Radixin inhibition decreases adult neural progenitor cell migration and proliferation in vitro and in vivo

Åsa Persson, Olle R. Lindberg and Hans G. Kuhn*
Center for Brain Repair and Rehabilitation, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

INTRODUCTION

In the adult rodent brain, neuronal progenitor cells, neuroblasts, migrate a long distance from the neurogenic subventricular zone (SVZ) through the rostral migratory stream (RMS) to their final destination in the olfactory bulb (OB) where they differentiate into mature neurons (Altman, 1969; Lois and Alvarez-Buylla, 1994). The significance and extent of adult neurogenesis in the human SVZ/RMS area is not clear, however, in rodents neuronal progenitors capable of long distance migration are produced throughout life in the subventricular zone (SVZ). Migration from the SVZ is carried out along a well-defined pathway called the rostral migratory stream (RMS). Our recent finding of the specific expression of the cytoskeleton linker protein radixin in neuroblasts suggests a functional role for radixin in RMS migration. The ezrin-radixin-moesin (ERM) family of proteins is capable of regulating migration through interaction with the actin cytoskeleton and transmembrane proteins. The ERM proteins are differentially expressed in the RMS with radixin and moesin localized to neuroblasts, and ezrin expression confined to astrocytes of the glial tubes. Here, we inhibited radixin function using the quinacrine analog DXS2-1 which resulted in reduced neuroblast migration in vitro, while glial migration remained unaltered. Furthermore, the morphology of neuroblasts was distorted resulting in a rounded shape with no or short polysialylated neural cell adhesion molecule positive processes. Intracerebroventricular infusion of the radixin inhibitor resulted in accumulation of neuroblasts in the anterior SVZ. Neuroblast chains were short and intermittently interrupted in the SVZ and considerably disorganized in the RMS. Moreover, we studied the proliferation activity in the RMS after radixin inhibition, since concentrated radixin expression has been demonstrated in the cleavage furrow of dividing cells, which indicates a role of radixin in cell division. Radixin inhibition decreased neuroblast proliferation, whereas the proliferation of other cells in the RMS was not affected. Our results demonstrate a significant role for radixin in neuroblast proliferation and migration.

Keywords: ezrin/radixin/moesin, subventricular zone, rostral migratory stream, neuronal migration, proliferation

Neuronal progenitors capable of long distance migration are produced throughout life in the subventricular zone (SVZ). Migration from the SVZ is carried out along a well-defined pathway called the rostral migratory stream (RMS). Our recent finding of the specific expression of the cytoskeleton linker protein radixin in neuroblasts suggests a functional role for radixin in RMS migration. The ezrin-radixin-moesin (ERM) family of proteins is capable of regulating migration through interaction with the actin cytoskeleton and transmembrane proteins. The ERM proteins are differentially expressed in the RMS with radixin and moesin localized to neuroblasts, and ezrin expression confined to astrocytes of the glial tubes. Here, we inhibited radixin function using the quinacrine analog DXS2-1 which resulted in reduced neuroblast migration in vitro, while glial migration remained unaltered. Furthermore, the morphology of neuroblasts was distorted resulting in a rounded shape with no or short polysialylated neural cell adhesion molecule positive processes. Intracerebroventricular infusion of the radixin inhibitor resulted in accumulation of neuroblasts in the anterior SVZ. Neuroblast chains were short and intermittently interrupted in the SVZ and considerably disorganized in the RMS. Moreover, we studied the proliferation activity in the RMS after radixin inhibition, since concentrated radixin expression has been demonstrated in the cleavage furrow of dividing cells, which indicates a role of radixin in cell division. Radixin inhibition decreased neuroblast proliferation, whereas the proliferation of other cells in the RMS was not affected. Our results demonstrate a significant role for radixin in neuroblast proliferation and migration.
morphological rearrangement, cell migration and signaling, have been demonstrated (Paglini et al., 1998; Castelo and Jay, 1999; Leecher et al., 2006; Haas et al., 2007; Parisiadou et al., 2009). In the adult brain, however, ERM proteins have been less studied. Although highly homologous, the ERM proteins seem to localize to different cell types in the adult brain, with ezrin expression in glial cells and radixin expression in neuronal cells (Paglini et al., 1998; Grönholm et al., 2005; Cleary et al., 2006; Persson et al., 2010). We recently described the specific expression of radixin in PSA-NCAM+ neuroblasts in the adult SVZ and RMS (Persson et al., 2010). Here we investigate the function of radixin in neuroblasts using the radixin inhibitor DX52-1, an aquinocarmycin analog. The inhibitor was recently shown to primarily target radixin and disrupt the ability for radixin to bind actin as well as transmembrane proteins, such as CD44 (Kabasi et al., 2006). In this study, we explore the effects of DX52-1 and radixin inhibition on neuroblasts in the adult SVZ and RMS using in vitro and in vivo approaches to analyze migration, proliferation, cell death, and proteomic changes.

