

NF- κ B controls functional circuit formation and tissue homeostasis in the adult dentate gyrus

Yvonne Imielski¹, Jens C. Schwamborn², Patrick Lüningschrör³, Peter Heimann³, Magdalena Holzberg¹, Hendrikje Werner^{4*}, Oliver Leske⁵, Andreas W. Püschel⁴, Sylvie Memet⁶, Rolf Heumann⁵, Alain Israel⁷, Christian Kaltschmidt³ and Barbara Kaltschmidt¹

¹Molecular Neurobiology, Faculty of Biology, University of Bielefeld, D-33602 Bielefeld, Germany

²AG Stammzellbiologie und Regeneration, Institut für Zellbiologie, ZMBE, D-48149 Münster, Germany

³Institut für Zellbiologie, Fakultät für Biologie, Universität Bielefeld, D-33602 Bielefeld, Germany

⁴Institut für Molekulare Zellbiologie, Westfälische Wilhelms-Universität Münster, Schlossplatz 5, D-48149 Münster, Germany

^{4*} Institute of Medical Biology, Immunos, 8A Biomedical Grove, Singapore 138648

⁵ Molekulare Neurobiochemie, Ruhr-Universität Bochum, D-44780 Bochum, Germany

⁶ Institut Pasteur, Unité de Mycologie Moléculaire, CNRS URA3012, Paris, France

⁷ Institut Pasteur, Unité de Signalisation Moléculaire et Activation Cellulaire, CNRS URA 2582, 75015 Paris, France

Summary

Cognitive decline during aging is correlated with a continuous loss of cells within the brain and especially within the hippocampus, which could be regenerated by adult neurogenesis. Here we show that genetic ablation of NF- κ B resulted in severe defects in the neurogenic region (dentate gyrus) of the hippocampus. Despite increased stem cell proliferation, axogenesis, synaptogenesis and neuroprotection were hampered, leading to disruption of the mossy fiber pathway and to atrophy of the dentate gyrus during aging. Here, NF- κ B controls the transcription of FOXO1 and PKA, regulating axogenesis. Structural defects culminated in behavioral impairments in pattern separation. Re-activation of NF- κ B resulted in integration of newborn neurons, finally to regeneration of the dentate gyrus, accompanied by a complete recovery of structural and behavioral defects. These data identify NF- κ B as a crucial regulator of dentate gyrus tissue homeostasis suggesting NF- κ B to be a therapeutic target for treating cognitive and mood disorders.

INTRODUCTION

The hippocampus is crucial for the formation of spatial or episodic memory (Squire et al., 2004). In a traditional view the hippocampus was seen as a static structure which is only modified during development. Recently, a new picture of hippocampal plasticity is emerging. Gross anatomical structural changes are induced not only during development but also in adulthood by physiological processes such as learning and pathological processes such as neurodegeneration. Pre-existing neuronal networks undergo modification in connectivity, that include changes in dendritic arborisation and more subtle changes in synaptic density. Completely novel

non pre-existing circuits are formed by the addition of adult newborn neurons due to neurogenesis. Thus, neurogenesis is a key-feature of the adult dentate gyrus, but the function of newborn neurons remains unclear (Deng et al., 2010). Recent studies suggest that the dentate gyrus (DG) subregion is involved in spatial pattern separation, a process of transforming similar representations or memories into highly dissimilar non-overlapping representations (Bakker et al., 2008; Leutgeb et al., 2007). Neurogenesis is continuing in two brain regions of adult rodent brain, the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal DG (Alvarez-Buylla et al., 2001; Doetsch et al., 2003; Gage F.H., 2000; McKay R., 1997; Temple, S., 2001; Ming and Song, 2005). While many studies have identified molecules and conditions that regulate neurogenesis, the transcriptional control of hippocampal neurogenesis during adulthood is less clear, see (Suh et al., 2009). Here, we investigated the role of NF- κ B in new circuit formation and structural plasticity.

NF- κ B is an inducible transcription factor important for immune function, cancer and anti-apoptotic cell protection (Hayden and Ghosh, 2008). NF- κ B is the generic name of dimeric combinations between 5 DNA-binding subunits: p50, p52, c-Rel, RelB, and p65 (RelA), the latter three having transactivation domains. NF- κ B comes in two flavours: a cytoplasmic non-DNA binding inducible form, which is a trimeric complex composed of two DNA-binding subunits and one inhibitory subunit of the I κ B family; an activated NF- κ B is a dimeric complex without the inhibitory subunit, it is transported into the nucleus, where it binds and activates the transcription of target genes. Activation takes place via a kinase cascade, which ends in the I κ B kinase complex (IKK; see (Karin, 2008) for review), which are specific kinases for the inhibitory I κ B subunits. Phosphorylation of I κ B- α by the IKK complex is a signal for ubiquitination by β -TrCP (for review see (Karin and Ben-Neriah, 2000). Ubiquitination

of κ B leads to its degradation by the proteasome, thus allowing nuclear translocation of NF- κ B dimers and activation of their target genes. Activation of NF- κ B is signal-driven e.g. by pro-inflammatory cytokines or pathogens.

Within the nervous system, NF- κ B is involved in neuroprotection/degeneration, synaptic plasticity (Kaltschmidt and Kaltschmidt, 2009; Mattson and Meffert, 2006) in neurite growth (Gavalda et al., 2009) and formation of functional dendritic spines (Boersma et al.). Recently, under pathological conditions such as acute immobilization stress NF- κ B could impair neurogenesis after IL-1 β -mediated activation (Koo et al., 2010). Whereas a lot of information on the role of NF- κ B in neuronal process growth is already available, one of the main questions that remained to be answered is: what is the physiological importance of the regulation of axonal growth by NF- κ B signaling for neuronal development in vivo (Gutierrez and Davies, 2011) This question will be addressed here in a special brain region, the dentate gyrus.

Previously, a genetic ablation of NF- κ B identified a neuronal target gene (Kaltschmidt et al., 2006), the catalytic subunit of protein kinase A (PKAc, Prkaca). Taken together some aspects of NF- κ B dependent signaling are well established (Kaltschmidt and Kaltschmidt, 2009), but its function in structural plasticity - that is reorganization of neural circuits (Leuner and Gould, 2009) by neurogenesis - still remains unclear.

Tissue homeostasis can be defined as the balance between cell death and neurogenesis (Henson and Hume, 2006), where stem cells can remodel relevant tissues in response to physiological change (Nakada et al., 2011). Here we provide evidence in support of the notion that NF- κ B regulates tissue homeostasis within the adult dentate gyrus. In the mouse model used here, NF- κ B fulfils a dual function: in neuronal progenitors NF- κ B is necessary for axogenesis and maturation, whereas in

mature granule cells NF- κ B regulates neuroprotection as well as synaptic transmission. Thus, inhibition of NF- κ B in both developmental stages leads to severe atrophy of the dentate gyrus in aging animals. These data might explain why the neuronal circuit is damaged upon inactivation of NF- κ B, because mossy fibers, the axons of granule cells, were degenerating and thus the dentate gyrus can not be regenerated by the addition of newborn neurons.

In this study the most fascinating finding is that re-activation of NF- κ B alone, can restore a functional dentate gyrus within the adult brain. Taken together our findings suggest that NF- κ B might have therapeutic potential for reversing dentate gyrus dysfunction as observed in Alzheimer's disease and mood disorders.

RESULTS

NF- κ B controls circuit formation of the mossy fiber pathway and replenishment of the dentate gyrus

In the mouse model used here, neuronal NF- κ B ablation can be established by CamKinasII promoter-driven tetracycline controlled transactivator (tTA) regulating the expression of Super-Repressor I κ B in mice (S1). A Super Repressor (SR) of NF- κ B was generated by mutating phosphorylation sites in I κ B- α . Transgene expression started in transient-amplifying neuronal progenitors (S2) that co-express the early neuronal marker doublecortin (DCX), so called type 2b cells (Kempermann et al., 2004). NF- κ B-dependent defects in structural plasticity were severe. Reduction was evident for granule cell axons, the mossy fibers (MF) connecting granule cells with pyramidal neurons of the CA3 region (Fig. 1a,b,c). The thickness of the dentate gyrus (DG) granular layer was reduced by about 50% (Fig. 1f). Pre-synaptic sites of MF were decreased in size and number (Fig. 1 d,e), suggesting an important role for

NF- κ B in regulating synapse density. Furthermore, we analyzed the morphology of mossy fiber synapses by electron microscopy. After NF- κ B ablation, size (by 24 %) and number of mossy fiber boutons (by 38 %) were reduced, as well as the number of synaptic contacts (by 31%; Fig. 1 g,h,i and S3). Structural NF- κ B dependent changes are summarized in Fig. 1 j,k. Thickness of the dentate gyrus was reduced by 50% in 6 months old mice (Fig. 1 a,b) and the mossy fiber projection was impaired, resulting in a reduced synapse density in the stratum lucidum.

We hypothesize that the observed structural changes detected here might be generated by a imbalance of cell death and neurogenesis.

