Dissociable effects of complement C3 and C3aR on survival and morphology of adult born hippocampal neurons, pattern separation, and cognitive flexibility in male mice.

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Abstract

Adult hippocampal neurogenesis (AHN) is a form of ongoing plasticity in the brain that supports specific aspects of cognition. Disruptions in AHN have been observed in neuropsychiatric conditions presenting with inflammatory components and are associated with impairments in cognition and mood. Recent evidence highlights important roles of the complement system in synaptic plasticity and neurogenesis during neurodevelopment and in acute learning and memory processes. In this work we investigated the impact of the complement C3/C3aR pathway on AHN and its functional implications for AHN-related behaviours. In $C3^{-/-}$ mice, we found increased numbers and accelerated migration of adult born granule cells, indicating that absence of C3 leads to abnormal survival and distribution of adult born neurons. Loss of either C3 or C3aR affected the morphology of immature neurons, reducing morphological complexity, though these effects were more pronounced in the absence of C3aR. We assessed functional impacts of the cellular phenotypes in an operant spatial discrimination task that assayed AHN sensitive behaviours. Again, we observed differences in the effects of manipulating C3 or C3aR, in that whilst $C3aR^{-/-}$ mice showed evidence of enhanced pattern separation abilities, $C3^{-/-}$ mice instead demonstrated impaired behavioural flexibility. Our findings show that C3 and C3aR manipulation have distinct effects on AHN that impact at different stages in the development and maturation of newly born neurons, and that the dissociable cellular phenotypes are associated with specific alterations in AHN-related behaviours.

1. Introduction

Adult hippocampal neurogenesis (AHN) is a unique process that recapitulates the steps of developmental neurogenesis in an otherwise anti-neurogenic brain, from precursor proliferation, fate specification, migration and differentiation to morphological and synaptic integration of newly born neurons¹. Originating from radial glial cells, neuronal progenitor cells are born in their thousands within the sub granular zone of dentate gyrus². While the majority of these cells undergo programmed cell death within the first few weeks of life³, surviving immature granule cell (GC) neurons progress through a multifaceted developmental process as they mature and integrate into the hippocampal circuitry⁴.

The function of AHN has been debated but current evidence suggests it is involved in information processing critical to adaptive behaviour across a number of areas including learning, memory and emotionality⁵⁻⁷. In particular, young adult born neurons are thought to perform pattern separation, the process that enables discrimination of highly similar, overlapping spatial and temporal stimuli, fostering the appropriate separation and storage of similar memory traces or engrams thereby reducing interference^{8,9}. Key evidence supporting a role for AHN in pattern separation comes from studies in which ablation of neurogenesis resulted in specific impairments in discriminating stimuli with a high degree of spatial similarity or overlap, but not stimuli that are more readily distinguished¹⁰⁻¹². Evidence also supports an important role for AHN in cognitive flexibility and the underlying processes of behavioural inhibition that allows flexible responding in the face of changing contingencies⁵, and elements of cognitive flexibility and behavioural inhibition have been shown to be sensitive to experimental manipulations of AHN¹³⁻¹⁷.

The process of AHN is subject to regulation by myriad factors, including microglia and immune signalling^{18,19}. The complement system consists of separate activation pathways which converge on a single molecule, C3, and then culminate in the terminal pathway²⁰. It is through C3 activation that the main effectors of the complement system are generated, including the anaphylatoxin C3a. This peptide signals via its cognate G protein-coupled C3a receptor (C3aR) to stimulate intracellular signalling pathways leading to the modulation of critical cellular functions²¹ and pro- and anti-inflammatory actions²². In recent years, prominent roles have emerged for the complement system in brain development, including synapse elimination²³ and embryonic neurogenesis²⁴, but also in neurodegeneration²⁵⁻²⁷ though how complement may influence ongoing neurogenesis and cognitive functions within the adult brain have received relatively little focus. Given converging evidence of aberrant adult neurogenesis^{28,29} and cognitive dysfunction³⁰⁻³² in neuropsychiatric disorders associated with abnormal complement activity^{33,34}, it is important that we improve our understanding of how complement may regulate ongoing neuroplasticity and neurodevelopment in the context of AHN.

Previous literature illustrates conflicting roles for different complement pathways in regulating basal AHN. Adult *C3*^{-/-} and *C3aR*^{-/-} mice, and wildtype mice in which C3aR was pharmacologically blocked, showed reduced numbers of adult born neurons³⁵, suggesting that the C3a-C3aR axis is a positive regulator of AHN. In contrast, another report focusing on the role of a different breakdown product of C3, found an inhibitory role of C3d/CR2 signalling in adult neurogenesis, whereby *CR2*^{-/-} mice showed elevated levels of basal neurogenesis in the adult hippocampus³⁶. Therefore, two opposing regulatory roles have been assigned to these separate breakdown products of C3, which suggest both pro and anti-neurogenic properties of the complement

system under physiological conditions. Furthermore, the functional consequences of these changes for cognition are unknown. Improved spatial memory recall and reversal learning have been described in *C3*^{-/-} mice^{37,38}, and blockade of the C3a receptor improved cognition in pathological contexts^{26,27,39}, but there have not yet been any direct investigations of AHN-associated cognition in complement knockout models.

Other important variables in the neurogenic process include the morphology and functional integration of newborn neurons into the existing hippocampal circuitry⁴⁰. In addition to the aforementioned roles of complement in sculpting neuronal circuitry in the developing brain^{23,41}, several studies have implicated the C3a/C3aR axis in modulating neuronal morphology *in vitro*^{39,42,43}. Whether complement is involved in the morphological maturation of adult born neurons *in vivo* within the hippocampal neurogenic niche has not yet been reported.

Using mice constitutively deficient in complement *C3* or *C3aR*, we investigated the impact of these pathways on discrete stages of the neurogenic process occurring in the adult hippocampus. We also assessed behaviour in a translational touch-screen visual discrimination task previously employed to demonstrate the involvement of AHN in pattern separation^{10,12,44}. Our findings reveal different effects of *C3* and *C3aR* manipulation on AHN and related behaviours. Cellular effects of *C3aR* deficiency were mainly limited to effects on the morphological complexity of adult born neurons, whilst *C3* deficiency impacted predominantly on their survival and ultimate location. The dissociations in cellular effects were associated with different behavioural outcomes and together the data suggest cellular mechanisms linking *C3aR* to pattern separation and *C3* to cognitive flexibility, respectively.

2. Materials and Methods

For full methods, see *Supplementary Information*.

2.1 Mouse models and husbandry

Wildtype and $C3^{-/-}$ strains were sourced in-house from Professor B. Paul Morgan and Dr Timothy Hughes (strains originally from The Jackson Laboratory; B6.PL-Thy1^a/CyJ stock#000406 and B6;129S4-C3tm1Crr/J stock#003641 respectively). $C3aR^{-/-}$ mice were provided by Professor Craig Gerard of Boston Children's Hospital, USA (mice subsequently provided to The Jackson Laboratory by Prof. Gerard, strain B6.129S4(C)- $C3ar1^{tm1Cge}$ /BaoluJ). $C3^{-/-}$ and $C3aR^{-/-}$ mice were maintained via homozygous breeding on a C57Bl/6J background and in all experiments were compared to wildtype mice also on a C57Bl/6J background. Male mice were kept in a temperature and humidity-controlled vivarium ($21 \pm 2^{\circ}\text{C}$ and $50 \pm 10\%$, respectively) with a 12-hour light-dark cycle (lights on at 07:00hrs/lights off at 19:00hrs). For ex vivo histological analyses, animals were between 8 and 12 weeks of age at the time of sacrifice. For behavioural testing, animals were habituated to daily handling by the experimenter for 2 weeks prior to beginning behavioral testing at 8 weeks of age and were approximately 22 weeks of age on completion. All procedures were performed in accordance with the requirements of the UK Animals (Scientific Procedures) Act (1986).

