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Abnormal Behavior and Neurotransmissions of Tenascin Gene Knockout Mouse

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To examine the role of tenascin (TN) *in vivo,* we have produced mice in which the TN gene is inactivated. In behavioral studies, TN-knockout mice showed abnormal behavior such as hyperlocomotion and poor swimming ability. Biochemical analysis revealed that serotonin (5-HT) and dopamine (DA) transmission was decreased in the cerebral cortex, the hippocampus, or the striatum of TN-knockout mouse brain. The intraperitoneal administration of the DA receptor agonist, LY171555 (0.5 mg/kg, BW), inhibited the hyperlocomotion, and swimming behavior was transiently improved by the treatment with the 5-HT receptor agonist, 1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride. These findings suggest that TN may play an important role in neurotransmissions related to behavior.

The extracellular matrix (ECM) is believed to play an important role in morphogenesis and molecular function, especially in the process of animal development (1,2). Tenascin-C (TN) is a large ECM glycoprotein and is highly expressed in the developing central nervous system (CNS), where it is involved in neuron-glia cell interactions, migration, axonal outgrowth, and tissue boundary formation (3,4).

To directly assess the function of TN *in vivo,* we introduced a null mutation in the TN gene by targeted mutagenesis. Matings of heterozygotes produce homozygous mutant offspring, indicating that embryos lacking TN gene products can develop to term normally (5). However, most homozygous mutant mice show hyperlocomotion, stereotyped turning behavior, or poor swimming.

Herein, the effects of the TN gene on neuronal transmissions involved in the abnormal behavior were examined. We analyzed the behavioral and neurochemical features of TN-knockout mice, and found that serotonin (5-HT) and dopamine (DA)-related agents improved upon these behavioral abnormalities.

EXPERIMENTAL PROCEDURES

Animals. We have used homologous recombination in embryonic stem cells to disrupt the TN gene and generate strains of mice deficient in the TN gene (5). The present study was carried out on postnatal TN−/TN− homozygous knockout mice which showed both hyperlocomotion and swimming abnormality, TN+/TN− heterozygote, and normal wild-type mice (age postnatal day 21–28). The mice were originally maintained under SPF conditions in the animal facility of Tsukuba Life Science Center, RIKEN (Ibaraki, Japan).

Behavioral studies. The mice, weighing 25–30g, at the start of the experiment, were housed individually and maintained in a 12h-light:12h-dark, temperature-controlled environment, with free access to food and water. For the measurement of the locomotor activity, a round open field or an empty plastic cage (16 × 22 cm) was used and the locomotion was recorded by SUPERMEX (Muromachi Kikai Co., LTD., Tokyo, Japan). The ambulation was scored by using a personal computer interfaced to the mouse body-temperature sensitive sensors.

Swimming test was carried out in the water tank (35 × 50 × 25 cm). The depth of water was such that the mice could not touch the bottom. The mice were put onto the water surface gently in the center of the pool and their swimming behavior

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was checked. If the mice appeared to be drowning, they were picked up immediately. If the mice did not drown for 3 min, they were regarded as normal mice as to the swimming test.

Chemicals. To examine the pharmacological effects on behavior, the animals received intraperitoneal injections of (+)SKF38393 (5 mg/kg), LY171555 (0.5 mg/kg), ketanserin (5 mg/kg), [1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride] (DOI) (3 mg/kg), or the same volume of vehicle as a control. All drugs used were purchased from Research Biochemicals, Inc. (Natick, USA).

Tissue preparation for biochemical analysis. After completion of behavioral testing, the animals remained on this regimen until they were killed. Animals were decapitated and the cerebral cortex, the striatum, the hippocampus and the cerebellum were rapidly removed under the stereoscopic microscope, and frozen in liquid nitrogen. Tissue samples were stored at −80°C until analyzed for ion-pair high performance liquid chromatography (HPLC) measurement of 5-HT, DA and their metabolites (6).

