

Manuscript Number: NSC-18-1935R2

Title: Reduced Hippocampal Neurogenesis in Mice Deficient in Apoptosis
Repressor with Caspase Recruitment Domain (ARC)

Article Type: Research Paper

Section/Category: Developmental Neuroscience

Keywords: disease-oriented adult neurogenesis
hippocampal neurogenesis
BDNF
apoptosis
nascent neurons
neuroprogenitors
neuroprogenitor proliferation
survival of neuroprogenitors
microglia

Corresponding Author: Professor Christoph Harms, MD

Corresponding Author's Institution: Charité-Universitätsmedizin Berlin

First Author: Golo Kronenberg, MD

Order of Authors: Golo Kronenberg, MD; Karen Gertz, MD; Ria Uhlemann, PhD; Melanie T Kuffner; Imke Kirste, PhD; Junfeng An, MD; Nafisa M Jadavji, PhD; Bjoern H Schott, MD; Thomas Scheffel, MD; Matthias Endres, MD; Rainer Hellweg, MD; Christoph Harms, MD

Abstract: In the adult hippocampal dentate gyrus (DG), the majority of newly generated cells are eliminated by apoptotic mechanisms. The apoptosis repressor with caspase recruitment domain (ARC), encoded by the Nrl3 gene, is a potent and multifunctional death repressor that inhibits both death receptor and mitochondrial apoptotic signaling. The aim of the present study was to parse the role of ARC in the development of new granule cell neurons. Nrl3 gene expression as revealed by in situ hybridization is present in the entire dentate granule cell layer. Moreover, a comparison of Nrl3 expression between FACS-sorted Sox2-positive neural stem cells and Doublecortin (DCX)-positive immature neurons demonstrates upregulation of Nrl3 during neurogenesis. Using ARC-deficient mice, we show that proliferation and survival of BrdU birth-dated cells is strongly reduced in the absence of ARC while neuronal-glial fate choice is not affected. Both the number of DCX-positive cells and the number of calretinin (CR)-positive immature postmitotic neurons is reduced in the hippocampus of ARC-/- mice. ARC knockout is not associated with increased numbers of microglia nor with microglia activation. However, hippocampal brain-derived neurotrophic factor (BDNF) protein content is significantly increased in ARC-/- mice, possibly representing a compensatory response. Collectively, our results suggest that ARC plays a critical cell-autonomous role in preventing cell death during adult granule cell neurogenesis.

Response to Reviewers: -----
Reviewers' comments:

Reviewer #1: Authors addressed all my concerns. I recommend to accept it.

Reviewer #2: Most of the points raised in my previous review have been addressed satisfactorily. Importantly, additional experiments aimed to address the role of ARC in proliferation of adult neuroblast (Ki67 staining), apoptosis (Casp3 staining), and microglial activation (Sholl analysis) have now been included in the study and greatly contribute to provide a more compelling evidence for a role of ARC in the regulation of survival of adult newborn neurons, the main conclusion of this study. There is however, a residual issue:

Figure 1C: please provide a more detailed description of the information that is presented in this plot. The figure legend indicates that the plot shows mRNA expression levels but the Y axis of the figure indicates "log2 fold change". I guess that points represent log2 fold change of Nl3 in Sox2+ and Dcx+ isolated cells but I miss what is the reference to which the mRNA expression levels have been compared to get the log2 fold change observed for each cell population (Sox2+ and Dcx+ cells). I could not find this information in the figure legend, methods or result section of the manuscript.

Response to Reviewer 2: We thank reviewer 2 for bringing up the point. We have now changed the y-axis title of figure 1C to log2 transformed + quantile normalized Nl3 mRNA. We have further changed the legend to figure 1 accordingly.

Please also note that your figures require some changes concerning the font sizes. Some lettering appear too small to be legible upon figure reproduction. Please size them as they will appear in the print version and determine that the font sizes are appropriately readable. we recommend using font sizes that are easily readable when the figures become sized for the print version, no smaller than 8 point for tick mark labels and legends, 10 or 11 point for axis labels and tick mark labels when there's no axis label, and 14 or 16 point for subplot labels (e.g., "A", "B", etc.).

Response to the Editor's advice: All figures have been adapted to increased font size as recommended.

CSB | Charité | Campus Mitte | 10117 Berlin

CharitéCentrum 15
Centrum für Schlaganfallforschung Berlin
Direktor: Prof. Dr. med. Andreas Meisel

Prof. Juan Lerma
Handling Editor

Prof. Artur Kania
Section Editor

Neuroscience

Prof. Dr. med. Christoph Harms
AG Molecular Stroke Research
Charitéplatz 1 | 10117 Berlin
Interne Geländeadresse:
Robert-Koch-Platz 4
3. OG | Raum 2830.3.10

Tel. +49 30 450 560 631
Fax +49 30 450 560 930

christoph.harms@charite.de
www.schlaganfallzentrum.de

Berlin, 2019-07-10

Dear Dr. Lerma, dear Dr. Kania,

Please find enclosed the revised manuscript entitled „Reduced Hippocampal Neurogenesis in Mice Deficient in Apoptosis Repressor with Caspase Recruitment Domain (ARC)“ in the category *Research paper*. We have changed the font size in all figures and followed the advice of reviewer 2 to change the y-axis title of figure 1C.

We thank you and the reviewers for your time.

Sincerely,



Christoph Harms

GEFÖRDERT VOM



Bundesministerium
für Bildung
und Forschung

MDC

MAX-DELBRÜCK-CENTRUM
FÜR MOLEKULARE MEDIZIN
BERLIN-BUCH
IN DER HELMHOLTZ-GEMEINSCHAFT e.V.



**HELMHOLTZ
GEMEINSCHAFT**

Reviewers' comments:

Reviewer #1: Authors addressed all my concerns. I recommend to accept it.

Reviewer #2: Most of the points raised in my previous review have been addressed satisfactorily. Importantly, additional experiments aimed to address the role of ARC in proliferation of adult neuroblast (Ki67 staining), apoptosis (Casp3 staining), and microglial activation (Sholl analysis) have now been included in the study and greatly contribute to provide a more compelling evidence for a role of ARC in the regulation of survival of adult newborn neurons, the main conclusion of this study. There is however, a residual issue:

Figure 1C: please provide a more detailed description of the information that is presented in this plot. The figure legend indicates that the plot shows mRNA expression levels but the Y axis of the figure indicates "log2 fold change". I guess that points represent log2 fold change of Nl3 in Sox2+ and Dcx+ isolated cells but I miss what is the reference to which the mRNA expression levels have been compared to get the log2 fold change observed for each cell population (Sox2+ and Dcx+ cells). I could not find this information in the figure legend, methods or result section of the manuscript.

Response to Reviewer 2: We thank reviewer 2 for bringing up the point. We have now changed the y-axis title of figure 1C to log2 transformed + quantile normalized Nl3 mRNA. We have further changed the legend to figure 1 accordingly.

Please also note that your figures require some changes concerning the font sizes. Some lettering appear too small to be legible upon figure reproduction. Please size them as they will appear in the print version and determine that the font sizes are appropriately readable. we recommend using font sizes that are easily readable when the figures become sized for the print version, no smaller than 8 point for tick mark labels and legends, 10 or 11 point for axis labels and tick mark labels when there's no axis label, and 14 or 16 point for subplot labels (e.g., "A", "B", etc.).

Response to the Editor's advice: All figures have been adapted to increased font size as recommended.

Highlights

- Reduced net hippocampal neurogenesis in ARC^{-/-} mice
- ARC knockout not associated with increased microglia density or activation
- Increased BDNF concentrations in microdissected left hippocampus of ARC^{-/-} mice

Abstract

In the adult hippocampal dentate gyrus (DG), the majority of newly generated cells are eliminated by apoptotic mechanisms. The apoptosis repressor with caspase recruitment domain (ARC), encoded by the *No/3* gene, is a potent and multifunctional death repressor that inhibits both death receptor and mitochondrial apoptotic signaling. The aim of the present study was to parse the role of ARC in the development of new granule cell neurons. *No/3* gene expression as revealed by in situ hybridization is present in the entire dentate granule cell layer. Moreover, a comparison of *No/3* expression between FACS-sorted Sox2-positive neural stem cells and Doublecortin (DCX)-positive immature neurons demonstrates upregulation of *No/3* during neurogenesis. Using ARC-deficient mice, we show that proliferation and survival of BrdU birth-dated cells is strongly reduced in the absence of ARC while neuronal-glial fate choice is not affected. Both the number of DCX-positive cells and the number of calretinin (CR)-positive immature postmitotic neurons is reduced in the hippocampus of *ARC^{-/-}* mice. ARC knockout is not associated with increased numbers of microglia nor with microglia activation. However, hippocampal brain-derived neurotrophic factor (BDNF) protein content is significantly increased in *ARC^{-/-}* mice, possibly representing a compensatory response. Collectively, our results suggest that ARC plays a critical cell-autonomous role in preventing cell death during adult granule cell neogenesis.

Key words: adult hippocampal neurogenesis, apoptosis, ARC, BDNF, microglia

Introduction

Apoptosis, i.e., programmed cell death, is a central mechanism in developmental neurobiology. In adulthood, apoptosis is strongly associated with brain areas of continuing neurogenesis (e.g., Petreanu & Alvarez-Buylla, 2002; Young et al., 1999). In the dentate gyrus (DG), the majority of TUNEL-positive cells are observed in the so-called 'neurogenic niche' at the border of the granule cell layer and the hilus (Biebl et al., 2000; Bengzon et al., 1997). The pronounced variation in the number of cells birth dated with synthetic nucleoside BrdU over time point to an important role of cell death among these newly produced cells in the DG (Nowakowski & Hayes, 2002).

Apoptosis repressor with a caspase recruitment domain (ARC) is a versatile and potent anti-apoptotic factor, which is highly expressed in the brain (Donath et al., 2006; Engidawork et al., 2001; Shelke & Leeuwenburgh, 2003). ARC, also known as nucleolar protein 3, is encoded by the *No/3* gene. ARC was initially recognized as an inhibitor of the extrinsic death receptor pathway. The caspase recruitment domain (CARD) of ARC exhibits homology to the prodomains of several apical caspases. By interacting with the initiator caspases 2 and 8, ARC protects against apoptosis induced by a variety of death receptors and their adaptors (Koseki et al., 1998). More recently, it has become apparent that through an interaction with Bax, ARC can also protect against cell death triggered by activation of the mitochondrial death pathway, preventing mitochondrial dysfunction and the intracellular release of pro-apoptotic molecules such as cytochrome c from the mitochondrion (Gustafsson et al., 2004). Moreover, ARC has recently been shown to inhibit TNF α induced regulated necrosis (Kung et al., 2014).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Its critical role in both the intrinsic and extrinsic apoptotic pathways alongside its robust expression in the brain make ARC a rather unique death repressor in the central nervous system (e.g., Donath et al., 2016). Considering the high proportion of newly generated cells in the DG that quickly undergo apoptosis (Nowakowski & Hayes, 2002), we hypothesized that ARC is an important regulator of hippocampal neurogenesis. We addressed this hypothesis by comprehensively assessing neurogenesis in ARC-deficient mice.

Materials and Methods

Animals and treatments

ARC-deficient mice (ARC^{-/-}) have been described in detail previously (Donath et al., 2006). In particular, knockout of both ARC mRNA and ARC protein has been demonstrated in heart, skeletal muscle, and brain (Donath et al., 2006). ARC null mice were backcrossed to C57/BL6 for more than ten generations. In brief, these animals are born and grow normally to adulthood without any overt abnormality in general health or appearance (Donath et al., 2006). Here, young adult (3-4 months) ARC^{-/-} mice and sex-matched wildtype littermates were used. Mice were maintained in a temperature (22°C ± 2°C) and humidity (55% ± 10%) controlled environment with a 12:12 h light-dark cycle and ad-libitum access to food and water. Thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) was administered intraperitoneally (i.p.) at a dose of 50 µg/g body weight at a concentration of 10 mg/ml (Kronenberg et al., 2010). All experimental procedures were approved by the relevant official committees and carried out in accordance with the Animal Welfare Act, the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the ARRIVE guidelines (Kilkenny et al., 2010).

Histological procedures

Animals were deeply anesthetized with ketamine and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were dissected and postfixed over night at +4°C. Brains were then transferred to a 30% sucrose solution (in 0.1 M phosphate buffer, pH 7.4) for dehydration. Subsequently, brains were frozen on dry ice and cut into 40-µm-thick coronal sections using a freezing microtome (Leica, Bensheim, Germany). All incubations and washes were performed

on 'free-floating' sections. BrdU antigen retrieval was done by incubation in 2 N HCl for 30 minutes at 37°C. Sections were then rinsed in 0.1 M borate buffer (pH 8.5) and thoroughly washed in Tris-buffered saline (TBS; pH 7.4). Primary and secondary antibodies were diluted in TBS supplemented with 0.1% Triton X-100, 0.1% Tween 20, and 3% donkey serum (TBS-plus). Labeled brain sections were mounted on microscope slides using a paintbrush.

