Distinct Expression and Distribution of Vesicular Proteins in the Hippocampus of TNFa-Deficient Mice During Development

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KEY WORDS Synaptotagmin; VAMP II; v-SNARE; neurogenesis

ABSTRACT Tumor necrosis factor alpha (TNFa) is a cytokine produced mainly by cells of the immune system. It is also expressed by brain neurons and glia. In the brain, TNFa governs synaptic plasticity, such as long-term potentiation and learning. Using TNFa-deficient mice (TNFa-KO) and immunohistochemical techniques, we resolved the spatio-temporal effect of TNFa on the expression of vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) in the presynaptic terminals of the hippocampus during the first month of development. During postnatal days 1–14, the levels of Synaptotagmin I and VAMP II were similar in the hippocampus of TNFa-KO and wild type (wt) mice. However, the levels of Syntaptotagmin II were reduced in the pyramidal cell layer of the CA1 region in TNFa-KO. At postnatal day 21, both proteins accomplished comparable levels in the hippocampus of TNFa-KO and wt mice. In addition, TNFa deficiency impairs the correlation of expression of Synaptotagmin I and II in CA1 region. The expression of those proteins in the CA1 stratum radiatum was uniform during development and similar in both mice groups. Higher expression of all examined proteins was demonstrated in dendritic fields of the CA3 region in TNFa-KO as compared to wt mice. We suggest that the impairment of synaptic plasticity by TNFa may be related to its modulation of synaptic vesicle proteins. **Synapse 53:6–10, 2004.**

INTRODUCTION

The central nervous system was considered for many years to be a privileged site, where cells of the immune system are absent. Recently, this misconception was shattered, and cells of the immune system and pro-inflammatory cytokines, such as TNFa and IL-1, were detected in the brain. Furthermore, TNFa overexpression in transgenic mice reduced spatial learning and memory in the Morris water maze (Aloe et al., 1999). We have recently demonstrated that a lack of TNFa in TNFa-deficient mice facilitated learning and memory in the same task (Golan et al., 2004). One cellular model for learning and memory is long-term potentiation (LTP). In several studies, it was demonstrated that exogeneous TNFa applied to hippocampal slices inhibited the induction of LTP in CA1 region of the hippocampus in a dose-dependent manner (Butler et al., 2004; Cunningham et al., 1996; Tancredi et al., 1992). These data suggest that the expression of TNFa in the brain may affect synaptic function, which may interplay with pre- or postsynaptic proteins. Evidence of the postsynaptic effects of TNFa were shown by Beattie et al. (2002). They demonstrated that the expression of AMPA receptors in the postsynaptic membrane in cultured hippocampal neurons and hippocampal slices increased following the application of exogenous TNFa. In the present study, we have examined the effects of TNFa on the expression levels of

