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Mutant mouse tottering: Selective increase of locus ceruleus axons in a defined single-locus mutation

(catecholamines/gene expression/terminal development/central nervous system/histofluorescence)

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ABSTRACT **The central catecholamine neuron system in the mutant mouse tottering was examined by fluorescence histochemistry and biochemical analysis of catecholamine content. This single-locus neurological mutation expresses a reproducible alteration in central nervous system physiology characterized by spontaneous spike-wave and focal motor seizures in the absence of any previously recognized disturbance of cellular organization or brain size. Histochemical analysis showed a significant increase in the number of noradrenergic axons in terminal fields innervated by the nucleus locus ceruleus when compared with the wild type. A concomitant 100–200% rise in norepinephrine levels is found in the same areas, including hippocampus, cerebellum, and dorsal lateral geniculate. Catecholamine fibers and transmitter content in areas innervated by a second major noradrenergic system arising from the brainstem lateral tegmental neurons are unaltered. The terminal axons and transmitter content were both unchanged in nuclei receiving a dense dopaminergic innervation. Despite the hypertrophy of the locus ceruleus axonal plexus, the number and size of locus ceruleus cell somata were identical in both wild-type and tottering mice. These findings are consistent with a specific gene-linked alteration of developmental events controlling the number of axons produced by a single neuronal population in the mammalian brain.**

Defined neurological mutants of the mouse (1) represent important models to analyze genetic and epigenetic factors involved in the complex processes of mammalian brain development (2). Morphological and biochemical studies have shown a variety of cytological effects of different single-gene mutations, ranging from selective alterations in restricted neural pathways to devastating structural deficits throughout the nervous system. Several mutants have provided highly reproducible genetic tools for the examination of one or a few principal developmental events governing cellular migration and synaptogenesis (2, 3).

Application of single-locus mutants to physiological investigations of central neurological disease states that may directly relate to inherited human clinical syndromes has been approached only recently (4). The mutant mouse tottering (*tg/tg*) is one example of an animal that has a well-defined alteration of central nervous system excitability, characterized by the spontaneous occurrence of seizures (5, 6). The phenotypic expression of this particular mutation is of considerable interest because of its stereotyped pathophysiology and delayed developmental onset, the existence of two additional mutant alleles, leaner (tg^{la}) (1) and Nagoya roller (tg^{rol}) (7, 8), and the unaltered regional brain morphology in the case of the tottering allele.

The organization and function of defined neurotransmitter pathways have yet to be studied in the *tg/tg* mouse. The central monoamine neuron system is the most extensively examined neurotransmitter system in the mammalian brain (9, 10) and is known to be susceptible to genetic determinants in controlling cell number, terminal axonal arbor and neurotransmitter, and synthetic enzyme levels when comparing different inbred strains (11–14). Mice homozygous for the tg^{rol} allele have been reported to have a relative increase in central catecholamine axons, although the cerebellum is reduced in size (7). The tg^{rol} homozygote can be recognized by its ataxic gait but does not exhibit the focal motor seizures seen in the *tg/tg* mutant.

To define more clearly any structural or biochemical alterations, fluorescence histochemistry and a sensitive liquid chromatographic procedure for the assay of catecholamines (CA) were used in a regional analysis of central CA pathways of *tg/tg* and wild-type (+/+) brains.

MATERIALS AND METHODS

Subjects. Matched pairs of homozygous mutant (*tg/tg*) and proven +/+ C57BL/6J mice, 4–24 weeks old, were used. Litters were housed with their mothers through the 21st postnatal day and maintained on a constant 12:12 light/dark cycle (lights on 0700–1900 hr). All animals were sacrificed between 0900 and 1100 hr. The earliest behavioral expression of the *tg* mutation occurs at 3–4 weeks postnatal, when affected mice can be readily distinguished from their normal littermates by a characteristic mild ataxic gait and a stereotyped pattern of spontaneous focal motor seizures (5, 6).

Fluorescence Histochemistry. CA-containing cell perikarya and processes were directly visualized by the sucrose/phosphate/glyoxylic acid fluorescence histochemical technique (15). In each age group, affected and +/+ mice were processed simultaneously. Every 15th coronal cryostat section (16 μm) from the frontal cortex to the lower medulla was collected, briefly incubated in the sucrose/phosphate/glyoxylic acid solution, dried in a cool air stream, and then heated for 2.5 min at 95°C. Coverslips were attached to the sections with light paraffin oil and examined on a Zeiss epifluorescence microscope. Adjacent sections were stained with cresyl violet. To objectively compare the extent of regional alteration in CA innervation, the microscopic analysis in both the mutant and control mice was performed without prior indication of tissue genotype.

