**EPITHELIAL SODIUM CHANNEL REGULATES ADULT NEURAL STEM CELL PROLIFERATION IN A FLOW-DEPENDENT MANNER**

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**SUMMARY**

The adult neurogenic niche of the subependymal zone (SEZ) is located at the interface with the lateral ventricle that is filled with circulating cerebrospinal fluid (CSF). While several components of the CSF have been shown to regulate adult neural stem cells (NSCs) behavior, flow and shear stress have not been considered so far. Here we show that conditional deletion of the epithelial sodium channel (ENaC) in NSCs and their progeny strongly impairs their proliferation and neurogenic output. Importantly, increased fluid flow promotes proliferation of NSCs and progenitor cells in an ENaC- and CRAC-dependent manner in a wholemount preparation, demonstrating a novel regulatory mechanism in this special niche.

**KEYWORDS**

Adult neurogenesis, proliferation, neural stem cells, ENaC, fluid flow

**HIGHLIGHTS**

1. ENaC is high in adult NSCs and neuroblasts
2. Knocking-out or blocking ENaC decreases proliferation of stem and progenitor cells.
3. Fluid flow promotes proliferation in ENaC-dependent manner.

**INTRODUCTION**

The subependymal zone (SEZ) provides a special niche for NSCs as they are embedded in a layer of ependymal cells with their apical ends projecting into the ventricular space filled with CSF (Doetsch et al., 1997; Mirzadeh et al., 2008; Ninkovic and Götz, 2015; Silva-Vargas et al., 2016). This cytoarchitecture places NSCs at the intersection between brain parenchyma and CSF allowing them to sense changes in both and then propagate this information in their syncytium (Lim and Alvarez-Buylla, 2014). Some key elements, such as Sonic Hedgehog, Wnt, or neuroendocrine peptides and trophic factors (Delgado et al., 2014; Huang et al., 2010; Lehtinen et al., 2011; Silva-Vargas et al., 2016), were identified in the CSF to regulate NSCs biology (Ihrie et al., 2011; Marinaro et al., 2012). Similarly, a gradient of CSF chemorepulsive factors was found to direct migration of neuroblasts (Sawamoto et al., 2006). However, other molecular sensors detecting e.g. the CSF flow remain so far unknown.

Ion channels are prime candidates for such sensor as they can merge different stimuli into a simple and fast signal, the change in electrochemical gradient of ions, such as sodium, across the plasma membrane. However, NSCs share most electrophysiological properties with astrocytes (Filippov et al., 2003; Fukuda et al., 2003; Liu et al., 2006; Steiner et al., 2006), including expression of common ion channels such as voltage-gated potassium channels (Butt and Kalsi, 2006; Pruss et al., 2011). To identify differences in ion channel expression between these cell types, we searched in our previous genome-wide expression analysis and identified the epithelium sodium channel (ENaC) as having higher expression in NSCs compared to parenchymal astrocytes in the diencephalon or cerebral cortex (Beckervordersandforth et al., 2010; Sirko et al., 2015).

ENaC is often located on the apical membrane (Enuka et al., 2012) and controls transepithelial flux of sodium in kidneys or lungs (Hanukoglu and Hanukoglu, 2016; Marunaka et al., 2011; Schild, 2010), where it is found also in stem cells (Liu et al., 2016). In the nervous system, it is expressed in brain centers controlling fluid volume or blood pressure (Amin et al., 2005; Teruyama et al., 2012; Wang et al., 2010), in retina, olfactory bulb (OB) and human cortex (Dyka et al., 2005) (Giraldez et al., 2007), in choroid plexus (Van Huysse et al., 2012) and mechanosensory neurons (Fricke et al., 2000). Among glial cells, ENaC has been described in retinal Müller glia (Brockway et al., 2002) and some glioma cells (Berdiev et al., 2003; Miller and Loewy, 2013). Because ENaC is regulated by fluid shear stress (Fronius et al., 2010; Wang et al., 2009), it may represent a bona fide candidate for the elusive molecular sensor detecting CSF flow and linking NSCs with activity of the ciliated ependymal cells that surround them in the SEZ (Mirzadeh et al., 2008).

Here, we show that aNSCs and their progeny in the SEZ express alpha subunit of ENaC and that genetic knock-down or pharmacological blocking of ENaC in cell cultures from adult SEZ reduces proliferation. *In vivo*, inducible knock-out of the alpha ENaC subunit in GLAST-expressing aNSCs and their progeny also leads to decreased proliferation. Importantly, we demonstrate for the first time that the increased fluid flow promotes proliferation of aNSCs and progenitors in ENaC-dependent manner. In summary, our results suggest that ENaC serves as an electrochemical sensor of aNSCs to regulate their proliferation.

