Neuronal NT-3 Is not Required For Synaptic Transmission or Long-Term Potentiation in Area CA1 of the Adult Rat Hippocampus

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Abstract

Neurotrophic factors, including BDNF and NT-3, have been implicated in the regulation of synaptic transmission and plasticity. Previous attempts to analyze synaptic transmission and plasticity in mice lacking the NT-3 gene have been hampered by the early death of the NT-3 homozygous knockout animals. We have bypassed this problem by examining synaptic transmission in mice in which the NT-3 gene is deleted in neurons later in development, by crossing animals expressing the CRE recombinase driven by the synapsin I promoter to animals in which the NT-3 gene is floxed. We conducted blind field potential recordings at the Schaffer collateral–CA1 synapse in hippocampal slices from homozygous knockout and wild-type mice. We examined the following indices of synaptic transmission: (1) input-output relationship; (2) paired-pulse facilitation; (3) post-tetanic potentiation; and (4) long-term potentiation: induced by two different protocols: (a) two trains of 100-Hz stimulation and (b) theta burst stimulation. We found no difference between the knockout and wild-type mice in any of the above measurements. These results suggest that neuronal NT-3 does not play an essential role in normal synaptic transmission and some forms of plasticity in the mouse hippocampus.

Introduction

Neurotrophic factors, including BDNF and NT-3, have been implicated in the regulation of synaptic transmission and plasticity (for a recent review, see Schuman 1999). For example, BDNF and NT-3 have been found to enhance synaptic transmission at the neuromuscular junction (Lohof et al. 1993) and in area CA1 of the hippocampus (Kang and Schuman 1995). In addition, the induction of long-term potentiation (LTP) leads to increases in both BDNF and NT-3 mRNA levels in area CA1 of the hippocampus (Patterson et al. 1992). Mice with a targeted disruption of the BDNF gene show diminished LTP in the hippocampus (Korte et al. 1995, 1996; Patterson 1996). In addition, application of TrkB-IgG fusion proteins can interfere with both early (Figurov et al. 1996; Kang et al. 1997) and late (Kang et al. 1997) LTP in the hippocampus and visual cortex (Akaneya et al. 1997). Unfortunately, similar studies using genetic or pharmacological disruptions of NT-3 function have not been reported owing to mice mortality and lack of reagent availability, respectively.

Although most of the homozygous NT-3 knockout mice die within a few days of birth (Ernfors et al. 1994), the heterozygous animals live throughout adulthood, permitting an analysis of synaptic transmission. At the lateral perforant path–granule cell synapse in the dentate gyrus, slices from heterozygous (+/−) knockout animals exhibited normal LTP, but diminished paired-pulse facilitation and post-tetanic potentiation (Kokaia et al. 1998). As of yet, synaptic transmission and plasticity in area CA1 of the hippocampus has not been examined in these mice.

NT-3 is expressed in both neurons and glia
(Tessarollo et al. 1993). Studies using pharmacological manipulations to examine the contribution of a particular molecule to plasticity are often compromised by an inability to address the source or site of action of the molecule of interest. A similar concern exists for genetic deletion experiments in which a gene is deleted in all cell types. By engineering a transgenic mouse in which the NT-3 gene is selectively deleted in neurons, the present study allowed us to address the contribution of neuronal NT-3 to both synaptic transmission and plasticity in the hippocampus.

Materials and Methods

PCR FOR NT-3-LOXP DELETION

PCR primer P1 5’-TTACCTGCTCATGAAGAAC-GCTTGGTGCAGC-3’ and primer P3 5’-GCATGGTTTCTGGCAGTCATAGATGCTTCC-3’ were used to detect the deletion of NT-3-loxp allele. Before deletion, these two primers were 5 kb apart and would not generate a visible band with the following cycling conditions. After deletion, these two primers would be only 200 bp apart and would give a 200-bp PCR band in 1.5% agarose gel. PCR cycling conditions were 94°C, 30 sec; 65°C, 30 sec; 72°C, 30 sec for 32 cycles.

GENERATION OF NT-3 CONDITIONAL KNOCKOUT MICE

Targeting vector construction, embryonic stem (ES) cell experiments, and chimeric mice will be described in detail elsewhere (L. Ma, J. Marth, and L.F. Parada, in prep.). After germ-line transmission of the NT-3-loxp allele was obtained, these mice were mated with different Cre-expressing mouse lines. In the present case, a transgenic mouse line expressing Cre recombinase under the control of the Synapsin I promoter (SynI-Cre) provided by Dr. J. Marth (University of California, San Diego), was used to generate a conditional knockout of NT-3 specifically in the central nervous system. lacZ reporter mice (Tsien et al. 1996) were used to detect the deletion of NT-3-loxp allele. Before deletion, these two primers were 5 kb apart and would not generate a visible band with the following cycling conditions. After deletion, these two primers would be only 200 bp apart and would give a 200-bp PCR band in 1.5% agarose gel. PCR cycling conditions were 94°C, 30 sec; 65°C, 30 sec; 72°C, 30 sec for 32 cycles.