Immunohistochemistry followed the peroxidase method with biotinylated secondary antibodies (all 1:500; Jackson ImmunoResearch Laboratories), ABC Elite reagent (Vector Laboratories), and diaminobenzidine (Sigma) as chromogen. Endogenous peroxidase activity was quenched with 0.6% H₂O₂ in TBS for 30 min. For immunofluorescence, FITC-, RhodX-, or Cy5-conjugated secondary antibodies were all used at a concentration of 1:250. Fluorescent sections were coverslipped in polyvinyl alcohol with diazabicyclooctane (DABCO) as antifading agent.

Primary antibodies were applied at the following dilutions: anti-BrdU (rat, 1:500; Harlan Seralab, Indianapolis, IN), anti-cleaved caspase-3 (rabbit, 1:100; Abcam, Cambridge, UK), anti-Ki67 (rabbit, 1:500; Novocastra, Newcastle upon Tyne, UK), anti-S100 β (rabbit, 1:2,500; SWant, Belinzona, Switzerland), anti-NeuN (mouse, 1:100; Chemicon, Temecula, CA), anti-doublecortin (goat, 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-calretinin (CR) (rabbit; 1:250; Swant), and anti-Iba1 (rabbit; 1:500; Wako Chemicals). Sections for Sholl analysis were counterstained with DAPI at a concentration of 1 μ g/ml.

Quantification and imaging

1 In order to quantify neurogenesis in the DG, cell counts were determined in one-in-six
2 series of sections covering the entire hippocampus in its rostrocaudal extent as
3 described in greater detail previously (Kempermann et al., 2003; Kronenberg et al.,
4 2003 and 2007). Briefly, cells located in the granule cell layer and adjacent
5 subgranular zone, defined as a two cell bodies wide zone at the granule cell
6 layer/hilus border, were counted (Brown et al., 2003). Cells in the uppermost focal
7 plane were excluded to avoid oversampling. The density of microglia in the
8 hippocampus was quantified using a stereology-based approach (Uhlemann et al.,
9 2016). Phenotypic analysis of BrdU-labeled cells was performed using a spectral
10 confocal microscope (TCS SP2; Leica). Appropriate gain and black level settings
11 were determined on control slices stained with secondary antibodies alone.

12 Sholl Analysis of microglia

13 Z-stacks of adjacent regions of the dentate gyrus were acquired with a spinning disk
14 confocal microscope (Nikon Ti2-scope equipped with with an Andor Revolution CSU-
15 X1 spinning disk unit and iXon DU-888 ultra EMCCD camera) using a 60x-oil
16 immersion objective (NA 1.4). DAPI and Iba1 were excited with 10% 405 nm (15
17 mW) and 20% 561 nm (15mW) and bandpass filtered (425/45 and 685/40,
18 respectively) before detection.

19 The images were segmented using the ImageJ Simple Neurite Tracing tool and
20 Hessian-based analysis (Longair et al., 2011). Microglial processes were traced in x,
21 y, and z direction starting from the soma center. The starting point was used as the
22 initial point for Sholl analysis. Analysis parameters were radius intervals of 2 μ m on a
23 standard axis and a linear profile. The automated calculation of the maximum branch
24 length and Schoenen ramification index was done by the Sholl Analysis Tool in

ImageJ (Norris et al., 2014; Ferreira et al., 2014). The resulting counts of 3 samples per animal were pooled and used for analysis.

Quantification of BDNF levels

After sacrifice, brains were quickly removed. The left hippocampus was dissected on a cold plate, snap-frozen in liquid nitrogen, and stored at -80 °C until further use. Endogenous levels of BDNF were measured in the thawed homogenates using a commercial ELISA in principle according to the manufacturer's instructions (Promega), but adapted to a highly sensitive fluorometric technique as described in detail earlier (Hellweg et al., 2007). BDNF content is expressed as equivalents of recombinant human BDNF. The detection limit of the assay was 1 pg/ml. Determinations of recovery and specific and unspecific neurotrophin binding (the latter against mouse IgG1 obtained from MOPC 21) involved triplicate fluorescence determinations for each sample. Using this optimized fluorometric ELISA, it was possible to measure BDNF in brain tissue with a minimal wet weight of approximately 5 pg (Hellweg et al., 2003 and 2006). BDNF levels are expressed as picograms per milligram of tissue (wet weight).

Statistical analysis

Experiments were performed in a blinded fashion. Data are presented as scatter plots with mean \pm 95% confidence interval (Figures 2-6) or as median with interquartile range (Figure 1C). Means were compared by two-tailed unpaired Student's t-test (Figures 2-6) or using false discovery rate (FDR) adjusted p-values as described for GSE21208 (Benjamini & Hochberg, 1995 and Bracko et al., 2012). Exact p values are provided in the figures. Statistical analysis was performed using GraphPad Prism version 6.07.

Results

Nucleolar protein 3 (apoptosis repressor with CARD domain) mRNA is strongly expressed in the dentate granule cell layer of adult mice

In situ hybridization with probes for *No/3* yields strong expression in the adult hippocampus (Figure 1A) and, in particular, in the entire DG (Figure 1B; image credit: Allen Institute [Lein et al., 2007]). Based on this observation, we wondered whether *No/3* mRNA expression is upregulated during granule cell neogenesis. We therefore examined *No/3* expression in a microarray dataset of FACS sorted neural stem cells and immature neurons (Bracko et al., 2012; GEO accession number GSE21208). Neural stem cells (NSCs) were isolated from the micro-dissected adult dentate gyrus using transgenic mice expressing GFP under the *Sox2* promoter (*Sox2*-positive cells). Immature neurons were identified using a *DsRed* reporter under control of the *DCX* promoter. Indeed, *No/3* is transcriptionally upregulated in *DCX*-positive immature neurons as compared to NSCs (Figure 1C; GSM530325, GSM50327, and GSM530328: Hip *Sox2* A-C versus GSM530320, GSM530322, and GSM530331: Hip *DCX* A-C).

*Reduced net neurogenesis in the hippocampus of *ARC*^{-/-} mice*

Neurogenesis in the adult DG is a unique form of plasticity that entails the generation of functional granule neurons from proliferating precursor cells (Kempermann et al., 2004; van Praag et al., 2002; Weber et al., 2013). Distinct developmental phases of hippocampal neurogenesis have been described (e.g., Kempermann et al., 2015). We started by investigating the effects of the *ARC* null genotype on precursor cell

1 proliferation and on the survival of newly generated BrdU-positive cells (Figure 2A,
2 B). ARC deficiency resulted in fewer BrdU-positive cells in the 'proliferation condition',
3
4 i.e., 24 h after a 5-day course of once daily i.p. BrdU (Figure 2G). Furthermore, lack
5
6 of ARC profoundly compromised the survival of newly generated cells as assessed
7
8 four weeks from the beginning of a 5-day course of once daily i.p. BrdU ('survival
9
10 condition'; Figure 2A, B, H).
11
12

13 Doublecortin (DCX) and calretinin (CR) are frequently used as BrdU independent
14
15 surrogate markers of neurogenesis (Brandt et al., 2003; Encinas et al., 2006;
16
17 Kronenberg et al., 2007). DCX expression signals neuronal lineage determination
18
19 (Couillard-Despres et al., 2003; Kronenberg et al., 2003; Rao & Shetti, 2004),
20
21 whereas CR represents a marker of early postmitotic granule cells (Brandt et al.,
22
23 2003). Again, in the absence of ARC, these 'milestones' of neurogenesis
24
25 (Kempermann et al., 2004) were significantly reduced (Figure 2C-F, I-J).
26
27
28
29
30

31 Next, we used Ki67, a cell cycle protein, as an endogenous marker of cell
32
33 proliferation in the hippocampal dentate gyrus (Figure 3A, B). This analysis provides
34
35 a more exact picture of cell proliferation in the neurogenic niche than the 5-day
36
37 course of once daily BrdU (Figure 2A) because quite significant early cell loss may
38
39 occur over this period of time. Interestingly, Ki67 immunohistochemistry did not yield
40
41 significant differences between genotypes (Figure 3A, B). After that, we used the
42
43 active form of caspase-3 as an immunohistological tool to detect apoptotic cells. We
44
45 detected more activated caspase 3-positive cells in the dentate gyrus of $ARC^{-/-}$ mice
46
47
48
49
50
51 than in the dentate gyrus of wildtype controls.
52
53
54
55

56 No significant difference in neuronal differentiation of newly generated cells between
57
58 $ARC^{-/-}$ and wildtype mice
59
60
61
62
63
64
65

Using triple immunofluorescence and confocal microscopy, we performed a phenotypic analysis of BrdU-positive cells in the survival condition (Figure 4). NeuN was used as a marker of neuronal differentiation (Figure 4A, B), while S100 β was used to identify new astrocytes (Mullen et al., 1992; Steiner et al., 2004; Kempermann et al., 2003; Figure 4C). Cells expressing neither NeuN nor S100 β were categorized as 'other' (Figure 4D). The fractions of newly born cells falling into each category did not differ significantly between ARC^{-/-} mice and wildtype controls (Figure 4B-D).

No significant difference in microglia density and microglia activation in hippocampus between ARC^{-/-} and wildtype mice

Pro-inflammatory signaling and microglia activation may profoundly inhibit hippocampal neurogenesis (e.g., Appel et al., 2018; Ekdahl et al., 2003; Monje et al., 2003; Woodbury et al., 2015). We therefore investigated the density of Iba1-positive microglia in hippocampus of ARC^{-/-} and wildtype mice (Figure 5A, B). No significant differences in Iba1 expression were detected between genotypes.

Microglia activation is reflected by morphological changes. Representative images of maximum intensity projections of confocal Z-stacks of wildtype controls and ARC^{-/-} mice showed no apparent morphological differences (Figure 5C, D). We performed a Sholl analysis of microglia using 3D tracing of confocal image stacks (Figure 5E). The total number of processes per microglia (Figure 5F), Schoenen Ramification Index (Figure 5G), and the number of intersections per microglia (Figure 5H) were measured. Again, comparison of ARC^{-/-} animals and wildtype controls yielded no significant differences. The length of the longest process determined by the maximal radius length (Figure 5I) also did not differ significantly between genotypes despite a strong tendency towards longer processes in ARC^{-/-} mice (p=0.051).

Increased BDNF protein concentrations in hippocampus of ARC^{-/-} mice

BDNF has been shown to be essential for the survival of neurons, specifically in populations that undergo continuous regeneration, i.e., in the DG and in the olfactory bulb (Linarsson et al., 2000). BDNF expression was measured at the protein level in dissected left hippocampus of ARC^{-/-} mice and wildtype controls. BDNF protein concentrations were significantly increased in ARC^{-/-} mice (Figure 6).

Discussion

ARC is a potent endogenous inhibitor of programmed cell death that blocks both the intrinsic and extrinsic apoptotic pathways as well as TNF α -induced regulated necrosis (Gustafsson et al., 2004; Koseki et al., 1998; Kung et al., 2014). Using constitutive ARC^{-/-} mice, we here investigated the neurogenesis phenotype in the absence of this versatile and potent CNS death repressor. Our study yielded the following major findings: (i) The proliferation and survival of BrdU birth-dated cells is strongly reduced in the absence of ARC, while neuronal-glial fate choice is not affected. (ii) Consequently, net neurogenesis in the DG is also robustly decreased in ARC knockout mice. This finding is reinforced by BrdU-independent evidence in that both the number of DCX-positive neuroblasts and, even more so, the number of CR-positive immature postmitotic neurons is reduced in the hippocampus of ARC^{-/-} mice. (iii) Remarkably, these effects of the ARC knockout are not associated with increased numbers of microglia in the hippocampus nor with microglia activation as assessed by Sholl analysis. (iv) An increase in activated caspase 3- positive cells indicates an anti-apoptotic action of ARC in early neuronal development while the number of actively proliferating Ki67-positive cells was not altered between genotypes. (v) BDNF expression measured at the protein level in dissected left hippocampus was significantly increased in ARC^{-/-} mice.

This report builds on earlier work investigating the role of apoptosis in hippocampal neurogenesis. Briefly, a synthesis of the existing knowledge paints the following picture: Broadly speaking, the density of dying cells is increased in neurogenic brain areas such as the olfactory bulb or the DG (Biebl et al., 2000). Interestingly, an

analysis of mRNA expression data from the Allen Brain Atlas (Lein et al., 2007) indicates strong *No/3* expression in these regions. Equally notably, the excessive cell death that occurs in mice lacking BDNF is also restricted to these same brain areas, supporting the concept that BDNF is essential for the survival of neurons specifically in populations that are continuously being regenerated in the mammalian brain (Linnarsson et al., 2000).