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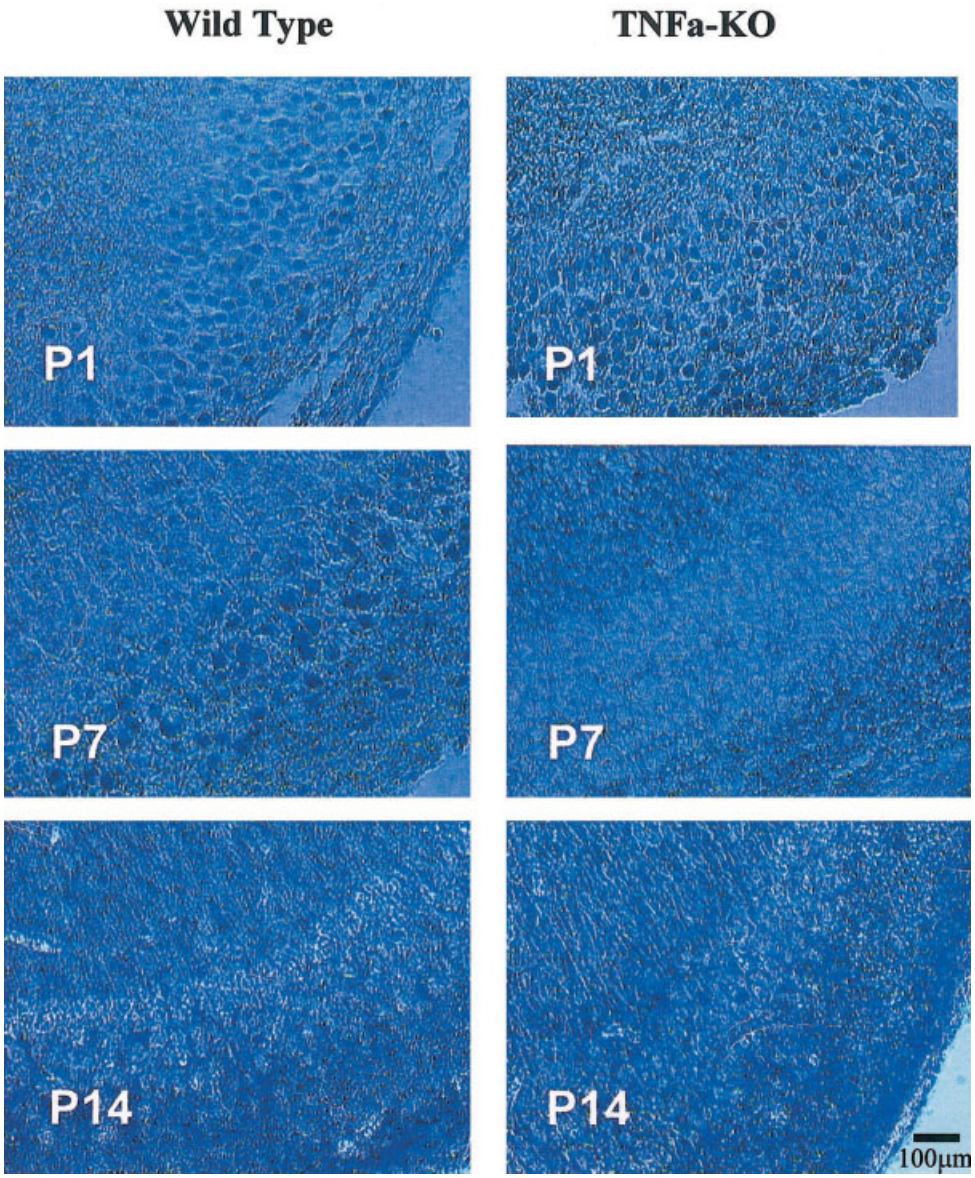

Fig. 1. Expression of VAMP II in the CA1-2 region of the hippocampus of TNFa-KO and wt newborn mice. IHC staining of VAMP II in the hippocampus of P1, P7, and P14 newborns of wt mice and TNFa-KO mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Synaptotagmin I, II (Synt I and II) and synaptobrevin/VAMP II, proteins located on synaptic vesicles in the presynaptic nerve terminal of the hippocampal formation. These proteins are expressed on the membrane of the synaptic vesicle and belong to the family of v-SNARE. Proteins in the Synaptotagmin family are one type of v-SNARE proteins, which are essential for the exocytosis of synaptic vesicles in the nerve terminal. Synt I was characterized as the calcium sensor that regulates fast synaptic transmissions (Fernandez-Chacon et al., 2001). It was recently shown that in the rat cerebral cortex, Synt I and its isoform Synt II are co-localized in the same synaptic vesicles. Moreover, Synt I and II heterodimerize in the presence of Ca^{+2} in a concentration-dependent manner (Osborne et al., 1999). An additional v-SNARE protein, synaptobrevin/VAMP II, participates in the fusion of synaptic vesicles to the membrane of the presynaptic nerve terminal (Jahn and Sudhof, 1999). VAMP II interacts with SNAP-25 and syntaxin to form the SNARE complex, which is a part of the complex essential for synaptic vesicles' docking and fusion to the nerve terminal membrane in the synapse. At least two types of interactions between VAMP II and Synt I were demonstrated (Fukuda, 2002). These interactions may be related to synaptic vesicles' trafficking or recycling.