MATERIALS AND METHODS

CHEMICALS

The quinocarmycin analog DX52-1 (generous gift from Prof. Gabriel Fenteany) was used to block radixin function as described previously (Lindberg et al., 2012). Briefly, osmotic pump preparations, animals were anesthetized using isofluorane (Bayer Healthcare AG, Tarrytown, NY, USA) anesthesia, and all efforts were made to minimize suffering. The animals were divided into two groups receiving either vehicle (0.05% DMSO in PBS) or the quinocarmycin analog DX52-1 (1.3 μg/day), for 4 days. The surgeries were performed under ketamine (33 mg/mL Ketalar, Pfizer, New York, NY, USA) and xylazine (6.67 mg/mL Rompun, Bayer Healthcare AG, Tarrytown, NY, USA) anesthesia, and all efforts were made to minimize suffering. The animals were divided into two groups receiving either vehicle (0.05% DMSO in PBS) or DX52-1 (1.3 μg/day), for 4 days. The surgeries were performed as previously described (Lindberg et al., 2012). Briefly, osmotic minipumps [Model 1002; Alzet-Durect, Cupertino, CA, USA] and infusion cannulas [Brain Infusion Kit 2; Alzet-Durect, Cupertino, CA, USA] were filled with vehicle or DX52-1. Cannulas were inserted intracerebroventricularly using a stereotoxic instrument [David Kopf, Tujunga, CA and Stoelting Co, Wood Dale, IL, USA, stereotoxic ap] and subsequently incubated for 24 h in 0.1 M phosphate buffer (pH 7.4). Brains were removed, postfixed for 24 h in 4% PFA (Thermo Fisher Scientific, Waltham, MA, USA) and thereafter kept in 30% sucrose at 4°C until further processed.

IMMUNOFUORESCENCE

The ipsilateral side of infused brains was cut in a sagittal plane and the contralateral side was cut coronally. Sagittal sections were cut at 25 μm and coronal sections were cut at 40 μm on a sliding microtome (Leica Microsystems, Wetzlar, Germany) followed by immunofluorescence. Immunostainings including radixin were preceded by antigen retrieval in sodium citrate, pH 6.0, for 20 min at 97°C followed by 15 min cooling at room temperature. Sections were blocked for 30 min in 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in 0.1% Tween 20, and then incubated for 48 h at 4°C in primary antibodies; monoclonal rabbit anti-radixin (Abcam, Cambridge, MA, USA), mouse anti-radixin (Abnova, Taipei City, Taiwan), rabbit anti-phosphorylated ezrin/radixin/moesin (Cell signaling, Danvers, MA, USA), rabbit anti-phosphorylated histone H3 (Upstate, Billerica, MA, USA), mouse IgM anti-PSA-NCAM (Chemicon International/Millipore, Billerica, MA, USA). ToPro-3 (Molecular Probes/Invitrogen, Carlsbad, CA, USA) was used as a nuclear counterstain. After rinsing in tris-buffered saline (TBS), sections were incubated for 2 h with Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and CF secondary antibodies (Biotium, Hayward, CA, USA). The sections were mounted on glass slides and coverslipped with ProLong Gold DAPI (Molecular Probes).

To study apoptotic cell death in the SVZ and RMS after DX52-1 infusion, the ApoTag Fluorescein Direct in situ Apoptosis Detection kit (Millipore) was used. Fixed free floating sections were mounted onto glass slides and pretreated with ethanol:acetic acid (2:1) for 5 min at ~20°C followed by a PBS washing step. After 1 h of incubation in terminal deoxynucleotidyl transferase at 37°C, the reaction was stopped by washing and the sections were incubated for 30 min with a Fluorescein-conjugated anti-digoxigenin antibody at room temperature and subsequently washed in PBS. The slides were coverslipped with ProLong Gold DAPI (Molecular Probes).

