Suppressor of Cytokine Signalling 2 (SOCS2) regulates numbers of mature newborn adult hippocampal neurons and their dendritic spine maturation

Running title: SOCS2, hippocampal neurogenesis and dendritic spines

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Number of text pages: 24

Number of Tables: 1

Number of Figures: 4 + 1 supplemental

Grant sponsor:

National Health and Medical Research Council of Australia; Project grant 1045125 and Fellowship (to AMT) 628344.

Stem Cells Australia

Key words: dentate gyrus, CA3, neural precursor cell, dendrite, nestin

Abstract

Overexpression of Suppressor of Cytokine Signalling 2 (SOCS2) has been shown to promote hippocampal neurogenesis *in vivo* and promote neurite outgrowth of neurons *in vitro*. In the adult mouse brain SOCS2 is most highly expressed in the hippocampal CA3 region and at lower levels in the dentate gyrus, an expression pattern that suggests a role in adult neurogenesis. Herein we examine generation of neuroblasts and their maturation into more mature neurons in SOCS2 null (SOCS2KO) mice. EdU was administered for 7d to label proliferative neural precursor cells. The number of EdU-labelled doublecortin⁺ neuroblasts and NeuN⁺ mature neurons they generated was examined at day 8 and day 35 respectively. While no effect of SOCS2 deletion was observed in neuroblast generation, it reduced the numbers of EdU-labeled mature newborn neurons at 35d. As SOCS2 regulates neurite outgrowth and dentate granule neurons project to the CA3 region, alterations in dendritic arborisation or spine formation may have correlated with the decreased numbers of EdU-labeled newborn neurons. SOCS2KO mice were crossed with Nes-CreER^{T2}/mTmG mice, in which membrane eGFP is inducibly expressed in neural precursor cells and their progeny, and the dendrite and dendritic spine morphology of newborn neurons were examined at 35d. SOCS2 deletion had no effect on total dendrite length, number of dendritic segments, number of branch points, or total dendritic spine density but increased the number of mature “mushroom” spines. Our results suggest that endogenous SOCS2 regulates numbers of EdU-labeled mature newborn adult hippocampal neurons, possibly by mediating their survival and that this may be via a mechanism regulating dendritic spine maturation.

Introduction

Neurogenesis or the birth of new neurons occurs throughout life in the adult brain. The sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) of the hippocampal dentate gyrus are the two major neurogenic niches of the adult brain. Neural precursor cells (NPCs) residing in the SVZ and SGZ proliferate and give rise to immature neurons called neuroblasts (Ming and Song 2011). SVZ derived neuroblasts leave the lateral ventricle and migrate to the olfactory bulb (OB) via the rostral migratory stream where they mature and integrate as various interneuron types (Lledo et al. 2006). SGZ derived neuroblasts remain within the dentate gyrus and migrate only a short distance to the granule cell layer (GCL), where they mature and integrate as dentate granule cells (Ming and Song 2011) whose dendritic arbors project to the CA3 region of the hippocampus.

Elucidation of the functional importance of endogenous adult neurogenesis remains an active area of research. Thus far, adult hippocampal neurogenesis has been implicated in roles such as the regulation of mood, learning and memory (Deng et al. 2009; Sahay and Hen 2007) and adult OB neurogenesis has importance in the regulation of olfactory circuit plasticity (Livneh et al. 2014). A role for endogenous adult neurogenesis in neural repair and regeneration is also suggested by increased NPC proliferation and/or ectopic NPC migration in animal models of neurotrauma (Ramaswamy et al. 2005). Indeed evidence for enhanced endogenous adult neurogenesis also exists in the human brain after trauma (Marti-Fabregas and Romaguera-Ros 2010; WeiMing et al. 2013). Disrupted adult neurogenesis has also been correlated with certain neurodegenerative disease states (Grote and Hannan 2007). Therefore, endogenous neurogenesis is an important process for the normal functioning of the adult brain.

Gaining a thorough understanding of factors that regulate aspects of NPC proliferation, differentiation and newborn neuron survival is important to allow us to explore strategies by which this process can be manipulated as a therapeutic strategy for neurodegenerative disease and/or trauma. Identification and characterisation of factors involved in the regulation of newborn neuron survival and/or neural differentiation is

particularly crucial with respect to the treatment of traumatic or ischemic brain injuries. This is because despite large increases in NPC proliferation following injury, very few of these cells survive to functional maturity and the remaining majority are directed towards a glial fate (Saha et al. 2013). One such factor which may be of importance in regulating newborn neuron survival and have potential anti-inflammatory roles in the broader context of the CNS is the suppressor of cytokine signalling-2 (SOCS2).

