

Injury-induced NF- κ B activation in the hippocampus: implications for neuronal survival

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ABSTRACT

Nuclear factor (NF)- κ B p50 protein is involved in promoting survival in hippocampal neurons after trimethyltin (TMT)-injury. In the current study, hippocampal NF- κ B activity was examined and quantitated from transgenic κ B-*lacZ* reporter mice after chemical-induced injury. NF- κ B activity was localized primarily to hippocampal neurons and significantly elevated over that in saline-treated mice between 4 and 21 days after TMT injection. Seven days after TMT injection, a timepoint of elevated NF- κ B activity, gene expression in the hippocampus was studied by microarray analysis through comparison of expression profiles between treated nontransgenic and p50-null mice with their saline-injected controls. Seventeen genes increased in nontransgenic TMT-treated mice relative to saline-treated as well as showing no increase in p50-null mice, indicating a role for p50 in their regulation. One of these genes, the Na⁺, K⁺-ATPase- γ subunit, was detected in brain for the first time. Several of the genes modulated by NF- κ B are potentially related to neuroplasticity, providing additional evidence that this transcription factor is a neuroprotective signal in the hippocampus.

Key words: signal transduction • Na⁺, K⁺-ATPase • neurodegeneration • NF- κ B p50 • transcription factors

The widely expressed transcription factor nuclear factor (NF)- κ B controls cell survival and has more target genes than any other reported to date. Aberrant activation of NF- κ B induces the expression of several antiapoptotic genes leading to neoplastic cell growth and correlates with resistance to some anticancer therapies (1, 2). Other human diseases such as AIDS, autoimmune and inflammatory disorders, heart disease, and some neuropathologies can also be linked to dysregulated NF- κ B signal transduction (3–7). In the brain, NF- κ B signaling is associated with survival of neurons, but in some cases of injury, such as ischemic insult, it has been linked to neuronal death (8).

Prototypical NF- κ B consists of a dimer of p50 and p65 proteins complexed with an inhibitory I- κ B α protein (I κ B α) (9), and stimulation of cells induces sequential phosphorylation, ubiquitination, and degradation of the I κ B (see ref 10 for review). After I κ B degradation, the nuclear localization sequence (NLS) of activated NF- κ B drives its nuclear accumulation. I κ B α also participates in the removal of NF- κ B from the nucleus (11, 12), and this property as an export chaperone appears to be required for NF- κ B sequestration in the cytoplasm (13). Activated NF- κ B complexes are localized throughout the neuron (14–16) and migrate to the nucleus to bind cognate κ B sequences on DNA of target genes and modulate gene transcription.

Brain-specific NF- κ B is activated during neuroplastic events, such as neuronal development, synaptic activity, injury, and memory formation (16–26). Stimulus-specific induction of different NF- κ B subunits has been demonstrated (27–29), and the p50 subunit is associated with enhanced neuronal survival in the intact brain (15, 16, 22). However, gene regulation that results from this activity has not been described in brain after injury.

The current study used κ B reporter transgenic mice, which contain the p105 promoter upstream of the lacZ reporter gene (30), to provide a detailed *in vivo* analysis of the timing of hippocampal NF- κ B activation after chemical-induced injury. NF- κ B activity was significantly increased during and after neurodegeneration, and the majority of this activity was localized to areas containing pyramidal hippocampal neurons. Microarray analysis identified genes regulated by NF- κ B p50 that are plasticity-associated genes.

MATERIALS AND METHODS

Animals and treatments

The University of South Florida's Institutional Animal Care and Use Committee approved all animals and procedures used in this study. All efforts were made to minimize the number of animals used and their suffering. κ B-dependent *lacZ* transgenic mice (p105*lacZ* line 189-4, which contains the p105 promoter upstream of the *lacZ* reporter gene line hereafter referred to as “ κ B mice”; Institut Pasteur, Paris, France), homozygous p50^{-/-} (“p50-null mice”), and p50^{+/+} (“nontransgenic mice”) (B6,129Nfkb1^{tm1Bal} and B6,129 2/J, Jackson Laboratory, Bar Harbor, ME) weighing 20–30 g were used in the experiments. Strategies used in generating the mice have been previously described (30, 31). Groups of three to four mice were housed on corn cob bedding in polycarbonate cages until treatment, when they were housed individually. Standard vivarium conditions included food and water available *ad libitum*, a 12-h light/dark cycle, 22 ± 3°C, and veterinary assistance available as needed. κ B and nontransgenic mice were given a single *i.p.* injection of 2.25 mg/kg trimethyltin-hydroxide (TMT-OH, ICN Biomedical, K&K Lab Division, Costa Mesa, CA), a concentration that produced histochemically detectable neuronal degeneration without seizure activity (16). p50-null mice were injected with 2.0 mg/kg in order to produce neuronal degeneration with a low mortality rate (see ref 16 for a complete discussion on the method used to determine dosing regimens). Nontransgenic mice at this dose did not show any neurodegeneration.

TMT, calculated as the free base, was prepared in 0.9% saline and administered in a volume of 10 μ l/g body weight. Control animals were injected with 0.9% saline in a volume of 10 μ l/g body weight. Mice were killed 1, 2, 3, 4, 7, 14, 21, and 28 days posttreatment. Animals used for immunohistochemical studies were transcardially perfused with 40 ml 0.9% saline followed by 40 ml 4% buffered paraformaldehyde, pH 7.4, while under deep pentobarbital anesthesia. Brains were removed and post-fixed overnight in 4% buffered paraformaldehyde, followed by cryoprotection in graded (10–30%) sucrose solutions prepared in PBS. Animals used for biochemical analyses were decapitated; brains were removed and hippocampi were immediately dissected, frozen, and stored at -80°C until used.