Radioligand receptor binding assays. The binding of [^{3}H]ketanserin ([ethylene-^{3}H]-ketanserin hydrochloride, specific activity, 80 Ci/mmol; New England, USA) was analyzed in the mouse frontal cortex by the method of Lysen et al. (7). Non-specific binding was defined with 10μ M methysergide. The maximum number of binding sites (Bmax) and the dissociation constants (Kd) were determined by Scatchard analysis. [^{3}H]spiperone binding assays were carried out following previously described methods (8). The specific binding was defined as the difference in binding obtained from the presence and absence of (±)sulpiride (10^{-5}M). The protein concentration was estimated by the method of Lowry et al. (9).

Statistical analysis. All the data are presented as the means ± SEM of the individual values of the mice from each group. Significance of differences between two groups was analyzed using the Student's *t*-test. Multiple group comparisons were made by the one-way analysis of variance followed by Duncan's test.

RESULTS

We have examined whether abnormal behaviors such as hyperlocomotion and poor swimming ability are due to the lack of tenascin molecule by means of backcross mating test and F2 progeny. The progeny (99%) of knockout male which was inbred 9 times by sib mating showed hyperlocomotion and swimming abnormality. They were mated with C57BL/6N female to make F1 progeny, and then F1 females were backcrossed to their father male. In order to get F2 progeny, these F1 were mated each other. The locomotion test was carried out by SUPERMEX, and swimming test was performed to put each backcross progeny into the water bath. They were allowed to swim for 3 min. In this test, complete drowned mice were counted as abnormal swimmer and other mice were counted as normal. Finally, their genotype were identified by PCR.

As a result, neither heterozygous nor wild-type mice showed any swimming abnormality and hyperlocomotion. On the other hand, the ratio of the abnormal mice were more than 50% of backcross homozygous mice. In F2 progeny, these abnormalities were observed only in homozygous, but not all of the homozygous mice. Thus, these findings indicate that these behavioral abnormalities are heavily linked to the lack of tenascin molecule. What all homozygous mice did not show the abnormal swimming may be due to the effect of the genetic background on the expression of phenotype. Fig. 1 shows the mouse locomotor activities detected by SUPERMEX under the light-dark transition design. In mutant mice, the regular circadian rhythm is ambiguous (Fig.1A), and locomotive activities are 2–3 folds higher than those of the wild mice (Fig.1B). Even in the day time, the mutant mice keep on moving (Fig.1A).

We administered various kinds of agonists or antagonists against neurotransmitter receptors to assess the effect of drugs on this hyperlocomotion of TN-mutant mice. Because DA or 5-HT receptor antagonists such as SCH 23390 (0.5 mg/kg), sulpiride (20 mg/kg), or mianserin (15 mg/kg) generally possess sedative effects on even wild mice as a control, we could not determine their action for hyperlocomotion of TN-mutant mice clearly. However, the selective DA D2/D3 receptor agonist, LY 171555 (0.5 mg/kg BW), prevented the emergence of this hyperlocomotion (Fig.2), but caused no behavioral change in heterozygous or wild mice. D1-mimetic agent, (+)SKF-38393 (5 mg/kg) and 5-HT antagonists, DOI (3 mg/kg) or ketanserin (5 mg/kg) failed to decrease the hyperlocomotion of TN-knockout mice (Fig.2).

In the swimming test, the normal mice swam along the frame and looked for a place to climb up. However, mutant mice were not good at swimming, went underwater and almost drowned within 1 minute. This poor swimming behavior was not improved through repeated training. The