2.2 Pulse-chase experiments

Animals were injected with 5-bromo-2'-deoxyuridine (Sigma Aldrich, UK) diluted in sterile Phosphate buffered saline (pH 7.4) at a dose of 100 mg/kg i.p either 6 hours

prior to sacrifice (short pulse BrdU) or once daily on 5 consecutive days, with sacrifice 30 days after the first dose (long pulse BrdU).

2.3 Tissue sampling and Immunohistochemistry

Paraformaldehyde fixed brain tissue was sectioned on a cryostat, with 6 sections collected per animal, spanning the longitudinal axis of the hippocampus beginning approximately -0.94 mm from bregma and ending at approximately -3.40 mm from bregma ⁴⁵. Sections were selected using a stereological sampling rate of 1/10. Free floating 40µm sections were used for immunohistochemistry (see Supplementary Information).

2.4 Microscopy

Tissue sections were imaged on an upright Leica DM6000B fluorescence microscope using tile scanning at 40x magnification to capture the entire left and right dentate gyri in each section. Experimenters were blinded to genotype during all analyses.

2.5 Cell counting and analyses

In order to estimate the total number of cells (e.g., DCX⁺, BrdU⁺, NeuN⁺) in the whole dentate gyri of each animal, the number of cells counted in the left and right dentate were summed and multiplied by the intersection interval (10) to obtain the total number of cells for the region spanning the current section until the next section. This was repeated for each of 6 sections, and the estimates obtained were summed in order to give a measure of the total number of cells present in the entire DG per animal. For migration analyses, the thickness of the GCL was measured in each section and divided into four equally sized bins, each approximately two cells thick and the first of which represented the sub granular zone (SGZ). The distance of individual BrdU⁺ cells

from the inner (hilar) portion of the SGZ was measured using ImageJ and the total number and proportions of cells per bin was calculated.

2.6 Sholl analyses

DCX⁺ cells were systematically randomly sampled and traced using Simple neurite tracer plugin⁴⁶ for ImageJ software (<https://imagej.net/Fiji>). Any cells with processes not entirely visible were excluded from analyses. For each cell, processes were traced manually. Primary and secondary path lengths were extracted, before Sholl analysis was performed. For all experiments detailed, radii separated by a distance of 10 μm were used. We compared sholl profiles using a single metric, the area under the curve (AUC). In addition, we analysed the Branching Index (BI), which produces a value that is proportioned to differences in the pattern of neurite ramification, and is relative to the amount of branches a neurite possesses⁴⁷. Data on primary path lengths (processes emanating directly from the soma) and branches (defined as any process originating from a primary process) was collected in addition to branch numbers.

2.7 Behavioural testing

All behavioural testing was conducted within the light phase. Genotypes were counterbalanced across morning and afternoon testing sessions. For the duration of testing, water was restricted to a two-hour access period per day. Body weight was monitored throughout the period of water deprivation, and animals were maintained at 90% of their free-feeding body weight prior to commencing water restriction. All subjects had *ad libitum* access to food in the home-cage throughout the behavioural testing period. All apparatus was cleaned thoroughly in between experimental subjects using 70% ethanol solution to remove odours.

2.7.1 Locomotor activity (LMA)

LMA was assessed in a 120-minute session conducted in the dark within a plexiglas chamber spanned transversely by two infrared beams.

2.7.2 Habituation to condensed milk

To habituate animals to the reward (condensed milk) to be used in the location discrimination task, animals were given the opportunity to consume either condensed milk or water. Preference for condensed milk was calculated as a percentage of total consumption for each of the five days during which water and milk were available.

2.7.3 Location discrimination task

To assess pattern separation, we used an adaptation of the Location Discrimination (LD) task as described in Hvoslef-Eide et al. (2013)⁴⁸ and originally reported by McTighe et al. (2009)⁴⁹. In order to facilitate daily testing of our large behavioural cohort, we implemented shorter, 20 minute test sessions rather than the 60 minute originally reported^{10,12,44,50,51}. Within each session, subjects could complete a maximum of 60 trials. The task was delivered in a touch screen operant chamber (Campden Instruments Ltd., UK; Figure 1A). Animals first went through a period of shaping (Figure 1B,i-ii), which accustomed them to making nose-poke responses to illuminated square stimuli at the front of the chamber, for which they received a reward of condensed milk. Having completed shaping, subjects were moved onto task training on a case-by-case basis to prevent overtraining⁴⁸.

Figure 1. Location discrimination (LD) task. **A)** Campden Instruments Ltd. mouse operant touchscreen chamber. The apparatus was housed inside sound-proof, sealed cubicles. The touchscreen was situated at the front of the chamber and black plastic masks with apertures were superimposed upon the touchscreen to direct nose pokes to the appropriate area of the screen on which stimuli were displayed. The mask used depended on the stage of training (see B). To the rear of the chamber, a magazine unit was integrated through which measured liquid rewards were delivered. In order to record the subject's movement, two photo-beams extended between the sidewalls parallel to the touchscreen and reward delivery area. The chamber floor was a perforated stainless steel raised above a tray filled with sawdust. **B)** Task structure. **i)** The initial stage of shaping consists of habituation and large squares. This stage teaches subjects to touch the screen to obtain reward. **ii)** The latter half of the shaping stage trains subjects to respond to smaller stimuli, to self-initiate trials and respond only to the illuminated stimulus. **iii)** After fulfilling criteria, subjects began training with stimuli at an intermediate separation. After reaching criterion at intermediate separation

training, subjects progress to task probe trials in which stimuli are presented at either close or far separations.

2.7.4 Task training

This stage saw the introduction of spatial discriminations, owing to the dual presentation of stimuli on the touchscreen, separated by an intermediate distance (response windows 2 and 5, Figure 1B,iii). One location (e.g., left) was designated as correct, whereas the other illuminated position (e.g., right) was incorrect. Animals were required to complete seven out of eight consecutive trials correct to reach criterion, after which the reward contingencies were reversed so that the previously incorrect location now became correct (Figure 2). Animals then had to achieve seven out of eight correct responses in the new location to attain criterion (reversal). Reward contingencies continued to reverse after each criterion was reached. The reversal element of the task reduces the likelihood that animals develop a side bias or use non-spatial strategies to complete the task^{12,48}. Between consecutive sessions (i.e., on subsequent days of training) the correct stimulus position remained consistent (e.g., if a session ended with the left stimulus being rewarded, the subsequent session, on the next day, would also begin with the left stimulus being rewarded). Subjects were considered to have completed training when they were able to achieve twenty trials or more per session, with a minimum of one reversal (i.e., reaching criterion twice) per session, on each of two consecutive days.

Figure 2. Trial structure for training stage and probe trials. Left panel shows a subject beginning a training session with the right-sided location designated correct. If an incorrect response is made, stimuli disappear and time-out occurs followed by a 10 second ITI, after which a new trial can be initiated. If a correct response is made, stimuli disappear, a reward can be collected and a new trial initiated after a 10 second ITI. If the subject makes 7 correct responses in 8 consecutive trials, the reward contingency is reversed and the left sided stimulus is now rewarded (right panel). The same process then ensues until the subject reaches criteria and another reversal occurs, after which the right side will be rewarded once again.