SVZ WHOLE MOUNT PREPARATION

After 4 days of vehicle or DX52-1 intracerebroventricular infusion, brains (n = 3) were removed and placed in 37°C warm Hank’s Balanced Salt Solution (HBSS, Invitrogen). The whole ventricular wall of the contralateral hemisphere, including the underlying parenchyma, was carefully dissected out and fixed in cold 4% PFA/0.1% Triton X-100 in PBS for 24 h before washing and blocking unspecific binding in 10% Donkey serum/2% Triton X-100 in PBS for 1 h (Mirzadeh et al., 2010). The whole-mount was incubated for 48 h with primary antibodies; goat anti-DCX (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-PhosH3 (Millipore), washed thoroughly in 0.1% Triton X-100 in PBS, and subsequently incubated for 24 h in Alexa Fluor secondary antibody at room temperature and subsequently washed in PBS.
antibodies (Molecular Probes). After completing the staining, a
crystal violet solution was used to counterstain the nuclei.

SVZ EXPLANTS
For explant cultures brains were rapidly removed and kept
in Hanks’s balanced salt solution (GBSS) on ice. One milli-
liter coronal brain slices were cut between anterior-posterior
coordinates Bregma −0.5 to 2.5 using a coronal brain matrix. The
slices were kept on ice while the lateral ventricle walls were dis-
sected and cut into approximately 100 μm diameter pieces. The
tissue pieces were resuspended in Neurobasal A medium (Invit-ogen), mixed 3:1 with Matrigel (BD Biosciences, San Jose, CA,
USA) and dispensed in 8-well chamber slides (BD Bioscience), fol-
lowed by 10 min polymerization at 37°C. Explants were cultured in
Neurobasal A medium, supplemented with B27 and Glutamax,
PenStrep (all Invitrogen) and a concentration series of the radixin
inhibitor DXS2-1 (Vehicle 0.0025% DMSO, 50, 100 or 250 nM) at
37°C in 5% CO2 and 1% O2 for 72 h. At the end of the experi-
ment the explants were fixed in 4% PFA for 20 min. After three
15 min washes in TBS, the explants were blocked for 3 h at room
temperature using 3% donkey serum and 0.2% Triton-X in TBS.
Explants were then incubated with primary antibodies for 48 h;
goat anti Sox2 (Santa Cruz Biotechnology), rabbit anti-GFAP (DakoCyto
tamination, Glostrup, Denmark), mouse IgM anti-PSA-
NCAM (Chemicon International) and with secondary antibodies
as described above.
Apoptosis and cell death was analyzed using Vybrants Apopto-
sis Assay kit 2 (Molecular Probes) on explants after 96 h in culture.
Explants were washed in cold PBS for 10 min and subsequently in
Annexin buffer for 15 min followed by incubation with Annexin
V conjugated with Alexa 488 and propidium iodide (PI) in room
Annexin buffer for 15 min followed by incubation with Annexin
alized with the nuclear stain T oPro-3 and
in migratory chains emerging from the explants. Cells were visu-
ized on a L TQ-Orbitrap-V elos (Thermo Fisher Scientific) before one wash in Annexin buffer
and subsequently coverslipped with ProLong Gold DAPI (Molecular Probes).

CONFOCAL MICROSCOPY AND QUANTIFICATIONS
Immunofluorescence labeling was imaged using confocal laser
scanning microscopy (Leica TCS SP2, Leica Microsystems, Wetzlar,
Germany), and at the Centre for Cellular Imaging, Zeiss LSM 710
and Zeiss LSM710, Carl Zeiss Microscopy GmbH, Jena, Germany). For
SVZ explant cultures, migration distance of migratory chains
was measured for PSA-NCAM+ cells. The three longest migratory
chains per explant were used to estimate the maximum migration
distance under DXS2-1 treatment (1–4 explants). Furthermore,
the percentage of PSA-NCAM+ and Sox2high cells leaving the
explants were quantified by counting the ratio of PSA-NCAM+ and Sox2high cells (total cells counted per condition: 549 ± 66)
in migratory chains emerging from the explants. Cells were visu-
alyzed with the nuclear stain ToPro-3 and n = 4 for all explant
quantifications.
For in vivo quantifications coronal sections from the contralat-
eral hemisphere were used after vehicle or DXS2-1 infusion. For
quantification of cell proliferation in the RMS, the total number of
PHH3+ cells, and PSA-NCAM+/PHH3+ double labelled cells,
in the RMS was acquired from 7 to 9 sections at a 1:12 inter-
val covering the RMS. Anterior RMS refers to antero-posterior
coordinates from 13.20 to 11.52 mm from interaural line and
posterior RMS refers to antero-posterior coordinates from 11.52
to 10.44 mm from interaural line. Double labeling was assumed
when cells exhibited direct co-localization or when nucleus and
cytoplasm or processes from the same cell were individually labeled.
Area/volume measurements and the number of ApopTag stained
cells were assessed using stereology software (Stereo Investigator;
MicroBrightField Inc., Williston, VT, USA).