NF- κ B deficient mice show increased apoptosis, neurogenesis and inflammation in the adult dentate gyrus

Degenerating neurons in the dentate gyrus were analysed by Fluoro-Jade staining (Fig. 2a,b). NF- κ B ablation resulted in an increase of degenerating neuronal nuclei (about 50%, $P = 0.0093$) and neurites (about 70%, $P = < 0.0001$, Fig. 2c,d), arguing for a defect in axonal outgrowth. The apoptotic marker cleaved Caspase-3 was increased about 3-fold (Fig. 2e, $P = 0.0003$). We conclude that the net granule cell number is reduced by apoptosis in I κ B/tTA mice. Based on previous findings showing that increased mitotic activity is observed after hippocampal damage and healthy mature neurons can inhibit neurogenesis (Hastings and Gould, 2003; Ming and Song, 2005) we next investigated changes in neurogenesis.

In controls, newborn neurons are located mainly in the subgranular zone (Fig. 2f), whereas I κ B/tTA mice contained misplaced neuronal progenitors (Fig. 2g). NF- κ B ablation led to about 30% increase of BrdU⁺ cells ($P = 0.0233$), which were positive for DCX (Fig. 2i) as well. DCX ($P = < 0.0001$) and calretinin positive cells ($P =$

0.0019) were increased in $\text{I}\kappa\text{B}/\text{tTA}$ mice (Fig. 2 h,j). Staining for Glial Fibrillary Acidic Protein (GFAP) in NF- κB ablated mice, revealed strongly increased numbers of activated astrocytes (Fig. 2 k,l), a hallmark of neuroinflammation. Within the neurogenic region of the dentate gyrus GFAP positive type 1 neural stem cells are localized in the subgranular zone. Here, GFAP positive cells were detected in an evenly spaced pattern near the dentate gyrus formation and showed a star-like morphology, suggesting that these cells are activated astrocytes.

The observed defects in mossy fiber projection can be explained by two hypotheses: Firstly, apoptosis of already connected mature granule cells or secondly, a defect in axonal specification in newborn neurons.

NF- κB regulates PKA and FOXO1 expression

To identify potential NF- κB target genes involved in these processes, we performed microarray analysis (Kaltschmidt et al., 2006) and examined the expression of two relevant NF- κB target genes, Foxo1 and the catalytic subunit of Protein kinase A (PKA) (HUGO gene name: Prkaca). While Foxo1 expression is restricted to the dentate gyrus in controls (Fig. 3A a,b), Foxo1 expression is no more detectable after NF- κB ablation (Fig. 3A c,d). Re-activation of NF- κB by doxycycline can transcriptionally switch on Foxo1 but extends the area of expression to CA3 and CA1 (Fig. 3A e,f). Interestingly, a recent report showed an involvement of Foxo1 in axonal outgrowth in hippocampal cultures and in the developing cerebellum (de la Torre-Ubieta et al., 2010). Furthermore, also PKA expression is regulated by NF- κB (Fig. 3B). PKA expression in controls was detected in all subfields of the hippocampus, whereas NF- κB ablation resulted in a complete loss of PKA in the dentate gyrus and in CA3. Re-activation of NF- κB by doxycycline resulted in expression of PKA similar

to controls. Since PKA plays a crucial role in axon fate determination (Shelly et al., 2007) and is a neuronal target gene of NF- κ B (Kaltschmidt et al., 2006) we have analysed PKA activity after NF- κ B ablation. Here we show that PKA activity is reduced by about 50% in the DG ($P = < 0.0001$) (Fig. 3C a,b,c). In extension, phosphorylation of the PKA substrate LKB1 at Ser 431 was reduced after NF- κ B ablation (Fig. 3Cd), correlating with a down-regulation of the catalytic subunit of PKA in this mouse model.

Cell autonomous role of NF- κ B in axogenesis

To analyse a potential cell autonomous function of NF- κ B, axogenesis in hippocampal cultures was studied. In accordance to the situation in vivo, I κ B expression inhibits axogenesis in vitro as well (Fig. 4a-d; h-i). The analysis of NF- κ B induced changes in morphology were examined by markers for neuronal polarity. Tau-1 immunoreactivity was used as an axonal marker, minor neurites (dendrites) were identified by anti-MAP2 labelling as described (Schwamborn and Puschel, 2004). Axon length was reduced in neurons transfected with super-repressor-I κ B (from $188 \mu\text{m} \pm 20 \mu\text{m}$ in the EGFP control to $87 \mu\text{m} \pm 10 \mu\text{m}$ after I κ B transfection) (Fig. 4 e). To a lesser extent shorter axons were observed in neurons transfected with the p50 subunit (Fig. 4 b, e). This might reflect the capability of p50 to act as a repressor, lacking its own transactivation domain. Thus super-repressor-I κ B expression resulted in neurons without Tau-positive axons (Fig. 4 d,i). Control neurons acquired a normal neuronal morphology (Fig. 4 a, g) and extended a single axon (1.1 ± 0.2) plus about 4 minor neurites. Transfection with NF- κ B DNA-binding subunits p50 and p65 did not induce a significant change in axon number (p50: 0.9 axons per cell ± 0.3 ; p65: 1.0 axons per cell ± 0.2), (Fig. 4 g). Camera lucida tracing

of neurons transfected with super-repressor-I κ B, revealed a clearly visible defect in axon formation (compare Fig. 4 h with 4 i). Taken together these data provide evidence, that NF- κ B controls axogenesis in vivo and in vitro. To study a role of the NF- κ B target gene PKA, we used again neuronal polarity assays in hippocampal cell cultures. Overexpression of PKAc in hippocampal neurons induced the formation of supernumerary axons (Fig. 4j and S4), consistent with the PKA-dependent phosphorylation of LKB1 (Shelly et al., 2007). Finally, co-expression of I κ B and PKAc led to a phenotype with multiple axons, arguing for a position of PKAc downstream of the NF- κ B pathway (Fig. 4 k).

NF- κ B dependent impairment of spatial pattern separation can fully be restored by NF- κ B reactivation

We hypothesized that the observed NF- κ B dependent structural plasticity in the DG affects behaviour. Therefore, we developed a novel challenging spatial task, based on the *Barnes Maze* (BM, (Barnes, 1979)), in which animals have to differentiate between locations with subtle differences (*Spatial-Pattern-Separation-BM: SPS-BM*). On the circular platform of *SPS-BM* mouse houses containing food were placed evenly, but only one house contained an accessible food reward (scheme in Fig. 5). Mice were analyzed in *SPS-BM* for seven days with one trial per day. I κ B/tTA mice needed considerably more time to reach the food house F (latency, $F_{1,6} = 40.94$, $P < 0.0001$), covered more distance ($F_{1,6} = 16.13$, $P = 0.0001$) and made more errors ($F_{1,6} = 7.244$, $P = 0.0085$) (Fig. 5, upper panel). Strikingly, after re-activation of NF- κ B by doxycycline treatment for four weeks, I κ B/tTA mice could learn the behavioural task as well as controls (latency, $F_{1,6} = 5.150$, $p = 0.0251$; errors $F_{1,6} = 1.784$, $p = 0.1841$; distance $F_{1,6} = 3.613$, $p = 0.0598$) (Fig. 5 lower panel) (see S7 movies). Taken

together these data argue for a functional recovery of the dentate gyrus by re-activation of NF- κ B signaling as measured by the SPS-BM. Furthermore we analyzed the cellular basis of this improvement in long-term memory. According to these results, *SPS-BM* seems to be a novel dentate gyrus-dependent behavioral task for spatial pattern separation.

To measure the general learning ability of $\text{I}\kappa\text{B}/\text{tTA}$ mice, we analyzed the mice in a classic BM task with visible external cues. One advantage of the *BM* is the possibility to analyze the animal's search strategies: random, serial and spatial (Barnes, 1979). Control mice learned the task by starting with random-, followed by a serial- and ending with spatial-search strategy (Fig. S7). In contrast, $\text{I}\kappa\text{B}/\text{tTA}$ mice used random and serial search strategies by chance, but never learned a spatial search strategy. Although the $\text{I}\kappa\text{B}/\text{tTA}$ mice with NF- κ B ablation were able to learn the classic *BM* and acquired spatial orientation (S6 a-c; latency $F_{1,7} = 1.585$, $P = 0.2111$, distance covered $F_{1,7} = 0.03702$, $P = 0.8478$ and errors made $F_{1,7} = 0.05903$, $P = 0.8086$), similar to controls. Taken together these data show that newborn neurons are required for spatial pattern separation and learning to navigate in a world with subtle environmental differences, processes where NF- κ B integrates structural changes with spatial pattern separation.

Reactivation of NF- κ B leads to regrowing of the dentate gyrus and recovery of structural defects

Recently several studies suggested, that addition of new cells by neurogenesis improved learning capacity in spatial pattern separation (Clelland et al., 2009). Therefore we tested in our mouse model, whether the behavioral benefit observed after reactivation of NF- κ B might have a cellular origin. Thus we studied the cellular

effects of NF- κ B reactivation in our mouse system where the expression of I κ B can be repeatedly turned on and off non-invasively. After four weeks of treatment with doxycycline, mice from the behavioral analysis were analyzed histologically. NF- κ B dependent re-growth of the dentate gyrus was detected by counting the number of nuclei (Fig. 6A,B) from seven animals. These mice, living for a period of 6 months without NF- κ B activity in the hippocampus, showed severe dentate gyrus atrophy (Fig. 6B (compare I κ B^{-/-} with I κ B/tTA, $p=0.0001$). Reactivation of NF- κ B led to a re-expression of the downstream targets Foxo1 and PKA (see Fig.3 A, B) and a significant ($p= 0.0002$) increase in cellular number by about 60% (Fig. 6A,B). Surprisingly, NF- κ B regulated re-growing of the dentate gyrus was still possible even in an already degenerating dentate gyrus. Doublecortin (DCX) positive cells were generated at large numbers after NF- κ B ablation, due to a disequilibrium between apoptosis and neurogenesis (see above). Therefore we analyzed now, whether NF- κ B activation could rescue this disequilibrium. We compared the amount of DCX positive cells after reactivation of NF- κ B in relation to controls (Fig. 6). In mice with NF- κ B ablation, DCX positive cells were dispersed all over the dentate gyrus (Fig. 6C, I κ B/tTA, arrows). However, reactivation of NF- κ B mediated the appropriate location of those DCX-positive cells within the subgranular zone (see arrows). From these data we conclude that NF- κ B directs migration of DCX-positive neuronal progenitors in the dentate gyrus. In addition to the appropriate position of DCX positive cells, their number was now strongly reduced (Fig. 6D) after reactivation of NF- κ B (by about 47 %, $p < 0.0001$). There was no significant difference compared to DOX treated controls.

