RESEARCH ARTICLE

Lateral line placodes of aquatic vertebrates are evolutionarily conserved in mammals

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ABSTRACT

Placodes are focal thickenings of the surface ectoderm which, together with neural crest, generate the peripheral nervous system of the vertebrate head. Here we examine how, in embryonic mice, apoptosis contributes to the remodelling of the primordial posterior placodal area (PPA) into physically separated otic and epibranchial placodes. Using pharmacological inhibition of apoptosis-associated caspases, we find evidence that apoptosis eliminates hitherto undiscovered rudiments of the lateral line sensory system which, in fish and aquatic amphibia, serves to detect movements, pressure changes or electric fields in the surrounding water. Our results refute the evolutionary theory, valid for more than a century that the whole lateral line system would be conserved in mammals. Instead, subsets of lateral line placodes have gone astray, to different degrees, in different gnathostome lineages (Northcutt, 1992, 1997; Schlosser, 2002a, 2006), and their order of ontogenetic appearance reveals interspecies variability (Northcutt and Brändle, 1995; Schlosser and Northcutt, 2000; Fig. 2C).

During the morphogenesis of epibranchial placodes, embryonic mice demonstrate functionally unexplained apoptosis in three main loci (Washausen and Knabe, 2013, 2017): (1) at the ventral margin of the otic pit [embryonic day (E) 9], (2) between the detachment site of the otic vesicle and the epibranchial placodes 1, 2, and/or 3 (E9.25–E9.5, peak period of apoptosis, Fig. 1A), and (3) in peripheral parts of the three mature epibranchial placodes (E9.5–E11.5). Surprisingly, these apoptotic events predominantly eliminate ectodermal cells that express the general placode marker Six1, a member of the Sine oculis homeobox (Six) family of transcription factors (Washausen and Knabe, 2013, 2017). We therefore aimed to determine the developmental potential of these physiologically eliminated placode precursor cells by pharmacologically inhibiting apoptosis in cultured mouse embryos. It turned out that, contrary to previous assumptions, amniotes have retained the capability to produce morphologically and molecularly typical lateral line placodes as well as the primordia of neuromasts. Available evidence suggests that apoptosis may eliminate vestigial lateral line placodes also in other amniotes [Washausen et al. (2005); our unpublished observations in chick embryos]. Our findings further support the hypothesis that lateral line placodes may constitute the default fate of the PPA.