2.7.5 Probe trials

Having met criteria on the training stage, subjects were advanced to a series of probe sessions (Figure 1B,iii). Trials followed the same structure as the training stage (Figure 2), except for the addition of a spatial discrimination manipulation; sessions featured

either far stimulus separations (positions 1 and 6 illuminated) or close stimulus separation (positions 3 and 4 illuminated; Figure 1B,iii). Within one session, all stimulus presentations were either close or far, and each subject received sessions of the same probe type on two consecutive days. The rewarded location did not change between day one and two (unless the subject had finished on a reversal), for example if a subject finished their session on day 1 with the left location being rewarded, they would continue with the left stimulus being rewarded on day 2 until they reached criterion and a reversal occurred. Animals starting on close or far, and with left and right rewarded stimuli, were counterbalanced across groups. Animals received two pairs of probe trials across 4 separate days, with this sequence being repeated twice, meaning that all animals experienced 4 sessions of each separation.

2.7.8 LD task data analysis

For data analysis, all performance metrics including trials to criterion and percentage correct responses to criterion were calculated across all 4 sessions of each separation. In the instance animals did not attain any criteria, which was more common under the close stimuli separation, the number of trials until the completion of probe trials of the same type was substituted to prevent missing data.

2.8 Statistics

All statistical analyses were carried out using GraphPad Prism 8.4.1 (GraphPad Software, CA, USA). Data was assessed for equality of variances using the Brown-Forsythe test and then appropriate parametric (t test, one-way or two-way ANOVA) or non-parametric (Kruskal-Wallis) tests used. The main between-subjects' factor for ANOVA analyses was GENOTYPE (WT, $C3^{-/-}$ or $C3aR^{-/-}$). For morphological analyses,

there was an additional within-subjects factor of BLADE (suprapyramidal, infrapyramidal) and for BrdU long-pulse migration analyses there was a within-subjects factor of BIN (1,2,3,4). For the condensed milk habituation experiment, there was a within-subject factors of SESSION (3-7) and for analysis of LD task probe trial data, there was an additional within-subjects factor of SEPARATION (Close, Far). As we predicted differences in the close but not the far condition, planned comparisons using Tukey posthoc tests were carried out to analyse probe trial performance between genotypes. For migration analyses, planned comparisons were used to compare the proportion of BrdU⁺ cells between genotypes at each bin. For all other analyses, posthoc pairwise comparisons were performed using the Tukey or Dunn's tests for parametric or non-parametric analyses, respectively. For all analyses, alpha was set to 0.05 and exact p values were reported unless p<0.0001. All p values were multiplicity adjusted⁵². Data are expressed as mean ± standard error of the mean.

3. Results

3.1 Lack of C3/C3aR does not affect progenitor cell proliferation or neuronal fate choice

Wildtype, *C3*^{-/-} and *C3aR*^{-/-} mice were injected with thymidine analogue BrdU (100mg/kg, I.P) 6 hours prior to sacrifice, and brain tissue harvested to quantify uptake (Figure 3A). We also co-stained tissue with the endogenous proliferation marker Ki67 (Figure 3B). There were no differences in the total number of BrdU⁺ cells in the granule cell layer (GCL; Figure 3C) or the total number of Ki67⁺ cells (Figure 3D), or in the distribution of these cells in dorsal and ventral regions of the hippocampus (Figure S1A). The mitotic index (BrdU⁺Ki67⁺/BrdU⁺) was also unaltered, suggesting no differences in cell cycle speed between genotypes (Figure 3E). Additionally, the area of the GCL (measured in both dentate gyri of each section) was equivalent between genotypes (Figure S2A). We also investigated the location of BrdU-labelled cells within the dentate. In the absence of C3 and C3aR, we did not see any differences in the number of ectopically located (either in the hilus or inner molecular layer) BrdU⁺ Ki67⁺ cells (Figure S2B).

Figure 3. C3/C3aR deficiency does not impact acute proliferation in the dentate gyrus. **(A)** Animals were injected with BrdU (100mg/kg, I.P) 6 hours prior to sacrifice, and brain tissue harvested to quantify BrdU uptake. **(B)** Immunohistochemistry was performed for BrdU and Ki67 reactivity. Panel contains representative example of BrdU and Ki67 staining in the granule cell layer. Arrow indicates double positive BrdU⁺ Ki67⁺ cells situated in the sub granular zone. **(C)** Total number of BrdU⁺ cells WT 779.33±60.01, C3^{-/-} 570.00±84.26, C3aR^{-/-} 782.6±60.28; $F_{2,13}=0.287$, $p=0.6926$. **(D)** Total number of Ki67⁺ cells; WT 1614.66±126.94, C3^{-/-} 1568.58±213.25, C3aR^{-/-} 1781.33±151.23; $F_{2,15}=0.445$, $p=0.6491$. **(E)** Proportion of BrdU⁺ proliferating (Ki67⁺) cells; WT 0.48±0.02, C3^{-/-} 0.43±0.07, C3aR^{-/-} 0.44±0.02; $F_{2,13}=0.390$, $p=0.6850$. Data are expressed as mean ± S.E.M. Wildtype $N=6$, C3^{-/-} $N=4$, C3aR^{-/-} $N=6$.

In a separate cohort of animals, we investigated the phenotype of proliferating cells (Ki67⁺) in the dentate gyrus using the glial marker GFAP and immature neuronal progenitor marker doublecortin (DCX; Figure 4A&E). In this cohort there were again no differences in the total number of Ki67⁺ cells (Figure 4B). There were no differences in the number of proliferating type 1 cells (Ki67⁺GFAP⁺ cells within the GCL with apical morphology; Figure 4C) and these cells accounted for a similar proportion of the total Ki67⁺ population between genotypes suggesting no differences in the proliferative stem cell pool (Figure 4D). The total number of proliferating type 2b/3 neuronal progenitor cells was also unchanged (Ki67⁺DCX⁺; Figure 4F) and these cells again accounted for a comparable proportion of the proliferative population across genotypes (Figure 4G) indicating that early neuronal fate choice was unaffected by absence of C3/C3aR.

Of the total neuronal progenitor (DCX⁺) population, significantly fewer of these were in the cell cycle in *C3aR*^{-/-} mice compared to wildtype ($p=0.0386$), whereas *C3*^{-/-} mice were unchanged (Figure 4H). Despite a trend towards slight decrease in the total number of DCX⁺ cells in *C3aR*^{-/-} mice (Figure 5A&B) this was not significantly different to WT or *C3*^{-/-} mice. Taken together, these findings indicate that the decreased growth fraction of neural progenitor cells in *C3aR*^{-/-} mice did not affect the overall production of neural progenitors. There were no genotype differences in the distribution of any of the aforementioned cell types along the hippocampal longitudinal axis (Figure S1 B-D).

