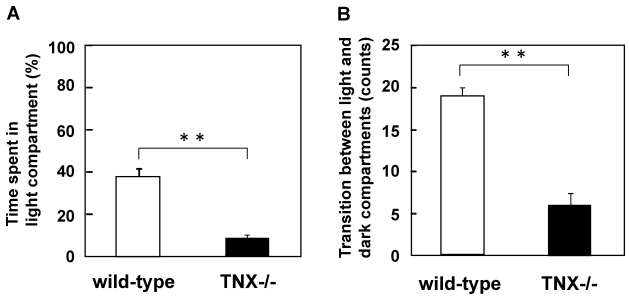

Fig. 1. Light–Dark Preference Test in TNX−/− and Wild-Type Mice

(A) Percentage of cumulative time spent in the light compartment during a period of 10 min. (B) Cumulative light and dark compartment transitions of mice during a period of 10 min. Data represent means±S.E.. ** $p<0.01$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=10$ mice per genotype.

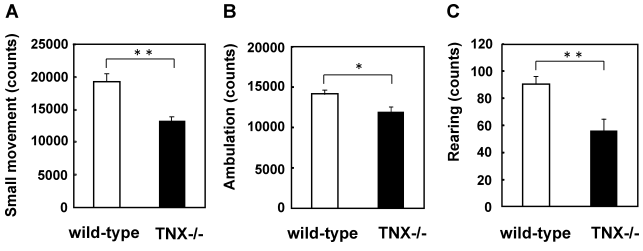

Fig. 2. Open-Field Test in TNX−/− and Wild-Type Mice

(A) Small movement. (B) Ambulation. (C) Rearing. Counts were done every 35 min. Data represent means±S.E. ** $p<0.01$ and * $p<0.05$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=10$ mice per genotype.

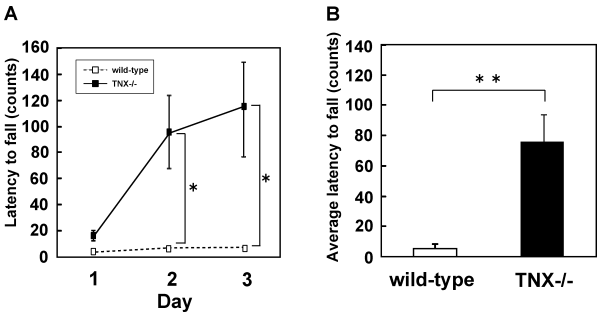

Fig. 3. Rotorod Test in TNX−/− and Wild-Type Mice

(A) Number of turns before mice fell from the rotating rod. Mice were tested once per day on three consecutive days. $*p<0.05$ *versus* age-matched C57BL/6J wild-type mice, Scheffé's test. (B) Average number of turns for three days. Data represent means±S.E. $**p<0.01$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=10$ mice per genotype.

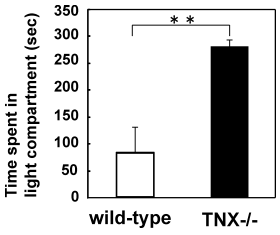

Fig. 4. Passive Avoidance Test in TNX−/− and Wild-Type Mice

After 24 h from electrical shock to mice, cumulative time spent in the light compartment is shown. $**p<0.01$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=6$—10 mice per genotype.

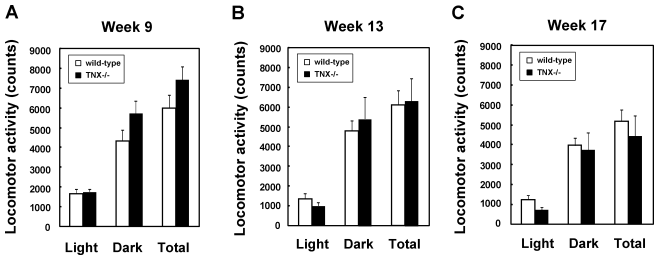

Fig. 5. Home-Cage Activity Test in TNX−/− and Wild-Type (C57BL/6J) Mice

Locomotor activities of free moving mice were counted using telemetry and a data acquisition system in the light and dark periods at the ages of 9 weeks (A), 13 weeks (B), and 17 weeks (C) for 12 h. Note that there is no genotype difference in locomotor activity at any age. $n=9$—10 mice per genotype.

This result indicated that TNX−/− mice have superior passive avoidance memory retention.

