Research report

Modulation of gap junction-associated Cx43 in neural stem/progenitor cells following traumatic brain injury

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ABSTRACT

Restoration of learning and memory deficits following traumatic brain injury (TBI) is attributed, in part, to enhanced neural stem/progenitor cell (NSPCs) function. Recent findings suggest gap junction (GJ)-associated connexin 43 (Cx43) plays a key role in the cell cycle regulation and function of NSPCs and is modulated following TBI. Here, we demonstrate that Cx43 is up-regulated in the dentate gyrus following TBI and is expressed on vimentin-positive cells in the subgranular zone. To test the role of Cx43 on NSPCs, we exposed primary cultures to the α-connexin Carboxyl Terminal (αCT1) peptide which selectively modulates GJ-associated Cx43. Treatment with αCT1 substantially reduced proliferation and increased caspase 3/7 expression on NSPCs in a dose-dependent manner. αCT1 exposure also reduced overall expression of Cx43 and phospho (p)-Serine368. These findings demonstrate that Cx43 positively regulates adult NPSCs; the modulation of which may influence changes in the dentate gyrus following TBI.

1. Introduction

Traumatic brain injury (TBI) affects over 1.7 million Americans each year and is a leading cause of long-term disability (Santopietro et al., 2015; Spitz et al., 2013; Sun, 2014; Swanson et al., 2017), including physical, cognitive, and emotional dysfunction ranging from headaches to memory loss and depression (Langlois et al., 2006; Rapoport et al., 2005; Siopi et al., 2012; Swanson et al., 2017). While the yearly incidence of TBI is more than triple that of most cancers (DeSantis et al., 2016), no effective therapies exist to prevent neural tissue damage and/or fully restore neurological function. Impairment in learning and memory is a major hallmark of the neurocognitive sequela of TBI (Arciniegas et al., 2002; Hamm et al., 1993; Whiting et al., 2006) and is partly attributed to hippocampal neuronal and neural progenitor cell loss (Blaiss et al., 2011; Rola et al., 2006; Whiting et al., 2006). Neural stem/progenitor cells (NSPCs) in the dentate gyrus (DG) play a key role in hippocampal function (Goncalves et al., 2016; Leuner and Gould, 2010; Zheng et al., 2013). Although NSPCs are initially vulnerable in this neurogenic region following TBI, subsequent promotion of neurogenesis could aid in replacing locally damaged neurons and enhancing neuronal circuit repair and restoration of neurocognitive decline (Han et al., 2011; Kernie and Parent, 2010; Kleindienst et al.,

2005; Sun et al., 2009; Wu et al., 2008; Yu et al., 2008). A comprehensive understanding of the cellular and molecular underpinnings controlling TBI-induced neurogenesis will promote future strategies targeting this adaptive mechanism after brain trauma.

Analysis of the neurogenic response in the human brain following trauma is severely limited due to lack of human tissue availability (Eriksson et al., 1998). Therefore, rodent models of reproducible injury have been widely used to address the cellular and molecular changes occurring in the DG following trauma (Johnson et al., 2015; Li et al., 2011; O'Connor et al., 2011). One such model, the moderate controlled cortical impact (CCI) injury results in a focal cortical contusion and selective cell loss in the hippocampus (Brickler et al., 2016; Johnson et al., 2015; Theus et al., 2014). Recent studies using a transgenic approach to ablate Nestin-expressing NSPCs directly linked TBI-induced neurogenesis to cognitive and motor functional recovery (Blaiss et al., 2011; Dixon et al., 2015; Yu et al., 2008). These findings emphasize the critical role of endogenous neurogenesis and underscore the importance of expanding our knowledge regarding the nuances regulating the complex milieu in the neurogenic niches of the brain following injury.

Gap junctional (GJ) communication plays a key role in proliferation and migration within the neurogenic zone during brain development by differentially coupling radial glial (RG)-like and dividing neural

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progenitor cells in the ventricular zone (Cina et al., 2009; Sutor, 2002; Sutor and Hagerty, 2005). Gap junctions are formed by connexin hemichannels and are specialized sites of cell–cell contact that allow the passage of ions, intracellular metabolites and messenger molecules (less than 2 kDa) as well as provide adhesion sites between adjacent cells (Contreras et al., 2004; Evans et al., 2006; Hoang et al., 2010; Saez et al., 2005). Clusters of neural progenitor and RG cells, but not migrating neuroblasts, exist within the developing ventricular zone (Freitas et al., 2012) and are differentially coupled by GJs depending on the phase of the cell cycle and the discrete stage of neurogenesis (Bittman et al., 1997). The modulation of gap junction-associated Cx43 during cell division is regulated, in part, at the level of phosphorylation (Laird et al., 1991; Solan and Lampe, 2005, 2009). Phosphorylation of serine368 is important for cell division and peaks during mitosis as gap junction communication ceases. Interestingly, Cx43 also displays channel-independent roles in regulating migration and adhesion (Elias et al., 2007). These findings suggest gap-junction associated Cx43 plays an important role in embryonic neurogenesis; however, no studies have addressed the expression and function of Cx43 in the adult neurogenic niches of the brain under homeostasis and following TBI.

To date it remains unclear whether modulation of GJ-associated Cx43 occurs in the clustered neural stem/progenitor cell niche of the SGZ of the DG and the effects of modulating Cx43 in these highly coupled cells. To address this question we evaluated the expression of Cx43 on vimentin-positive NSPCs in the SGZ in sham and CCI-injured mice at 4-days using confocal imaging microscopy. To further address the role of GJ-associated Cx43 in NSPC activities we exposed primary NSPC cultures to a mimetic peptide of Cx43, called α-connexin Carboxyl Terminal 1 (αCT1), (Ghatnekar et al., 2009; Soder et al., 2009), and found increased Caspase 3/7 activation and reduced BrdU incorporation in a dose-dependent manner. Treatment with αCT1 reduced total levels of Cx43 expression on NSPCs at 8 and 24 h post-treatment. Our findings suggest that Cx43 plays a positive role in regulating NSPC survival and proliferation, the modulation of which could influence key neurogenic changes following TBI.