In the present study, we have tested the involvement of TNFa in the regulation of the spatio-temporal ex-

pression of Synt I, Synt II, and VAMP II in the hippocampal formation of developing mice.

MATERIALS AND METHODS

The experimental animals included C57BL wild type mice (wt) and TNF-KO of the same genetic background. The TNFa-KO mice were generously provided to us by Prof. George Kollias, Hellenic Pasteur Institute, Athens, Greece (Pasparakis et al., 1996). The absence of the TNFa gene was confirmed by PCR. The mice colony was maintained in a 12:12 light dark schedule, and 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 of the Negev Animal Care Committee. A single newborn per mother was drawn from any single litter in postnatal days: 1, 7, 14, and 21. Four newborns, in average, from each age group of wt and KO mice, were analyzed. One- and seven-day-old mice were anesthetized by cooling, while older mice were anesthetized with i.p. administration of Ketamine (0.8 mg/g) concomitantly with Rompun (0.16 mg/g). After anesthesia was achieved, the mice were perfused (trans-cardiac) with saline fluid followed by 4% paraform-aldehyde (PFA) for 5–10 min. The brains were removed and kept overnight in 4% PFA. Medial hippocampus transverse paraffin sections, four microns thick, were stained. Before the primary antibodies were applied for 90 min at room temperature, nonspecific staining was blocked with a serum and albumin-containing blocking solution (Synaptotagmin-IgG, 1:100 and VAMP II, 1:200, Alomone Labs, Jerusalem, Israel; Synaptotagmin II-IgG, 1:100, Santa-Cruz Biotechnology, Inc., Santa Cruz, CA). The biotinylated antibody and the avidin–biotin conjugate were applied according to the suppliers' directions. Blocking of the endogenous peroxidase, with 3% H_2O_2 in 80% methanol for 15 min, was carried out before the avidin-biotin-peroxidase conjugate was applied. Development was performed with 0.06% DAB + H_2O_2, intensified by $CoCl_2$. As for a negative control, primary antibodies were pre-incubated with recombinant protein. Hippocampal sections were sampled on an Olympus IX-70 microscope at a magnification of $\times$20 and via a Super-Cam video camera (Applitec, Israel) with constant illumination. Staining intensity was analyzed for the CA1-2 and CA3 stratum pyramidalis (SP), stratum radiatum (SR), and stratum oriens (SO), and for the dentate gyrus (DG) granular cell layer. The analysis of staining density (optical density, OD) was done using NIH image software. Each "region of interest" (50 μm $\times$ 50 μm) within each of these areas was measured three times and averaged for each sample, then the measure of staining density (OD) in the negative control section was subtracted from the staining density measured. Thus, staining intensity = (sample OD − negative control OD for the same region of interest).