SOUTHERN BLOT HYBRIDIZATION

Mouse brain genomic DNA (10–30 µg) from 5-week-old animals was digested with EcoRI and resolved on 0.8% agarose gel, blotted onto nylon membranes (Amersham), and hybridization was performed using either 2.2-kb 5’ or 1.4-kb 3’ external probes as detailed in Figure 1a. The filters were then washed once with 1× SSC, 0.1% SDS at 65°C for 1 hr, then once with 0.5× SSC, 0.1% SDS at 65°C for 30 min (Sambrook et al. 1989).

NORTHERN BLOT HYBRIDIZATION

Mouse brain total RNAs from 5-week-old animals were prepared using Trizol (GIBCO-BRL) and separated on 2.2 m formaldehyde and 1% agarose gels. Northern blot was performed on Nylon membranes. An NT-3 cDNA fragment (0.8 kb) was used as probe for hybridization. Washing conditions were as described for Southern blot hybridization (above). After exposure for NT-3 mRNA signals, the membrane was stripped and rehybridized with GAPDH probe (Sambrook et al. 1989).

PHOSPHORIMAGER QUANTIFICATION

Either Southern or Northern membranes were exposed to PhosphorImager screens and the signal intensity of each band was measured as described by the manufacturer (Bio-Rad). Percentage of NT-3-loxp (cko−) deletion or NT-3 mRNA reduction was calculated based on the relative signal intensities.

X-GAL STAINING OF BRAIN SECTIONS

PO mouse pups were perfused with 4% paraformaldehyde. Whole brains were embedded in chicken albumin and sectioned as 100-mm coronal sections with a vibratome. The brain sections were stained in 1× PBS, 2 mM MgCl₂, 50 mM potassium ferri/ferrocyanide, 1 mg/ml X-gal at 25°C for 48 hr.

ELECTROPHYSIOLOGICAL RECORDING AND ANALYSIS

Hippocampal slices (400 µm) were prepared from young adult (31- to 45-day-old) male mice us-
ing a vibratome. Immediately after dissection, individual slices were placed in a well of a 24-well plate containing oxygenated artificial cerebral spinal fluid (ACSF). The plate was stored in an enclosed chamber saturated with 95% O₂ and 5% CO₂. The ACSF was then replaced by 250 µl of oxygenated slice medium consisting of ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 2.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, 20.0 mM glucose) supplemented with B-27 (GIBCO). Slices were incubated for at least 2 hr before recording. For electrophysiological recordings, slices were submerged in a stream of ACSF maintained at 29 ± 1°C and gassed with 95% O₂ and 5% CO₂. Field excitatory postsynaptic potentials/currents (EPSPs) measured in stratum radiatum were evoked by stimulation of the Schaffer collateral–commissural afferents at a rate of 0.033 Hz using bipolar stimulating electrodes. To be included for further analysis baseline field EPSPs of slope −0.2 to −0.3 mV/msec had to be elicited with stimulation currents ≤50µA and have fiber volley amplitudes ≤0.15 mV. Extracellular recording electrodes were filled with 3M NaCl. The initial slope of the EPSP was measured for all experiments. Input–output (I/O) relations were monitored by measuring the EPSP slope in response to increasing stimulation currents. Paired-pulse facilitation was measured at four different intervals: 10, 25, 50, and 100 msec. Tetanic stimulation was delivered at the test intensity in 1-sec trains at 100 Hz, with two trains delivered 15 sec apart. Theta-burst stimulation (TBS) consisted of one episode of the following: 10 bursts of stimuli, each of four pulses at 100 Hz; interburst interval, 200 msec. Paired or unpaired t-tests were used to calculate the statistical significance of within group or between group comparisons, respectively. A one-way analysis of variance was used to calculate the statistical significance of potential differences between the I/O curves. P values greater than 0.05 were considered not significant (N.S.). All of the electrophysiology and analysis were conducted with the experimenter blind to the genotype of the animal.