2. Materials and methods

2.1. Animals

All mice were generated and housed in an AAALAC approved, virus/specific antigen-free facility with a 12 h light-dark cycle; food and water *ad libitum*. CD1 mice were purchased from Charles Rivers and bred until desired numbers were generated for experimentation. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #15-063) and the Virginia Maryland Regional College of Veterinary Medicine.

2.2. CCI injury

Male CD1 mice at 8–12 weeks of age were anesthetized with ketamine and xylazine intraperitoneal (i.p.) injection and positioned in a stereotaxic frame (Baumann et al., 2013; Theus et al., 2010, 2014). Body temperature was monitored with a rectal probe and maintained at 37 °C with a controlled heating pad (homeothermic blanket system; Harvard Apparatus). A 5 mm craniotomy was made using a portable drill over the right parietal-temporal cortex (− 2.5 mm A/P and 2.0 mm lateral from bregma). Injury was induced by moderate CCI using the eCCI- 6.3 device (Custom Design & Fabrication; 3 mm impounder) at a velocity of 3.5 m/s, depth of 1.0 mm, and 150 ms impact duration (Baumann et al., 2013; Theus et al., 2010). Sham controls received anesthesia and craniotomy only. Following injury, the incision was closed using Vetbond tissue adhesive (3 M, St. Paul, MN, USA) and the animals were placed into a heated cage to maintain body temperature

for 1 h post-injury followed by daily monitoring for 4 days or until the day of euthanization.

2.3. Tissue sectioning and immunostaining

Brains were dissected from the skull and embedded into tissue freezing medium (Optical Cutting Temperature (OCT) Compound, Fisher Healthcare) before immediately snap frozen. Five serial, coronal sections (30 μm in thickness) were collected spaced 300 μm apart using a cryostat (Thermo Fisher Cryostar NX50, Waltham, MA, USA). To label for neural stem/progenitor cells undergoing apoptosis, slides were fixed in 10% formalin (Fisher Chemicals, Pittsburgh, PA, USA) for 10 min, washed 3 times with 1X PBS, and permeabilized in 2:1 Ethanol:Acetic Acid for 10 min, washed 3 times in 1X PBS then incubated with 0.4% Triton X-100 for 5 min and washed with 1X PBS. Slides were incubated in primary antibody using block overnight at 4 °C at 1:500 anti-Cx43 (Santa Cruz Biotechnology) and anti-vimentin (Santa Cruz Biotechnology, Dallas, TX, USA). Sections were washed with 1X PBS then incubated with anti-rabbit Alexa Fluor 594-conjugated and Alexa Fluor 488-conjugated anti-rabbit or Alexa Fluor 488-conjugated anti-goat (Molecular Probes, Carlsbad, CA) in block for 1 h at RT.

2.3.1. Peptide sequences

The inhibitor peptide, αCT1, includes the Cx43 C-terminal amino acids [374]RPRPDDLEI[382] that encompass the ZO-1-binding sequence. A control peptide was generated by reversing the Cx43 amino acid sequence (IELDDPRPR), and is termed "Reverse" throughout the manuscript. Both peptides contain a 16-amino acid antennapedia internalization vector (RQPKIWFPNRRKPWKK) linked to the N terminus of the Cx43 (or reversed Cx43) sequence; peptides were N-terminally biotinylated. These peptides were dissolved and used as previously described (Hunter et al., 2005).

2.3.2. Neural stem cells assays

Neural stem/progenitor cells were isolated from the SVZ of adult CD1 mice (2–4 months old) and grown as monolayer cultures as previously described (Theus et al., 2012). Once stem cells reached 70–80% confluency, they were passaged and 20,000 cells were plated in a 96-well plate using monolayer culture media (DMEM/F12 (Hyclone, Logan, Utah) containing N2 supplement (Life Technologies Corporation, Grand Island, NY), 35 μg/mL bovine pituitary extract (Sigma-Aldrich, St. Louis, MO, USA), 1X pen-strep glutamine (100X stock, Life Technologies Co., Grand Island, NY), 5% fetal bovine serum, and 20 ng/mL each epidermal growth factor (EGF, Chemicon International, Temecula, CA, USA) and basic fibroblast growth factor (EGF, Chemicon International, Temecula, CA, USA) in the presence of 150 μM, 75 μM and 37.5 μM Reverse or αCT1 peptide. After 8 or 24 h, cells were fixed for 10 min using 10% buffered formalin. For immunostaining, wells were washed 3 times in PBS before blocking in 2% fish gelatin with 0.1% Triton X-100 (2% fish gelatin/0.1% Triton-X100) for 1 h at RT before primary incubation in 1:500 (rabbit anti-Cx43 polyclonal, Santa Cruz, Dallas, TX, USA) and 1:100 (mouse anti-Nestin, Santa Cruz Biotechnology, Dallas, TX, USA) for 1–2 h at room temperature. Wells were washed with 1X PBS then incubated in anti-mouse Alexa-Flour 488-conjugated secondary antibody (1:250) or anti-rabbit Alexa-flour 594-conjugated secondary antibody (1:500) (Invitrogen) along with DAPI (1:2000, Cell Signaling, Danvers, MA, USA) for 1 h at RT.