Figure 4. C3/C3aR deficiency does not impact the proliferation of type 1 radial glial cells whereas C3aR specifically reduces the proportion of neuroblasts that are proliferating. In a further cohort of animals, immunohistochemistry was performed for Ki67, GFAP and doublecortin (DCX) reactivity. **(A)** Representative example of Ki67 and GFAP staining. Ki67⁺ GFAP⁺ cells were only counted if they demonstrated the apical morphology characteristics of type 1 radial glial cells and were situated in the granule cell layer (as indicated by white arrow). **(B)** Total number of Ki67⁺ cells. WT 1606.00±172.00, C3^{-/-} 1993.00±178.6, C3aR^{-/-} 1749.00±188.7; F_{2,15}=1.19, p=0.3321. **(C)** Total number of proliferating type 1 radial glial cells (Ki67⁺ GFAP⁺); WT 238.50±48.80, C3^{-/-} 357.90±63.91, C3aR^{-/-} 292.80±60.30, F_{2,15}=1.06, p=0.3703. **(D)** Proportion of Ki67⁺ GFAP⁺ cells in total Ki67⁺ population; WT 0.15±0.02, C3^{-/-} 0.17±0.02, C3aR^{-/-} 0.16±0.01; F_{2,15}=0.143, p=0.8678. **(E)** Representative example of Ki67 and DCX staining. Yellow arrow indicates a cluster of double positive cells. **(F)** Total number of immature neuronal progenitor cells in the cell cycle (Ki67⁺ DCX⁺). WT 557.10±58.87, C3^{-/-} 719.00±67.24, C3aR^{-/-} 647.60±77.54; F_{2,15}=1.41, p=0.2744. **(G)** Proportion of Ki67⁺ DCX⁺ cells in total Ki67⁺ population. WT 0.37±0.02, C3^{-/-} 0.39±0.03, C3aR^{-/-} 0.39±0.01; F_{2,15}=0.278, p=0.7614. **(H)** Proportion of Ki67⁺ DCX⁺ cells in total DCX⁺ population. WT 0.05±0.006, C3^{-/-} 0.06±0.01, C3aR^{-/-} 0.03±0.007; H₂=7.45, p=0.0172. Data are mean ± S.E.M. Wildtype N =6, C3^{-/-} N =6, C3aR^{-/-} N =6. * = p≤0.05 for *post-hoc* genotype comparisons.

Figure 5. The total number of immature neuronal progenitor cells is unaffected by lack of C3 and C3aR. (A) Representative example of DCX staining in different genotypes. **(B)** Total number of DCX⁺ cells. WT 12904±1323, C3^{-/-} 13502±1530, C3aR^{-/-} 10344±1912; $F_{2,14}=1.08$, $p=0.3678$. Data are mean ± S.E.M. Wildtype $N=6$, C3^{-/-} $N=5$, C3aR^{-/-} $N=6$.

3.2 Lack of C3 results in increased neurogenesis due to greater survival of newborn neurons

In order to track the maturation and survival of new neurons born in the dentate gyrus, mice were injected with BrdU (100mg/kg, i.p) daily for 5 days and sacrificed 30 days later (Figure 6A). Tissue was processed for BrdU and NeuN immunoreactivity to detect surviving adult-born neurons (Figure 6B&C). We observed a significantly greater number of BrdU⁺ cells throughout the hippocampus (Figure S1 E) in C3^{-/-} mice compared to both wildtype ($p=0.0007$) and C3aR^{-/-} mice ($p=0.0051$; Figure 6D). There

444 were also a greater total number of cells double positive for BrdU and NeuN (Figure
445 6E) in $C3^{-/-}$ mice compared to both wildtype ($p=0.0022$) and $C3aR^{-/-}$ mice ($p=0.0139$;
446 Figure 6E). The proportion of BrdU labelled cells adopting neuronal fate ($BrdU^{+} NeuN^{+}$)
447 was unaltered between genotypes (Figure 6F).

448

449 Given that the total number of proliferating cells (Figure 3), the number of proliferating
450 cells that were expressing DCX (Figure 4F&G) and the proliferation dynamics of
451 immature neurons (Figure 4H) were all unchanged in $C3^{-/-}$ mice, we were confident
452 that there were no changes in early neuronal differentiation or proliferation and
453 therefore the net increase we observed of $NeuN^{+}BrdU^{+}$ cells in $C3^{-/-}$ mice (Figure 6)
454 suggested a specific effect of C3 on the survival of adult born GCs.

Figure 6. C3 influences the number of surviving adult-born neurons but does not affect fate choice. (A) Animals were injected with BrdU (100mg/kg, I.P) once daily for 5 days and sacrificed 30 days later. (B) Brain tissue was sectioned at 40 μ m and processed for BrdU and NeuN immunoreactivity. Micrograph shows representative staining. Yellow bow indicates example of BrdU⁺ NeuN⁺ cell magnified

in. **(C)** Representative example of BrdU⁺ NeuN⁺ double positive cell. **(D)** Total number of BrdU⁺ cells. WT 1096±60.9, C3^{-/-} 2152±253, C3aR^{-/-} 1344±100, F_{2,16}=12.0, p=0.0006. **(E)** Total number of BrdU⁺ NeuN⁺ cells. WT 439±26.0, C3^{-/-} 932±134, C3aR^{-/-} 559±56.5; F_{2,16}=9.25, p=0.0021. **(F)** Proportion of total BrdU⁺ cell population double positive for NeuN⁺ (neuronal fate). F_{2,16}=0.792, p=0.4700. Data are mean ± S.E.M. Wildtype N =6, C3^{-/-} N =6, C3aR^{-/-} N =7. *, ** and *** represent p≤0.05, p≤0.01 and p≤0.001 for *post-hoc* genotype comparisons, respectively.

3.3 Lack of C3 results in accelerated migration of surviving new neurons outside the sub granular neurogenic niche

As adult-born neurons mature, they migrate from the sub granular zone (SGZ) deeper into the granule cell layer (GCL)⁵³ as they extend their apical dendrites further into the molecular layer⁵⁴. To measure the migration of surviving neurons labelled in the 30-day BrdU pulse-chase experiment, we measured the distribution of BrdU⁺ cells within the GCL. We divided the GCL into four equal bins, the innermost of which corresponded to the SGZ, the band approximately two cell bodies thick that follows the GCL-hilar border. The subsequent two bins (1 & 2) represented the middle layers of the GCL, whereas bin 3 consisted of the outermost portion of the GCL proximal to the inner molecular layer (Figure 7A). There were no significant differences in the thickness of the GCL between wildtype and C3^{-/-} mice (p=0.2622) although C3aR^{-/-} mice did have a thicker GCL than wildtypes (p=0.0407; Figure S3) by approximately ~3 µm, less than one cell width. We analysed the number of BrdU⁺ cells that had migrated beyond the SGZ (i.e., occupied bins 1-3) and found that significantly more BrdU⁺ cells had migrated in C3^{-/-} mice compared to wildtype mice (p=0.0009) and C3aR^{-/-} mice (p=0.0146; Figure 7B). The proportion of BrdU⁺ cells that had migrated

was also significantly increased in $C3^{-/-}$ mice compared to wildtype ($p=0.0055$) but not compared to $C3aR^{-/-}$ mice ($p=0.1367$; Figure 7C), suggesting that the increased numbers of migrated cells in $C3^{-/-}$ mice were not merely due to surplus cells migrating normally, but instead due to altered migratory patterns of newborn neurons. Since we anticipated differences in the proportion of cells that migrated in $C3^{-/-}$ mice, we conducted planned comparisons of BrdU⁺ cells within the SGZ or bins 1-3. In $C3^{-/-}$ mice, we found an exaggerated proportion of cells in bin 1 compared to wildtype ($p=0.0119$) and $C3aR^{-/-}$ mice ($p=0.0312$) suggesting accelerated migration of surviving immature GC neurons in the absence of C3.

