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Regular Article

Title:

Alteration of NO-producing system in the basal forebrain and hypothalamus of Ts65Dn mice: An immunohistochemical and histochemical study of a murine model for Down syndrome

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Abstract

Ts65Dn mice have been developed as a model for Down syndrome (DS). Because of its involvement in complex behaviors, including sexual and aggressive behaviors, we investigated the nitric oxide (NO) system in specific brain regions of these mutant mice (TS) after isolation-induced aggression. Male TS mice displayed significantly higher aggression than wild type (WT) mice and the comparison of the NO system, both with immunohistochemical and histochemical methods, resulted in robust differences between TS and WT mice in the hypothalamic paraventricular nucleus, in the nucleus of the diagonal band and in the medial septum, but not in the striatum of TS mice. In conclusion, we document alterations in the neuronal NO system of the TS mouse model of DS, suggesting a correlation of the behavioral aggressiveness with deficient NO production.

Keywords: Down syndrome, Ts65Dn mouse, Aggression, Nitric oxide, Paraventricular nucleus, Diagonal band, Medial septum, Caudate-Putamen complex

Introduction

Down syndrome (DS) is determined by a common chromosomal aberration that involves the total or partial trisomy of chromosome 21 in humans. DS patients are characterized by a distinctive phenotype that can be diagnosed at birth or shortly thereafter. The mental retardation is accompanied by learning and memory deficits, high intensity of repetitive behaviors, impairment in adaptive behavior, and premature senility associated with neuropathological characteristics of Alzheimer disease (Chapman and Hesketh, 2000; Cosgrave et al., 1999; Evans and Gray, 2000; Nadel, 1994). At present, few studies have been conducted on the anatomical and functional correlates in humans (Seidl et al., 2001).

The mouse chromosome 16 carries the most homologous sequences to those on the so-called “obligate region” of human chromosome 21. Because mice with total trisomy of this chromosome die *in utero* (Lacey-Casem and Oster-Granite, 1994), a partially trisomic mouse was then developed in which only the segment of mouse chromosome 16 syntenic to human chromosome 21 was triplicated (Ts65Dn) (Davisson et al., 1990). Ts65Dn mice have several behavioral alterations resembling those observed in DS patients including deficits in learning and memory (Demas et al., 1998; Demas et al., 1996; Escorihuela et al., 1998; Reeves et al., 1995), equilibrium and motor coordination (Costa et al., 1999), hyperactivity (Coussons-Read and Crnic, 1996; Escorihuela et al., 1995), as well as altered sexual and aggressive behaviors (Klein et al., 1996), suggesting deficits in adaptive behavior. It was therefore proposed that these mice might serve as a useful animal model for the study of DS (see review at (Dierssen et al., 2001).

The nitric oxide (NO) system has been implicated in the control of many of the impaired behaviors described above (Demas et al., 1997; Prast and Philippu, 2001; Vincent and Kimura, 1992). Indeed, male mice with targeted disruption of the neuronal NO synthase (nNOS) gene display inappropriate aggressiveness and increased sexual behavior (Nelson et al., 1995). Pharmacological suppression of nNOS activity in male WT mice also induces elevated aggression (Demas et al., 1997). Several of these NO-related behaviors have been reported in both DS patients and Ts65Dn mice, suggesting a possible involvement of nNOS-derived NO in DS. In the present study, we compared the distribution of nNOS immunoreactivity (nNOS-IR) and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity in serial adjacent sections from different regions of the basal forebrain: 1) the nucleus of the diagonal band of Broca (DB) and the medial septum (MS), cholinergic nuclei largely colocalized with nNOS and implicated in the control of movement; 2) the

caudate-putamen complex (CPu), also involved in the control of movement, displaying a large population of nitrinergic interneurons (Schober et al., 1989) not colocalized with cholinergic neurons; 3) the hypothalamic paraventricular nucleus (PVN), well known for its projections to the neurohypophysis, considered as a visceral effector nucleus. The PVN has a key role in the integration between autonomic and neuroendocrine functions (Kiss, 1988; Swanson and Sawchenko, 1983), and the presence of nitrinergic neurons is well documented (Arévalo et al., 1992; Miyagawa et al., 1994).

Materials and methods

Animals

Twelve segmental trisomic mice (Ts65Dn, TS) and 14 control B6C3HF1 strain male house mice (*Mus musculus*, WT) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) when two months old, and maintained at The Johns Hopkins University vivarium (Baltimore, MD, USA) until 3 months of age. They were housed individually in polycarbonate cages (28 x 17 x 12 cm), and were maintained in LD 14:10 photoperiods (lights on 0700 h EST) at $20 \pm 2^\circ\text{C}$ with relative humidity of $50 \pm 5\%$. Food and tap water were available *ad libitum* for the duration of the study. Young adult male CD-1 mice (Charles River Breeding Laboratories, USA) socially housed in groups of five per cage were used as intruders in the behavioral testing. All care and handling procedures were in accordance to institutional and government guidelines from both the USA and European Communities Council.

Behavioral testing

Aggression Test (Resident–Intruder Paradigm). The home cages of resident mice (TS or WT) were not changed or cleaned during the final week of the isolation period of 3 weeks. Early in the testing day, each animal was weighed and left undisturbed in their home cages. In the afternoon, a young male adult stimulus mouse (intruder) was introduced into the home cage of each resident, and the latency to first attack bite, the total number of attack bites, and the total duration of attack episodes (bursts of bites) initiated by the resident mouse were recorded for 900 sec. (for details see (Chiavegatto et al., 2001)). Each intruder mouse was used only once. The test was videotaped overhead and scored by two observers unaware of the genotype. The body weight data were analyzed by two-tailed *t* test. The data from the different

parameters of the aggression tests were not normally distributed, so the nonparametric Mann-Whitney test was employed. Differences were considered statistically significant if $p < 0.05$.

Fixation and tissue preservation

Two hours after the end of the behavioral tests the mice were deeply anaesthetized with xilazine-ketamine and perfused through the heart with saline solution (0.9%) until the return blood was clear and then with 400 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35). Brains were dissected out of the skull, post-fixed for 2 h in the same fixative, and rinsed in 0.01 M saline phosphate buffer (PBS). They were then placed overnight in a 30% sucrose solution in PBS, frozen in liquid isopentane at -35°C and stored in a deep freezer at -80°C until sectioning. All processing of the brain tissues was done at the University of Torino (Torino, Italy).

Brains were serially cut in the coronal planes at 25 μm thickness with a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the mouse brain atlas (Franklin and Paxinos, 1997). Sections were collected in a cryoprotectant solution (Watson et al., 1986) at -20°C . Every fourth section (a section every 100 μm) was processed for nNOS. Alternate sections were stained for ND. Brains were always stained in groups containing both WT and TS sections, so that between-assays variance could not cause systematic group differences.

nNOS immunocytochemistry

The sections were immunostained for nNOS after an overnight wash in PBS at pH 7.2. The free floating sections were first washed in PBS containing 0.2% Triton X-100 for 30 min and then treated to inhibit endogenous peroxidase activity with a solution of methanol/hydrogen peroxide for 20 min. Sections were incubated for 30 min with normal goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at room temperature with nNOS rabbit antibody (DiaSorin, MN, USA) diluted 1:12000 in PBS, pH 7.3-7.4, containing 0.1-0.2% Triton X-100. On the next day a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) was then used at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by a biotin-avidin system (Vectastain ABC Kit Elite, Vector Laboratories) with incubation for 60 min. The peroxidase activity was visualized with a solution containing 0.187 mg/ml 3,3'-diamino-benzidine (Sigma, Milan, Italy) and 0.003% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene and cover

slipped with Entellan (Merck, Milan, Italy). In a control procedure, the primary antibody was omitted and the cells presented unstained.

ND histochemistry

The sections were stained for ND after an overnight wash in 0.1 M phosphate buffer (PB). For detection of ND activity we employed the histochemical technique described in detail by (Alonso et al., 1995). In brief, free floating sections were incubated in a solution containing 1 mM reduced β -NADPH, 0.8 mM nitroblue tetrazolium and 0.05% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8), at 37°C for 1 hour. In the absence of ND activity the cells were not stained. The reagents were obtained from SIGMA (Milan, Italy). The course of the reaction was followed under the microscope and blocked by immersion of the sections in PB. Sections were then mounted according to the same procedure as described for nNOS immunocytochemistry.