2.3.3. Caspase 3/7 analysis

To assess cellular apoptosis *in vitro*, NSPCs were plated in a 96-well plate and treated with peptides as described above. After 8 h or 24 h, CellEvent Caspase-3/7 Green Detection Reagent (ThermoFisher Inc, Waltham, MA, USA) was added to each well at a final concentration of 4 μM and incubated for 30 min at 37 °C. To quantify Caspase 3/7 expression, each well was imaged at 4X and overall expression was measured using mean intensity in ImageJ. To analyze mean intensity in
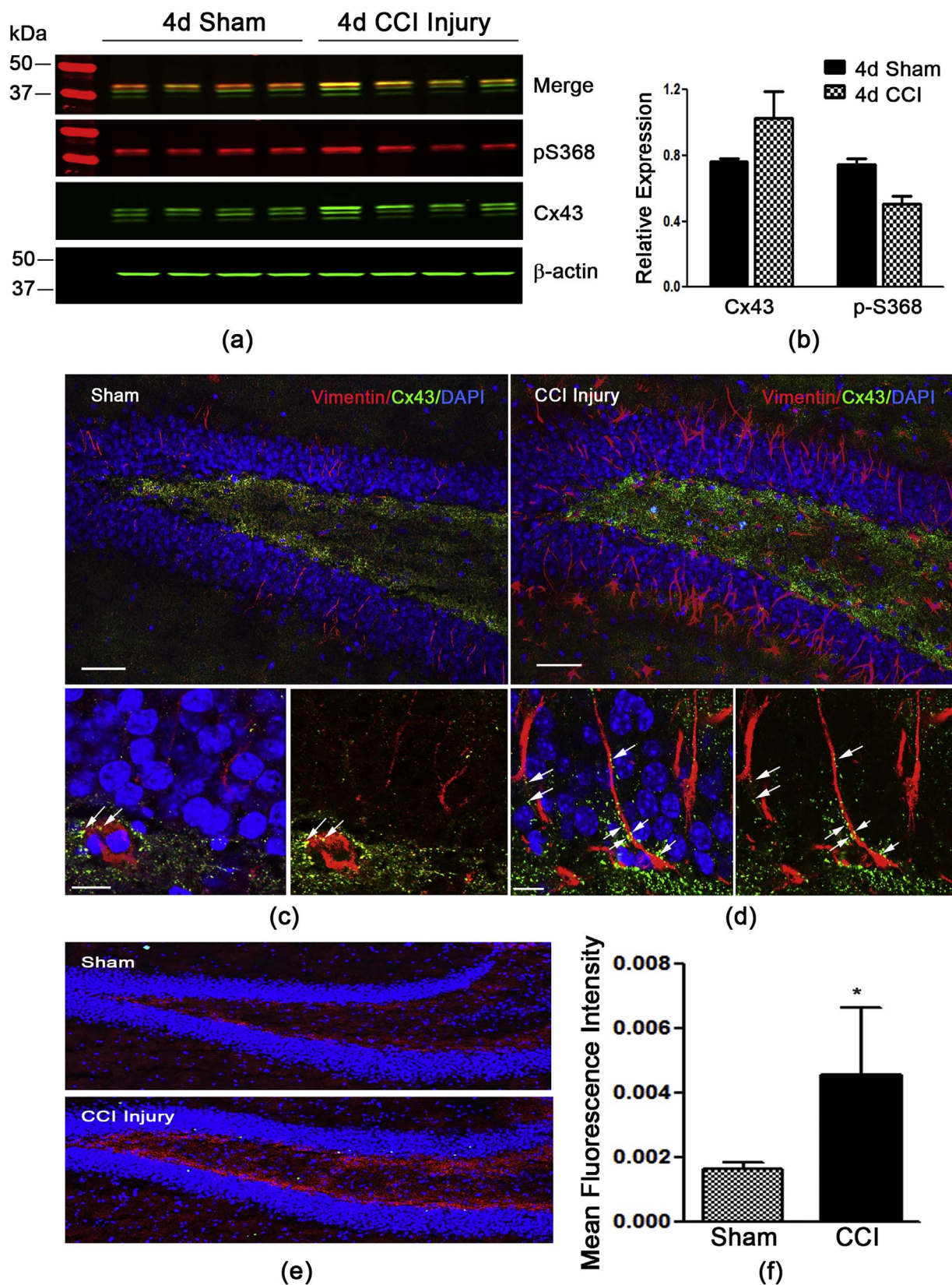
Fig. 1. Cx43 expression in the hippocampus 4 days post-CCI injury. (a) Western blot analysis showing total Cx43 and phospho (p) serine 368 protein levels in the whole hippocampus at 4 days post-sham or CCI injury. (b) Quantified data showing the fluorescence intensity of Cx43 relative to β-actin and p-S368 relative to total Cx43. (c) Immunohistochemistry for vimentin (red) and Cx43 (green) expression in the dentate gyrus (DG) of sham injury mice at 4 days post-sham compared to CCI injury (d). Increased expression by immunofluorescence is seen in the DG following CCI injury (e). (f) The mean fluorescence intensity of Cx43 expression in the region of the subgranular layer of the DG was significantly increased after CCI injury. *P < 0.01 compared to sham injury. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Image J, we opened the image, selected "Analyze > Measure" and recorded the "Mean" value for mean intensity.

2.3.4. BrdU analysis

To assess proliferation we performed BrdU incorporation, which identifies cells in the s-phase of the cell cycle, after NSPCs were plated in a 96-well plate and treated as described above. After 8 h or 24 h of αCT1 treatment, 10 μM BrdU was added for 20 min before cells were fixed with 10% formalin and washed 3 times with 1X PBS. Cells were incubated in 1N HCL for 1 h at 37 °C to denature the DNA and the pH was neutralized in 0.1M-borate buffer at pH 8.5 and washed in 1X PBS. Cells were blocked in 2% fish gelatin/0.1% Triton-X100 for 1 h before incubating with rat anti-BrdU antibody 1:2000 (LifeTechnologies, Eugene, OR, USA) in block overnight at 4 °C. The following day, each well was washed in 1X PBS then incubated in anti-rat Alexa-Flour 594-conjugated secondary antibody (1:500) (Invitrogen, Inc) were added with DAPI (1:2000, Cell Signaling Inc., Dallas, TX, USA) for 1 h at RT. After washing in 1X PBS, the ratio of BrdU-positive cells to total nuclei was determined by imaging each well 2 times at 10X and using the Cell Counter on ImageJ (NIH) to quantify total cell numbers. Each experiment was repeated 4–5 times with 4–5 wells per treatment.