**RESULTS**

**αENaC is Expressed in NSCs and Progenitors in the SEZ**

To determine the localization of ENaC in the SEZ of adult mice, we immuno-stained for the pore-forming alpha subunit of ENaC (αENaC) with cell type-specific antibodies (Figure 1) and observed αENaC in NSCs labeled by the expression of the green fluorescent protein (GFP) driven by the promoter of human glial fibrillary acidic protein (GFAP, Figure 1A) and nestin-GFP (nestin-GFP; (Yamaguchi et al., 2000) Figure S1A) or GFAP and Sox2-immunostaining (Bracko et al., 2012; Lopez-Juarez et al., 2012), Figure S1B). Indeed, αENaC-staining was also present in the NSCs located in the core of the pinwheel structures labeled by β-catenin and hGFAP-GFP (Figure 1B, Figure S1C) where NSCs are located (Beckervordersandforth et al., 2010; Mirzadeh et al., 2008). In addition to NSCs αENaC-immunostaining was also contained in proliferating cells labeled with Ki67 (Scholzen and Gerdes, 2000) and Doublecortin (DCX)+ neuroblasts (Gleeson et al., 1999; Nacher et al., 2001). Interestingly, this was the case only in the SEZ (Figure 1C,D), and not in the rostral migratory stream (RMS, Figure S1D). Thus αENaC is confined to NSCs and their immediate progeny in the SEZ while niche cells are immunonegative (Figure S1E for oligodendrocyte progenitors, Figure S1F for niche astrocytes), except weak staining ependymal cells (Figure 1B).

The hippocampal subgranular zone (SGZ) showed rather more restricted αENaC-immunostaining with rare GFAP+, nestin-GFP+ or Sox2+ NSCs and some DCX+ neuroblasts (Figure S1H-J), while parenchymal astrocytes were ENaC-negative (Figure S1G).

We also checked mRNA levels of α, β and γENaC subunits from dissected SEZ by the reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and found αENaC expressed much higher than β or γENaC (Figure S1K). The comparison of relative mRNA expression (2-ΔΔCt; (Livak and Schmittgen, 2001) revealed 15 times more αENaC in the SEZ than in the OB, but 8.96 ± 0.19 times less when compared to kidney. When SEZ cells were isolated by fluorescence-activated cell sorting (FACS) as previously described (Codega et al., 2014; Fischer et al., 2011), αENaC but not β or γ expression was detected in quiescent and activated NSCs and ependymal cells (Figure 1E). Neuroblasts had higher αENaC mRNA levels, while virtually nothing was detectable in transiently amplifying progenitors (TAPs) despite the presence of the ENaC protein in these cells (Figure 1C). This suggests either fast down-regulation of ENaC mRNA upon dissociation and FACS or a high protein stability and low turn-over with low mRNA levels in these cells. In order to test if αENaC forms a functional channel in the SEZ, we performed whole-cell patch-clamp recordings and successfully isolated currents sensitive to the ENaC-specific blocker, Benzamil (Benz) (Alvarez de la Rosa et al., 2013) (Figure 1H-J) in NSCs identified by Aldh1l1-GFP+ (Heintz, 2004) and a radial morphology (Figure 1F,G and S1L).

**ENaC is Critical for SEZ Cell Proliferation *In Vitro***

To investigate the functional role of ENaC, we first examined neurospheres from adult SEZ subjected to pharmacological inhibition by ENaC-specific blockers Benz or amiloride (Alvarez de la Rosa et al., 2013) or to genetic knock-down (KD) by esiRNA against αENaC. Incubation of the neurospheres in proliferating conditions (of which 77% cells express αENaC, Figure S2A,B) in Benz (Figure 2A-F) or amiloride for 24 hours (Figure S2C) significantly reduced the number of cells in a concentration-dependent manner (for example, 10-100 μM Benz) at 72 hours (h) after the block. Similarly, αENaC KD, which shows about 60% reduction in ENaC-immunopositive cells (Figure S2D-F), resulted in significantly fewer cells at 72 h after transfection (Figure 2G-K). In order to determine if this reduction in cell number is caused by reduced proliferation and/or increased cell death, we followed single cells and their progeny for 85 h by time-lapse imaging of primary SEZ cells as previously described (Ortega et al., 2011). Both αENaC KD (Figure 2L-N) and Benz incubation (Figure 2O) significantly decreased the number of divisions and cells generated per individual cell lineage when compared to controls. Moreover, bath application of Benz induced rapid cell death (average time 14.2±1.6 hours) in about 60% of cells while control cells died in only 6% of all cases (Figure S2G-I). Thus, reducing function or protein levels of αENaC impairs proliferation and survival of primary SEZ cells. Similarly, also SEZ neurosphere-derived cells died as indicated by an increase in TUNEL+ cells upon Benz treatment (Figure S2J-L) or KD of αENaC (Figure S2M). Interestingly, αENaC also plays a role in differentiation as blocking or KD of ENaC significantly decreased the proportion of βIII-tubulin+ neurons from primary or secondary neurospheres (Figure 2P-R; Figure S3A-F) and also after shorter (6 h) incubation in the ENaC blockers (Figure S3G-I). Taken together, limiting ENaC function or protein *in vitro* impairs survival, proliferation and neurogenesis.