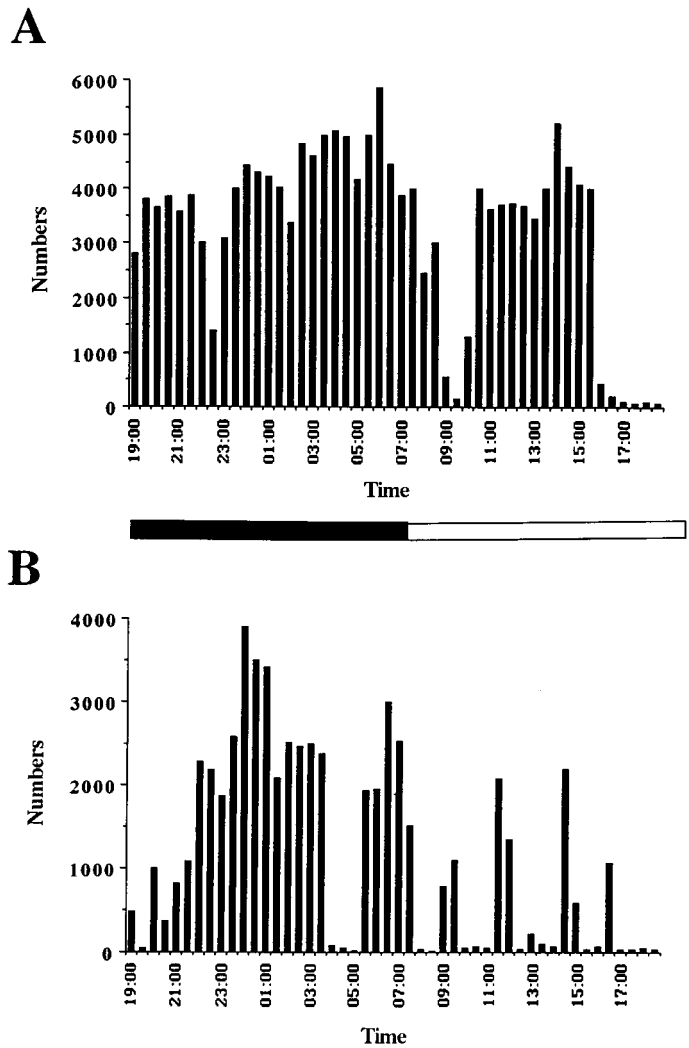

FIG. 1. Typical mouse locomotor activities under light–dark cycle. Mice were housed individually in transparent cages, kept on a 12-hour artificial light/12-hour dark cycle (dark on 19:00–07:00), and given food and water ad libitum. Each value represents accumulative scores for 30 min. (A): TN-mutant mice, (B): normal wild mice.

intraperitoneal injection of 5-HT2A/2C receptor agonist, DOI hydrochloride, made about 85% of the abnormal swimming mice improve this swimming behavior and this effect lasted several hours. Dopaminergic, adrenergic, cholinergic, histaminergic, and other drugs which were given had no effect on this abnormal swimming behavior.

The levels of 5-HT, DA and their metabolites, 5-HIAA and DOPAC, respectively, from the discrete regions of the brain were monitored using HPLC-ECD. 5-HIAA/5-HT and DOPAC/DA ratios are considered to be indexes of 5-HT or DA utilization in the synaptic cleft, respectively (10). In the mice with abnormal behavior, 5-HT turnover rate was decreased in the cerebral cortex and the hippocampus (Fig. 3A), and DA turnover rate was significantly reduced in the striatum and the hippocampus (Fig. 3B).

The binding of [^{3}H]ketanserin to the cerebral cortex, and [^{3}H] spiperone to the striatum were saturable, reversible and of high affinity (data not shown). In the [^{3}H]ketanserin binding assay, the Kd was not markedly changed, but the Bmax was increased in the cerebral cortex of the knockout

