

Involvement of Tumor Necrosis Factor Alpha in Hippocampal Development and Function

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Tumor necrosis factor alpha (TNF α) is a cytokine produced mainly by cells of the immune system. It is also expressed by brain neurons and glia. The physiological role of TNF α in the brain is not yet fully clear. Using TNF α -deficient mice, we have examined its role in hippocampal development and function. We report here that TNF α is involved in the regulation of morphological development in the hippocampus. TNF α -deficient mice exhibited an accelerated maturation of the dentate gyrus region and smaller dendritic trees in CA1 and CA3 regions in young mouse. In addition to its involvement in hippocampal morphogenesis, TNF α deficiency specifically improved performance of affected mice in behavioral tasks related to spatial memory. Moreover, lack of TNF α increased the expression of nerve growth factor (NGF), but not brain-derived neurotrophic factor (BDNF), following performance of the learning task. Our results suggest that TNF α actively influences hippocampal development and function. In adult mice, TNF α may interfere with memory consolidation, perhaps by regulating NGF levels.

Introduction

The central nervous system was considered for many years as an immunologically privileged site, i.e. where cells of the immune system are absent. Recently, this concept was called into question when immune cells, as well as pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNF α) and interleukin-1 (IL-1), were detected in the brain. In fact, it is now known that TNF α acts in the brain, through its receptor, TNFRI, a 55 kDa protein, to promote apoptosis and, through an isoform of the receptor, TNFRII (75 kDa) as a trophic factor (Neumann *et al.*, 2002). Neurotrophins such as nerve growth factor (NGF), operating through their tyrosine kinase (Trk) receptors, block TNF α -induced apoptosis. According to the neurotrophic hypothesis, newly differentiating neurons compete for neurotrophins that are in limited supply and only those that succeed in establishing correct synaptic connections will acquire the trophic support to allow their survival (Levi-Montalcini, 1987). Thus, both death signals and survival signals could play a key role during neurogenesis. Recently, it was demonstrated that this death is induced partly by TNF α (Barker *et al.*, 2001).

In the present study, we have examined the involvement of TNF α in the development and function of the central nervous system (CNS), concentrating on the hippocampal region. Using TNF α -knock-out (TNF-KO) mice, we have demonstrated, for the first time, that basal levels of TNF α in the brain regulates both NGF and brain-derived neurotrophic factor (BDNF) levels during hippocampal development. Modulation of these factors governs granular cell migration in the dentate gyrus and dendritic tree formation by pyramidal cells in regions CA1 and CA3. Furthermore, we have demonstrated that TNF α may

specifically suppress hippocampal function in adult mice, through suppression of NGF expression.

Materials and Methods

Jackson Black C-57 mice and TNF-KO on JB C-57 background mice were used in the study. The TNF-KO mice (Pasparakis *et al.*, 1996) were generously provided by Professor George Kollias, Hellenic Pasteur Institute, Athens, Greece. Absence of the TNF α gene was confirmed by polymerase chain reaction (PCR) and by specific mTNF α enzyme-linked immunosorbent assay (ELISA) to serum of lipopolysaccharide (LPS)-injected mice (3 h after i.p. injection of 20 μ g/ml of LPS) or to conditioned media of spleen cells activated with LPS (10 μ g/ml) for 24 h. The mouse colony was maintained in a 12:12 light:dark schedule; food and water were provided *ad libitum*. All procedures were performed according to guidelines from the Israeli Council on Animal Care and approved by the Ben-Gurion University animal care and use committee.

Phenotype Aspects in the TNF-KO Mice

Mice were tested daily during the first month of life for general phenotypic and morphogenic aspects: body wt and length; brain length and width; hair growth; and date of eyelid opening and tooth eruption. A single animal per group was drawn from any single litter. Following phenotype examination, mice were anesthetized and thereafter killed. Brains were rapidly removed into ice-cold artificial cerebrospinal fluid (ACSF) and deeply frozen at -80°C for later analysis. Mice aged 1–7 days were anesthetized by hypothermia, while older mice were anesthetized by i.p. administration of ketamine and rompun.

Murine TNF α , NGF and BDNF ELISA

Immunoreactive murine TNF α was examined in serum and conditioned media of spleen cells activated with LPS; NGF and BDNF were examined in homogenates of hippocampi using a specific ELISA (R&D Systems, Minneapolis, MN). The sensitivity of the ELISA was 4 pg/ml for TNF and NGF and 47 pg/ml for BDNF.

Dendritic Tree Staining and Sampling

Four-day-old wild type (*wt*) and newborn TNF α -KO mice were cold-anesthetized, brains were rapidly removed to cold ACSF, hippocampi were isolated and transverse slices (300 μ m thick) prepared with a tissue chopper. Following a 1 h incubation at 4°C , slices were placed in petri dishes coated with micro-crystals of the vital fluorescent dye DiI_{C18} (Molecular Probes), covered with a drop of modified Eagle medium (MEM) medium and incubated at 37°C , 5% CO₂ for 10 min. After a brief rinse, the slices were incubated until appropriate staining was achieved, then fixed in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS) and sampled. Neurons that could be separately visualized were sampled in a confocal laser scanning microscope (LSM510) at a magnification of 20 \times . Images were flattened and analyzed using NIHimage. Dendritic branch length and number of branch points per neuron were obtained for the apical dendrite.