Assay for CA. *tg/tg* and +/+ mice, 8 weeks old, were used for simultaneous measurement of dopamine and norepinephrine (NE) with a liquid chromatographic–electrochemical technique (16). The lower absolute detection limit of the assay system is 10–20 pg. Tissue samples containing predetermined CA projection fields were collected by free-hand dissection with the aid of an atlas of the mouse brain (17), rapidly weighed, and frozen on dry ice. Nine standardized areas ($n = 6$ for each) were

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Abbreviations: *tg/tg*, tottering; +/+, wild type; CA, catecholamine; NE, norepinephrine; LC, locus ceruleus; LT, lateral tegmental.

selected for assay. Each sample circumscribed the nucleus or an area listed in Table 1.

RESULTS

Pattern of CA Axons in Terminal Fields of the +/+ Mouse. The innervation patterns of CA axons in most areas of the mouse brain were found to be almost identical to those described in great detail in the rat (for review, see refs. 9, 10, 18). The normal adult pattern of innervation is present in all the nuclei examined in the mouse by 4 weeks. In general, three groups of CA terminal axons are readily distinguished and conform closely to those described in rat (18–20). The first group, NE-containing axons that have fine intervaricose segments and regularly spaced varicosities, arises from the NE-containing neurons of the nucleus locus ceruleus (LC) and innervates specific terminal fields in a diffuse highly collateralized pattern. This axon type is found in the neocortex, hippocampus (Fig. 1*E*), dorsal lateral geniculate nucleus (Fig. 1*C*), cerebellar cortex (Fig. 1*A*), cochlear nucleus (Fig. 2*A*), and anterior hypothalamus.

The second group of NE-containing axons arise from the brainstem NE cells outside the LC, designated as the lateral tegmental (LT) NE cell group (10). These axons are of either large or small caliber and contain irregularly spaced large and small varicosities. Many nuclei in the tubular and posterior hypothalamus, basal forebrain, and brainstem (Fig. 2*C*) are densely innervated by these axons.

The third group is present in dopamine-containing terminal fields, such as the neostriatum, olfactory tubercle, and septal area. The axons are either finely branching and highly varicose or long and thin with little branching and infrequent varicosities. Both types arise from mesencephalic dopamine neurons (19, 21).

Pattern and Density of CA Innervation in Terminal Fields of the *tg/tg* Mouse. With the single and striking exception of the NE axons of the first group, the character and pattern of the CA innervation are essentially identical in the brains of +/+ and mutant mice. The profuse LT noradrenergic projections to the hypothalamus and brainstem cranial nerve nuclei are unaffected in the mutant (Fig. 2*D*). In addition, there are no significant differences in axon number or pattern in nuclei that receive a dense dopaminergic innervation, in particular the neostriatum, nucleus accumbens, olfactory tubercle, and amygdala.

In contrast, all terminal fields that receive an LC-type axonal innervation show clear alterations in fiber number in the *tg/tg* mutant. This change was seen uniformly in the youngest mice examined (4 weeks) through those 24 weeks of age. Two- to 3-fold increases in the number of fluorescent axons are apparent in all LC terminal fields, including neocortex, hippocampus (Fig. 1*F*), dorsal lateral geniculate nucleus (Fig. 1*D*), cerebellar cortex (Fig. 1*B*), and cochlear nucleus (Fig. 2*B*). The dimensions and gross cellular organization of these target nuclei in the mutant appear unchanged from the +/+. The fluorescence intensity of individual axons also appears subjectively brighter in the *tg/tg*, although the specific pattern of innervation and axonal morphology is unchanged. Alterations in the number of NE axons in nuclei that also receive a very dense dopamine input could not be resolved by the histochemical method. Therefore, a quantitative biochemical assay for catecholamines was performed to verify the morphological observations in the LC terminal fields and the other unaltered target areas.