Tissue processing and immunohistochemistry

Fixed, frozen brain sections from κB mice (4 saline-treated, 17 TMT-treated) were cut in the coronal plane at 30 μm on a cryostat. Immunohistochemistry was performed on free-floating sections using a modification of methods described previously (16). Endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide for 20 min. Tissue was blocked for 1 h in a solution containing 10% goat serum, 0.3 M lysine, 0.3% Triton X-100, and 5% skim milk made in TBS. Next, sections were incubated overnight at 4°C in primary antibody (rat polyclonal anti-GFAP 1:10,000, Zymed Laboratories, Inc., San Francisco, CA; rabbit polyclonal anti- β -galactosidase 1:10,000 dilution, Biogenesis, Kingston, NH). The antibody dilution buffer consisted of TBS containing 5% skim milk, 2% normal serum, and 0.3% Triton X-100. Sections were washed in TBS, followed by incubation for 1 h in either biotinylated goat-anti-rabbit or goat-anti-rat secondary antibody (1:300; Vector Laboratories, Burlingame, CA). After additional washes in TBS, sections were incubated for 1 h in avidin-biotin horseradish peroxidase macromolecular complex (ABC Elite, Vector Laboratories), and then washed again. Sections were incubated in metal-enhanced 3',3-diaminobenzidine (DAB) in 0.03% buffered hydrogen peroxide (Pierce, Rockford, IL) to produce a brown color. For double-labeling experiments, DAB staining was followed by Vector SG (Substrate Kit for Peroxidase; Vector Laboratories) as the chromogen, yielding a blue-gray stain, following the manufacturer's instructions. After three final washes, sections were mounted onto glass slides (Colorfrost/Plus, Fisher Scientific, Pittsburgh, PA) and air-dried. Slide-mounted sections were dehydrated through graded ethanols, cleared in xylene, and placed on coverslips using Cytoseal-60. Sections simultaneously processed without primary antibody were negative for staining.

Image acquisition and processing

Photomicrographs were captured through a Zeiss Axioskop 2 microscope using an AxioCam digital camera (Carl Zeiss Vision, München-Hallbergmoos, Germany) and *OpenLab* software (Improvision, Boston, MA). Final images were processed for publication using Adobe Photoshop 6.0 software (Adobe Systems, Inc., San Jose, CA).

β -Galactosidase enzyme activity assay

β -Galactosidase activity was analyzed using the β -Galactosidase Enzyme Assay System from Promega (Madison, WI) following the manufacturer's instructions, with slight modifications for

hippocampal tissue. Hippocampi from saline- and TMT-treated κ B mice were homogenized in 300 μ l 1 \times reporter lysis buffer (three to five animals for each time point). Lysates were placed into microcentrifuge tubes and incubated at room temperature for 15 min. Tubes were inverted several times during the incubation. After vortexing for 10–15 s, lysates were centrifuged at top speed for 2 min at 4°C. The supernatant was transferred to fresh tubes and diluted with 100 μ l RLB. Protein concentrations were determined using the method of Bradford (32). Promega Assay 2 \times buffer (150 μ l) (200 mM PBS [pH 7.3], 2 mM MgCl₂, 100 mM β -mercaptoethanol, 1.33 mg/ml *o*-nitrophenyl- β -D-galactopyranoside) was added to 150 μ l cell lysate for each sample. Samples were mixed briefly by vortexing and then incubated at 37°C for 40 min. Reactions were stopped by adding 500 μ l 1 M sodium carbonate. After vortexing briefly, the absorbance of each sample was read on a spectrophotometer at 420 nm and then compared with a standard curve for determination of milliunits of β -galactosidase. Values were normalized to the concentration of protein for each sample and then divided by the densitometric units of neuron-specific enolase in the same lysates, yielding an accurate representation of NF- κ B activity in surviving neurons (33).

Affymetrix GeneChip Expression Analysis System

Using the RNeasy Mini Kit (Qiagen, Valencia, CA) per the manufacturer's instructions, total RNA was isolated from hippocampi of 12 mice: three each from TMT and saline-treated wild-type at the 7-day survival time point, and three each from TMT and saline-treated p50-null mice at the same time point. Extracted RNA was pooled for each genotype and condition to yield four samples. This process was repeated with a different set of mice to produce the second array. RNA processing and analysis was carried out by the H. Lee Moffitt Microarray Core Facility using standard protocols established by Affymetrix (34) and four Murine Genome MG-74A gene chips. This chip was selected because the majority of genes on it are characterized (Affymetrix).

Confirmation of gene chip results: reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 12 mouse hippocampi (three each from TMT and saline-treated wild-type at the 7-day survival time point, and three each from TMT and saline-treated p50-null mice at the same time point) using the RNeasy Mini Kit per the manufacturer's instructions. cDNA was synthesized from 5 μ g of the total RNA by extension of random primers with the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, Inc., Foster City, CA), using the two-step procedure for RT-PCR in the manufacturer's protocol. The 40 μ l reaction mix consisted of 15.4 μ l RNase-free water, 8 μ l of 5X RT-PCR buffer, 4 μ l of 25 mM MgCl₂, 4 μ l of 10 mM dNTP blend, 1 μ l RNase inhibitor, 4 μ l 100 mM DTT, 1 μ l random hexamer blend, and 0.6 μ l of MultiScribe reverse transcriptase and 2 μ g RNA. The reaction was carried out in a standard thermocycler, with 10 min at 25°C, 12 min at 42°C, and 5 min at 99°C to denature the reverse transcriptase, with a final drop to 4°C to hold the reaction until storage at -20°C

Real-time quantitative (RTQ) PCR

Reactions were set up using SYBR Green PCR Master Mix (Qiagen) with published primers for 18S rRNA (35) and custom-designed primers for the mouse γ -subunit of the Na⁺, K⁺ ATPase (SeqWeb Wisconsin Package, Version 10.3, Accelrys, Inc., San Diego, CA). The following sequences were used: 18S forward primer-5'GTAACCCGTTGAACCCATT; 18S reverse primer-5'CCATCCAATCGGTAGTAGCG. ATPase- γ forward primer-5'GCCTCCTCATCATTCTCAG and ATPase- γ reverse primer-5'GCCTATGTTTCTTACCGCC (for 18S, accession no., 18S X00686.1; forward primer, 1577-1596; reverse primer, 1727-1708. For ATPase, accession no., ATPase X70060.1; forward primer, 98-116; reverse primer, 154-136).