SOCS2 is one of eight members of the SOCS protein family and was originally characterised for its role in the periphery as a negative regulator of growth hormone (GH) with SOCS2KO mice having an overgrowth phenotype (Metcalf et al. 2000). SOCS2 has since also been identified as an important regulator of lipoxin mediated anti-inflammatory responses in the periphery (Machado et al. 2006) and to have roles both dependent and independent of GH in the CNS (Rico-Bautista et al. 2006). In the developing CNS, SOCS2 has been shown to overcome the inhibitory effects of GH signalling on SVZ-derived neurogenesis by regulating expression of the neurogenic basic helix- loop-helix (bHLH) transcription factor neurogenin-1 (Ngn1); SOCS2KO mice showed decreased cortical neuron density (Turnley et al. 2002). SOCS2 has also been identified as a downstream regulator of Epidermal Growth Factor (EGF) and Nerve Growth Factor (NGF), promoting neurite outgrowth in cultured cortical and dorsal root ganglia neurons, respectively (Goldshmit et al. 2004a; Goldshmit et al. 2004b; Uren et al. 2014). Further, brains of adult SOCS2 overexpressing transgenic (SOCS2Tg) mice had increased cortical neuron density coupled with increased dendrite arborisation (Ransome and Turnley, 2005). They also show increased adult hippocampal neurogenesis, with enhanced survival of newborn adult hippocampal neurons both under basal physiological and voluntary running conditions, independent of GH signalling (Ransome and Turnley 2008). In addition to EGF and NGF signalling, SOCS2 has been shown to associate with the Brain Derived Neurotrophic Factor (BDNF) receptor TrkB (unpublished observations) and to modulate erythropoietin signalling, both of which have pro-neurogenic roles and may play a role in SOCS2 regulation of neurogenesis (Uren and Turnley 2014; Wang et al. 2004; Wei et al. 2015; Yu et al. 2002).

In SOCS2Tg mice, SOCS2 expression is driven by the Ubiquitin C promoter and, as such, is expressed in most cell types (Greenhalgh et al. 2002). However, in the normal adult brain SOCS2 is predominantly expressed in the CA3 region and more weakly in the dentate gyrus (Polizzotto et al. 2000) (Supp. Fig. 1). Such specific endogenous SOCS2 expression suggests it plays a role in normal hippocampal neurogenesis but it remains to be determined whether the pro-survival phenotype observed in SOCS2Tg mice was the result of an NPC-specific effect or due to a pro-neuron survival effect of the hippocampal microenvironment generated by global SOCS2 overexpression.

Functional integration of newborn neurons is required for newborn neuron survival (Turnley et al. 2014) and this is at least in part regulated by dendritic arborisation and synapse formation. As SOCS2 overexpression enhances neurite outgrowth of primary neurons (Goldshmit et al. 2004a; Uren et al. 2014) and neurons differentiated from SOCS2Tg NPCs (Scott et al. 2006) *in vitro*, it is possible that the enhanced newborn adult hippocampal neuron survival observed in SOCS2Tg mice (Ransome and Turnley 2008) may be mediated by improved integration of those cells by increased dendritic arborisation *in vivo*.

In this manuscript we examine whether there is a role for endogenous SOCS2 in adult hippocampal neurogenesis and/or newborn neuron survival by comparing neurogenesis in wildtype mice with mice in which the SOCS2 gene has been deleted, SOCS2 knockout (SOCS2KO) mice. We observed that there were decreased numbers of newborn mature neurons in the SOCS2KO mice, the opposite to what we observed previously in mice which over-expressed SOCS2 (SOCS2Tg) (Ransome and Turnley 2008). Given that overexpression of SOCS2 enhances neurite outgrowth *in vitro*, as described above, we examined whether or not differences in dendritic tree arborisation or dendritic spine morphology in newborn hippocampal neurons of SOCS2KO mice may provide a potential mechanism for the decreased newborn neuron survival. While no differences in dendrite morphology or dendritic spine density were observed there was an increase in the proportion of more mature “mushroom” spines.

Materials and Methods

Animals

Mice were obtained from breeding colonies maintained at University of Melbourne. Animals were housed in a specific pathogen free facility in individually ventilated cages with food and water ad libitum and kept on a constant 12h light/dark cycle. All mouse strains were on the C57Bl/6 background. The use of experimental animals was approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health, University of Melbourne, Australia. Male and female mice between 8-12 weeks of age were used. The mouse strains used are listed below.

SOCS2KO mice

SOCS2 null (knockout; KO) mice do not express SOCS2 as they lack the entire SOCS2 coding region (Metcalf et al. 2000). They are available via the Australian Phenome Bank: ID 1812. Mice were bred and maintained as a homozygous KO x KO colony, therefore aged matched wildtype (WT) C57Bl/6 controls obtained from the Animal Resource Centre, Western Australia were used as controls. These C57Bl/6 mice were also used to refresh the SOCS2KO colony on a regular basis.