Finally, we investigated circuit formation by NF- κ B dependent axonal outgrowth of mossy fibers (Fig. 6E). A significant ($p < 0.0366$) increase of neurofilament positive mossy fibers was detected in mice after NF- κ B reactivation (I κ B/tTA +DOX). There

was no significant difference when compared to DOX treated controls. Taken together the NF- κ B dependent rebuilding of the dentate gyrus and its mossy fiber projection suggests a cellular basis for the observed improvements in behavior.

DISCUSSION

NF- κ B controls circuit formation and tissue homeostasis

Here we used behavioral analysis, involving a newly developed SPS Barnes maze and morphological analysis to study the role of NF- κ B in the hippocampus. We found that NF- κ B signaling is crucial for tissue homeostasis and functional circuitry of the dentate gyrus during adulthood. Immunocytochemistry and electron microscopy of the mossy fiber projections and synaptic contacts with CA3 dendrites point to a role of NF- κ B in axogenesis and synaptogenesis in vivo and in vitro. Formation of mossy fiber boutons was significantly reduced in size and number after NF- κ B ablation. Neuronal NF- κ B might have two functions: In immature neurons NF- κ B is necessary for axon formation, survival and integration into the neuronal network, whereas in mature neurons NF- κ B is important for survival and synaptic activity (Kaltschmidt and Kaltschmidt, 2009; Mattson, 2005).

Inhibition of both functions resulted in severe defects of tissue homeostasis during aging in the dentate gyrus. A rescue by increased neurogenesis did not regenerate the dentate gyrus, since NF- κ B ablation blocked the differentiation from a type 2b neuronal precursor to a mature neuron, resulting in a net reduction in cell number.

Recently, the role of NF- κ B p50 in neurogenesis was analyzed (Denis-Donini et al., 2008), in these p50^{-/-} mice the net rate of neural precursor proliferation was unchanged, but only 50 % of newborn neurons survived in the DG and a defect in spatial short term memory was observed. Because in p50^{-/-} mice only one NF- κ B

subunit is deleted in all cell types, we used in this study a neuronal-specific ablation of all NF- κ B subunits. We confirmed in part the results of (Denis-Donini et al., 2008) for a role of NF- κ B in neurogenesis and extended that conclusion to a function of NF- κ B in tissue homeostasis, axogenesis and spatial pattern separation. In contrast, here we find an increased rate of proliferating DCX-positive granule cell progenitors, presumably due to the high rate of apoptosis observed in DG (see (Gould, 2007) for discussion of feedback mechanisms). These data suggest, that regrowing after re-activation of NF- κ B has a major impact on integration and survival of newborn DCX⁺ neurons. The regulation of adult neurogenesis by transcription factors is still a matter of debate (Mu et al., 2010) and our data show that the transcription factor NF- κ B is a crucial regulator of neurogenesis, essential for axogenesis and integration of newborn neurons.

Molecular mechanism controlling NF- κ B directed axogenesis

We uncovered a NF- κ B dependent signaling pathway relevant for axogenesis (see Fig. 7), involving the forkhead transcription factor Foxo1 and a PKA kinase cascade, which might integrate both: axogenesis and neuronal survival. The NF- κ B target gene PKAc phosphorylates CREB (Fridmacher et al., 2003), which was shown to increase survival of newly generated granule cells (Jagasia et al., 2009). Additionally, LKB1 is a substrate of PKA and is an important player in axogenesis (Shelly et al., 2007) (Barnes et al., 2007). Phosphorylation of LKB1-S431 by PKA, which is a crucial step for initiation of axon outgrowth, is decreased in NF- κ B ablated mice. In this line we show that Foxo1 exclusively is expressed in immature and mature granule cells of the dentate gyrus and its expression is strongly dependent on NF- κ B signaling. FOXO1 signaling together with other members of the FOXO family was

recently reported to regulate axogenesis, both in cerebellar granule cells and in hippocampal cell cultures and within the cerebellar cortex (de la Torre-Ubieta et al., 2010). In addition recent reports suggest an involvement of FOXO in neural stem cell homeostasis and energy balance (Paik et al., 2009; Renault et al., 2009). Thus, NF- κ B might function as a master regulator upstream of several other transcription factors such as CREB and FOXO. The strong effect of NF- κ B on tissue homeostasis of the dentate gyrus might occur only if two circumstances happen together: enhanced cell death coupled with a blockade in neuronal progenitor differentiation, which normally could improve the deficit. That might be the reason why NF- κ B dependent tissue homeostasis was observed in the nervous system for the first time. Recently, it was shown that NF- κ B regulates tissue homeostasis in several other organs such as skin, liver and gut (Pasparakis, 2009).

Taken together we show that NF- κ B plays an important role at three consecutive stages of neurogenesis: proliferation/differentiation of neural progenitor cells, axon specification and integration of young neurons and survival of mature granule cells. A similar conserved function at different stages of neurogenesis has been previously described for members of the TRIM-NHL family (Schwamborn et al., 2009) (Khazaei et al.).

Currently two different models are discussed for the function of new neurons and memory capacity (Deng et al., 2010) a “replacement model” where a newborn neuron replaces an already existing one and a second “addition model”, where new neurons are added to the existing neuronal network. Our data suggest a function of NF- κ B in favor of the “replacement model”. Despite the vast increase in newborn neurons, DG tissue homeostasis is disturbed, resulting in a net reduction of granule cell number by about 50% and finally to an atrophy of DG during adult life. NF- κ B ablation was not toxic in vivo (Bhakar et al., 2002; Fridmacher et al., 2003) and in vitro on its own, but

granule cell precursors do not survive in vivo presumably due to a failure in axon formation by reduction of phospho-LKB1.

NF- κ B dependent integration of new born neurons is crucial for spatial pattern separation

The observed NF- κ B dependent structural defects resulted in a behavioural phenotype. Recently, behavioural tests (Clelland et al., 2009) were developed to measure memory of subtle differences in spatial environment. The authors suggest that neurogenesis enhanced the recognition of lowly separated cues. In addition (Sahay et al., 2011) have shown that Bax ablation increased neurogenesis and resulted in improved discrimination between similar contexts. In this line, we developed a special behavioral test (*SPS-BM*), which is able to measure spatial pattern separation with the advantage to analyze search strategies. $\text{I}\kappa\text{B}/\text{tTA}$ mice revealed a severe impairment in this *SPS-BM*. They progressed only through the first two steps of learning, using random and serial search strategies by pure chance, but failed to use the spatial strategy consistently. In contrast a spatial search strategy might require the mouse to learn multiple relationships among extramaze cues to guide it to its target, the food house.

On the other hand $\text{I}\kappa\text{B}/\text{tTA}$ mice could learn tasks such as the classic *BM* and the *Morris water maze (MWM)* to some extent (Kaltschmidt et al., 2006). This is in accordance with previous results showing that the DG is mainly responsible for spatial pattern separation tasks (Deng et al., 2010), but is not involved in navigation via the temporoammonic pathway (Brun et al., 2002).

The mouse model described here is a phenocopy of Alzheimer's disease (AD) in three aspects: reduced NF- κ B activity as in AD brains (Kaltschmidt et al., 1999) and increased proliferation of immature DCX⁺ neuronal precursors and progressive cell

loss (Jin et al., 2004) coupled with strong neuroinflammation (Sturchler-Pierrat et al., 1997). Thus NF- κ B activation in transplanted neuronal precursors might be a therapeutic target in defeating AD.

Experimental Procedures

Animals

Mice were kept under specific pathogen free conditions as defined by the Federation European Laboratory Animal Science Association (FELASA) in the central animal facility of Bielefeld University. Mice were kept in standard cages in a temperature and humidity controlled (22°C) room under diurnal condition (12h light/dark cycle), with HEPA filtered air. Food and water were provided *ad libitum*. I κ B/tTA mice were described in (Fridmacher et al., 2003; Kaltschmidt et al., 2006), genotyping was done by PCR. To reduce individual variability only males with an age difference of less than 2 weeks were used in behavioural experiments. For NF- κ B re-activation experiments, doxycycline (Dox) was administered in drinking water (2 mg/ml w. 2,5% sucrose) for 14 days.