Quantitative analysis

For the quantitative analysis, we assessed three different nuclei or complexes: the DB and the MS (thereafter DB-MS), the PVN, and the striatum, or CPu. These regions were identified following the nomenclature of the stereotaxic mouse brain atlas (Franklin and Paxinos, 1997). For each animal we selected the sections in which the corresponding nuclei were present. Sections were examined with a Leitz Laborlux microscope equipped with a *camera lucida* by using x10 objective. Finally, we counted all the positive neurons (identified for the presence of a clearly labeled cell body), within the boundaries of each nucleus, in the nNOS and ND stained sections.

For the PVN we assessed all the sections containing the nucleus, and the positive elements were investigated within the four large subdivisions: the ventral part (PaV); the lateral magnocellular part (PaLM); the anterior parvicellular part (PaAP), and the medial parvicellular part (PaMP). The DB and the MS (DB-MS) were counted jointly; the count was performed at three representative levels (A, rostral; B, intermediate; C, caudal) of the DB-MS complex. In addition to the total number of cells, we have here considered differences in cell staining. We have identified two types of cells (Fig. 4): type I cells, weakly stained, and type II cells, intensely stained with unstained nucleus. For the CPu, a more extensive area, we selected one corresponding level from each animal.

During the various reactions a few sections were lost and consequently some animals were excluded from the quantitative analysis. Therefore, we compared 8 WT mice to 7 TS mice for the DB-MS, and 6 WT mice were compared to 6 TS mice for both CPu and PVN.

Collected data were analyzed by an unpaired *t*-test (comparison between WT and TS animals for nNOS-IR or ND histochemistry) and paired *t*-test (comparison between the two type of reactions inside each group of mice) using the software StatView 5 (Abacus Concepts, Inc., Berkeley, CA, USA). Differences were considered statistically significant if $p < 0.05$. Sections were photographed with a Zeiss Axioplan microscope, equipped with a Nikon Coolpix 990 digital photcamera connected to an Apple G4 Macintosh. Digital images were processed using Adobe PhotoShop 6.0.

Results

Behavior

The 3-month-old TS male mice were smaller than WT male mice at the same age. This was reflected in their body weights (WT= 28.64 g \pm 0.89, n= 14; TS= 23.73 g \pm 0.95, n= 12, SEM, $p < 0.001$; Fig 1 A).

In the aggression test, the isolated resident TS mice displayed increased number of attack bites against the opponent compared to the WT residents during the 900 sec test (WT= 3.08 \pm 1.69, n= 13; TS= 12.09 \pm 3.59, n= 11, SEM, $p < 0.05$; Fig 1 B). Neither the total duration of attack episodes (WT= 16.92 sec \pm 6.73; TS= 33.36 sec \pm 12.77, $p > 0.05$; Fig 1 B) nor the latency to first bite (WT= 638.23 sec \pm 95.92; TS= 560.27 sec \pm 88.79, $p > 0.05$; Fig. 1C) were statistically significant. Of the total TS mice, 72.7%, as compared to 38.5% of WT mice, exhibited aggressiveness during the test.

Qualitative neuroanatomical results

Wild type mice. Because there is no clear anatomical separation between the DB and the MS, in the present study they are considered together. However, there are differences in the intensity of both immunohistochemical and histochemical reactions: a dense cluster of strongly stained neurons was observed prevailing in the nucleus of the DB (Fig. 2); the MS appeared more clear with isolated, moderately stained neurons (Fig. 3). We have therefore characterized two types of cells (Fig. 4): type I cells, weakly stained, generally small sized

with processes and nucleus unstained, and type II cells, intensely stained with nucleus clearly visible and processes of various lengths.

Many neurons are intensely stained in the PVN: these cells are mainly situated in the dorsal part and around the fornix (Fig. 5, 6, 7). The striatum (CPu) contains a scattered population of large multipolar type II neurons and a dense fiber network within the striatal neuropil (Fig. 8).

The morphology of the cells observed in the considered areas with both immunohistochemical (nNOS) and histochemical (ND) methods is similar. In the DB-MS we can discriminate the two different types of cells in both cases; however, the immunohistochemical method emphasizes the fiber networks within the neuropil.

The distribution of these two markers in the mouse brain areas is consistent with previous studies on the distribution of NO-producing system in the rat brain (Vincent and Kimura, 1992; Yamada et al., 1996). In the DB-MS complex, as well as in the PVN nucleus, the number of nNOS positive elements appears greater than of the visible ND cells (Fig. 2, 3, 5). Among the subdivisions of the PVN, most nNOS cells are in the magnocellular part, around the fornix (Fig. 5). However, the decrease of ND cells is uniform in the different parts of the nucleus. In the CPu a quantitative difference between nNOS positive and ND cells is not clearly visible.

Trisomic mice. The general morphology of the cells (observed both with nNOS-IR and ND histochemical methods) is similar to that of WT mice; no obvious differences are clearly distinguishable between WT and TS type of cells. In the DB-MS we can also discriminate the two different types of cells in both methods.

The TS mice show a small but visible reduction of nNOS-IR in the DB (Fig. 2), MS (Fig. 3), and in the PVN (Fig. 5, 6, 7) compared to WT mice.

On the other hand, several distinct differences were observed between WT and TS mice in the ND histochemistry: a robust reduction in the number of cells are observed in the DB (Fig. 2), MS (Fig. 3), and in the PVN (Fig. 5, 6, 7) of TS mice. In the CPu, no differences can be seen between WT and TS mice in either the distribution of nNOS and ND cells (Fig. 8).

Finally, at high magnification, reduced ND-fibers and ND-punctuate structures close to the cells in the TS mice were observed in all areas examined. In the CPu, this decrease was also visible using the nNOS methods.

Quantitative results

DB-MS - Total and Partial results

Despite the observable differences in stained cells in the DB-MS complex, a quantitative analysis in these two regions did not show significant differences between WT and TS mice in the total nNOS-IR ($p = 0.9475$) or in ND histochemistry ($p = 0.2002$; Fig. 9C). Therefore, we repeated the quantification considering the different type of cells (type I and type II, Fig. 9D) in the DB-MS. The weakly stained type I cells were much more numerous compared with the intensely stained type II cells in both nNOS and ND reactions.

- *nNOS-IR*: there was no significant difference between WT and TS mice in both type I ($p = 0.2572$) and type II cells ($p = 0.2488$; Fig. 9D).
- *ND histochemistry*: no significant difference was found in the weakly stained type I cells ($p = 0.1197$), but the intensely stained type II neurons were reduced in the TS mice ($p = 0.0184$; Fig. 9D).

These data confirm and extend the initial interpretation of the qualitative data, in which a decrease of ND cells in TS mice was clearly visible. This effect was mainly due to fewer ND type II cells.

PVN – Total results

There was a marked decrease of nNOS-IR cells ($p = 0.0069$; Fig 9A, left columns) and ND positive elements ($p = 0.0005$; Fig. 9A, right columns) in the PVN of TS mice compared to WT mice.

PVN – Partial results

The distribution of nNOS and ND positive cells within the different subdivisions of the PVN was not homogeneous, being the highest density in the PaLM and the lowest in the PaAP and PaMP (Fig. 10).

- **PaLM.** This area contained the highest number of cells from the PVN. In the TS mice there was a striking reduction in the number of neurons with either nNOS ($p=0.0116$) or ND staining ($p = 0.0127$; Fig. 10).
- **PaV.** In this subdivision, the number of nNOS cells in the TS mice was not significantly decreased ($p = 0.0707$); however, ND cell numbers were dramatically reduced ($p = 0.0011$; Fig. 10).
- **PaAP-PaMP.** nNOS positive cells were marginally reduced ($p = 0.0523$) but ND staining was severely impaired in the TS compared to WT mice ($p = 0.0024$; Fig. 10).

CPu – Total

The quantitative analysis confirmed the lack of difference between WT and TS mice in both nNOS ($p = 0.9409$) and ND staining ($p = 0.6927$; Fig. 9B).