SOCS2KO x Nes-CreER^{T2} 5.1/mTmG mice

Nes-CreER^{T2} 5.1/mTmG animals (Nes-CreER^{T2}/mTmG) are the progeny of a cross between Nes-CreERT2 5.1 and mTmG expressing mice. In Nes-CreER^{T2} 5.1 mice, the neural precursor cell specific promoter Nestin (Nes) drives the expression of Cre recombinase (CreER^{T2}) (Imayoshi et al. 2006). The recombinase function of CreER^{T2} is activated upon Tamoxifen administration enabling excision of loxP flanked transgenes in NPCs. In mTmG mice, there is global expression of a loxP flanked membrane targeted tdTomato fluorescent protein (mT) followed by a CreER^{T2} inducible membrane targeted eGFP (mG) (Muzumdar et al. 2007). Thus, in Nes-CreER^{T2}/mTmG mice, activation of CreER^{T2} by Tamoxifen leads to the excision of the mT sequence and induction of mG expression in NPCs. Membrane targeted fluorescent proteins are useful for clear resolution of dendrite and spine structures of neurons facilitating analysis of various aspects of neuron morphology. Therefore, to label and study the morphology of newborn adult

hippocampal neurons of SOCS2KO mice, they were crossed with Nes-CreER^{T2}/mTmG animals (a gift from Dr R. Kageyama, Kyoto University and Dr T. Merson, Florey Institute of Neuroscience and Mental Health) to obtain the SOCS2KO x Nes-CreER^{T2}/mTmG mouse lines. SOCS2KO and littermate WT mice used were hemizygous for Nes-CreER^{T2}/mTmG.

Cell Labelling

EdU labelling of proliferative cells

To label proliferating cells in the brain the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen (Life technologies), Melbourne, Australia) was used. EdU incorporates into the DNA of dividing cells during the S-phase of mitosis (Chehrehasa et al. 2009). All animals were administered EdU via intra-peritoneal injection using a 30G insulin syringe (BD, Sydney, Australia) at a dose of 50mg/kg in saline. Animals were administered one daily dose of EdU for 7 days and tissues taken either at 8 days to assess Neural Precursor Cell (NPC) proliferation and neuroblast generation or at 35 days to assess survival of newborn neurons.

NPC specific eGFP induction with Tamoxifen

To induce eGFP reporter expression in the Nes-CreER^{T2}/mTmG strains described above, 8 week old mice were administered one dose of 20mg/kg of Tamoxifen in corn oil (Sigma). Tamoxifen was delivered by oral gavage using disposable plastic 20G x 30mm gavaging needles (Walker Scientific, Joondalup DC, WA). Animals were perfused at 35 days post-Tamoxifen delivery for assessment of newborn adult hippocampal neuron morphology.

Brain tissue dissection and sectioning

Transcardial perfusion and brain dissection

Mice were anaesthetised with 300µl of equal parts Lethobarb (Virbac, Sydney, Australia) and saline. Upon absence of a foot pinch reflex mice were secured on their back onto a Styrofoam block using pins. The ribcage was carefully cut away to reveal the heart. A 25G butterfly infusion needle connected to a Masterflex perfusion pump (John Morris Scientific Pty Ltd, Sydney, Australia), was inserted into the left ventricle. The right atrium cut and then 0.1M Phosphate Buffered Saline (20mL) was perfused through to flush out

the blood, followed by 4% paraformaldehyde (PFA; Sigma) in PBS (20mL) for fixing the tissue. The brain was dissected out and placed in 4% PFA at 4°C overnight followed by 30% sucrose (Chem-Supply Pty Ltd.) in PBS at 4°C for 48h.

Brains were hemisected with the aid of a mouse brain blocker (David Kopf Instruments, Sydney, NSW). The coronal cut was made at slot number 5 such that the front half of the tissue block contained lateral ventricles and the other half the hippocampus. Brains were placed cut side down into plastic Tissue-Tek® Cryomolds (Grale Scientific Pty Ltd., Australia), covered in Tissue-Tek® optimum cutting temperature compound (O.C.T.; Grale Scientific Pty Ltd.), frozen in isopentane cooled over dry ice and stored at -80°C until use.

Brain tissue sectioning

Sectioning of brain tissue was done using a Leica cryostat. All free floating sections were collected in 24 well plates containing 500µl PBS. For SOCS2KO brains, free floating serial coronal sections, 40µm thick were collected through the hippocampus (bregma -1.34mm to -2.54mm). For each animal, 6 wells containing 6 sections spaced 240µm apart were collected. For Nes-CreER^{T2}/mTmG brains, 4 adjacent coronal hippocampal sections of 80µm thickness were collected (bregma -1.5mm to -1.82mm). Following collection of all free floating sections, PBS was replaced with 500µl of anti-freeze (15% sucrose (Chem-Supply Pty Ltd.) and 30% Ethylene Glycol (Chem-Supply Pty Ltd.) in PBS) and sections stored at -20°C until staining.