In the DG, the number of BrdU-labeled cells declines sharply at early time points after injection (e.g., Kempermann et al., 2004; Kronenberg et al., 2003; Sierra et al., 2015). Moreover, most TUNEL-positive cells reside at the border between the hilus and the granule cell layer (Biebl et al., 2000), giving further credence to the notion that the bulk of newly generated cells is eliminated at early developmental stages while still in close proximity to the neurogenic niche (Biebl et al., 2000). Transgenic mice overexpressing the anti-apoptotic protein Bcl-2 under control of the neuron-specific enolase promoter exhibit a doubling in the rate of net hippocampal neurogenesis (Kuhn et al., 2005) – a finding that further illustrates the size of the effect that programmed cell death has on neurogenesis. Finally, more recently, ramified (i.e., not activated) microglia have been shown to remove apoptotic newborn cells rapidly through phagocytosis in the intact DG (Sierra et al., 2010). Our results indicate that the increased loss of newborn cells in *ARC*^{-/-} mice is also insufficient to elicit an increase in microglia density or any discernible microglia activation.

Exciting new research involving live imaging of radial glia-like cells and their progeny suggests that the early wave of cell death during hippocampal neurogenesis is governed by as yet unidentified intrinsic mechanisms (Pilz et al., 2018). After a limited number of cell divisions resulting in a transient burst in neurogenic activity, neural stem and progenitor cells are rapidly lost (Pilz et al., 2018). Our results

highlight the crucial importance of ARC as a key anti-apoptotic molecule that effectively tips the scales toward early neuronal survival. The observation that *No/3* mRNA expression is upregulated in DCX-DsRed positive immature neurons compared to Sox2-GFP positive NSCs is in line with the idea that cell-autonomous activation of anti-apoptotic pathways (i.e., relating to the mitochondrial death pathway) at a very early stage is a crucial step in hippocampal neurogenesis. Interestingly, the effects of ARC knockout seem to accrue over the course of neurogenesis. Descriptively, the reduction in BrdU-positive cells in *ARC^{-/-}* mice was more pronounced in our survival condition than in our proliferation condition. Similarly, the difference between genotypes appears somewhat stronger in the case of postmitotic CR-positive neurons than in the case of more immature DCX-positive neuroblasts. These observations are in good agreement with earlier studies that have described an early wave of cell death within the initial days of cell birth and a second delayed phase of cell loss or survival that is input-dependent and involves integration of surviving cells into hippocampal circuitry (Pilz et al., 2018; Sierra et al., 2010; Tashiro et al., 2006). Most likely, ARC, as a multifunctional death repressor, confers protection against both the initial and the delayed phases of programmed cell death over the entire course of neurogenesis.

BDNF is generally held to promote neurogenesis. However, even today, the precise mechanisms controlling BDNF levels in brain remain to be better understood. Exercise and antidepressants have been shown to increase hippocampal BDNF transcription (e.g. Neeper et al., 1995; Russo-Neustadt et al., 1999). Mice with either reduced BDNF concentrations (*BDNF^{+/-}* mice) or impaired tyrosine receptor kinase B (TrkB) activation (*trkB.T1*-overexpressing mice) show reduced long-term survival of new neurons (Sairanen et al., 2005). Furthermore, ablation of TrkB in neural

precursor cells leads to impaired proliferation and neurogenesis alongside behavioral insensitivity to antidepressants or exercise (Li et al., 2008). Seemingly paradoxical increases in BDNF content have been reported under pathophysiological conditions such as in mice lacking brain serotonin (Kronenberg et al., 2016) or in aged APP23 transgenic mice (Burbach et al., 2004). It is tempting to speculate that our finding of increased hippocampal BDNF protein content in $ARC^{-/-}$ mice may also represent a form of compensatory counterregulatory mechanism in the face of increased cell death. However, we acknowledge that our study does not provide further mechanistic insights into the link between absence of ARC and increased BDNF concentrations in hippocampus.

In summary, there are several novel findings from this study. Most importantly, we demonstrate that ARC imparts anti-apoptotic properties to newborn cells over the course of hippocampal neurogenesis, thereby increasing the number of newly generated granule neurons. ARC knockout does not lead to microglia activation or increased microglia density. Increased hippocampal BDNF content in $ARC^{-/-}$ mice may represent a compensatory mechanism.

Acknowledgements

The authors thank Annemarie Bunge, Melanie Kroh, Janet Lips, and Stefanie Balz for excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB TRR43 and Exc257 to M.E.; KR 2956/4-1 to G.K.; GE 2576/2-1 to K.G.; HA 5741/5-1 to C.H. and Berlin-Brandenburg School for Regenerative Therapies GSC 203), the Bundesministerium für Bildung und Forschung (CSB 01 EO 1301 to M.E., K.G., G.K., and CH.), the German Center for Neurodegenerative Diseases (DZNE to M.E.), the German Center for Cardiovascular Research (DZHK to M.E.), and the Corona Foundation (to M.E.). Berlin Institute of Health (TRG7 to C.H.). We thank the Advanced Medical Bioimaging Core Facility (AMBIO) of the Charité for support in the acquisition of imaging data.

References

Appel JR, Ye S, Tang F, Sun D, Zhang H, Mei L, Xiong WC (2018) Increased Microglial Activity, Impaired Adult Hippocampal Neurogenesis, and Depressive-like Behavior in Microglial VPS35-Depleted Mice. *J Neurosci* 38(26):5949-5968

Bengzon J, Kokaia Z, Elmér E, Nanobashvili A, Kokaia M, Lindvall O (1997) Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7

Benjamini, Y, Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 57:289-300.

Biebl M, Cooper CM, Winkler J, Kuhn HG (2000) Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neurosci Lett* 291:17-20

Bracko O, Singer T, Aigner S, Knobloch M, Winner B, Ray J, Clemenson GD, Jr., Suh H, Couillard-Despres S, Aigner L, Gage FH and Jessberger S (2012) Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. *J Neurosci* 32:3376-87

Brandt MD, Jessberger S, Steiner B, Kronenberg G, Reuter K, Bick-Sander A, von der Behrens W, Kempermann G (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. *Mol Cell Neurosci* 24: 603-613.

1
2 Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG
3
4 (2003) Transient expression of doublecortin during adult neurogenesis. J Comp
5
6 Neurol 467(1):1-10.
7
8
9

10
11 Burbach GJ, Hellweg R, Haas CA, Del Turco D, Deicke U, Abramowski D, Jucker M,
12
13 Staufenbiel M, Deller T (2004) Induction of brain-derived neurotrophic factor in
14
15 plaque-associated glial cells of aged APP23 transgenic mice. J Neurosci
16
17 24(10):2421-30
18
19
20
21
22

23
24 Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N,
25
26 Bogdahn U, Winkler J, Kuhn HG, Aigner L. 2005. Doublecortin expression levels in
27
28 adult brain reflect neurogenesis. Eur J Neurosci 21: 1-14.
29
30
31
32

33
34 Donath S, Li P, Willenbockel C, Al-Saadi N, Gross V, Willnow T, Bader M, Martin U,
35
36 Bauersachs J, Wollert KC, Dietz R, von Harsdorf R; German Heart Failure Network
37
38 (2006) Apoptosis repressor with caspase recruitment domain is required for
39
40 cardioprotection in response to biomechanical and ischemic stress. Circulation
41
42 113(9):1203-12
43
44
45
46
47

48
49 Donath S, An J, Lee SL, Gertz K, Datwyler AL, Harms U, Müller S, Farr TD,
50
51 Fächteimeier M, Lättig-Tünnemann G, Lips J, Foddiss M, Mosch L, Bernard R, Grittner
52
53 U, Balkaya M, Kronenberg G, Dirnagl U, Endres M, Harms C (2016) Interaction of
54
55 ARC and Daxx: A Novel Endogenous Target to Preserve Motor Function and Cell
56
57 Loss after Focal Brain Ischemia in Mice. J Neurosci 36(31):8132-48
58
59
60
61
62
63
64
65

1
2 Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O (2003) Inflammation is
3
4 detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A 100(23):13632-
5
6
7 7.
8
9

10
11 Encinas JM, Vaahtokari A, Enikolopov G (2006) Fluoxetine targets early progenitor
12
13 cells in the adult brain. Proc Natl Acad Sci 103: 8233-8238
14
15
16

17
18 Engidawork E, Gulesserian T, Yoo BC, Cairns N, Lubec G (2001) Alteration of
19
20 caspases and apoptosis-related proteins in brains of patients with Alzheimer's
21
22 disease. Biochem Biophys Res Commun 281(1):84-93.
23
24
25

26
27 Ferreira TA, Blackman AV, Oyrer J, Jayabal S, Chung AJ, Watt AJ, Sjostrom PJ, van
28
29 Meyel DJ (2014) Neuronal morphometry directly from bitmap images. Nat Methods,
30
31 11 (2014) 982-984.
32
33
34

35
36 Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA (2004) Apoptosis
37
38 repressor with caspase recruitment domain protects against cell death by interfering
39
40 with Bax activation. J Biol Chem 279:21233–21238.
41
42
43

44
45 Hayes NL, Nowakowski RS (2002) Dynamics of cell proliferation in the adult dentate
46
47 gyrus of two inbred strains of mice. Brain Res Dev Brain Res 134(1-2):77-85.
48
49
50

51
52 Hellweg R, Lohmann P, Huber R, Kühl A, Riepe MW (2006) Spatial navigation in
53
54 complex and radial mazes in APP23 animals and neurotrophin signaling as a
55
56 biological marker of early impairment. Learn Mem 13(1):63-71
57
58
59
60
61

Hellweg R, von Arnim CA, Büchner M, Huber R, Riepe MW (2003) Neuroprotection and neuronal dysfunction upon repetitive inhibition of oxidative phosphorylation. *Exp Neurol* 183(2):346-54

Hellweg R, Zueger M, Fink K, Hörtnagl H, Gass P (2007) Olfactory bulbectomy in mice leads to increased BDNF levels and decreased serotonin turnover in depression-related brain areas. *Neurobiol Dis* 25(1):1-7

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* 130(2):391-9

Kempermann G, Jessberger S, Steiner B, Kronenberg G. 2004. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27: 447-452

Kempermann G, Song H, Gage FH (2015) Neurogenesis in the Adult Hippocampus. *Cold Spring Harb Perspect Biol* 7(9):a018812

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8: e1000412

Koseki T, Inohara N, Chen S, Nunez G (1998) ARC, an inhibitor of apoptosis expressed in skeletal muscle and heart that interacts selectively with caspases. *Proc Natl Acad Sci U S A* 95:5156 -5160

Kronenberg G, Lippoldt A, Kempermann G (2007) Two genetic rat models of arterial hypertension show different mechanisms by which adult hippocampal neurogenesis is increased. *Dev Neurosci* 29(1-2):124-33

Kronenberg G, Mosienko V, Gertz K, Alenina N, Hellweg R, Klempin F (2016) Increased brain-derived neurotrophic factor (BDNF) protein concentrations in mice lacking brain serotonin. *Eur Arch Psychiatry Clin Neurosci* 266(3):281-4

Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G (2003) Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. *J Comp Neurol* 467(4):455-63

Kronenberg G, Gertz K, Baldinger T, Kirste I, Eckart S, Yildirim F, Ji S, Heuser I, Schröck H, Hörtnagl H, Sohr R, Djoufack PC, Jüttner R, Glass R, Przesdzing I, Kumar J, Freyer D, Hellweg R, Kettenmann H, Fink KB, Endres M (2010) Impact of actin filament stabilization on adult hippocampal and olfactory bulb neurogenesis. *J Neurosci* 2010 Mar 3;30(9):3419-31

Kuhn HG, Biebl M, Wilhelm D, Li M, Friedlander RM, Winkler J (2005) Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. *Eur J Neurosci* 22(8):1907-15

Kung G, Dai P, Deng L, Kitsis RN (2014) A novel role for the apoptosis inhibitor ARC in suppressing TNF α -induced regulated necrosis. *Cell Death Differ* 21(4):634-44

1 Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski
2 MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook
3 BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare
4 TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin
5 LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN,
6 Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP,
7 Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramée
8 AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan
9 JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak
10 TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR,
11 Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivasay T,
12 Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR,
13 Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR,
14 Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu
15 MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR (2007)
16 Genome-wide atlas of gene expression in the adult mouse brain. *Nature*
17 445(7124):168-76
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42

43 Linnarsson S, Willson CA, Ernfors P (2000) Cell death in regenerating populations of
44 neurons in BDNF mutant mice. *Brain Res Mol Brain Res* 75(1):61-9
45
46
47
48
49
50

51 Longair MH, Baker DA, Armstrong JD (2011) Simple Neurite Tracer: open source
52 software for reconstruction, visualization and analysis of neuronal processes.
53 *Bioinformatics*, 27 (2011) 2453-2454.
54
55
56
57
58
59
60
61
62
63
64
65