Further consideration between nNOS and ND staining

There was a difference between the nNOS-IR and ND stained neurons in the PVN and in the DB/MS complex within each genotype. This difference is consistently shown by fewer ND cells in respect to the nNOS neurons (within WT mice: PVN $p = 0.0116$, and DB/MS $p = 0.0229$; within TS mice: PVN $p = 0.0026$, and DB/MS $p = 0.0039$; Fig. 11).

Discussion

Male TS mice are obviously smaller in size than the age-matched control B6C3HF1 mice. The reduced size is reflected in their significantly reduced body mass. Short stature is a common trait shared by young DS patients (Toledo et al., 1999) and TS mice (Davisson et al., 1990; Escorihuela et al., 1995).

We assessed aggressive behavior of isolated TS mice in a resident-intruder test. Social isolation in mice represents a stressful situation and induces several behavioral and neurochemical alterations, particularly elevated aggressiveness (Chiavegatto et al., 2001; Garattini et al., 1967; Miczek and O'Donnell, 1978; Nelson and Chiavegatto, 2001; Valzelli, 1973). The TS resident mice are more aggressive, displaying more attacks with bites against an intruder in their home cages compared to the WT residents. Approximately 75% of the TS mice tested showed aggressive behavior, whereas only about 40% of the WT mice displayed aggressiveness. These results are consistent with studies that reported increased offensive aggression in paired or grouped-housed male TS mice (Klein et al., 1996).

Elevated aggressive behavior was reported in male mice lacking the gene for nNOS (Nelson et al., 1995), or in mice with pharmacological inhibition of nNOS activity (Demas et al., 1997). Neurons positive for nNOS or expressing ND activity are distributed throughout the vertebrate brain (Bredt et al., 1991; Dawson et al., 1991). These neurons are particularly numerous in regions implicated in the control of motor pathways, in the cholinergic system, hippocampus, olfactory system (Vincent and Kimura, 1992), and in the hypothalamic regions involved in the control of sexual behavior, including the ventromedial nucleus (Ceccatelli et al., 1996) and preoptic area (Du and Hull, 1999). Accordingly, we investigated the brain NOS

system in isolated TS and WT mice, two hours after the completion of behavioral testing. We used two methods to visualize the NO system. nNOS-IR was used to investigate the tissue and cellular localization of the nNOS protein in the brain, and ND histochemistry was used to identify topographically the nNOS protein possessing enzymatic activity. This latter reaction was used as an indirect marker for the production of NO. The distribution of the NO-producing system in the basal forebrain of B6C3HF1 mice (WT), observed in the present study, is generally consistent with the results of previous studies in rats (Vincent and Kimura, 1992; Yamada et al., 1996) and C57BL/6 mice (Cork et al., 1998). Also, using nNOS-IR, we observed a reduction in the total number of nitrinergic neurons in the total PVN, mainly represented by the PaLM and PaAP-PaMP regions, but not in the DB-MS or in the striatum (CPu complex) of TS mice compared to the WT mice (Figs. 9 and 10).

Despite a similar topographic distribution, the number of ND positive neurons is significantly lower than nNOS-IR neurons in the PVN, as well as in the DB-MS complex (Fig. 11). This observation suggests that only a fraction of the nNOS protein present in these regions displays enzymatic activity under our experimental conditions. The decreased nNOS activity is found in both WT and TS mice, although it is more pronounced in the TS mice. This relationship does not occur in the CPu, where the nNOS protein level is similar to its activity as demonstrated by the ND histochemistry in both genotypes.

Although the correlation with behavioral aggressiveness has not been established in the present study, our data suggest that the isolation-induced aggression in TS mice is associated with decreased activity of nNOS in selected forebrain areas. The data from the ND histochemistry reflect the decreased production of neuronal NO in isolated mice two hours after the exposure to the opponent. This finding is consistent with the elevated aggression in mice after 7-NI treatment, a selective inhibitor of nNOS activity (Demas et al., 1997). In the present study, the aggressive TS mice displayed both reduction in nNOS protein and additional reduction of its activity in the PVN and DB-MS. This reduction was not observed in the CPu, suggesting that the striatum is not an area directly involved with aggressive behavior modulated by nNOS-derived NO. Thus, the partial trisomy of chromosome 16 in the TS mice markedly disturbs the neuronal NO system in selected brain areas.

The decrease in NO production may cause possible physiological consequences due to pleiotropic effects. Accordingly, we have previously reported a selective dysfunction in the 5-HT neurotransmission in some forebrain areas of nNOS^{-/-} male mice (Chiavegatto et al., 2001). The highly aggressive phenotype of these mutant mice was eliminated in a dose-dependent manner by augmenting 5-HT neurotransmission in the brain (Chiavegatto et al.,

2001). Although one report of an absence of difference in the 5-HT-immunoreactive cells in the medial and dorsal raphe of Ts65Dn mice, their forebrain areas were not investigated (Megias et al., 1997). Interestingly, a 5-HT dysfunction in the caudate nucleus, temporal cortex, amygdala and cingulate cortex in adult humans with DS has been reported (Seidl et al., 1999; Yates et al., 1986), indicating that an association of reduced NO and 5-HT disturbance in DS warrants further investigation.

Similar to people with DS, TS mice have a triplication of the Sod-1 gene, leading to its increased expression in the forebrain (Holtzman et al., 1996). It may be possible that the NO-alteration is a direct consequence of the aberrant production of SOD-1 protein, possibly affecting catabolism, or synthesis of NO (Schmidt et al., 1996).

Few neuroanatomical studies have been reported for TS mice. Previously, it was demonstrated that this murine model has a precocious loss (around 6 months of age) of cholinergic neurons in the basal forebrain coincident with the period of cognitive decline of these mice (Granholm et al., 2000). The mice used in our study were 3-months old, an age at which these immunohistochemical alterations are not evident. Alterations of ND activity of type II cells, and consequently NO production, in the DB-MS complex, where nNOS and acetylcholine are colocalized, may represent a very early signal of degeneration of the cholinergic system. Interestingly, in the striatum, where a large population of cholinergic and nitrinergic neurons are not colocalized, there is no modification in the NOS system.

The marked impairment of neuronal NOS in the PVN of TS mice is intriguing because the PVN is an essential site for autonomic and endocrine homeostasis, comprising an important area for central NO actions. However, the cellular targets and mechanisms mediating NO actions within the PVN are not completely understood. The PVN has been associated with maternal aggressive behavior (Consiglio and Lucion, 1996; Gammie and Nelson, 2000), but our data also implicate this brain area in the isolation-induced aggressive behavior of male mice. Reduced nNOS-IR in the PVN of humans is correlated with affective disorders (Bernstein et al., 1998), whereas increased nNOS expression and/or activity in the PVN is observed in response to several types of acute stressors in rodents (Calzà et al., 1993; de Oliveira et al., 2000; Sanchez et al., 1999).

In conclusion, our data provide the first evidence of disturbed neuronal NO system in the segmental trisomic mouse model of DS (Ts65Dn); taken together, the disturbance of the neuronal NO system has potential conceptual and clinical significance for this disorder. This impairment is selective to some brain areas, especially the hypothalamic PVN. We suggest a correlation of the behavioral aggressiveness of these mice with the deficiency of NO

production in this brain region. Additional work is necessary to elucidate the molecular mechanisms and to investigate the contribution of the impaired neuronal NO system in specific phenotypes of DS patients.

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Figure legends

Fig. 1 Body mass and aggressive behavior of male adult TS mice. Mean \pm SEM, A) TS $n= 12$ and WT $n= 14$; t test ** $p < 0.01$. B) TS $n= 11$ and WT $n= 13$; Mann-Whitney test * $p < 0.05$.

Fig. 2 Photomicrographs illustrating the nNOS-immunoreactive neurons (nNOS) on the left and the NADPH-diaphorase histochemistry (ND) on the right, in the diagonal band of Broca (DB) from WT (top panels) and TS (bottom panels) male mice. The number of cells stained by ND is reduced in the TS mice. Magnification bar = 150 μm in each panel.

Fig. 3 Photomicrographs of sections showing the nNOS-immunoreactivity (nNOS) on the left and the NADPH-diaphorase histochemistry (ND) on the right, in the medial septum (MS) from WT (top panels) and TS (bottom panels) male mice. The number of cells in the ND reaction is significantly reduced. Magnification bar = 150 μm in each panel.