Immunohistochemistry and cell staining

Single marker immunohistochemistry

Floating serial sections were washed in PBS three times for at least 5min at RT. The 40µm thick sections were blocked with 5% normal goat serum (Invitrogen) or normal donkey serum (Sigma) and 0.02% Triton X-100 (Sigma) in PBS for at least 1h at RT. Sections were incubated with primary antibody diluted in blocking solution overnight at 4°C. The primary antibodies used were goat anti-doublecortin (DCX) (Santa Cruz, sc-8066; 1:200; Antibody Registry ID AB_2088494) or mouse anti-NeuN (Chemicon, MAB377; 1:500;

Antibody Registry ID AB_10048713). Sections were then washed in PBS and incubated with Alexafluor⁴⁸⁸-conjugated donkey anti-goat or goat anti-mouse IgG diluted in PBS, for 2h at RT. For anti-GFP staining of 80µm thick Nes-CreER^{T2}/mTmG floating sections, PBS wash duration was increased to 10min per wash and sections blocked for at least 3h with an increased Triton X-100 concentration of 1.5%. For overnight primary antibody incubation using chicken anti-eGFP (Aves Labs Inc., GFP-1020; 1:2000; Antibody Registry ID AB_10000240), Triton X-100 concentration was reduced to 0.5%, followed by incubation in FITC-conjugated donkey anti-chicken IgY(IgG)(H&L) (Jackson ImmunoResearch) for 3h.

Sections were given a final wash with DAPI (1:5000, Invitrogen D1306) in PBS, slide mounted and coverslipped using Mowiol or Dako fluorescence mounting medium (Dako, Sydney, Australia).

Double labelling with EdU

EdU labelling was performed following cell type specific antibody staining detailed above. After secondary antibody labelling, sections were washed in PBS and blocked with 5% bovine serum albumin (Sigma) and 0.5% Triton X-100 (Sigma) in PBS for 30min. Sections were washed in PBS and incubated in a reaction cocktail prepared from the Click-iT[®] EdU Alexa Fluor[®] 555 Imaging Kit (Molecular probes (Life technologies), Melbourne, Australia) for 45min at 4°C. Components of the Click-iT[®] reaction cocktail were prepared as per manufacturer's instructions however a reduced total volume of 300µl per tissue well was used. Also, the Alexa Fluor[®] 555 azide dilution was increased by dissolving the stock in 140µl of DMSO instead of 70µl and using 1.25µl of working stock per ml of reaction cocktail instead of 2.5µl. Additionally, component D (1x Click-iT[®] reaction buffer) was replaced with PBS. Sections were given a final wash with DAPI in PBS and slide mounted as above.

Neuroblast and newborn mature neuron counts

All standard fluorescence and bright field imaging was performed using an Olympus 1X81 inverted microscope with a mercury burner and white light source. Cell counts were

performed using ImageJ (NIH, Maryland, USA) with the aid of the cell counter plugin and the hyper-stack channel tool for determining co-localisation of Dcx and EdU (neuroblasts) or NeuN and EdU (mature newborn neurons), essentially as we have previously described (Ransome and Turnley 2008). The dentate gyrus was imaged at 10x magnification from 6 serial sections and the volume of the SGZ, GCL and total dentate gyrus (between bregma -1.34mm to -2.54mm) calculated. The dentate gyrus was defined as the dense DAPI positive region of the superior and inferior blades and the SGZ as the area 16µm (approximating 2-cell widths) either side of the junction between the (GCL) and the hilus. For each section, the area of the dentate gyrus, or SGZ and GCL was measured on each side of the brain using ImageJ and the volume of each region calculated using Cavalieri's formula: $\text{volume} = \sum A \times I_{SF} \times t$, where A is region area, I_{SF} is the inverse of the sampling fraction (1 in 6 series), and t is section thickness (40µm). Counts of co-labelled cells or EdU⁺ cells alone were performed for each section and the total cell count per dentate gyrus or SGZ and GCL calculated (average of left and right hemisphere counts) and averaged for each animal. For a group the results are expressed as total cells per dentate gyrus, SGZ or GCL ± SEM.

Assessment of adult dentate granule cell morphology

Confocal imaging of spines and dendritic trees

Imaging was performed using a Zeiss LSM 780 upright confocal microscope maintained by the Florey Microscopy Platform at the University of Melbourne. Images of spines were acquired using a x63/1.4 Plan-Apochromat oil objective, 0.09µm-x/y by 0.2µm-z voxel size and 512x128 pixel frame size. Eight strongly labelled dendrite segments located at 110-130µm from the cell body, one from each dentate gyrus from four 80µm sections of medial hippocampus per animal were chosen for analysis of spine morphology. Images of dendritic trees were acquired as a 3x3 tile with 10% overlap using a x40/1.4 Plan-Apochromat oil objective, 0.07µm-x/y by 0.2µm-z voxel size and 1024x1024 pixel frame size. Granule cells with cell bodies that had migrated at least half way up into the GCL, located midsection and were mostly isolated from other GFP labelled cells were chosen for dendritic tree imaging. Acquired images of spines and dendrites were de-convolved

using Huygens Remote Manager v3.1.1 (Scientific Volume Imaging B.V., Hilversum, Netherlands) before processing on Imaris 3D.