The identical cDNA sample was used for amplification of both genes, and the Bio-Rad (Hercules, CA) iQ model iCycler was programmed and run following the manual (http://www.biorad.com/LifeScience/pdf/Bulletin_2500D.pdf). Specific cycling parameters were: 15 min at 95°C, then 45 cycles of 15 s at 95°C, 60 s at 52°C, and 20 s at 72°C. This was followed by a melt-curve procedure beginning at 60°C and heating to 90°C in 0.5°C increments.

Sequencing of RTQ-PCR products

Amplification products were isolated from primers and dNTPs using Qiagen MinElute spin columns. DNA sequencing was performed by the Core Sequencing Facility at the H. Lee Moffitt Cancer Research Center on an ABI Prism 377 Sequencing System. ABI PRISM BigDye Terminator v 3.0 Ready Reaction Cycle Sequencing Kits with AmpliTaq DNA polymerase were used, following the manufacturer's protocol for cycle sequencing. Unincorporated dye terminators were removed by gel filtration using Performa DTR Gel Filtration Cartridges from Edge BioSystems (Gaithersburg, MD).

Statistical analyses

In all cases, significance was set at $P \leq 0.05$. All results are expressed as the mean \pm SE for each treatment group or survival time point. Statistical difference between groups was assessed using one-way ANOVA. For analysis of β -galactosidase assays, we used pairwise multiple comparisons to assess specific relationships of each survival time point to the saline-treated group. For microarray studies, scanned output files were visually inspected for hybridization artifacts. Arrays were scaled to an average intensity of 150 then analyzed using Affymetrix Microarray 5.0 software. Genes were considered up-regulated if the expression was changed >1.5-fold relative to saline-treated wild-type mice, the baseline control sample in both microarray analyses. For RT-PCR confirmation of microarray data, Ct threshold cycle determinations for the Na⁺, K⁺-ATPase- γ subunit, were imported into Microsoft Excel files and analyzed by comparison with control values after normalizing to the 18S levels for each sample.

Immunoblotting

Whole-cell lysates (25 μ g protein) from the β -galactosidase enzyme assay were separated by SDS-PAGE on 10% gels for 1 h at 100 V. Proteins were transferred onto nitrocellulose

membranes (Bio-Rad) in transfer buffer (1 mM Tris, 0.1 M glycine, 10% methanol) overnight at 25 V and then blocked with 5% skim milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 2 h at room temperature. The membrane was incubated for 2 h at room temperature with primary antibody diluted in blocking buffer (rabbit polyclonal anti-neuron-specific enolase [NSE], 1:5000 dilution, Polysciences, Inc., Warrington, PA). After washing with TBST (four times, 15 min each), the blots were incubated for 1 h at room temperature with goat anti-rabbit or horse anti-goat IgG coupled to horseradish peroxidase (Vector, 1:5000) in blocking buffer and then washed again in TBST. Following incubation with enhanced chemiluminescent substrate (ECL, Pierce, Rockford, IL), membranes were exposed to film, and the film was developed. Films were analyzed using scanning densitometry.

RESULTS

Localization of NF- κ B activity

To determine the cellular localization of NF- κ B activity (represented by β -galactosidase protein in κ B-*lacZ* transgenic mice), we doubly labeled hippocampal sections from saline- and TMT-treated mice with anti- β -galactosidase (brown) followed by anti-GFAP (gray; marker for astrocytes) (Fig. 1). The 7-day (Fig. 1D–F) time point represents localization of β -galactosidase during marked astrogliosis and is the time point at which ensuing microarray analyses were performed. The representative 28-day section (Fig. 1G–I) demonstrates that NF- κ B activity did not shift to astrocytes at later time points. GFAP-labeled astrocytes did not contain appreciable β -galactosidase immunoreactivity, whereas the majority of immunoreactivity resided in the neuronal layers of the hippocampus.

Activation of NF- κ B induced by TMT continues for at least 28 days

β -Galactosidase enzyme activity (conversion of ONPG to *o*-nitrophenol) was quantitated in hippocampal extracts from 43 κ B mice to elucidate the duration and relative level of NF- κ B activation in the hippocampus after TMT-induced injury. Activity was measured after survival times of 1, 2, 3, 4, 7, 14, 21, and 28 days, expressed as milliunits of β -galactosidase and was then normalized to the amount of NSE protein in the same lysates (see Materials and Methods). β -Galactosidase activity in TMT-treated animals differed significantly from that in saline-treated animals from the 4- to 21-day time points (Fig. 2, $P \leq 0.05$). However, the activity remained elevated at the time points at 2 and 28 days but did not reach statistical significance because of variation between the samples.

Expression of genes dependent on p50 expression and TMT treatment

The 7-day time point was chosen on the basis of robust activation of NF- κ B p50 (16). Two separate microarray analyses were performed in which RNA from three individual mice were pooled into each group. The Affymetrix chip MG-A was used because it contained more characterized genes than the B or C chips. ESTs were discarded. Genes were identified with expression profiles showing no change in TMT-treated p50-null mice but that did increase in nontransgenic TMT-treated mice relative to nontransgenic saline-treated animals on both arrays.

These genes were considered to be modulated, either directly or indirectly, by NF- κ B p50. [Table 1](#) shows 14 genes that were induced at least 2.0-fold (as analyzed using Affymetrix Microarray 5.0 software) and three genes with between 1.5- and 2.0-fold increase with expression that did not change in p50-null mice after TMT treatment relative to the nontransgenic saline-treated reference sample.

The identified 17 genes were grouped into five functional categories as shown in [Figure 3](#). The group of lysosomal and other enzymes contained six genes in which cathepsin C, D, Z, β -glucuronidase, and endoplasmic reticulum degradation enhancing α mannosidase-like protein are located in the lysosome, whereas the Na⁺, K⁺-ATPase- γ subunit is a component of an ionic pump. CD45, CD52, CD53, and TYRO-3 are all cell surface proteins. Calcium/calmodulin-dependent kinase II δ and regulator of G-protein signaling 10 (RGS10) are involved in signal transduction. Three immune-associated proteins—complement C1qB, complement C3a receptor, and chemokine CXC ligand 13—are increased in this model of brain injury. Finally, two heat shock-related proteins, HRP12 and Hsc70t, are modestly increased in our array results.