RESULTS

Inhibition of apoptosis reveals rudiments of lateral line placodes in mice

In order to learn how apoptosis contributes to placode morphogenesis in the PPA, mouse embryos were removed from the uterus immediately prior to the peak period of apoptosis, and were exposed for 12–36 h in whole embryo culture to the pan-caspase inhibitor Q-VD-OPh (n=164). Compared with control embryos (see the Materials and Methods), treatments with 50 µM Q-VD-OPh and, more pronounced, with 100 µM Q-VD-OPh reveal a significant reduction of apoptotic cells in the PPA (Fig. 1). In their places, all embryos treated with 50 or 100 µM Q-VD-OPh (n=156)
Fig. 1. Pharmacological inhibition of apoptosis in the posterior placodal area (PPA) of embryonic mice. (A) Summary scheme of in utero-developed control embryos including ectoderm (light grey), otic vesicle with detachment site (dark grey), epibranchial placodes (orange), and apoptosis (purple) demonstrates the peak of PPA apoptosis (compiled from Washausen and Knabe, 2013, 2017; n=44 body sides). (B) Levels of apoptosis in the PPA (black contour in the schematized embryo; section interval evaluated=10 µm) of in utero-developed embryos (n=20 body sides) or specimens developed for 24 h in whole embryo culture (wec). Embryos were cultured either in the presence of only the solvent DMSO (control; n=20), or in the presence of 10–100 µM of the pan-caspase inhibitor Q-VD-OPh (n=8 for 10, 20, or 50 µM, respectively; n=20 for 100 µM), or in the presence of 200 µM of the more narrow spectrum caspase inhibitor Z-VAD-fmk (n=12). It turned out that Q-VD-OPh treatment reduces PPA apoptosis in a dose-dependent manner. Furthermore, inhibition with 50 µM Q-VD-OPh or 200 µM Z-VAD-fmk is significantly less efficient compared with treatments using 100 µM Q-VD-OPh. Significant differences were measured with unpaired Mann–Whitney test (*P<0.001). Box plots indicate medians (centre lines), 25th and 75th percentiles (box limits), lower and upper extremes (whiskers), data points evaluated separately for each body side (purple dots), and outliers (open circles). (C) Micrographs (standardized sectioning plane) taken from anti-cleaved caspase-3 (Casp3) stained serial sections of mouse embryos treated with 10, 20, 50 or 100 µM Q-VD-OPh or 200 µM Z-VAD-fmk. It turned out that the more effective reduction of PPA apoptosis (arrowheads) is, the better rudiments of the lateral line placodes are preserved. Scale bars: 50 µm (overviews) and 10 µm (magnified insets). E, embryonic day; e1, e2, e3, epibranchial placodes 1, 2, 3, respectively; ot, otic anlage; ov, optic vesicle; p1, p2, pharyngeal pouch 1, 2, respectively.
without exception generated morphologically typical lateral line placodes. To demonstrate that formation of these supernumerary placodes depends on the inhibition of caspase-dependent cell death, we performed several additional experiments. First, dose-dependency was demonstrated by treating mouse embryos with 10 or 20 µM Q-VD-OPh. In these cases, significant reductions of apoptotic cells in the PPA and typical lateral line placodes were absent (Fig. 1B,C). Second, other embryos were treated with the more narrow spectrum caspase inhibitor Z-VAD-fmk [less potent caspase-2 and -6 inhibition; Chauvier et al. (2007)]. As expected, application of its common maximum dose [200 µM, whole embryo culture; Massa et al. (2009)] significantly reduces the level of PPA
apoptosis and generates supernumerary placodes. However, similar to application of 50 µM Q-VD-OPh, caspase inhibition by 200 µM Z-VAD-fmk is less efficient than inhibition with 100 µM Q-VD-OPh (Fig. 1B,C). Thus, in all further assays, embryos were exposed to 100 µM Q-VD-OPh and, for the purpose of simplification, are further referred to as ‘Q-VD-OPh-treated’ embryos. In Q-VD-OPh-treated embryos, supernumerary lateral line placodes either reside between the detachment site of the otic vesicle and epibranchial placode 1 (anterodorsal lateral line placode) or, respectively, epibranchial placode 2 (middle lateral line placode), and/or immediately posterior to epibranchial placode 2 (posterior lateral line placode) (Fig. 2A,B; Figs S1–S9). Among 100 reconstructed embryos (18, 24, or 36 h in culture), bilateral presence was found in 48% (anterodorsal placode), 100% (middle placode), or 66% (posterior placode) of all cases. At least unilateral development was observed in 87% (anterodorsal placode) or 89% (posterior placode) of all studied embryos (for subgroup analysis, see Fig. 2D).

### Structural properties of lateral line placodes and neuromasts in Q-VD-OPh-treated embryos

Lateral line placodes of Q-VD-OPh-treated mice consist of cubic to columnar, single-row to pseudostratified epithelium (Fig. 3A). In addition, they contain various maturation stages of neuromast primordia (Northcutt, 1992; Northcutt et al., 1994; Sato, 1976; Schlosser, 2002b; Stone, 1933; Winklbauer, 1989). Low to moderately differentiated neuromast primordia were identified by their rosette-like arrangement of typically no more than 5–10 epithelial cells per section. Additionally, these forms may reveal pit-like structures.
depressions and/or mantle cells with crescent-shaped nuclei that ensheath centrally located, more spherical cells (Figs 3E, 4D, 5B,D,F; Fig. S10A,D). More complex forms (approximately 10–20 epithelial cells per section) also demonstrate peripherally located mantle cells but, in their centres, allow discrimination between support cells and spherical hair cell precursors placed on top of the support cells (Fig. 3B–D, also for a comparison with axolotl embryos). Furthermore, a few hair cell kinocilia were found (Fig. 3C;)

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Fig. 4. See next page for legend.
Fig. 4. Lateral line placodes of Q-VD-OPh-treated mice reveal the specific molecular properties of posterior placodes.