Fig. 4 Photomicrographs illustrating the two types of perikarya in the diagonal band of Broca. Type I cells are weakly stained, generally small sized with processes and nucleus unstained. Type II cells are intensely stained with nucleus clearly visible and processes of various lengths. Both types of cells are found in WT and TS mice. Magnification bar = 50 μm in each panel. ND= NADPH-diaphorase, nNOS= neuronal nitric oxide synthase.

Fig. 5 Photomicrographs of sections from the paraventricular hypothalamic nucleus (PVN) demonstrating the nNOS-immunoreactivity (nNOS) on the left and the NADPH-diaphorase histochemistry (ND) on the right, from WT (top panels) and TS (bottom panels) male mice. ND labeling is markedly decreased in the TS mice (f = fornix; * = 3rd ventricle). Magnification bar = 300 μm in each panel.

Fig. 6 Panels illustrating the nNOS immunolabeling (left) and NADPH diaphorase staining (right) in the anterior parvicellular part of the paraventricular hypothalamic nucleus (PaAP). The WT mice sections are on the top and the TS on the bottom panels. The decrease in staining of TS neurons can be easily discerned. (* = 3rd ventricle). Magnification bar = 100 μm in each panel.

Fig. 7 Photomicrographs illustrating the nNOS immunolabeling (left) and NADPH diaphorase staining (right) in the lateral magnocellular part of the paraventricular hypothalamic nucleus (PaLM). The WT mice sections are on the top and the TS on the bottom panels. The ND histochemistry from TS mice is markedly reduced. Magnification bar = 50 μ m in each panel.

Fig. 8 Photomicrographs of sections showing the nNOS-immunoreactivity (nNOS) on the left and the NADPH-diaphorase histochemistry (ND) on the right, in the caudate-putamen complex (CPu) from WT (top panels) and TS (bottom panels) male mice. In both types of reactions WT and TS mice showed the same number of cells. Magnification bar = 100 μ m in each panel.

Fig. 9 Quantitative analysis of total number of cells stained by nNOS immunoreaction and NADPH diaphorase histochemistry in the WT and TS mice. A) PVN- Total; B) CPu - Total; C) DB-MS - Total; D) DB-MS - Partial. Number of type I cells (weakly stained) and type II cells (strongly stained with clear nucleus) in three sections of different levels, similarly for each genotype. Mean \pm SEM; Student *t* test; * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to WT group in the same staining.

Fig. 10 Quantitative analysis of nNOS and ND positive cells in the 3 subdivisions of the PVN. *Left*: PaAP and PaMP. *Middle*: PaV. *Right*: PaLM. Mean \pm SEM; Student *t* test; * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to WT group in the same staining.

Fig. 11 Analysis of the total number of cells stained by nNOS or ND in the three considered areas: PVN, CPu, DB-MS within each genotype. A) WT mice; B) TS mice. Mean \pm SEM; paired *t* test; * $p \leq 0.05$; ** $p < 0.01$ compared to nNOS group in the same area.

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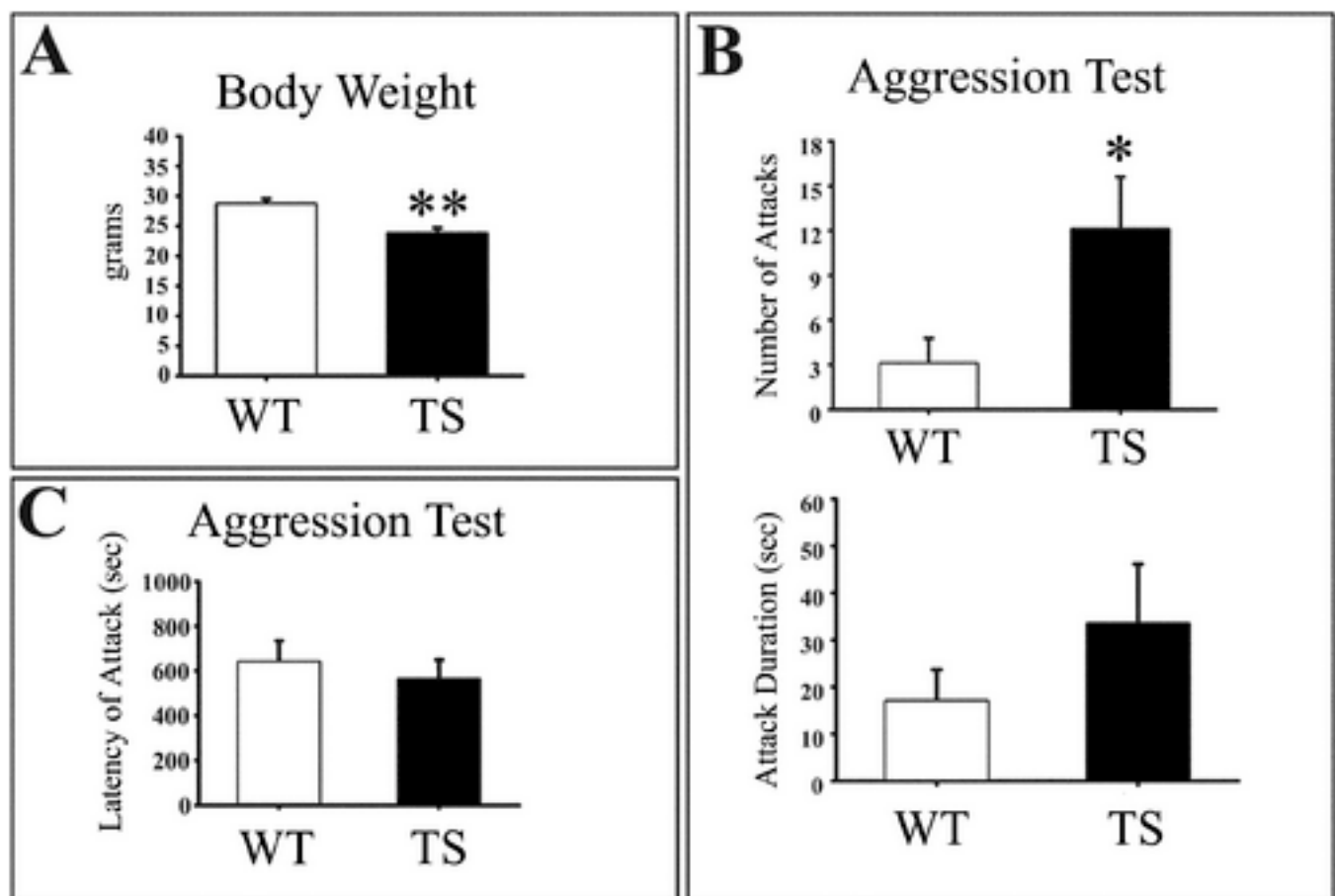