**Conditional Deletion of** α**ENaC In NSCs *In Vivo* Reduces SEZ Cell Number and Proliferation**

Next, we examined the role of αENaC *in vivo* by deleting αENaC selectively in NSCs and their progeny using GLASTCreERT2/CAG-GFP mice (Mori et al., 2006; Ninkovic et al., 2007) crossed with Scnn1aflox/flox mice (Hummler et al., 2002) to obtain GLASTCreERT2/CAG-GFP/Scnn1awt/wt (control) and GLASTCreERT2/CAG-GFP/Scnn1aflox/flox (iENaC) knock-out (KO) mice. Injections of tamoxifen (Tam, (Petrik et al., 2012)) lead to deletion of Scnn1a and GFP expression in GLAST-expressing cells and their progeny. First, we examined when ENaC protein is lost in the recombined SEZ cells at different times after induction. At 5 days post Tam (DPT) the proportion of αENaC+ cells amongst recombined GFP+ cells was already reduced in the SEZ of iENaC KO, but reached even lower levels by 10 DPT (Figure S4D-F). As the loss of ENaC protein was virtually complete at 10 DPT with only about 5% of GFP+ ENaC+ cells (Figure S4E,F), we chose to examine this time-point for a phenotype.

To examine if cell number, proliferation and neurogenesis were affected *in vivo*, we performed stereological and proportional analyses of GFP+ cells double-stained for Ki67 and DCX at 10DPT (Figure 3A,B). Consistent with the *in vitro* results, the absolute number of GFP+ cells was significantly reduced in the iENaC KO SEZ versus control (Figure 3C). As the detection of fewer GFP+ cells in the iENaC KO may be due to a lower recombination rate we determined the recombination efficiency by assessing the number of recombined GFP+ astrocytes (that are ENaC-negative and hence should not be affected, see Figure S1G) in the cerebral cortex in controls and iENaC KO at 10 DPT. As recombined cell numbers in control and iENaC cerebral cortex were well comparable (Figure S4A-C), this suggests a similar level of recombination efficiency between genotypes. Indeed, the decrease in recombined cells in the SEZ resulted in reduced SEZ thickness (measured as the dense band of DAPI+ cells directly adjacent to the lateral ventricle) in iENaC KO compared to controls (Figure 3F), demonstrating a loss of cells rather than reduced recombination. Importantly these results also indicate that the phenotype in the recombined cells is not compensated by surrounding non-targeted cells.

Next we examined whether GFP+ cells in iENaC SEZ were reduced by defects in proliferation, survival or fate change. We found fewer proliferating cells (Ki67+GFP+), both progenitors (Ki67+DCX-GFP+) and proliferating neuroblasts (Ki67+DCX+GFP+) in the SEZ of iENaC KO compared to control (Figure 3C). In order to discriminate whether all cell types are affected equally (possibly due to defects in the NSCs) or whether some cell types are specifically affected, we also quantified the proportion of progenitors, proliferating and non-proliferating NBs among GFP+ cells (Figure 3D,E). Interestingly, this analysis showed a significant reduction of all proliferating cells (progenitors and proliferating NBs), while the other populations were relatively less affected in iENaC KO versus control. These data suggest a particularly pronounced effect of ENaC deletion on proliferation.

To determine if also aNSC numbers are reduced upon ENaC deletion, we discriminated Sox2+ (GFAP-) progenitors and Sox2+GFAP+ NSCs (Figure 3G). Indeed, also the NSC number (Sox2+GFAP+GFP+ cells) was significantly reduced in the iENaC KO SEZ (Figure 3H), suggesting that both NSCs and their proliferating progeny are affected by ENaC ablation.

To address if the reduction in NSCs may be due to an altered fate, we quantified ependymal cells by S100β staining and their position lining the ventricle (Figure 3I). However, their proportion was similar between control and iENaC KO (Figure 3J), suggesting that the reduction in NSCs is not due to conversion into ependyma (Conover et al., 2000), for label-retaining cell measurements, see below). As staining for activated caspase 3 showed no increase in iENaC KO at both 5 and 10 DPT (Figure S4G and Figure 3K), apoptosis does not seem to contribute to the *in vivo* phenotype, suggesting that mostly reduced proliferation causes the severe cellular depletion of the SEZ upon ENaC deletion. Importantly, this also resulted in reduced OB neurogenesis as the number of GFP+ cells in the iENaC KO OB was reduced compared to control (Figure 3L-N).