(A–C,G) Micrographs taken from serially sectioned mouse embryos, with their positions being shown in the preceding reconstructions that demonstrate ectoderm (light grey), otic pit (A,C) or otic vesicle (B) with detachment site (dark grey), epibranchial placodes (orange), lateral line placodes (blue), plane of sectioning (black line in reconstructions). (A) Pax8 immunopositivity is present in epibranchial placode 1, in the prospective placodes (blue), plane of sectioning (black line in reconstructions). (A) Pax8 demonstrate ectoderm (light grey), otic pit (A,C) or otic vesicle (B) with their positions being shown in the preceding reconstructions that

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Figs S4F, S5F, S6L). Among 100 reconstructed embryos, left- and right-sided PPPAs revealed a total of 211 or 231 neuromast primordia, respectively. Fifty-three (12%) of these 442 cases represented complex primordia reveal an apical cavity covered by flat superficial cells that

Secondly, anamniote secondary neuromasts develop from the mantle zones of primary neuromasts (Northcutt, 1997; Schlosser, 2002). Correspondingly, pairs or triplets of adjacent neuromast primordia were observed in Q-VD-OPh-treated mice (Fig. S10A). Thirdly, in the lateral line system of zebrafish, cell proliferation peaks (1) during gastrulation, (2) within individualized placodes or migrating primordia, and (3) among support cells of the sensory organs (Laguerre et al., 2005). Accordingly, in Q-VD-OPh-treated mice, phospho-Histone H3 immunopositive (pH3+) cells are present among neuromast support cells as well as in non-neuromast bearing parts of the lateral line placodes (Fig. S10B).

Molecular signature of lateral line placodes and neuromasts in Q-VD-OPh-treated embryos

Lateral line placodes of Q-VD-OPh-treated mice also exhibit the molecular signature of PPA derivatives (Schlosser, 2006, 2010). Firstly, they express the general placode marker Six1 (Fig. 3B; Fig. S8I–K). This retrospectively clarifies why apoptosis, seemingly located ‘interplacodally’ between the otic and epibranchial placodes of in utero-developed mice (Washausen and Knabe, 2013, 2017) (Fig. 1A), predominantly removed Six1 immunopositive (Six1+) cells (Washausen and Knabe, 2017). Secondly, they spring from paired homeobox (Pax) 2 and/or Pax8 expressing parts of the PPA but, under experimental conditions, lose protein expression of these specific posterior placode markers shortly thereafter (Fig. 4A,B). Thirdly, they develop in tandem with the otic placode, as is apparent from their common expression of Sox10 (Schlosser, 2010), a member of the group E Sox (sex determining region Y-box) family of transcription factors. Prior to the peak period of PPA apoptosis, this common expression domain was observed in both control and Q-VD-OPh-treated embryos. Furthermore, Sox10+ epithelial rosettes indicate the onset of neuromast formation (Fig. 4C,D). During the peak period of PPA apoptosis, control embryos reveal disorganized Sox10+ epithelia, decreased Sox10 immunoreactivity, and massive apoptosis in the positions of ‘dormant’ lateral line placodes (Fig. 4E,F). In contrast, lateral line placodes of Q-VD-OPh-treated mouse embryos escape apoptosis and demonstrate mosaics of viable Sox10+/Sox10− cells as well as intact Sox10− neuromast primordia (Fig. S10C,D). Fourthly, some but not all lateral line placodes and/or neuromasts of Q-VD-OPh-treated mouse embryos express mosaics of Tbx3+/Tbx3− cells (Fig. 4G−I). This T-box transcription factor is specifically upregulated in the lateral line placodes of anamniotes (Schlosser, 2006, 2010). Furthermore, Q-VD-OPh-treated (Fig. 4G) as well as in utero-developed mice (Bollag et al., 1994) express Tbx3 in the otic vesicle.

As a next step, we examined whether the lateral line placodes of Q-VD-OPh-treated mice express Sox2 which maintains the proliferative status and determines the neural fate of placode precursor cells (Schlosser, 2010). In zebrafish, Sox2 immunopositivity was found in neuromast mantle and support cells, with the latter serving as the source for hair cell replacement (Hernández et al., 2007). Correspondingly, neuromast primordia of Q-VD-OPh-treated mice show Sox2+ mantle and support cells (Fig. 5A,B). Altogether, Sox2+ cells were observed in 36 out of 66 embryos (Figs S1–S6F), and up to 24 Sox2+ cells were present in the lateral line placodes of a single individual (Fig. S2A).