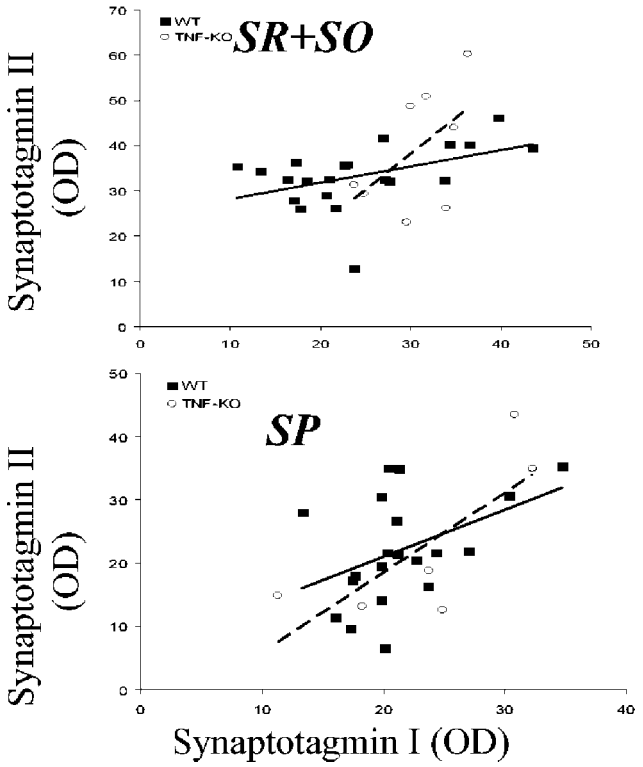

Fig. 2. The correlation between Synt I and Synt II levels in the CA1-2 region was impaired in TNFa-deficient mice. Correlation between Synt I and Synt II OD in the dendritic fields SR+SO of CA1-2 region. Correlation between Synt I and Synt II OD in the SP.

Statistical analyses were performed using SPSS software. A multi-factorial-model was used to compare the effect of (age $\times$ KO) or (KO) on vesicular protein expression. The Pearson correlation was used to evaluate the correlation between the expressions of pairs of proteins.

RESULTS AND DISCUSSION

The expression of Synt I, Synt II, and VAMP II in the CA1-2 pyramidal layer of wt newborns was usually higher in P1 and decreased during the first postnatal month, as depicted in Figure 1 for VAMP II. On day P14, pyramidal neuron somas did not stain for v-SNAREs. However, positive staining was observed, at this age, in the region of the dendritic fields (SR, SML, and SO), where synaptic input to the pyramidal neurons is concentrated. The expression of Synt I and VAMP II in CA1-2 SP was identical in the TNFa-KO and the wt mice. In contrast, the density of the Synt II was significantly reduced during the first 2 weeks in TNFa-KO mice, as compared to wt mice (n = 13–26, $P = 0.017$, F = 6.33, ANOVA). Synt II staining density in the SR and SO regions had no constant trend of change with age. The expression of all the tested proteins in the TNFa-KO mice was similar to that measured in the wt mice on days P1 and P7. However, lower levels of Synt II and higher levels of VAMP II