Immunohistochemical and Cell Nuclei Staining

Sections for immunohistochemical staining were prepared from paraformaldehyde-fixed (4%), paraffin-embedded tissue. Transverse sections (4 μ m thick) of medial hippocampus, were mounted on saline-coated slides, dried at 37°C for 48 h and stored at room temperature. Before the primary antibodies were applied, endogenous peroxidase

was quenched by incubation in 3% H₂O₂ in 80% methanol for 15 min and non-specific staining blocked with a serum and albumin-containing solution. This solution was also used to dilute the primary antibodies and for washing the sections. Primary antisera (BDNF-IgG, R&D Systems, Minneapolis, MN; NGF-IgG, Alomone Labs, Jerusalem, Israel) were applied at dilutions of 1:100 and 1:50, respectively. The biotinylated antibodies and avidin-biotin conjugate were applied according to the suppliers' directions. Development was performed with 0.06% 3',3'-diaminobenzidine + H₂O₂, with or without cobalt enhancement; Mayers haematoxylin was used to counterstain cell nuclei in corresponding sections. The sections were mounted with Eukitt. For immunofluorescence localization of BDNF, a secondary antibody conjugated to Cy-3 was used. For a negative control, normal serum replaced the primary antibodies. For cell density analysis, images of the dentate gyrus region of the hippocampus were sampled in an Olympus IX-70 microscope at 400× equipped with a SuperCam video camera (Applitec, Israel). Analysis was performed using NIHimage (Wayne Rasband, NINDS, NIH). The number of cell nuclei/(pixel)² was determined in transverse sections through the approximate mid-rostral-caudal hippocampus of P1, P7, 14 mice. Cell density was categorized to: (i) more than one cell per 1000 pixel², which represents the non-developed hilus and (ii) less than one cell per 1000 pixel², which represents the normal development in the hilus of adult mice.

Behavior

Reflex Development in Newborn Mice

Righting reflex was measured as the time, in seconds, required for a newborn laying on its back to right itself on four limbs (Crawley, 1999). Rotaroad (adjusted for neonates) tests the ability to hold a rotating bar at a rate of four cycles per minute. Latency to fall from the rotarod was recorded. Locomotion on inclination—the ability of newborn mice to climb on an inclined board—was examined at slopes of 45, 70 and 90°.

Behavior of Adult Mice

Open Field Task. Performance was tested in an open field arena: a 65 cm diameter gray wall, 30 cm high, over a period of 2 min. The following variables were observed: rearing (times), grooming (times), center/perimeter time and quantity of defecation delivered (Henderson, 1967).

Elevated Plus Maze. A 'plus maze', 15 cm high and with 40 cm long arms, was used to measure the time (s) spent in the open versus the closed arms (Lister, 1987a).

Hole Board. The number of holes into which the mice inserted their noses during a given 2 min interval was measured (Lister, 1987b).

Morris Water Maze. Mice were trained to swim to a hidden platform in the water maze (Morris, 1984). The maze consisted of a 70 cm diameter, 30 cm high, circular pool filled with milk and maintained at 25–26°C. The 10 cm diameter platform was 0.5–1 cm below the surface of the milk. The pool was placed in the center of a room with white walls and a colored geometric figure on each of the walls (triangle, square, circle, strip) that served as spatial cues. One day before the training, the mice were placed on the platform for 10 s, if they failed, they were replaced on the platform. Twelve male mice of each group (90 days old) were tested in a blind analysis and were randomly assigned for training to find the platform in a given quadrant. For three consecutive days, each mouse was placed in the pool six times, starting in a random order of direction – north, south, east or west – with 30 min intervals between trials. After locating the platform, the mice were dried and returned to their cages. If, after 60 s in the pool, the mice could not locate the platform, the trial was stopped and the escape latency recorded as 61 s. A probe test was conducted as follows. Following the last trial on the third day, the platform was removed from the pool. The mice were placed in the pool, starting in the location opposite the platform and allowed to swim for 60 s. The time spent in swimming in each quadrant was recorded. The amount of time spent swimming in the quadrant where the platform had been located was recorded as another index of spatial learning. All data were recorded on

videotape and analyzed blind. Mice were housed in individual cages on a 12 h light:dark cycle and were tested between 9:00 and 16:00, daily.

Visible Platform Training. Spatial cues were removed and the location of the platform made visible by use of clear water, marking of the submerged platform by blue paint and elevating it 1 cm above the surface of the water. The remaining details were similar to those described for the hidden platform test.

Results

Effects of TNF α -deficiency on Mice Phenotype Development

The number of offspring per litter in the TNF-KO mice was similar to the *wt* mice (7 ± 2.74 , $n = 22$ and 8.5 ± 2.9 , $n = 16$, respectively; mean \pm SD). TNF-KO mice were analyzed for gross developmental consequences through the first postnatal month. The mice so examined developed normal phenotypes with respect to body wt, body length and general brain size (depicted in Fig. 1). The days of eyelid opening, hair growth and teeth eruption were considered as the days in which 50% of newborn fit the criteria (Table 1). No significant differences were observed in these parameters.

In addition, development of locomotion reflexes, muscle strength and balance in the rotarod and inclining slope were similar in both groups. Righting reflex was fully developed in *wt* mice between postnatal days 2 and 9. However, in the TNF-KO newborns, it was fully developed by postnatal day 5 in all of the mice pups. Moreover, TNF-KO pups that succeed in righting themselves by postnatal days 4 and 5 were significantly faster than those in the *wt* group as depicted in Figure 1c ($P < 0.01$, *t*-test). In general, except for their superiority in the development of the righting reflex, TNF-KO mice exhibited phenotype and reflex development at the same rate as *wt* mice.

NGF and BDNF are differentially expressed in the hippocampus of TNF α -deficient and *wt* mice. Development of the hippocampus begins in the embryo and continues through the first month after birth (Bayer, 1980). One of the crucial molecules regulating neuronal survival and hippocampal morphogenesis is the neurotrophin, NGF (Levi-Montalcini, 1987). Hippocampal levels of NGF in rodents increase throughout the first month of life. In the present study, NGF protein was measured in hippocampal homogenates from both *wt* and TNF-KO mice at P1, P7, P14, P21 and P180 (depicted in Fig. 2b). During the first postnatal month, the NGF levels were significantly higher in the hippocampi of TNF-KO mice than in *wt* mice, as illustrated by immunohistochemical staining of the CA1 region of the hippocampus and neocortex (Ctx; Fig. 2a). NGF immunostaining was weaker in both CA1 and cortex of *wt* mice compared to TNF-KO mice (upper panels of Fig. 2a). Quantification of NGF in the homogenates of these tissues by ELISA confirmed the immunostaining results (Fig. 2b; $P < 0.05$, $F = 3.8$, one-way analysis of variance). This developmental difference was not observed in adult mice (Fig. 2b).