2.3.5. Confocal imaging and cx43 quantification

In order to analyze images taken on Zeiss LSM 880 confocal laser scanning microscope, three series coronal sections per brain were imaged using z-stack at 20X and 40 X magnifications. To analyze z-stacks as one image rather than several images individually, we used Fiji by ImageJ (NIH) (Schneider et al., 2012) to project the z-stack images into a 2D plane. We analyzed mean intensity expression on each 2D plane by placing a triangle shaped contour around the dentate gyrus, then using the Fiji by ImageJ program to calculate the "Mean" value for mean intensity by selecting "Analyze" > "Measure". This recorded the mean intensity for Cx43 expression inside the contour of the dentate gyrus.

2.3.6. Western blot analysis

Protein of NSC cultures or hippocampal brain was extracted by lysing cells in RIPA buffer (1% NP-40, 1% sodium-deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, and 0.01 M sodium phosphate) in the presence of complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktail 2 (Sigma, St. Louis, MO, USA). Supernatant was collected by centrifuging at 13,000 *g* for 20 min at 4 °C and the Lowry assay was used for determination of protein concentration (Pierce, Rockford, IL). Cell lysates (50 μg) were resolved on 10% SDS-PAGE gels and blotted onto PVDF membranes, blocked with 5% bovine serum albumin (BSA) in TBST buffer (20 mM Tris, 137 mM NaCl and 0.1% tween) then incubated in block overnight at 4 °C with primary antibody against Cx43 (rabbit polyclonal, Santa Cruz, Dallas, TX, USA), p-Cx43 (s368, Cell Signaling, Danvers, MA, USA), or β-actin (mouse, 1:5000 Cell Signaling, Danvers, MA). Secondary antibodies (donkey anti-rabbit IRDye 800CW and donkey anti-mouse IRdye 680; Licor) were applied at 1:5000 to the membrane for 3 h at room temperature before scanning the membranes with the Odyssey Fc imaging system (Licor, Lincoln, NE). Blots were quantified by densitometry using acquisition into Adobe Photo Shop (Apple, Cupertino, CA, USA). The level of protein expression was normalized according to β-actin control levels. Samples were run in duplicate.

2.4. Statistical analysis

Data was graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). Student's two-tailed *t*-test was used for comparison of two experimental groups. Multiple comparisons were done by using one-way ANOVA where appropriate followed by *post hoc* Tukey test for multiple pairwise examinations. Differences were identified as significant if P < 0.05. Mean values were reported together with the standard error of mean (SEM).

3. Results

3.1. Connexin43 expression in the hippocampus and dentate gyrus following controlled cortical impact

Our preliminary findings demonstrate that Cx43 is highly expressed in the neurogenic compartments of the adult brain, specifically the subventricular zone and the subgranular zone (SGZ) of the dentate gyrus (DG) (Supplementary Fig. S1). Cx43 expression has been shown to be increased after TBI, although it is unclear whether these changes occur in the hippocampus, an area vulnerable to brain trauma. To examine this we isolated protein from the hippocampus 4 days following sham or controlled cortical impact (CCI) injury and evaluated levels of Cx43 and pS368 using Western blot analysis. Using antibodies against Cx43 and pS368, we found no statistically significant difference in the expression of Cx43 and reduced *p*-S368 (Fig. 1a and b) (P > 0.05 for both). To evaluate more cell-specific changes, we used serial coronal tissue sections of sham and CCI-injured brains and assessed Cx43 expression in the DG on vimentin-positive cells using immunohistochemistry. Vimentin is a type III intermediate filament protein present on neural stem cells and is upregulated in the DG following CCI injury (Fig. 1d) compared to sham (Fig. 1c). This upregulation coincides with an increase in Cx43 immunostaining in the SGZ and hilus of the DG following CCI injury (Fig. 1d–f) compared to sham (Fig. 1c, e and f). Vimentin is also upregulated on reactive astrocytes and is associated with astroglial proliferation. Therefore, Cx43 expression is present on both neural stem cells and reactive astrocytes in the DG after CCI injury.

3.2. Modulation of cx43 on primary adult NSPCs using αCT1

Modulation of Cx43 using αCT1 has been shown to increase Cx43 gap junctional activity and impair proliferation and survival of breast cancer cells (Grek et al., 2015). To investigate the role of gap junction-associated Cx43 on adult NSPC behavior, we exposed primary murine NSPCs (Baumann et al., 2013; Dixon et al., 2015; Theus et al., 2010) to the Cx43 mimetic peptide αCT1. Using BrdU incorporation, we found a significant reduction in proliferation in a dose dependent manner (Fig. 2d). At 24 h post-treatment, the lowest concentration (37.5 μM) showed a significant reduction in proliferation (p < 0.05) between cells treated with αCT1 (11.37 ± 1.923% positive BrdU cells) (Fig. 2d) compared to Reverse control peptide (19.06 ± 1.957% BrdU-positive cells) (Fig. 2a). At 75 μM there was also a significant difference in proliferation (p < 0.001) between αCT1 (5.482 ± 1.356% BrdU-positive cells) (Fig. 2e) and Reverse 16.88 ± 1.765% positive BrdU cells) (Fig. 2b) treated cells. At the highest concentration (150 μM), αCT1 (2.397 ± 0.440% BrdU-positive cells) inhibits proliferation (p < 0.01) (Fig. 2f) in comparison with the Reverse control peptide (7.567 ± 1.683% BrdU-positive cells) (Fig. 2c) however, at this concentration the reverse also appears to have some suppressive effect on proliferation. To determine whether this effect was occurring earlier than 24 h we examined BrdU incorporation at 8 h post-αCT1 or −Reverse control treatment. We found no significant changes in the percent of BrdU-positive cells between 150 μM (F = 13.65, p = 0.7057), 75 μM (F = 1.389, p = 0.7831), or 37.5 μM (F = 8.740, p = 0.1013) αCT1 and Reverse (Fig. 2h). These results demonstrate that modulation of Cx43 using the selective inhibitor αCT1 negatively affect NSPC proliferation.