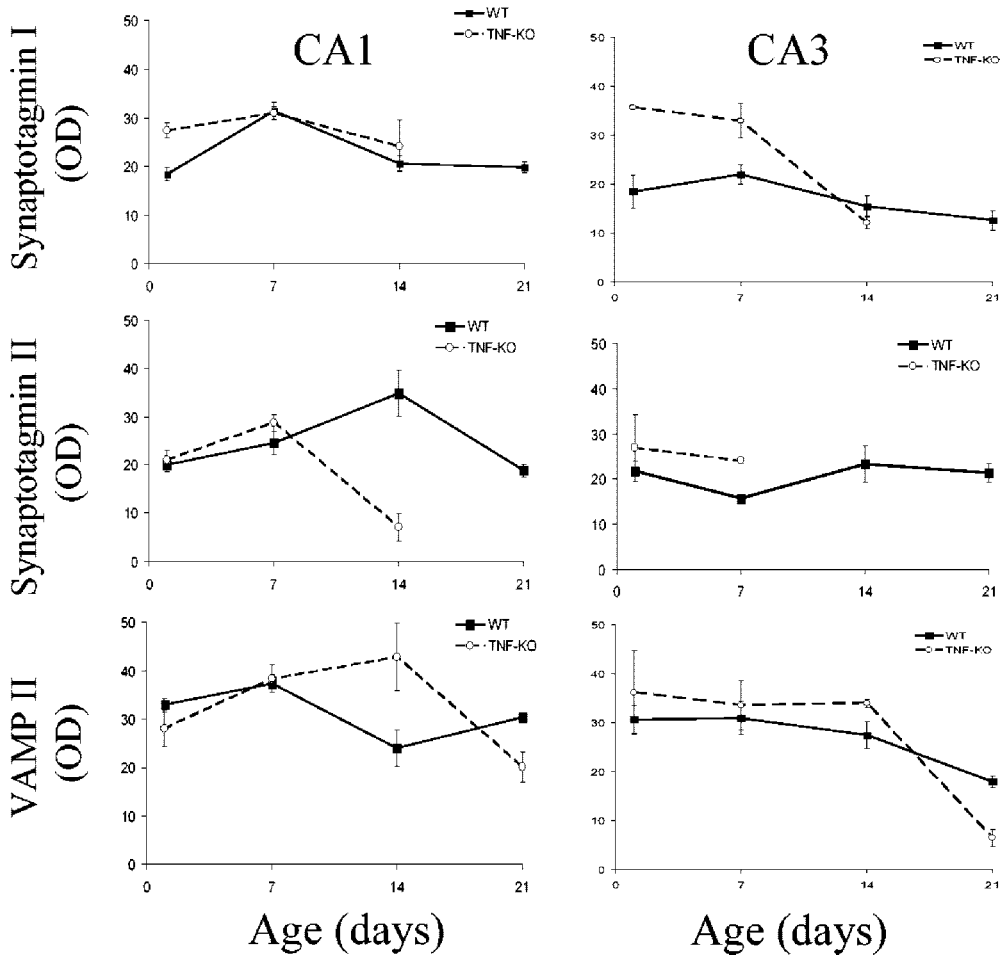

Fig. 3. Developmental profile of vesicular proteins expression in SR and SO of CA1-2 and CA3 regions in the hippocampus of TNFa-KO and wt newborn mice. **Left:** Quantification of IHC OD in the dendritic areas: SR and SO, of CA1-2 region. Synt I, $P = 0.02$, F = 5.5 (KO). Synt II, $P = 0.001$, F = 11.04 (KO). **Right:** Quantification of synaptic vesicle proteins in the dendritic fields of CA3 region. Synt I, $P = 0.021$, F = 6.5 (KO). Synt II, $P = 0.045$, F = 5.12 (KO). VAMP II, $P = 0.058$, F = 2.8 (KO × age). Synt I, n(wt) = 10–24, n(KO) = 4–12, Synt II, n(wt) = 12–24, n(KO) = 6–18, VAMP II, n(wt) = 6–24, n(KO) = 6–18.

were observed at P14. The correlation between the levels of these proteins was evaluated for each examined sample. Figure 2 depicts a high correlation (0.65, $P = 0.001$, Pearson correlation) between the levels of Synt I and Synt II in the SR and SO regions in wt mice. However, TNFa-KO mice show no correlation in the levels of these proteins in the same regions. In contrast, the CA1-2 pyramidal layer in wt mice shows no correlation in the level of these proteins, whereas, a trend for a high correlation 0.95 in this region was observed in the TNFa-KO mice ($P = 0.07$, Pearson correlation). In region CA3, all the examined vesicular proteins (Synt I, Synt II, and VAMP II) were expressed at a higher intensity on the P1 and P7 days in TNFa-KO mice, as compared to wt mice. This difference was absent for Synt I at P14 and for VAMP II at P21 (Fig. 3). The effect of a TNFa deficiency on the expression of Synt I was larger ($P = 0.02$, F = 6.5, n = 5–19, ANOVA) than its effect on VAMP II, which was near significance ($P = 0.058$, F = 2.8, n = 10–26, ANOVA). The expression of these proteins in SP in the CA3 region was similar for wt and TNFa-deficient mice (data not shown). Thus, increased protein levels in the TNFa-KO mice were directly reflected in the synaptic regions and not at their site of synthesis.

TNFa deprivation during the embryonic period and first postnatal month of development affected the levels and distribution of Synt I, Synt II, and VAMP II in the different sub-fields of the hippocampus. A distinct effect was observed for the different v-SNAREs examined. Nevertheless, there was no general trend of change in v-SNARE levels in the TNFa-KO mice. Reduction in Synt II levels in the CA1-2 region and an increase in Synt I and VAMP II in the CA3 region were observed. The present results suggest another role for TNFa in hippocampal morphological maturation, in addition to the differences in granular cell migration and pyramidal cell arborization, shown by us previously (Golan et al., 2004). The high correlation observed in the wt mice in the major input region of CA1