Determination of Regional CA Content in +/+ and *tg/tg* Brain. Nine areas were selected for assay (Table 1). Nuclei were chosen that in the rat have been shown to receive either NE afferents exclusively from LC neurons (occipital cortex, lateral geniculate, cerebellar cortex, hippocampus), LT neurons (motor trigeminal nucleus, nucleus of the solitary tract), primarily dopamine neuronal input (neostriatum, olfactory tubercle), or nuclei that share a mixed innervation (anterior hypothalamus, olfactory tubercle).

Nuclei that receive NE input arising solely from the LC all show 100–200% increases in transmitter content, although the elevation in the occipital cortex is statistically significant but less striking (27%). With this single exception, the increased levels of NE in the LC terminal fields parallel the increased number and intensity of terminal axons visualized histochemically. NE levels measured in LT-innervated regions, such as the motor trigeminal nucleus and the nucleus of the solitary tract, show slight elevations but are not statistically significant (Table 1). Dopamine content in the neostriatum and olfactory tubercle is unchanged in *tg/tg* mice. All other areas assayed contain only precursor levels of dopamine except the hypothalamic sample, which may be due to the inclusion of the caudal septal area in the dissection. This dopamine increase may be a precursor-related reflection of the large NE increase in the mutant, rather than a dopamine neurotransmitter increase in the axons of the septal area.

Preliminary Cell Counts of the LC in +/+ and *tg/tg* Mice. The evidence for a selective alteration of the LC neuron system suggested that the fiber and neurotransmitter increases might be due to either an absolute increase in the total number of LC neurons in the mutant or to the hypertrophy of the axonal plexus among a constant number of cells. Cell counts of paired LC nuclei were obtained in cresyl violet-stained serial polyester wax sections (12 μm) taken from one adult *tg/tg* mouse and from an age-matched +/+ mouse. Using the cell nucleolus as a marker, the counts were corrected by standard methods (22). The calculated number of LC cells after correction in the +/+ (1570) and *tg/tg* (1425) mice do not differ significantly and fall within the normal range calculated (1408 $\pm$ 49) for this nucleus in the C57BL/6J inbred strain (13). The diameters of LC perikarya in both animals were also found to be identical, ranging from 20- to 35 μm. By fluorescence (Fig. 2 *E* and *F*) and light microscopic appearance of cresyl violet-stained sections, the cells of the mutant LC are indistinguishable from those of the normal animal.

DISCUSSION

Selective Hypertrophy of LC Axonal Plexus. Our observations indicate that a single gene mutation (tottering) can selectively alter the LC NE neuronal system while sparing other neuronal systems that share the same class of neurotransmitter. The distinction can be made on an anatomical and biochemical basis by considering the discrete segregation of the LC and LT projections (see *Results* and refs. 10 and 20). First, the number of NE-containing axons visualized histochemically increases greatly in all the LC terminal fields examined in the *tg/tg* mouse. Second, there is a variable but striking concomitant increase of assayed NE in the same LC-innervated nuclei. Finally, fluorescence histochemistry showed no discernable alteration in the number of NE-containing axons in the LT-innervated nuclei. NE content in the LT-innervated nuclei does increase moderately but with a variance too large for the changes to be statistically significant. When analyzed in the same manner, the dopamine neuron system was also unaltered in *tg/tg* mice. The possibility that the large number of fibers seen in the *tg/tg* mouse is due to a simple increase in the NE content within axons not normally visualized in the +/+ brain is extremely unlikely because the histofluorescence method used is sufficiently sensitive to visualize even the finest (1-μm) noradrenergic axons (23). Moreover, the number and patterns of the LC NE-containing axons visualized in the +/+ tissue correspond closely