1 Monje ML, Toda H, Palmer TD (2003) Inflammatory blockade restores adult
2 hippocampal neurogenesis. *Science* 302(5651):1760-5
3
4
5

6
7 Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in
8 vertebrates. *Development* 116(1):201-11
9
10

11
12
13
14 Neeper SA, Gómez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain
15 neurotrophins. *Nature* 373(6510):109.
16
17
18

19
20
21 Norris G, Derecki N, Kipnis J (2014) Microglial Sholl Analysis. 26 August 2014,
22 PROTOCOL (Version 1) available at Protocol Exchange
23 [+<https://doi.org/10.1038/protex.2014.029+>]
24
25
26
27

28
29
30
31 Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb
32 granule neurons: role of olfaction. *J Neurosci* 22(14):6106-13.
33
34
35

36
37
38 Pilz GA, Bottes S, Betizeau M, Jörg DJ, Carta S, Simons BD, Helmchen F,
39 Jessberger S (2018) Live imaging of neurogenesis in the adult mouse hippocampus.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

1 Russo-Neustadt A, Beard RC, Cotman CW (1999) Exercise, antidepressant
2 medications, and enhanced brain derived neurotrophic factor expression.
3
4 Neuropsychopharmacology 21(5):679-82
5
6
7

8
9 Sairanen M, Lucas G, Ernfors P, Castrén M, Castrén E (2005) Brain-derived
10 neurotrophic factor and antidepressant drugs have different but coordinated effects
11 on neuronal turnover, proliferation, and survival in the adult dentate gyrus. J Neurosci
12
13 25(5):1089-94
14
15
16
17
18

19
20
21 Shelke RR, Leeuwenburgh C (2003) Lifelong caloric restriction increases expression
22 of apoptosis repressor with a caspase recruitment domain (ARC) in the brain. FASEB
23
24 J 17(3):494-6
25
26
27
28

29
30
31 Sierra A, Martín-Suárez S, Valcárcel-Martín R, Pascual-Brazo J, Aelvoet SA, Abiega
32 O, Deudero JJ, Brewster AL, Bernalles I, Anderson AE, Baekelandt V, Maletić-Savatić
33 M, Encinas JM (2015) Neuronal hyperactivity accelerates depletion of neural stem
34 cells and impairs hippocampal neurogenesis. Cell Stem Cell 16(5):488-503
35
36
37
38
39
40

41
42
43 Steiner B, Kronenberg G, Jessberger S, Brandt MD, Reuter K, Kempermann G
44 (2004) Differential regulation of gliogenesis in the context of adult hippocampal
45 neurogenesis in mice. Glia. 2004 Apr 1;46(1):41-52.
46
47
48
49
50

51
52
53 Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH (2006) MDA-receptor-mediated,
54 cell-specific integration of new neurons in adult dentate gyrus. Nature 442(7105):929-
55
56
57
58 33
59
60
61
62

Uhlenmann R, Gertz K, Boehmerle W, Schwarz T, Nolte C, Freyer D, Kettenmann H,
Endres M, Kronenberg G (2016) Actin dynamics shape microglia effector functions.
Brain Struct Funct 221(5):2717-34

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002)
Functional neurogenesis in the adult hippocampus. Nature 415(6875):1030-4

Woodbury ME, Freilich RW, Cheng CJ, Asai H, Ikezu S, Boucher JD, Slack F, Ikezu
T (2015) miR-155 Is Essential for Inflammation-Induced Hippocampal Neurogenic
Dysfunction. J Neurosci 35(26):9764-81

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental
enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective.
Nat Med 5(4):448-53

Legends

Figure 1. Nucleolar protein 3 mRNA expression in the hippocampal dentate gyrus

(A) In situ hybridization (ISH) in male P56 mice demonstrates presence of *No/3* (ARC) in brain regions with continuing adult neurogenesis (hippocampus, subventricular zone, rostral migratory stream, and olfactory bulb). Image credit: Allen Institute. (B) Higher magnification demonstrates *No/3* expression throughout the entire granule cell layer. Image credit: Allen Institute. (C) Array data of neural stem cells (Sox2 positive) and their early neuronal progeny (DCX-positive) isolated from the micro-dissected dentate gyrus of 6- to 8-week-old mice have recently been published by Bracko and co-workers (Bracko et al., 2012). Using Geo2R, we examined *No/3* mRNA expression. *No/3* was significantly increased in DCX-positive immature neurons relative to Sox2-positive NSCs (false discovery rate adjusted p-value, $n = 3$, **log2 transformed and quantile normalized *No/3* mRNA**, gene ID 7842, GEO accession number GSE21208). Scale bar in (A), 1400 μm . Scale bar in (B), 100 μm .

Figure 2. Milestones of hippocampal neurogenesis in *ARC*^{-/-} mice

To quantify the survival of newly generated cells, mice were killed four weeks from the beginning of a 5-day course of once daily BrdU. Representative images of this staining are shown in (A, B) and quantified in (H). Reduced numbers of DCX-positive cells (C, D, I) and of CR-positive cells in *ARC*^{-/-} mice (E, F, J). $N = 8-9$ animals per group. Scale bar in (F), 200 μm . In the absence of ARC, proliferation (G) and survival (H) of BrdU birth-dated cells are significantly reduced. Cell proliferation in the dentate

gyrus was quantified after a 5-day course of once daily intraperitoneal BrdU. Mice were killed on day 6 (G).

Figure 3. Cell proliferation and apoptosis

The absence of ARC did not exert a significant effect on the number of Ki67-positive cells (A, B). The number of activated caspase 3-positive cells was significantly increased in ARC^{-/-} mice (A, C). N=6-7 mice per group. Scale bar 200 μ m.

Figure 4. Phenotypic analysis of newly generated cells

The phenotypes of newly generated cells were analyzed four weeks from the beginning of a 5-day course of once daily BrdU. Neuronal marker NeuN (green in A) and astrocytic marker S100 β (blue in A) were used for phenotypic analysis of BrdU-positive (red in A) cells. BrdU cells designated as “other” lacked expression of NeuN and S100 β . N = 8 animals per group. Scale bar in (A) 50 μ m.

Figure 5. Iba1 expression and Sholl analysis of microglia

The density of Iba1-expressing microglia in hippocampus did not differ between ARC^{-/-} mice and wildtype mice. N=6-7 mice per group. Scale bar 100 μ m (A, B). Sholl analysis of microglia at the border of the dentate gyrus (C – I). Iba1 staining with DAPI counterstaining of wildtype (C) and ARC^{-/-} mice (D). Representative image of three dimensional traced microglial processes (pink) in a 3D reconstruction of a Z-stack image of microglia (E). There were no significant changes in the number of processes, ramification index, mean intersections per cell, and maximal process length. N= 6-7 mice per group. The total number of processes per microglia were almost identical between genotypes (F). The Schoenen ramification index is a marker of microglial branching and is dependent on the maximal number of crossings in a

given Sholl radius (G). The number of mean intersections per cell represents the total number of intersections in one microglia divided by the number of intersecting radii (H). Maximal process length shows a trend toward longer processes in $ARC^{-/-}$ animals ($p = 0.051$; I).

Figure 6. BDNF protein concentration in dissected hippocampus

BDNF protein concentration was quantified using ELISA. N=9-12 mice per group.

Figure 1

[Click here to download high resolution image](#)

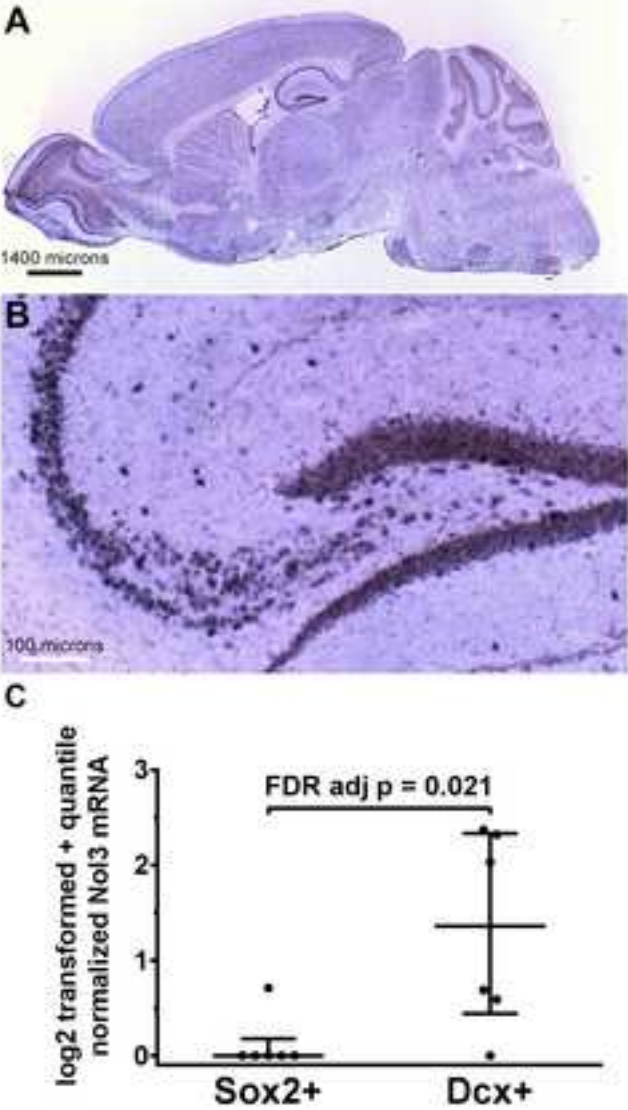


Figure 2
[Click here to download high resolution image](#)

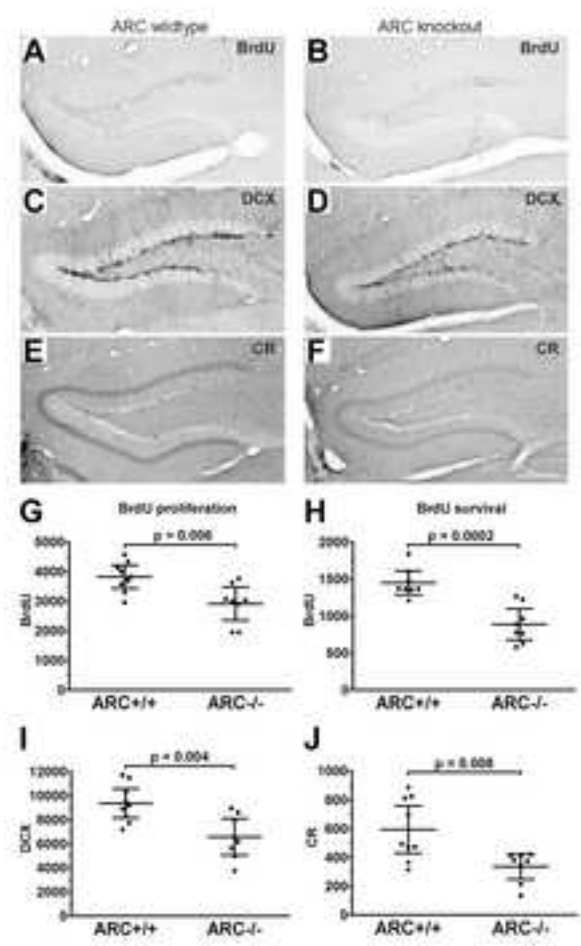


Figure 3
[Click here to download high resolution image](#)