Analysis of spine and dendritic tree morphology with Imaris 3D

Spine and dendrite morphology were analysed on Imaris 3D (Bitplane, Zurich, Switzerland) using the Filament Tracer tool. The semi-automated AutoDepth algorithm of Filament Tracer was used for tracing spines or dendrites from confocal z-stack images. AutoDepth allows manual tracing of the length of a spine or dendrite from any point of a 3D rendered image while automatically detecting depth based on local intensity of the fluorescent label. The Filament Tracer tool automatically computes statistics for dendritic tree or spine tracings.

For analysis of spines, AutoDepth was used to trace the dendrite segment of interest following which the Creation Wizard was used to automatically detect spines along it. Following spine detection, any incorrectly identified or missing spines were manually deleted or added respectively. Once the spine tracing was corrected the statistical data for the trace was exported from Imaris as a Microsoft Excel file. Data for maximum spine length, maximum spine diameter, maximum spine neck diameter and branching value was used to classify the spines into mushroom, thin, stubby and branched morphologies. Spine morphologies were assigned based on criteria adapted from Harris et al. (1992) (Harris et al. 1992) and are listed in **Table 1**. Classification of spines was automated using the IF function in Microsoft Excel. The IF function classifies a given criteria as true or false and the formulas used for spine classification are detailed in **Table 1**. The formulas were set up such that when a criterion was proven true it was assigned the value 1 and when proven false it was assigned the value 0. A given spine could only be proven true for one of the four morphology classifications. The true and false values were summed for each classification to obtain a total number of branched, stubby, mushroom or thin spines. Total spine number was divided by the length of the dendrite analysed to obtain a spine density per μm of dendrite. For a group the results are expressed as spine density/ $\mu\text{m} \pm \text{SEM}$.

For analysis of dendritic trees, AutoDepth was used to trace all dendritic segments of a granule cell starting from the cell body. The statistical data for the trace was exported from Imaris as a Microsoft Excel file. Data for total dendrite length (μm) calculated by sum of individual dendrite segments, number of dendrite segments and number of dendritic branch points was graphed for analysis.

Statistical analysis

GraphPad Prism 5 was used for all statistical analyses and graph generation. For SOCS2KO neurogenesis experiments all data was analysed using the un-paired t-test, with $n=4-8$ mice/group. SOCS2KO spine density data was analysed using 2-way analysis of variance (ANOVA) and Bonferroni post-hoc testing, using $n=5-6$ mice/group. For all experiments results were considered significant when $p < 0.05$.

Results

Adult SOCS2KO mice and WT controls were administered EdU daily for 7d and brains analysed at day 8 and day 35 to study NPC proliferation/ neuroblast generation and newborn neuron survival, respectively. Adult SOCS2KO and SOCS2Tg mice on a Nes-CreER^{T2}/mTmG background were also used in this study to examine newborn adult dentate granule cell morphology. These mice were administered a single dose of tamoxifen for induction of NPC-specific membrane bound GFP and taken at 35d post induction for morphological analysis of newborn mature eGFP expressing dentate granule neurons.

Numbers of newborn neuroblasts at 8d was similar in SOCS2KO and WT mice

To assess whether NPC proliferation in the dentate gyrus was affected in SOCS2KO mice, tissue taken at 8d post EdU labelling was immunostained for EdU and doublecortin (Dcx; immature neuron marker) (**Fig. 1A**). Cells positive for EdU only (EdU⁺) or double positive for EdU and Dcx (EdU⁺Dcx⁺) were counted. There were no significant differences in the total number of EdU⁺ cells per dentate gyrus (**Fig. 1B**; $p=0.599$), the number of EdU⁺Dcx⁺ cells per dentate gyrus (**Fig. 1C**; $p=0.96$) or in the proportion of EdU⁺ cells that co-labelled with Dcx⁺ (**Fig. 1D**; $p=0.41$). However, the dentate gyrus in SOCS2KO mice showed a 20% decrease in volume compared to WT mice (**Fig. 1E**; $p=0.0001$).

Numbers of EdU-labeled newborn adult hippocampal neurons at 35d are reduced in SOCS2KO mice

Tissue taken at 35d was labelled for EdU and NeuN (mature neuron marker) to assess the survival of mature newborn dentate granule cells (**Fig. 2A**). As observed in the 8d cohort of animals, SOCS2KO mice in the 35d cohort were also found to have a reduced dentate gyrus volume (17% decrease) compared to WT (**Fig. 2E**; $p=0.039$). There was no statistically significant difference in the number of EdU⁺ cells in the SGZ between genotypes (**Fig. 2B**; $p=0.41$). However, SOCS2KO mice displayed a 46% decrease in the number of EdU⁺ cells in the GCL compared to WT (**Fig. 2C**; $p=0.005$), which equated to a similar decrease in EdU⁺/NeuN⁺ newborn neurons per GCL (**Fig. 2D**; $p=0.005$), as virtually

100% of EdU⁺ cells in the GCL co-labelled with NeuN in both genotypes (WT 100+/-0 versus SOCS2Tg 99.44+/-0.48%; $p=0.34$).