PROTEOMIC ANALYSIS
The proteomic analysis was performed by the Proteomics core
group (Thermo Fischer Scientific), where the reporter mass is used for semi quantitative identification of pro-
teins with tandem mass spectrometry. The ipsilateral SVZ was
microdissected from rats (n = 3) after intracerebroventricular
infusion of DXS2-1 or vehicle. Tissue samples were lysed in a
buffer containing: 50 mM TEAB, 8 M Urea, 4% Chaps, 0.2%
SDS, 5 mM EDTA, pH 8.5. Total protein concentration was
determined using Pierce 660 nm Protein Assay (Thermo Fisher
Scientific). 100 μg protein per sample were incubated with TCEP
(tris(2-carboxyethyl)phosphine), alkylated with MMTS (methyl
methanethiosulfonate) and digested with trypsin, after a four-fold
dilution, in 0.5 M TEAB ratio 1:25 over night in 37°C.
TMT 6-plex reagents (126–131) were dissolved in ACN and
added to the respective sample according to manufacturer’s pro-
tocol (Thermo Fisher Scientific). After labeling and quenching
of the reagents, the samples were combined and concentrated.
TMT-labeled peptides were separated with strong cation exchange
chromatography (SCX). The 18 peptide containing fractions were
desalted on PepClean C18 spin columns according to manufac-
turer’s instructions (Thermo Fisher Scientific). The desalted and
dried fractions were reconstituted into 0.1% formic acid and ana-
yzed on a LQ-Orbitrap-V elos (Thermo Fisher Scientific) inter-
faced with an in-house constructed nano-LC column. Two-micro
liter sample injections were made with an Easy-nLCautosampler
(Thermo Fisher Scientific), running at 200 nL/min. The peptides
were trapped on a precolumn (45 × 0.075 mm i.d.) and sepa-
rated on a reversed phase column, 200 × 0.075 mm, packed
in-house with 3 μm Reprosil-Pur C18-AQ particles. The gradi-
ent was as followed; 0-60 min 5-25% acetonitrile (ACN), 0.1% formic
acid, 60–75 min 25-40% ACN, 0.1% formic acid and the
last 15 min at 90% ACN, 0.1% formic acid. LTQ-Orbitrap-V elos
settings were: spray voltage 1.4 kV, 1 microscan for MS1 scans at
60,000 resolutions (m/z 400), full mass spectrometry (MS) mass
range m/z 400–1,800. The LTQ-Orbitrap-V elos was operated in a
data-dependent mode with one MS1 FTMS scan precursor ions
followed by HCD (high energy collision dissociation), MS2 scans of
the 10 most abundant protonated ions in each FTMS scan. The
mass spectrometry raw data files from all SCX fractions for the
TMT set were merged for relative quantification and identification


using Proteome Discoverer version 1.3 (Thermo Fisher Scientific). Database search was performed by Mascot search engine using the following criteria: Swissprot rat protein database; MS peptide tolerance as 10 ppm, MS/MS tolerance as 0.1 Da, trypsin digestion allowing one missed cleavages with variable modifications; methionine oxidation, cysteine methylthiol, and fixed modifications; N-terminal TMT-label, lysine TMT-label. The detected protein threshold in the software was set to 99% confidence and identified proteins were grouped by sharing the same sequences to minimize redundancy.

For TMT quantification, the ratios of TMT reporter ion intensities in MS/MS spectra (m/z 126.12, 127.13, 128.13, 129.14, 130.14, 131.14) from raw data sets were used to calculate fold changes between samples. The average of all three reporters for the control group were used as the denominator. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. The resulting ratios were then exported into Excel for data interpretation. The total group of 32 significantly changed proteins were analyzed using the software Ingenuity Pathway Analysis (IPA, Ingenuity systems, Redwood City, CA, USA).