Figure 7. Lack of C3 alters the migration of adult born neurons into the granule cell layer. (A) Example of how we divided the GCL into four equal portions or bins. The inner-most bin, proximal to the hilus, comprised the sub granular zone (SGZ).

Bin 1 and 2 represented the middle of the GCL, whereas bin 4 represented the outermost layer of the GCL proximal to the inner molecular layer. **(B)** Total number of BrdU⁺ cells that had migrated beyond the SGZ (i.e., in bin 1-3). Wildtype 271±20.8, C3^{-/-} 648±97.2, C3aR^{-/-} 356±56.9, $F_{2,16}=10.8$, $p=0.0011$. **(C)** Migrated cells (BrdU⁺ cells found beyond the SGZ) as a proportion of total GCL BrdU⁺ cells. Wildtype 0.22±0.02, C3^{-/-} 0.34±0.01, C3aR^{-/-} 0.27±0.02, $F_{2,16}=7.18$, $p=0.0059$. **(D)** BrdU⁺ cells found in each bin as a proportion of total GCL BrdU⁺ cells. Main effect of BIN; $F_{1.72, 27.5}=88.1$, $p<0.0001$; GENOTYPE $F_{2,16}=0.139$, $p=0.8711$; BIN × GENOTYPE $F_{6,48}=1.51$, $p=0.1936$. Data are mean ± S.E.M. A total of 576 BrdU⁺ cells were analysed from $N=6$ wildtype animals, 1139 BrdU⁺ cells from $N=6$ C3^{-/-} animals and 869 BrdU⁺ cells from $N=7$ C3aR^{-/-} animals. *, ** and *** represent $p\leq 0.05$, $p\leq 0.01$ and $p\leq 0.001$ for *post-hoc* genotype comparisons, respectively.

3.4 Lack of C3/C3aR reduces morphological complexity of immature neuronal progenitor cells

We next used Sholl analysis to investigate the morphology of immature DCX⁺ neurons with vertically oriented apical processes in the GCL. These cells are typically type 3 cells², the stage at which the greatest morphological changes occur⁵³. Due to the known differences in the dendritic trees of immature neurons located in the suprapyramidal blade versus the infrapyramidal blade of the dentate⁵⁵, we analysed cells from each blade separately (Figure 8A). Cells were systematically randomly sampled, and their dendritic trees manually traced and subjected to Sholl analysis (Figure 8B). The resulting Sholl profiles indicated differences in both knockouts in the

524 suprapyramidal blade beyond ~70 μm from the cell soma (Figure 8C) but not the
525 infrapyramidal blade (Figure 8D). This was confirmed by analysis of area under the
526 curve, which revealed that both $C3^{-/-}$ and $C3aR^{-/-}$ DCX⁺ cells had a reduced AUC in
527 the suprapyramidal, but not the infrapyramidal blade, compared to wildtypes (Figure
528 8E).

529 At the outset, these differences could reflect both neurite length and branching⁴⁷ and
530 we therefore employed further measures to differentiate these possibilities. The
531 branching index⁴⁷ (BI; a specific measure of neurite ramification) of $C3aR^{-/-}$ DCX⁺ cells
532 was reduced in the suprapyramidal blade compared to that of wildtype and $C3^{-/-}$
533 (Figure 8F) suggesting less complex dendritic branching. In addition, there was a trend
534 towards reduced primary path length of suprapyramidal DCX⁺ cells in $C3aR^{-/-}$ cells
535 compared to wildtype (Figure S4 A). A similar trend was observed in average branch
536 length (Figure S4 B) and $C3aR^{-/-}$ DCX⁺ cells had significantly fewer branches
537 compared to wildtype and $C3^{-/-}$ cells, regardless of blade (Figure S4 C). These results
538 indicated an overall reduced dendritic complexity in the absence of C3aR, reflected by
539 decreased branch length, number and ramification in $C3aR^{-/-}$ DCX⁺ cells.

Figure 8. Absence of C3/C3aR influences the morphology of immature neurons.

(A) Location of suprapyramidal (most dorsal) and infrapyramidal (most ventral) blades.

(B) Left panel shows a representative example of a DCX⁺ immature neuron selected at random for Sholl analysis. Middle panel shows the tracing in progress, with primary process in green and branches in magenta. Right panel depicts the resulting trace, upon which Sholl analyses are based. (C) Sholl curve for cells in suprapyramidal blade (D) Sholl curve for cells in infrapyramidal blade. (E) Area under the curve (AUC) analysis. WT 202.41±11.74, C3^{-/-} 169.41±8.97, C3aR^{-/-} 160.91±9.25. BLADE × GENOTYPE interaction F_{2,386}=4.68, p=0.0098 [WT vs. C3^{-/-} p=0.0167, WT vs. C3aR^{-/-} p=0.0017, C3^{-/-} vs. C3aR^{-/-} p=0.7573] and main effect of BLADE; F_{1,386}=65.9, p<0.0001. (F) Branching Index (BI); WT 117.39± 10.88, C3^{-/-} 107.39±11.35, C3aR^{-/-} 79.98±9.79; main effect of GENOTYPE F_{2,272}=4.29, p=0.0147 *post hoc* WT vs. C3aR^{-/-} p=0.0076, C3^{-/-} vs. C3aR^{-/-} p=0.0556, WT vs. C3^{-/-} p=0.7494. Data are mean ± S.E.M. Analyses were of 131 cells from N =6 wildtype animals, 127 cells from N =5 C3^{-/-} animals, and 131 cells from N =6 C3aR^{-/-} animals. * and ** represent p≤0.05 and p≤0.01 respectively for *post-hoc* genotype comparisons.

Overall, our histological data indicated that lack of C3 specifically increased the survival of adult born neurons and accelerated their migration. Lack of either C3 or C3aR had effects on the morphological development of adult born neurons, though this was more pronounced in the absence of C3aR. We next assessed the functional relevance of these phenotypes for pattern separation, the computational process thought to supported by adult born neurons that is important for distinguishing between similarly encoded spatial or contextual stimuli⁸.

3.5 *C3aR*^{-/-} mice demonstrate improved pattern separation whereas *C3*^{-/-} show evidence of impaired cognitive flexibility

We investigated AHN-related behaviours in a reward-based visual discrimination task using a paradigm previously shown to be sensitive to manipulations of AHN in assaying pattern separation^{44,50,56}. The task required discrimination of stimuli with varying degrees of spatial proximity with closer stimuli placing a greater demand upon pattern separation^{10,12,44}. Mice were initially trained to discriminate the correct response location between two white square stimuli (located in two of six possible locations; Fig 9A) at an intermediate level of spatial separation to a baseline performance criterion of 7 correct responses out of 8. Task difficulty was manipulated from this baseline by introducing probe trials where the stimuli were either further apart (reducing pattern separation requirements) or closer together (increasing pattern separation requirements) than the baseline discrimination (see Fig 9A, probe trials). Prior to beginning training in the touch-screen apparatus, we established equivalent locomotor activity between genotypes (Figure S5A), adapted mice to a water deprivation schedule to motivate reward-orientated behaviour (Figure S5B) and established equal preferences for condensed milk, the liquid foodstuff to be used as reward in the discrimination task (Figure S5C).