**Effects of ENaC Deletion *In Vivo* Cannot Be Compensated at Later Stages**

In order to monitor the behavior of NSCs upon ENaC deletion by their label retaining hallmark and to follow the phenotype for longer, we examined the SEZ at 30 DPT (Figure 4). As expected, we found a larger number of GFP+ cells in the control SEZ compared to 10 DPT as further progeny has been generated from the genetically traced NSCs in the controls, while there was virtually no increase in GFP+ cell number in the iENaC KO (compare Figures 3C and 4C). Indeed, proliferation was drastically reduced at 30 DPT (Figure 4A-E). To determine if this is due to a depletion of NSCs we used the label-retaining paradigm by giving BrdU, a thymidine analog, in drinking water for two weeks starting at 3 DPT and analyzing GFP+ BrdU+ and GFAP+ NSCs at 24 DPT (8 days after BrdU, Figure 4F). Indeed, the proportion of BrdU+GFAP+GFP+ cells was significantly reduced in iENaC KO compared to control (Figure 4G), suggesting that NSCs either fail to become activated and proliferate after αENaC deletion or die. Given their small number, the latter is rather difficult to assess. However, we could test whether the phenotype involves already previously activated NSCs by labeling proliferating NSCs prior to Tam treatment by giving BrdU for 2 weeks before Tam and analyzing 7 days later. Even in this paradigm, there was a smaller proportion of BrdU+GFAP+GFP+ cells in iENaC KO versus control (Figure 4H) demonstrating that also previously activated NSCs are reduced after ENaC deletion. Thus, proliferation and activation of previously and newly active NSCs is reduced in the absence of ENaC demonstrating the crucial contribution of this constitutively open channel to the proliferative activity of NSCs. As a consequence of decreased proliferation of stem and progenitor cells persisting after ENaC depletion, the number of newly generated neurons also remains significantly reduced in the OB of iENaC KO compared to control (Figure 4I-K), demonstrating that ENaC is essential for adult neurogenesis.

**ENaC is Required in Fast Proliferating SEZ Progenitors *In Vivo* as Revealed by Their Selective Targeting**

To discriminate between the effects of ENaC deletion in NSCs and their progeny, we selectively targeted the rapidly proliferating progenitor cells by MLV-based retroviral delivery of NLS-Cre recombinase (Colak et al., 2008) injected into the SEZ of ENaCwt/wt (WT) and ENaCflox/flox mice. Already 3 days post injection (dpi) Ki67+ cells were reduced to almost half amongst all GFP+ cells in the SEZ of ENaCflox/flox, as compared to WT mice (Figure S5A-C), demonstrating a very fast effect of ENaC loss on progenitor proliferation without affecting the proportion of apoptotic cells (Figure S5D,E). Thus, the iENaC KO phenotype is not exclusively due to the reduction in aNSC numbers but rather ENaC also directly regulates fast proliferating cells, progenitors and NBs.

**Fluid Flow Induces Proliferation in SEZ in an ENaC-Dependent Manner**

ENaC channels are constitutively open, but their opening probability can be further increased by various gating mechanisms (Boscardin et al., 2016), including fluid flow (Fronius et al., 2010; Wang et al., 2009). To examine the effects of fluid flow in the SEZ, we used SEZ whole-mounts and subjected them to control or elevated ACSF flow (see Methods for calculations of shear stress) for 4 hours (Figure 5A), after which the SEZ was stained and the number of Ki67+ and DCX+ cells was quantified (Figure 5B,C). Strikingly, the number of total Ki67+ proliferating cells and proliferating NBs was significantly increased in the SEZ wholemount exposed to elevated fluid flow when compared to control (Figure 5D). Thus, increased flow induces a fast proliferation response.

To determine to which extent ENaC contributes to this fast response, we repeated the experiment with ACSF containing Benz. Excitingly, Benz completely abrogated the flow-induced proliferation response (Figure 5D). To determine if ENaC in NSCs and their progeny is responsible for the flow-induced proliferation, we utilized the controls and iENaC KO SEZ at 10DPT. Indeed, while high flow increased the proportion of proliferating (Ki67+ or Ki67+DCX+) GFP+ progenitors and NBs in controls, there was no difference between control and elevated ACSF flow in iENaC KO (Figure 5E-G) clearly demonstrating that ENaC is required for this response.

To understand possible downstream signaling pathways mediating the proliferative response to the elevated fluid flow via ENaC, we considered the mitogen-activated protein kinases (Bodart, 2010) as prime candidates given their recent implication as mediators between mechanical stimuli and cell division via the Piezo channels in other cell types (Gudipaty et al., 2017). SEZ wholemounts from WT mice were subjected to control or elevated ACSF flow as described above and stained for the activated (phosphorylated) form of Erk kinase (pErk; Figure 5H-I). High flow increased number of pERK+Ki67+ and pErk+ proliferating NBs (Figure 5J). This increase was reversed in the presence of Benz suggesting that elevated fluid flow requires ENaC to activate Erk kinase, presenting a novel mechanism to regulate adult neurogenesis.