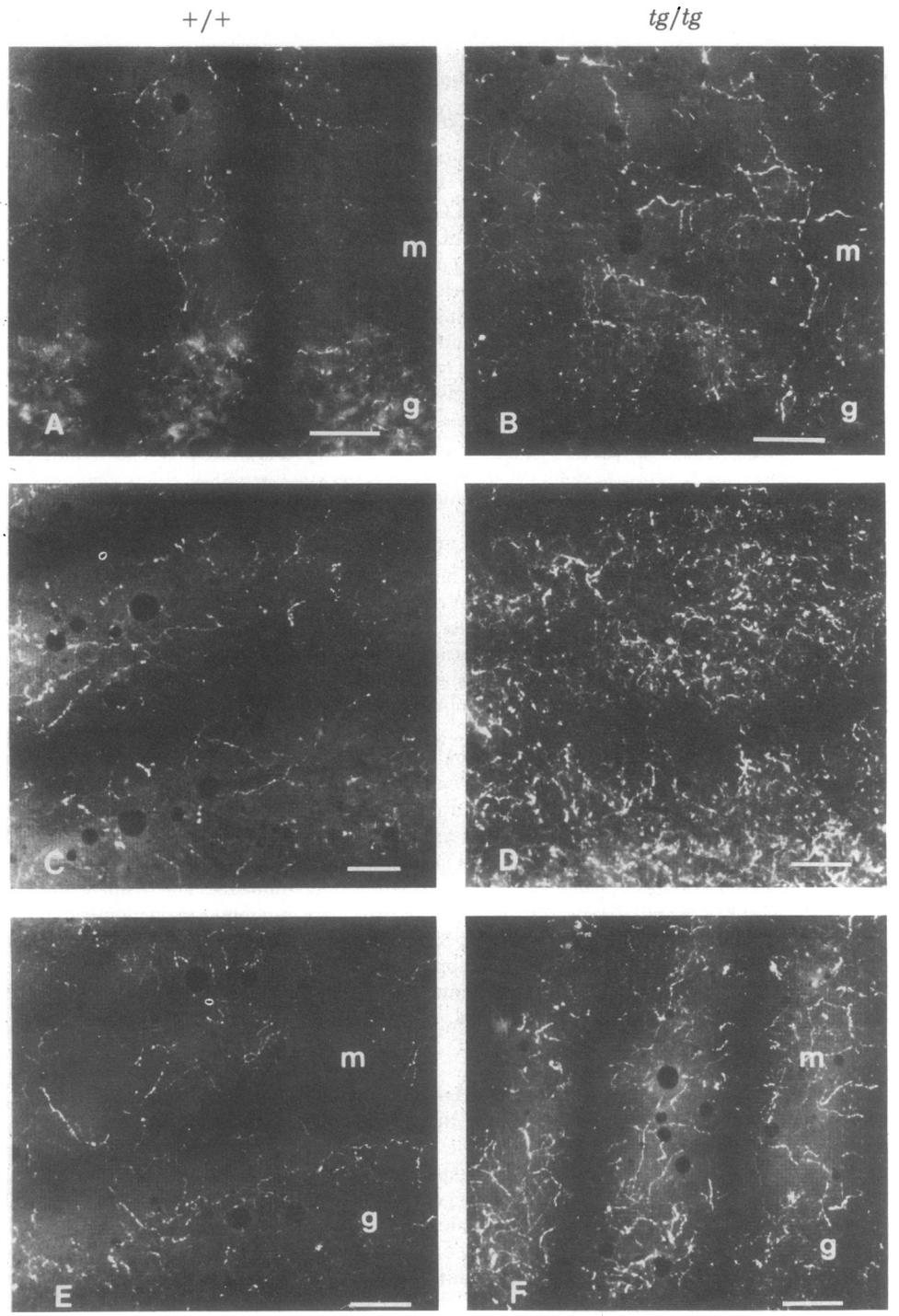

FIG. 1. Fluorescence histochemical patterns illustrating the LC NE innervation in cerebellum (*A* and *B*), dorsal lateral geniculate nucleus (*C* and *D*), and area dentata of the hippocampus (*E* and *F*) in age-matched +/+ and *tg/tg* mice. In each region, there is a striking hypertrophy of the NE axonal plexus in the *tg/tg* mouse. The typical LC-type axonal morphology and innervation pattern are preserved. Axons in the *tg/tg* cerebellum (*B*) and hippocampus (*F*) are increased in number in the molecular (m) and granule cell (g) layers. The fibers in the dorsal lateral geniculate increase throughout (*D*). (Bars = 50 μm.)

to those in recent studies using fluorescence histochemistry and the highly sensitive immunocytochemical technique using antibodies to the synthetic enzyme dopamine-β-hydroxylase (24–28). Thus, the data show a specific hypertrophic effect on one group of CA neurons that are anatomically and functionally distinct from all other CA cells in the rodent brain. It is worth noting that this selectivity resides in the central CA neuron system and does not exclude the possible alteration of other neurotransmitters in the *tg/tg* nervous system that might be expressed either *en cascade* or as independent but gene-linked events.