The Na⁺, K⁺-ATPase- γ has not been described previously in rodent brain, and the TMT-induced increase was attenuated in NF- κ B p50-null mice. We therefore chose Na⁺, K⁺-ATPase- γ for further analysis. RTQ-PCR on RNA extracted from nontransgenic and p50-null mice was performed, confirmed by sequencing, and results are presented in [Figure 4](#).

Gene promoter sequences of 10 of the 17 genes are published and were inspected for potential NF- κ B recognition sites. None of these genes contained identifiable NF- κ B sites in the putative promoter sequences. However, all of the gene promoters either contained GC-rich regions (3), no TATA box (1), or both (6) as shown in [Table 2](#).

DISCUSSION

A systemic injection of TMT kills certain populations of neurons, whereas surviving neurons express activated NF- κ B p50 (16, 36–38). NF- κ B activation in the brain has been linked to transcription of genes involved in neuronal plasticity, remodeling, repair, and survival (17, 39–41). Further evidence that NF- κ B is involved in neuroprotection is evinced by increased neurodegeneration in mice lacking the p50 subunit of NF- κ B after chemical-induced hippocampal injury (16, 42). The bulk of TMT-induced neuronal damage occurs in the dentate gyrus of nontransgenic mice, where immunoreactivity of activated p50 is low. However, activated p50 is detectable in surviving hippocampal neurons for at least 28 days after a TMT-induced lesion (16) correlating with the κ B activity demonstrated in the current study. This prolonged activation parallels changes in the expression of NF- κ B-modulated genes related to plasticity and repair reported by others (17, 39).

The time course of NF- κ B activity through 28 days after hippocampal lesioning was examined indirectly as β -galactosidase enzyme activity in neurons of mice carrying an NF- κ B-responsive *lacZ* reporter gene. β -Galactosidase immunoreactivity was localized primarily in the neuronal layers of the hippocampus, with little immunoreactivity observed in astrocytes at any point after lesioning. The number of hippocampal neurons decreases after TMT treatment (36, 43–46),

correlating with declining levels of NSE. To ensure that β -galactosidase activity accurately represented that in neurons, we normalized levels of the amount of NSE in each hippocampal extract, and this more sensitive strategy revealed temporally different peaks of activity.

TMT differs from other models of brain injury in that the blood-brain barrier remains intact and there is no increase in cytokine expression, one of the major inducers of NF- κ B (47, 48). This lack of cytokine induction may explain why NF- κ B activation is observed at low or undetectable levels in glial cells ([Fig. 1](#)). Glial cells respond as in any injury model, with microglia becoming reactive to the injury by 2 days and returning to their resting state at 7 days posttreatment (49). Reactive astrocytes appear in the hippocampus at about 3–4 days post-TMT treatment, coinciding with the onset of neurodegeneration, and remain reactive for at least 36 days after TMT treatment, long after neuronal death has ceased (46). Although cytokines are not involved in the NF- κ B activation, reactive astrocytosis does occur in correlation, suggesting that these cells, perhaps through release of neurotrophins, are stimulating this signal transduction pathway in surviving neurons.

The role of NF- κ B in the response to brain injury has not been resolved and is likely dependent on the brain region in which the transcription factor is expressed. NF- κ B transcription factors regulate processes involved in central nervous system (CNS) plasticity, including synaptic activity, learning, memory, survival, and apoptosis (15–17, 50–55). p50 intensifies the DNA binding affinity of p65, and p65 strongly transactivates NF- κ B-responsive genes (56–58). Lack of p50 expression and concomitant reduction of NF- κ B activity increases neurodegeneration in the hippocampus (16, 42). However, NF- κ B activity has been linked to neuronal death in the striatum after excitotoxicity and ischemia (8, 51). Thus, the role of NF- κ B in the brain appears to be region-specific and possibly injury-specific, accounting for the discrepancies in the literature.

Hippocampal neurons surviving TMT-induced injury contain p50 protein as a protective response (16), which correlates with NF- κ B activity reported in this study. Gene expression induced by NF- κ B in this model, therefore, is likely to be involved in promoting neuronal resistance to impairment and facilitating recovery from injury. To identify NF- κ B p50 modulated genes, we compared microarray analysis of RNAs from mice lacking p50 expression with those in nontransgenic mice after TMT-induced injury of the hippocampus. Only genes that showed a profile of being increased in nontransgenic mice after TMT treatment but not in mice lacking p50 expression after TMT or saline treatment or in saline-injected nontransgenic mice in both microarray studies are reported, with 17 genes meeting these criteria. Interestingly, these genes are easily assembled into five functional groups of genes, some having potential roles in neuronal plasticity.

Genes related to immune function are increased in brain injury and are detected in our microarray results. Complement C1qB expression is elevated during Alzheimer's disease, amyotrophic lateral sclerosis, and excitotoxic brain injury (59, 60). Hippocampal neurons surviving glutamate toxicity contain increased levels of C1qB (60). Interestingly, C1qB is expressed during brain development and appears to have a novel role unrelated to cytotoxicity (61). As with C1qB, complement receptor is expressed by neurons and is up-regulated after ischemic injury (62). Whereas the chemokine CXC ligand 13 is uncharacterized, chemokines are

known to be increased during brain injury (63). Chemokine receptor activation has been shown to regulate both survival and apoptotic signaling (64), and chemokines direct the migration of developing neurons for proper cellular positioning (65). Injury to the hippocampus enhances neurogenesis in the dentate gyrus (66, 67), and chemokines may play a role in the migration of these progenitors.

Among potential neuroprotective genes, heat shock proteins are molecular chaperones assisting in protein folding, transport, and assembly into complexes, and their increased expression in neurons enhances survival (68, 69). Their expression is increased in a variety of brain injury models (70, 71) and after ischemic preconditioning, which is protective for ischemic insult (72). Our array results yielded two of them, but both genes were found to be increased only modestly (150–200%). Nevertheless, their expression profiles were very similar in both array runs. HRP12 is a relatively uncharacterized heat-responsive gene isolated from the liver (73), and microarray analysis detecting genes required for induction, growth, and maintenance of the lens included this gene (74). Hsc70t, a homologue of the well-characterized Hsp70, has been exclusively studied in the context of spermatogenesis (75). Although these genes have not been characterized in the brain, they are both associated with development and growth in other cell types, suggesting that they could be performing similar functions in the brain injury response.

