Video Article

Methods for the Modulation and Analysis of NF- κ B-dependent Adult Neurogenesis

Darius Widera¹, Janine Müller², Yvonne Imielski², Peter Heimann¹, Christian Kaltschmidt¹, Barbara Kaltschmidt²

¹Cell Biology, University of Bielefeld

²Molecular Neurobiology, University of Bielefeld

Correspondence to: Barbara Kaltschmidt at barbara.kaltschmidt@uni-bielefeld.de

URL: <http://www.jove.com/video/50870>

DOI: [doi:10.3791/50870](https://doi.org/10.3791/50870)

Keywords: Neuroscience, Issue 84, NF- κ B, hippocampus, Adult neurogenesis, spatial pattern separation-Barnes maze, dentate gyrus, p65 knock-out mice

Date Published: 2/13/2014

Citation: Widera, D., Müller, J., Imielski, Y., Heimann, P., Kaltschmidt, C., Kaltschmidt, B. Methods for the Modulation and Analysis of NF- κ B-dependent Adult Neurogenesis. *J. Vis. Exp.* (84), e50870, doi:10.3791/50870 (2014).

Abstract

The hippocampus plays a pivotal role in the formation and consolidation of episodic memories, and in spatial orientation. Historically, the adult hippocampus has been viewed as a very static anatomical region of the mammalian brain. However, recent findings have demonstrated that the dentate gyrus of the hippocampus is an area of tremendous plasticity in adults, involving not only modifications of existing neuronal circuits, but also adult neurogenesis. This plasticity is regulated by complex transcriptional networks, in which the transcription factor NF- κ B plays a prominent role. To study and manipulate adult neurogenesis, a transgenic mouse model for forebrain-specific neuronal inhibition of NF- κ B activity can be used.

In this study, methods are described for the analysis of NF- κ B-dependent neurogenesis, including its structural aspects, neuronal apoptosis and progenitor proliferation, and cognitive significance, which was specifically assessed via a dentate gyrus (DG)-dependent behavioral test, the spatial pattern separation-Barnes maze (SPS-BM). The SPS-BM protocol could be simply adapted for use with other transgenic animal models designed to assess the influence of particular genes on adult hippocampal neurogenesis. Furthermore, SPS-BM could be used in other experimental settings aimed at investigating and manipulating DG-dependent learning, for example, using pharmacological agents.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50870/>

Introduction

Ontologically, the hippocampus is one of the oldest anatomical brain structures known. It is responsible for diverse complex tasks, such as pivotal functions in the regulation of long-term memory, spatial orientation, and formation and consolidation of the respective memory. Anatomically, the hippocampus consists of pyramidal cell layers (*stratum pyramidale*) including the cornu Ammonis (CA1, CA2, CA3, and CA4) regions and the dentate gyrus (*gyrus dentatus*), which contains granule cells and a few neuronal progenitors within its subgranular zone. The granule cells project towards the CA3 region via the so-called mossy fibers (axons of granule cells).

Until the end of the last century, the adult mammalian brain was believed to be a static organ lacking cellular plasticity and neurogenesis. However, during the last two decades, a growing amount of evidence clearly demonstrates adult neurogenesis taking place in at least two brain regions, the subventricular zone (SVZ) and the subgranular zone of the hippocampus.

Our previous studies, and those of other groups, have shown that the transcription factor NF- κ B is one of the crucial molecular regulators of adult neurogenesis, and that its de-regulation results in severe structural hippocampal defects and cognitive impairments¹⁻⁶. NF- κ B is the generic name of an inducible transcription factor composed of different dimeric combinations of five DNA-binding subunits: p50, p52, c-Rel, RelB, and p65 (RelA), the latter three of which have transactivation domains. Within the brain, the most abundant form found in the cytoplasm is a heterodimer of p50 and p65, which is kept in an inactive form by inhibitor of kappa B (I κ B)-proteins.

To study and directly manipulate NF- κ B-driven neurogenesis, we use transgenic mouse models to enable simple inhibition of all of the NF- κ B subunits, specifically in the forebrain⁷ (see **Figure 1**). For this purpose, we cross-bred the following transgenic mouse lines, I κ B^{-/-} and -/tTA. The transgenic I κ B^{-/-} line was generated using a trans-dominant negative mutant of NF- κ B-inhibitor I κ B α (super-repressor I κ B α -AA1)⁸. In contrast to the wild-type I κ B α , I κ B α -AA1 has two serine residues mutated to alanines (V32 and V36), which hinder the phosphorylation and subsequent proteasomal degradation of the inhibitor. For forebrain neuron-specific expression of the I κ B α -AA1-transgene, I κ B^{-/-} mice were cross-bred with mice harboring a calcium-calmodulin-dependent kinase II α (CAMKII α)-promoter that can be driven by tetracycline trans-activator (tTA)⁹.

p65 knock-out mice have an embryonic lethal phenotype, due to massive liver apoptosis¹⁰, so the approach shown here provides an elegant method for investigating the role of NF- κ B in postnatal and adult neurogenesis.