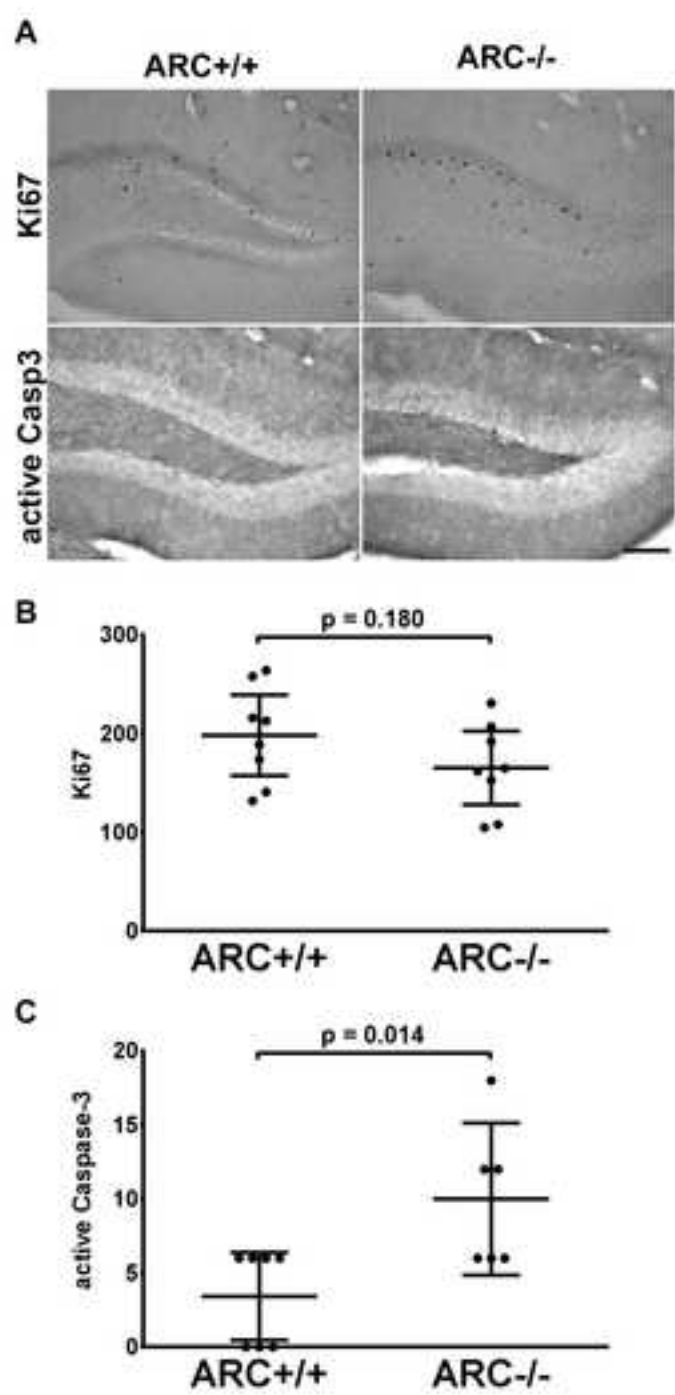


Figure 4

[Click here to download high resolution image](#)

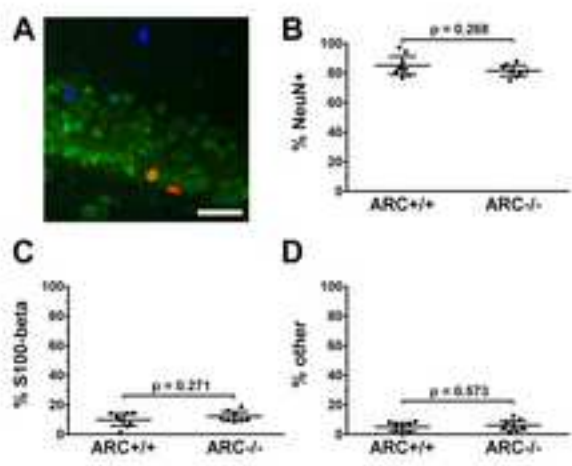


Figure 5
[Click here to download high resolution image](#)

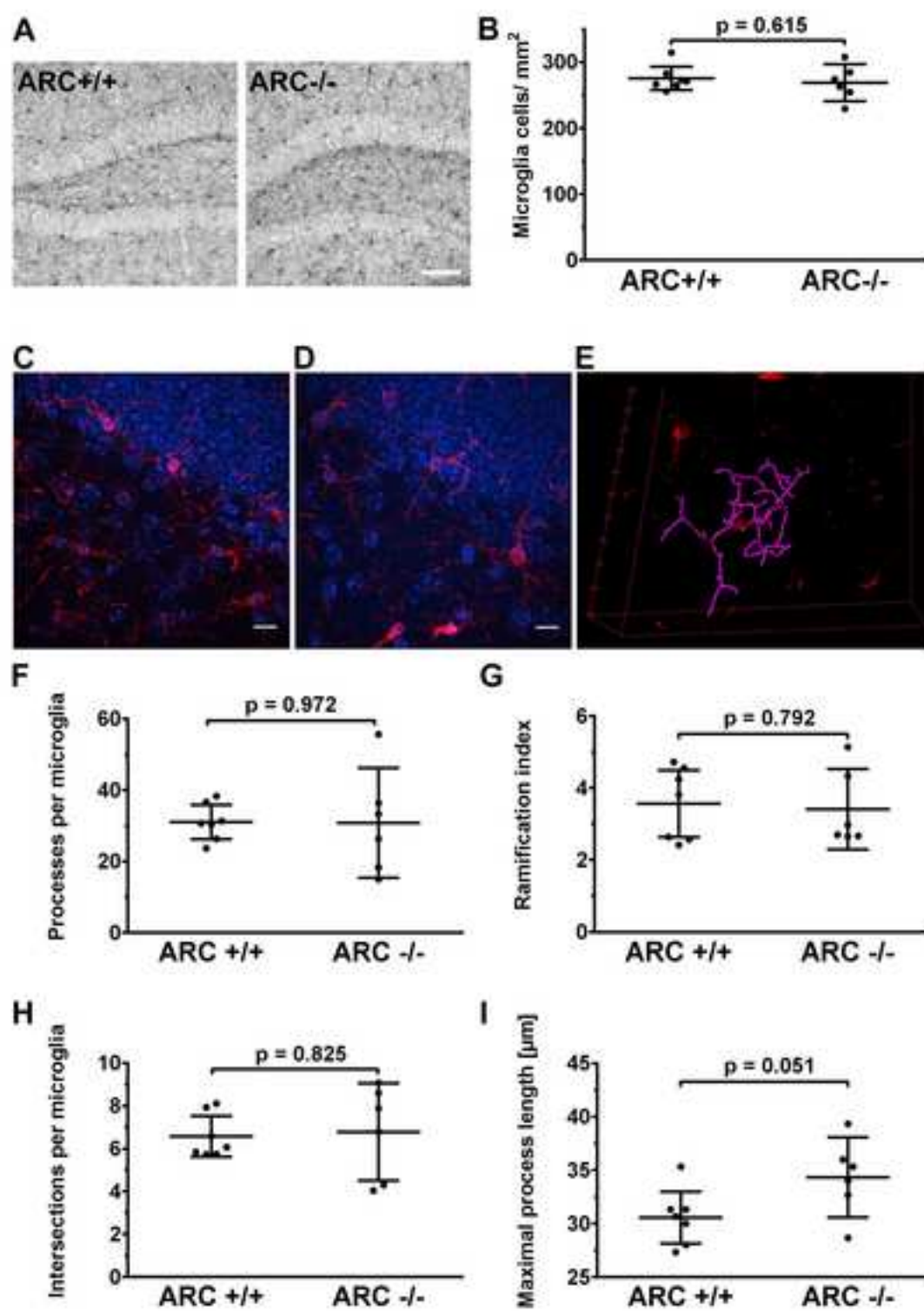
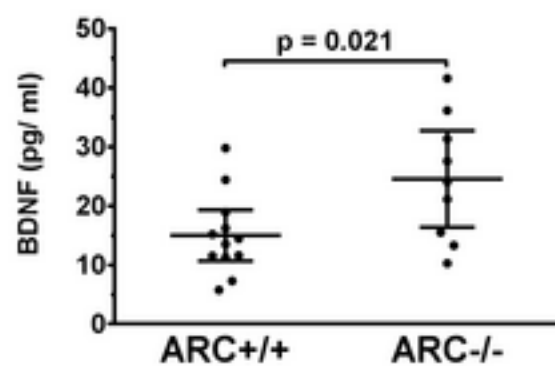


Figure 6
[Click here to download high resolution image](#)



Legends

Figure 1. Nucleolar protein 3 mRNA expression in the hippocampal dentate gyrus

(A) In situ hybridization (ISH) in male P56 mice demonstrates presence of *No/3* (ARC) in brain regions with continuing adult neurogenesis (hippocampus, subventricular zone, rostral migratory stream, and olfactory bulb). Image credit: Allen Institute. (B) Higher magnification demonstrates *No/3* expression throughout the entire granule cell layer. Image credit: Allen Institute. (C) Array data of neural stem cells (Sox2 positive) and their early neuronal progeny (DCX-positive) isolated from the micro-dissected dentate gyrus of 6- to 8-week-old mice have recently been published by Bracko and co-workers (Bracko et al., 2012). Using Geo2R, we examined *No/3* mRNA expression. *No/3* was significantly increased in DCX-positive immature neurons relative to Sox2-positive NSCs (false discovery rate adjusted p-value, $n = 3$, log 2 fold change, gene ID 7842, GEO accession number GSE21208). Scale bar in (A), 1400 μm . Scale bar in (B), 100 μm .

Figure 2. Milestones of hippocampal neurogenesis in *ARC^{-/-}* mice

To quantify the survival of newly generated cells, mice were killed four weeks from the beginning of a 5-day course of once daily BrdU. Representative images of this staining are shown in (A, B) and quantified in (H). Reduced numbers of DCX-positive cells (C, D, I) and of CR-positive cells in *ARC^{-/-}* mice (E, F, J). $N = 8-9$ animals per group. Scale bar in (F), 200 μm . In the absence of ARC, proliferation (G) and survival (H) of BrdU birth-dated cells are significantly reduced. Cell proliferation in the dentate gyrus was quantified after a 5-day course of once daily intraperitoneal BrdU. Mice were killed on day 6 (G).

Figure 3. Cell proliferation and apoptosis

The absence of ARC did not exert a significant effect on the number of Ki67-positive cells (A, B). The number of activated caspase 3-positive cells was significantly increased in ARC^{-/-} mice (A, C). N=6-7 mice per group. Scale bar 200 μ m.

Figure 4. Phenotypic analysis of newly generated cells

The phenotypes of newly generated cells were analyzed four weeks from the beginning of a 5-day course of once daily BrdU. Neuronal marker NeuN (green in A) and astrocytic marker S100 β (blue in A) were used for phenotypic analysis of BrdU-positive (red in A) cells. BrdU cells designated as “other” lacked expression of NeuN and S100 β . N = 8 animals per group. Scale bar in (A) 50 μ m.

Figure 5. Iba1 expression and Sholl analysis of microglia

The density of Iba1-expressing microglia in hippocampus did not differ between ARC^{-/-} mice and wildtype mice. N=6-7 mice per group. Scale bar 100 μ m (A, B). Sholl analysis of microglia at the border of the dentate gyrus (C – I). Iba1 staining with DAPI counterstaining of wildtype (C) and ARC^{-/-} mice (D). Representative image of three dimensional traced microglial processes (pink) in a 3D reconstruction of a Z-stack image of microglia (E). There were no significant changes in the number of processes, ramification index, mean intersections per cell, and maximal process length. N= 6-7 mice per group. The total number of processes per microglia were almost identical between genotypes (F). The Schoenen ramification index is a marker of microglial branching and is dependent on the maximal number of crossings in a given Sholl radius (G). The number of mean intersections per cell represents the total number of intersections in one microglia divided by the number of intersecting radii

(H). Maximal process length shows a trend toward longer processes in $ARC^{-/-}$ animals ($p = 0.051$; I).

Figure 6. BDNF protein concentration in dissected hippocampus

BDNF protein concentration was quantified using ELISA. N=9-12 mice per group.

Reduced Hippocampal Neurogenesis in Mice Deficient in Apoptosis Repressor with Caspase Recruitment Domain (ARC)

Golo Kronenberg¹, Karen Gertz², Ria Uhlemann², Melanie T. C. Kuffner², Imke
Kirste³, Junfeng An^{2,4}, Nafisa M. Jadavji², Bjoern H. Schott^{5,6}, Thomas Scheffel^{7,8},
Matthias Endres², Rainer Hellweg⁸, Christoph Harms^{*2}

1. University of Leicester and Leicestershire Partnership NHS Trust, Leicester, United Kingdom

2. Charité - Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Klinik und Hochschulambulanz für Neurologie und Centrum für Schlaganfallforschung Berlin (CSB), 10117 Berlin, Germany

3. CRTD, DFG Research Center for Regenerative Therapies Dresden, 01307 Dresden, Germany

4. Medical Research Centre, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

5. Klinik für Psychiatrie und Psychotherapie, Universitätsklinikum Göttingen, 37075 Göttingen, Germany

6. Leibniz Institute for Neurobiology, 39118 Magdeburg, Germany

7. Klinik für Psychiatrie, Psychotherapie und Psychosomatik, Medizinische Hochschule Brandenburg, Campus Neuruppin, 16816 Neuruppin, Germany

8. Charité - Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Klinik und Poliklinik für Psychiatrie und Psychotherapie, 10117 Berlin, Germany

*Corresponding author

Prof Christoph Harms, Klinik und Hochschulambulanz für Neurologie, Abteilung für Experimentelle Neurologie und Centrum für Schlaganfallforschung Berlin, Charité - Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin.