Lysosomal enzymes are essential molecules in the brain's response to injury in degradation of potentially toxic molecules, and several lysosomal enzymes are up-regulated in response to TMT-induced injury. β -Glucuronidase is considered neuroprotective because its expression confers resistance to amyloid- β peptide toxicity (76). Inhibition of lysosomes in hippocampal slices increases protein accumulation in an Alzheimer-like fashion, contributing to synaptic decline. Elevated expression of β -glucuronidase and cathepsins clear these proteins, resulting in normal synaptic functioning (77). Cathepsin C is up-regulated in the cortex 24 h after ischemic insult but not earlier, suggesting a role in tissue repair and functional recovery (78). However, cathepsins, in particular D, are associated with neuronal death (79). This protease can generate pathogenic A β peptides from its precursor in vitro, suggesting a role in the pathology of Alzheimer's disease (80), and it is up-regulated in tangle-bearing hippocampal neurons in Alzheimer's disease brains (81). Therefore, as with many other brain injury response proteins, cathepsins are implicated in both neuronal death and survival processes.

Three of the cell surface proteins are lymphoid-associated, and CD45 has been shown to be expressed by microglia (82). Because p50 has not been observed to be expressed in microglia in this model, expression of microglial-associated genes suggests that neurons are influencing microglia to further enhance repair and survival after injury. Stimulation of CD53 expressed on immune cells increases cellular glutathione to improve the probability of surviving oxidant-induced apoptosis (83, 84) and activates survival pathways (85). Tyro-3, a receptor protein tyrosine kinase, is a neural cell adhesion protein, which is up-regulated postnatally in the CNS coincident with the period of active synaptogenesis (86, 87). Similarly, calcium/calmodulin-dependent protein kinase II δ is expressed in developing CNS (88). Its activity induces neurite outgrowth (89), and it is localized in developing axons (90). Thus, the array results show an overlap of genes related to survival with others related to CNS repair. Future microarray

analyses at earlier time points will determine whether survival- and repair-associated genes are concurrently induced.

The sodium pump controls Na^+ and K^+ concentration gradients across the plasma membranes of eukaryotic cells, and its inhibition has been linked to accumulation of extracellular K^+ and neuronal dysfunction (91–95). Dysregulation or inhibition of the sodium pump in the hippocampus negatively influences cationic equilibrium and recovery of resting membrane potential, promotes apoptosis, facilitates membrane failure, and leads to synaptic dysfunction and reduction in neuritic outgrowth (96–100). Restoration of efficient neuronal ATPase function after injury could be vital to successful recovery. The γ subunit of Na^+ , K^+ ATPase, which is expressed mainly in kidney, as of this writing had not been described in brain (101), specifically regulates Na^+ , K^+ -ATPase by associating with α subunits to increase ATP affinity (102–104). Gamma induction was not detected in p50-null mice after neurotoxicant exposure, and neurodegeneration is profoundly increased in these mice (16, 58, 105, 106). This lack of γ gene induction implies that an increased affinity of the sodium pump for ATP is necessary to neuronal survival and indicates that p50 is required for such induction after neuronal injury. Increased affinity of the sodium pump for available ATP may be a critical determinant of neuronal fate when mitochondrial dysfunction results in reduced ATP production (107–110). In surviving neurons, NF- κ B p50-mediated expression of Na^+ , K^+ -ATPase- γ subunit may counteract injury-induced perturbations of homeostasis. This is currently being assessed.

It is interesting that in an array designed to discover NF- κ B p50-dependent genes, there is a preponderance of genes with GC-rich, TATA-less promoters (111–119). In fact, all the 10 characterized genes but 1 (CD45) (120) contain GC-rich regions or are dependent on SP1 sequences for transcription. This result may indicate the involvement of a dominant promoter type in the injury response. Although the promoters did not contain any apparent NF- κ B regulatory sites, this does not preclude a role of NF- κ B in their transcription, because it may act kilobases away from the characterized promoter region. We concede, however, that this analysis will potentially highlight not only those genes directly controlled by the p50 subunit of NF- κ B, but also genes and pathways that are secondarily affected by the loss of NF- κ B signaling. Future arrays will determine whether GC-rich, TATA-less genes are p50-mediated expressed at other time points after TMT-induced injury.

In summary, NF- κ B is activating gene transcription before, during, and long after neurodegeneration has ceased, strongly suggesting that it is involved in neuroprotective, neurorepair, and other neuroplastic changes related to injury response. Supporting this contention, several genes dependent on p50 expression and up-regulated during hippocampal injury are associated with neurosurvival, neurite outgrowth, and synaptogenesis. Although more array studies are needed for a complete gene expression time course, this analysis provides insight into the NF- κ B-mediated expression of genes whose combined expression leads to enhanced neuronal survival and repair.

ACKNOWLEDGMENTS

This work was supported by National Institute of Neurological Diseases and Stroke NS39141-01A2 and American Heart Association National 9930072N to K.R.P. Thanks to the Sequencing and Microarray Core at the Moffitt Cancer Center for their technical assistance.

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Received September 22, 2003; accepted December 15, 2003.

Table 1**Genes that increased in nontransgenic mice but did not change in p50-null mice 7 days after TMT injection**

Genes that increased >2.0fold		Genes that increased between 1.5- and 2.0-fold	
U77461	Complement C3a Receptor	X68378	Cathepsin D
M22531 AF024637 AF030636	Complement C1qB TYRO-3 Chemokine CXC ligand 13	U50631 AF109906	HRP12 Hsc70t
X70060	Gamma subunit of Na ⁺ , K ⁺ -ATPase		
X97227	CD53		
M55561	CD52		
M14343 AF059029	CD45 Calcium/calmodulin-dependent protein kinase II delta		
A1847399 M19279	Regulators of G-Protein Signaling 10 β -Glucuronidase		
AJ242663 U74683 AW212878	Cathepsin Z Cathepsin C Endoplasmic Reticulum Degradation Enhancing α Mannosidase-like		