**Results**

**GENERATION OF NT-3 CONDITIONAL KNOCKOUT MICE**

The NT-3 conditional knockout mice were generated as illustrated in Figure 1 and will be de-

Figure 1: Strategy for generating NT-3 conditional knockout mice. (a) Genomic structure of mouse NT-3 gene. (b) Homologous recombinant allele. Targeting vector was transfected into CJ7 ES cells and the details of selection were as described previously (Tessarollo et al. 1994). Homologous recombination was screened with Southern blot using both 5′ and 3′ probes. (c) Generation of cko⁺ allele. Recombinant ES clones were transfected with a CMV–Cre-expressing plasmid to delete the TK-neo selection cassette (Kuhn et al. 1995; Ramirez-Solis et al. 1995). Southern blot was carried out to screen for NT-3 allele (NT-3–loxp allele) flanked by two loxp sites. ES clones with NT-3–loxp were injected into blastocysts for germ-line transfer. (d) Generation of cko⁻ allele. Mice with NT-3–loxp allele were then crossed with Synapsin 1–Cre (Syn–Cre) (Schoch et al. 1996) transgenic mice to produce NT-3 conditional knockout mice with cko⁻ allele. From a–d, restriction sites are (B) BamHI, (E) EcoRI.
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scribed in greater detail elsewhere (L. Ma, J. Marth, and L.F. Parada, in prep.) The design of the recombination cassette provided for the single NT-3 coding exon, as well as the TK-neo selection cassette, to be flanked by loxP sites (Fig. 1b). Removal of the TK-neo cassette was achieved by transient transfection of the homologous recombinant ES cells with a Cre-expressing vector pMC-Cre followed by infection of the homologous recombinant ES cells to be flanked by lacZ-containing reporter gene expression in other tissues (i.e., heart and kidney).

Figure 2a,c, we estimate that as much as 60% of the cko− may reflect leakiness of the tissue preparations with sympathetic or parasympathetic neurons (Fig. 2a). Figure 2a indicates that dramatic reduction of NT-3 mRNA compared to cko+ (Fig. 2c). Finally, a reporter mouse strain designed to activate the β-galactosidase gene when Cre is expressed (Tsien et al. 1996) was crossed with SynI-Cre mice to analyze the expression pattern of Cre in the brain. As shown in Figure 2f and g, lacZ reporter gene expression was observed in cortex, hippocampus, and diencephalon. Although neurons appear to be stained uniformly, it is also likely that some non-neural cells have also ablated the NT-3 gene in these mice.

SYNAPTIC TRANSMISSION AND PLASTICITY ARE NORMAL IN THE NT-3 CONDITIONAL KNOCKOUT MICE

To test the role of neuronal NT-3 in synaptic transmission and plasticity, we examined several indices of synaptic transmission in the conditional knockout (SynI-Cre/cko−) and control mice (cko+/cko+) at the Schaffer collateral-CA1 synapse in the hippocampus. Basal synaptic transmission in slices from the conditional knockout mice was indistinguishable from that in the control mice (Fig. 3). This was monitored by measuring the slope of the fEPSP (field EPSP) in response to electrical stimulation of varying current amplitudes. As shown in Figure 3, the mean responses obtained from conditional knockout slices were not significantly different from those obtained from control slices (N.S.).

In addition, two measures of short-term synaptic plasticity, paired-pulse facilitation (PPF) and post-tetanic potentiation (PTP), were also examined. PPF, observed at many different synapses, involves an augmentation of the synaptic response to the second of two closely spaced stimuli. It is likely mediated by residual intraterminal Ca2+ at the time of the second stimulus (e.g., Katz and Miledi 1968). The PPF observed in slices from the NT-3 knockout animals did not differ significantly from the controls at any of the interstimulus intervals (ISI) examined (Fig. 4) [PPF, mean percent of first response at 10 msec ISI: knockout, 142.5 ± 4.2% (n = 5); control, 142.2 ± 10.1% (n = 5); (P = N.S.); 25 msec ISI: knockout, 144.9 ± 4.7% (n = 8); control, 149.9 ± 9.1% (n = 8); (P = N.S.); 50 msec ISI knockout, 151.7 ± 4.2%
(n = 20); control, 142.6 ± 2.5% (n = 21; P = N.S.); 100 msec ISI knockout, 141.7 ± 2.4% (n = 20); control, 137.2 ± 1.7% (n = 21; P = N.S.). In addition, analysis of the synaptic response immediately after 100 Hz or TBS (PTP) revealed no significant difference between slices from the knockout and control animals [mean percent of baseline after 100 Hz: knockout 255.2 ± 15.3% (n = 11); control, 275.8 ± 15.6% (n = 10) (P = N.S.); following theta burst: knockout, 211.1 ± 18.1% (n = 9); control, 241.9 ± 24.9% (n = 11) (P = N.S.)]. We then addressed whether long-lasting plasticity was intact in slices from the neuronal NT-3 knockout mice. We found that two trains of 100 Hz stimulation (1 sec each) produced LTP in the knockout slices that was indistinguishable from that seen in the control slices (Fig. 5) mean percent of baseline 50–60 min after tetanus: knockout, 159.4 ± 8.2% (n = 11); control, 169.1 ± 7.3% (n = 10) (P = N.S.). A recent study of the BDNF/TrkB dependence of LTP dem-
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Figure 3: Input–output relations are normal in hippocampal slices from conditional neuronal NT-3 mutant mice. Shown are the mean fEPSP responses to stimuli of increasing strengths for both the knockout (SynI–cre/cKO) and control (cKO+/cKO) slices. There was no significant difference between the two groups. (n) Number of slices. The number of animals from which the slices were taken was eight and eight, for the knockout and controls, respectively.