E-mail: christoph.harms@charite.de

Abstract

In the adult hippocampal dentate gyrus (DG), the majority of newly generated cells are eliminated by apoptotic mechanisms. The apoptosis repressor with caspase recruitment domain (ARC), encoded by the *No/3* gene, is a potent and multifunctional death repressor that inhibits both death receptor and mitochondrial apoptotic signaling. The aim of the present study was to parse the role of ARC in the development of new granule cell neurons. *No/3* gene expression as revealed by in situ hybridization is present in the entire dentate granule cell layer. Moreover, a comparison of *No/3* expression between FACS-sorted Sox2-positive neural stem cells and Doublecortin (DCX)-positive immature neurons demonstrates upregulation of *No/3* during neurogenesis. Using ARC-deficient mice, we show that proliferation and survival of BrdU birth-dated cells is strongly reduced in the absence of ARC while neuronal-glial fate choice is not affected. Both the number of DCX-positive cells and the number of calretinin (CR)-positive immature postmitotic neurons is reduced in the hippocampus of *ARC^{-/-}* mice. ARC knockout is not associated with increased numbers of microglia nor with microglia activation. However, hippocampal brain-derived neurotrophic factor (BDNF) protein content is significantly increased in *ARC^{-/-}* mice, possibly representing a compensatory response. Collectively, our results suggest that ARC plays a critical cell-autonomous role in preventing cell death during adult granule cell neurogenesis.

Key words: adult hippocampal neurogenesis, apoptosis, ARC, BDNF, microglia

Introduction

Apoptosis, i.e., programmed cell death, is a central mechanism in developmental neurobiology. In adulthood, apoptosis is strongly associated with brain areas of continuing neurogenesis (e.g., Petreanu & Alvarez-Buylla, 2002; Young et al., 1999). In the dentate gyrus (DG), the majority of TUNEL-positive cells are observed in the so-called 'neurogenic niche' at the border of the granule cell layer and the hilus (Biebl et al., 2000; Bengzon et al., 1997). The pronounced variation in the number of cells birth dated with synthetic nucleoside BrdU over time point to an important role of cell death among these newly produced cells in the DG (Nowakowski & Hayes, 2002).

Apoptosis repressor with a caspase recruitment domain (ARC) is a versatile and potent anti-apoptotic factor, which is highly expressed in the brain (Donath et al., 2006; Engidawork et al., 2001; Shelke & Leeuwenburgh, 2003). ARC, also known as nucleolar protein 3, is encoded by the *No/3* gene. ARC was initially recognized as an inhibitor of the extrinsic death receptor pathway. The caspase recruitment domain (CARD) of ARC exhibits homology to the prodomains of several apical caspases. By interacting with the initiator caspases 2 and 8, ARC protects against apoptosis induced by a variety of death receptors and their adaptors (Koseki et al., 1998). More recently, it has become apparent that through an interaction with Bax, ARC can also protect against cell death triggered by activation of the mitochondrial death pathway, preventing mitochondrial dysfunction and the intracellular release of pro-apoptotic molecules such as cytochrome c from the mitochondrion (Gustafsson et al., 2004). Moreover, ARC has recently been shown to inhibit TNF α induced regulated necrosis (Kung et al., 2014).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Its critical role in both the intrinsic and extrinsic apoptotic pathways alongside its robust expression in the brain make ARC a rather unique death repressor in the central nervous system (e.g., Donath et al., 2016). Considering the high proportion of newly generated cells in the DG that quickly undergo apoptosis (Nowakowski & Hayes, 2002), we hypothesized that ARC is an important regulator of hippocampal neurogenesis. We addressed this hypothesis by comprehensively assessing neurogenesis in ARC-deficient mice.

Materials and Methods

Animals and treatments

ARC-deficient mice (ARC^{-/-}) have been described in detail previously (Donath et al., 2006). In particular, knockout of both ARC mRNA and ARC protein has been demonstrated in heart, skeletal muscle, and brain (Donath et al., 2006). ARC null mice were backcrossed to C57/BL6 for more than ten generations. In brief, these animals are born and grow normally to adulthood without any overt abnormality in general health or appearance (Donath et al., 2006). Here, young adult (3-4 months) ARC^{-/-} mice and sex-matched wildtype littermates were used. Mice were maintained in a temperature (22°C ± 2°C) and humidity (55% ± 10%) controlled environment with a 12:12 h light-dark cycle and ad-libitum access to food and water. Thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) was administered intraperitoneally (i.p.) at a dose of 50 µg/g body weight at a concentration of 10 mg/ml (Kronenberg et al., 2010). All experimental procedures were approved by the relevant official committees and carried out in accordance with the Animal Welfare Act, the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the ARRIVE guidelines (Kilkenny et al., 2010).

Histological procedures

Animals were deeply anesthetized with ketamine and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were dissected and postfixed over night at +4°C. Brains were then transferred to a 30% sucrose solution (in 0.1 M phosphate buffer, pH 7.4) for dehydration. Subsequently, brains were frozen on dry ice and cut into 40-µm-thick coronal sections using a freezing microtome (Leica, Bensheim, Germany). All incubations and washes were performed

on 'free-floating' sections. BrdU antigen retrieval was done by incubation in 2 N HCl for 30 minutes at 37°C. Sections were then rinsed in 0.1 M borate buffer (pH 8.5) and thoroughly washed in Tris-buffered saline (TBS; pH 7.4). Primary and secondary antibodies were diluted in TBS supplemented with 0.1% Triton X-100, 0.1% Tween 20, and 3% donkey serum (TBS-plus). Labeled brain sections were mounted on microscope slides using a paintbrush.

Immunohistochemistry followed the peroxidase method with biotinylated secondary antibodies (all 1:500; Jackson ImmunoResearch Laboratories), ABC Elite reagent (Vector Laboratories), and diaminobenzidine (Sigma) as chromogen. Endogenous peroxidase activity was quenched with 0.6% H₂O₂ in TBS for 30 min. For immunofluorescence, FITC-, RhodX-, or Cy5-conjugated secondary antibodies were all used at a concentration of 1:250. Fluorescent sections were coverslipped in polyvinyl alcohol with diazabicyclooctane (DABCO) as antifading agent.

Primary antibodies were applied at the following dilutions: anti-BrdU (rat, 1:500; Harlan Seralab, Indianapolis, IN), anti-cleaved caspase-3 (rabbit, 1:100; Abcam, Cambridge, UK), anti-Ki67 (rabbit, 1:500; Novocastra, Newcastle upon Tyne, UK), anti-S100 β (rabbit, 1:2,500; SWant, Belinzona, Switzerland), anti-NeuN (mouse, 1:100; Chemicon, Temecula, CA), anti-doublecortin (goat, 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-calretinin (CR) (rabbit; 1:250; Swant), and anti-Iba1 (rabbit; 1:500; Wako Chemicals). Sections for Sholl analysis were counterstained with DAPI at a concentration of 1 μ g/ml.

Quantification and imaging

1 In order to quantify neurogenesis in the DG, cell counts were determined in one-in-six
2 series of sections covering the entire hippocampus in its rostrocaudal extent as
3 described in greater detail previously (Kempermann et al., 2003; Kronenberg et al.,
4 2003 and 2007). Briefly, cells located in the granule cell layer and adjacent
5 subgranular zone, defined as a two cell bodies wide zone at the granule cell
6 layer/hilus border, were counted (Brown et al., 2003). Cells in the uppermost focal
7 plane were excluded to avoid oversampling. The density of microglia in the
8 hippocampus was quantified using a stereology-based approach (Uhlemann et al.,
9 2016). Phenotypic analysis of BrdU-labeled cells was performed using a spectral
10 confocal microscope (TCS SP2; Leica). Appropriate gain and black level settings
11 were determined on control slices stained with secondary antibodies alone.

12 Sholl Analysis of microglia

13 Z-stacks of adjacent regions of the dentate gyrus were acquired with a spinning disk
14 confocal microscope (Nikon Ti2-scope equipped with with an Andor Revolution CSU-
15 X1 spinning disk unit and iXon DU-888 ultra EMCCD camera) using a 60x-oil
16 immersion objective (NA 1.4). DAPI and Iba1 were excited with 10% 405 nm (15
17 mW) and 20% 561 nm (15mW) and bandpass filtered (425/45 and 685/40,
18 respectively) before detection.

19 The images were segmented using the ImageJ Simple Neurite Tracing tool and
20 Hessian-based analysis (Longair et al., 2011). Microglial processes were traced in x,
21 y, and z direction starting from the soma center. The starting point was used as the
22 initial point for Sholl analysis. Analysis parameters were radius intervals of 2 μ m on a
23 standard axis and a linear profile. The automated calculation of the maximum branch
24 length and Schoenen ramification index was done by the Sholl Analysis Tool in

ImageJ (Norris et al., 2014; Ferreira et al., 2014). The resulting counts of 3 samples per animal were pooled and used for analysis.

Quantification of BDNF levels

After sacrifice, brains were quickly removed. The left hippocampus was dissected on a cold plate, snap-frozen in liquid nitrogen, and stored at -80 °C until further use. Endogenous levels of BDNF were measured in the thawed homogenates using a commercial ELISA in principle according to the manufacturer's instructions (Promega), but adapted to a highly sensitive fluorometric technique as described in detail earlier (Hellweg et al., 2007). BDNF content is expressed as equivalents of recombinant human BDNF. The detection limit of the assay was 1 pg/ml. Determinations of recovery and specific and unspecific neurotrophin binding (the latter against mouse IgG1 obtained from MOPC 21) involved triplicate fluorescence determinations for each sample. Using this optimized fluorometric ELISA, it was possible to measure BDNF in brain tissue with a minimal wet weight of approximately 5 pg (Hellweg et al., 2003 and 2006). BDNF levels are expressed as picograms per milligram of tissue (wet weight).

Statistical analysis

Experiments were performed in a blinded fashion. Data are presented as scatter plots with mean \pm 95% confidence interval (Figures 2-6) or as median with interquartile range (Figure 1C). Means were compared by two-tailed unpaired Student's t-test (Figures 2-6) or using false discovery rate (FDR) adjusted p-values as described for GSE21208 (Benjamini & Hochberg, 1995 and Bracko et al., 2012). Exact p values are provided in the figures. Statistical analysis was performed using GraphPad Prism version 6.07.

Results

Nucleolar protein 3 (apoptosis repressor with CARD domain) mRNA is strongly expressed in the dentate granule cell layer of adult mice

In situ hybridization with probes for *No/3* yields strong expression in the adult hippocampus (Figure 1A) and, in particular, in the entire DG (Figure 1B; image credit: Allen Institute [Lein et al., 2007]). Based on this observation, we wondered whether *No/3* mRNA expression is upregulated during granule cell neogenesis. We therefore examined *No/3* expression in a microarray dataset of FACS sorted neural stem cells and immature neurons (Bracko et al., 2012; GEO accession number GSE21208). Neural stem cells (NSCs) were isolated from the micro-dissected adult dentate gyrus using transgenic mice expressing GFP under the *Sox2* promoter (*Sox2*-positive cells). Immature neurons were identified using a *DsRed* reporter under control of the *DCX* promoter. Indeed, *No/3* is transcriptionally upregulated in *DCX*-positive immature neurons as compared to NSCs (Figure 1C; GSM530325, GSM50327, and GSM530328: Hip *Sox2* A-C versus GSM530320, GSM530322, and GSM530331: Hip *DCX* A-C).

*Reduced net neurogenesis in the hippocampus of *ARC*^{-/-} mice*

Neurogenesis in the adult DG is a unique form of plasticity that entails the generation of functional granule neurons from proliferating precursor cells (Kempermann et al., 2004; van Praag et al., 2002; Weber et al., 2013). Distinct developmental phases of hippocampal neurogenesis have been described (e.g., Kempermann et al., 2015). We started by investigating the effects of the *ARC* null genotype on precursor cell

1 proliferation and on the survival of newly generated BrdU-positive cells (Figure 2A,
2 B). ARC deficiency resulted in fewer BrdU-positive cells in the 'proliferation condition',
3
4 i.e., 24 h after a 5-day course of once daily i.p. BrdU (Figure 2G). Furthermore, lack
5
6 of ARC profoundly compromised the survival of newly generated cells as assessed
7
8 four weeks from the beginning of a 5-day course of once daily i.p. BrdU ('survival
9
10 condition'; Figure 2A, B, H).
11
12

13 Doublecortin (DCX) and calretinin (CR) are frequently used as BrdU independent
14
15 surrogate markers of neurogenesis (Brandt et al., 2003; Encinas et al., 2006;
16
17 Kronenberg et al., 2007). DCX expression signals neuronal lineage determination
18
19 (Couillard-Despres et al., 2003; Kronenberg et al., 2003; Rao & Shetti, 2004),
20
21 whereas CR represents a marker of early postmitotic granule cells (Brandt et al.,
22
23 2003). Again, in the absence of ARC, these 'milestones' of neurogenesis
24
25 (Kempermann et al., 2004) were significantly reduced (Figure 2C-F, I-J).
26
27
28
29
30

31 Next, we used Ki67, a cell cycle protein, as an endogenous marker of cell
32
33 proliferation in the hippocampal dentate gyrus (Figure 3A, B). This analysis provides
34
35 a more exact picture of cell proliferation in the neurogenic niche than the 5-day
36
37 course of once daily BrdU (Figure 2A) because quite significant early cell loss may
38
39 occur over this period of time. Interestingly, Ki67 immunohistochemistry did not yield
40
41 significant differences between genotypes (Figure 3A, B). After that, we used the
42
43 active form of caspase-3 as an immunohistological tool to detect apoptotic cells. We
44
45 detected more activated caspase 3-positive cells in the dentate gyrus of $ARC^{-/-}$ mice
46
47
48
49
50
51 than in the dentate gyrus of wildtype controls.
52
53
54
55

56 No significant difference in neuronal differentiation of newly generated cells between
57
58 $ARC^{-/-}$ and wildtype mice
59
60
61
62
63
64
65

Using triple immunofluorescence and confocal microscopy, we performed a phenotypic analysis of BrdU-positive cells in the survival condition (Figure 4). NeuN was used as a marker of neuronal differentiation (Figure 4A, B), while S100 β was used to identify new astrocytes (Mullen et al., 1992; Steiner et al., 2004; Kempermann et al., 2003; Figure 4C). Cells expressing neither NeuN nor S100 β were categorized as 'other' (Figure 4D). The fractions of newly born cells falling into each category did not differ significantly between ARC^{-/-} mice and wildtype controls (Figure 4B-D).