**ENaC Loss Affects Flow-Induced Changes in Calcium Dynamics in NSCs and Progenitors**

To further understand how ENaC may convey the information about the fluid flow to the cells, we performed calcium imaging of the SEZ during low and high flow of ACSF using two-photon microscopy. Acute brain sections from control and iENaC KO mice at 10 DPT (Figure 6A) were loaded with the calcium indicator Oregon Green Bapta 1 (OGB1) and calcium oscillations were first monitored in baseline ACSF flow (Figure 6B,C). After 15 minutes, we increased the ACSF flow and continuously recorded the calcium events for another 15 minutes followed by baseline ASCF flow recovery. In the post-hoc analysis, we evaluated calcium events in GFP+ cells that were pooled into cell classes by their morphology and anatomical location as arbitrary adult neural stem cells/progenitors and ependymocytes. We used an automated detection of calcium events to determine the frequency of calcium oscillations and overall type of responsiveness of cells to elevated shear stress (Figure 6C). When cells were classified into positive responders (showing higher frequency of calcium oscillations in elevated fluid flow), non-responders (no change in frequency) and negative responders (reduced frequency) we observed a trend towards more negative and non-responders in the GFP+ stem cells/progenitors of iENaC KO compared to controls (Figure 6D,E). Importantly, the GFP+ stem cells/progenitors from control mice displayed an increase in the frequency of calcium oscillation in response to elevated ACSF flow, a feature that was not observed in GFP+ stem cells/progenitors from iENaC KO (Figure 6F). Thus, cells lacking ENaC also lack the capacity to respond to increased flow by increasing proliferation, pERK and Ca-signals.

Notably, however, cells from iENaC KO showed higher frequency of Ca signals at baseline ACSF flow when compared with controls (Figure 6D, F). This could be due to the loss of ENaC dysregulating calcium handling in stem and progenitor cells under the baseline flow. Importantly, ENaC involvement in calcium signals was specific to stem cells/progenitors because there was no difference in Ca signals between control and iENaC KO and between baseline and elevated flow in cells classified as ependymocytes (Figure 6G). To identify the possible source of calcium, we exposed SEZ wholemounts to a specific blocker of the store-operated Ca release-activated Ca (CRAC) channels, BTP-2 (YM-58483; (Parekh, 2010), given the relatively slow nature of the flow induced Ca-signals. Similar to Benz (Figure 5D), BTP-2 also abrogated the flow-induced increase in proliferating cells or proliferating NB in SEZ (Figure 6H-J) suggesting that CRAC channels are a likely source of calcium in this context.

**DISCUSSION**

**αENaC Channel Activity is a Key Regulator of the SEZ Neurogenic Activity**

Here, we provide for the first time a role for a voltage- and ligand-independent ion channel in adult neurogenesis. Our results show that αENaC is expressed in NSCs and their progeny in the SEZ, where it is critical for their proliferation. Finally, we suggest that ENaC allows the NSCs and progenitors to sense fluid flow or shear stress as a novel regulator of the neurogenic process.

While ENaC is an essential component of epithelial cells in the peripheral organs (Hanukoglu and Hanukoglu, 2016), this channel has hardly been studied in the brain let alone in NSCs. Both qPCR and immunohistochemistry showed the predominant expression of the alpha subunit in both the SEZ tissue and in sorted NSCs and neuroblasts, which is the subunit essential for channel function. While ENaC channels have been suggested to exist as heterotetrameric, heterotrimeric, or homotrimeric channels *in vitro* (Anantharam and Palmer, 2007; Staruschenko et al., 2005; Staruschenko et al., 2004; Stewart et al., 2011), only channels containing αENaC, either alone or with other subunits can form functional channels (Canessa et al., 1993; Fyfe and Canessa, 1998). ENaC subunits show different expression profiles in cell-dependent context. For example, colonic epithelia or Műller glia express α, but not β or γ ENaC subunits (Asher et al., 1996; Brockway et al., 2002) similar to our findings in NSCs. Thus, the lack of β or γ ENaC subunits does not preclude ENaC currents. Indeed, we recorded Benz-sensitive currents in Aldh1l1-GFP+ cells with radial glia morphology in the SEZ suggesting functional ENaC currents in NSCs. Our observation that ENaC blockers at high concentrations (Hirsh et al., 2004) were needed to induce proliferation deficits similar to αENaC knock-down *in vitro* is consistent with the native ENaC channels containing only α or β subunits being less sensitive to these blockers (McNicholas and Canessa, 1997). Moreover, ENaC may form chimeras with other members of the ENaC/Degenerin family such as the acid-sensing ion channels (ASICs), which were demonstrated in astrocytes and gliomas as having lower sensitivity to blockers than the original ENaC channels (Kapoor et al., 2011; Meltzer et al., 2007). Importantly, however, αENaC is the subunit essential for channel function, which is why we targeted it for deletion here.