To test whether αCT1 reduces proliferation due to changes in cell survival, we assessed Caspase 3/7 activation, which is indicative of cells undergoing apoptosis. At 24 h post-Reverse or − αCT1 peptide treatment we found a significant increase in Caspase 3/7 activation (p < 0.001) at 150 μM αCT1 (2.427 ± 0.2943 mean intensity) (Fig. 3f and g) treated cells compared to 150 μM Reverse (0.9813 ± 0.05619 mean intensity) (Fig. 3c and g), and in 75 μM αCT1 (1.244 ± 0.08346 mean intensity) (Fig. 3e and g) compared to 75 μM Reverse
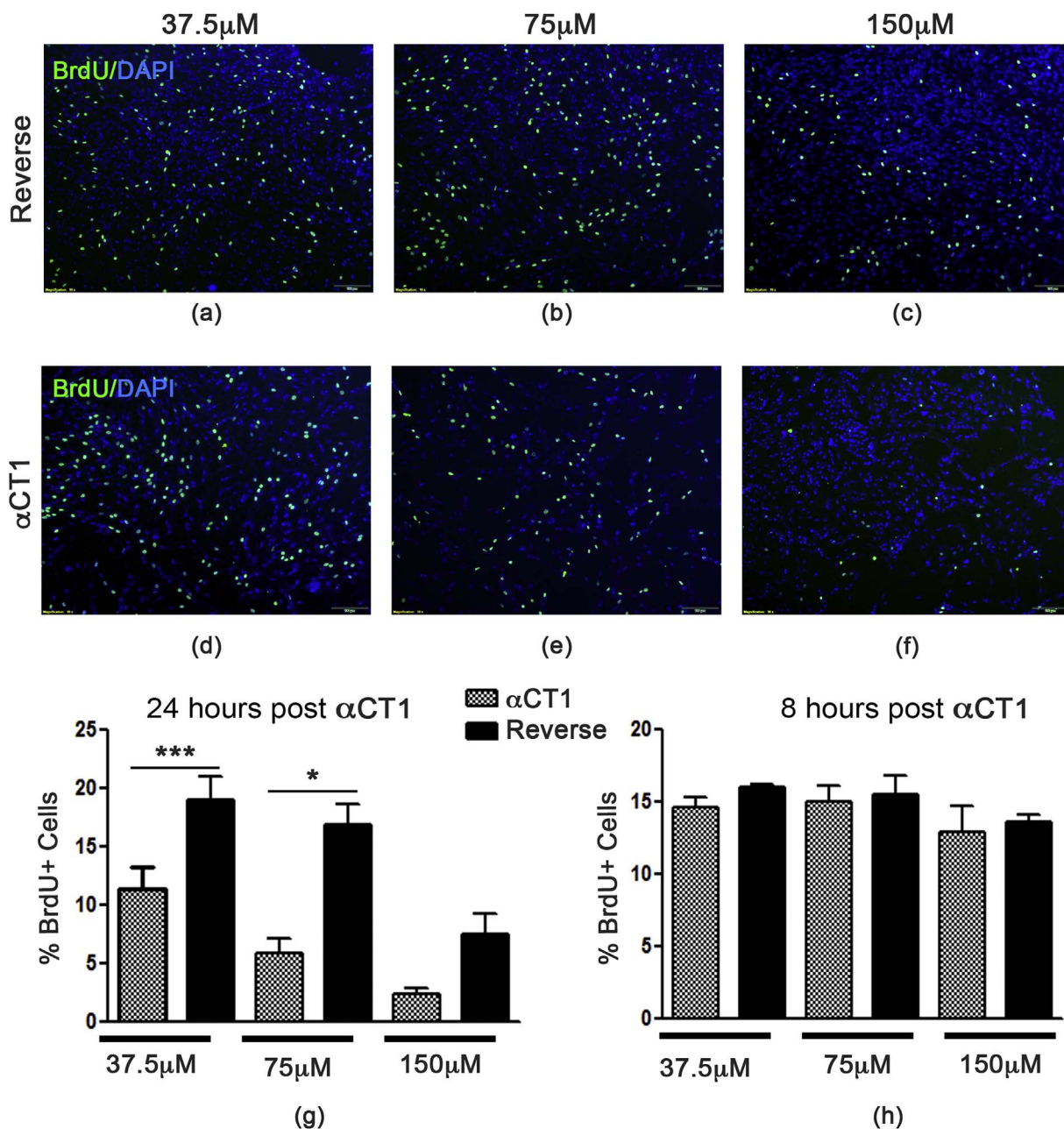
Fig. 2. Proliferation of NSPCs is reduced following αCT1treatment. Exposure of NSPCs to selective inhibitor αCT1 (d–f) reduces BrdU incorporation in a dose dependent manner following 24 h incubation compared to the control Reverse control peptide (a–c). (g) Quantified data showing a significant reduction in the percentage of BrdU-positive cells at 37.5 and 75 μm αCT1 exposure compared to Reverse. Non-specific effects of Reverse at 150 μm were also seen. (h) Quantified data showing no changes in NSPC proliferation are evident at 8 h of αCT1 treatment. *P < 0.01; ***P < 0.001 compared to Reverse control peptide.

(0.7774 ± 0.02735 mean intensity) (Fig. 3b and g) treated cells. We did not see any significant changes (F = 2.160, p = 0.1293) between 37.5 μM αCT1 (0.8428 ± 0.04075 mean intensity) (Fig. 3d and g) and 37.5 μM Reverse (0.08158 ± 0.05118 mean intensity) (Fig. 3a and g) treatments. Under phase contract microscopy, cells treated with 150 μM αCT1 were visibly unhealthy showing numerous pyknotic cells when compared to 37.5 μM (Supplementary Fig. S2). In addition, cells were fixed and immunostained for antibodies against Nestin and Cx43 at 24 h after Reverse (Fig. 4a and b) or αCT1 (Fig. 4c and d) peptide exposure. Using fluorescence image analysis we found reduced Cx43 aggregates on the cell surface of Nestin-positive, αCT1 treated NSPCs compared to Reverse. Nestin expression also revealed significant morphological changes such as reduced extensions or processes in the αCT1 treatment groups compared to Reverse controls. Together, these results show that modulation of Cx43 suppresses the growth and survival of

adult NSPCs.