No significant difference in microglia density and microglia activation in hippocampus between ARC^{-/-} and wildtype mice

Pro-inflammatory signaling and microglia activation may profoundly inhibit hippocampal neurogenesis (e.g., Appel et al., 2018; Ekdahl et al., 2003; Monje et al., 2003; Woodbury et al., 2015). We therefore investigated the density of Iba1-positive microglia in hippocampus of ARC^{-/-} and wildtype mice (Figure 5A, B). No significant differences in Iba1 expression were detected between genotypes.

Microglia activation is reflected by morphological changes. Representative images of maximum intensity projections of confocal Z-stacks of wildtype controls and ARC^{-/-} mice showed no apparent morphological differences (Figure 5C, D). We performed a Sholl analysis of microglia using 3D tracing of confocal image stacks (Figure 5E). The total number of processes per microglia (Figure 5F), Schoenen Ramification Index (Figure 5G), and the number of intersections per microglia (Figure 5H) were measured. Again, comparison of ARC^{-/-} animals and wildtype controls yielded no significant differences. The length of the longest process determined by the maximal radius length (Figure 5I) also did not differ significantly between genotypes despite a strong tendency towards longer processes in ARC^{-/-} mice (p=0.051).

Increased BDNF protein concentrations in hippocampus of ARC^{-/-} mice

BDNF has been shown to be essential for the survival of neurons, specifically in populations that undergo continuous regeneration, i.e., in the DG and in the olfactory bulb (Linarsson et al., 2000). BDNF expression was measured at the protein level in dissected left hippocampus of ARC^{-/-} mice and wildtype controls. BDNF protein concentrations were significantly increased in ARC^{-/-} mice (Figure 6).

Discussion

ARC is a potent endogenous inhibitor of programmed cell death that blocks both the intrinsic and extrinsic apoptotic pathways as well as TNF α -induced regulated necrosis (Gustafsson et al., 2004; Koseki et al., 1998; Kung et al., 2014). Using constitutive ARC^{-/-} mice, we here investigated the neurogenesis phenotype in the absence of this versatile and potent CNS death repressor. Our study yielded the following major findings: (i) The proliferation and survival of BrdU birth-dated cells is strongly reduced in the absence of ARC, while neuronal-glial fate choice is not affected. (ii) Consequently, net neurogenesis in the DG is also robustly decreased in ARC knockout mice. This finding is reinforced by BrdU-independent evidence in that both the number of DCX-positive neuroblasts and, even more so, the number of CR-positive immature postmitotic neurons is reduced in the hippocampus of ARC^{-/-} mice. (iii) Remarkably, these effects of the ARC knockout are not associated with increased numbers of microglia in the hippocampus nor with microglia activation as assessed by Sholl analysis. (iv) An increase in activated caspase 3- positive cells indicates an anti-apoptotic action of ARC in early neuronal development while the number of actively proliferating Ki67-positive cells was not altered between genotypes. (v) BDNF expression measured at the protein level in dissected left hippocampus was significantly increased in ARC^{-/-} mice.

This report builds on earlier work investigating the role of apoptosis in hippocampal neurogenesis. Briefly, a synthesis of the existing knowledge paints the following picture: Broadly speaking, the density of dying cells is increased in neurogenic brain areas such as the olfactory bulb or the DG (Biebl et al., 2000). Interestingly, an

analysis of mRNA expression data from the Allen Brain Atlas (Lein et al., 2007) indicates strong *No/3* expression in these regions. Equally notably, the excessive cell death that occurs in mice lacking BDNF is also restricted to these same brain areas, supporting the concept that BDNF is essential for the survival of neurons specifically in populations that are continuously being regenerated in the mammalian brain (Linnarsson et al., 2000).

In the DG, the number of BrdU-labeled cells declines sharply at early time points after injection (e.g., Kempermann et al., 2004; Kronenberg et al., 2003; Sierra et al., 2015). Moreover, most TUNEL-positive cells reside at the border between the hilus and the granule cell layer (Biebl et al., 2000), giving further credence to the notion that the bulk of newly generated cells is eliminated at early developmental stages while still in close proximity to the neurogenic niche (Biebl et al., 2000). Transgenic mice overexpressing the anti-apoptotic protein Bcl-2 under control of the neuron-specific enolase promoter exhibit a doubling in the rate of net hippocampal neurogenesis (Kuhn et al., 2005) – a finding that further illustrates the size of the effect that programmed cell death has on neurogenesis. Finally, more recently, ramified (i.e., not activated) microglia have been shown to remove apoptotic newborn cells rapidly through phagocytosis in the intact DG (Sierra et al., 2010). Our results indicate that the increased loss of newborn cells in *ARC*^{-/-} mice is also insufficient to elicit an increase in microglia density or any discernible microglia activation.

Exciting new research involving live imaging of radial glia-like cells and their progeny suggests that the early wave of cell death during hippocampal neurogenesis is governed by as yet unidentified intrinsic mechanisms (Pilz et al., 2018). After a limited number of cell divisions resulting in a transient burst in neurogenic activity, neural stem and progenitor cells are rapidly lost (Pilz et al., 2018). Our results

1 highlight the crucial importance of ARC as a key anti-apoptotic molecule that
2 effectively tips the scales toward early neuronal survival. The observation that *No/3*
3 mRNA expression is upregulated in DCX-DsRed positive immature neurons
4 compared to Sox2-GFP positive NSCs is in line with the idea that cell-autonomous
5 activation of anti-apoptotic pathways (i.e., relating to the mitochondrial death
6 pathway) at a very early stage is a crucial step in hippocampal neurogenesis.
7 Interestingly, the effects of ARC knockout seem to accrue over the course of
8 neurogenesis. Descriptively, the reduction in BrdU-positive cells in $ARC^{-/-}$ mice was
9 more pronounced in our survival condition than in our proliferation condition.
10 Similarly, the difference between genotypes appears somewhat stronger in the case
11 of postmitotic CR-positive neurons than in the case of more immature DCX-positive
12 neuroblasts. These observations are in good agreement with earlier studies that have
13 described an early wave of cell death within the initial days of cell birth and a second
14 delayed phase of cell loss or survival that is input-dependent and involves integration
15 of surviving cells into hippocampal circuitry (Pilz et al., 2018; Sierra et al., 2010;
16 Tashiro et al., 2006). Most likely, ARC, as a multifunctional death repressor, confers
17 protection against both the initial and the delayed phases of programmed cell death
18 over the entire course of neurogenesis.
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45

46 BDNF is generally held to promote neurogenesis. However, even today, the precise
47 mechanisms controlling BDNF levels in brain remain to be better understood.
48 Exercise and antidepressants have been shown to increase hippocampal BDNF
49 transcription (e.g. Neeper et al., 1995; Russo-Neustadt et al., 1999). Mice with either
50 reduced BDNF concentrations ($BDNF^{+/-}$ mice) or impaired tyrosine receptor kinase B
51 (TrkB) activation ($trkB.T1$ -overexpressing mice) show reduced long-term survival of
52 new neurons (Sairanen et al., 2005). Furthermore, ablation of TrkB in neural
53
54
55
56
57
58
59
60
61
62
63
64
65

precursor cells leads to impaired proliferation and neurogenesis alongside behavioral insensitivity to antidepressants or exercise (Li et al., 2008). Seemingly paradoxical increases in BDNF content have been reported under pathophysiological conditions such as in mice lacking brain serotonin (Kronenberg et al., 2016) or in aged APP23 transgenic mice (Burbach et al., 2004). It is tempting to speculate that our finding of increased hippocampal BDNF protein content in $ARC^{-/-}$ mice may also represent a form of compensatory counterregulatory mechanism in the face of increased cell death. However, we acknowledge that our study does not provide further mechanistic insights into the link between absence of ARC and increased BDNF concentrations in hippocampus.

In summary, there are several novel findings from this study. Most importantly, we demonstrate that ARC imparts anti-apoptotic properties to newborn cells over the course of hippocampal neurogenesis, thereby increasing the number of newly generated granule neurons. ARC knockout does not lead to microglia activation or increased microglia density. Increased hippocampal BDNF content in $ARC^{-/-}$ mice may represent a compensatory mechanism.

Acknowledgements

The authors thank Annemarie Bunge, Melanie Kroh, Janet Lips, and Stefanie Balz for excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB TRR43 and Exc257 to M.E.; KR 2956/4-1 to G.K.; GE 2576/2-1 to K.G.; HA 5741/5-1 to C.H. and Berlin-Brandenburg School for Regenerative Therapies GSC 203), the Bundesministerium für Bildung und Forschung (CSB 01 EO 1301 to M.E., K.G., G.K., and CH.), the German Center for Neurodegenerative Diseases (DZNE to M.E.), the German Center for Cardiovascular Research (DZHK to M.E.), and the Corona Foundation (to M.E.). Berlin Institute of Health (TRG7 to C.H.). We thank the Advanced Medical Bioimaging Core Facility (AMBIO) of the Charité for support in the acquisition of imaging data.

References

Appel JR, Ye S, Tang F, Sun D, Zhang H, Mei L, Xiong WC (2018) Increased Microglial Activity, Impaired Adult Hippocampal Neurogenesis, and Depressive-like Behavior in Microglial VPS35-Depleted Mice. *J Neurosci* 38(26):5949-5968

Bengzon J, Kokaia Z, Elmér E, Nanobashvili A, Kokaia M, Lindvall O (1997) Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7

Benjamini, Y, Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 57:289-300.

Biebl M, Cooper CM, Winkler J, Kuhn HG (2000) Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neurosci Lett* 291:17-20

Bracko O, Singer T, Aigner S, Knobloch M, Winner B, Ray J, Clemenson GD, Jr., Suh H, Couillard-Despres S, Aigner L, Gage FH and Jessberger S (2012) Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. *J Neurosci* 32:3376-87

Brandt MD, Jessberger S, Steiner B, Kronenberg G, Reuter K, Bick-Sander A, von der Behrens W, Kempermann G (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. *Mol Cell Neurosci* 24: 603-613.