STATISTICS

Statistical differences of in vivo quantifications were analyzed using the 2-tailed Student's t-test. For in vitro experiments, one-way ANOVA and Bonferroni post hoc test were employed. All error bars represent standard error of the mean (s.e.m.). All statistical calculations and graphical visualizations, except for the proteomics analysis, were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). For the proteomic analysis Welch t-test was used. Differences of p < 0.05 were considered statistically significant (*).
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**FIGURE 1** | Dose-dependent decrease in migration distance of neuronal progenitor cells from SVZ explants under radixin inhibition. Neural and glial progenitor cells are visualized by PSA-NCAM and Sox2 immunofluorescence. (A) Color separation for PSA-NCAM (top row) and Sox2 (middle row) expressing cells under increasing concentrations of the radixin inhibitor DX52-1 (Vehicle, 10, 50, 100, and 250 nM). (B) Bottom row depicts color merge of PSA-NCAM (green), Sox2 (red) immunoreactivity with Topro-3 as a nuclear stain (blue). (C) Migration distance (μm) of PSA-NCAM+ cells under DX52-1 treatment. (D) Migration distance (μm) of Sox2high glial cells under DX52-1 treatment. The fraction of cells expressing PSA-NCAM+ (black line) and Sox2high (red line) in the population that migrated from the explants (*p < 0.05, **p < 0.01, ***p < 0.001). Scale bar = 100 μm.

**FIGURE 2** | No increase in cell death of progenitor cells in vitro or in vivo under radixin inhibition. Paired results from four different experiments showed no significant difference in the percentage of cells that migrated out from SVZ explant cultures being AnnexinV positive (A) or PI positive (B) under different concentrations of the radixin inhibitor DX52-1 (Vehicle, 50, 100, and 250 nM). (C) The number of TUNEL positive cells was quantified per volume in the RMS after 4 days of intracerebroventricular infusion of vehicle or DX52-1 (n = 5).
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FIGURE 3 | Cell populations migrating in SVZ explant cultures. (A) Percentage of cells positive for the markers PSA-NCAM, Sox2 and GFAP in the SVZ explant experiment. All analyzed cells migrating out from the explants expressed Sox2, in low or high levels. Similar proportions of neuronal (PSA-NCAM+) and glial (GFAP+) cells migrated in the SVZ explants cultures and the majority of the GFAP+ cells expressed Sox2high. (B) Examples of Sox2low (arrows) and Sox2high (arrowheads) cells. Scale bar in (B) = 20 μm.

To assess induction of apoptosis due to the DX52-1 treatment in vivo we analyzed the number of TUNEL positive cells in the RMS; however, no significant difference was detected (Figure 2C).

ALTERED PROTEIN EXPRESSION AFTER RADIXIN INHIBITION

To study protein expression changes after treatment with DX52-1, the ipsilateral SVZ was dissected after 4 days of intracerebroventricular infusion of DX52-1 or vehicle (each n = 3). Using isobaric labeling and LC-MS/MS, 32 proteins were identified with significantly changed expression levels after treatment (Table 1). Functional analysis of all significantly changed proteins was performed using IPA (Ingenuity Systems, Redwood City, CA, USA) identifying two associated functional networks: (1) cell morphology, cellular development, small molecule biochemistry (enrichment score = 48); (2) cell-to-cell signaling and interaction, cellular development, developmental disorder (enrichment score = 30). The protein list was enriched for proteins involved in a number of basic molecular and cellular functions including cellular assembly and organization, which correlates well with the predicted functions of radixin (Figure 7).

A number of proteins involved in cell-to-cell signaling and interaction changed expression levels, such as the metabotropic glutamate receptor 5 (mGlur5), arrestinβ1 and adenylatecyclase 5. The cytoskeleton components dynactin and alpha tubulin were enriched after DX52-1 treatment. Furthermore, proteins involved in molecular and vesicular transport, such as mitochondrial import inner membrane translocase, Schl617, syntaxin-12, synapsin-2, presynosomal membrane protein PEX14 and flotillin-1 were altered. The suppressor of G2 allele SKP1 homolog, a protein regulating the transition from G2 to M-phase, was reduced, corroborating the in vivo results of decreased proliferation after radixin inhibition.

Altered proteins involved in protein metabolism include cytoplasmic tryptophanyl-tRNA synthetase, V-type proton ATPase, cytoplasmic aspartate aminotransferase, mitochondrial ATP synthase-coupling factor 6, and heterogeneous nuclear riboprotein D0.