The classic behavioral test to study spatial learning and memory was described in the 1980s by Richard Morris, a test known as the Morris water-maze (MWM)¹¹. In this open-field water-maze, animals learn to escape from opaque water onto a hidden platform based on orientation and extra-maze cues. A dry variant of MWM is the so-called Barnes maze (BM)¹². This test utilizes a circular plate with 20 circular holes arranged at the border of a plate, with one defined hole as an escape box, and visual extra-maze cues for orientation. Both experimental paradigms rely on the flight behavior induced by a rodent's aversion to water, or open, brightly illuminated spaces. Both tests allow an investigation of spatial orientation, and the related memory performance. Although the hippocampus plays a general and essential role in the spatial memory formation, the hippocampal regions involved differ depending on the test applied. The memory tested in BM arises from neuronal activity between the entorhinal cortex (EC) and the pyramidal neurons located in the CA1-region of the hippocampus without a contribution of the DG¹³⁻¹⁶. In particular, the classic BM mainly relies on navigation via the monosynaptic temporo-ammonic pathway from EC III to CA1 to EC V. Importantly, the DG is crucially involved in the so-called spatial pattern recognition¹⁷, which implies not only the processing of visual and spatial information, but also the transformation of similar representations or memories into dissimilar, nonoverlapping representations. This task requires a functional tri-synaptic circuit from EC II to DG to CA3 to CA1 and EC VI, which cannot be tested in the BM¹⁸.

To address these challenges, we have devised SPS-BM as a behavioral test to specifically test dentate gyrus-dependent cognitive performance in control animals, and in the Ikb/tTA super-repressor model following NF- κ B inhibition. Importantly, in contrast to the MWM or the BM, the SPS-BM can reveal subtle behavioral deficits resulting from impairment of neurogenesis. Since spatial-pattern-separation is strictly dependent on a functional circuit between EC II and DG and CA3 and CA1 and EC VI, this test is highly sensitive to potential changes in neurogenesis, modifications of the mossy fiber pathway or alterations of tissue homeostasis within the DG.

Technically, the set-up of our test is based on the study by Clelland *et al.*, in which the spatial separation pattern was tested using a wooden 8-arm radial arm maze (RAM)¹⁹. In our modified set-up, the eight arms were replaced by seven identical yellow food houses. In summary, the methods shown here, including analysis of doublecortin-expressing (DCX+) cells within the hippocampus, the mossy fiber projections, neuronal cell death and particularly the SPS-BM presented here, can be applied to investigations of other mouse models incorporating transgenes that have an impact on adult neurogenesis. Further applications may include the study of pharmacological agents and measuring their impact on DG and spatial pattern separation.

Protocol

Ethics statement

This study was carried out in strict accordance with the regulations of the governmental animal and care use committee, LANUV of the state North Rhine-Westphalia, (Düsseldorf, Germany). All animal experiments were approved by LANUV, Düsseldorf under the license number 8.87-51.04.20.09.317 (LANUV, NRW). All efforts were made to minimize distress and the number of animals required for the study.

1. Animal Care and Housing

1. All animals used in the protocols described herein should be kept under specific pathogen-free conditions, as defined by the Federation European Laboratory Animal Science Association (FELASA).
2. Mice should be kept in standard cages in a temperature- and humidity- controlled (22 °C) room under diurnal conditions (12 hr light/dark cycle) with HEPA filtered air.
3. Standardized food and water should be provided *ad libitum*.
4. If Ikb/tTA and Ikb/- control mice are used, PCR-based genotyping should be performed for each animal, as described in^{7,18}.
5. Male animals with an age difference of less than four days should be used to reduce individual variability.
6. (OPTIONAL): For NF- κ B reactivation experiments, doxycycline must be administered in drinking water (2 mg/ml with 2.5% sucrose) for at least 14 days.