In many single-locus mutants, the neuropathological severity of the genetic lesions in the central nervous system has presented a considerable problem in isolating direct neurotransmitter-related changes when compared with their +/+ controls (29–31), as in the case of the tottering allele, tg^{rol} (7). The unaltered size of target brain nuclei in the *tg/tg* mouse has enabled the changes in transmitter content and axon number to be directly correlated with those of the +/+ mouse. The unambiguous increase of LC axons expressed by the *tg* gene is genuine and cannot be accounted for by differences in brain size or gross organization between the mutant and the +/+.

Site of Gene Expression Producing Increased LC Axonal Plexus. Although monoaminergic cell number generally appears to be under genetic control (12–14), the axonal hypertrophy in the *tg/tg* mouse is not due to alterations in cell number or size; cell counts and perikaryal size measurements of the LC in the *tg/tg* and +/+ mice are identical. Therefore, expression

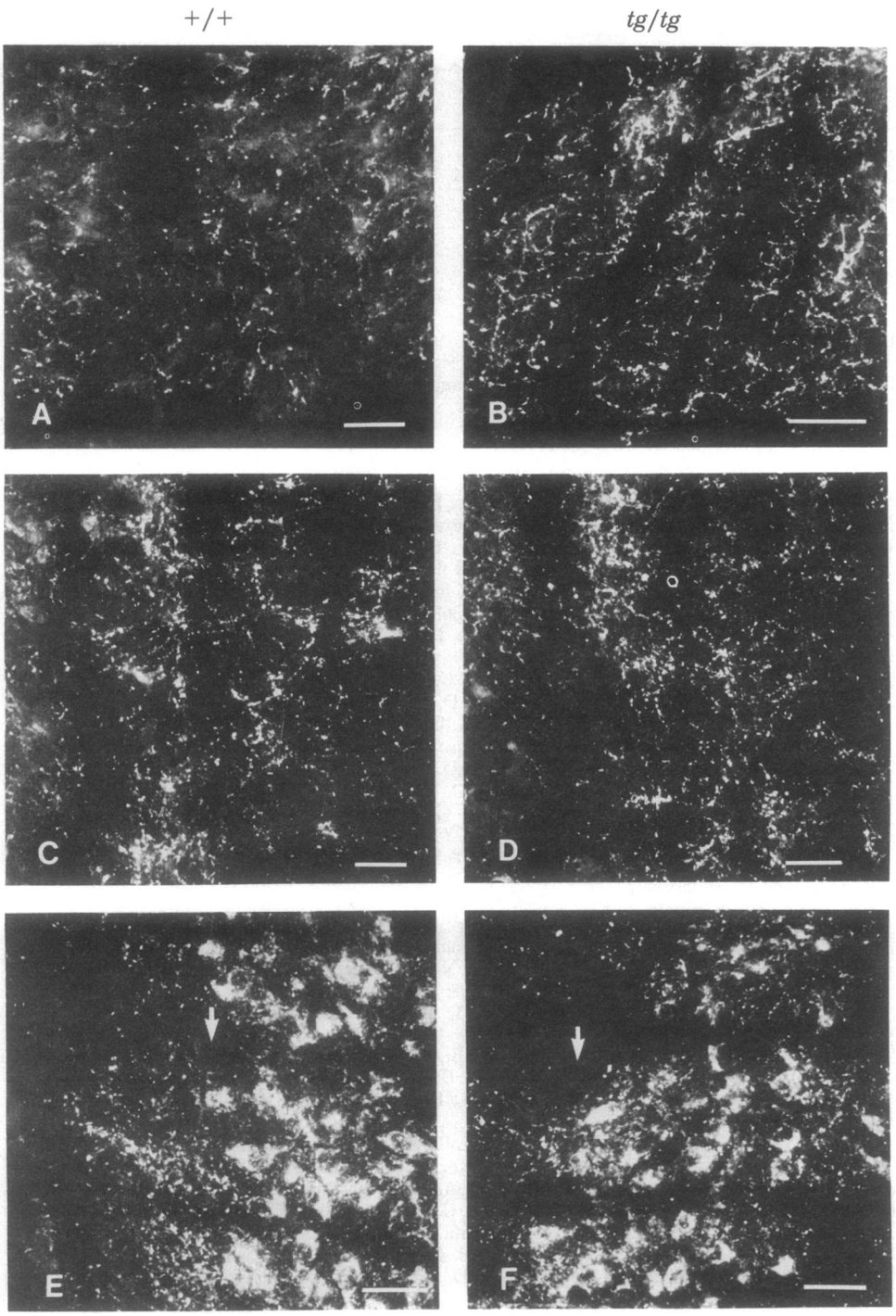

FIG. 2. Fluorescence photomicrographs of the LC innervation in the ventral cochlear nucleus and the LT NE input to the facial motor nucleus in +/+ and *tg/tg* mice. Relative hypertrophy of the LC axonal plexus in the *tg/tg* cochlear nucleus is evident (*A* and *B*). In contrast, there is no obvious difference between +/+ and *tg/tg* LT NE innervation in the facial motor nucleus (*C* and *D*). (*E* and *F*) LC cell perikarya in the +/+ and *tg/tg* mouse. The nucleus is situated medial to nonfluorescent cells in the mesencephalic trigeminal nucleus (arrows). LC cell size, number, and general histochemical appearance are identical in both animals. (Bars = 50 μm.)

of the mutant gene at the cellular level could act either on mechanisms *intrinsic* to LC cells controlling the amount of axonal arbor that an individual LC neuron produces or on those *extrinsic* to LC cells that are common to the specific target nuclei. Previous reports have shown that a substantial size reduction of an LC target area during development by chemical (32) or genetic (29) lesions affects the pattern of NE axonal innervation but not the fiber number or transmitter levels. These data suggest that factors extrinsic to LC neurons may be more involved in defining the spatial distribution or "fine tuning" of the axonal arbor (2, 32), leaving the specification of the absolute axon quantity to genetic processes intrinsic to the parent neuron. The present study provides further evidence in support of this view and indicates that the expression of a terminal field of defined proportions can be directed by a single gene.