DISCUSSION

The data presented in the current study suggest a role for radixin in neuronal progenitor migration and proliferation. We have
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FIGURE 5 | Decreased phosphorylation of radixin in the RMS after DX52-1 treatment. (A) Merged image of radixin and phosphorylated ezrin/radixin/moesin (pERM) immunoreactivity in a coronal section of the control RMS. (B) pERM immunoreactivity in the control RMS. (C) Merged image of radixin and phosphorylated ezrin/radixin/moesin (pERM) immunoreactivity in a coronal section in the DX52-1 treated RMS. (D) pERM immunoreactivity in the DX52-1 treated RMS. Radixin/pERM immunopositive circular structures in B and D are likely residual ependymal cells originating from the wall of the collapsed olfactory ventricle (Peretto et al., 1997). Ependymal cells are known to express high levels of ezrin which explains sustained pERM expression in this area in both control and DX52-1 treated RMS. Scale bar = 50 μm.

previously shown that radixin is specifically expressed in migrating neuroblasts in the RMS of the adult rodent brain (Persson et al., 2010). The current study confirms the expression of radixin in PSA-NCAM + migratory cells, both in vivo and in vitro. Blocking radixin with the quinocarmycin analog DX52-1 in vitro resulted in a dose-dependent downregulation of neuroblast migration from SVZ explants. Under control conditions similar numbers of PSA-NCAM + neuroblasts and Sox2 high expressing glial cells migrate from the explants. The motility of glial cells was not affected by the inhibitor, supporting previous results describing the expression of ezrin, but not radixin, in glial cells (Cleary et al., 2006; Persson et al., 2010).

DX52-1 specifically binds radixin at low concentration and inhibits its binding to F-actin and the transmembrane protein CD44 (Kahsai et al., 2006). At concentrations above those used in our study, DX52-1 are reported to interact with additional proteins, such as the other ERM proteins, ezrin and moesin, and galectin-3 (Kahsai et al., 2006). Treatment with a low dose of DX52-1 in Madin-Darby canine kidney (MDCK) epithelial cell cultures revealed a decreased ability for wound closure after radixin inhibition (Kahsai et al., 2006), suggesting a role for radixin in migration and/or proliferation of epithelial cells. The selective inhibition of neuroblasts in our study confirms the specificity of DX52-1 for radixin, since Sox2 high glial cells remained migratory in the migration assay. In addition, continued migration of glial cells indicates that the inhibitor is not generally toxic to cells. Furthermore, we can exclude DX52-1 toxicity in the neurogenic niche since the rate of apoptosis or cell death was not increased, neither in vitro nor in vivo.

Intracerebroventricular infusion of DX52-1 resulted in distortion of neuroblast chain formation in the SVZ and the

FIGURE 6 | Decreased neuroblast proliferation and accumulation of neuroblasts in the posterior RMS after radixin inhibition. (A, B) PSA-NCAM immunoreactivity in the anterior RMS after 4 days of DX52-1 intracerebroventricular infusion of vehicle (A) or DX52-1 (B). (C, D) PSA-NCAM immunoreactivity in the posterior RMS after 4 days of DX52-1 intracerebroventricular infusion of vehicle (C) or DX52-1 (D). There was no difference in the volume of the anterior RMS of vehicle and DX52-1 treated animals. (I) The volume of the posterior RMS was increased after infusion of DX52-1. (G, H) The number of PHH3 and PSA-NCAM double positive cells in the anterior (G) and posterior (H) RMS was decreased after 4 days of DX52-1 intracerebroventricular infusion. However, there was no difference in the number of PHH3 positive/PSA-NCAM negative cells in the anterior (I) or posterior RMS (K) (*p < 0.05, **p < 0.01). Scale bar in (A) = 50 μm, scale bar in (E) = 10 μm.
Table 1 | Proteins with altered expression level after radixin inhibition in the SVZ as detected by isobaric labeling and mass spectrometry.