Neurogenesis in lateral line placodes and ganglia of Q-VD-OPh-treated mice

We then sought to determine whether Q-VD-OPh-treated mice produce neuroblasts destined to populate lateralis ganglia. In zebrafish, X. tropicalis and X. laevis these premigratory neuroblasts express Neurogenin1 (Ngn1) (Andermann et al., 2002; Nieber et al., 2009; Sarrazin et al., 2006), a proneural gene of the basic helix-loop-helix (bHLH) family of transcription factors. Accordingly, Ngn1+ neuroblasts were observed in the lateral line placodes of 6 out of 9 Q-VD-OPh-treated mouse embryos (Fig. 5C,D; Fig. S6G–L and data not shown). However, the numbers were extremely low (no more than 8 Ngn1+ cells per single individual: Fig. S6K), and Ngn1− lateralis ganglia were not identified by us. Nevertheless, in rare cases, we found groups of up to 5 β-Tubulin-III immunopositive (Tubb3+) neurons in the mesenchyme beneath the lateral line placodes that may represent candidates for such rudimentary ganglia (Fig. 5G; Fig. S10E). Murine epibranchial placodes contain both Ngn1+ and Ngn2+ premigratory neuroblasts (Fode et al., 1998), in this again resembling X. tropicalis and X. laevis (Nieber et al., 2009). Since the lateral line placodes of Q-VD-OPh-treated mice completely lack Ngn2+ neuroblasts (n=15 out of 15 embryos; Fig. 5E,F; Figs S7 and S8A–C), differential expression patterns of Ngn1 and Ngn2 help to distinguish between closely apposed Ngn1+/Ngn2− lateral line placodes and Ngn1+/Ngn2− epibranchial placodes (Fig. 4C–F).

In zebrafish, lateral line placodes, neuromasts and ganglia express the bHLH transcription factor NeuroD1 (Neurogenic differentiation 1) downstream to Ngn1. Furthermore, neuromast hair cells co-express NeuroD1 and the proneural gene Atoh1 (Atonal homolog 1) that, in hair cells of the inner ear, is activated by interactions between Sox2, Six1 and its transcriptional co-factor Eya1 (Eyes absent 1)
Ahmed et al., 2012; Andermann et al., 2002; Itoh and Chitnis, 2001; Sarrazin et al., 2006). Lateral line placodes of Q-VD-OPh-treated mice are immunonegative both for NeuroD1 and Atoh1 (Fig. S8D–H) but, like epibranchial placodes, are partly immunopositive for the LIM-homeodomain protein Isl1 (Islet 1: Fig. S10F) which terminates the expression of Ngn1 and, in turn, NeuroD (Sun et al., 2008). Correspondingly, anamniote lateral line and epibranchial placodes express Isl1 (Park and Saint-Jeannet, 2010).

**DISCUSSION**

Glücksmann (1951) distinguished between three categories of ontogenetic degenerations: ‘morphogenetic’ ones which precede
lateral line placodes represent a new case of here provide evidence that, in normally developing mouse embryos, lateral line placodes from known signalling centres in the hindbrain and pharyngeal pouches (Schlosser, 2010). Accordingly, lateral line placodes of Q-VD-OPh-treated mice develop in the absence of externally supplied inducing signals. However, only very recently it was demonstrated that, in zebrafish, anterior lateral line placodes require Fgf signalling, whereas posterior lateral line placodes depend on retinoic acid that inhibits Fgf signalling (Nikaido et al., 2017). Nevertheless, the additional finding that ectopic activation of Fgf or Wnt signalling suppresses posterior lateral line placodes but increases the size of the otic placode is consistent with the hypothesis that (posterior) lateral line placodes may represent the default fate of the PPA (Nikaido et al., 2017).

In view of the fact that culture of E9 mice should not markedly exceed 18–24 h to keep the embryos healthy (Martin and Cockcroft, 1999), it is presently impossible to fully explore the developmental potential of vestigial lateral line precursor cells. Thus, for instance, we were unable to determine whether, in embryonic mice, rudiments of the lateral line system can generate migratory primordia, or whether neuronomasts principally arise in un migrating lateral line placodes. Nor can we comment on whether the observed rare occurrence of hair cell kinocilia may be due to culture conditions, or whether these supernumerary placodes normally do not complete the full lateral line differentiation pathway apart from rare exceptions. Both scenarios could explain why we found no evidence for the expression of Atoh1, which is thought to be required for hair cell differentiation (Sarmzin et al., 2006). However, Atoh1 may well have been expressed in the precursors of the few hair cells that differentiated.

