Supplementary Methods

Constructs and Transfection

The GFPp65 construct was also put into a PUC backbone under control of the neuronal-
specific Thy-1 promoter (Thy-1 promoter was a kind gift from the laboratories of J.Sanes and
P.Caroni)\textsuperscript{51}. We chose the p65 NF-κB subunit for this construct because only p65 is capable
of upregulating its own inhibitor, IκBα. This might alleviate potential problems that could be
created if the fusion construct were over-expressed. A direct GFP-p65 fusion was chosen
because the sequence in linkers have sometimes been found to result in aberrant subcellular
localization. Tests of the GFPp65 construct in NIH 3T3 cells revealed that the GFP tag did
not interfere with normal cytoplasmic localization or nuclear translocation following TNF-α
stimulation.

Electrophoretic Mobility Shift Assay

EMSA and extract preparations were optimized using previous findings\textsuperscript{52}. Several protease
inhibitors were included in extract buffers: leupeptin, aprotinin, pepstatin (all 5 μg / ml),
para-aminobenzamidine (0.5 mM) and PMSF (1mM). Supershift analysis was performed by
incubating monoclonal antibodies (Santa Cruz) for specific Rel family members in a pre-
binding reaction and then looking for band shifts on EMSA. Protein levels were made
equivalent in all binding reactions. DNA probes containing the following wild-type and
mutant κB consensus sequence were labeled with α-\textsuperscript{32}P using a Klenow fill-in reaction:

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>GGG GAC TTT CC</th>
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<tr>
<td></td>
<td>CCC CTG AAA GG</td>
</tr>
<tr>
<td>Mutant</td>
<td>GTT GAC TTT CC</td>
</tr>
<tr>
<td></td>
<td>CAA CTG AAA GG</td>
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Permeability and efficacy of the CaMKII inhibitor, antCNt, have been verified by
phosphorylation assays measuring inhibition of CaMKII activity after extracellular
application to HEK293 cells (effective inhibition was observed in the low μM range, J.Tsui
and K.U.Bayer, personal communication) and antCNt also provided inhibition in cellular
assays measuring CaMKII effect on dendritic mobility\textsuperscript{19}. For experiments testing the effects
of constitutive CaMKII, T286D α-CaMKII (kindly provided by K.U. Bayer, Schulman
laboratory) was transfected into day 2 hippocampal cultures (Lipofectamine 2000,
Invitrogen), and crude nuclear extracts harvested for EMSA 72 hrs post-transfection.

Ribonuclease Protection Assay
Total RNA was prepared from neuronal cultures at selected time points following stimulation by immediate homogenization in Tri Reagent (Molecular Research Center) and subsequent RNA isolation. RPA probes were purchased (Pharmingen) and RPA performed according to manufacturers instructions (Riboquant, Pharmingen). No binding was seen to yeast tRNA used as a control for specificity. IκBα test probe was combined with probes for L32 and GAPDH housekeeping genes. In the data analysis, this allowed the expression levels of each sample (in each lane) to be normalized to the average of the two housekeeping genes. Percent induction over non-stimulated controls was then calculated using normalized values.

**Microscopy**
FRAP was used to examine the dynamics of NF-κB in GFPp65 transfected neurons (36). Neurons expressing relatively high levels of GFPp65 were found to contain both cytoplasmic and nuclear GFP fluorescence, presumably because high expression overwhelmed the endogenous capacity of binding proteins and/or IκB production. This could be largely avoided by either co-coating the microcarrier beads with both GFPp65 and IκBα constructs, or by titrating down the amount of GFPp65 DNA that was coated onto beads. We chose the latter method to avoid introducing possible artifacts. Expression in the appropriate range was judged by fluorescence intensity and the absence of visible nuclear fluorescence in unstimulated cells. In neurons with high GFPp65 expression, FRAP experiments could not be performed because GFP fluorescence diffused into the photobleached region even without stimulation.

**Reporter Assay**
Virus was produced in 293 cells co-transfected with helper plasmids and supernatant harvested and concentrated 48 hrs post-transfection. Neurons were co-infected using master mixes of reporter and LacZ virus for 24 hrs, incubated for 48 hrs or more, and stimulated in triplicate. Viral infection had no apparent toxicity for neuronal cultures and expression could be maintained for >3 weeks (the longest time examined) in healthy cultures as assessed using a GFP-containing version of the lentivirus.