Table 2*Characterized gene promoters*

Gene	TATA Box	GC Rich Sequence
Hsc70t	No	Yes
HRP12	No	Yes
CD52	No	Yes
CD53	Yes	Yes
CD45	No	No
Cathepsin D	No	Yes
Cathepsin Z	No	Yes
Complement C3a receptor	No	Yes
β -Glucuronidase	Yes (murine not human)	Yes
Na ⁺ , K ⁺ -ATPase γ	Yes (one of two promoters)	Yes

Fig. 1

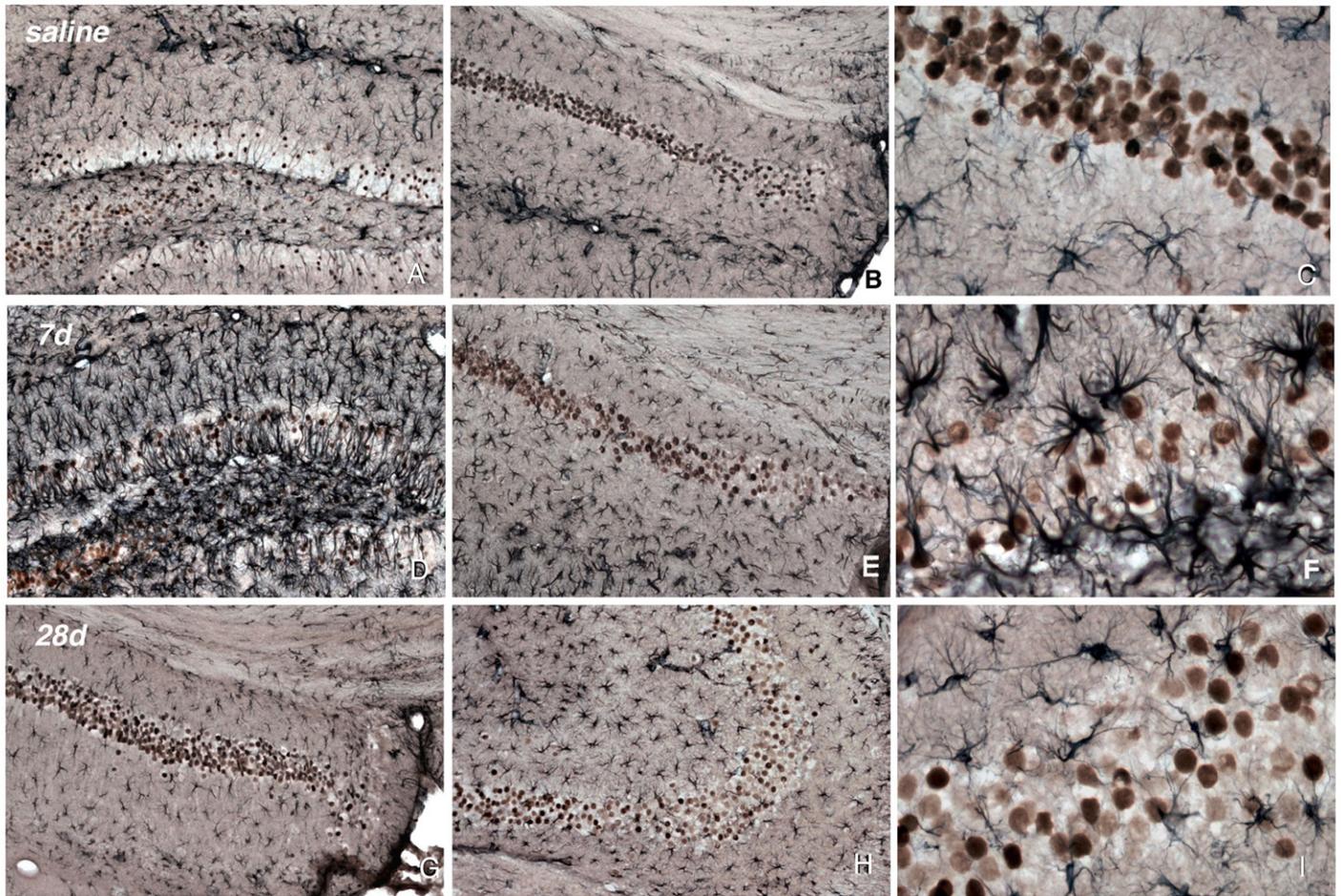


Figure 1. NF- κ B activity is not localized in astrocytes. Mice carrying a κ B-dependent *lacZ* reporter gene were injected with saline or 2.25 mg/kg TMT and killed after survival times of 1–28 days. Coronally sliced hippocampal sections were consecutively labeled with antibodies against β -galactosidase (brown) and GFAP (dark gray). Basal levels of β -galactosidase and GFAP are shown on sections from saline-treated mice (**A–C**) in CA4/dentate gyrus (**A**) and CA1 (**B, C**) hippocampal subregions. At the 7-day survival point, marked reactive astrogliosis is observed in the CA4/dentate gyrus (**D, F**) and CA1 (**E**) subregions, without colocalization of GFAP and β -galactosidase. By the 28-day survival timepoint, GFAP still does not colocalize with β -galactosidase in any subregion, including CA1 (**G**) and CA3 (**H, I**). Magnification: $\times 100$ (**A, B, D, E, G, H**) and $\times 400$ (**C, F, I**).