Home-Cage Activity Test To assess diurnal locomotor activity in free moving TNX−/− and wild-type mice, telemetry and a data acquisition system were used. All of the mice used for this test appeared lively throughout the study, and we observed no behavioral differences compared with mice without transmitters. In consideration of circadian rhythms in locomotor activity, locomotor activities were separately recorded during the light period (07:00 to 19:00 h) and during the dark period (19:00 to 07:00 h). Figure 5 shows the locomotor activity at indicated ages. Although circadian rhythms in locomotor activity were observed in both genotypes (low during the light period and high during the dark period), there was no difference in locomotor activity between the two genotypes at any age.

DISCUSSION

Our data indicated that TNX deficiency alters measure of anxiety. TNX−/− mice showed hypoactivity and increased anxiety-like behavior in the two anxiety tests used (light–dark preference and open-field tests). The knockout mice also displayed superior emotional learning and memory in the passive avoidance test and superior sensorimotor ability in the rotorod test. However, no difference in diurnal locomotor activity was detected in TNX−/− mice in the home-cage test.

Among the tenascin family members, TNC has been reported to influence cerebellar granule cell migration and guide postnatal granule cell neurons from the external to the internal cell layer in the cerebellum.[20] Behavioral abnormalities such as hyperlocomotion have been observed in TNC−/− mice due to a decreased level of dopamine transmission in the brain.[21,22] Furthermore, TNR plays an important role in neurite outgrowth, axon targeting, neural cell adhesion, and migration and differentiation during nervous morphogenesis in the central nervous system.[23] TNR−/− mice showed alterations of the extracellular matrix and decreased axonal conduction velocities in the central nervous system.[24] On the other hand, although the localization of TNX in the cerebral cortex has not been disclosed, TNX is localized in the leptomeningeal trabecula and in the connective tissue of the choroid plexus in the brain.[14] In the peripheral nervous system, TNX is localized in the perineurium and endoneurium of nerve fibers.[19] Although we previously showed that individual axons in the sciatic nerves of TNX−/− mice do not differ from those of wild-type mice in ultrastructure,[19] morphological and biochemical analyses of the brains of TNX−/− mice have not been done. Interestingly, SNP analysis in human showed that TNX is significantly associated with schizophrenia.[17,18] This evidence indicates an important role of TNX in the central nervous system.

It is known that a hyperlocomotive and anxiolytic-like phenotype is characteristic of rodent models of schizophrenia[25] and could correspond to psychomotor agitation present in schizophrenic patients. In contrast to the behaviors exhibited in schizophrenia model animals, TNX−/− mice showed reverse phenotypes such as increased hypoactivity and anxiety-like behavior. SNP analysis in schizophrenia patients suggested an important non-synonymous substitution such as Glu2578Gly located in exon 23 of *TNX*.[17,18] Thus, such point mutation in *TNX* might be necessary for a factor of schizophrenic illness rather than the null mutation in the TNX-deficient mice. In TNX−/− mice, some abnormalities such as collagen deposition alteration,[10] enhanced activation of MMP,[12,26] triglyceride accumulation and altered composition of triglyceride-associated fatty acids,[12] have been reported. The behavior alterations in TNX−/− mice in this paper might appear as comprehensive outcome of some abnormalities to be seen in TNX−/− mice. Further biochemical and morphological studies are needed to elucidate the relationship between schizophrenia and function of TNX.

In conclusion, this study suggests an important role for TNX in anxiety-like behavior, emotional learning and memory, and sensorimotor ability. Future biochemical and pharmacological studies should be done to reveal the precise

mechanism underlying the effects of TNX on behavior. Morphological and biochemical analyses of the brains of TNX−/− mice would be also useful for this purpose.