The levels of BDNF in the hippocampus were similar in both groups in the first postnatal week. However, in adult mice, levels of BDNF were lower, though not reaching statistical significance. That could be seen in the immunofluorescent staining of hippocampal sections (Fig. 2c) and ELISA of homogenates (Fig. 2d) from P7 mice.

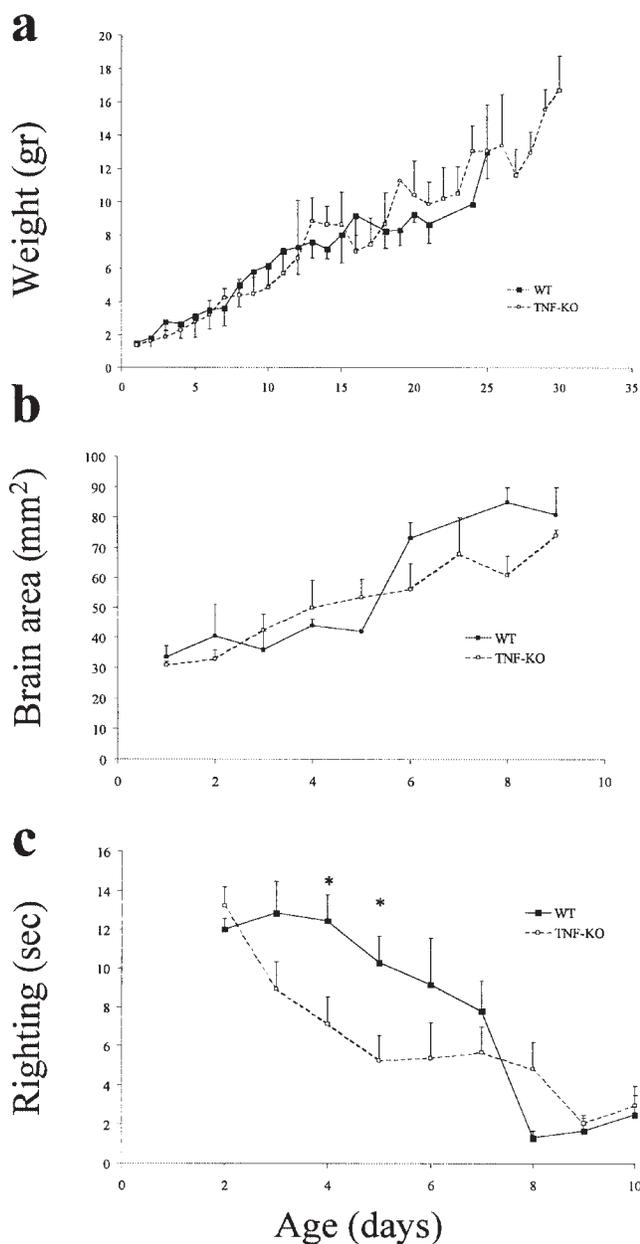


Figure 1. The following parameters were compared between early postnatal wild-type (*wt*, filled squares) and TNF-KO (TNF-KO, open squares) mice. (a) Body weight (g). (b) Brain area (mm²), based on length and width measurements. (c) Righting reflex (s) — time required for newborn mice to right themselves, at different postnatal days, until the reflex is fully developed (1 s). One pup per litter was sampled on any given day; $n = 10\text{--}20$ for all data points, $P < 0.01$.

Involvement of TNF α in the Regulation of Hilar Maturation (Morphogenesis)

Since NGF and BDNF are involved in various aspects of neurogenesis and in our present study we found that these factors are differently expressed in TNF-KO and *wt*, we therefore asked whether loss of TNF α also affects hippocampal morphogenesis. The dentate gyrus of the hippocampus is an area that undergoes extensive development postnatally (Bayer, 1980). During the first postnatal month and even later, cells in the

Table 1
Developmental phenotype

Parameter	<i>wt</i>	TNF-KO
Tooth eruption	6	5
Hair	3	4
Eye opening	12	13

Postnatal day in which 50% of newborns fit criteria, $n = 10\text{--}30$.

hilus continue to divide, differentiate and migrate toward the stratum granulosum layer (Gould *et al.*, 1998). Hence, at birth, the hilus is densely populated with cell somata, which, over the following days, with the end of mitosis and continued cell migration, reduces, indicating maturation of the area. In the present study, we used cell density in the hilar region as an indicator of the state of dentate gyrus development and maturation. Figure 3*a-c* depicts the dentate gyrus at the different stages of postnatal development in *wt* mice. The right-hand panels (Fig. 3*d,e*) depict representative images of this area in the TNF-KO mice of the same ages. The percentage of mice with mature hippocampal hilus (see Materials and Methods) was significantly higher in the TNF-KO mice compared to *wt* during the first week (P1 and P7, $P = 0.0004$ and 0.001 , respectively). However, by P14, both groups had achieved equivalent developmental stages (Fig. 3*f*). This would appear to suggest that at birth, the dentate gyrus and hilus of the TNF-KO mice are already at advanced maturational stages.