An initial finding was that in the earliest stages of behavioural shaping (Figure S6) and training to baseline criterion (Figure 9B), *C3*^{-/-} mice displayed a relative enhanced performance, requiring fewer trials to reach criterion. Upon reaching baseline performance each animal was subjected to the probe trials. As predicted, task difficulty was increased in the close stimuli separation condition, an effect seen across genotypes as evidenced by an overall reduced percentage of correct responses made

in reaching the criterion of 7/8 correct responses (Figure 9D), fewer mean criteria achieved (where the mice could continue responding beyond reaching the first criterion following a reversal of the rewarded stimuli position; Figure 9E) and increased latencies for both correct and incorrect responses (Figure 9H).

As we anticipated differences in the effects of the close and far probes, we carried out planned comparisons using *posthoc* Tukey tests that revealed an enhanced performance of *C3aR*^{-/-} mice in reaching the first criterion on close probe trials compared to wildtype and *C3*^{-/-} mice. *C3aR*^{-/-} mice were relatively unperturbed by the close separation probe, and under that condition required fewer trials to reach the first criterion compared to both wildtype and *C3*^{-/-} mice (Figure 9C; WT vs *C3aR*^{-/-} $p=0.0150$, *C3*^{-/-} vs. *C3aR*^{-/-} $p=0.0004$). *C3aR*^{-/-} mice also made a greater percentage of correct responses in achieving the first criterion in the close separation probe compared to wildtypes (Figure 9D; WT vs *C3aR*^{-/-} $p=0.0235$, *C3*^{-/-} vs. *C3aR*^{-/-} $p=0.2673$). Although there was a trend towards *C3*^{-/-} mice requiring more trials to reach criterion, their performance was not significantly different to wildtype mice on either the number of trials to first criterion (WT vs. *C3*^{-/-} $p=0.3267$; Figure 9C) or the percentage of correct responses before achieving first criterion (WT vs. *C3*^{-/-} $p=0.5790$; Figure 9D). There were no significant differences between genotypes in the far separation condition in either trials to first criterion or percentage correct responses to first criterion (Figure 9C,D).

After animals reached the first criterion in either the close or far condition, the spatial position of the rewarded stimulus was reversed and relearning of the contingency was required to reach subsequent criteria. In reality animals rarely reached criterion within

the same session especially under the close condition, so we took account of all probe trial sessions of each separation type to calculate the mean number of criteria achieved. Mirroring to an extent the enhanced performance of $C3^{-/-}$ mice at the baseline training stage (Figure 9B), where stimuli were separated by an intermediate distance, $C3^{-/-}$ mice achieved significantly more criteria across sessions than wildtype and $C3aR^{-/-}$ mice under the far separation (Figure 9E; $C3^{-/-}$ vs. WT $p=0.0001$, vs. $C3aR^{-/-}$ $p=0.0019$,) but not however at the close separation ($C3^{-/-}$ vs WT $p=0.3077$; vs. $C3aR^{-/-}$ $p=0.6302$). This was an important distinction since it implied that the apparent enhanced performance in the far separation condition was not due to an effect on pattern separation, since any advantage would be manifest in the close but not far separation condition.

We observed that the behaviour of the $C3^{-/-}$ mice was characterised by a general increase in responding resulting in a significantly greater number of trials across all sessions regardless of stimulus separation (Figure 9F). We also noted that $C3^{-/-}$ mice showed a different response style compared to wildtype and $C3aR^{-/-}$ mice, demonstrating decreased response latencies, regardless of accuracy or stimulus separation, although these differences were particularly pronounced at close separations due to the slowing of responses in wildtype and $C3aR^{-/-}$ mice (Fig 9H; correct responses at close $C3^{-/-}$ vs. WT $p=0.1126$; vs. $C3aR^{-/-}$ $p=0.0295$; and incorrect responses at close $C3^{-/-}$ vs. WT $p=0.0011$ vs. $C3aR^{-/-}$ $p=0.0133$). In addition, $C3^{-/-}$ mice made a greater number of inter-trial interval touches across conditions (Figure 9I; Close; $C3^{-/-}$ vs. WT $p=0.0073$; vs. $C3aR^{-/-}$ $p=0.0025$; Far $C3^{-/-}$ vs. WT $p=0.0039$, vs. $C3aR^{-/-}$ $p=0.0150$).

The pattern of behaviour in the $C3^{-/-}$ mice was reminiscent of impaired behavioural inhibition, that is, a relative inability to suppress inappropriate responding. To test this further we took advantage of the reversal manipulation component of the paradigm and analysed the number of incorrect responses made post-reversal as an index of perseverative responding having first adjusted this for the number of criteria achieved per subject. We found that $C3^{-/-}$ mice made significantly more perseverative errors per criteria when stimuli were close together (Figure 9G; $C3^{-/-}$ vs. WT $p=0.0008$, vs. $C3aR^{-/-}$ $p=0.0142$) but not far apart ($C3^{-/-}$ vs. WT $p=0.6512$, vs. $C3aR^{-/-}$ $p=0.8060$). These data were consistent with a deficit in behavioural inhibition manifest under conditions of increased task difficulty where there were increased demands on pattern separation.

In summary, $C3aR^{-/-}$ showed enhancements in pattern separation abilities, evidenced by improved discrimination performance under conditions where stimuli were highly spatially similar. In contrast, $C3^{-/-}$ behaviour was consistent with deficits in cognitive flexibility arising from impaired behavioural inhibition that were especially marked under conditions of increased task difficulty.

Figure 9. Performance on the location discrimination task. (A) Subjects were first trained on stimuli separated by an intermediate distance, before moving onto probe trials in which the spatial separation between stimuli was manipulated to tax pattern separation. Criteria was reached when subjects made 7 out of 8 correct responses on

665 consecutive trials, after which the reward contingency was reversed, and subjects
 666 were required to re-learn the contingency to achieve a subsequent criterion. **(B)** Mean
 667 number of trials needed to complete training. $H_2=5.59$, $p=0.0610$. **(C)** Trials to first
 668 criterion. Planned comparison of genotypes at close; WT 31.08 ± 4.31 vs $C3aR^{-/-}$
 669 17.00 ± 2.93 $p=0.0150$, $C3^{-/-}$ 38.33 ± 4.87 vs. $C3aR^{-/-}$ $p=0.0004$, WT vs. $C3^{-/-}$ $p=0.3084$
 670 and far; WT 28.50 ± 3.43 vs $C3aR^{-/-}$ 17.90 ± 2.47 $p=0.0851$, $C3^{-/-}$ 22.17 ± 1.82 vs. $C3aR^{-/-}$
 671 $p=0.6957$, WT vs. $C3^{-/-}$ $p=0.4238$. **(D)** Percentage of correct responses made in the
 672 run up to first criterion; main effect of SEPARATION $F_{(1,28)}=4.64$, $p=0.0400$. Planned
 673 comparisons of genotypes at close: WT 46.16 ± 5.98 vs $C3aR^{-/-}$ 67.03 ± 6.05 $p=0.0235$,
 674 $C3^{-/-}$ 54.08 ± 4.55 vs. $C3aR^{-/-}$ $p=0.2673$ and far; WT 60.04 ± 3.44 vs $C3aR^{-/-}$ 72.86 ± 8.29
 675 $p=0.7731$, $C3^{-/-}$ 65.46 ± 3.34 vs. $C3aR^{-/-}$ $p=0.6440$, WT vs. $C3^{-/-}$ $p=0.7731$. **(E)** Mean
 676 criteria achieved across sessions; main effect of SEPARATION $F_{(1,28)}=49.5$, $p<0.0001$.
 677 Planned comparisons at close; $C3^{-/-}$ 1.06 ± 0.19 vs WT 0.54 ± 0.20 $p=0.3077$; vs. $C3aR^{-/-}$
 678 0.75 ± 0.20 $p=0.6302$; WT vs. $C3aR^{-/-}$ $p=0.8528$ and far; $C3^{-/-}$ 5.11 ± 0.63 vs WT
 679 2.17 ± 0.30 $p=0.0001$, vs. $C3aR^{-/-}$ 2.60 ± 0.56 $p=0.0019$, WT vs. $C3aR^{-/-}$ $p=0.7646$. **(F)**
 680 Total trials completed across sessions. Close WT 82.67 ± 9.60 , $C3^{-/-}$ 142.78 ± 11.31 ,
 681 $C3aR^{-/-}$ 67.70 ± 11.75 ; Far WT 89.08 ± 9.16 , $C3^{-/-}$ 153.56 ± 12.60 , $C3aR^{-/-}$ 89.60 ± 16.73 .
 682 Main effect of GENOTYPE; $F_{(2,28)}=10.6$, $p=0.0004$ [*posthoc* $C3^{-/-}$ vs WT $p=0.0015$;
 683 vs. $C3aR^{-/-}$ $p=0.0007$; WT vs. $C3aR^{-/-}$ $p=0.8877$]. **(G)** The number of perseverative
 684 errors (incorrect responses made after a reversal) were calculated and adjusted to
 685 account for differences in the number of criteria reached per animal. Planned
 686 comparisons at close; $C3^{-/-}$ 27.64 ± 4.52 , vs. WT 10.71 ± 3.09 $p=0.0008$, vs. $C3aR^{-/-}$
 687 15.07 ± 3.34 $p=0.0142$; and far $C3^{-/-}$ 10.91 ± 1.32 , vs. WT 7.81 ± 1.09 ; $p=0.6512$, vs.
 688 $C3aR^{-/-}$ 13.30 ± 3.11 $p=0.8060$. **(H)** Latencies of correct and incorrect responses.