2. Spatial Pattern Separation-Barnes Maze (SPS-BM)

1. All behavioral testing should be carried out according to international and local guidelines.
2. IMPORTANT! Use only male mice with an age of six months or older. The age difference between the animals of one test series should be less than four days. The testing must be performed by the same operator for each series. The mice should receive a standard diet prior to the testing to further increase the motivation partially driven by a sweet food reward.
3. Set up a white circular plate made from hard plastic (diameter 120 cm, see **Figure 5A**) in a humidity- and temperature- controlled room (22 °C), illuminated with at least 4 x 80 W and 3 x 215 W neon fluorescent lamps. IMPORTANT! Ensure the correct illumination is used, as the motivation of the mice to enter the food houses is partially driven by their aversion to bright, exposed places.
4. Set up the video-tracking system. The camera should be placed 115 cm above the center of the plate (see **Figure 5A**).
5. Attach multicolored extra-maze cues (EMC) to a white-colored cloth in positions easily visible for the animals, approximately 100 cm from the border of the plate (see **Figure 5A**).
6. Carefully clean the plate with a rapid disinfectant that can remove any odor from the experimental set-up.
7. Place seven identical yellow food houses (12 cm x 7 cm x 8 cm, see **Figure 5A**) on the white plate. The positions should be unequivocally marked (see **Figure 5A**).
8. Place sweet food pellet rewards (a quarter of a Kellogg's Froot Loop/food house) inside all food houses on defined positions (see **Figure 5A**) with only one defined food house being freely accessible to the animal (location F, see **Figure 5A**). Close the nontarget food houses with transparent foil.
9. Prior to the test, perform habituation (one day before starting the task). Make all food houses freely accessible and allow the mice to explore the maze freely and to retrieve a food pellet reward (10 min/animal).
10. Switch the computer and the camera on and start the video-tracking system software.
11. Start the recording.

12. Place the animal at the defined start point on the circular plate (**Figure 5A**, S: start position) and allow the mice to search for the target food house for 10 min.
13. Stop the video-tracking.
14. IMPORTANT! Clean the circular plate and the food houses after each trial with rapid disinfectant.
15. Repeat the test daily for seven consecutive days (for each animal).
16. Analyze the results (latency, distance covered and errors) using appropriate statistics software. Define errors as approaching the wrong food house and / or contact with the proper box without entering and retrieving the food pellet reward. For grouped analysis use two-way ANOVA with Bonferroni post-hoc test.
17. (OPTIONAL) Sacrifice the mice and analyze the hippocampi as described below.

3. BrdU Labeling

1. Inject intraperitoneally 50 mg/kg i.p. BrdU once daily for 3 days (analysis of differentiation and integration) or 200 mg/kg i.p. for a single injection (analysis of proliferation).
2. Sacrifice the animals, dissect the hippocampus and prepare 40 μ m sections as described below.
3. Denature the sections with 2 M HCl for 10 min and incubate in 0.1 M borate buffer for 10 min.
4. Label a one-in-twelve series of 40 μ m sections (240 μ m apart) from each animal immunohistochemically, as described below using antibodies directed against BrdU.
5. Quantify the labeled cells by confocal microscopy analysis throughout the rostrocaudal extent of the granule cell layer and subgranular zone. Multiply the resulting numbers by 12 to obtain the estimated total number of BrdU-labeled cells per hippocampus and divide by two to obtain the total number of labeled cells per DG.

4. Removal of the Brains and Preparation of Cryosections from Nonperfused Animals

1. Observe locally approved procedures for euthanizing animals. Mice may be directly euthanized by cervical dislocation.
2. (OPTIONAL) Animals can be anesthetized before euthanization according to local and international guidelines, e.g. by intraperitoneal injection of 0.8 ml Avertin for a 33 g mouse (freshly made by mixing 150 ml stock solution made of 2.2 mg 2,2,2-tribromoethanol in 1 ml isoamyl ethanol with 1.85 ml of saline or physiological buffer).
3. Sterilize the head with Betadine (10% povidone-iodine)-soaked gauze and swab subsequently with gauze soaked in 70% ethanol.
4. Decapitate the animal using appropriate surgical scissors and pull the skin aside.
5. (OPTIONAL) Fixators can be applied to avoid folding back of the retarded skin.
6. Make a midline incision in the skull and carefully pull the skull fragments aside.
7. Carefully remove the whole brain using an appropriate surgical instrument (e.g. Moria Spoon).
8. Precool 25 ml of 2-methylbutane in a 50 ml beaker (e.g. Schott Duran) to -30 to -40 °C on dry ice.

Note: For free-floating staining of "thick" sections which are ideally suited for confocal laser scanning microscopy, remove brain, wash 3 times in buffer and store at 4 °C in phosphate buffered 30 % sucrose solution in 50 ml tube until brain has sunk down to bottom (typically overnight).

9. Carefully place brain on a piece of Nescofilm (Parafilm) and cover with ample amount of TissueTek OCT compound, freeze on Nescofilm in 2-methylbutane, store at -80 °C until use.

Note: For long-time storage at -20 °C store in 9% sucrose, 7 mM MgCl₂, 50 mM phosphate buffer, 44% glycerol.

10. Cut the brain into 10-12 μ m thick sections on appropriate cryomicrotome.

Note: For thick, free-floating sections, freeze brain on cryotome stage of appropriate cryotome (e.g. Reichert Jung, Frigomobil) and cut 40 μ m horizontal sections at -20° to -25 °C. Collect sections from knife with fine brush and collect in buffer or keep in storage solution (step 4.9) at -20 °C for long-time storage.