Decreased Arborization of the Apical Dendrites in CA1 and CA3 of TNF-KO Mice

At birth, pyramidal neurons in the CA1 region of the hippocampus vary in size and dendritic tree arborization. Some cells present only short apical dendrites, while others present a more developed dendritic tree (Tyzio *et al.*, 1999). The number of branch points in the apical dendrites of pyramidal neurons and the length of dendritic branch segments (as shown in Fig. 4*bii*) were employed as measures of development. As depicted in Figure 4*c*, most of the cells in the CA1 region of P4 mice of both groups developed, on average, <10 branch points per neuron. On the other hand, in the *wt* group, 37% of the neurons developed >10 branching points per neuron compared with 5% in the TNF-KO group (Fig. 4*c*, $n = 27, 49$, $P < 0.004$, χ^2 test). A similar trend was observed in CA3. The number of neurons with a small number of branch points in CA3 was significantly higher in the TNF-KO mice compared to *wt* mice ($n = 52, 47$, respectively, $P < 0.0001$, χ^2 test). In the *wt* mice, 60% of neurons developed dendritic trees with >10 branch points. However, only 15% of neurons from the KO mice exhibited the same number of branch points. Thus, the pyramidal neurons of both CA1 and CA3 from TNF-KO mice were less arborized than those from *wt* mice.

The average lengths of segments of dendritic tree branches are shown in Figure 4*e,f* as percentages of total segments of branches. No significant difference was observed in this parameter in CA1 and CA3 regions of both study groups. Thus, although no significant differences were observed between *wt* and TNF-KO in gross phenotype and development of some motor reflexes during the first month of life, the TNF-KO

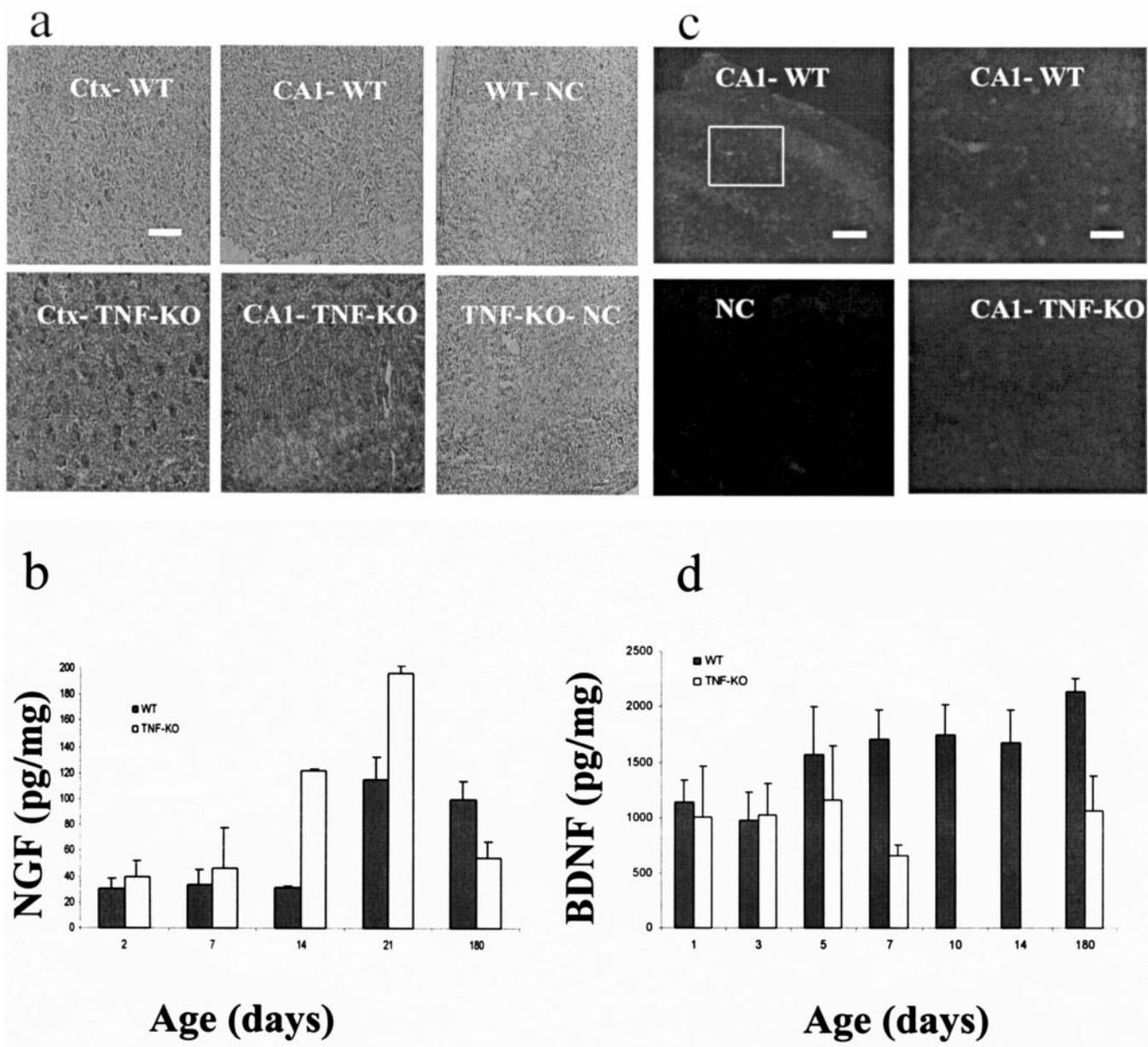


Figure 2. Expression levels and cellular localization of NGF and BDNF in hippocampus during development. (a) Cellular localization of NGF in (left panels) the cerebral cortex from *wt* mice (Ctx-*wt*), and in TNF-KO mice (Ctx-TNF-KO). Middle panels: CA1 region from *wt* (CA1-*wt*) and TNF-KO (CA1-TNF-KO). In the right panels, negative control staining of hippocampus from *wt* (*wt*-NC) or from TNF-KO mice (TNF-KO-NC) are presented. Magnification 200 \times . (b) Expression levels of NGF in the hippocampus of *wt* and TNF-KO mice as measured by ELISA and normalized to total protein. (c) cellular localization of BDNF in the CA1 region from *wt* mice (CA1-*wt*) (upper left panel 20 \times , calibration bar = 100 μ m). Higher magnification image (calibration bar = 20 μ m) of the region in the square is seen in the upper right panel (CA1 - *wt*). The lower right panel shows BDNF expression in a similar region from a TNF-KO mouse hippocampus (200 \times ; CA1-TNF-KO). The lower left panel shows the negative control staining for *wt* mouse (NC). (d) Expression levels of BDNF in the hippocampus of *wt* and TNF-KO mice. Calibration bar = 20 μ m. See Supplementary Material for the color version of this figure.

animals did exhibit specific differences in morphological development.