Preparation of tissue

For frozen sections animals were decapitated, brains were removed and embedded in TissueTek OCT compound, frozen in -20°C cold 2-methylbutane and cut horizontally on a Leica cryotome. Sections of 12 μ m thickness were mounted on Superfrost slides, dried for 5 min at room temperature and stored at -80°C until use.

For fixed sections, animals were anesthetized with Avertin and transcardially perfused with phosphate buffered saline (PBS) containing heparine (0.025g/100 ml PBS) and procaine (0.5 g/100 ml PBS) followed by 4% paraformaldehyde in PBS. Subsequently brains were postfixed in 4% paraformaldehyde at 4°C for 24h and dehydrated in 30% sucrose in phosphate buffer saline at 4°C. 40 μ m horizontal sections were cut on a Leica Frigocut cryotome and stored in cryoprotectant solution (0.1M phosphate buffer, 50% glycerol, 0,14% MgCl₂, 8.6% sucrose) at -20°C until use.

Immunofluorescence

For immunohistochemistry, frozen sections were dried at ambient temperature for 5 min followed by postfixation with -20°C cold methanol for 10 min. Slides were rinsed with PBS and blocked with 5% normal serum (from the species which was used for raising the secondary antibody) for 30 min and incubated with primary antibodies over night at 4°C and secondary antibodies at room temperature for one hour. Antibodies used were: anti-Neurofilament-M (2H3, Developmental Studies Hybridoma Bank), anti-active Caspase-3 (#9664; Cell Signaling), anti-PKA substrate (RRXS/T, #9624; Cell Signaling).

Fixed brain sections were labelled free-floating in primary antibodies overnight at 4°C, secondary antibodies at room temperature for three hours. Antibodies were diluted in PBS containing 0.3% Triton-X. Antibodies used were: anti-Calretinin (6B3, SWANT), anti-doublecortin DCX (sc-8066; Santa Cruz), anti-GFP (ab290; abcam), secondary antibodies: Alexa-555 and Alexa-488 (Invitrogen - Molecular Probes). Nuclei were stained with Sytox or Hoechst stain.

Analysis of neuronal morphology

Neuronal morphology was analysed by staining with anti-Tau-1 and anti-MAP2 antibodies (Chemicon) and using the WASABI software (Hamamatsu), ImageJ (NIH), and Adobe Photoshop. The development of axonal fate was analyzed as described previously (Schwamborn et al., 2004). The length of axons and dendrites was determined by Spot software (Diagnostic Instruments). The Student's *t*-test was used to test statistical significance, when analyzing morphology in vitro.

BrdU labelling

To measure proliferation, mice were injected with BrdU 50 mg/kg i.p. once daily for 3 days as previously described (Lafenetre et al., 2010). The hippocampus was dissected, frozen and double labeling was done on free floating cryosections with antibodies directed against BrdU (Clone BU1/75; Accurate Chemical) and anti-doublecortin (sc-8066; Santa Cruz). Before labelling sections were denatured with 2M HCl for 10 min and incubated in 0,1 M borate buffer for 10 min. Quantification of labelled cells was done as described (Clelland et al., 2009). One-in-seven of 40 µm thick serial sections (280 µm apart) from each brain was immunohistologically stained (see above) and analyzed by fluorescent confocal microscopy. Immunolabelled cells were counted using a 40x objective (Zeiss). Total number of cells was extrapolated by multiplication of the counted numbers of cells with 60 (60 slices per hippocampus).

Western Blotting

Protein extracts were prepared from 10 hippocampi, using 100 µl buffer (1% NP-40, 50mM NaCl, 50 mM Hepes) with “Halt” phosphatase inhibitor cocktail (Pierce) and proteaseinhibitors (aprotinin, leupeptin and pepstatin). Tissue was homogenized using an Ultra-Turrax (IKA) for 15 sec. on ice. After centrifugation supernatant was collected, protein concentration was determined (RotiNanoquant, Roth) and 30 µl (about 80 µg total protein) per lane was separated on a SDS gel. Gels were semi-dry blotted onto nitrocellulose (Pall). and were incubated with antibodies against LKB1 (D-19; Santa Cruz), anti-phospho-LKB1 (Ser 431; Santa Cruz) and anti-GAPDH (6C5; Santa Cruz).

Assay for apoptosis and dying neurons

Apoptosis was detected immunohistochemically by staining for active (cleaved) caspase-3 on frozen sections, Fluoro-Jade-C (Histo-Chem) staining was performed on fresh frozen, unfixed slices as adapted from (Schmued et al., 1997).

Electron microscopy

Mice were anaesthetized and transcardially perfused according to local institutional guide lines in 3 steps slightly modified according to (Forssmann et al., 1977) with 3% paraformaldehyde, 3% glutaraldehyde, 0,5% picric acid in 0.1 M sodium phosphate buffer, pH 7.4 for 10 minute. The hippocampus was dissected and fixed in the same solution for additional 1-2 hours at 4°C, postfixed in 2% osmium tetroxide (2h, 4°C) and embedded in *Araldite*. For light microscopical identification of target region sections of 1.5 µm thickness were stained with Richardson's blue (1% w/v methylene blue, 1% w/v Azur II) for 1 min, 80°C. 60-80 nm sections (stained for 40 min in uranyl acetate and 7 min in lead citrate) were used for electron microscopy (Zeiss EM 109). Only those boutons were examined that established contacts with dendritic excrescences of CA3 pyramidal neurons. For quantification of mossy fiber boutons at least 8 independent, non-overlapping analog photographs of the *stratum lucidum* were made at a primary magnification of 3440x of coded sections by an unbiased observer. Each photograph represented an area of 360 µm². Care was taken to avoid areas containing structures not of interest e.g. nuclei or bigger blood vessels. High resolution scans of negatives were made with an EPSON 4990 photo scanner, these scans could be loss-free zoomed in up to 12x and were evaluated with Adobe-Photoshop. To quantify the number of synaptic contacts per section of a bouton, 40 mossy-fiber boutons per animal (n=3) were evaluated. Synaptic contacts, in contrast to puncta adherentia, were characterized if applicable by 3 criteria: presence of synaptic vesicles close to the presynaptic density, asymmetry between pre- and

postsynaptic density and widening of the synaptic cleft. Due to non-ideal orientation of the synaptic cleft, often only the first criterion was applicable.

To determine numbers of synaptic vesicles per bouton, 25 photographs per animal (n=3) were taken at a primary magnification of 13.000x. Evaluation of eight different areas per animal, each 360 μm^2 was done. Areas were measured with IMAGE J (NIH; Washington, D.C.) and vesicles were counted with the Image J cell counter plug-in. Statistical evaluation was performed with the Mann-Whitney-U-test.

Neuronal culture and transfection

Cultures of dissociated hippocampal neurons were prepared and transfected as described previously (Schwamborn et al., 2004). Briefly, the hippocampus was dissected from E18 rat embryos, dissociated, and neurons plated onto glass coverslips coated with polyornithine (Sigma) at a density of 800,000 cells per coverslip. Attached neurons were transfected 2 h after plating using Lipofectamine 2000 (Invitrogen) as described. After an incubation for 2 h, the transfection medium was replaced by Neurobasal medium (supplemented with B27, 0.5mM glutamine, and 100 U/ml penicillin/streptomycin; Invitrogen). The cells were detached after the transfection by moderate pipetting and replated onto new coverslips at a lower density (40,000-60,000 cells per coverslip in a 24-well plate). Neurons were fixed at 3 d.i.v. with 4% paraformaldehyde and 15% sucrose in phosphate buffered saline (PBS) for 20 min at 4°C. To analyse the establishment of neuronal polarity, neurons were stained with the Tau-1 (as a marker for axons) and an anti-MAP2 antibody (minor neurites). Processes showing Tau-1 immunoreactivity in their distal segments were counted as axons, MAP2-positive neurites longer than one cell diameter as dendrites. Three independent transfection experiments were conducted. The following numbers of neurons were analyzed, EGFP: n= 54; p50: n= 37; p65: n= 35;

I κ B-AA1: n= 42. Means \pm s.e.m.; ** p<0.001 compared to EGFP; 3 independent experiments; * p<0.05 compared to EGFP; 3 independent experiments. For the PKAc experiments the following numbers of neurons were analyzed in three independent experiments; EGFP control : n=46; PKAc: n= 58; I κ B-AA1: n=64; PKAc + I κ B-AA1: n=56.

Statistical evaluation

For analysis of immunohistological stainings Student's *t*-test was used. The evaluation of the behavioral tests occurred using two-way ANOVA. Errors bars indicate SEM. ; **P* < 0.05, ***P* < 0.01, ****P* < 0.0001.

Behavioral assays

Spatial pattern separation-Barnes Maze (SPS-BM)

Since behavioral deficits resulting from impairment of neurogenesis may be subtle (Clelland et al., 2009; Rola et al., 2004) we designed a new challenging task, the *SPS-BM*. On a circular plate made from hard-plastic (diameter 120 cm) seven rectangular houses of the same colour, size and shape (see rectangles in Fig. 4a) were placed. Only one of the houses was freely accessible and contained a food pellet reward (Kellogs Froot Loops). During seven consecutive days of training (one trial per day, 10 min.) the mice had to find the food house (location F) using distal extramaze cues. Start position is indicated by the letter S. To avoid odour as a facilitatory intramaze cue, the circular plate and the food house was cleaned after each trial with Buraton rapid disinfectant (Schülke & Mayr GmbH). In addition a food pellet is deposited in every house to avoid orientation by odour. Only male mice with an age of six month were tested. Two different genotypes were compared, mice with

NF- κ B ablation ($I\kappa B/tTA$, $n = 9$) and control mice ($I\kappa B/-$, $n = 8$). Habituation occurred one day before starting the task, during 10 minutes all houses were freely accessible and the animals had time to explore the environment and the houses. The performance of the mice was documented by a video-tracking system (TSE VideoMot 2, Bad Homburg) and analyzed by measuring latency, errors and distance covered before arriving at the food house.