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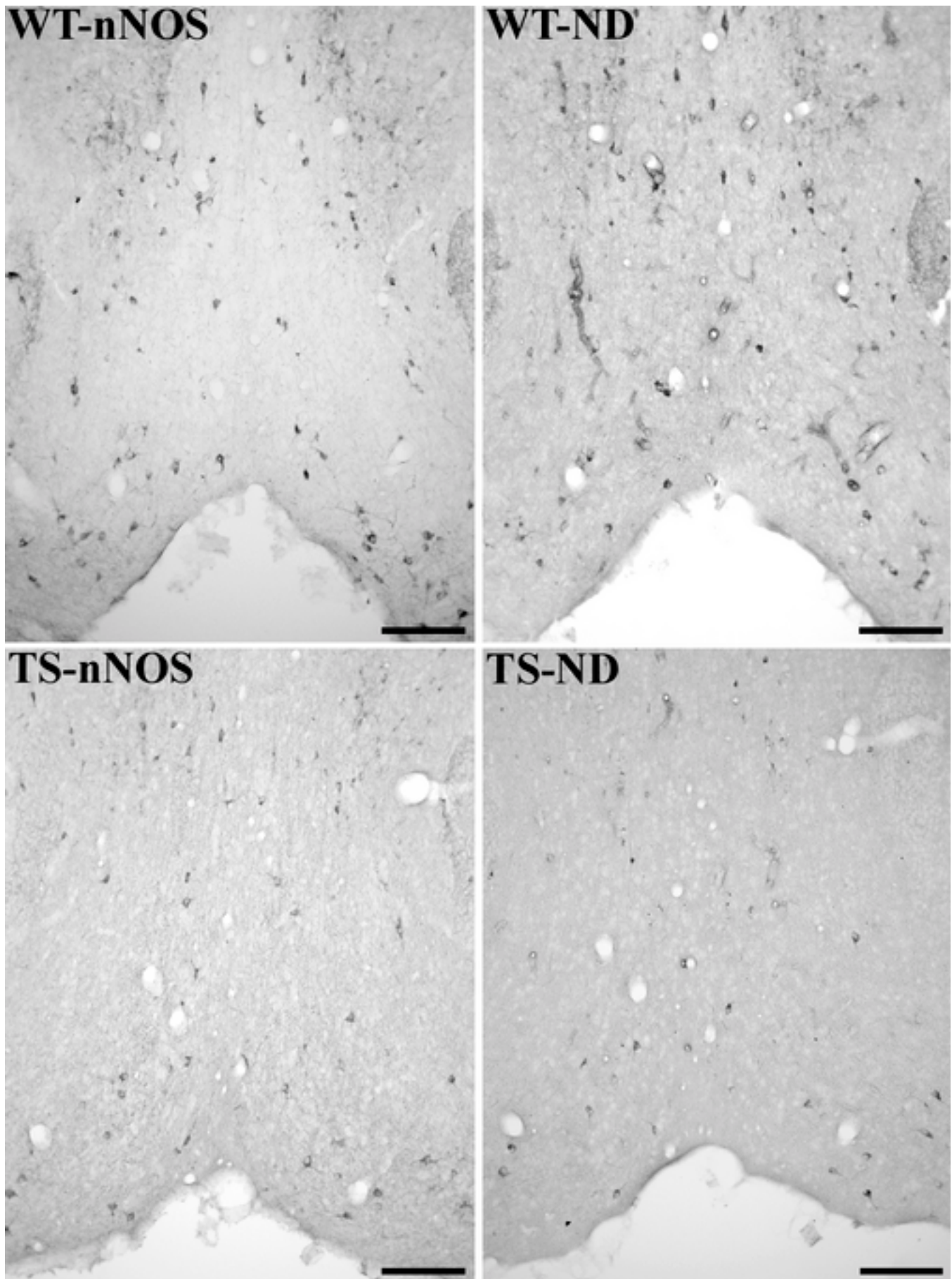


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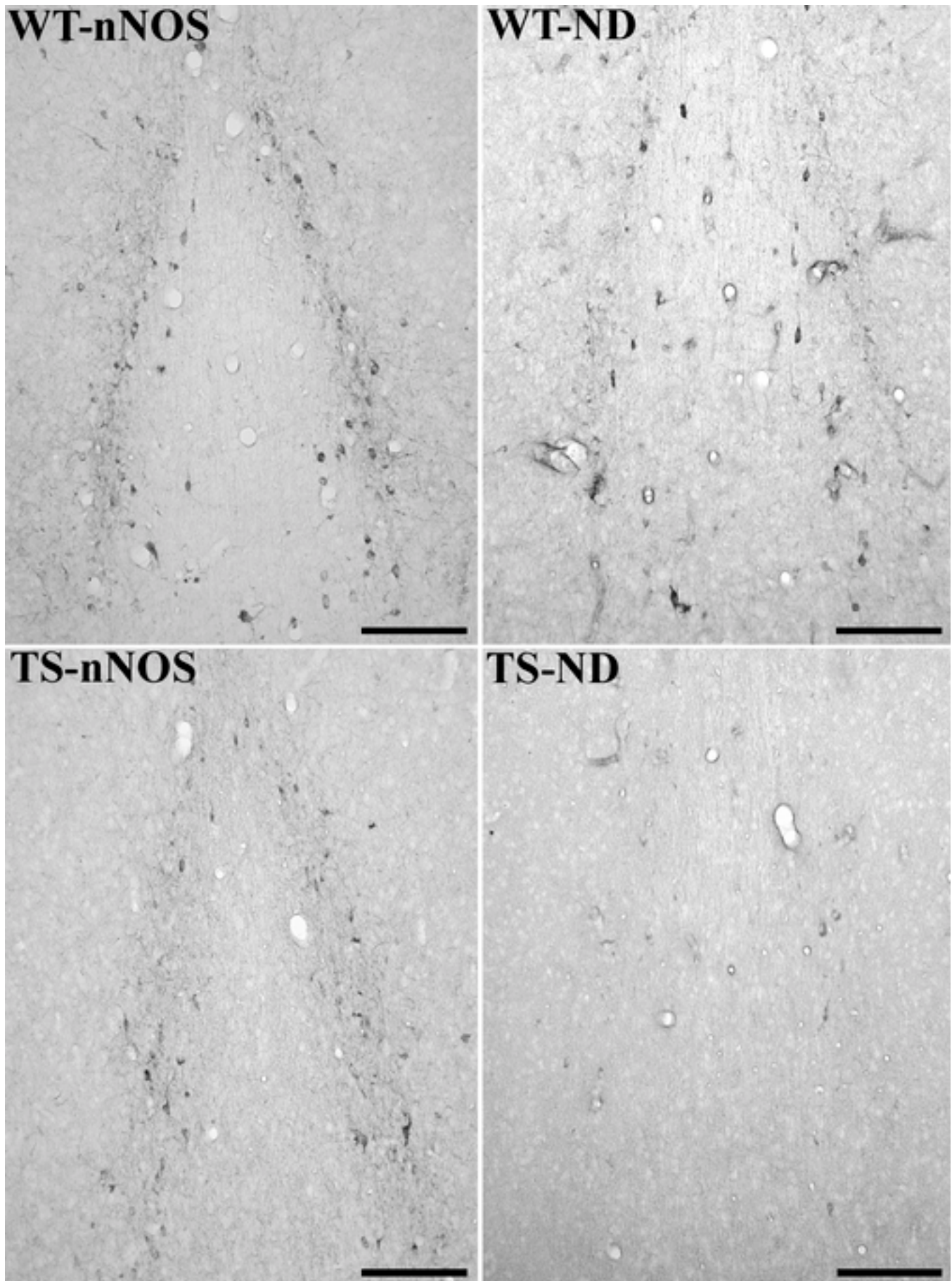


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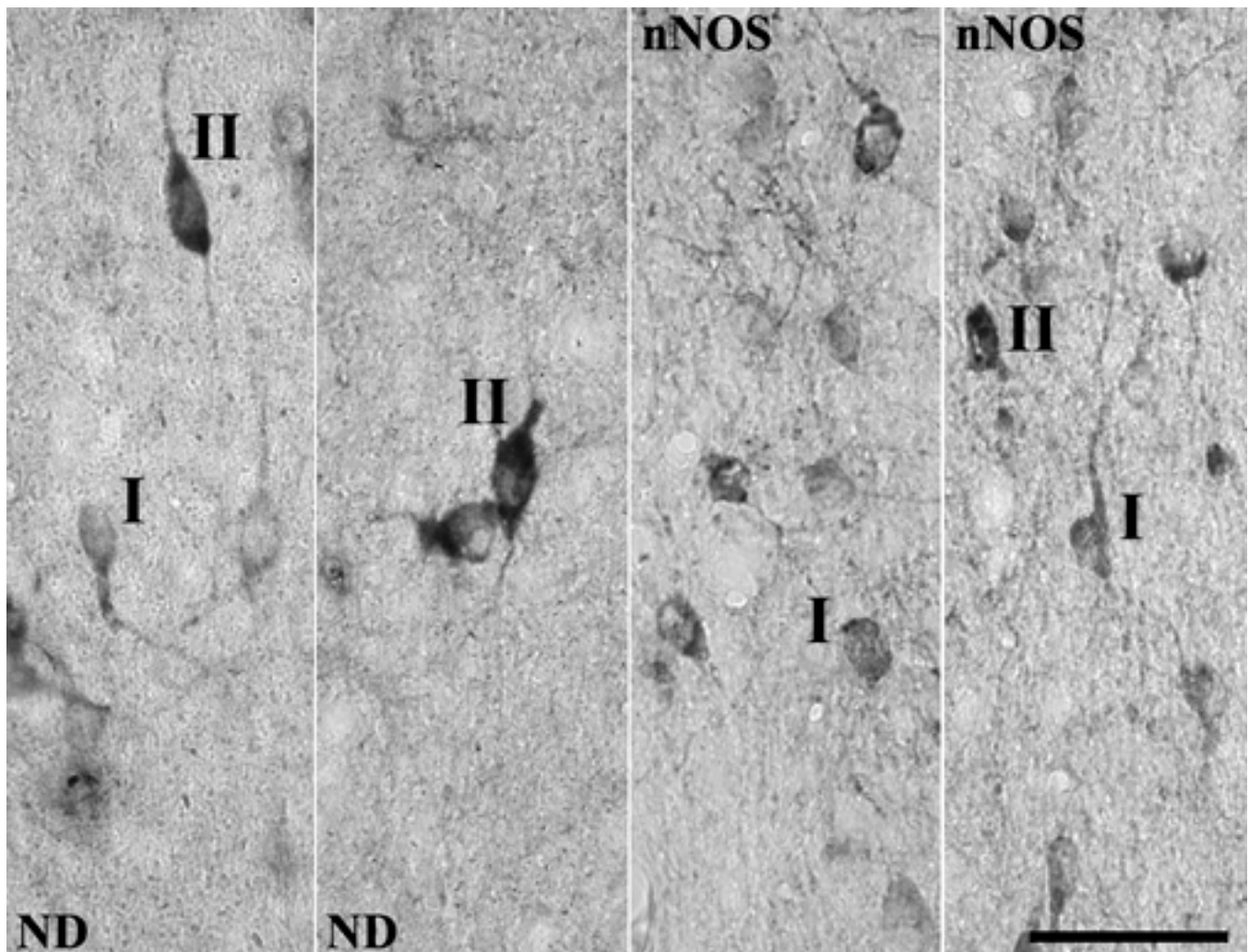