Effect of TNF α deficit on Behavior in Adult Mice

We have examined whether the developmental differences observed in hippocampi of *wt* and TNF-KO mice could lead to differences in their behavior as adults. In the open field arena (Fig. 5a), TNF-KO and *wt* mice were found to be similar in their rearing and grooming behaviors. Nevertheless, the ratio of perimeter versus center time was higher, 0.83 ± 0.17 (mean \pm

SD), for the TNF-KO compared to 0.63 ± 0.2 for the *wt* mice ($n = 12$, $P = 0.03$, *t*-test; Fig. 5a) and they produced a larger numbers of fecal pellets (2.25 ± 1.4 and 1.16 ± 1.3 , respectively, $n = 12$, $P = 0.03$, *t*-test). These results may indicate that the TNF-KO mice are more distressed than the *wt* mice when exposed to an open field environment. This possibility was assessed in the elevated plus maze (Fig. 5b). The time that mice from both groups spent in the dark arms did not differ (Fig. 5c). In order to evaluate the basal activity levels in addition to the exploratory behavior of the mice; activity was examined in the

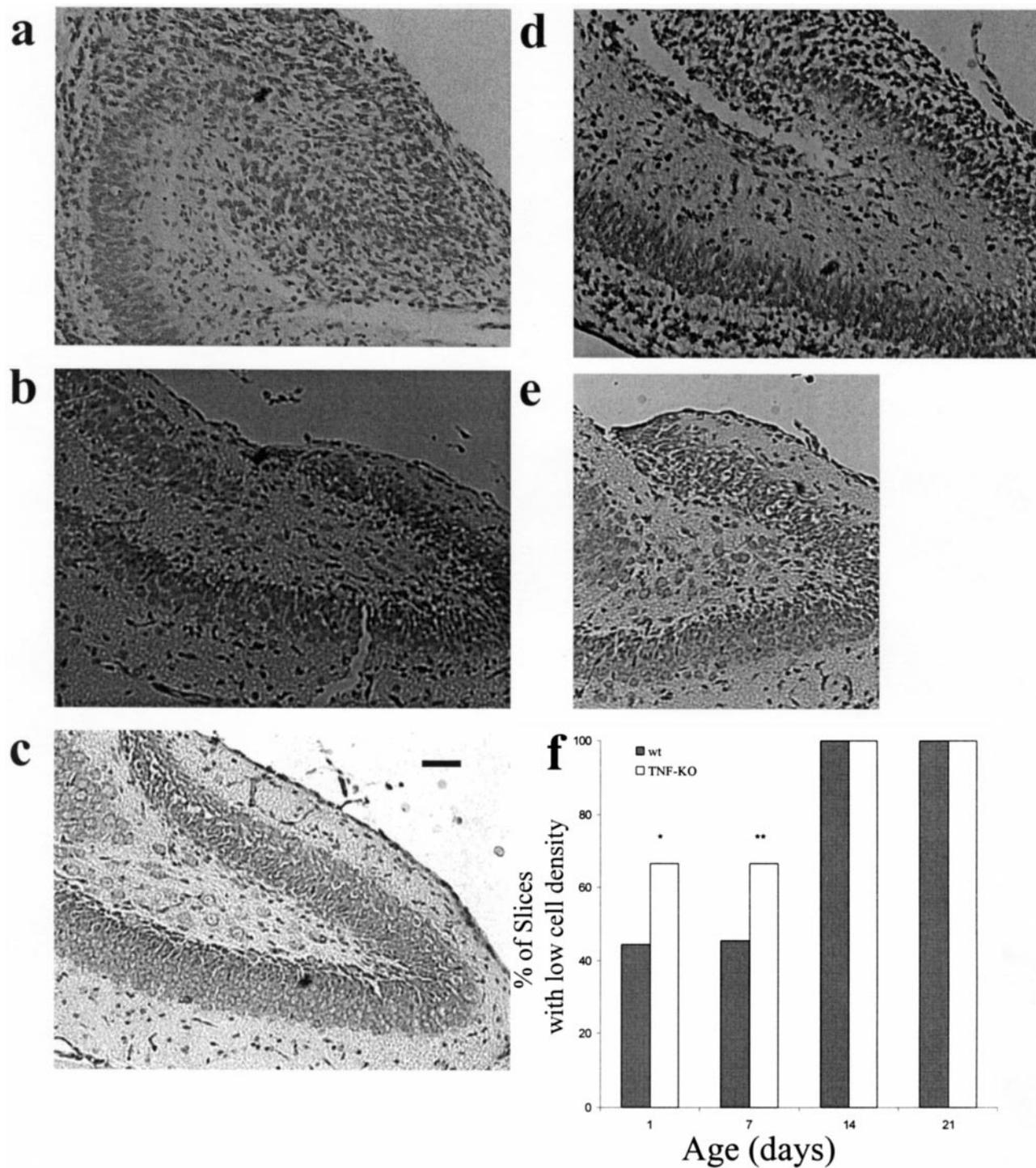


Figure 3. Effect of TNF α -knockout on dentate gyrus development. Hematoxylin-stained nuclei of neurons from the dentate gyrus of wt [(a) P1, (b) P7, (c) P14] and TNF-KO [(d) P1, (e) P7]. (f) Quantification of dentate gyrus maturation as estimated by cell density in the hilus. A hilus that exhibited low cell density contained less than one cell per 1000 pixel². The percentage of slices with low cell density is presented for each age of wt (gray) and TNF-KO (pattern) mice. * $P = 0.0004$; ** $P = 0.001$, χ^2 . Calibration bar = 20 μm for all panels.