11. Carefully mount two slices on single microscope slides. The use of Superfrost UltraPlus slides is highly recommended to maximize adhesion of the sections.
12. Dry the slides for 5 min at room temperature. Slides can be stored at -80 °C until use.

5. Preparation of Sections from Perfused Animals

1. Anesthetize the animals as described above (step 4.2).
2. Carefully perfuse the animal transcardially with phosphate buffered saline (PBS) containing heparin (0.025 g/100 ml PBS) and procaine (0.5 g/100 ml PBS) for 2-4 min, followed by 4% paraformaldehyde in PBS for 10-15 min. Perfusion is optimally performed by perfusing via the left ventricle and opening of the right atrium using a hydrostatic pressure of approximately 1.2-1.4 m or utilizing a peristaltic pump set to approximately 15 ml/min. Optimal perfusion results in a pale white brain with no red blood vessels being visible.
3. Dissect and post-fix the brains in 4% paraformaldehyde at 4 °C for 24 hr.
4. Cryoprotect the brains in 30% sucrose in PBS at 4 °C for at least 24 hr.
5. Freeze the brains on the cryotome stage.
6. Prepare 40 μ m horizontal sections from entire forebrains using an appropriate cryotome.
7. The slides can be stored in cryoprotectant solution (0.1 M phosphate buffer, 50% glycerol, 0.14% MgCl₂, 8.6% sucrose) at -20 °C until use.

6. Immunohistochemistry of Brain Sections of Nonperfused Animals

1. Post-fix the cryosections, prepared as described above, using -20 °C cold methanol for 10 min.

2. Block with 5% normal serum containing 0.3% Triton-X (from the species which was used for raising the secondary antibody) overnight at 4 °C.
3. Rinse 3x with 1x PBS and incubate with primary antibodies overnight at 4 °C.
4. Rinse 3x with 1x PBS and incubate with secondary antibodies at room temperature for one hour.
5. Rinse 3x with 1x PBS
6. Counterstain the section for DNA using SYTOX green, or DAPI (or an alternative DNA dye).
7. Rinse 3x with 1x PBS
8. Embed the sections in an aqueous embedding medium.
9. Let the embedding medium polymerize for at least 48 hr.

7. Immunohistochemistry of Brain Sections of Perfused Animals

1. Block as described in step 6.2 and subsequently incubate the fixed brain sections in primary antibody solution diluted in PBS containing 0.3% Triton-X (free-floating) overnight at 4 °C.
2. Rinse three times with 1x PBS and incubate in secondary antibody solution diluted in PBS containing 0.3% Triton-X (free-floating) at room temperature for 3 hr.
3. Rinse 3x with 1x PBS
4. Counterstain the section for DNA using SYTOX green or DAPI (or an alternative DNA dye).
5. Rinse three times with 1x PBS
6. Embed the sections in an aqueous embedding medium.
7. Let the embedding medium polymerize for at least 48 hr.

8. Investigation of Mossy Fiber Projections

1. Sacrifice the animals, prepare 40 µm coronal sections as described above and stain the hippocampal section using antibody for neurofilament M.
2. Scan the sections at the appropriate wavelength using a confocal laser scanning microscope to visualize mossy fibers and nuclei. Start the scanning at low magnification.
3. Use the low magnification images for orientation and target the hippocampus with mossy fiber projections.
4. Scan the sections (z-sectioning) at high resolution and high magnification.
5. Analyze the morphological appearance and connectivity of the mossy fibers visualized via staining for NF-M.
6. (OPTIONAL) Analyze the size and volume of hippocampal blades. Use the DNA signal for the measurements.

9. Fluoro-Jade C Assay (Neuronal Cell Death)

1. Sacrifice the animals, dissect the hippocampus, and prepare 12 µm sections as described above.
2. Let the sections dry for 30 min at room temperature.
3. Fix the sections in 4% PFA for 40 min at room temperature.
4. Briefly wash the sections 3x with ddH₂O.
5. Incubate the sections with 0.06 % potassium permanganate (KMnO₄) for 10 min under continuous, gentle shaking.
6. Wash the sections 3x with ddH₂O.
7. Incubate the sections with 0.002% Fluoro-Jade C solution for 20 min at room temperature.
8. (OPTIONAL) For simultaneous nuclear stainings, 0.002% Fluoro-Jade C solution can be supplemented with 10 µg/ml DAPI.
9. Briefly wash the sections 3x with ddH₂O.
10. Let the sections dry for 30 min at room temperature.
11. Incubate the sections with Xylene (1 min)
12. Mount the sections using a Xylene-based mounting medium (D.P.X.).