1
2 Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG
3
4 (2003) Transient expression of doublecortin during adult neurogenesis. J Comp
5
6 Neurol 467(1):1-10.
7
8
9

10
11 Burbach GJ, Hellweg R, Haas CA, Del Turco D, Deicke U, Abramowski D, Jucker M,
12
13 Staufenbiel M, Deller T (2004) Induction of brain-derived neurotrophic factor in
14
15 plaque-associated glial cells of aged APP23 transgenic mice. J Neurosci
16
17 24(10):2421-30
18
19
20
21
22

23
24 Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N,
25
26 Bogdahn U, Winkler J, Kuhn HG, Aigner L. 2005. Doublecortin expression levels in
27
28 adult brain reflect neurogenesis. Eur J Neurosci 21: 1-14.
29
30
31
32

33
34 Donath S, Li P, Willenbockel C, Al-Saadi N, Gross V, Willnow T, Bader M, Martin U,
35
36 Bauersachs J, Wollert KC, Dietz R, von Harsdorf R; German Heart Failure Network
37
38 (2006) Apoptosis repressor with caspase recruitment domain is required for
39
40 cardioprotection in response to biomechanical and ischemic stress. Circulation
41
42 113(9):1203-12
43
44
45
46

47
48 Donath S, An J, Lee SL, Gertz K, Datwyler AL, Harms U, Müller S, Farr TD,
49
50 Fächteimeier M, Lättig-Tünnemann G, Lips J, Foddiss M, Mosch L, Bernard R, Grittner
51
52 U, Balkaya M, Kronenberg G, Dirnagl U, Endres M, Harms C (2016) Interaction of
53
54 ARC and Daxx: A Novel Endogenous Target to Preserve Motor Function and Cell
55
56 Loss after Focal Brain Ischemia in Mice. J Neurosci 36(31):8132-48
57
58
59
60
61
62
63
64
65

1
2 Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O (2003) Inflammation is
3
4 detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci U S A* 100(23):13632-
5
6
7 7.
8
9

10
11 Encinas JM, Vaahtokari A, Enikolopov G (2006) Fluoxetine targets early progenitor
12
13 cells in the adult brain. *Proc Natl Acad Sci* 103: 8233-8238
14
15
16

17
18 Engidawork E, Gulesserian T, Yoo BC, Cairns N, Lubec G (2001) Alteration of
19
20 caspases and apoptosis-related proteins in brains of patients with Alzheimer's
21
22 disease. *Biochem Biophys Res Commun* 281(1):84-93.
23
24
25

26
27 Ferreira TA, Blackman AV, Oyrer J, Jayabal S, Chung AJ, Watt AJ, Sjostrom PJ, van
28
29 Meyel DJ (2014) Neuronal morphometry directly from bitmap images. *Nat Methods*,
30
31 11 (2014) 982-984.
32
33
34

35
36 Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA (2004) Apoptosis
37
38 repressor with caspase recruitment domain protects against cell death by interfering
39
40 with Bax activation. *J Biol Chem* 279:21233–21238.
41
42
43

44
45 Hayes NL, Nowakowski RS (2002) Dynamics of cell proliferation in the adult dentate
46
47 gyrus of two inbred strains of mice. *Brain Res Dev Brain Res* 134(1-2):77-85.
48
49
50

51
52 Hellweg R, Lohmann P, Huber R, Kühl A, Riepe MW (2006) Spatial navigation in
53
54 complex and radial mazes in APP23 animals and neurotrophin signaling as a
55
56 biological marker of early impairment. *Learn Mem* 13(1):63-71
57
58
59
60

Hellweg R, von Arnim CA, Büchner M, Huber R, Riepe MW (2003) Neuroprotection and neuronal dysfunction upon repetitive inhibition of oxidative phosphorylation. *Exp Neurol* 183(2):346-54

Hellweg R, Zueger M, Fink K, Hörtnagl H, Gass P (2007) Olfactory bulbectomy in mice leads to increased BDNF levels and decreased serotonin turnover in depression-related brain areas. *Neurobiol Dis* 25(1):1-7

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* 130(2):391-9

Kempermann G, Jessberger S, Steiner B, Kronenberg G. 2004. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27: 447-452

Kempermann G, Song H, Gage FH (2015) Neurogenesis in the Adult Hippocampus. *Cold Spring Harb Perspect Biol* 7(9):a018812

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8: e1000412

Koseki T, Inohara N, Chen S, Nunez G (1998) ARC, an inhibitor of apoptosis expressed in skeletal muscle and heart that interacts selectively with caspases. *Proc Natl Acad Sci U S A* 95:5156 -5160

Kronenberg G, Lippoldt A, Kempermann G (2007) Two genetic rat models of arterial hypertension show different mechanisms by which adult hippocampal neurogenesis is increased. *Dev Neurosci* 29(1-2):124-33

Kronenberg G, Mosienko V, Gertz K, Alenina N, Hellweg R, Klempin F (2016) Increased brain-derived neurotrophic factor (BDNF) protein concentrations in mice lacking brain serotonin. *Eur Arch Psychiatry Clin Neurosci* 266(3):281-4

Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G (2003) Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. *J Comp Neurol* 467(4):455-63

Kronenberg G, Gertz K, Baldinger T, Kirste I, Eckart S, Yildirim F, Ji S, Heuser I, Schröck H, Hörtnagl H, Sohr R, Djoufack PC, Jüttner R, Glass R, Przesdzing I, Kumar J, Freyer D, Hellweg R, Kettenmann H, Fink KB, Endres M (2010) Impact of actin filament stabilization on adult hippocampal and olfactory bulb neurogenesis. *J Neurosci* 2010 Mar 3;30(9):3419-31

Kuhn HG, Biebl M, Wilhelm D, Li M, Friedlander RM, Winkler J (2005) Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. *Eur J Neurosci* 22(8):1907-15

Kung G, Dai P, Deng L, Kitsis RN (2014) A novel role for the apoptosis inhibitor ARC in suppressing TNF α -induced regulated necrosis. *Cell Death Differ* 21(4):634-44

1 Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski
2 MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook
3 BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare
4 TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin
5 LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN,
6 Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP,
7 Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramée
8 AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan
9 JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak
10 TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR,
11 Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivasay T,
12 Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR,
13 Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR,
14 Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu
15 MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR (2007)
16 Genome-wide atlas of gene expression in the adult mouse brain. *Nature*
17 445(7124):168-76
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42

43 Linnarsson S, Willson CA, Ernfors P (2000) Cell death in regenerating populations of
44 neurons in BDNF mutant mice. *Brain Res Mol Brain Res* 75(1):61-9
45
46
47
48
49
50

51 Longair MH, Baker DA, Armstrong JD (2011) Simple Neurite Tracer: open source
52 software for reconstruction, visualization and analysis of neuronal processes.
53 *Bioinformatics*, 27 (2011) 2453-2454.
54
55
56
57
58
59
60
61
62
63
64
65

1 Monje ML, Toda H, Palmer TD (2003) Inflammatory blockade restores adult
2 hippocampal neurogenesis. *Science* 302(5651):1760-5
3
4
5

6
7 Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in
8 vertebrates. *Development* 116(1):201-11
9
10

11
12
13
14 Neeper SA, Gómez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain
15 neurotrophins. *Nature* 373(6510):109.
16
17
18

19
20
21 Norris G, Derecki N, Kipnis J (2014) Microglial Sholl Analysis. 26 August 2014,
22 PROTOCOL (Version 1) available at Protocol Exchange
23 [+<https://doi.org/10.1038/protex.2014.029+>]
24
25
26
27

28
29
30
31 Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb
32 granule neurons: role of olfaction. *J Neurosci* 22(14):6106-13.
33
34
35

36
37
38 Pilz GA, Bottes S, Betizeau M, Jörg DJ, Carta S, Simons BD, Helmchen F,
39 Jessberger S (2018) Live imaging of neurogenesis in the adult mouse hippocampus.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

1 Russo-Neustadt A, Beard RC, Cotman CW (1999) Exercise, antidepressant
2 medications, and enhanced brain derived neurotrophic factor expression.
3
4 Neuropsychopharmacology 21(5):679-82
5
6
7

8
9 Sairanen M, Lucas G, Ernfors P, Castrén M, Castrén E (2005) Brain-derived
10 neurotrophic factor and antidepressant drugs have different but coordinated effects
11 on neuronal turnover, proliferation, and survival in the adult dentate gyrus. J Neurosci
12 25(5):1089-94
13
14
15
16
17
18

19
20
21 Shelke RR, Leeuwenburgh C (2003) Lifelong caloric restriction increases expression
22 of apoptosis repressor with a caspase recruitment domain (ARC) in the brain. FASEB
23 J 17(3):494-6
24
25
26
27
28

29
30
31 Sierra A, Martín-Suárez S, Valcárcel-Martín R, Pascual-Brazo J, Aelvoet SA, Abiega
32 O, Deudero JJ, Brewster AL, Bernalles I, Anderson AE, Baekelandt V, Maletić-Savatić
33 M, Encinas JM (2015) Neuronal hyperactivity accelerates depletion of neural stem
34 cells and impairs hippocampal neurogenesis. Cell Stem Cell 16(5):488-503
35
36
37
38
39
40
41

42
43 Steiner B, Kronenberg G, Jessberger S, Brandt MD, Reuter K, Kempermann G
44 (2004) Differential regulation of gliogenesis in the context of adult hippocampal
45 neurogenesis in mice. Glia. 2004 Apr 1;46(1):41-52.
46
47
48
49
50
51

52
53 Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH (2006) MDA-receptor-mediated,
54 cell-specific integration of new neurons in adult dentate gyrus. Nature 442(7105):929-
55 33
56
57
58
59
60
61
62
63
64
65

1 Uhlemann R, Gertz K, Boehmerle W, Schwarz T, Nolte C, Freyer D, Kettenmann H,
2 Endres M, Kronenberg G (2016) Actin dynamics shape microglia effector functions.
3
4 Brain Struct Funct 221(5):2717-34
5
6
7
8

9 van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002)
10 Functional neurogenesis in the adult hippocampus. Nature 415(6875):1030-4
11
12
13
14
15

16 Woodbury ME, Freilich RW, Cheng CJ, Asai H, Ikezu S, Boucher JD, Slack F, Ikezu
17 T (2015) miR-155 Is Essential for Inflammation-Induced Hippocampal Neurogenic
18
19 Dysfunction. J Neurosci 35(26):9764-81
20
21
22
23
24

25 Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental
26 enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective.
27
28
29
30
31 Nat Med 5(4):448-53
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Legends

Figure 1. Nucleolar protein 3 mRNA expression in the hippocampal dentate gyrus

(A) In situ hybridization (ISH) in male P56 mice demonstrates presence of *No/3* (ARC) in brain regions with continuing adult neurogenesis (hippocampus, subventricular zone, rostral migratory stream, and olfactory bulb). Image credit: Allen Institute. (B) Higher magnification demonstrates *No/3* expression throughout the entire granule cell layer. Image credit: Allen Institute. (C) Array data of neural stem cells (Sox2 positive) and their early neuronal progeny (DCX-positive) isolated from the micro-dissected dentate gyrus of 6- to 8-week-old mice have recently been published by Bracko and co-workers (Bracko et al., 2012). Using Geo2R, we examined *No/3* mRNA expression. *No/3* was significantly increased in DCX-positive immature neurons relative to Sox2-positive NSCs (false discovery rate adjusted p-value, $n = 3$, log2 transformed and quantile normalized *No/3* mRNA, gene ID 7842, GEO accession number GSE21208). Scale bar in (A), 1400 μm . Scale bar in (B), 100 μm .

Figure 2. Milestones of hippocampal neurogenesis in $\text{ARC}^{-/-}$ mice

To quantify the survival of newly generated cells, mice were killed four weeks from the beginning of a 5-day course of once daily BrdU. Representative images of this staining are shown in (A, B) and quantified in (H). Reduced numbers of DCX-positive cells (C, D, I) and of CR-positive cells in $\text{ARC}^{-/-}$ mice (E, F, J). $N = 8-9$ animals per group. Scale bar in (F), 200 μm . In the absence of ARC, proliferation (G) and survival (H) of BrdU birth-dated cells are significantly reduced. Cell proliferation in the dentate

gyrus was quantified after a 5-day course of once daily intraperitoneal BrdU. Mice were killed on day 6 (G).

Figure 3. Cell proliferation and apoptosis

The absence of ARC did not exert a significant effect on the number of Ki67-positive cells (A, B). The number of activated caspase 3-positive cells was significantly increased in ARC^{-/-} mice (A, C). N=6-7 mice per group. Scale bar 200 μ m.

Figure 4. Phenotypic analysis of newly generated cells

The phenotypes of newly generated cells were analyzed four weeks from the beginning of a 5-day course of once daily BrdU. Neuronal marker NeuN (green in A) and astrocytic marker S100 β (blue in A) were used for phenotypic analysis of BrdU-positive (red in A) cells. BrdU cells designated as “other” lacked expression of NeuN and S100 β . N = 8 animals per group. Scale bar in (A) 50 μ m.

Figure 5. Iba1 expression and Sholl analysis of microglia

The density of Iba1-expressing microglia in hippocampus did not differ between ARC^{-/-} mice and wildtype mice. N=6-7 mice per group. Scale bar 100 μ m (A, B). Sholl analysis of microglia at the border of the dentate gyrus (C – I). Iba1 staining with DAPI counterstaining of wildtype (C) and ARC^{-/-} mice (D). Representative image of three dimensional traced microglial processes (pink) in a 3D reconstruction of a Z-stack image of microglia (E). There were no significant changes in the number of processes, ramification index, mean intersections per cell, and maximal process length. N= 6-7 mice per group. The total number of processes per microglia were almost identical between genotypes (F). The Schoenen ramification index is a marker of microglial branching and is dependent on the maximal number of crossings in a

given Sholl radius (G). The number of mean intersections per cell represents the total number of intersections in one microglia divided by the number of intersecting radii (H). Maximal process length shows a trend toward longer processes in $ARC^{-/-}$ animals ($p = 0.051$; I).

Figure 6. BDNF protein concentration in dissected hippocampus

BDNF protein concentration was quantified using ELISA. N=9-12 mice per group.