Classic Barnes Maze test (BM)

The BM was developed to test the ability of spatial learning of rodents (Barnes, 1979). Around the perimeter of a circular plate made out of hard-plastic (diameter 120 cm) there are forty circular holes, one having a tunnel where the mouse has the possibility to hide. During eight consecutive days of training (one trial per day, 30 min) the mice had to find the tunnel with the help of distal extramaze cues. Start position is indicated by the letter S. To avoid odour as a facilitatory intramaze cue, the circular plate and the tunnel was cleaned after each trial with Buraton. Only six months old males were tested. Two different genotypes were compared, mice with NF- κ B ablation ($I\kappa B/tTA$, $n = 7$) and control mice ($I\kappa B/-$, $n = 7$). Habituation took place one day before starting the test. During that phase animals had ten minutes to explore the environment without the tunnel. Performance was documented and evaluated as defined above.

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FIGURES

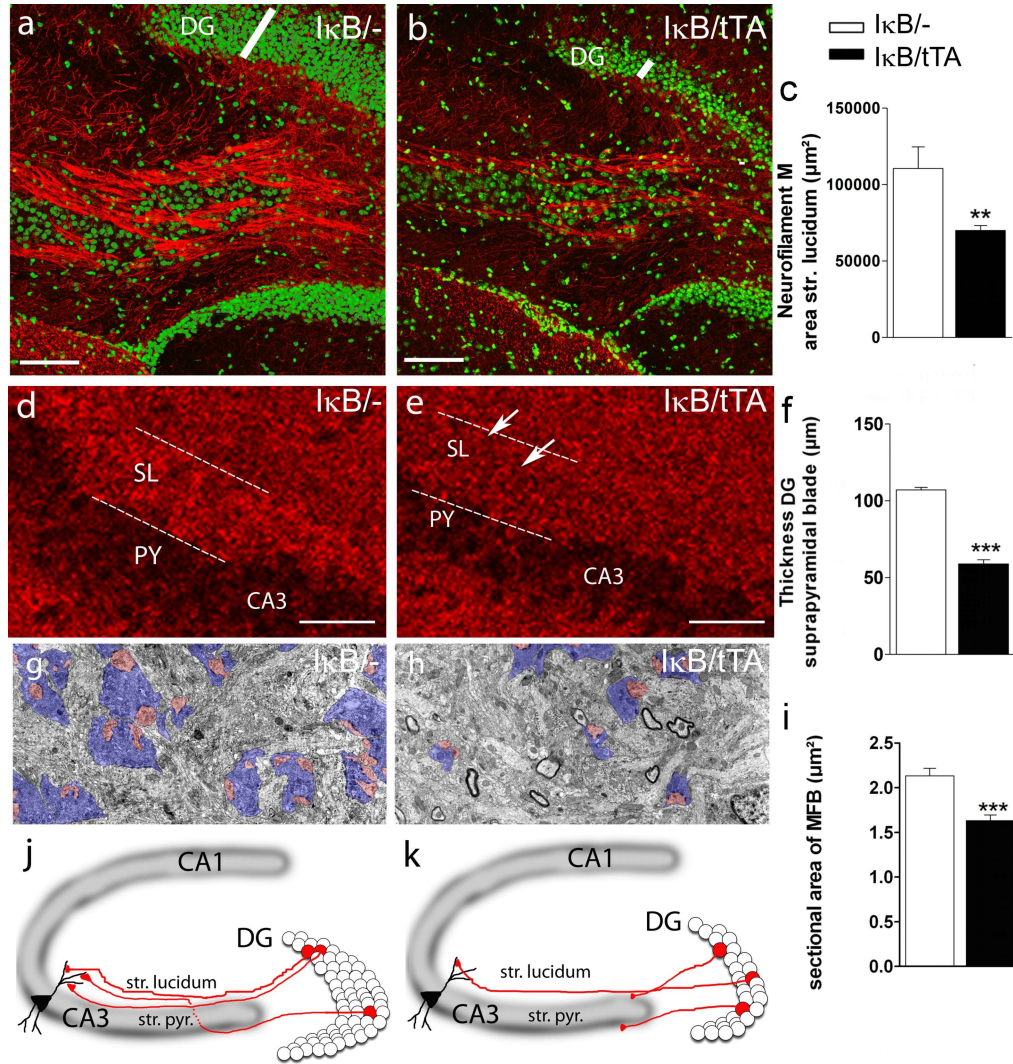


Fig. 1 NF- κ B regulates mossy fiber projections to CA3 and replenishment of the dentate gyrus

- (a) Immunostaining for neurofilament M (NF-M) in control mice ($I\kappa B^{-/-}$) reveals a fasciculated organisation of mossy fibers, connecting granule cells to their target cells in CA3. (b) Impairment of mossy fiber projections after neuronal NF- κ B ablation ($I\kappa B/tTA$). (c) Statistical analysis of NF-M staining shows significantly reduced mossy fiber bundles in $I\kappa B/tTA$ mice ($n = 8$) as compared to controls ($n = 4$) ($P = 0.0034$); (d) Synapses stained for synaptophysin are organised in a laminated fashion. Strongest staining was visible in the stratum lucidum (SL), which corresponds to the termination field of mossy fibers on dendrites of CA3 neurons. (e) In contrast, staining is strongly reduced in the SL after NF- κ B ablation, suggesting an important role of NF- κ B in regulating synaptic density. (f) NF- κ B ablation results also in a

significantly decreased dentate gyrus size (white bar in **a**, **b**, $n = 9$) compared to control mice ($n = 6$) ($P = < 0.0001$). (**g**, **h**) Electron microscopy shows that size and frequency of mossy fiber boutons (MFB) are reduced after NF- κ B inhibition (blue, mossy fiber boutons; red, dendritic excrescences). (**i**) The size of MFB is reduced in I κ B/tTA mice. (**j**, **k**) Scheme of structural defects after NF- κ B ablation. Scale bars (a,b) 100 μ m, (d,e) 20 μ m, (g,h) 2 μ m); DG-dentate gyrus, SL-stratum lucidum, PY-pyramidal layer, MFB-mossy fiber boutons. Error bars, SEM. ** $P < 0.01$; *** $P < 0.0001$; Unpaired t-test, two tailed.

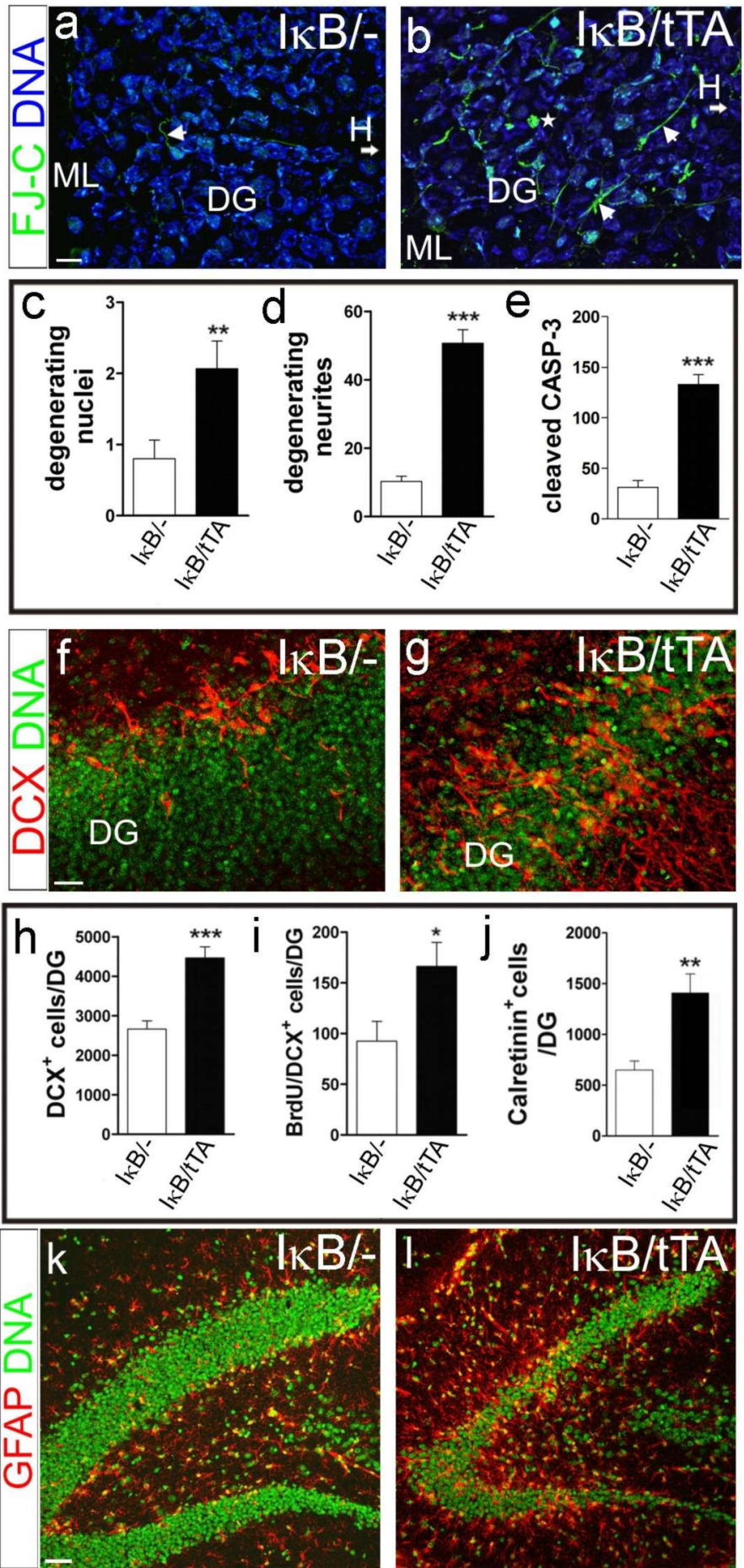


Fig. 2 Increased apoptosis, neurogenesis and inflammation in the dentate gyrus of NF- κ B deficient mice