Considering that, compared with mice, largely identical patterns of apoptosis were observed in the PPA of the primate-related Tupaia belangeri (Tupaiaidae, Scandentia, Mammalia) (Waschawski et al., 2005) and chick embryos (our unpublished data), apoptotic elimination of vestigial lateral line placodes may prove a widespread phenomenon among amniotes. Our results also shed new light on the possible developmental origin of the mechanosensory paratympanic and spiracular organs (Baker et al., 2008; O’Neill et al., 2012). Recent evidence suggests that the amniote paratympanic and the amniote spiracular organs are homologous. Furthermore, a Sox2+ placode that resides dorsally adjacent to the first epibranchial placode could be identified as the source of the paratympanic organ in chicken embryos (O’Neill et al., 2012). Whether this previously undiscovered placode and, thus, paratympanic and spiracular organs develop independent of both lateral line and epibranchial placodes is not yet fully resolved. Our finding that, in embryonic mice, a latent conservation of mechanisms exists to develop lateral line placodes which are able to recapitulate at least part of the lateral line developmental program increases the probability of a lateral line origin of both organs. Modified versions of our experimental setting may provide an innovative possibility to further explore hitherto unknown developmental links between lateral line, otic and epibranchial placodes (Baker et al., 2008), and the molecular mechanisms that underlie placode morphogenesis in the PPA.
using ImageJ (Rasband, 1997-2016). According to these measurements, somite stages were determined with the help of mouse developmental tables (van Maele-Fabry et al., 1993). Embryos with 9–15 somite pairs [embryonic day (E) E8.5 to less advanced E9] were selected for wec only on the condition that neither their yolk sacs nor their ectoplacental cones had been damaged. Dissected embryos were transferred to the roller culture system using sterile transfer pipettes, and were randomly assigned either to Q-VD-OPh treatment or to one of the above specified control groups 1, 2 or 3 (2–4 embryos per bottle, 1 embryo/ml culture medium). At constant gas supply (40% O2, 5% CO2, 55% N2, gas flow rate: 25 ml/min), embryos were then incubated at 37.5°C and 30 rpm for 12, 18 or 24 h in the dark. For extended incubation periods (30 or 36 h in the dark), gas supply was modified to 70% O2, 5% CO2, and 25% N2 (gas flow rate: 25 ml/min) following 22 h in wec. At the end of culture, embryos were transferred to HBSS (37.5°C) and carefully examined for viability and developmental stage using established morphological criteria and measurements (van Maele-Fabry et al., 1990, 1993). Embryos from all experimental and control groups were selected for further analysis only on the condition that an appropriate developmental status had been reached compared with the corresponding in utero-developed embryos.

**Histological procedures**

Embryos were removed from the yolk sacs and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) for 24 h at room temperature. Following two washes in PBS for 30 min, specimens were dehydrated via an ascending ethanol series. Hereby, embryos were pre-temperatures. Following two washes in PBS for 30 min, specimens were

Embryos were serially sectioned at 5 µm using rotary microtomes (RM2245, RM2265, Leica). Serial sections were consecutively placed on two sets of slides (Knabe et al., 2002) that were used either for Mayer’s haematoxylin staining (Romeis, 1948) or for immunostainings with different primary antibodies.

**Antibodies**

In the following, each primary antibody is specified along with the corresponding dilution, the incubation parameters applied in our experiments, and with a reference providing details on the antibody specificity. Primary antibodies included: mouse anti-Atonal homolog 1 (Atoh1; Atoh1 supernatant, Developmental Studies Hybridastrum Bank, Iowa City, USA, lot 6/2013, RRID: AB_10805299; 1:50, overnight, 4°C) (Ahmed et al., 2012; Gowan et al., 2001), mouse anti-β-Tubulin-III (Tubb3; clone SDL.3D10, T8660, Sigma-Aldrich, lot 073K4835, Leica). Finally, embryos were serially sectioned at 5 µm using rotary microtomes (RM2245, RM2265, Leica). Serial sections were consecutively placed on two sets of slides (Knabe et al., 2002) that were used either for Mayer’s haematoxylin staining (Romeis, 1948) or for immunostainings with different primary antibodies.