Figure 4: Paired-pulse facilitation is normal in hippocampal slices from conditional neuronal NT-3 mutant mice. Shown is the mean for three different interstimulus intervals. There was no significant difference between the knockout (solid bars) and control (open bars) slices at any of the intervals tested. The number of slices used for each interval is as follows (knockout/control): 10 msec (5,5); 25 msec (8,8); 50 msec (20,21); 100 msec (20,21). The number of animals from which the slices were taken was 8 and 8 for the 50 and 100 msec ISI, 3 and 3 for the 25 msec ISI, and 2 and 2 for 10 msec ISI for the knockout and controls, respectively.

Demonstrated that the stimulation parameters used to induce LTP are an important determinant of whether TrkB is involved (Kang et al. 1997). LTP induced by 100 Hz stimulation was not blocked by function-blocking TrkB antibodies, whereas LTP induced by TBS was inhibited (Kang et al. 1997). As such, we also tested whether LTP induced by TBS (see Methods) was sensitive to neuronal NT-3 deletion. We found, however, that TBS-induced LTP was also normal in the NT-3 knockout slices [mean percent of baseline 50–60 min after TBS: knockout, 147.0 ± 10.2% (n = 9); control, 142.1 ± 9.8% (n = 11) (P = N.S.)]. Taken together, these results indicate that neuronal NT-3 is not required for synaptic transmission or short- or long-term plasticity at the Schaffer collateral–CA1 synapse in the mouse hippocampus.

Discussion

Some of our SynI–Cre/cKO colony have surpassed a year of age with no apparent deleterious phenotypes (behavioral or pathological) resulting from ablation in most neurons and in a proportion of additional neural cells. We have also maintained NT-3 compound heterozygotes for the conditional and null alleles in the context of the Syn–Cre transgene. These mice have a complete somatic ablation of one NT-3 allele in addition to the ablation in all neurons and some glial cells of the second cko− allele. We have yet to observe any overt pathological or behavioral phenotype in these mice (L. Ma and L. Parada, pers. comm.). Recently, Bates et al. (1999) reported the generation of a similar conditional NT-3 mutant mouse. Through use of a nestin promoter to drive Cre expression, NT-3 conditional deletion in the central nervous system and additional sites was reported. Unlike the Syn–Cre/cKO− described here, Bates and colleagues observed gait and behavioral abnormalities that they associated with cerebellar malfunction. We have not assessed directly the cerebellar morphology of our Syn–Cre/cKO− mice to determine the extent of neural and non-neural ablation in this tissue.

The lack of effect of neuronal NT-3 deletion on synaptic transmission or plasticity differs from recent observations in NT-3+/− mice, in which a decrease in paired-pulse facilitation was observed in the dentate gyrus (Kokaia et al. 1998). These NT-3+/− mice show ~30% reduction in NT-3 mRNA
levels in the dentate granule neurons (Elmer et al. 1997), but NT-3 levels in area CA1 have not been reported. Taken together these results suggest, however, that neuronal NT-3 plays different roles at Schaffer collateral–CA1 synapses versus dentate granule synapses. This is further substantiated by the observation that LTP induction results in up-regulation of NT-3 in CA1 neurons (Patterson et al. 1992), but results in NT-3 down-regulation in dentate granule cells (Castren et al. 1993).