Representative Results

Cross-breeding of the *IkB α* - and *tTA* transgenic mouse lines leads to conditional inhibition of NF- κ B activity in the hippocampus.

To investigate the expression of the *IkB α* -AA1-transgene in the double transgenic mouse (**Figure 1A**), brains were isolated, cryosectioned and stained using an antibody against GFP (green fluorescent protein). Confocal laser scanning microscopy revealed high expression of the transgene in the CA1 and CA3 regions, and in the DG (**Figure 1B**).

The *IkB α* -AA1-transgene is expressed in type-2b progenitors, and is active in intermediate immature states and in mature granule cells.

To determine the earliest cell type expressing the CAMKII α -driven *IkB α* -AA1 in the hippocampal neurogenic region, cryosections of hippocampi from *IkB/tTA* animals were stained for doublecortin (DCX) and the transgene (GFP). Confocal analysis revealed GFP-signals in young DCX-positive granule cells, indicating induction of the expression of the transgene by CAMKII α -activity (**Figure 2**, arrows).

Neuronal NF- κ B inhibition via *IkB α* -AA1 reduces mossy fiber projections, and the thickness and volume of the DG.

Coronal sections of the hippocampus of $\text{I}\kappa\text{B}/\text{tTA}$ and $\text{I}\kappa\text{B}/-$ mice were stained for neurofilament M (NF-M) to visualize mossy fiber projections, and reveal complex and fascicular organization (**Figure 3**). In $\text{I}\kappa\text{B}/\text{tTA}$ mice, the mossy fiber projections were severely impaired. In addition, NF- κB inhibition led to a significantly reduced suprapyramidal blade thickness and a smaller DG volume (**Figure 3B**).

Neuronal inhibition of NF- κB in $\text{I}\kappa\text{B}/\text{tTA}$ mice leads to elevated neuronal degeneration, increased neurogenesis, and a disturbed migration of DCX-expressing progenitors.

To study the potential reasons for the dramatic structural defects, fresh, unfixed hippocampal slices from $\text{I}\kappa\text{B}/\text{tTA}$ mice were stained with Fluoro-Jade C, which allows the specific detection of neuronal cell death (**Figure 4A**). Here, a dramatically increased number of degenerating neurites were observed in double transgenic animals, compared with single transgenic controls. Moreover, a significant increase of cleaved caspase-3-positive apoptotic cells was detected in $\text{I}\kappa\text{B}/\text{tTA}$ mice. By contrast, immunohistochemical staining against DCX revealed significantly increased amounts of DCX+ cells in the brains of $\text{I}\kappa\text{B}/\text{tTA}$ mice. Furthermore, DCX+ cells in $\text{I}\kappa\text{B}/\text{tTA}$ -hippocampi were not arranged exclusively in the subgranular zone, but were also found in the deeper regions of the DG (**Figure 4B**), whereas the DCX+-progenitors in the control animals were localized nearly exclusively within the subgranular zone. To test if the increased amount of DCX-expressing cells is a result of maturation of earlier progenitors or directly due to proliferation of DCX+ cells, BrdU was injected into the hippocampi of $\text{I}\kappa\text{B}/\text{tTA}$ and control mice, which were then cryosectioned and stained with DCX and BrdU (**Figure 4**). For analysis of proliferation, BrdU was injected once (see **Figure 4B**, scheme), followed by analysis after 24 hr. For analysis of differentiation, three injections were performed daily, followed by analysis after seven days. After a single BrdU-injection, no significant differences in the total number of BrdU-positive cells between $\text{I}\kappa\text{B}/\text{tTA}$ - and control animals were observed, whereas the three serial injections-approach revealed a clearly increased amount of BrdU/DCX+ cells in the DG of $\text{I}\kappa\text{B}/\text{tTA}$ mice. This indicates that disturbed differentiation or integration of DCX-expressing cells may have caused the increased numbers of DCX+ cells, and suggests that type-3 cells were involved.

Neuronal inhibition of NF- κB results in severe learning defects, as demonstrated by the Spatial Pattern Separation-Barnes Maze test. To specifically test the behavior of the double transgenic mice in a DG-dependent task, we used the SPS-BM, whereby *animals have to differentiate between locations with subtle differences* (**Figure 5**). In this behavioral test, the $\text{I}\kappa\text{B}/-$ control animals explored the food houses serially on the first day of the experiment (**Figure 5**). After seven days of training, the animals were able to find the open food house directly. By contrast, the $\text{I}\kappa\text{B}/\text{tTA}$ animals continued to explore the food houses serially, even after the training (**Figure 5B**). The observation of significant increases in the distances covered, and higher latencies and error rates in the $\text{I}\kappa\text{B}/\text{tTA}$ animals, compared with the $\text{I}\kappa\text{B}/-$ control animals, suggests that these animals had severe learning defects (**Figure 5C**).