**Immunohistochemistry**

Deparaffinised and rehydrated sections were washed in Tris-buffered saline (TBS: 0.05 M Tris, 0.15 M NaCl, pH 7.4). Next, antigen retrieval was performed by high-pressure cooking in citrate buffer (0.01 M, pH 6). Following cooling to room temperature, endogenous peroxidase activity was blocked and sections were permeabilized by incubation with 1% H2O2 and 0.3% Triton X-100 in TBS for 30 min. Thereafter (and in between all following incubation steps), sections were washed three times in TBS (5 min each). For anti-Ngn2 staining, reagents and procedures from the mouse-on-mouse kit (BMK-2002, Vector Laboratories) were used to block the sections and to perform the primary and secondary antibody incubation steps. For all other immunostainings, primary antibodies were diluted in Dako REAL antibody diluent which contains background reducing agents (S202230-2, Agilent Technologies, Waldbronn, Germany). Immunoreacted sections were incubated with the appropriate biotinylated secondary antibody diluted 1:100 in TBS with 2% normal serum of the same species as the secondary antibody (S1000, S2000, Vector Laboratories) for 1 h at room temperature. For all immunostaining, sections were finally incubated with the avidin-biotin peroxidase complex (Elite ABC reagent, PK-7100, Vector Laboratories) for 1 h at room temperature. Peroxidase reactions were developed with 0.06% 3,3'-diaminobenzidine (DAB; D5637, Sigma-Aldrich) and 0.007% H2O2 in Tris-HCl buffer (0.1 M, pH 7.6). Afterwards, sections were thoroughly rinsed in distilled water, counterstained with Mayer’s haematoxylin (Romeis, 1948), and, following dehydration and clearance, embedded with DePeX mounting medium (18243, Serva, Heidelberg, Germany). Negative controls were performed by omission of the primary antibody and resulted in the absence of immunostaining.

**Histological analysis**

Embryos were taken from the developmental period between E9 and E10.5. Embryo numbers in the Q-VD-OPh and control groups are consistent with, or considerably exceed, those employed in previous studies which were based on comparable methodologies (Huang et al., 2012; Lassiter et al., 2009; Massa et al., 2009). During all histological examinations, the group allocations were not blinded to the investigators. However, analysis was performed according to predefined, objective criteria, and findings were evaluated independently by the two authors.

Immunohistochemistry with antibodies against the transcription factors Atoh1, Isil1, NeuroD1, Ngn1, Ngn2, Pax2, Pax8, Six1, Sox2, Sox10, and Tbx3 reveals nuclear staining patterns. In cases where immunolabelling was absent from the lateral line anlagen (Atoh1, NeuroD1, Ngn2), internal positive controls were observed as follows: anti-Atoh1 labelling in the dorsal neural
transferase-mediated dUTP nick end-labelling) method, and structural against cleaved caspase-3, (2) the TUNEL (terminal deoxynucleotidyl been determined by using combinations of (1) immunohistochemistry (Kiernan et al., 2005), Sox10 expression in neural crest cells (Adameyko et al., 2012), and Tbx3 expression in the ventral pharyngeal endoderm, neural crest-derived mesenchyme, and the otic vesicle (Bollag et al., 1994; Mesbahi et al., 2012). To ensure specificity of anti-Pax2, anti-Pax8, and anti-Six1 immunostainings, internal positive and negative controls were applied as previously described (Washausen and Knabe, 2017). Anti-Tubb3 immunohistochemistry reveals neuronal cell bodies, dendrites and axons in the central and peripheral nervous system of E9.5 mouse embryos (Liu et al., 2007). Anti-phH3 staining detects chromatin condensations of mitotic cells as well as weak chromatin condensations of the late G2 phase and G2/M transition (Loponen et al., 2011).