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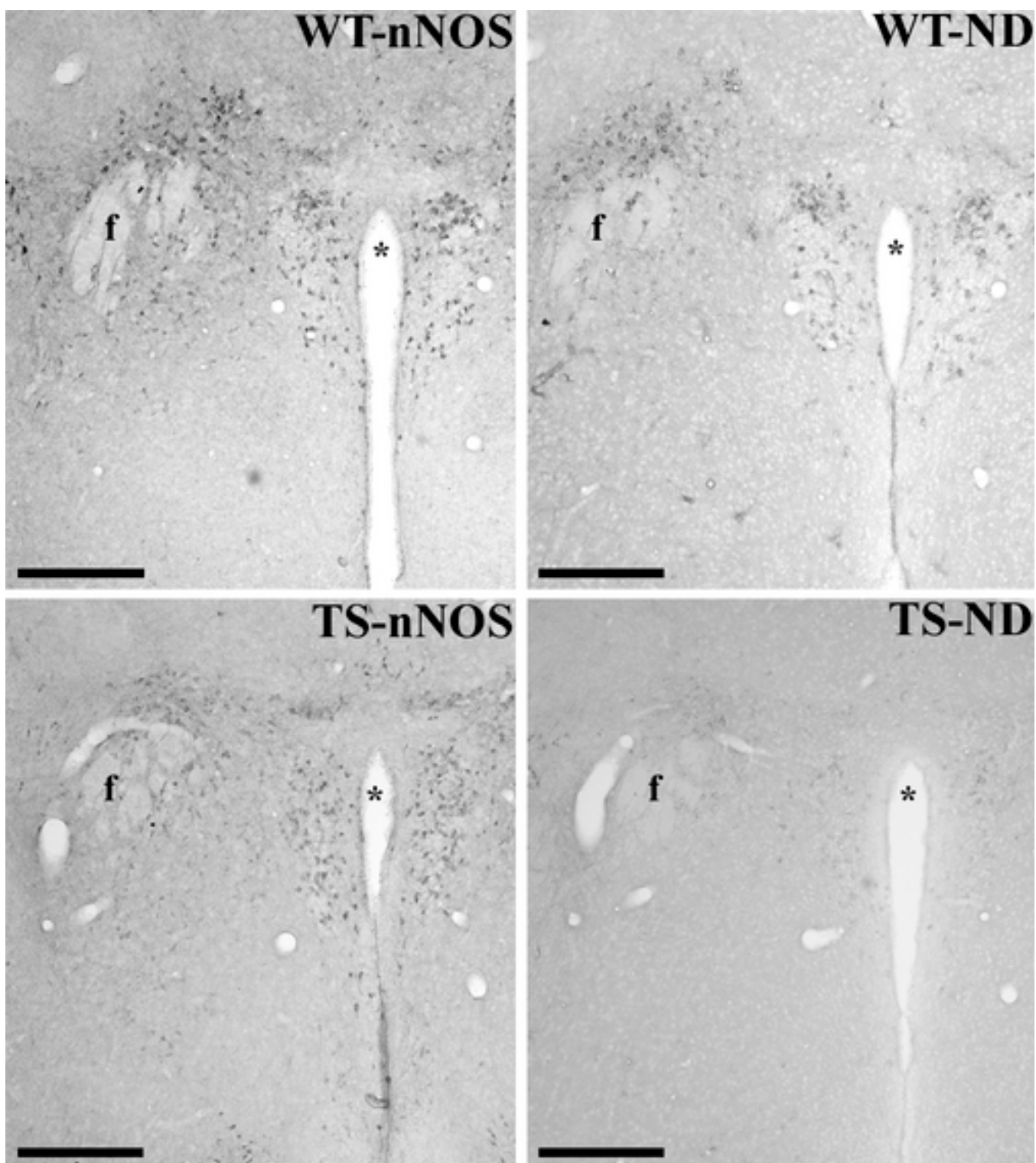


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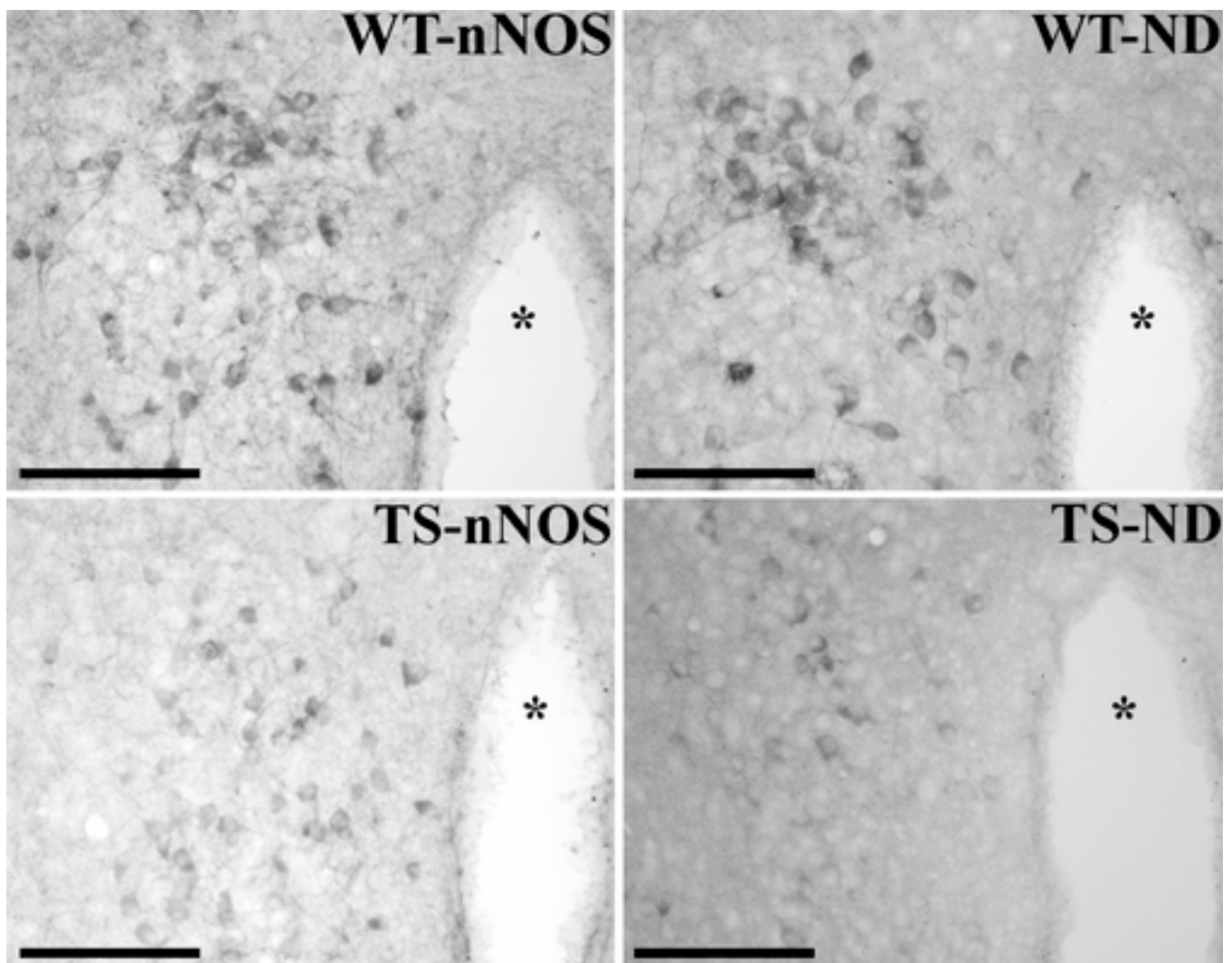