Newborn neuron dendritic tree arborisation was not altered at 35d in SOCS2KO mice

SOCS2 overexpression or absence has been shown to enhance or reduce neurite outgrowth of cortical neurons (Goldshmit et al. 2004a; Ransome et al. 2004; Ransome and Turnley 2005). To determine if SOCS2 may regulate newborn adult hippocampal neuron survival *in-vivo* through modulation of newborn neuron morphology, confocal z-stacks of newborn GFP-expressing adult dentate granule cells from SOCS2KO x Nes-CreER^{T2}/mTmG mice and their WT littermates were analysed in a semi-automated manner using Imaris 3D.

The dendritic tree morphology of newborn adult hippocampal neurons from SOCS2KO x Nes-CreER^{T2}/mTmG animals showed no significant differences in the extent of dendritic arborisation, as assessed by measuring the total dendritic length (calculated by the addition of individual dendrite segment lengths; $p=0.395$), number of dendritic segments ($p=0.284$) and number of dendritic branch points for each neuron ($p=0.233$) (**Fig. 3**).

Mushroom spine density was increased in newborn hippocampal neurons at 35d in SOCS2KO mice

Spine morphology and density was assessed for SOCS2KO x Nes-CreER^{T2}/mTmG animals (Fig. 4; see Table 1 for criterion of morphological classification). The density of spines of different morphologies varied (ANOVA; $p<0.0001$) and branched, stubby, thin and total spines were present in similar densities to littermate WT animals (Fig. 4B). However, a significantly greater density of mushroom spines was present in the SOCS2KO x Nes-CreER^{T2}/mTmG animals compared to WT (Fig. 4B; $p<0.05$).

Discussion

This study examined endogenous adult hippocampal neurogenesis and newborn adult hippocampal neuron morphology in SOCS2KO mice. While no deficits in NPC proliferation or differentiation were observed, SOCS2 deficiency resulted in reduced numbers of

mature EDU-labeled newborn adult hippocampal neurons. SOCS2 deficient newborn adult hippocampal neurons also showed an increase in mushroom spine density but no statistically significant differences in dendritic tree arborisation were observed compared to WT animals.

SOCS2 regulates newborn neuron maturation and/or survival

SOCS2 deficiency did not appear to affect NPC proliferation or survival, as assessed by total EdU⁺ cell counts per dentate gyrus, nor did it appear to affect the extent to which labelled EdU⁺ NPCs differentiated into Dcx⁺ neuroblasts. NPC proliferation was similarly not altered in the dentate gyrus under SOCS2 overexpressing conditions (Ransome and Turnley 2008).

In support of the previously defined role for SOCS2 in newborn adult hippocampal neuron survival (Ransome and Turnley 2008), in which SOCS2 overexpression promoted survival, a significant reduction in EdU⁺NeuN⁺ cells was observed in the dentate gyrus of SOCS2KO animals at 35d after initiation of EdU labelling. This result was accompanied by the concurrent finding of a reduced proportion of EdU⁺ cells co-labelled with NeuN in the SOCS2KO dentate gyrus. Further, longer term studies are required to determine whether SOCS2 deletion is leading to a delayed maturation of Dcx⁺ neuroblasts into NeuN⁺ neurons rather than an effect on their survival *per se*. However, as the average size of the dentate gyrus, as assessed by measuring the volume of DAPI-stained dentate granule cell nuclei, was decreased in the SOCS2KO animals at both timepoints, this may have been due to a deficit in mature neuronal numbers. Overall, these findings suggest an important role for SOCS2 in the regulation of newborn adult hippocampal neuron production by modulating aspects of newborn neuron maturation and/or mature neuron integration.

The mechanism through which SOCS2 may be regulating adult hippocampal neurogenesis remains to be elucidated. GH signalling, which is regulated by SOCS2 during developmental SVZ-derived cortical neurogenesis (Turnley et al. 2002), was previously found to not play a role in SOCS2 mediated newborn adult hippocampal neuron survival (Ransome and Turnley 2008). However, delivery of exogenous GH to adult animals has

been shown to have positive effects on adult neurogenesis (Aberg et al. 2009; Devesa et al. 2011). Whether this is through a direct or indirect action of GH remains to be determined as exogenous GH has been suggested to induce pro-neurogenic factors such as EPO and BDNF (Arámburo et al. 2014). In the current study, SOCS2KO mice, which display excessive GH signalling (Metcalf et al. 2000), were found to have decreased adult hippocampal neurogenesis, suggesting other factors may play a role.