To more quantitatively assess the effects of αCT1 treatment on Cx43 expression, we collected protein from αCT1 or Reverse treated cells at 24 h and performed Western blot analysis (Fig. 5). We found a significant decrease in Cx43 expression in cells treated with 150 μM αCT1 (0.325 ± 0.005 relative to β-actin) compared to 150 μM Reverse peptide (2.24 ± 0.01 relative to β-actin) (Fig. 5a and b) and an overall decrease in the total levels of phosphorylated serine 368 (pS368) (Fig. 5a). However, relative to the total Cx43 levels, pS368 was significantly increased in αCT1 (2.25 ± 0.02 relative to Cx43) compared to Reverse (0.55 ± 0.02 relative to Cx43) treated NSPCs. Moreover, we observed a trend towards reduced expression of p-Akt (1.01 ± 0.03 Reverse vs 0.74 ± 0.01 αCT1 relative to total Akt) and Bcl-2 (0.31 ± 0.001 Reverse vs 0.17 ± 0.02 αCT1 relative to β-actin), although the results did not reach statistical significance. We also
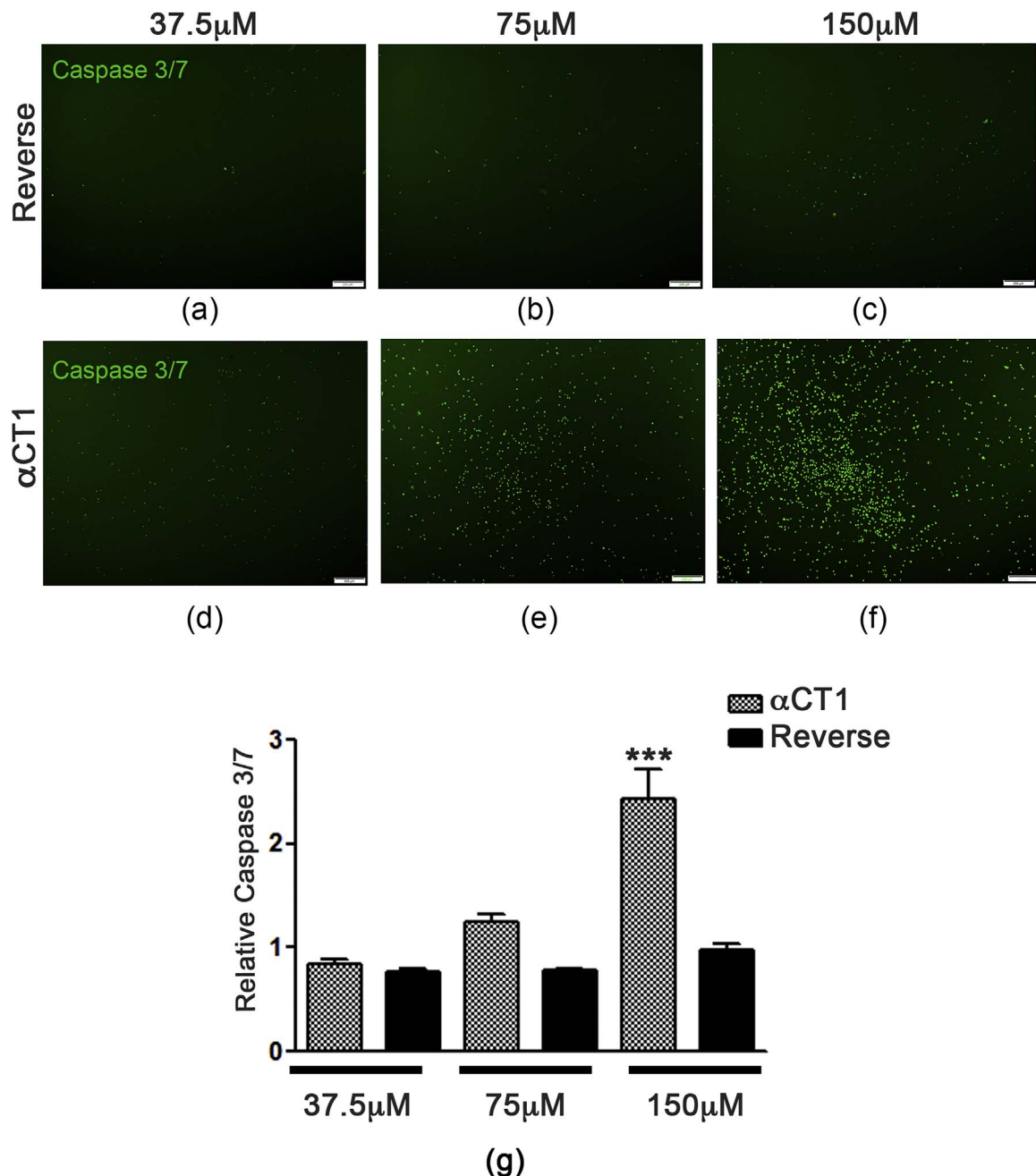
Fig. 3. Modulation of Cx43 induces apoptosis in NSPCs. Compared to Reverse control peptide (a–c), αCT1 dose dependently increased caspase 3/7 activation following 24 h incubation (d–f). (g) Quantified data showing a significant increase in caspase 3/7 mean intensity following 150 μm αCT1 exposure compared to Reverse and an increase trend at 75 μm αCT1. ***P < 0.001 compared to Reverse.

analyzed Cx43 and pS368 expression by Western blot at 8 h post-treatment and found a significant reduction in both pS368 and total Cx43 expression and no change in *p*-AKT or bcl-2 (data not shown). This correlates with previous reports showing overexpression of Cx43 stimulated proliferation of neural progenitor cells, however, chemical-induced uncoupling of gap junctions reduced proliferation and increased cell death (Cheng et al., 2004). Our findings suggest that αCT1 negatively affects NSPC survival and proliferation by reducing total cellular Cx43.