(a, b) Neuronal cell death visualized by Fluoro Jade-C. Degenerating neurites (arrows) and nuclei (asterisk) are significantly increased in I κ B/tTA mice ($n = 3$) as compared to control mice ($n = 3$) (c, d). (e) Increased cleaved caspase-3 positive cells after NF- κ B ablation ($n = 3$). (f, g) DCX immunoreactivity is significantly increased in the DG after NF- κ B ablation. Note that DCX⁺ cells are not arranged in the subgranular zone alone but migrate deeper into the DG. (h, j) Total number of DCX⁺ and Calretinin⁺ cells is increased in I κ B/tTA mice ($n = 3$) compared to controls ($n = 3$). (i) Increased BrdU/DCX⁺ cells in the DG seven days after the last BrdU injections ($n = 3$). (k, l) Representative images of the distribution of glial fibrillary acidic protein (GFAP) in the hippocampus. Note significantly increased numbers of GFAP positive cells in I κ B/tTA mice, particularly within the DG. Scale bars (a, b) 10 μ m, (f, g) 25 μ m, (k, l) 50 μ m; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; Error bars: SEM; DG dentate gyrus, GFAP glial fibrillary acidic protein, ML molecular layer, H hilus, DCX doublecortin, FJ-C fluoro Jade-C, BrdU bromodeoxyuridine

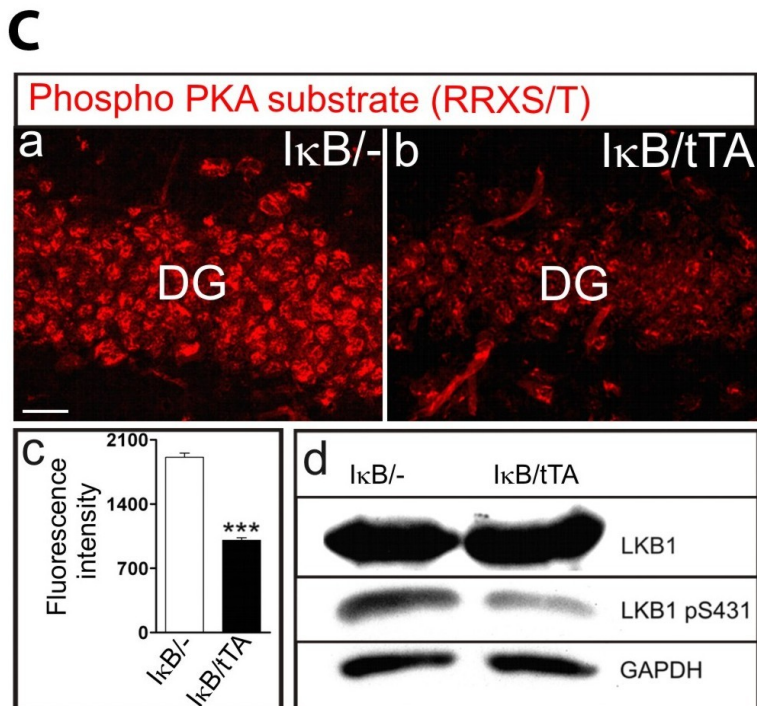
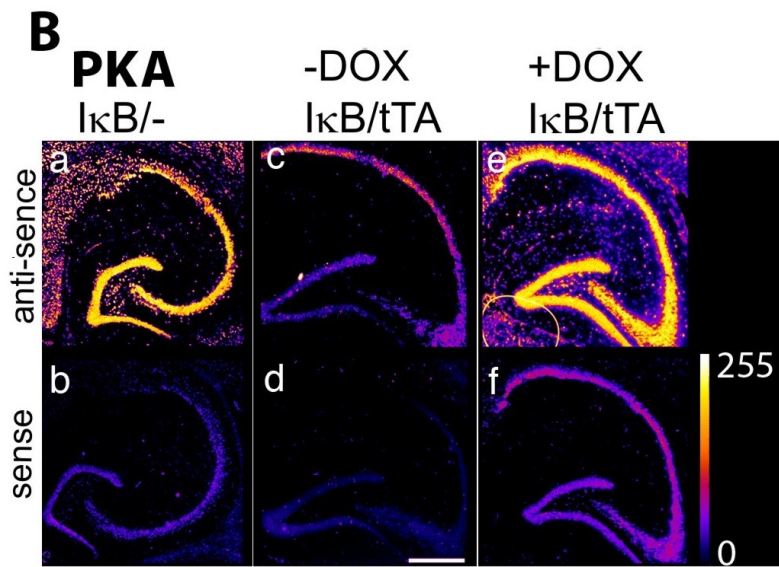
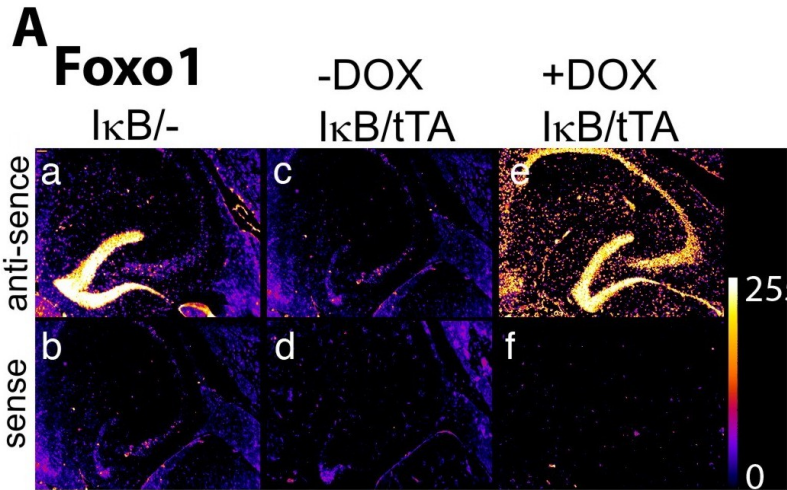


Fig. 3 NF- κ B dependent regulation of PKA and FOXO1

A (a, b) Forkhead box protein O1 (FOXO1) is expressed in the DG in control mice (I κ B^{-/-}), but is downregulated in NF- κ B deficient mice (**c, d**). (**e, f**) Doxycycline induced reactivation of NF- κ B leads to re-expression of the transcription factor Foxo1 as shown by in situ hybridisation (encoded in false colour).

B (a, b) The catalytic subunit alpha of Protein kinase A (PKA) is expressed in the DG of control mice (I κ B^{-/-}), but is strongly downregulated in NF- κ B deficient mice (**c, d**). Reactivation of NF- κ B by doxycycline treatment results in re-expression of PKA (**e, f**); as shown by in situ hybridisation (encoded in false colour).

C (a, b) Phosphorylated PKA substrates are reduced in granule cells of the dentate gyrus as detected by phospho-specific antibody. (**c**) Fluorescence intensity is significantly decreased in I κ B/tTA mice ($n = 4$) as compared to controls ($n = 4$) ($P = < 0.0001$) (**d**) Phosphorylation of LKB1, a substrate of PKA, is reduced in hippocampal brain extracts from I κ B/tTA mice as detected by Western blotting. Scale bar (a, b) 20 μ m; Error bars: SEM; DG dentate gyrus