SOCS2 has also been implicated in regulation of EPO mediated adult SVZ NPC differentiation (Wang et al. 2004). Evidence for such an interaction between EPO and SOCS2 has not been found in the hippocampus, however EPO stimulates adult SGZ NPC proliferation (Chen et al. 2007; Ransome and Turnley 2007), a process which was unaffected in SOCS2KO animals. BDNF has a better defined role in adult hippocampal neurogenesis and specifically in newborn hippocampal neuron survival (Matteo et al. 2008; Wei et al. 2015). There is recent evidence for the regulation of the BDNF receptor, TrkB, by SOCS2 (Uren and Turnley 2014). Therefore, modulation of TrkB signalling may be a plausible mechanism by which SOCS2 regulates newborn adult hippocampal neuron survival/maturation. Furthermore, BDNF regulates dendritic morphology and dendritic spine formation (reviewed in (Sala and Segal 2014)), which may correlate with enhanced dendritic spine maturation observed in the SOCS2KO animals.

An additional contributor to the observed altered mature newborn neuron numbers may be an altered neuroinflammatory environment. SOCS2 has been shown to regulate aspects of the neuroinflammatory response (Machado et al. 2006) and more recently we have shown that SOCS2 regulates the microglial response after traumatic brain injury in SOCS2Tg mice (Basrai et al. 2016). Although we did not find any differences in microglia under non-injured conditions, further studies are required to determine whether microglial subtypes, and hence the cytokines they produce, may be altered in SOCS2 mutant mice leading to an altered micro-environment that may modulate newborn neuron survival or apoptosis.

Altered mushroom spine density of newborn adult hippocampal neurons of SOCS2KO mice may be a secondary response to aberrant neurogenesis

Given the role for SOCS2 in the regulation of EGF and NGF mediated neurite outgrowth (Goldshmit et al. 2004a; Goldshmit et al. 2004b; Uren et al. 2014), development of the mature granule cell dendritic arbour was proposed as a mechanism by which SOCS2 may regulate newborn neuron survival in the hippocampus. However, no defects in dendritic arborisation were detected in the newborn adult hippocampal neurons of SOCS2KO mice but there was a significant increase in mushroom spine density. Mushroom spines are considered the most stable subtype of spines and are found in increasing abundance as a granule cell matures (Chunmei et al. 2006). Also, mushroom spine density is positively correlated with the synaptic activity of a neuron (Bourne and Harris 2007). Therefore, greater numbers of mushroom spines on SOCS2KO newborn adult hippocampal neurons would suggest increased synaptic activity of those cells compared to WT. However, the correlation between increased synaptic activity and reduced newborn adult hippocampal neuron numbers in SOCS2KO mice seems counterintuitive. This is because increased synaptic activity would at first instance suggest an improved capacity for neuronal circuit integration and in turn increased rather than reduced survival of newborn neurons. Thus, the potentially increased synaptic activity of SOCS2KO newborn neurons may be a secondary response to reduced adult hippocampal neurogenesis to perhaps functionally compensate for fewer mature neurons in the dentate. Alternatively, this may be a neuroprotective response to a potentially increased neuronal work load. Indeed increased spine density is hypothesised to be neuroprotective under conditions of excessive electrical stimulation (Segal 2010). Future electrophysiological studies may help to unravel what differences, if any, SOCS2 deletion incurs on hippocampal circuitry.

Mechanisms by which SOCS2 could regulate dendritic spine maturation

While the mechanism by which SOCS2 could regulate dendritic spine maturation remains to be determined, possibilities include downstream effects on expression of synaptic proteins or activation of Rho GTPases. SOCS2 regulates signalling downstream of multiple growth factors, including GH, EGF and neurotrophins. Administration of GH has been shown to enhance hippocampal synaptic function in the context of reduced GH levels induced by sleep deprivation (Kim et al. 2010) and SOCS2KO mice are hyper-responsive to GH signalling (Metcalf et al. 2000). However, neurotrophins, and in particular BDNF, are

better known for their role in regulation of dendritic spine morphology and may be more likely candidates to play a role in SOCS2-mediated changes. Future studies will address this possibility.

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Acknowledgments

We thank Dr Clare Faux, University of Melbourne, for performing SOCS2 in situ hybridisations and Dr R. Kageyama, Kyoto University and Dr T. Merson, Florey Institute of

Neuroscience and Mental Health for provision of the Nestin-CreER^{T2} mice. We thank Stem Cells Australia for additional support.