Acknowledgments We thank Hitomi Arauchi for her contribution to preparing some figures. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Tucker R. P., Drabikowski K., Hess J. F., Ferralli J., Chiquet-Ehrismann R., Adams J. C., *BMC Evol. Biol.*, **6**, 1—17 (2006).
2) Burch G. H., Gong Y., Liu W., Dettman R. W., Curry C. J., Smith L., Miller W. L., Bristow J., *Nat. Genet.*, **17**, 104—108 (1997).
3) Schalkwijk J., Zweers M. C., Steijlen P. M., Dean W. B., Taylor G., van Vlijmen I. M., van Haren B., Miller W. L., Bristow J., *N. Engl. J. Med.*, **345**, 1167—1175 (2001).
4) Zweers M. C., Hakim A. J., Grahame R., Schalkwijk J., *Arthritis Rheum.*, **50**, 2742—2749 (2004).
5) Minamitani T., Ikuta T., Saito Y., Takebe G., Sato M., Sawa H., Nishimura T., Nakamura F., Takahashi K., Ariga H., Matsumoto K., *Exp. Cell Res.*, **298**, 305—315 (2004).
6) Egging D., van den Berkmortel F., Taylor G., Bristow J., Schalkwijk J., *Arch. Dermatol. Res.*, **298**, 389—396 (2007).
7) Veit G., Hansen U., Keene D. R., Bruckner P., Chiquet-Ehrismann R., Chiquet M., Koch M., *J. Biol. Chem.*, **281**, 27461—27470 (2006).
8) Margaron Y., Bostan L., Exposito J. Y., Malbouyres M., Trunfio-Sfarghiu A. M., Berthier Y., Lethias C., *Biophys. Chem.*, **14**, 87—91 (2010).
9) Zweers M. C., van Vlijmen-Willems I. M., van Kuppevelt T. H., Mecham R. P., Steijlen P. M., Bristow J., Schalkwijk J., *J. Invest. Dermatol.*, **122**, 885—891 (2004).
10) Mao J. R., Taylor G., Dean W. B., Wagner D. R., Afzal V., Lotz J. C., Rubin E. M., Bristow J., *Nat. Genet.*, **30**, 421—425 (2002).
11) Matsumoto K., Takayama N., Ohnishi J., Ohnishi E., Shirayoshi Y., Nakatsuji N., Ariga H., *Genes Cells*, **6**, 1101—1111 (2001).
12) Matsumoto K., Sato T., Oka S., Orba Y., Sawa H., Kabayama K., Inokuchi J., Ariga H., *Genes Cells*, **9**, 737—748 (2004).
13) Matsumoto K., Saga Y., Ikemura T., Sakakura T., Chiquet-Ehrismann R., *J. Cell Biol.*, **125**, 483—493 (1994).
14) Imura K., Sato I., *Ann. Anat.*, **190**, 324—328 (2008).
15) Geffrotin C., Garrido J. J., Tremet L., Vaiman M., *Eur. J. Biochem.*, **231**, 83—92 (1995).
16) Deckner M., Lindholm T., Cullheim S., Risling M., *Exp. Neurol.*, **166**, 350—362 (2000).
17) Wei J., Hemmings G. P., *Am. J. Med. Genet. B. Neuropsychiatr. Genet.*, **125**, 43—49 (2004).
18) Tochigi M., Zhang X., Ohashi J., Hibino H., Otowa T., Rogers M., Kato T., Okazaki Y., Kato N., Tokunaga K., Sasaki T., *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **144B**, 305—309 (2007).
19) Matsumoto K., Sawa H., Sato M., Orba Y., Nagashima K., Ariga H., *Acta Neuropathol.*, **104**, 448—454 (2002).
20) Husmann K., Faissner A., Schachner M., *J. Cell Biol.*, **116**, 1475—1486 (1992).
21) Fukamauchi F., Mataga N., Wang Y.-J., Sato S., Yoshiki A., Kusakabe M., *Biochem. Biophys. Res. Commun.*, **221**, 151—156 (1996).
22) Cifuentes-Diaz C., Velasco E., Meunier F. A., Goudou D., Belkadi L., Faille L., Murawsky M., Angaut-Petit D., Molgó J., Schachner M., Saga Y., Aizawa S., Rieger F., *Cell. Mol. Biol.*, **44**, 357—379 (1998).
23) Pesheva P., Probstmeier R., *Prog. Neurobiol.*, **61**, 465—493 (2001).
24) Weber P., Bartsch U., Rasband M. N., Czaniera R., Lang Y., Bluethmann H., Margolis R. U., Levinson S. R., Shrager P., Montag D., Schachner M., *J. Neurosci.*, **19**, 4245—4262 (1999).
25) Gainetdinov R. R., Mohn A. R., Bohn L. M., Caron M. G., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 11047—11054 (2001).
26) Matsumoto K., Minamitani T., Orba Y., Sato M., Sawa H., Ariga H., *Exp. Cell Res.*, **297**, 404—414 (2004).