holes board (Fig. 5c). Again, no statistical difference was noted between the groups (24 ± 8.9 and 30.66 ± 9.4 holes/2 min, for the wt and TNF-KO mice, respectively). Since developmental differences were observed in the hippocampi of young mice, another task, based on the neural circuitry of the hippocampus, i.e. spatial learning in the Morris water maze, was

examined. Twelve mice from each group (each one from a different litter) were examined in the maze (Fig. 5d,e). The performances of the TNF-KO mice, on the first day, were better than performances of the wt mice. No errors in platform finding occurred during the first trial, compared to 5/12 errors for the wt mice (Fig. 5d). Performance of the task by the KO

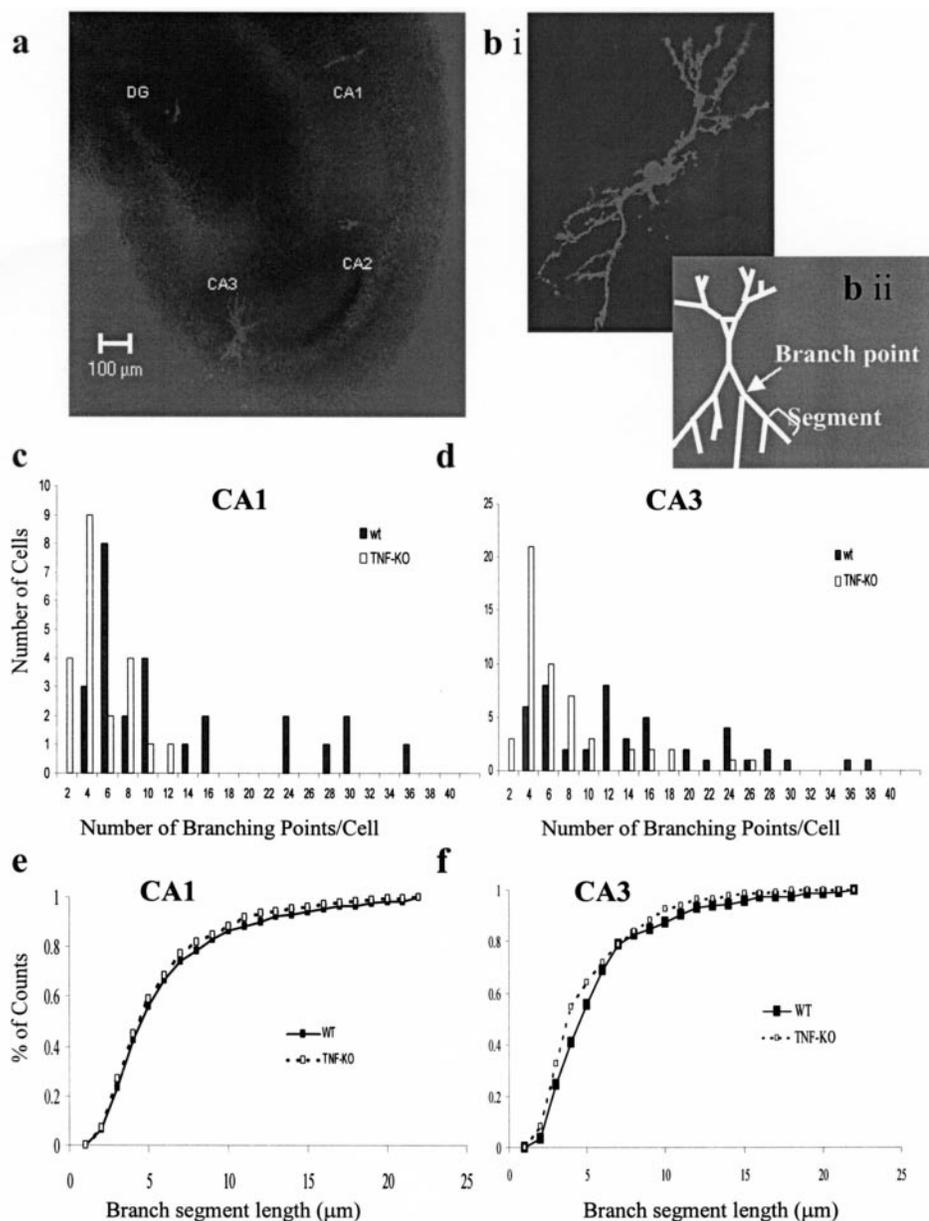


Figure 4. Effect of TNF α -knockout on dendrite arborization of CA1 and CA3 pyramidal cells. Morphological analyses were performed following Dil staining. Overview of a stained slice and an example of a stained neuron are presented (*a, b*). Number of branching points/cell distribution as measured in the apical dendrite of (*c*) CA1 of *wt* ($n = 27$) and TNF-KO ($n = 49$; $P < 0.004$) and (*d*) CA3 of *wt* ($n = 47$) and TNF-KO ($n = 52$; $P < 0.001$). The cumulative distributions of dendrite segment lengths were normalized to the total number of branches. (*e*) CA1 and (*f, d*) CA3; n as in (*c, d*). See Supplementary Material for the color version of this figure.

group was also characterized by shorter latency in finding the platform (26.4 ± 6.6 s, compared to 44.1 ± 6.7 s for the *wt*, $n = 12$, $P < 0.05$, *t*-test, Fig. 5*e*). On days 2 and 3, these differences were masked, due apparently to the learning process in the *wt* group. Though not demonstrated by latency, the TNF-KO mice better remembered the platform location, as indicated by the results of the probe test conducted at the end of the experiment. In these experiments, the TNF-KO mice spent a significantly longer time (32.2 ± 5.6 s) in the correct quarter compared to the *wt* mice (20.4 ± 5.7 s) ($P < 0.01$, *t*-test). No differences were observed between the two groups in the performance of the cued version of this task.