Analysis of apoptosis

To verify the diagnosis of apoptotic cells in tissue sections, a multiparametric approach has to be applied (Stadelmann and Lassmann, 2000; Taatjes et al., 2008). Accordingly, previously published patterns of apoptosis in the PPA of C57BL/6N mice (E8.5 to E11.5, n=65 embryos) had been determined by using combinations of (1) immunohistochemistry against cleaved caspase-3, (2) the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method, and structural diagnosis in haematoxylin-stained (3) paraffin or (4) ‘semithin’ (1 µm thick) resin sections (Washausen and Knabe, 2013, 2017). It turned out that the peak period of PPA apoptosis takes place in embryos of approximately 19–27 somite pairs that had been classified either to E9.25 (detaching otic vesicle exhibits a small pore) or E9.5 (a solid stalk connects the closed otic vesicle to the overlying ectoderm) (Washausen and Knabe, 2013, 2017). To provide a baseline for the present experimental work, a summary scheme depicting PPA apoptosis during the peak period was compiled from previously studied embryos (Washausen and Knabe, 2013, 2017) (n=44 body sides; Fig. 1A). In the current work, apoptotic cells were identified by immunohistochemistry with antibodies against cleaved caspase-3 as well as by established structural criteria (Hacker, 2000; Sanders and Wride, 1995). Furthermore, representative sections were subjected to TUNEL staining according to the protocol published previously (Washausen and Knabe, 2013).

Reconstructions of the PPA

Fine-grained schematic reconstructions were performed by transferring histological and/or immunohistochemical data from the PPAs of completely serially sectioned Q-VD-OPh-treated or control embryos to basic schemes of the embryonic head that had been generated in CorelDRAW X4 (Corel, Unterschleißheim, Germany) using scanning electron micrographs and three-dimensional reconstructions of the corresponding embryonic stages as a reference (Tamarin and Boyd, 1977; Verwoerd and van Oostrom, 1979; Washausen et al., 2005). Depending on the embryonic stage and/or the plane of sectioning, 60 up to 240 serial sections (section thickness=5 µm) were evaluated per PPA. Case-dependent, either complete series (section interval=5 µm) or every second section (interval=10 µm) were used to reconstruct the PPA. The plane of sectioning was determined according to the positions of various topographical landmarks (e.g. optic and otic vesicles, branchial membranes). Epibranchial placodes were identified structurally as patches of high-grade thickened, pseudostratified epithelium located adjacent to the branchial membranes (Washausen and Knabe, 2013, 2017). Additionally, the otic anlage and, if present, the otic detachment site were mapped. Diagnosis of lateral line placodes and neuromast primordia was based on the criteria which have been previously established in amnionites (Northcutt, 1992; Northcutt et al., 1994; Sato, 1976; Schlosser, 2002b; Schlosser and Northcutt, 2000; Stone, 1933; Winblauer, 1989).

Photomicrographs

Histological sections were examined under an Axioskop 2 MOT microscope (Carl Zeiss, Göttingen, Germany). Micrographs were captured with an Axioscam HR digital camera (Carl Zeiss) and the KS400 image analysis software (v3.0, Carl Zeiss). Following shading correction in KS400, images were cropped, resized, and adjusted for brightness (including slight gamma changes), colour balance, and sharpness in Corel Photo-Paint X4. All adjustments were applied to the whole image and no specific features within the photographs were modified, removed, or inserted.

Statistics

Statistical analysis was performed using STATISTICA software (v12.0, StatSoft, Hamburg, Germany). Since the numbers of apoptotic cells in the PPA were not normally distributed (Kolmogorov–Smirnov test) and variances between the three groups were not homogeneous (Levene’s test), the non-parametric Mann–Whitney test was used to compare the levels of PPA apoptosis between in utero-developed embryos, cultured embryos incubated with DMSO, and cultured embryos treated with Q-VD-OPh or Z-VAD-fmk, respectively (Fig. 1B). All tests were two-sided, and P values <0.05 were considered statistically significant. Boxplots of PPA apoptosis were created using STATISTICA software. Diagrams demonstrating the frequency of unilaterally or bilaterally developed lateral line placodes were produced in Microsoft Excel (Fig. 2D).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.W., W.K.; Methodology: S.W., W.K.; Validation: S.W., W.K.; Formal analysis: S.W., W.K.; Investigation: S.W., W.K.; Writing – original draft: S.W., W.K.; Writing - review & editing: S.W., W.K.; Visualization: S.W., W.K.; Supervision: W.K.; Project administration: W.K.

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