Int. J. Devl Neuroscience 41 (2015) 98–104

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Signaling via the transcriptionally regulated activin receptor 2B is a novel mediator of neuronal cell death during chicken ciliary ganglion development

S. Koszinowski[a,b,*], K. Buss[a], K. Kaehlcke[a], K. Krieglstein[a,**]

[a] Department of Molecular Embryology, Institute of Anatomy and Cell Biology, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany
[b] Faculty of Biology, Albert-Ludwigs-University Freiburg, Schaenzlestrasse 1, D-79104 Freiburg, Germany

ARTICLE INFO

Article history:
Received 10 January 2015
Received in revised form 30 January 2015
Accepted 31 January 2015
Available online 3 February 2015

Keywords:
TGF-β
Activin
BMP
Ontogenetic cell death
Apoptosis
Ciliary ganglion

ABSTRACT

The TGF-β ligand superfamily members activin A and BMP control important aspects of embryonic neuronal development and differentiation. Both are known to bind to activin receptor subtypes IIA (ActRIIA) and IIB, while in the avian ciliary ganglion (CG), so far only ActRIIA-expression has been described. We show that the expression of ACVR2B, coding for the ActRIIB, is tightly regulated during CG development and the knockdown of ACVR2B expression leads to a deregulation in the execution of neuronal apoptosis and therefore affects ontogenetic programmed cell death in vivo. While the differentiation of choroid neurons was impeded in the knockdown, pointing toward a reduction in activin A-mediated neural differentiation signaling, naturally occurring neuronal cell death in the CG was not prevented by follistatin treatment. Systemic injections of the BMP antagonist noggin, on the other hand, reduced the number of apoptotic neurons to a similar extent as ACVR2B knockdown. We therefore propose a novel pathway in the regulation of CG neuron ontogenetic programmed cell death, which could be mediated by BMP and signals via the ActRIIB.

1. Introduction

The transforming growth factor (TGF)-β superfamily, comprising TGF-βs, bone morphogenetic proteins (BMPs), activins and related proteins, is a key regulator of various developmental processes in the nervous system, including the proliferation of neural precursors, neuronal differentiation and ontogenetic programmed cell death (Böttner et al., 2000; Unsicker and Krieglstein, 2002). While TGF-βs are established regulators of neuronal apoptosis (Krieglstein et al., 2002), the role of activins in cell growth inhibition and apoptosis have mostly been shown in non-neural systems, such as the immune system and carcinogenesis (Shav-Tal and Zipori, 2002; Chen et al., 2006). In the nervous system, activins are rather known to promote neuronal survival and regeneration after trauma, as seen in models of cerebral ischemia and excitotoxic neurodegeneration (Mukerji et al., 2007; Abdipranoto-Cowley et al., 2009) as well as neuronal differentiation (Rodríguez-Martínez et al., 2012). Nevertheless, in the oligodendroglial progenitor cell line OLI-neu, activin A can mediate apoptosis, in a pathway distinct from TGF-β1-induced cell death (Schulz et al., 2008). BMPs are expressed in the nervous system throughout development and exert pivotal functions, starting from the induction of the neuroectoderm and the neural crest, giving rise to the peripheral nervous system, via the induction of dorsal cell types in the neural tube, to cell fate regulation in the postnatal and adult brain (Hegarty et al., 2013). Apart from neural crest induction, BMP signaling also promotes the onset of neural crest cell migration and can mediate apoptosis in the rhombencephalic neural crest (Graham et al., 1994; Sela-Donenfeld and Kalcheim, 1999).

All TGF-β family members bind to type II receptors that recruit the type I cell surface receptors with serine/threonine kinase activity and mediate their effects via the canonical Smad signaling pathway (Böttner et al., 2000). Activins bind to the activin receptors (ActR) type IIA or IIB and recruit ActRIA/B. BMPs are also known to bind ActRIIA/B, but other than activin signaling, which recruits receptor Smads 2 and 3, BMPs signal via Smads 1, 5 and 8 (Krieglstein et al., 2011). In the chicken ciliary ganglion (CG), a classical model for the study of ontogenetic neuronal programmed cell death (Dryer, 1994), ActRIIA mRNA has been detected in CG

* Corresponding author at: Department of Molecular Embryology, Institute of Anatomy and Cell Biology, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany. Tel.: +49 761 203 5384.
** Corresponding author. Tel.: +49 761 203 5087.
E-mail addresses: sophie.koszinowski@anat.uni-freiburg.de (S. Koszinowski), kerstin.krieglstein@anat.uni-freiburg.de (K. Krieglstein).

http://dx.doi.org/10.1016/j.ijdevneu.2015.01.006

neurons during development (Kos and Coulombe, 1997) and activin A, produced by the choroid tissue, has been shown to induce somatostatin/cortistatin expression in the choroid neuron population of the ganglion (Darland et al., 1995; Nishi et al., 2010). In a genome-wide transcriptomic screen, performed between embryonic day (E) 6 and E14 in the chicken CG, we have recently detected that the transcription of the ACVR2B gene, coding for ActRIIB, is dynamically regulated, with a significant up-regulation of ACVR2B expression at E7, an early stage of development prior to ontogenetic cell death (Landmesser and Pilar, 1974), and a fast decline thereafter. In mice and rats, neuronal cell death peaks around E10–E18, but still continues in the postnatal period (Lossi and Merighi, 2003). In the human fetus, apoptotic cell death that coincides with neuronal differentiation and synaptogenesis increases around midgestation and lasts until late gestation (Chan et al., 2002), while around birth, a stable number of cortical neurons is achieved (Rabinowicz et al., 1996). The incubation period of the chicken is 21 days and neuronal programmed cell death in the CG is completed within the course of seven days until E14, and therefore long before hatching (Dryer, 1994), making the CG an attractive model for the study of cell death.