NGF and BDNF are Differently Regulated by TNF α in Brains of Adult Mice Following Learning

Neurotrophins, in addition to supporting survival and growth of neurons, are also involved in learning and memory consolidation (Woolf *et al.*, 2001). Therefore, we have examined NGF and BDNF levels in adult mouse brains in both groups following learning in the Morris water maze. Twenty-four hours following the probe test, NGF levels in mouse brains were determined by ELISA. Left and right hippocampi were examined separately and the frontal cortex was analyzed as a negative control area, since it is not primarily involved in the spatial learning processes. NGF levels in TNF-KO hippo-

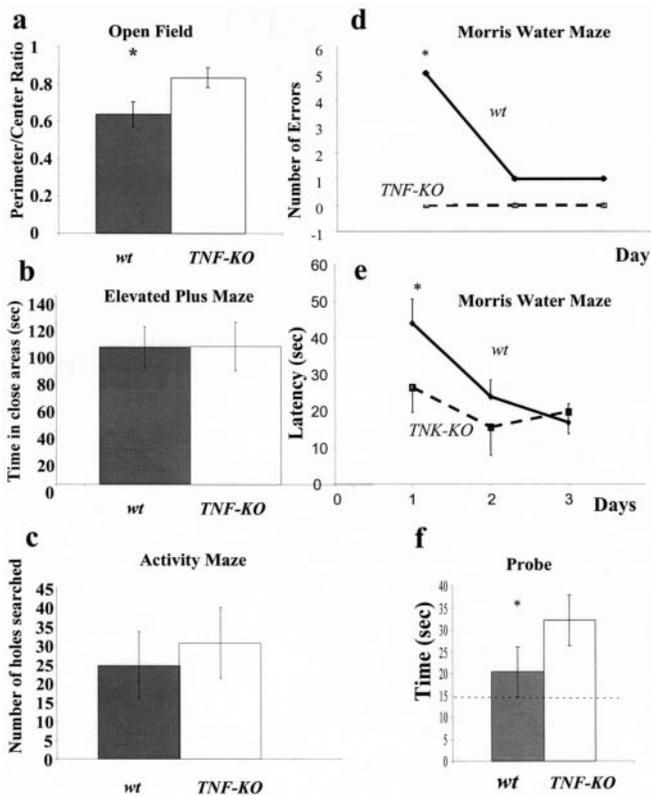


Figure 5. Behavioral comparison of *wt* and TNF-KO mice. Open field: (a) ratio between time spent at the margins and the time spent in the center of the arena within the first 2 min of the trial ($n = 12$, $P < 0.05$). Activity maze: (b) number of holes into which the mice insert their nose within a 2 min trial ($n = 12$). Elevated plus maze: (c) time spent in dark areas within first 2 min of a trial ($n = 12$). Morris water maze: (d) number of times mice failed to find the platform within 60 s; (e) latency in finding the platform in the first trial each day of training ($n = 12$, $P < 0.05$). Memory retention at the probe test: (f) 24 h following the last trial, time spent swimming in the correct quarter where the platform had previously been, the dashed line represent random searching; $P < 0.01$.

ampi were two to three fold higher than those in the *wt* mice ($n = 5$, $P < 0.01$, *t*-test, for NGF in whole hippocampus of *wt* as compared to TNF-KO). This change is apparently region specific, since no difference in NGF levels was observed in the frontal cortex (Fig. 6). In contrast, 24 h after learning, BDNF levels did not differ in the two groups, either in hippocampus or frontal cortex (Fig. 6b). This might suggest that expression of NGF protein is specifically regulated in the hippocampus by TNF α during memory consolidation.

Discussion

TNF α is Involved in Hippocampal Development

TNF α deprivation during embryonic and early postnatal development did not result in gross phenotypic alterations. However, it did affect protein levels of neurotrophic factors and resulted in morphological variation in the hippocampus. The major roles of neurotrophic factors in neurogenesis are well established. They are involved in cell differentiation, migration and morphogenesis (for reviews, see Huang and Reichardt, 2001; Sofroniew *et al.*, 2001). Since NGF was shown to induce cell differentiation, it is possible that elevated levels

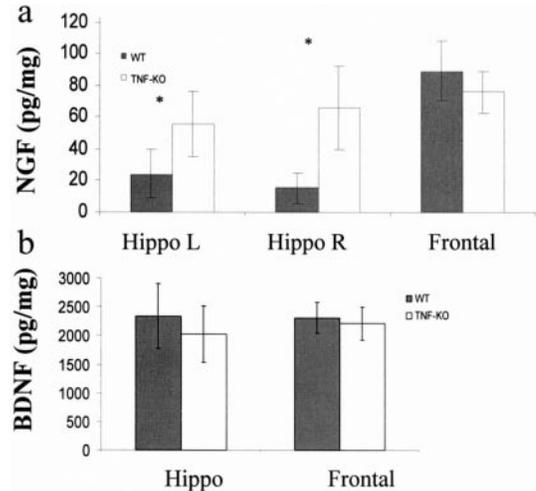


Figure 6. Expression levels of BDNF and NGF in the hippocampus and frontal cortex of the brain of *wt* and TNF-KO mice following learning. NGF and BDNF levels were examined in the hippocampus and frontal cortex of the mice 24 h after completing a learning paradigm. (a) NGF levels in the right and left hippocampus and in the frontal cortex ($n = 5$), $P < 0.01$ for NGF in whole hippocampus of *wt* compared to TNF-KO. (b) BDNF levels in the right hippocampus and frontal cortex ($n = 5$).