Figure 1. Generation of a conditional forebrain-specific inhibition of NF- κB activity. **A.** Schematic drawing of the transgenic models used for studying the role of NF- κB in adult hippocampal neurogenesis. Calcium-calmodulin-dependent kinase II α (CAMKII α) promoter-driven tetracycline transactivator (tTA) mice were used to regulate the expression of a trans-dominant negative mutant $\text{I}\kappa\text{B}\alpha$ ($\text{I}\kappa\text{B}\alpha$ -AA1 super-repressor in $\text{I}\kappa\text{B}$ mice) combined with a green fluorescent protein tracer (GFP). In $\text{I}\kappa\text{B}/\text{tTA}$ mice, NF- κB is kept in the cytoplasm in its inactive state by nondegradable $\text{I}\kappa\text{B}\alpha$ -AA1, which can be detected via GFP-fluorescence. The inhibition can be reversed by doxycycline, which inhibits tTA. **B.** Representative transgene expression in different hippocampal regions of $\text{I}\kappa\text{B}/\text{tTA}$ mice (CA1, CA3 and DG). Cryosections were prepared from isolated brains of $\text{I}\kappa\text{B}/-$ and $\text{I}\kappa\text{B}/\text{tTA}$ mice, and were fixed and stained using an antibody against GFP. Note that the transgene is highly expressed within the DG. De: Dendrites, DG: Dentate Gyrus, ML: molecular layer; SL: Stratum lucidum. [Click here to view larger image.](#)

Figure 2. The IκBα-AA1 transgene is expressed in DCX-positive, type-2b progenitors and is active in further intermediate states and in mature granule cells. **A.** Cryosectioned hippocampi of IκB/tTA animals with doublecortin (DCX) staining and GFP expression reveal expression of the transgene in young DCX-positive granule cells (arrows). DG: Dentate Gyrus, ML: molecular layer. **B.** Schematic drawing of transgene expression in various developmental stages during granule cell development. IκBα-AA1-transgene expression was detected in the DCX-positive, type-2b progenitors, all intermediate immature states and in mature granule cells. By contrast, early type-1 and type-2a progenitors (lacking DCX-expression) did not express the transgene, and were therefore not affected by NF-κB inhibition. Modified after Kempermann *et al.*²⁰ [Click here to view larger image.](#)

Figure 3. NF- κ B inhibition leads to impaired mossy fiber projections and to a reduced DG thickness and volume in $\text{I}\kappa\text{B}/\text{tTA}$ mice, compared to single transgenic control ($\text{I}\kappa\text{B}^{-/-}$). **A.** Immunostaining of cryosectioned hippocampi for neurofilament M (NF-M) to visualize mossy fiber projections. In control mice ($\text{I}\kappa\text{B}^{-/-}$), a complex and fascicular organization of mossy fibers connecting granule cells to their target cells in CA3 is evident, which was impaired after neuronal inhibition of NF- κ B in $\text{I}\kappa\text{B}/\text{tTA}$ mice. Furthermore, expression of the super-repressor led to a significantly reduced suprapyramidal blade thickness (compare arrow in left and right panel) and a diminished DG volume. **B.** Quantification and statistical analysis of structural defects resulting from NF- κ B inhibition. Unpaired t-test, two-tailed. Data taken from the OA-version of Imielski *et al.*² [Click here to view larger image.](#)

Figure 4. Inhibition of NF- κ B in I κ B/tTA mice leads to increased neuronal cell death, enhanced neurogenesis, and a disturbed migratory pattern of DCX-expressing cells. **A.** Neuron-specific Fluoro-Jade C staining and increased numbers of cleaved caspase-3-positive cells in hippocampal sections reveals a higher level of neuronal cell death in I κ B/tTA mice than in the I κ B^{-/-} controls. Degenerating neurites are marked by arrows, and nuclei are indicated by asterisks. Right site: quantification of cleaved caspase-3-positive cells within the hippocampi suggests highly increased apoptosis in double transgenic animals. **B.** Staining of hippocampal cryosections for DCX reveals an increased number of DCX-expressing cells in the double transgenic animals. Note that DCX⁺ cells in I κ B/tTA-hippocampi are not arranged exclusively in the subgranular zone, but migrated deeper into the DG than in the I κ B^{-/-} control. **C. Left panel:** To test the influence of the transgene on proliferation, a single BrdU-injection was performed, which resulted in no significant differences in the number of BrdU-positive cells between I κ B/tTA and controls. **Right panel:** Statistical evaluation revealed a significant increase in DCX-expressing cells in I κ B/tTA animals after three daily BrdU-injections. The analysis was performed seven days after the last injection (test of differentiation and integration). A significant increase in BrdU/DCX⁺ was detected in the DG of I κ B/tTA mice ($n = 3$). Data partially taken from the OA-version of Imielski *et al.*² [Click here to view larger image.](#)