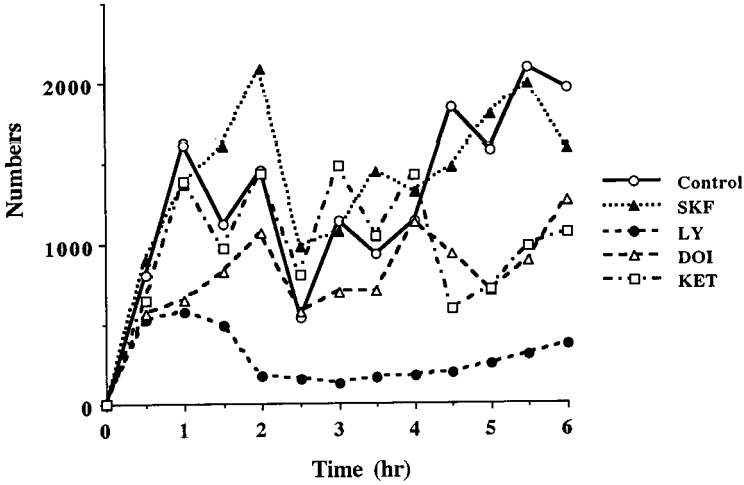

FIG. 2. Effects of serotonergic and dopaminergic agents on hyperlocomotion shown by TN-knockout mice. Each value represents the mean of data obtained on 5 or 6 rats. For the sake of clarity SEM are not shown. The scores of LY171555-challenged mice were significantly lower than those of vehicle-treated mice from 1 to 6 hr ($p < 0.01$). (SKF: (+) SKF38393 (5 mg/kg), LY: LY171555 (0.5 mg/kg), DOI (3 mg/kg), KET: ketanserin (5 mg/kg)).

mice (Table 1). On the other hand, [^{3}H]spiperone binding of mouse striatum was significantly increased (Table 2). Ketanserin is a specific 5-HT2A receptor antagonist and [^{3}H] spiperone is thought to reflect dopamine D2 receptor binding.

DISCUSSION

Knocking out genes by homologous recombination may help to elucidate the complex activities in the CNS. Mice disrupting TN gene showed the behavioral abnormalities described above and the rate of the behavioral abnormalities gradually increased by means of inbreeding to enrich this phenomenon. Neither the heterozygous nor normal wild-type mice showed abnormal behavior. It may be helpful to focus on specific molecules in the synapse, such as transmitters and receptors to analyze the nature of the molecular-behavioral interface. Previous reports showed that catecholamine and indolamine as a neurotransmitter are deeply associated with behavior and emotion. In our present study, the selective DA D2/D3 receptor agonist, LY 171555, inhibited the hyperlocomotion without affecting non-hyperlocomotive mice. On the other hand, 5-HT2A/2C receptor agonist, DOI improved the swimming behavior.

From the biochemical analysis, 5-HT turnover rate of behavioral abnormal mice was significantly decreased in the cerebral cortex and the hippocamopus, and the DA turnover rate was lowered in the striatum and the hippocampus, compared to heterozygote or wild-type mice. Fur-

TABLE 1
The Binding of [^{3}H]Ketanserin to Mouse Cerebral Cortex

mice	N	Kd (nM)	Bmax (fmol/mg protein)
+/+	7	0.34 ± 0.02	253.1 ± 4.8
+/−	6	0.33 ± 0.01	286.8 ± 5.5
−/−	6	0.36 ± 0.02	316.1 ± 10.6*

5-HT2 receptor binding sites in the mouse cerebral cortex. Values are means ± SEM.

* $p<0.05$ compared with the wild mice. Correlation coefficient: (+/+), 0.995; (+/−), 0.992; (−/−), 0.991.