4. Discussion

The gap junction (GJ)-associated protein Cx43 has been implicated in the response to TBI (Frantseva et al., 2002; Ohsumi et al., 2010; Sun et al., 2015; Sun et al., 2014). Previous studies suggest that radial glial-like stem cells in the adult dentate gyrus (DG) form Cx43 associated GJs, which is necessary to maintain adult neurogenesis (Kunze et al., 2009; Lemcke and Kuznetsov, 2013a; Naus et al., 2016). Our findings demonstrate that Cx43 is up-regulated in the subgranular zone of the dentate gyrus, specifically on vimentin-positive cells and is expressed by both horizontal and radial glia (RG)-like neural stem cells in the adult DG (Seri et al., 2004). To identify the role of Cx43 in adult NSPC function, we exposed cultured NSPCs to the α-connexin carboxyl-terminal peptide, αCT1, (Ghatnekar et al., 2009; Hunter et al., 2005) which resulted in an overall reduction in Cx43 expression at 8 and 24 h which coincided with a dose-dependent reduction in proliferation and increase in apoptotic expression of Caspase 3/7. These effects were both present at the highest dosage (150 μM). While there was no significant difference in Caspase 3/7 activation at 37.5 μM or 75 μM, there is still a significant reduction in proliferation at these dosages. This specific
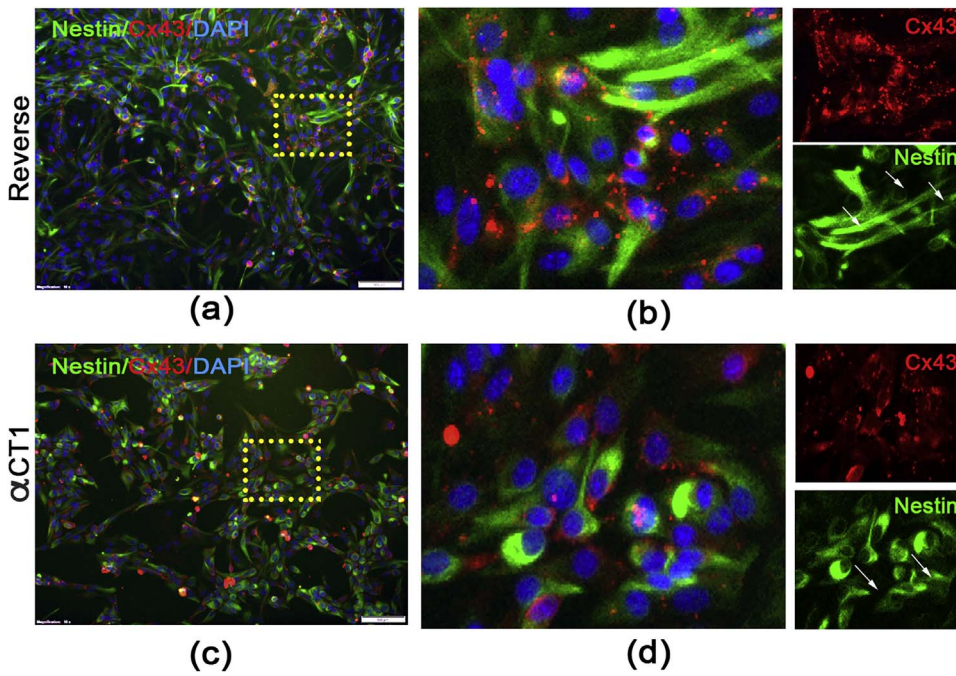
Fig. 4. αCT1 reduces Cx43 expression and induces changes in NSPC morphology. (a–b) Immunocytochemistry using antibodies against Cx43 (red) and Nestin (green) on monolayer cultures of NSPCS treated with 150 μm Reverse control peptide for 24 h. (c–d) Significant morphological changes are seen following 150 μm αCT1 exposure, demonstrated by nestin staining showing NSPCs are more rounded with fewer protrusions. The density and aggregate number and size of Cx43 is also visibly reduced compared to Reverse control peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
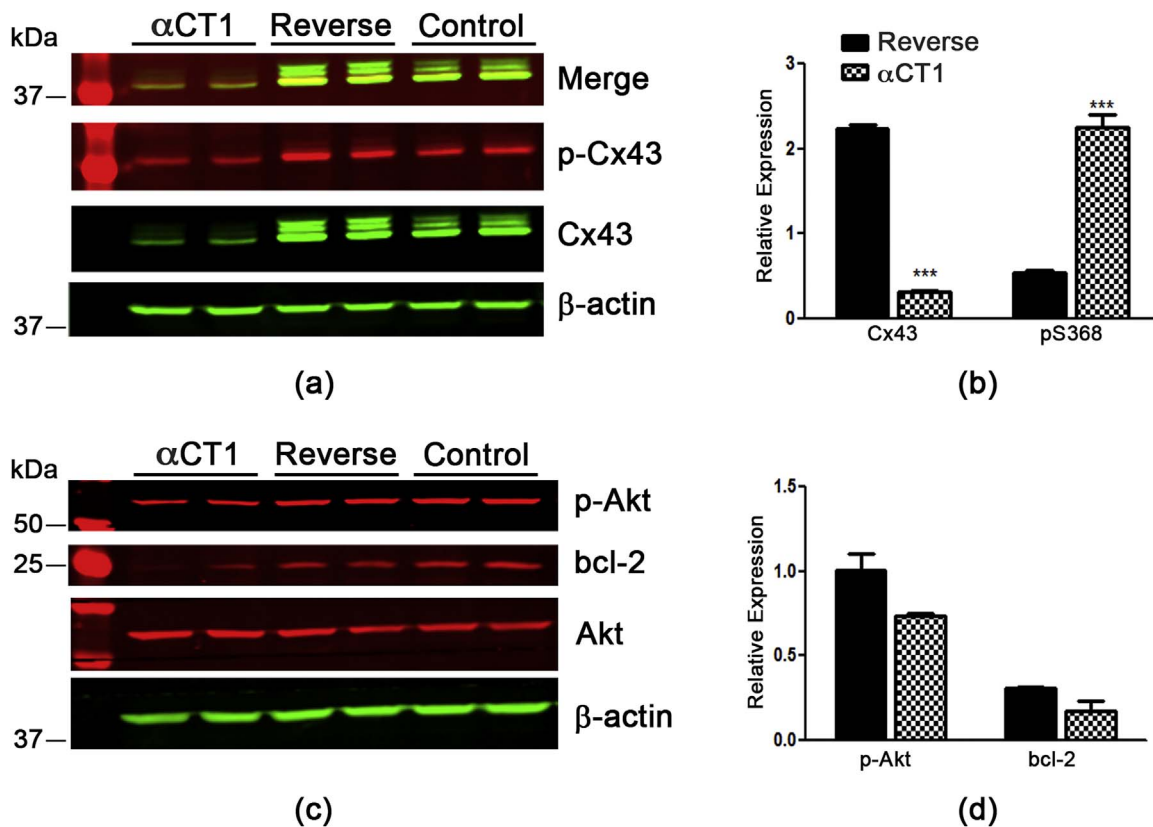
Fig. 5. αCT1 reduces Cx43 protein expression. (a–b) Using Western blot analysis we find a significant reduction in Cx43 expression at 24 h post-αCT1 treatment compared to Reverse. Quantified expression of Cx43 is graphed relative to β-actin levels. Although overall pS368 is reduced, the expression relative to the total levels of Cx43 is increased following 150 μm αCT1 exposure compared to 150 μm Reverse. (c–d) Expression of p-Akt (relative to total Akt) and Bcl-2 (relative to β-actin) show a trend towards reduced levels in αCT1 compared to Reverse. ***$P < 0.001$ compared to Reverse control peptide.