| Accession no. | Description                                                                 | Foldchange | p-value | CV (%) |
|---------------|------------------------------------------------------------------------------|------------|---------|--------|
| Q9WV97        | Mitochondrial import inner membrane translocase subunit Tim9                  | 1.21       | 0.05    | 8.13   |
| P61327        | Ras-related protein Rap-2b                                                   | 1.20       | 0.05    | 5.73   |
| Q6A9H5        | Dynactin subunit 2                                                           | 1.19       | 0.02    | 5.45   |
| P31682        | Orphan sodium- and chloride-dependent neurotransmitter transporter NTT4 (Slc6a17) | 1.13       | 0.05    | 4.58   |
| G3V7P1        | Syntaxin-12                                                                  | 1.13       | 0.03    | 1.65   |
| Q63537        | Synapsin-2                                                                  | 1.12       | 0.02    | 3.91   |
| Q8P9V9        | Tubulin alpha-1 chain                                                        | 1.12       | 0.02    | 3.60   |
| P9P7BO        | Tryptophanyl-tRNA synthetase, cytoplasmic                                    | 1.12       | 0.01    | 1.65   |
| P65408        | Vinylglycine-ATPase subunit F                                                 | 1.11       | 0.00    | 1.91   |
| Q3K11         | Rab11, Rab11-related GTPase A                                                | 1.11       | 0.03    | 2.31   |
| P13721        | Aspartate aminotransferase, cytoplasmic                                      | 1.10       | 0.02    | 3.11   |
| P21571        | ATP synthase-coupling factor 6, mitochondrial                                | 1.10       | 0.02    | 2.76   |
| P54757        | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)                             | 1.10       | 0.01    | 2.37   |
| Q5E824        | DnaJ homolog subfamily A member 2                                            | 1.09       | 0.05    | 3.08   |
| P54636        | Mutated dehydrogenase, mitochondrial                                         | 1.08       | 0.02    | 2.64   |
| Q0S3T1        | UMP-CMP kinase                                                               | 1.08       | 0.03    | 1.81   |
| Q8C8F2        | Cell division control protein 42 (Cdc42)                                     | 1.08       | 0.01    | 0.98   |
| Q63569        | 26S protease regulatory subunit 6A                                           | 1.04       | 0.02    | 0.90   |
| P27139        | Carbonyl reductase 2                                                         | 0.97       | 0.00    | 0.59   |
| Q62950        | Dihydroxyacetone synthase-related protein-1                                  | 0.95       | 0.04    | 1.85   |
| Q69F4D        | Activin-related protein 2/3 complex subunit 1A                               | 0.94       | 0.01    | 2.03   |
| Q56829        | Phosphoryl-CoA hydroxylase-interacting protein                               | 0.91       | 0.02    | 3.26   |
| O70196        | Prolyl endopeptidase                                                         | 0.90       | 0.01    | 2.25   |
| Q04400        | Adenylyl cyclase type 5                                                      | 0.89       | 0.02    | 3.34   |
| Q9E58S        | N-terminal EF-hand calcium-binding protein 1 (Necab1)                        | 0.87       | 0.03    | 3.62   |
| Q64204        | Peroxinsomal membrane protein PEX14                                           | 0.86       | 0.02    | 4.39   |
| Q8211E        | Fltillin-1/Reggic-2                                                          | 0.85       | 0.03    | 4.19   |
| B0B985        | Suppressor of G2 allele of SKP1 homolog                                      | 0.85       | 0.01    | 3.49   |
| P20666        | Beta-arrestin-1                                                              | 0.84       | 0.01    | 3.56   |
| P31424        | Metabolotropic glutamate receptor 5                                           | 0.82       | 0.01    | 5.03   |
| Q64537        | Carpan small subunit 1                                                       | 0.80       | 0.04    | 9.91   |
| Q64384        | Heterogeneous nuclear ribonucleoprotein D                                    | 0.80       | 0.04    | 9.27   |

Coefficient of variance, CV; n = 3, (p < 0.05).

RMS. The accumulation of neuroblasts in the posterior parts of the RMS suggests that fewer neuroblasts migrate through the RMS. This was corroborated by an increased volume in the posterior RMS. However, this accumulation was not sufficient to cause any significant decrease in the volume of the anterior RMS indicating that neuroblast migration in the SVZ and posterior RMS may be hampered although sufficient to proceed through the RMS. A longer infusion period than 4 days may be required to reveal an effect along the entire RMS. Phosphorylation of radixin enables its binding to the actin cytoskeleton under control conditions. After DX52-1 treatment in vivo, the level of phosphorylated radixin immunoreactivity was low in the RMS. Thus, the aberrant neuroblast migration could be a result of decreased phosphorylation of radixin.

Furthermore, radixin has been shown to concentrate in the cleavage furrow of dividing cells (Sato et al., 1991) and may have a role in proliferation. We demonstrate a selective decrease in neuroblast proliferation in the RMS after intracerebroventricular infusion of DX52-1. Proliferation of other cell types (PSA-NCAM negative) was not affected. These data are in accordance with a study that tested DX52-1 as a chemotherapeutic agent after a screening for molecules affecting growth of melanoma cells, which also express radixin (Plowman et al., 1995).