Correct response latencies; main effect of SEPARATION $F_{(1,28)}=13.9$, $p=0.0009$;
Incorrect response latencies; main effect of SEPARATION $F_{(1,28)}=18.3$, $p=0.0002$.
Planned comparison of correct responses at close $C3^{-/-}$ 6.35 ± 0.95 s, vs. WT
 11.42 ± 2.35 s; $p=0.1126$; vs. $C3aR^{-/-}$ 13.16 ± 1.95 $p=0.0295$; WT vs. $C3aR^{-/-}$ $p=0.7531$
and incorrect responses at close $C3^{-/-}$ 5.57 ± 0.68 s, vs. WT 15.62 ± 3.01 s; $p=0.0011$;
vs. $C3aR^{-/-}$ 13.66 ± 2.52 $p=0.0133$; WT vs. $C3aR^{-/-}$ $p=0.7278$. (II) Touches made to the
screen in the inter-trial interval (ITI). Planned comparisons at close; $C3^{-/-}$ 11.86 ± 2.13
s, vs. WT 4.96 ± 1.22 s; $p=0.0073$; vs. $C3aR^{-/-}$ 3.83 ± 1.03 $p=0.0025$; WT vs. $C3aR^{-/-}$
 $p=0.8558$; and far $C3^{-/-}$ 13.22 ± 1.98 , vs. WT 5.83 ± 1.26 s; $p=0.0039$, vs. $C3aR^{-/-}$
 6.63 ± 1.75 $p=0.0150$; WT vs. $C3aR^{-/-}$ $p=0.9267$. Data are mean $\pm$ S.E.M. $N=12$ wildtype
animals, $N=9$ $C3^{-/-}$ animals and $N=10$ $C3aR^{-/-}$ animals. *, ** and *** = $p\leq0.05$, $p\leq0.01$
and $p\leq0.001$ for *post-hoc* genotype comparisons respectively.

4. Discussion

Using knockout mouse models, we conducted an investigation of the impact of signalling through the C3a/C3aR axis on adult hippocampal neurogenesis and demonstrated separable effects of these proteins in regulating cellular, morphological and functional aspects of neurogenesis. C3 impacted upon the survival and migration of new neurons in the GCL, and while both C3 and C3aR influenced the morphology of immature neurons, the effect was most evident in the total absence of C3aR signalling. Lack of C3aR enhanced pattern separation without influencing other aspects of cognition probed in the discrimination task, whereas the increased neurogenesis and altered migration seen in *C3^{-/-}* mice was associated with impairments in cognitive flexibility under conditions placing demands upon pattern separation.

Our findings of enhanced survival in absence of C3 align with a prior report focusing on C3d/CR2 signalling³⁶. Consideration of these findings alongside ours may provide clues as to the specific pathways responsible for C3 effects upon cell survival. Moriyama et al (2011)³⁶ demonstrated an inhibitory role for the C3d/CR2 axis in AHN, reporting increases in immature GCs (BrdU⁺ NeuN⁺) in *CR2^{-/-}* mice using a pulse-chase paradigm equivalent to our own. Our observation of increased basal neurogenesis in *C3^{-/-}* mice but not *C3aR^{-/-}* mice is consistent with this since C3d is derived from C3, thus *C3^{-/-}* mice lack C3d/CR2 signalling whereas *C3aR^{-/-}* mice do not. This suggests that the survival effects we have seen may be due to a lack of signaling through CR2, which under physiological conditions is expressed on neural progenitor cells³⁶. Since other C3 breakdown products and their receptors, including C5a/C5aR⁵⁷, C3b/CR3³⁶ and according to our data C3a/C3aR, do not modulate net

levels of adult neurogenesis³⁶, it is likely that C3d/CR2 signalling constitutes a novel immune mechanism to maintain homeostatic levels of neurogenesis.

Our findings seem at odds with another report which showed a positive role for the C3a/C3aR axis in regulating adult neurogenesis, evidenced by reduced numbers of neural progenitors (BrdU⁺ DCX⁺ cells) and committed immature GCs (BrdU⁺ NeuN⁺) in both C3^{-/-} and C3aR^{-/-} mice and wildtype mice treated with a C3aR antagonist³⁵. While we did see a significant decrease in BrdU⁺ DCX⁺ cells in C3aR^{-/-} mice compared to wildtypes, this was not seen in C3^{-/-} mice and our main finding of increased neurogenesis (BrdU⁺ NeuN⁺ cells) is inconsistent with these results. However, the use of different pulse-chase intervals in the previous study makes interpretation challenging in terms of disentangling their effects on early progenitor proliferation dynamics versus our effects on neuronal survival. Moreover, a subsequent study from the same group using C3a-overexpressing mice did not support a positive role for C3a/C3aR signalling in regulating basal neurogenesis⁵⁷. Instead, our work using both a short-term and a long-term labelling procedure permits clear dissociation of effects on acute proliferation versus long-term survival, demonstrating a prominent role for C3 fragments in negatively regulating survival without affecting proliferation or neuronal lineage commitment.