effect on cell cycle arrest might be attributed to time difference in activation of Caspase 3/7 versus exiting the s-phase of the cell cycle at the lower dosages. Alternatively, a dose dependent effect of αCT1 on overall Cx43 expression could exist, making the cells more vulnerable to apoptosis at higher doses. This dosage response could also regulate differential effects on cell signaling pathways important for survival

and/or proliferation (Eungdamrong and Iyengar, 2004). Moreover, it is unlikely, these effects influenced the production of doublecortin-positive neuroblasts in the NSPC cultures. The defined cell culture conditions used generally do not allow for the differentiation of doublecortin positive cells as the NSPCs are kept in a tight self-renewal state. It is also unlikely that after 8 or 24 h of αCT1 treatment this would change given

that differentiation is a much slower event.

Although not significant, a trend toward reduced expression of anti-apoptotic protein Bcl-2 and p-Akt growth signaling could suggest that Cx43 may positively regulate NSPC survival and proliferation by mediating these downstream pathways. Additionally, the mTOR pathway, which regulates the trafficking of Cx43, could also be disrupted by αCT1 treatment (Smyth and Shaw, 2013). Immunocytochemistry revealed that αCT1 reduced the density and size of Cx43 aggregates and caused a morphological change in cell shape. These findings correlate with previous studies showing high levels of Cx43 and GJ communication are required for maintaining NSPCs in a proliferative state (Cheng et al., 2004; Lemcke and Kuznetsov, 2013a; Liebmann et al., 2013) and suggest that up-regulation of Cx43 on NSPCs may represent a compensatory mechanism to induce proliferation and cellular replacement in the DG after TBI. The role of Cx43 on NSPC proliferation and survival appear to be regulated, in part, by epidermal growth factor receptor signaling (EGF/EGFR), which has a modulatory effect on the functional activity and expression of connexin proteins (Lemcke and Kuznetsov, 2013b; Maldonado et al., 1988). However, the impact of EGF/EGFR signaling axis on GJ coupling and Cx43 expression appears to be cell-type dependent. For example, EGF mediates down-regulation of Cx43 on cortical rodent astrocytes (Ueki et al., 2001) and inhibits Cx43-mediated GJ communication in HEK cells and liver epithelial cells (Cameron et al., 2003; Leithe and Rivedal, 2004). On the other hand, EGF/EGFR signaling enhances GJ communication and Cx43 expression in kidney epithelial cells and granulosa cells (Kennedy et al., 2003; Vikhamar et al., 1998), as well as embryonic-derived neural progenitor cells (Cheng et al., 2004). Although we did not test Cx43 expression in adult NSPCs following withdrawal of EGF, we did observe enhanced cell death and growth restriction when cells were exposed to αCT1 in the absence of EGF and FGF (data not shown). Overall, these data suggest that adult NSPCs are highly vulnerable to changes in Cx43 expression, and high level of Cx43 and strong cell–cell coupling may be required to maintain them in a healthy, proliferative state.

It remains unclear whether αCT1 may also disrupt GJ-independent actions of Cx43 such as hemi-channel-related cell survival signaling cascades (Sun et al., 2012). Cx43 hemichannels can transduce survival signals in response to extracellular cues via ERK anti-apoptotic pathways (Plotkin et al., 2002). Likewise, αCT1 may be mediating translocation of Cx43 to the mitochondria of NSPCs, where Cx43 was previously shown in pancreatic cancer cells to interact with Bax and initiate the mitochondria apoptotic pathway (Sun et al., 2012; Zhou and Jiang, 2014). Future studies elucidating these and other pathways are currently underway. To conclude, we find that adult NSPCs are selectively vulnerable to changes in Cx43 expression and that selective inhibition of Cx43 using the αCT1 peptide resulted in the induction of apoptosis and reduced proliferation of NSPCs. It is possible these effects may be mediated by both GJ-dependent and independent mechanisms. Although up-regulation of Cx43 following TBI has been linked to neural damage, the functional consequence of Cx43 dysregulation in the brain may be cell-type specific. The preservation and maintenance of Cx43 expression on NSPCs in the neurogenic compartment may confer protection of selective progenitor cell populations and be necessary and sufficient to enhance the neurogenic response following TBI although future studies will elucidate these cell-type specific longitudinal changes in Cx43 expression.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainresbull.2017.06.016.

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