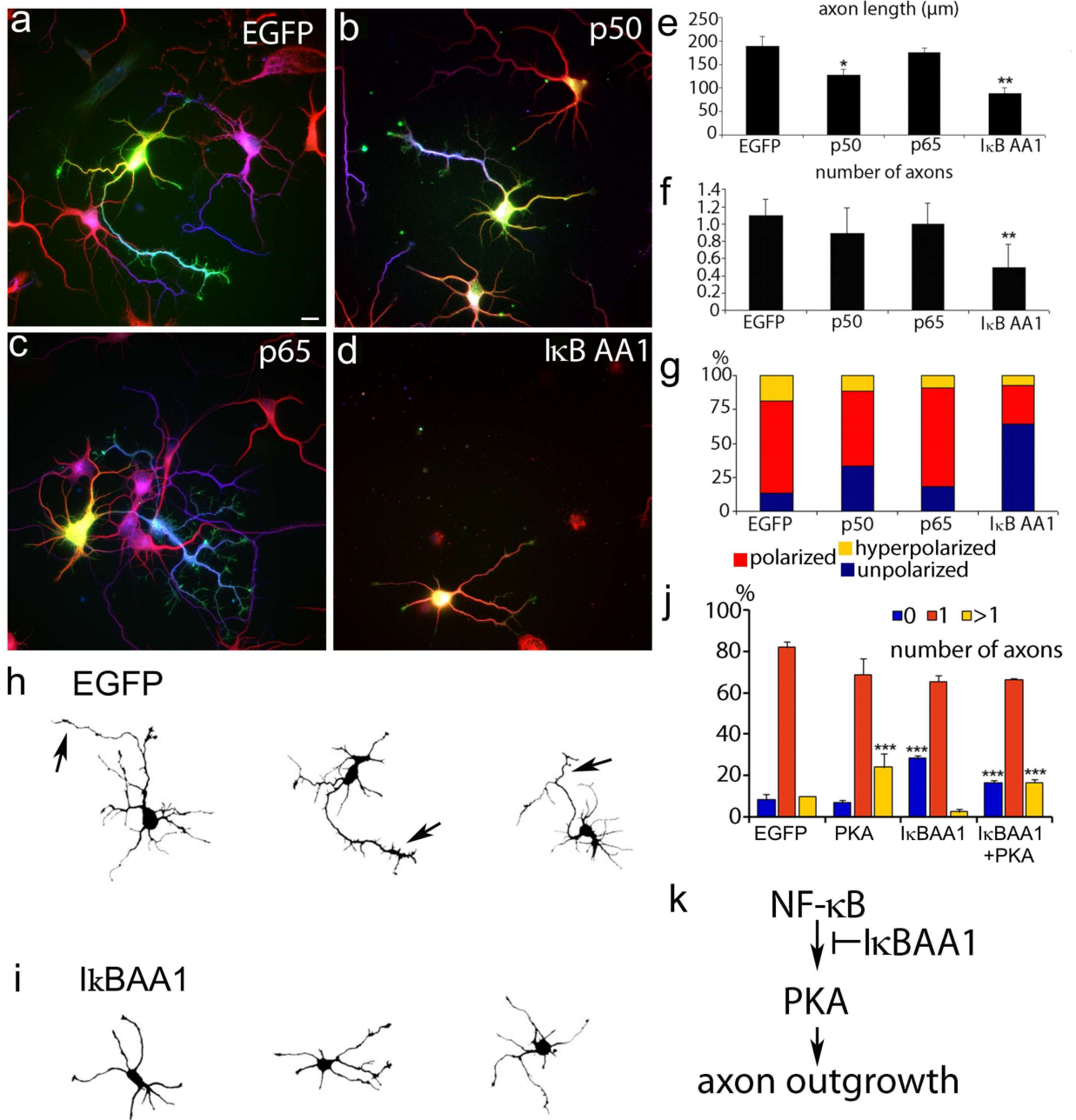


Fig. 4 NF-κB dependent changes in axonal fate determination in vitro

Effect of NF-κB inhibition in hippocampal neurons transfected 2 hours after plating with expression vectors for EGFP and NF-κB subunits as indicated. The analysis of NF-κB induced changes in morphology was done by immunostainings with markers for neuronal polarity. Tau-1 immunoreactivity was used as axonal marker, minor neurites (dendrites) were identified by anti-MAP2 labelling.

Axon length was reduced in neurons transfected with super-repressor-IκB (from 188 μm ± 20 μm in the EGFP control to 87 μm ± 10 μm after IκB transfection (d, e). To a

lesser extend shorter axons was observed in neurons transfected with the p50 subunit (**b**, **e**). Thus super-repressor-I κ B expression resulted in neurons without Tau-positive axons (**d**). Control neurons acquired a normal neuronal morphology (**a**, **g**) and extended a single axon (1.1 ± 0.2) plus about 4 minor neurites as shown before (Schwamborn et al., 2007; Schwamborn and Puschel, 2004). (**g**) Transfection with NF- κ B DNA-binding subunits p50 and p65 did not induce a significant change in axon number (p50: 0.9 axons per cell \pm 0.3; p65: 1.0 axons per cell \pm 0.2). (**h**, **i**) Representative samples of reconstructed hippocampal neurons. Neurons transfected with control plasmids (**h**) and with super-repressor I κ B α -AA (**i**). Note the strongly reduced complexity of neuronal processes in NF- κ B ablated cells, resembling an immature developing neuron. (**j**) Furthermore we tested, if the effect on neuronal morphology could be rescued by the NF- κ B downstream target gene PKAc. Indeed, overexpression of PKAc in hippocampal neurons showed hyperpolarized neurons with multiple axons, consistent with the recent observation of PKA dependent phosphorylation of the downstream kinase LKB1, which is necessary for axon differentiation. Coexpression of super-repressor-I κ B and PKAc led to a hyperpolarized phenotype. Transfected cells were identified via EGFP fluorescence. Development of neuronal polarity was analyzed by determining the length of minor neurites per cells (**e**). Error bars: SEM; *** $p < 0.0001$ compared to EGFP; $n = 3$ independent experiments. (**k**) Model of a transcriptional network regulating axon outgrowth of the granule cells. NF- κ B can activate the transcription of the catalytic subunit PKAc alpha, which is necessary for axon formation. NF- κ B activation can be inhibited by overexpression of super-repressor I κ B α -AA, which interferes with PKA transcription and axon formation. Error bars: SEM; $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$

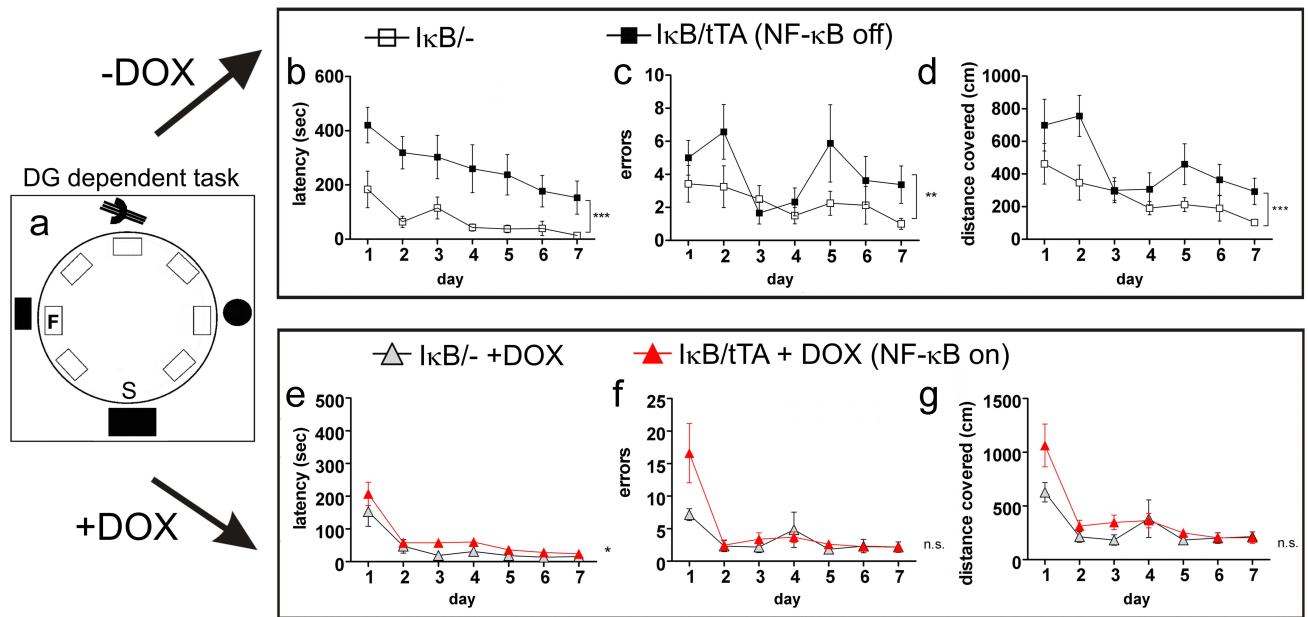


Fig. 5 Impaired NF- κ B signalling interferes with spatial pattern separation

A *spatial pattern separation-Barnes Maze (SPS-BM)* was developed to test DG dependent spatial pattern separation (a). During consecutive days of training the mice had to find the food house (location F), but only one of the identical seven houses on the plate is freely accessible. Dependent on distal extramaze cues the animals have to find the open food house. The presentation of several identical objects in an environment should lead to overlapping activation in place cells. The analysis of the SPS-BM shows highly significant memory deficits in NF- κ B ablated mice ($n = 9$) as compared to controls ($n = 8$). Latency (b), distance (d) and error rate (c) were significantly increased in I κ B/tTA mice.