Figure 5. Set-up and typical results of spatial pattern separation-Barnes maze (SPS-BM). **A.** Left: Schematic drawing of the experimental set-up. Seven identical food houses (same color, size and shape) were placed symmetrically on defined spots of a round, home-made plate built from hard inert plastic (diameter of 120 cm), with one free spot (starting point, S). Multicolored extra-maze cues (EMC) are attached in front of a white-colored cloth in positions easily visible to the animals, approximately 100 cm from the border of the plate. To avoid orientation by odor, food pellets are deposited in every house, but only one food house is accessible (closure made of transparent foil). Right: Video camera focused on the plate (distance to plate: 115 cm), and a photograph of the set-up including the extra-maze cues. **B.** Typical experimental outcome of a SPS-BM test. The *IkB*^{-/-} control animal explores the food houses serially on the first day of the test series, and the open food house is found immediately after seven days of training. In contrast, *IkB*^{tTA} mice reveal poorer learning, as demonstrated by serial exploration even after the training phase. **C.** Comparison of parameters measured in the SPS-BM in *IkB*^{-/-} and *IkB*^{tTA} animals. Double transgenic mice show significant memory deficits ($n = 9$) compared to controls ($n = 8$) with respect to latency, distance covered and error rate. Two-way ANOVA evaluation; error bars: SEM. Data in B and C were taken from the OA-version of Imielski *et al.*² [Click here to view larger image.](#)

Discussion

Adult neurogenesis, and the possibility of its manipulation via inhibition of NF- κ B in neurons, and its later reactivation via doxycycline, offers a fascinating system for investigations into newborn neurons in the adult brain, as well as into neuronal de- and re-generation. The beauty of this system is that NF- κ B signaling pathway inhibition in neurons not only results in changes in neuronal cell death, progenitor proliferation and migration, and severe structural and anatomical changes, but also in obvious learning defects. Importantly, the phenotype of the *IkB*^{tTA} animals

can be experimentally reversed and rescued after administration of doxycyclin. This reversion of the phenotype includes structural aspects, and can also lead to significant improvement in DG-dependent learning².

To ensure a interpretable outcome for the behavioral experiments, it is critical that only male animals with an age difference of less than four days be used. Furthermore, all test series should be performed by the same operator. With regard to the testing room, we strongly recommend performing the test in an acoustically insulated environment to avoid stress and loss of attention resulting from external noise sources. To prevent orientation based on odor detection instead of extra-maze cues, the closed food houses should not be accessible to the animal, and normal air and odor circulation should be permitted. In addition, the plate used for the test should be built of odor-neutral and detergent-resistant material, and should be carefully cleaned between experiments. A further potential source of experimental variation is room illumination. The light source should be bright enough to allow image acquisition, the recognition of the extra-maze cues, and to induce flight behavior, but should not be of an intensity to blind the animal. Moreover, we suggest performing the tests for each animal at the same time of day to avoid circadian variations.

Mus musculus has a fairly good vision ability which is, in natural conditions, among others used to detect territorial boundaries at visually striking features (visual cues like trees) (Latham *et al.*, *Appl. Animal Behav. Sci.* **86**(3-4), 261-289, 2004). In a classic experiment Balkema *et al.* (*J. Neurophysiol.* **48**(4), 968-980, 1982) placed +6, 0, and -7 diopter lenses in front of mouse eyes and observed no significant changes of receptive field sizes in retinal ganglion cell. Due to this enormous depth of field visual stimuli (e.g. extra-maze cues) can be placed over a large range of distances in front of mice and remain in focus. In our approach, the distance of extra-maze cues from the starting point was 30 cm from the border of the plate, which is in accordance with the literature (e.g. 140 cm in Wong *et al.*, *Genes Brain Behav.* **5**(5), 389-403, 2006)

Rodents have the tendency to remain close to the walls of an open field. This behavior is defined as thigmotaxis (Barnett, SH, *The rat: a study in behavior*, Aldine publishing, Chicago, pp 31-32, 1963). A recent study by O'Leary *et al.* (*Behav. Brain Res.* **216**(2), 531-542, 2011) demonstrated that the C57BL/6J mice showed better learning in a Barnes Maze compared to 12 other mice strains. 28% of the mice tested used spatial search, whereas 64% used serial-thigmotactic strategy. This data holds true for small maze with a diameter of 69 cm with a 15 cm wall-surrounding. However, mice tested on a large maze without walls or intramaze cues, as our setup (diameter of 120 µm) have reliably shown evidence for the use of mainly extra-maze visual cues (see e.g. Bach *et al.*, *Cell.* **81**, 905-915, 1995). To avoid further physical cues e.g. caused by microstructure differences, the plate was rotated after each experiment.