α**ENaC Regulates Proliferation of NSCs and their Progeny**

Our *in vitro* and *in vivo* results jointly point towards a conclusion that ENaC regulates cell proliferation in the SEZ. Blocking or knocking-down ENaC in neurosphere-derived or primary SEZ cells decreases the proliferation as shown by live imaging and immunostaining for Ki67+. Similarly, knocking-out αENaC in NSCs *in vivo* leads to fewer BrdU+ NSCs and their progeny in the SEZ. Furthermore, Cre delivered selectively to the rapidly proliferating cells by viral vectors (Colak et al., 2008) revealed that αENaC is also required for proliferation in progenitors and NBs. Thus αENaC acts in a bimodal manner regulating proliferation of both actively dividing NSCs as well as their progeny. So far, only few reports link ENaC activity to cell cycle progression. For example, blocking ENaC had anti-tumor effects hinting possible anti-proliferative effects (Matthews et al., 2011) and incubation of glioma cells in higher concentrations of Benz halted the cell cycle in G0/G1 phase (Rooj et al., 2012). In addition, there is evidence for ENaC affecting cell migration, e.g. upon knockdown of chimeric channels composed of ENaC and ASIC subunit in glioblastoma (Kapoor et al., 2009; Kapoor et al., 2011) or by pharmacological block of ENaC decreasing keratinocyte migration (Yang et al., 2013). Notably, NB migration was not affected by ENaC deletion as determined by the localization of cells targeted by the Cre virus (data not shown). Thus, in adult neurogenesis ENaC mostly acts by regulating proliferation, demonstrating a key role of this constitutively open, voltage- and ligand-independent ion channel in regulating adult neurogenesis.

Notably, the effects of blocking or KD of ENaC *in vitro* included (besides effects on proliferation and neurogenesis) also cell death. As we could not detect any increase in apoptotic cells at 5 or 10 DPT in iENaC KO and at 3 days post injection of retroviruses we would conclude that the cell death elicited by loss of ENaC function or protein *in vitro* can be compensated by the niche environment *in vivo*. However, even by affecting apparently largely proliferation only ENaC exerts a powerful control over NSC and NB numbers as its deletion leads to a fast depletion of SEZ cells. Thus, ENaC is a novel and potent regulator of adult neurogenesis determining proliferation of NSCs and their progeny.

**Fluid Flow Regulates Proliferation via ENaC in the Whole-Mount SEZ**

Our findings that fluid flow stimulates cell proliferation in SEZ in an ENaC-dependent manner suggest that ENaC may serve as a sensor of environmental changes. Indeed, this sensory role for ENaC was previously suggested in taste, mechanosensing or circumventricular organs of the brain (Ben-Shahar, 2011; Chandrashekar et al., 2010; Miller and Loewy, 2013). The laminar shear stress elicited by fluid flow increases open probability and thus ENaC currents (Althaus et al., 2007; Karpushev et al., 2010), a phenomenon also observed in native tissues such as endothelial cells in blood vessels or tubules in kidney nephrons (Morimoto et al., 2006; Satlin et al., 2001; Wang et al., 2009). In line with these findings, our results demonstrate that shear stress caused by physiological forces previously shown to stimulate ENaC (Althaus et al., 2007) cause an increase in the number of proliferating cells including NBs in the SEZ, which is diminished by Benz in WT tissue or does not occur in GFP+ cells lacking αENaC. Finally, the elevated fluid flow activates Erk kinase in ENaC-dependent fashion suggesting that it connects fluid flow with proliferation. Thus, our results not only imply for the very first time fluid flow and hence physical forces as a regulator of NSCs and progenitor proliferation in the SEZ, but also identify the channel ENaC as a central mediator of this response. This is important given the expression of various other mechanosensitive channels in NSCs, such as Piezo and TRP channels (Beckervordersandforth et al., 2010; Blumenthal et al., 2014; Codega et al., 2014).

Why would NSCs and progenitors need to sense fluid flow? One possibility is that sensing fluid flow may serve as a yard stick to detect their proximity to the ventricle. Indeed, it has been suggested that radial glial cells contacting the ventricle would act as NSCs while those located deeper in the tissue would act as niche astrocytes (Lim and Alvarez-Buylla, 2014). A similar scenario could account for regulating NB proliferation as these cells proliferate as long as they are in the SEZ while exiting towards the RMS is accompanied by loss of ENaC as well as slowing and eventually termination of proliferation. Thus, we would suggest that SEZ cells utilize the constant fluid drag forces to sense their distance to the ventricle, rather than actually measuring fluid flow speed as endothelial or kidney cells. In addition, it is important to recognize that ENaC is also regulated by a wide variety of factors other than mechanical forces, such as extracellular proteases and ions or cytoplasmic kinases and links to the cytoskeleton (Boscardin et al., 2016), which may also be involved in its function in the SEZ. Indeed, reducing ENaC function in conditions with negligible fluid flow, such as *in vitro* preparations, still affects proliferation and viability of NSCs, because ENaC is constitutively open and does not need mechanical forces to be activated. Thus, shear stress is unlikely to be the exclusive mechanism required for ENaC-dependent regulation of proliferation in the SEZ, but it is a fascinating novel one.

**ENaC Function Bridges Fluid Flow and Calcium Signaling**

Besides the up-stream regulators of ENaC function, we also examined down-stream effectors. We found that ENaC deletion changes calcium signaling and response to increased fluid flow. Likewise, blocking Orai/STIM CRAC channels, that regulate proliferation in the SEZ (Somasundaram et al., 2014), abrogated the flow-induced increase in cell proliferation. Indeed, the calcium oscillations we recorded in the SEZ were relatively slow (lasting on average around ten seconds) consistent with the store-operated calcium sources (Toth et al., 2016) that were observed before in NSCs of the SEZ (Young et al., 2014). Calcium signaling regulates multiple cellular functions during neurogenesis and in stem cells, including proliferation (Deng et al., 2015; Toth et al., 2016). To our knowledge, however, we demonstrated for the first time here that a ligand- and voltage-independent, constitutively active ion channel is required for shear stress-dependent regulation of calcium signaling in NSCs and their progeny.