Our earlier studies demonstrated that application of either BDNF or NT-3 to hippocampal slices is sufficient to enhance synaptic transmission (Kang and Schuman 1995, 1996). To what extent does the neurotrophin-induced synaptic enhancement involve cellular mechanisms that overlap with LTP? Early experiments (Kang and Schuman 1995) examined the extent to which previous potentiation by either BDNF or NT-3 influenced the subsequent ability to obtain LTP at the same synapses. These experiments showed that LTP (induced by several trains of 100-Hz stimulation) could still be elicited at synapses previously potentiated by neurotrophin. This implied that neurotrophin-induced potentiation and LTP used at least partially independent mechanisms. A later study (Kang et al. 1997) revealed, however, that the degree to which BDNF/TrkB-dependent potentiation

Figure 5: LTP is normal in hippocampal slices from conditional neuronal NT-3 mutant mice. (a) Ensemble averages for slices exposed to two epochs of 100 Hz stimulation (tetanus) for 1 sec each. Both the control and the mutant mice exhibited LTP, which was of a similar magnitude. In the mutant mice the mean fEPSP slope was $-0.25 \pm 0.02$ mV/msec and $-0.38 \pm 0.02$ mV/msec, before and after LTP induction by 100 Hz. These slices exhibited significant potentiation ($P < 0.001$). In the control mice the mean fEPSP slope was $-0.24 \pm 0.01$ mV/msec and $-0.41 \pm 0.02$ mV/msec, before and after LTP induction by 100 Hz. These slices also exhibited significant potentiation ($P < 0.001$). (n) Number of slices. The number of animals from which the slices were obtained was four and four for knockout and control mice, respectively. (b) Ensemble averages for slices exposed to TBS. Both the control and the mutant mice exhibited LTP, which was of a similar magnitude. In the mutant mice the mean fEPSP slope was $-0.25 \pm 0.02$ mV/msec and $-0.36 \pm 0.03$ mV/msec, before and after LTP induction by TBS. These slices exhibited significant potentiation ($P < 0.001$). In the control mice the mean fEPSP slope was $-0.23 \pm 0.02$ mV/msec and $-0.32 \pm 0.03$ mV/msec, before and after LTP induction by TBS. These slices also exhibited significant potentiation ($P < 0.001$). Bar, 0.5 mV/100 msec. (n) Number of slices. The number of animals from which the slices were obtained was four and four for knockout and control mice, respectively.
overlaps with LTP depends on the particular stimulation protocols used to induce LTP. That is, LTP induced by 100-Hz stimulation was found to be independent of TrkB function, whereas LTP induced by more "subtle" stimulation protocols such as TBS or pairing required TrkB function (Kang et al. 1997). It was with this subtlety in mind that we tested both 100-Hz and TBS-induced LTP in the NT-3 neuronal knockout. We found, however, that LTP was intact in both cases, indicating that neuronal NT-3 is not required for these forms of LTP.

Taken together with our earlier work (Kang and Schuman 1995), these data imply that NT-3 is sufficient, but not necessary, for synaptic potentiation in area CA1 of the hippocampus. One caveat concerns the species differences in these two sets of studies: NT-3-induced potentiation has been observed in rat hippocampus, but has not yet been reported in mouse hippocampus. An additional potentially noteworthy feature of our previous work with neurotrophins in hippocampal slices is the degree to which the potentiation elicited by either BDNF or NT-3 appeared to use similar cellular mechanisms. For example, both neurotrophins decreased PPF (Kang and Schuman 1995), required receptor tyrosine kinase activity (Kang and Schuman 1995a) and protein synthesis (Kang and Schuman 1996) for potentiation, and did not require NMDA receptor function for potentiation (H. Kang and E.M. Schuman, unpubl.). Although the synaptic enhancement elicited by BDNF is prevented completely by TrkB receptor blockade (Kang et al. 1997), preliminary experiments indicated that NT-3-induced potentiation is prevented only partially by blockade of TrkC function (H. Kang and E.M. Schuman, unpubl.). This raises the possibility that some of the potentiation produced by the application of exogenous NT-3 may be mediated by interactions with TrkB. If so, this could explain why the absence of neuronal NT-3 did not affect LTP in the present study, as other TrkB ligands, such as BDNF, could compensate.

The hippocampus is a prominent site of NT-3 expression (Ernfors et al. 1990). Our Northern blot analysis indicates that the conditional knockout resulted in ~75% reduction of the NT-3 mRNA in brain. Moreover, the lacZ expression pattern in the reporter mouse (Fig. 2f,g) indicates that the Cre recombinase was expressed robustly in the pyramidal cell layers of areas CA3 and CA1. These data, however, do not exclude the possibility that a non-neuronal source could provide the NT-3 required to sustain both synaptic transmission and plasticity at the Schaffer collateral–CA1 synapse. An analysis of mice in which NT-3 is deleted in glia will likely provide the answer to this question. In addition, given the abundance of neuronal NT-3 in the hippocampus, a more thorough analysis of the present conditional knockout mice may elucidate a previously unappreciated function for NT-3 in synaptic transmission.

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