We thus set out to elucidate the role of ACVR2B expression in CG neuronal development, investigating its function in neuronal differentiation and ontogenetic cell death.

2. Materials and methods

2.1. Embryos, fixation and histology

Fertilized white leghorn chicken eggs (Gallus gallus domesticus) were obtained from a local farm and incubated at 38.5 °C and 70% humidity. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). At the desired stage, embryos were killed by decapitation and the heads were fixed in 4% paraformaldehyde, dehydrated in ascending ethanol concentrations and embedded in paraffin. 10 μm microtome sections were collected for hematoxylin eosin (HE) staining, immunohistochemistry and in situ hybridization. In order to be able to compare equivalent cross-sections of the ganglion, sections were consecutively mounted in a series of ten slides, each comprising 4–5 single sections covering the entire ganglion. Neurons were counted on HE stainings as described before (Oppenheim et al., 1989).

2.2. Immunohistochemistry

Sections were deparaffinized and heated in citrate buffer for improved antigen retrieval and further incubated with anti-islet-1/2 (40.2D6) 1:50; anti-gag (AMV3C2, 1:200; both antibodies from Developmental Studies Hybridoma Bank, University of Iowa), anti-somatostatin (Millipore; 1:100); anti-BrdU (Sigma; 1:1000); anti-active caspase 3 (R&D Systems, Wiesbaden, Germany; 1:500) and visualized using biotinylated secondary antibodies (donkey-anti-mouse; -anti-rabbit; -anti-rat; 1:100; Dianova, Hamburg, Germany) and diaminobenzidine (Vectastain Peroxidase ABC-kit 6100, Vector Laboratories, CA, USA). Active caspase-3 and BrdU-positive neurons were counted, taking every cell into account showing the dark DAB precipitate. Every tenth cross-section of the entire ganglion was counted and the mean number of positive neurons/section was calculated.

2.3. In situ hybridization

Sections were deparaffinized and in situ hybridization and preparation of the digoxigenin-labeled probes for Cash1 and ACVR2B were performed as described previously (Ernsberger et al., 1997). The Cash1 probe was generously provided by K. Huber (Institute of Anatomy, Albert Ludwigs University Freiburg). Chicken ACVR2B was obtained as a chicken EST clone (ChEST296F4) from the EST library of the University of Manchester, UK (Boardman et al., 2002).

2.4. Microarray

Ciliary ganglia were dissected from E6 to 10 and E14 chicken embryos. For each analyzed time point, ganglia from at least 40 embryos were pooled for RNA extraction using the Qiagen RNeasy micro kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Four replicate sample of each time point were obtained. For transcriptome analysis, 4×44 K Agilent (Santa Clara, CA, USA) whole transcriptome arrays were used. The raw intensity reads for each array were background corrected by spatial detrending and subsequently quantile normalized for comparison across arrays using the limma package from Bioconductor (http://www.bioconductor.org). Probes with low interquartile range across arrays and probes with low intensities were filtered out. Subsequent analysis considered only genes having a corresponding EntrezID annotation. If multiple probes mapped to the same EntrezID, the probe having the largest interquartile range was chosen and all others discarded, leaving 13,533 transcripts for analysis.

2.5. Production of shRNA virus

RCAS(BP)B virus carrying an shRNA construct against ACVR2B was produced according to (Das et al., 2006). The primers used for the ACVR2B hairpin in the cloning oligos were shACVR2B forward: gagaggtgctgctgagcgggacgtcttgctgaagaacgacttagtgaagccacagatgta and sh ACVR2B reverse: attcaccaccactaggcaaacgtcttgctgaagaacgacttacatctgtggcttcact. DF-1 cells were transfected with the RCAS(BP)B sh ACVR2B plasmids and RCAS(BP)B plasmids without an shRNA insert using Lipofectamine 2000 (Invitrogen, Life Technologies, Darmstadt, Germany). Virus was concentrated from the culture medium using Amicon ultra-4 centrifugal filter units (Merck Millipore, Darmstadt, Germany) with a 100 kDa cutoff. Virus stock aliquots were stored at −20° until use.

2.6. Injection into chick embryos

For virus injection, eggs were incubated until the desired stage (HH9). Virus stock was mixed with a vital dye (Fast Green, Sigma) and backfilled into a glass capillary, prepared in a Flaming/Brown micropipette puller (Shutter Instruments Co., USA). The virus was injected into the neural tube of the embryo at the level of the mesencephalon, where the progenitors of the CG neurons will delaminate (Narayanan and Narayanan, 1978). The eggs were further incubated and the embryos were sacrificed by decapitation at E14. BrdU injections were performed according to (Striedter and Keefer, 2000), shortly, BrdU (Sigma) was injected at a concentration of 20 μg/μl into one of the embryoís vitelline veins. The embryos were further incubated for 3 h before fixation at E7 and E9. Recombinant noggin and follistatin (SRP4675, SRP3045, Sigma) were injected into a vitelline vein once every day between E6 and E8 at a concentration of 0.2 μg/μl. Embryos were fixed at E9 and processed for active caspase 3 staining.

2.7. Statistics

Data are expressed as means + SEM. Two-group analysis was performed using Student's *t*-test. *P* values for multiple comparisons were calculated using repeated measure one-way ANOVA with Dunnett's multiple comparisons post-hoc test. Values of $p < 0.05$ were considered as statistically significant. All statistical analyses