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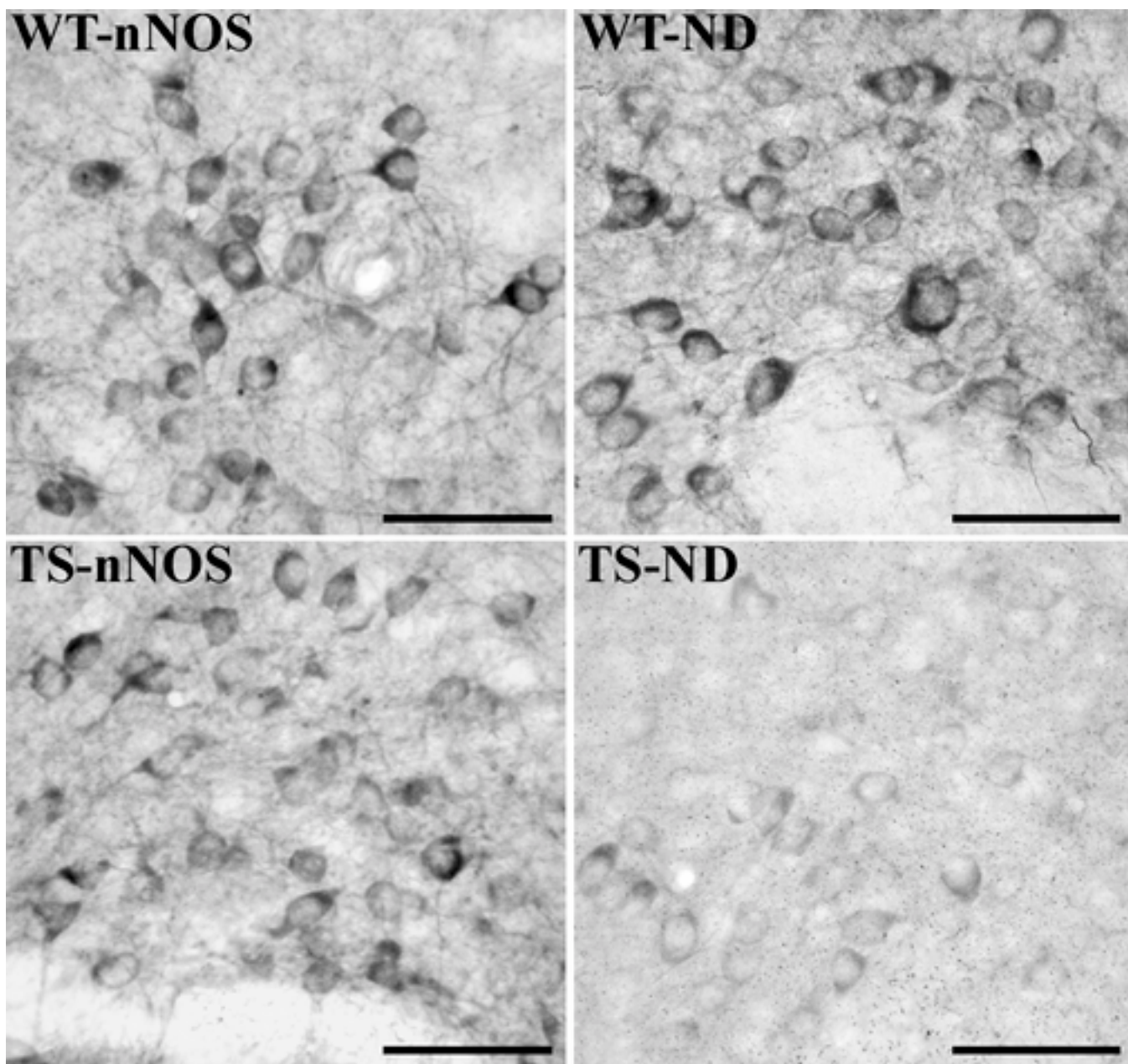


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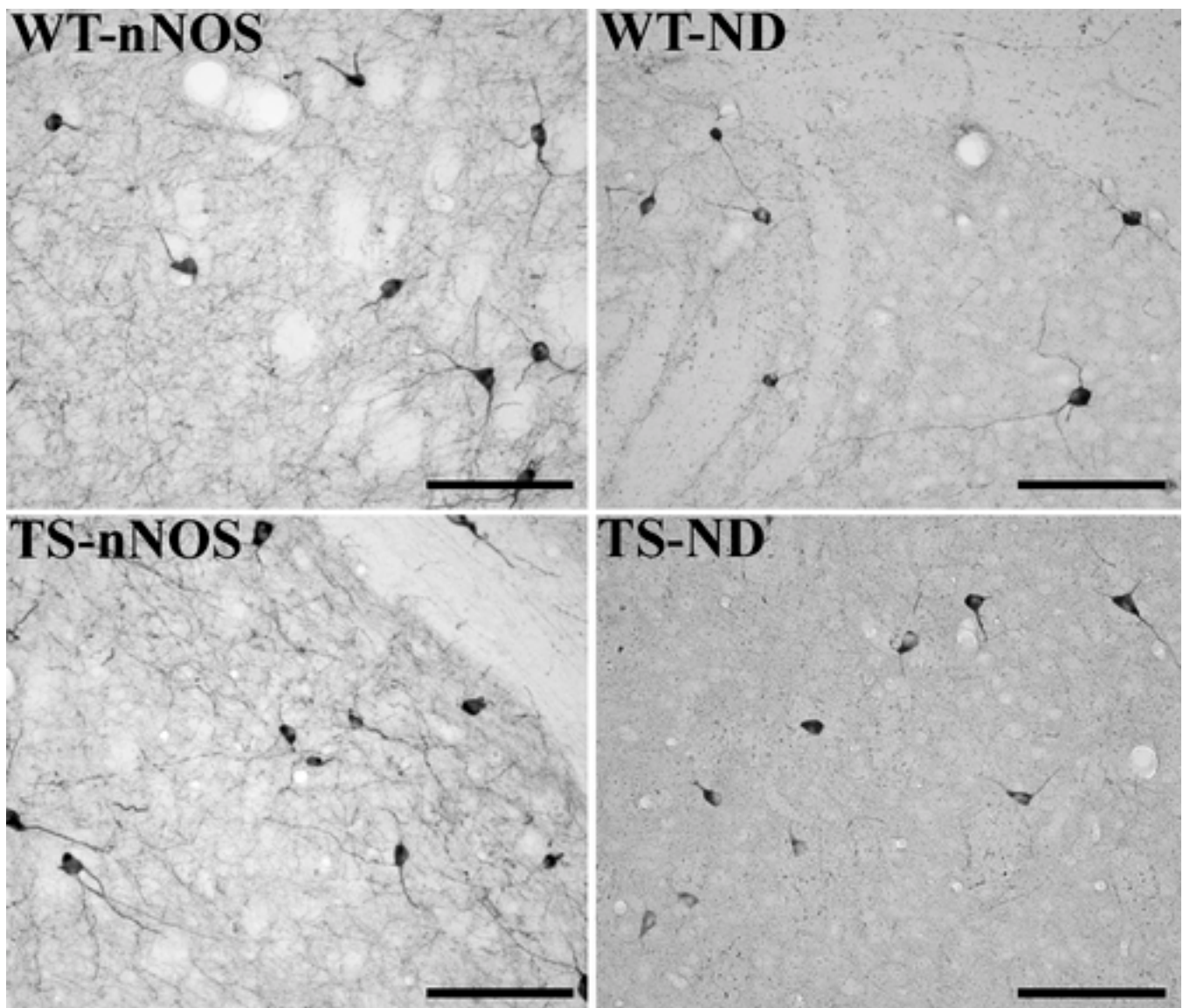