Fig. 2

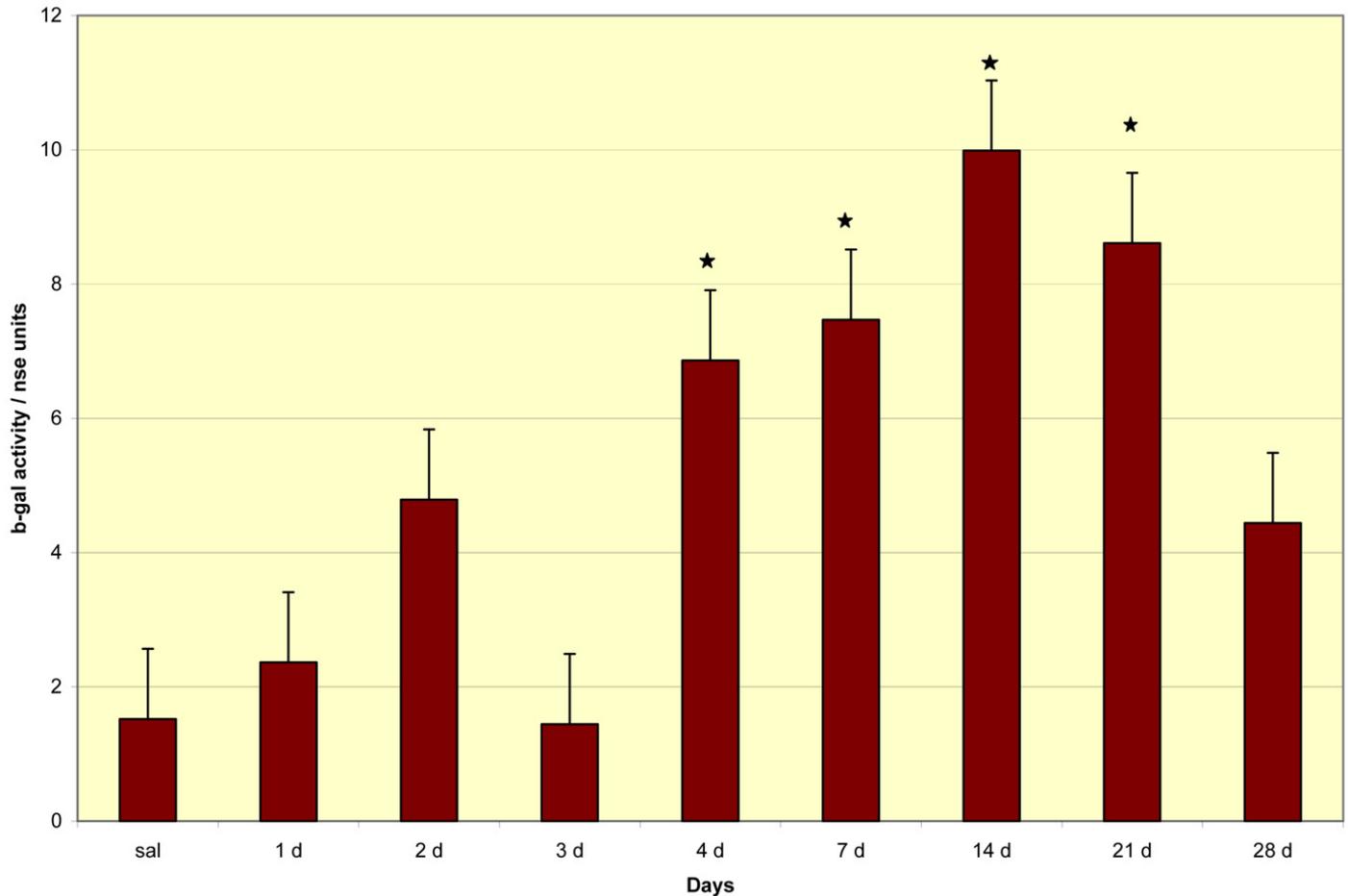


Figure 2. β -Galactosidase activity (representing NF- κ B activation) was measured in whole-cell hippocampal lysates in κ B reporter mice treated from 1 to 28 days with TMT and saline. The mU of β -galactosidase activity adjusted to total protein was normalized to neuron-specific enolase protein expression in each of the lysates. Activity in the TMT-treated (for 4–21 days) mice differed significantly from activity in saline-treated mice ($P \leq 0.05$) as tested by ANOVA followed by Fischer's LSD post hoc test.