The behavioral test can be simply adapted for investigating the impact of other transcription factors on adult hippocampal neurogenesis, using appropriate mouse models. However, it is mainly designed to test DG-dependent learning (spatial pattern separation), and may not be appropriate for the investigation of neurogenesis or neuronal degeneration in other brain regions. In this respect, the use of SPS-BM is further limited by its DG-specificity (strict dependence on a functional circuit between EC II and DG and CA3 and CA1 and EC VI), and should therefore not be applied for investigating tasks that involve the monosynaptic EC III - CA1 - EC V - circuit.

Future applications of the methods described herein may include investigating the effects of stem cell transplantations, pharmaceutical treatments on adult hippocampal neurogenesis, and tissue homeostasis within the hippocampus.

Disclosures

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Angela Kraleman-Köhler for excellent technical support. Experimental work described herein was performed in our laboratory and was supported by grants of the German Research Council (DFG) to CK and BK and a grant of the German Ministry of Research and Education (BMBF) to BK.

References

- Gutierrez, H. & Davies, A. M. Regulation of neural process growth, elaboration and structural plasticity by NF-kappaB. *Trends Neurosci.* **34**, 316-325, doi:10.1016/j.tins.2011.03.001 (2011).
- Imielski, Y. *et al.* Regrowing the adult brain: NF-kappaB controls functional circuit formation and tissue homeostasis in the dentate gyrus. *PLoS One.* **7**, e30838, doi:10.1371/journal.pone.0030838 (2012).
- Zheng, M. *et al.* Intrahippocampal injection of A beta(1-42) inhibits neurogenesis and down-regulates IFN-gamma and NF-kappaB expression in hippocampus of adult mouse brain. *Amyloid.* **20**, 13-20, doi:10.3109/13506129.2012.755122 (2013).
- Denis-Donini, S. *et al.* Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. *J. Neurosci.* **28**, 3911-3919 (2008).
- Bracchi-Ricard, V. *et al.* Astroglial nuclear factor-kappaB regulates learning and memory and synaptic plasticity in female mice. *J. Neurochem.* **104**, 611-623, doi:10.1111/j.1471-4159.2007.04993.x (2008).
- Koo, J. W., Russo, S. J., Ferguson, D., Nestler, E. J. & Duman, R. S. Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 2669-2674, doi:10.1073/pnas.0910658107 (2010).
- Fridmacher, V. *et al.* Forebrain-specific neuronal inhibition of nuclear factor-kappaB activity leads to loss of neuroprotection. *J. Neurosci.* **23**, 9403-9408 (2003).
- Whiteside, S. T. *et al.* N- and C-terminal sequences control degradation of MAD3/I kappa B alpha in response to inducers of NF-kappa B activity. *Mol. Cell. Biol.* **15**, 5339-5345 (1995).
- Mayford, M. *et al.* Control of memory formation through regulated expression of a CaMKII transgene. *Science.* **274**, 1678-1683 (1996).
- Rosenfeld, M. E., Prichard, L., Shiojiri, N. & Fausto, N. Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. *Am. J. Pathol.* **156**, 997-1007, doi:10.1016/S0002-9440(10)64967-X (2000).
- Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods.* **11**, 47-60 (1984).

12. Barnes, C. A. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. *J. Compar. Physiol. Psychol.* **93**, 74-104 (1979).
13. Brun, V. H. *et al.* Place cells and place recognition maintained by direct entorhinal-hippocampal circuitry. *Science*. **296**, 2243-2246, doi:10.1126/science.1071089 (2002).
14. Meshi, D. *et al.* Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. *Nat. Neurosci.* **9**, 729-731, doi:10.1038/nn1696 (2006).
15. Bakker, A., Kirwan, C. B., Miller, M. & Stark, C. E. Pattern separation in the human hippocampal CA3 and dentate gyrus. *Science*. **319**, 1640-1642, doi:10.1126/science.1152882 (2008).
16. Dupret, D. *et al.* Spatial relational memory requires hippocampal adult neurogenesis. *PLoS One*. **3**, e1959, doi:10.1371/journal.pone.0001959 (2008).
17. Leutgeb, J. K., Leutgeb, S., Moser, M. B. & Moser, E. I. Pattern separation in the dentate gyrus and CA3 of the hippocampus. *Science*. **315**, 961-966, doi:10.1126/science.1135801 (2007).
18. Kaltschmidt, B. *et al.* NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling. *Mol. Cell. Biol.* **26**, 2936-2946 (2006).
19. Clelland, C. D. *et al.* A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science*. **325**, 210-213, doi:10.1126/science.1173215 (2009).
20. Kempermann, G., Jessberger, S., Steiner, B. & Kronenberg, G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27**, 447-452 (2004).