Figure 9

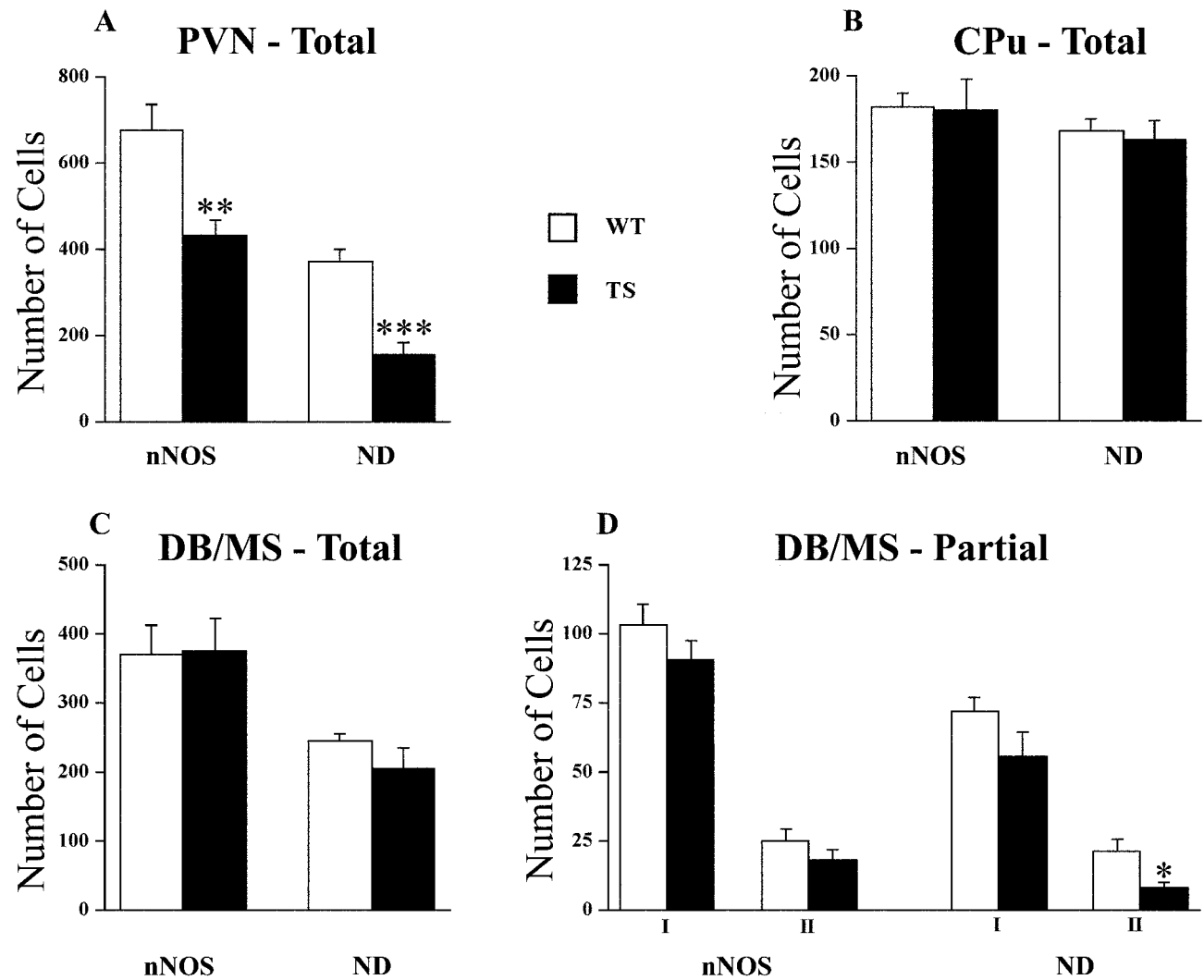


Figure 10

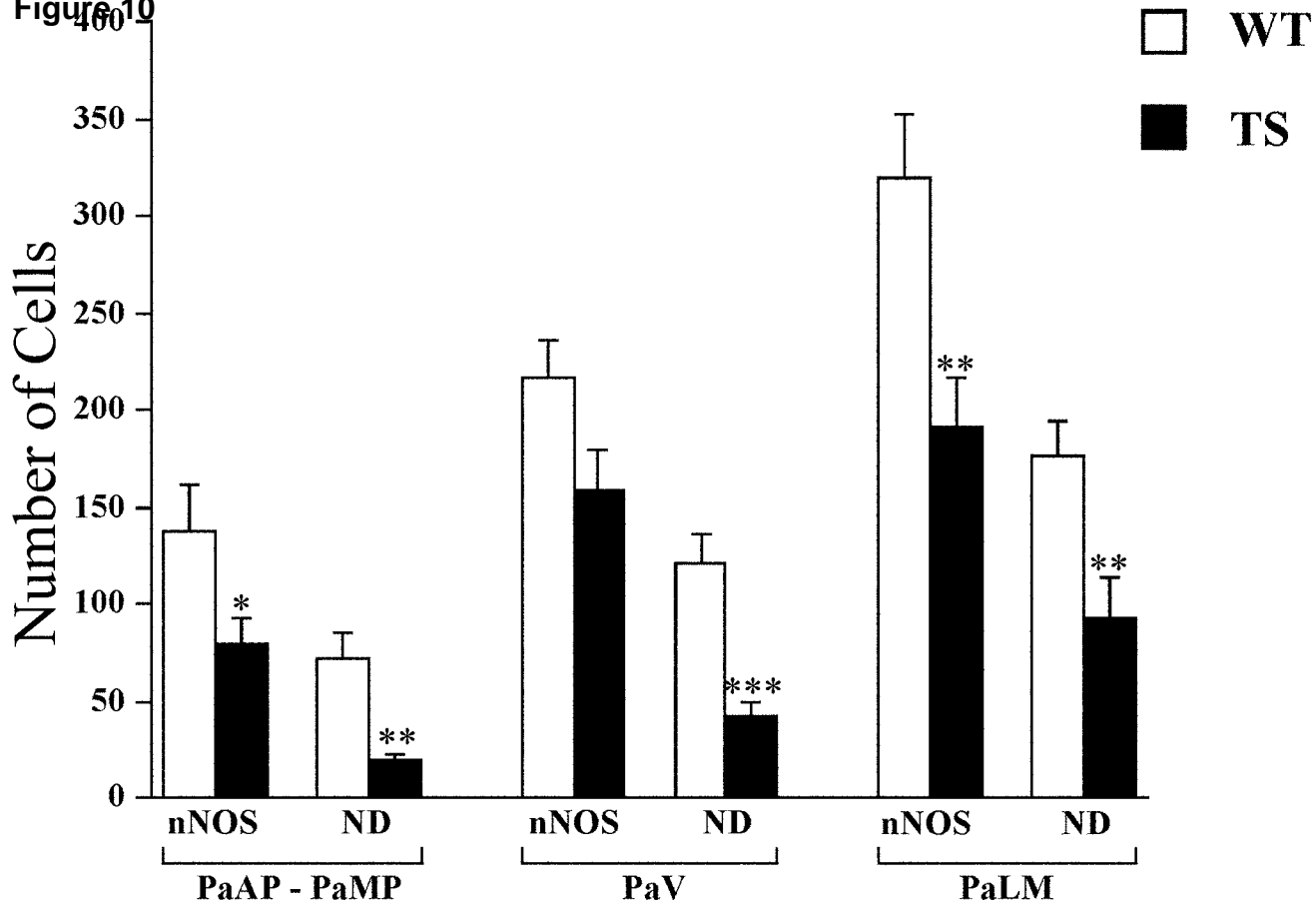


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