The described changes in *tg/tg* mice represent novel observations with respect to neurological mutants and the general phenomenon of CA neuron system plasticity. Specific cell populations or fiber systems are usually atrophic in neurological mutant mice. The increased number of LC axons in the *tg/tg* mouse is the first example of change in a direction of growth. This unusual growth may be related to the capacity of central CA neurons for fiber plasticity in developing and adult animals, as shown by the proliferation of surviving CA axons after chemical or mechanical axotomy (33–35). However, the sprouting in these systems is dependent on the destruction of a portion of the terminal axonal arbor. Experimental manipulation of the CA system has yet to elicit a response that is characterized by a

Table 1. CA content of brain nuclei in +/+ and *tg/tg* mice

Region analyzed	Dopamine, ng/g of tissue			Norepinephrine, ng/g of tissue		
	+/+	*tg/tg*	% difference	+/+	*tg/tg*	% difference
Hippocampus	ND	ND		285 ± 38	566 ± 66*	99
Occipital cortex	ND	ND		251 ± 15	320 ± 43†	27
Dorsal lateral geniculate nucleus	ND	ND		384 ± 89	1057 ± 135*	175
Cerebellar cortex				446 ± 55	984 ± 108*	121
Neostriatum	2065 ± 118	2360 ± 216	14	227 ± 29	642 ± 88*	183
Olfactory tubercle	3735 ± 423	4032 ± 260	8	280 ± 34	535 ± 59*	91
Anterior hypothalamus	213 ± 12	439 ± 40*	100	621 ± 67	2027 ± 255*	226
Motor trigeminal nucleus	ND	ND		702 ± 167	968 ± 84	38
Nucleus of the solitary tract	ND	ND		512 ± 73	649 ± 128	27

Results represent mean ± SEM. ND, dopamine content is <5% of NE content and thus considered precursor level.
* $P < 0.01$ (two-tailed t test).
† $P < 0.05$.

general hypertrophy of an entire NE projection system in the absence of any apparent neuropathological lesion, as occurs in the adult *tg/tg* mouse. These selective neuroanatomical and biochemical changes in response to this inherited lesion make the *tg/tg* mouse a valuable model for developmental studies of mechanisms that govern the growth of central axons. It appears that the altered LC system and the focal motor seizures in the *tg/tg* mouse may be only indirectly linked, since the tottering allele tg^{rol} expresses a relative NE axonal proliferation in the absence of motor seizures. However, the relationship of the changes in the *tg/tg* LC system to the remainder of the pathophysiological syndrome, in particular the stereotyped pattern of hypersynchronous cortical spike-wave discharges with behavioral "absence" seizures, remains to be explored.

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