The mechanisms through which C3 may influence cell survival are unclear. Only a small percentage of newborn cells are incorporated into the hippocampal circuitry and ~70% are eliminated at the immature neuron stage^{61,62} via apoptosis-coupled microglial phagocytosis. One possibility is that C3 signalling promotes programmed cell death, either via cell-intrinsic or extrinsic mechanisms. Similar mechanisms have been described for other complement factors implicated in developmental neurogenesis⁸⁷. Another possibility relates to opportunities for synaptic

integration. During development, programmed cell death is necessary for optimal matching of the neuronal population with available synaptic targets⁵⁸ and this is recapitulated within the adult neurogenic niche, where important determinants of survival include the success of immature GCs in forming synaptic contacts within the molecular layer. Increased synapse density has been found in this area of the $C3^{-/-}$ brain^{38,59}, presumably due to the absence of normal complement-mediated developmental synaptic elimination²³. It is therefore possible that increased survival of newborn cells in $C3^{-/-}$ mice is facilitated by increased availability of synaptic contacts, less competition and greater opportunity for integration and survival. The accelerated migration of BrdU-labelled cells we observed in $C3^{-/-}$ mice is consistent with this notion, since migration into the GCL permits maturation via further reaching synaptic contacts⁵³.

We also demonstrated effects of C3 and C3aR on the morphological complexity of neuronal progenitor cells. Our results, showing reduced dendritic arborisation primarily in the absence of C3aR, support prior *in vitro* studies implicating a necessary role for C3aR in normal neuronal morphology^{39,42,43} and is the first to demonstrate this relationship is maintained *in vivo*, within the adult neurogenic niche. While these previous studies have mostly focused on either inhibition of C3aR, or addition of C3a, the fact that our effects were most pronounced in the absence of C3aR suggests redundancy of C3a in mediating morphological development. One possible explanation is that VGF-deprived peptide TLQP-21, a known C3aR ligand^{60,61}, contributes to the maturation of immature neurons in the hippocampal neurogenic niche, and further investigations are required to address this possibility. Interestingly, we found effects of C3/C3aR on the morphology of neurons in the suprapyramidal, but not infrapyramidal blade. How such specificity might come about is unclear and there

is scarce information relating to the transverse hippocampal axis. In a small number of studies that have examined the two blades, differential neurogenesis, in addition to connectivity, dendritic tree size and synapse density of GC neurons has been demonstrated⁶²⁻⁶⁴. Functionally, although suprapyramidal GCs are known to respond preferentially to spatial exploration^{65,66}, how their morphology contributes to the functional properties of immature neurons in encoding spatial information is unknown.

To assess whether the changes we observed in AHN were associated with functional changes in pattern separation, we used an established discrimination task previously utilised to detect changes in pattern separation after pro-neurogenic⁴⁴ and anti-neurogenic manipulations^{10,12,49}. Typically, enhancing adult neurogenesis is associated with improved ability to discriminate highly similar stimuli or contexts^{44,67,68} although this has not been entirely consistent across the literature^{69,70}. Despite increased levels of neurogenesis, $C3^{-/-}$ mice showed impairments in spatial discrimination performance, but only post-reversal, indicating impairments in cognitive flexibility as opposed to pattern separation per se. Interestingly, it has been reported that manipulations of neurogenesis only affected performance on trials after a learned discrimination was reversed, rather than during the initial spatial discrimination¹⁴. Additionally, abnormally enhanced neurogenesis in $C3^{-/-}$ mice may be responsible for impairments in cognitive flexibility. Consistent with our data in $C3^{-/-}$ mice showing elevated levels of neurogenesis alongside impaired reversal learning in the face of increased spatial discrimination demands, ablating neurogenesis has led to improvements in spatial working memory when animals were required to discriminate highly similar cues presented in short succession or when animals needed to suppress conflicting responses⁷¹, conditions akin to our task.

Despite the impaired performance of $C3^{-/-}$ mice in the reversal element of the task, $C3^{-/-}$ mice paradoxically achieved the greatest number of criteria (and thus reversals) across groups. We believe this is an epiphenomenon due to these mice completing a greater number of trials throughout training and probe trials. These mice therefore had more opportunity than other groups to reach criterion, even if achieved in a less efficient manner. In the absence of a general hyperactivity phenotype, this may indicate changes in motivation for reward. Moreover, decreased response latencies and increased inappropriate responding in $C3^{-/-}$ indicated a failure of behavioural inhibition and a potential impulsivity phenotype. It is unlikely that increased speed of responding was due to a decreased perceived difficulty of the task for these animals, given that their accuracy of responding was not improved. Using tasks designed to specifically probe the influence of C3 on cognitive flexibility, motivational behaviour and impulsivity will be a priority for future work.

$C3aR^{-/-}$ mice on the other hand were superior in their pattern separation ability and were unimpaired by the reversal component of the task. If the cellular phenotypes we have observed in these mice are linked to behavioural phenotypes, it seems paradoxical that decreased morphological complexity of immature neurons in the suprapyramidal blade would benefit function. Indeed, spatial learning has been shown to increase dendritic complexity of immature neurons⁷². While it is known that younger neural progenitor cells mediate pattern separation⁷³, it has also been demonstrated that less morphologically complex immature DCX cells may be more important for pattern separation and cognitive flexibility than more mature DCX cells and GC neurons ($BrdU^+ NeuN^+$)⁷⁴. Additional factors likely of relevance include the fact that $C3aR$ is also part of a signalling pathway that mediates synaptic strength^{75,76} which likely influences the synaptic connectivity both within and beyond the neurogenic

niche, consistent with evidence of altered cognition in *C3aR*^{-/-} mice from a variety of contexts^{26,39,77,78}.

An important consideration is our use of constitutive knockouts. Knocking out complement components such as C1q, C3 or CR3 results in sustained deficits in synaptic connectivity^{23,33,38,79} which may alter the wider environment in which adult neurogenesis takes place. Furthermore, increased neurogenesis is not always beneficial and is often associated with abnormal neuronal maturation, integration⁸⁰ and migration, as we have seen. Therefore, despite the increased levels of neurogenesis we report in *C3*^{-/-} mice, it is possible that their surplus adult born neurons integrate into altered hippocampal circuit and this may have greater effects on cognition than the impacts on GC survival per se.

Limitations of our study include our initial focus on male subjects due to known fluctuations in AHN attributable to sex hormones⁸¹. Given important sexual dimorphism in hippocampal cognition⁸² and complement activity⁴⁸, this will be a priority in our future work. Finally, we had to adapt our analysis of the location discrimination task due to the considerable difficulty subjects experienced in reaching criterion within a single session, a known challenge of this task when applied in mice as opposed to rats^{10,12,44,49}. Instead, we analysed performance across rather than within sessions of each condition and as such there is likely to be more variability in our data introduced by delays between individual sessions than in some other reports^{10,12,44,49}.

In conclusion, we show novel, negative regulatory roles for complement C3 and C3aR in the survival and morphology of adult born neurons in the adult hippocampus, findings consistent with the detrimental impact of excessive inflammation on neurogenesis in neuropathologies such as temporal lobe epilepsy, where complement activation is associated with chronically reduced neurogenesis⁸³⁻⁸⁵. We demonstrate

for the first time that C3 and C3aR influence neurogenesis-associated cognitive processes of relevance to a range of neuropsychiatric disorders in which abnormal neurogenesis and complement activity are implicated^{28,86} and add to the growing body of evidence literature demonstrating intriguing non-canonical roles of complement in the brain.

Author contributions

The study was designed by LJW, TH, BPM, MZ, WPG and LSW. LJW and TH performed behavioural experiments. Molecular analyses were performed by LJW, NH, CE and OM. Data interpretation were carried out by LJW, JH, NH, TRH, MZ, BPM, LSW, TH and WPG. The manuscript was drafted by LJW, TH, WPG and LSW. All authors approved the final manuscript.

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Competing financial interests

The authors declare no competing financial interests.

Materials and correspondence

All data from this study are available from the corresponding authors upon reasonable request.

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