We showed that elevated fluid flow increased the frequency of calcium oscillations in control mice but not in iENaC KO animals. Surprisingly, SEZ cells lacking αENaC displayed increased frequency oscillations under the baseline fluid flow when compared to controls. While this may be counter-intuitive because the absence of sodium inward conductance through ENaC should promote hyperpolarization and thus attenuate calcium oscillations, it is important to realize that our analysis was performed at 10 DPT, i.e. sometime after loss of ENaC. Thus, the long-term lack of ENaC may lead to dysregulation of calcium signaling, which is complex.

Our data suggest that shear stress is one of the regulators of ENaC that triggers alterations in calcium signaling requiring CRAC channels. This leads (directly or indirectly) to Erk phosphorylation that regulates proliferation. While the mechanosensing is certainly only one of the possible activator mechanisms of ENaC, it represents an entirely novel mode adult neurogenesis regulation. The involvement of ENaC in NSCs and progenitors could have medical repercussions since ENaC blockers are used as diuretics to treat certain types of hypertension, can cross the blood brain or CSF barrier (Alvarez de la Rosa et al., 2013) and may hence have potent side effects on neurogenesis, which provides new striatal neurons from SEZ in humans (Ernst et al., 2014). This highlights how important it is to understand novel regulators of adult neurogenesis and avoid unintended pharmacological side effects.

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**AUTHOR CONTRIBUTIONS**

M.G. conceived the project. D.P. and M.G. conceptualized the project. D.P., S.G., M.M., B.G. and M.G. designed experiments. D.P. performed all experiments and analyzed all results other than the following. S.G. performed stereotaxic surgeries. M.P. performed FACS sorting. D.P. and M.M. performed experiments and analyzed results for calcium imaging. D.P. and M.G. wrote the paper with feedback from B.G. M.M. and S.G.

**FIGURE LEGENDS**

**Figure 1. ENaC Expression in Subependymal Zone**

(A) Microphotograph of staining for αENaC and hGFAP-GFP in SEZ (lv = lateral ventricle). (B) αENaC staining in β-catenin pinwheel structures of SEZ whole-mounts. (C) Ki67-positive (Ki67+, arrowhead) and Ki67+DCX+ (arrow) cells in SEZ express αENaC. (D) In SEZ, αENaC overlaps with DCX+ cells. (E) Relative mRNA fold change of αENaC when compared to GAPDH in cDNA from sorted quiescent adult neural stem cells (qNSCs), activated aNSCs (aNSCs), progenitors (Progen.) , neuroblasts (NBs), and ependymocytes (EC). Microphotographs in bright field (F) and fluorescence (G) showing a patch-clamp electrode on a GFP+ cell on the very edge of SEZ. (H) Patch-clamp experimental paradigm. (I) Voltage-command and an example of whole-cell currents elicited by a -90 mV rectangular pulse (40 ms long) before (black) and 6 minutes after Benz perfusion (red). (J) Benz-sensitive currents at -90 mV. Scale bars, all 10 μm. Animals were tested at 6-8 weeks of age. N = 10 for each FACS sorting. N = 3 for patch-clamp recordings.

Data are presented as mean ± SEM.

**Figure 2. Reduced Proliferation and Survival upon Blocking or Knocking-down of ENaC in Primary SEZ or Neurosphere-derived Cells**

(A) Schematics of passage and treatment of SEZ neurosphere-derived cells in proliferating conditions. Representative images of neurospheres 24 h (B,C) and 72 h (D,E) after Benz (bottom row) or in control (upper row). (F) Quantification of dissociate neurosphere cells after 72 h of Benz. Representative images of neurospheres after transfection with control esiRNA (G,I) or αENaC esiRNA (H,J) at two time points. (K) Dissociate neurosphere cell number quantification after esiRNA knock-down (KD) of αENaC. Example cell division trees from time-lapse imaging in control (L) or αENaC esiRNA (M) during 84 h after transfection. Quantification of the number of cell divisions and cells per division tree in αENaC KD (N) or ENaC-blocking (O) experiments. Representative images of primary SEZ cells grown in differentiating conditions stained as indicated after transfection with control (P) or αENaC (Q) esiRNA. (R) Quantification of proportion of cells positive for GFAP or βIII-tubulin. Scale bars, 100 μm (B-E, G-J) and 50 μm (P,Q). Animals were tested at 6-8 weeks of age. N = 3-6 for cell cultures. N = 12-16 for lineage trees. \* P < 0.05; \*\* P < 0.01. Data are presented as mean ± SEM.