Fig. 3

Day 7 Upregulated Genes

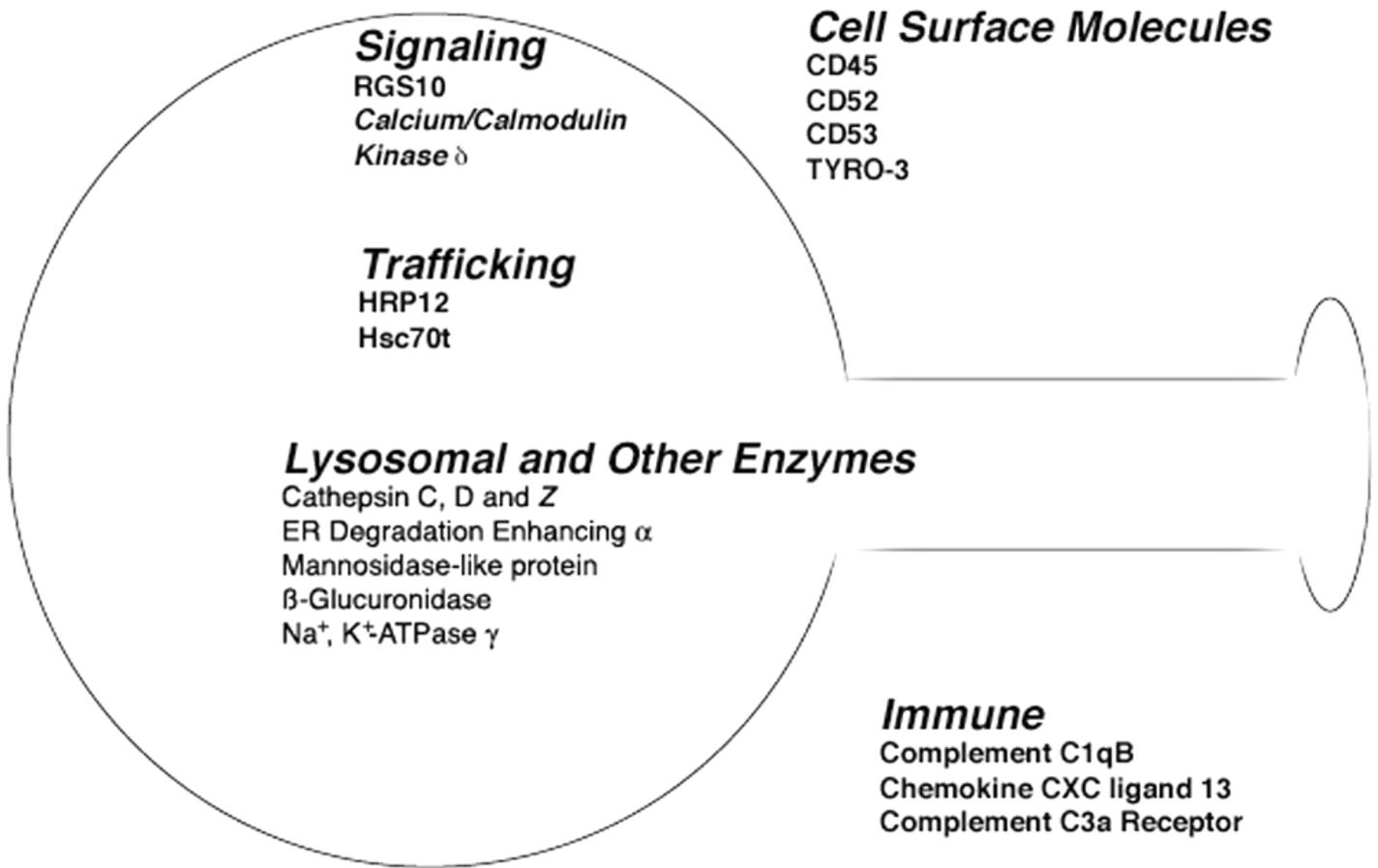


Figure 3. NF- κ B p50-modulated genes are increased in nontransgenic mice after TMT treatment. Microarray analysis identified 14 genes with expressions that were up-regulated >2.0-fold and 3 genes up-regulated between 1.5- and 2.0-fold in nontransgenic mice 7 days after TMT but did not change in NF- κ B p50-null mice. These genes were characterized into five functional groups.

Fig. 4

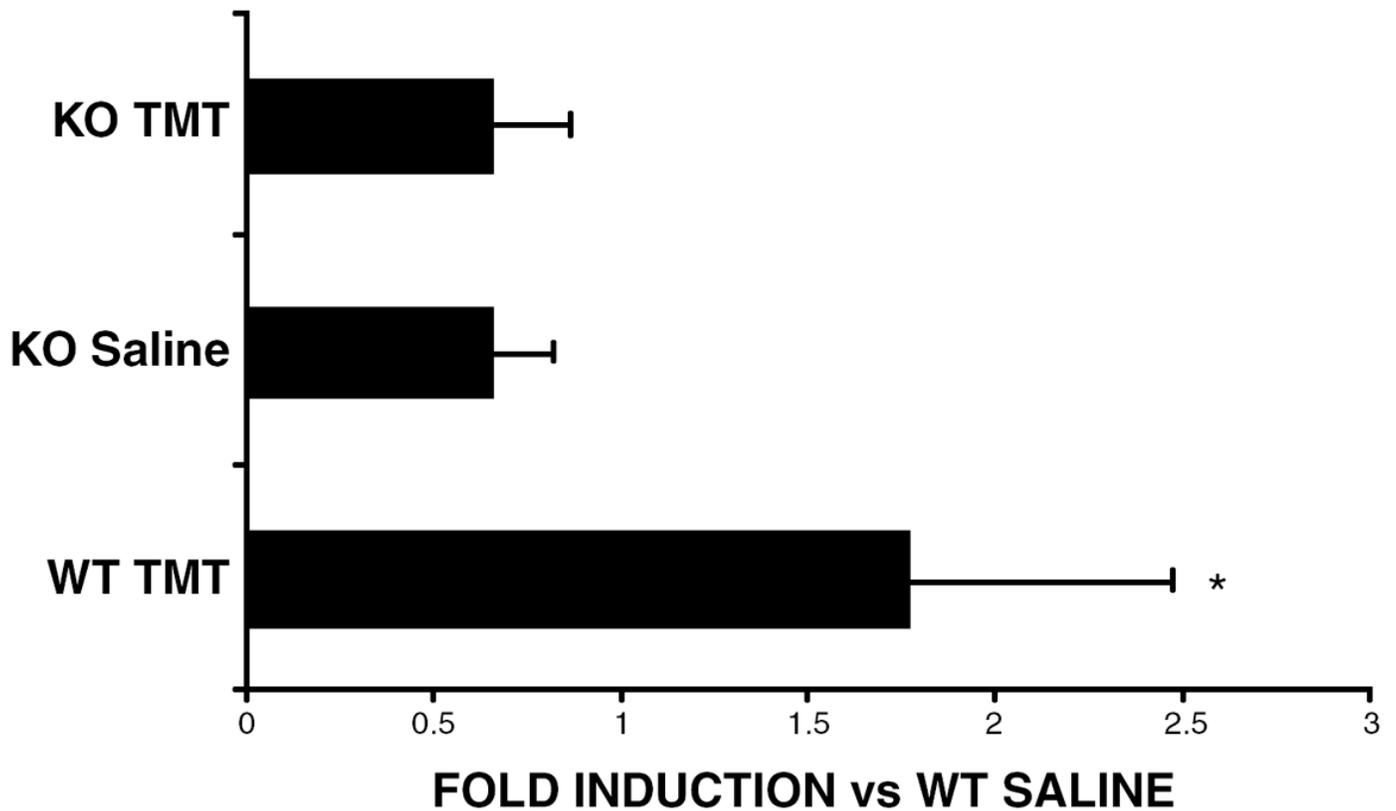


Figure 4. Na^+ , K^+ -ATPase γ gene expression requires NF- κ B p50. Nontransgenic (WT) and p50-null mice (KO) were treated with either saline or TMT and then killed 7 days later. RNA from hippocampi of three animals from each genotype and treatment group was extracted and reverse transcribed, and the resultant cDNA from each sample was used as a template in subsequent PCR reactions. The Na^+ , K^+ -ATPase γ gene was amplified concurrently with 18S RNA from the same samples. Differences in ratios of γ gene expression to 18S RNA among the samples are represented as fold change relative to nontransgenic saline (WT saline). Na^+ , K^+ -ATPase γ gene expression in nontransgenic TMT-treated mice was significantly elevated over that in nontransgenic saline-treated mice (ANOVA followed by Fischer's LSD post hoc test, $P < 0.05$). Na^+ , K^+ -ATPase γ gene expression did not change in p50-null mice after TMT treatment.