Figure Legends

Fig 1 No difference in the generation of neuroblasts in the dentate gyrus of SOCS2KO or WT animals

Immunohistochemical staining with the immature neuron marker doublecortin (Dcx; green) and EdU (red) was performed on coronal hippocampal sections of WT and SOCS2KO animals at 8 days post EdU labelling (A). Small arrows in top panels indicate EdU and Dcx co-labelled cells, scale bar 100µm; bottom panels show an enlargement of the top panels, with boundaries indicated by the long arrows, scale bar 50µm. No significant difference in total numbers of EdU⁺ cells was seen in the dentate gyrus of SOCS2KO animals (B), no differences were present in the number of EdU⁺ cells co-labelled with Dcx (C) nor in the proportion of EdU⁺ cells that were Dcx⁺ (D). SOCS2KO animals showed a significantly reduced dentate gyrus volume (E). Results show mean ± SEM; *n*=4 WT and 8 SOCS2KO mice/group; ****p*<0.001 (Unpaired Student's t-test; B-E).

Fig 2 Survival of newborn neurons in the dentate gyrus of SOCS2KO animals

Immunohistochemical staining with NeuN (green) and EdU (red) was performed on coronal hippocampal sections of WT and SOCS2KO animals 35 days post EdU labelling (A). Small arrows in top panels indicate EdU and NeuN co-labelled cells, scale bar 100µm; bottom panels show an enlargement of the top panels, with boundaries indicated by the long arrows, scale bar 50µm. No differences were seen in EdU⁺ cell number in the SGZ of SOCS2KO and WT animals (B) but a significant decrease was present in the number of EdU⁺ cells in the GCL (C), which was essentially the same as EdU⁺ cells co-labelled with NeuN in the GCL (D). SOCS2KO animals also showed a significantly reduced dentate gyrus volume (E). Results show mean ± SEM; *n*=6 WT and 8 SOCS2KO mice/group; **p*<0.05, ***p*<0.01 (Unpaired Student's t-test; B-E).

Fig 3 Analysis of newborn adult hippocampal neuron dendritic tree morphology of SOCS2KO animals

The dendritic tree morphologies of newborn adult hippocampal neurons from SOCS2KO x Nes-CreER^{T2}/mTmG and WT littermate animals were analysed. For each animal 3-4 neurons were imaged, traced on Imaris 3D and statistics generated for analysis. An example of a

dendritic tree tracing is shown (A, scale bar = 20 μ m). No significant differences were found between SOCS2KO and WT animals in total dendritic length (B), number of dendritic segments (C) and number of dendritic branch points (D). Results show mean $\pm$ SEM (B-D), $n=5$ WT and 6 SOCS2KO mice/group.

Fig 4 Analysis of dendritic spine morphologies in newborn adult hippocampal neurons of SOCS2KO animals

The spine morphologies of newborn adult hippocampal neurons from SOCS2KO x Nes-CreER^{T2}/mTmG animals were analysed compared to WT littermates. For each animal 6-8 dendrite segments were imaged, spines traced on Imaris 3D and statistics generated for analysis. Representative confocal z-stack images of dendrite segments analysed for spine analysis are shown, scale bar = 2 μ m (A). SOCS2KO x Nes-CreER^{T2}/mTmG animals showed no differences in branched, stubby, thin or total spine density but had a significant increase in mushroom spine density compared to WT (B). Results show mean $\pm$ SEM; $n=6$ WT and 5 SOCS2KO mice/group, $*p<0.05$ (Two-way ANOVA with Bonferroni post hoc test).

Supplemental Figure 1: In situ hybridization showing SOCS2 expression in the CA3 region and dentate gyrus of adult hippocampus. The highest level of SOCS2 expression in the adult brain is in the CA3 region with lower expression in the dentate gyrus. Panel B is an enlargement of box in panel A and arrows show examples of granule cells expressing SOCS2, as indicated by a thin rim of cytoplasmic staining. Scale bars, A) 500 μ m; B) 100 μ m.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1. Criteria for classification of spine morphology on Microsoft Excel from Imaris 3D generated statistics

<i>Morphology</i>	<i>Criteria</i>	<i>IF Function formula</i>
Branched	If branching value ≥ 1	=IF(branching value ≥ 1 , 1, 0)

Stubby	If branching value = 0 If maximum spine diameter = maximum spine neck diameter If maximum spine length < 1 μ m	$=IF(AND(branching\ value=0, maximum\ spine\ diameter\ value=maximum\ spine\ neck\ diameter\ value, maximum\ spine\ length\ value<1), 1, 0)$
Mushroom	If branching value = 0 If maximum spine diameter > maximum spine neck diameter If maximum spine length < 1 μ m	$=IF(AND(branching\ value=0, maximum\ spine\ diameter\ value>maximum\ spine\ neck\ diameter\ value, maximum\ spine\ length\ value<1), 1, 0)$
Thin	If branching value = 0 If maximum spine diameter $\geq$ maximum spine neck diameter If maximum spine length > 1 μ m	$=IF(AND(branching\ value=0, maximum\ spine\ diameter\ value\geq maximum\ spine\ neck\ diameter\ value, maximum\ spine\ length\ value>1), 1, 0)$

Supplemental Figure 1