of NGF in the TNF α -deprived mice may result in an accelerated maturation of the dentate gyrus region. The appearance of a 'mature' dentate gyrus in the P1 TNF-KO newborn may reflect acceleration of embryonic development. Similarly, exogenous BDNF has been shown, in various brain areas, to enhance axonal growth and branching (McAlister *et al.*, 1995) and regulation of growth and maturation of the dendritic tree in developing cerebral cortex (McAlister *et al.*, 1995, 1997) and also to influence synaptogenesis in the hippocampus (Martinez *et al.*, 1998). Our finding that TNF α deprivation reduced hippocampal BDNF levels and the number of branching points of the apical dendrite of CA1 and CA3 pyramidal cells is consistent with these reports. Although, TNF α was previously shown to be involved in the regulation of NGF and BDNF (Aloe *et al.*, 1999), we report here the first *in vivo* evidence linking TNF α to hippocampal morphogenesis and, more specifically, to dentate gyrus maturation and dendritic tree arborization in CA1 and CA3. A recent paper reported that TNF α has a direct effect on dendritic branching in cultured neurons (Neumann *et al.*, 2002), probably through its TNFR2 receptor, which is known to transduce the trophic effect of TNF α (Yang *et al.*, 2002). However, it is unclear if the basal levels of TNF α in these regions are sufficient to activate this receptor. Moreover, since we observed significant changes in neurotrophin levels, we suggest that TNF α could have a direct influence on the morphological changes we observed, which might be effected in a synergistic manner with the neurotrophins. Reduction in the branching of the pyramidal cell dendrites of TNF-KO mice contradicts the suppressive effect of TNF α on dendritic branching reported by Neumann *et al.* (2002). This could be explained by the difference in the culture (i.e. *in vitro*) versus the intact brain (i.e. *in vivo*) environment. TNF α induction of AMPA receptor surface expression was recently reported (Beattie *et al.*, 2002). However, its biological relevance to our findings is not yet clear. All the above developmental changes may alter the performance in hippocampus-dependent tasks, such as those observed in water maze test.

Additional evidence for TNF α involvement in the development of neuronal circuits was demonstrated in the righting reflex. Accelerated reflex development was observed in the TNF α -deficient mice, possibly due to earlier maturation of cerebellar circuits. We suggest that TNF α had no effect on the musculature development, since similar performance to the *wt* was observed in the rotaroad task and inclining slope locomotion, both tests involving muscle strength.

Involvement of TNF α in Learning and Memory

Our data indicate specific involvement of TNF α in spatial learning and memory as measured in the Morris water maze and possibly on other behavioral aspects. These findings are in agreement with a recent report by Aloe *et al.* (1999), who demonstrated, in two lines of mice that over-expressed human recombinant TNF α , a significant impairment in spatial learning, as demonstrated by the Morris water maze. In addition, our data are also in agreement with several other studies that have examined the suppressive effect of TNF α on synaptic plasticity in the hippocampus. In those studies, application of exogenous TNF α to hippocampal slices suppressed LTP (Tancredi *et al.*, 1992; Cunningham *et al.*, 1996), a cellular manifestation of learning and memory. Besides the specific improvement in performance of the spatial learning task, TNF α -deficient mice in the present study showed a region-specific increase in NGF levels following learning. It was previously reported that NGF levels correlate with an animal's capacity for spatial learning (Henriksson *et al.*, 1992) and that NGF infusion facilitates spatial memory performance (Janis *et al.*, 1995). Involvement of NGF in memory consolidation was also reported by Woolf *et al.* (2001). However, in that study, elevated levels of NGF in rats were obtained only a week following the contextual learning paradigm. Comparing NGF levels in the adult mice before (Fig. 2b) and after learning (Fig. 6a) indicates that in the *wt* mice, there was a 70% suppression of NGF production after learning. In contrast, in the TNF-KO mice, NGF levels were 26% higher following learning as compared to levels in adult mice (Fig. 2b). Thus, it is possible that under normal conditions (*wt* mice), basal levels of TNF α partially impede plasticity of hippocampal circuitry and that removal of this suppressing factor (TNF-KO mice) enables exposure of a larger portion of its capacity.

Besides the specific increase in NGF levels in the hippocampus of TNF-KO mice, no differences were observed in BDNF levels in either hippocampus or frontal cortex. An increase in BDNF levels in rat hippocampus following a spatial learning task was previously reported following intensive training in an eight-arm radial maze (Mizuno *et al.*, 2000). It is possible to suggest that the relatively short training period required for the Morris water maze was not sufficient to induce an increase in BDNF protein level. No differences were observed in BDNF levels in either group, suggesting that TNF α regulation during neuronal activity could be specifically associated with NGF levels.

The present data suggest that basal levels of TNF α may be specifically involved in the regulation of hippocampus development and function. The interaction between TNF α and NGF may indicate that the levels of NGF in young mice may regulate higher sensitivity to NGF in adults, for example, by up-regulating expression of Trk A.

Our results indicate that TNF α specifically and significantly affects the hippocampus. They further support the possible

involvement of TNF α in the regulation of learning and memory under physiological and pathological conditions. Since TNF α has been implicated in the development of some neurodegenerative diseases related to memory, such as Alzheimer's, it is possible that regulation of TNF α may be considered in development of new therapeutic strategies.

Supplementary Material

Supplementary material can be found at:
<http://www.cercor.oupjournals.org>

Notes

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