Reactivation of NF- κ B in I κ B/tTA animals by doxycycline treatment ($n = 13$; lower panel) improved the performance considerably. Mice showed significantly decreased latency (e), the error rate (f) and distance covered (g) was comparable to control mice ($n = 6$). two-way ANOVA evaluation; error bars: SEM; S = Start position; F = food house

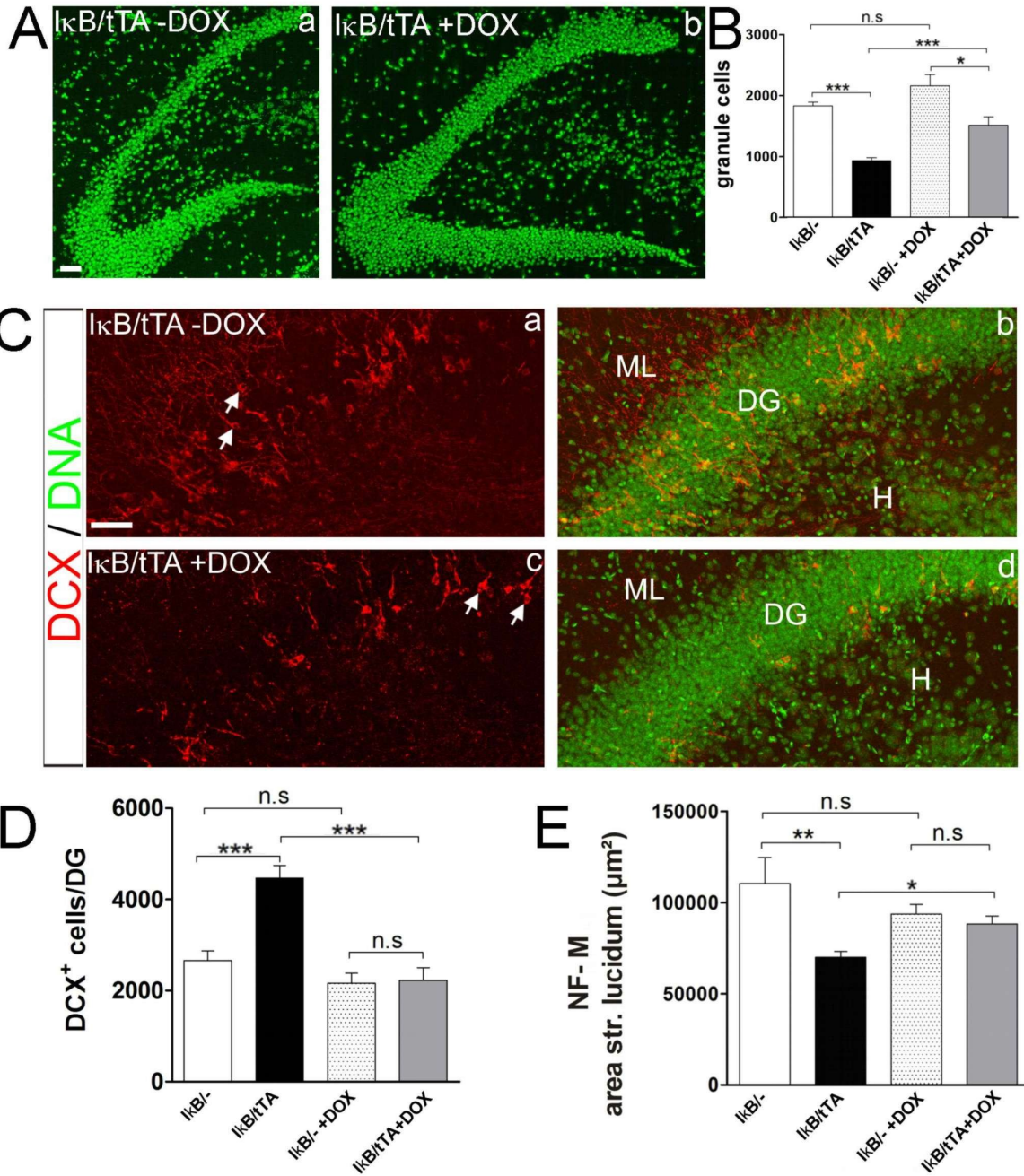


Fig. 6 Reactivation of NF- κ B leads to regrowing of the dentate gyrus

(A) (a) The size of the dentate gyrus (DG) in NF- κ B ablated mice is strongly reduced. Reactivation of NF- κ B leads to increased number of granule cells within the DG **(b)**. **(B)** Statistical analysis shows a significant decrease of granule cells after NF- κ B ablation (I κ B/tTA mice, $n = 9$; as compared to control mice I κ B^{-/-}; $n = 3$, ($P = < 0.0001$)). Note, after reactivation of NF- κ B by doxycycline treatment the number of

granule cells is increased in I κ B/tTA mice ($n = 7$) ($P = 0.0002$). No difference in the control groups I κ B/- ($n = 3$) and I κ B/- +DOX ($n = 3$) was detected ($P = 0.0605$)

(C) Doublecortin (DCX) staining marking neuronal precursors. After doxycycline treatment the number of DCX positive cells is strongly decreased in I κ B/tTA mice ($n = 3$) **(c, d)** as compared to NF- κ B ablated group ($n = 3$) **(a, b)**

(D) Statistical evaluation shows significantly less DCX positive cells after doxycycline treatment in I κ B/tTA mice ($n = 3$) as compared to untreated I κ B/tTA group ($n = 3$) ($P = < 0.0001$). No difference in control groups I κ B/- ($n = 3$) and I κ B/- +DOX ($n = 3$) was detected ($P = 0.1063$).

(E) Statistical analysis of neurofilament-M (NF-M) staining shows significantly reduced mossy fiber bundles in I κ B/tTA ($n = 8$) mice as compared to controls ($n = 4$) ($P = 0.0034$). Reactivation of NF- κ B results in mossy fiber outgrowth of newborn neurons, thus increasing the area of mossy fibers in doxycycline treated I κ B/tTA mice as compared to the untreated group ($P = 0.0366$). No difference in the control groups I κ B/- ($n = 4$) and I κ B/- +DOX ($n = 3$) was detected ($P = 0.1897$). Error bars: SEM; $P < 0.05$; $**P < 0.01$; $***P < 0.0001$

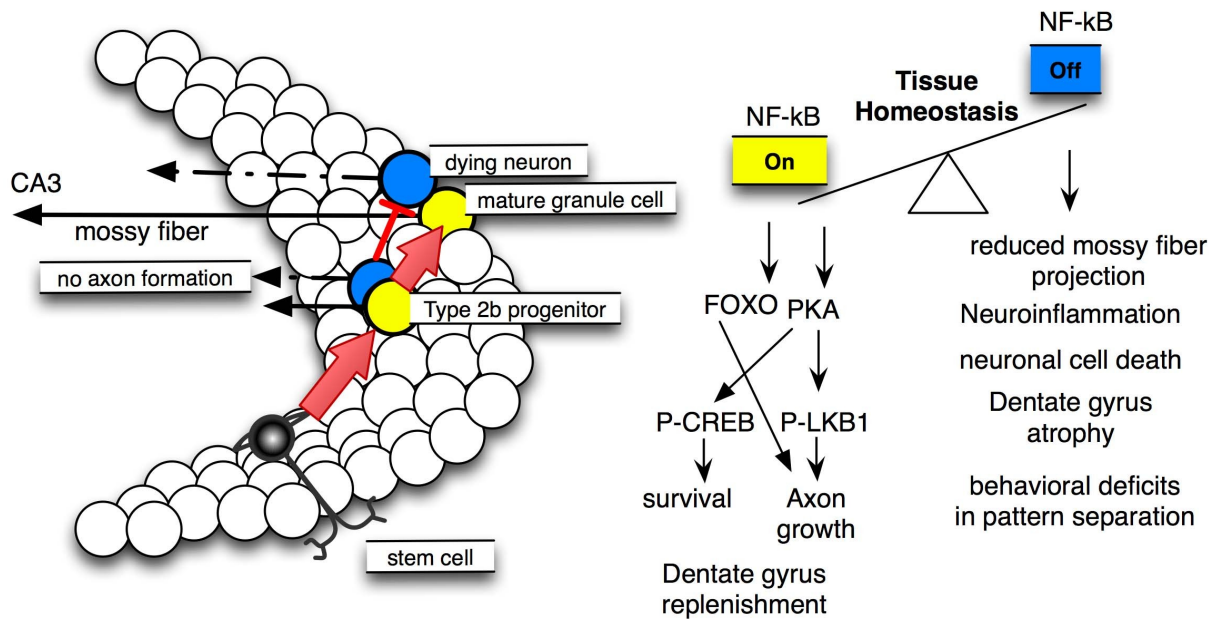


Fig. 7 NF- κ B signalling can play different roles in dentate gyrus tissue homeostasis

The dentate gyrus (left) is built from stem cells that develop to neuroblasts and type 2b progenitors that still can divide. These progenitors give rise to mature granule cells, connecting CA3 pyramidal neurons via mossy fibers. NF- κ B signalling plays an important role at least in two of the processes that regulate the balance between degeneration and regeneration (tissue homeostasis) of the dentate gyrus. That is firstly axon formation and maturation from a immature type 2b neuronal progenitor (DCX⁺) to a mature granule cell neuron and secondly regulation of survival and energy balance of mature granule cells. yellow = cells with NF- κ B on; blue= NF- κ B ablated cells, NF- κ B off

Right side, molecular mechanisms: Our results point to a cell autonomous mechanism by which NF- κ B regulates axogenesis in newborn granule cells. Critically

involved are the FOXO transcription factors and the bona fide NF- κ B target gene PKA (Prkaca). A second role for NF- κ B in mature granule cells is neuroprotection presumably transmitted by a PKA – phospho-CREB pathway. Ablation of NF- κ B (NF- κ B off) results in severe structural and behavioural defects described above.

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