**Behavioral Testing**
Although gross examination and immunostaining failed to distinguish brain tissue of TNFRp65−− and TNFRp65−+ mice, it remains possible that subtle differences in neuronal
architecture and/or connectivity exist. When maintained in a reduced pathogen environment these mice appeared to develop normally, survived for normal lifespans (>1 year), and did not exhibit abnormal behavior. Matings of both heterozygous and homozygous mice were successful in producing litters with genotypes at approximately expected Mendelian ratios.

Mice to be tested on the radial arm maze were transferred from Caltech to UCLA, allowed one week to habituate to the new surroundings, and then begun on a week-long gradual food deprivation period to achieve the target 85% of free-feeding weight. During food deprivation, mice received familiarization by feeding in the maze room and, on the last three days of this period, were each handled by the experimenter for 2 min. Mice were weighed daily and free-feeding time adjusted to maintain target weight. Experimenters were blind to the genotypes of mice tested. Equal numbers of p65 wildtype and deficient mice were randomly selected and evaluated for infection by nasopharyngeal and cecal cultures (diagnostics performed by RADIL) at the conclusion of the experiments. There were no positive cultures.

The spatial version of the maze task consisted of two trials per day. The first trial of each day was a sample trial in which the doors to four of the eight maze arms were open and these arms were baited with food pellets. The second trial of the day was the test trial where the doors to all arms were open and the four arms blocked in the sample trial were now baited with food pellets. On both trials, the mouse was placed in the center platform and allowed to explore freely until either all reward pellets were eaten or 5 min elapsed. During the 5 min intertrial interval, mice were placed in a holding room while the maze was cleansed to remove olfactory cues. Mice were tested for 20 days and performance was scored for both within-trial errors (re-entry into an arm previously entered on the same test trial) and between-trial errors (entry on the test trial to an arm previously baited on that day's sample trial). Mice were also scored for the percent of correct (baited) arms entered in the first four arm choices of the test trial; there are a total of four baited arms in this trial. Both the order of individual mouse testing and the maze arms blocked on the sample varied and were chosen randomly prior to the task.

The cued version of the maze task consisted of two identical trials per day. In each trial the doors to all arms were open and four randomly selected arms were lit and baited with food pellets. On both trials, the mouse was placed in the center platform and allowed to explore freely until either all reward pellets were eaten or 5 min. elapsed. Each
time a mouse correctly entered a lit arm and consumed the food reward, the arm light was promptly extinguished. During the 5 min intertrial interval, mice were placed in a holding room while the maze was cleansed to remove olfactory cues. Mice were tested for 24 days and performance was scored for the percent of correct (lit and baited) arms entered in the first four arm choices of the second trial of each day; there are a total of four lit and baited arms in this trial. Following this testing period, a probe trial was conducted in which each mouse was given 8 consecutive 2 min trials in which only one arm was lit and baited; the last four of these trials were scored for percent correct (Fig 4d). Both the order of individual mouse testing and the maze arms lit and baited varied and were chosen randomly prior to the task.

In the spatial version of the RAM, attention to extramaze cues is required to avoid both within trial and between trial errors. While p65-deficient mice achieved a within trial error performance equivalent to p65-wildtype mice by day 9-10, they could not consistently achieve equivalent performance of between trial errors until day 17 – 18 (Fig. 4a,b). This discrepancy is much greater than that seen for p65-wildtype mice (where the two tasks are mastered within 2 days of each other) and suggests that a learning deficit is more likely to account for the difference than a deficit in attending to extramaze cues.

Nuclear translocation of NF-κB

Our FRAP experiments do not specifically exclude either diffusion or directed movement as the mechanism of p65 translocation, although the finding of polarized movement does suggest directed transport. Rates of p65 movement calculated from FRAP experiments (e.g. between 5 - 10 min Fig.2c) are not wholly consistent with diffusional (proportional to t1/2) or constant velocity (proportional to t) movement. Several explanations might account for this finding. FRAP could depend upon multiple variable time constants, such as the pathway leading to the asynchrononous phosphorylation and degradation of IκB. Alternatively, the movement of individual molecules might occur not constantly, but intermittently, such as if NF-κB were to bind on and off a transport protein, for example. It is also of interest that molecules of p65 which reach the nucleus from distal processes following stimulation must escape re-binding of IκB en-route. It is possible that the IκB-binding site of NF-κB is concealed during translocation, perhaps by binding of the NLS to molecular transporters. Mutation of the NLS of p65 does appear to interfere with nuclear translocation in neurons83.
Supplementary References