We have determined the effects on neuroblast migration and proliferation in vivo after DX52-1 infusion. However, the
compound may have additional effects which are not related to neuroblast migration and proliferation. We could discern a moderate morphological change in the microglia population and an increase in the immunoreactivity of GFAP in the SVZ and corpus callosum (data not shown). Microdissection of DX52-1 treated brains indicated affected areas outside the SVZ/RMS and we observed vasculature changes in the thalamus, occasionally ventricle enlargement and softening of white matter tissue (data not shown). This may be explained by altered functions in non-neuroblast radixin expressing cells.

Considering recent evidence for regulation of cell functions by radixin other than migration (Loebrich et al., 2006; Tang et al., 2007; Valderrama et al., 2012), we quantified protein changes in the SVZ using a proteomic approach to identify biological functions affected by radixin inhibition in the neurogenic niche. It is important to consider that the proteomics analysis was based on a material of mixed cell types, and includes both intra- and extracellular proteins. This approach enables detection of general protein changes, including both primary and secondary events to the treatment. Our results show that the majority of the altered proteins have a role in cellular morphology and cellular assembly and organization which match our in vitro and in vivo results of radixin inhibition. Of the altered proteins 31% are abundantly expressed in the RMS according to the Allen brain atlas (http://mouse.brain-map.org), for example Retinol 4/Nogo-A, Dynactin 2, and suppressor of G2 allele of SKP homolog. Cdc42 and Rac1 are members of the Rho family of small GTP-binding proteins, and radixin is known to interact with several Rho GTP-binding proteins and to regulate Rac1 activity (Takahashi et al., 1998; Hamada et al., 2001; Valderrama et al., 2012). Both Cdc42 and Rac1 are involved in neuronal embryonic migration but may have different roles (Konno et al., 2005). Cdc42 is for instance important for the guiding cues of the Slit-Robo pathway (Wong et al., 2001), suggesting a specific role in RMS migration. Calpain small subunit 1 is present in both calpain 1 and 2 and calpains regulate cell migration and adhesion (Huttenlocher et al., 1997; Dourdin et al., 2001). Recently, calpain 1 expression was shown to be high in neural stem cells and decreased during differentiation and calpain 1 inhibition increased neural stem cell differentiation (Santos et al., 2012). Contrary, calpain 2 was increased during neuronal stem cell differentiation (Santos et al., 2012). Furthermore, arrestins were initially acknowledged for their role in receptor internalization; however, recent evidence suggest a role for β-Arrestin-2 in promoting actin polymerization and migration of leukocytes (Zoudilova et al., 2010). The interaction of radixin with proteins identified in the proteomics analysis may be direct or indirect, or induced as a compensatory action to radixin inhibition. Future studies will have to address these issues. Our data suggest that radixin likely interacts with several different signaling or scaffolding proteins to mediate and/or regulate dynamic actin rearrangement.

FIGURE 7 | Schematic figure categorizing cellular functions affected in the SVZ under radixin inhibition according to IPA analysis of the altered proteins as detected by proteome analysis. Proteins allocated to each cellular function group are presented and the associated p-value for their enrichment in the group of altered proteins.

Cdc42
Retinol 4/Nogo-A
Dynactin 2
RAP 2b
Arrestin beta1
CRMP1
FLOT1
Calpain small subunit 1
Synapsin-2

Cdc42
Retinol 4/Nogo-A
Dynactin 2
RAP 2b
Arrestin beta1
CRMP1
FLOT1
Calpain small subunit 1
Synapsin-2

Cdc42
Retinol 4/Nogo-A
Dynactin 2
RAP 2b
Arrestin beta1
CRMP1
FLOT1
Calpain small subunit 1
Synapsin-2

Cdc42
Arrestin beta1
CRMP1
FLOT1
Calpain small subunit 1
Synapsin-2

Arp2/3
Cdc42
Retinol 4/Nogo-A
Dynactin 2
RAP 2b
Arrestin beta1
CRMP1
FLOT1
Calpain small subunit 1
Synapsin-2

p-value = 0.0002 - 0.0475
p-value = 0.0002 - 0.0421
p-value = 0.0004 - 0.0457
p-value = 0.0003 - 0.0460
p-value = 0.0003 - 0.0465
The effects of acute radixin inhibition in this study suggest that the functions of radixin in neuroblasts are not compensated by other actin binding proteins described in RMS neuroblasts, as suggested by others (Gronholm et al., 1999; Castelo et al., 2006). We conclude from this that radixin is a key component of the ERM family of proteins and mediates their function in neuroblast migration and cellular motility.

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