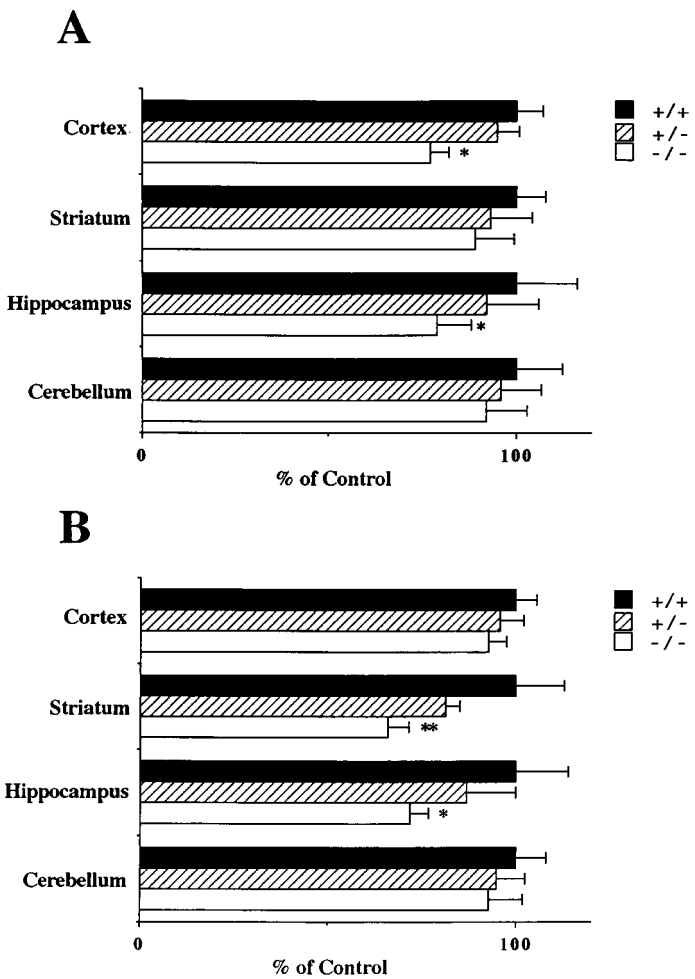

FIG. 3. 5-HIAA/5-HT (A) and DOPAC/DA (B) ratio in the discrete brain regions of (−/−) homozygous, (+/−) heterozygous, and (+/+) wild type mice measured by HPLC. Each value is the mean with SEM of 6–7 determinations. *$p < 0.05$, **$p < 0.01$ when compared to the wild mice.

thermore, binding experiments indicated that 5-HT2A binding sites detected by [^{3}H]ketanserin induced a significant increase of Bmax value in the cerebral cortex of the knockout mice, and D2 binding sites assessed by [^{3}H]spiperone similarly elevated Bmax in the striatum. Our interpretation is that these up-regulations of 5-HT2A or D2 binding sites may have developed in TN mutant mice

TABLE 2
The Binding of [^{3}H]Spiperone to Mouse Striatum

mice	N	Kd (nM)	Bmax (fmol/mg protein)
+/+	7	0.27 ± 0.03	195.6 ± 8.9
+/−	6	0.25 ± 0.02	222.1 ± 10.8
−/−	6	0.26 ± 0.03	266.9 ± 12.6*

D2 receptor binding sites in the mouse striatum. Values are means ± SEM.

* $p<0.05$ compared with the wild mice. Correlation coefficient: (+/+), 0.993; (+/−), 0.991; (−/−), 0.989.

as a compensatory mechanism to adapt to a lower level of 5-HT or DA neurotransmission in each area. Taken together, at least, poor swimming may be related to 5-HT2A receptor and hyperlocomotive activities through the D2 receptor.

The capability of receptors to change transmitter release rates gives enormous flexibility to synaptic transmission and is one source of the synaptic plasticity that underlies changes in the CNS. 5-HT has been implicated in the pathogenesis of depression, anxiety, panic disorder, or migraine (11). Manipulation of the 5-HT2 receptor with selective agents is associated with striking changes in behavior in experimental animals (12). Moreover, 5-HT2A/2C receptors may be related to feeding behavior, ACTH release, and circadian rhythm (11).

On the other hand, the DA system is involved in movement control typically seen in Parkinson's disease, thought processing, fear, anxiety, learning, and memory. The D2 receptor gene may be relevant to the augmentation, modification, or clinical onset of psychosis (13).

Thus, both the 5-HT and DA system are closely relevant to behavior and emotion. Our concern is whether these behavioral abnormalities result from motor function or unusual emotionality. The precise mechanism as to how TN modulates the neurotransmission and abnormal behavior remains unknown. There might be receptor abnormalities in sensitivity or response to the ligand, in addition to the attenuated utilization of neurotransmitters. ECM like TN acts on cytoskeletal organization via its receptors. In turn, the cytoskeleton has been demonstrated to alter gene expression at the transcription or post-transcription level (14,15). In this manner, the arrangement of the cytoskeleton, synaptic vesicle transport, or exocytosis of neurotransmitters may be affected. Further studies should be required to clarify the neural mechanisms of emotion and related behavior in terms of the signal transduction.

Finally, since the behavior of TN-knockout mice is similar to Rett's syndrome in some respects such as purposeless movements, stereotyped behavior, the difficulty of adaptability to new circumstances and insomnia, TN-knockout mice could be a useful model for this syndrome.

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