sub-fields is in accordance with a previous report that showed a high co-localization of Synt I and II in synaptic vesicles (Osborne et al., 1999). Nevertheless, in the TNFa-KO mice, there was no correlation in the levels of Synt I and II in the SR + SO regions. The biological meaning of this ratio is yet unclear. However, it may contribute to the kinetics and efficacy of neurotransmitter release and its viability for modulation. Disturbance of the equilibrium in the levels of different v-SNAREs may also explain the suppressive effect of TNFa on long-term potentiation of synaptic potentials, as shown previously (Cunningham et al., 1996; Tancredi et al., 1992). Recently, TNFa was shown to inhibit neurite outgrowth in cultured neurons; this effect was dependent on RhoA activation (Neumann et al., 2002). Neurite outgrowth also depends on Synt I/II as shown by Fukuda and Mikoshiba (2000). Thus, it is possible that the activation of RhoA by TNFa and the balance between Synt I/II are related. Although it is not a direct piece of evidence, it is possible that the modulation of Synt I and/or VAMP II expression by TNFa during development is mediated by RhoA activation.

In conclusion, the differences in the expression of Synt I and VAMP II and the difference in the relative proportions of these proteins in the hippocampus of TNFa-KO mice may suggest a possible mechanism for TNFa effect on synaptic plasticity.

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REFERENCES

Aloe L, Properzi F, Probert L, Akassoglou K, Kassiotis G, Micera A, Fiore L. 1999. Learning abilities, NGF and BDNF brain levels in two lines of TNFalpha transgenic mice, one characterized by neurological disorders, the other phenotypically normal. Brain Res 840:125–137.

Beattie E, Stellwagen D, Morishita W, Bresnahan J, Ha B, Von Zastrtrow M, Beattie M, Malenka R. 2002. Control of synaptic strength by glial TNFa. Science 295:2282–2285.

Butler MP, O'Connor JJ, Moynagh PN. 2004. Dissection of tumor-necrosis factor-a inhibition of long-term potentiation (LTP) reveals A p38 mitogen-activated protein kinase-dependent mechanism which maps to early-but not late-phase LTP. Neuroscience 124: 319–326.

Cunningham AJ, Murray CA, O'Neill LAJ, Lynch MA, O'Connor JJ. 1996. IL-1beta and TNF inhibit LTP in the rat dentate gyrus in vitro. Neurosci Lett 203:17–20.

Fukuda M. 2002. Vesicle-associated membrane protein-2/synaptobrevin binding to synaptotagmin I promotes o-glycosylation of synaptotagmin I. J Biol Chem 277:30351–30358.

Fukuda M, Mikoshiba K. 2000. Expression of synaptotagmin I or II promotes neurite outgrowth in PC12 cells. Neurosci Lett 295:33–36.

Fernandez-Chacon R, Konigstorfer A, Gerber SH, Gracia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmind C, Sudhof TC. 2001. Synaptotagmin I function as a calcium regulator of release probability. Nature 410:41–49.

Golan H, Levav T, Mendelsohn A, Huleihel M. 2004. TNF alpha involvement in hippocampus development and function. Cereb Cortex 14:97–105.

Jahn R, Sudhof TC. 1999. Membrane fusion and excocytosis. Ann Rev Biochem 68:863–911.

Neumann H, Schweigreiter R, Yamashita T, Rosenkranz K, Wekerle H, Barde Y. 2002. Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by Rho-dependent mechanism. J Neurosci 22:854–862.

Osborne SJ, Herreros J, Bastiaens PIH, Schiavo G. 1999. Calcium-dependent oligomerization of synaptotagmin I and II. J Biol Chem 274:59–66.

Pasparakis M, Alexopoulous L, Episkopou V, Kollias G. 1996. Immune and inflammatory responses in TNFa-deficient mice: a critical requirement for TNFa in the formation of primary B cells follicles, follicular dendritic cell networks and germinal cancers, and in the maturation of the humoral immune response. J Exp Med 184:1397–1411.

Tancredi V, D'Arcangelo G, Grassi F, Tarroni P, Palmieri G, Santoni A, Eusebi F. 1992. Tumor necrosis factor alters synaptic transmission in rat hippocampal slice. Neurosci Lett 146:176–178.