**Figure 3. Phenotype of αENaC Knock-out in GLAST-expressing NSCs and Their Progeny in SEZ at 10 days After Tamoxifen**

Representative images of SEZ from control (A) or iENaC KO (B) brains stained as indicated. In (C), stereological analysis of absolute number of GFP+ cell subpopulations in SEZ: all proliferating cells (Ki67+), proliferating neuroblasts (Ki67+DCX+), progenitors (Ki67+DCX-), and neuroblasts (Ki67-DCX+). Proportional analysis of Ki67+ and/or DCX+ cells in the GFP+ cell population in control (D) or iENaC KO (E). (F) Measurements of average SEZ thickness in μm. (G) Representative image of SEZ stained for GFAP, GFP and Sox2. (H) Quantification of number of Sox2+GFP+ progenitors and Sox2+GFAP+GFP+ NSCs. (I) Representative image of staining for S100β+ and GFP+ ependymal cells in SEZ. (J) Quantification of proportion of S100β+GFP+ cells out GFP+ cells in control and iENaC KO mice. (K) Number of activated caspase 3 (AC3)-positive cells in both genotypes. Representative images of the olfactory bulb stained as indicated in control (L) or iENaC KO (M). Quantification of number of GFP-positive cells per mm3 of olfactory bulb tissue (N), lv = lateral ventricle. Scale bars, 50 μm (A-B, L-M) and 20 μm (G, I). Animals were tested at 9-10 weeks of age, N = 5. \* P < 0.05; \*\* P < 0.01. Data are presented as mean ± SEM.

**Figure 4. Phenotype of αENaC Knock-out in SEZ at 30 Days After Tamoxifen**

Representative images of SEZ stained as indicated in control (A) and iENaC KO (B). (C) Stereological quantification of GFP+ cells labeled as in Figure 3C. Proportional analysis of Ki67+ and/or DCX+ GFP-expressing cells in SEZ of control (D) or iENaC KO (E). (F) SEZ stained for GFAP, GFP and BrdU. (G) Schematics of tamoxifen (Tam) administration before BrdU and quantification of proportion of GFP+ cells positive for BrdU and GFAP. (H) Schematics of BrdU administration before Tam and proportional quantification. Olfactory bulb from Control (I) and iENaC KO (J) stained as indicated. (K) Quantification of GFP+ cells in different layers of olfactory bulb cortex as indicated. Note that cell numbers were quantified by stereology, while in Figure 3N the cells were quantified in mm3 of OB core tissue. Scale bars, 50 μm (A-B, I-J), 20 μm (F). Animals were tested at 11-13 weeks of age. N = 6-7 for Tam-treated. N = 3-4 for BrdU-treated. \* P < 0.05; \*\* P < 0.01. Data are presented as mean ± SEM.

**Figure 5. Effects of Fluid Flow on Cell Proliferation in SEZ**

(A) Schematic depicting the experimental design for flow manipulation with SEZ whole-mounts (anatomical directions: P = posterior, D = dorsal, A = anterior, V = ventral). Representative images of en-face SEZ stained as indicated after being subjected to control (B) or high (C) ACSF flow. (D) Quantification of number of Ki67+ and Ki67+DCX+ (Neuroblast) cells per mm3 of SEZ tissue without or with benzamil. Representative images SEZ stained as indicated in iENaC KO mice at 10 DPT with control (E) and high fluid flow (F). Experimental design and cell quantification of proliferating cells and neuroblasts in control and iENaC KO mice under control and high flow. (G). Representative images of SEZ stained as indicated (H,I). Quantification of pErk+ cells per mm3 of SEZ tissue without or with benzamil (J). Scale bars, 20 μm. Animals were tested at 7-10 weeks of age, N = 3-4. \* P < 0.05. Data are presented as mean ± SEM.

**Figure 6. Calcium Signaling in SEZ Under Different Flow Conditions**

(A) The experimental design for calcium imaging. (B) Schematic representation of an acute brain slice subjected to ACSF. (C) Representative still image of SEZ cells loaded with Oregon Green Bapta 1 (OGB-1) with regions of interests depicted as circles. (D) Representative traces of change in fluorescence (ΔF/F) as a function of time in OGB1-loaded SEZ cells from control (top 3 traces) and iENaC KO (bottom 3 traces) at 10 DPT. The red bar indicates exposure to high flow. (E) Number of stem cells/progenitors sorted by their responsiveness to elevated shear stress as indicated in the legend in control and iENaC KO. (F) Frequency of calcium events per minute in baseline or elevated shear stress in GFP-positive NSCs/progenitors of control and iENaC. (G) Frequency of calcium events per minute in ependymocytes. Animals were tested at 9-10 weeks of age, N = 3-4. Representative images of en-face SEZ stained as indicated after being subjected to control (H) or high flow with BTP-2 (I). (J) Quantification of number of Ki67+ and Ki67+DCX+ cells per mm3 of SEZ tissue. Animals were tested at 7-10 weeks of age, N = 3. \*\*\* P < 0.001. Data are presented as mean ± SEM.

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