

Caspase-mediated apoptosis induction in zebrafish cerebellar Purkinje neurons

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ABSTRACT

The zebrafish is a well-established model organism in which to study *in vivo* mechanisms of cell communication, differentiation and function. Existing cell ablation methods are either invasive or they rely on the cellular expression of prokaryotic enzymes and the use of antibiotic drugs as cell death-inducing compounds. We have recently established a novel inducible genetic cell ablation system based on tamoxifen-inducible Caspase 8 activity, thereby exploiting mechanisms of cell death intrinsic to most cell types. Here, we prove its suitability *in vivo* by monitoring the ablation of cerebellar Purkinje cells (PCs) in transgenic zebrafish that co-express the inducible caspase and a fluorescent reporter. Incubation of larvae in tamoxifen for 8 h activated endogenous Caspase 3 and cell death, whereas incubation for 16 h led to the near-complete loss of PCs by apoptosis. We observed synchronous cell death autonomous to the PC population and phagocytosing microglia in the cerebellum, reminiscent of developmental apoptosis in the forebrain. Thus, induction of apoptosis through targeted activation of caspase by tamoxifen (ATTACTM) further expands the repertoire of genetic tools for conditional interrogation of cellular functions.

KEY WORDS: Zebrafish, Cerebellum, Purkinje cells, Cell ablation, Apoptosis, Inducible caspase

INTRODUCTION

In vivo cell ablation is a useful research method with which to study the development, function, interaction and regeneration of cells or tissues in a multicellular context. Working with zebrafish embryos, one can combine *in vivo* cell ablation with multicolor live-imaging techniques and numerous forms of genetic or pharmaceutical manipulations due to the aquatic lifestyle, small size, external development and transparency of these embryos (Lieschke and Currie, 2007; Weber and Köster, 2013). To obtain spatial and temporal control over the cell type to be ablated, and to avoid

collateral tissue damage that is associated with invasive ablation techniques, inducible genetic cell ablation methods were developed. Cell type-specific or subcellular-specific expression of the phototoxic fluorescent proteins KillerRed (KR) (Bulina et al., 2006) or SuperNova (Takemoto et al., 2013) can be used to generate radicals upon excitation, which either results in cell death or in destruction of the targeted organelle (Teh et al., 2010; de Anda et al., 2010; Korzh et al., 2011). However, its use is restricted to tissues accessible by the often shallow light penetration. Expression of the bacterial enzyme nitroreductase (NTR) does not require light but instead uses the pro-drug uptake of the expressing tissue to elicit induction of cell death through enzyme-mediated pro-drug conversion into a p53-independent DNA-damaging compound (Cui et al., 1999; Djeha et al., 2005). Depending on the pro-drug used, cell death will be restricted to the NTR-expressing cell population, as is the case for the antibiotic metronidazole. Alternatively, with the pro-drug CB1954, NTR non-expressing neighboring cells are killed by the spread of the toxic compound, a process called ‘bystander effect’, which is desirable in cancer therapy applications (Bridgewater et al., 1997; Djeha et al., 2005; Lipinski et al., 2006). NTR-mediated metronidazole-induced cell ablation has been widely used in zebrafish in several tissues, including rod photoreceptors, pancreatic β -cells and hepatocytes (Curado et al., 2007; Davison et al., 2007; Pisharath, 2007; Chen et al., 2011; White and Mumm, 2013). An improved NTR variant with faster kinetics was recently reported in zebrafish, increasing the effectiveness and versatility of this ablation system (Mathias et al., 2014).

However, if two cell types are to be ablated independently, a second NTR-independent ablation system is necessary. It would be desirable to complement existing methods with a cell ablation system in zebrafish that ideally directly activates apoptosis as a non-lytic endogenous cellular mechanism of cell death and uses well-established drugs with high activity in almost all tissues.

The small synthetic compound FK1012 can trigger the homotypic dimerization of a fusion protein of the FKBP dimerization domain and the initiator caspase Caspase 8, thereby activating apoptotic cell death. This method is termed apoptosis through targeted activation of Caspase 8 (ATTAC) (Pajvani et al., 2005). However, the pharmacology of the ligand is unknown and harbors an immunosuppressive activity. Analogous to this method, we have recently shown that Caspase 8 fused to the mutated tamoxifen-interacting estrogen receptor ligand-binding domain (ER^{T2}) can be activated by tamoxifen to induce apoptosis in a wide range of different cultured cell types that express this ‘inducible caspase’ (Chu et al., 2008). In the past, tamoxifen has been proven to activate protein function in tissues of all germ layers in aquatic vertebrates and mammalian model organisms. It works in embryos and adults, and passes across the blood-brain barrier. Tamoxifen is an estrogen analogue, but the three known estrogen

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receptors found in zebrafish do not respond to tamoxifen (Bardet et al., 2002).

We have now transferred this ablation system to living zebrafish, pairing it with the co-expression of a fluorescent protein to monitor the time course and extent of ablation. We targeted differentiating cerebellar Purkinje cells (PCs), as they display a highly polarized morphology and form a single cell layer, which allows for a good score of their survival and healthiness, and easy bioimaging (Hibi and Shimizu, 2012). Moreover, PCs are the sole output neurons of the cerebellum, which is affected by several degenerative diseases (Fernandez-Gonzalez et al., 2002). This transgenic PC-ATTACTM (ATTAC induced by tamoxifen) zebrafish strain will thus be a powerful tool with which to study the consequences of PC ablation with respect to cerebellar neuronal survival, regeneration and function in the near future.

RESULTS

Inducible caspase under transient transgenic conditions

Tamoxifen is usually dissolved in ethanol (EtOH) and used in zebrafish at concentrations of 5 μ M (Mosimann et al., 2011). In order to address whether EtOH or tamoxifen treatment alone induces cell death, zebrafish embryos were incubated in either EtOH alone or tamoxifen for 10 h starting at 24 hpf using staurosporine as positive control (Fig. S1A). Acridine Orange (AO) staining detected a sevenfold increase in the number of dead cells upon staurosporine treatment, whereas neither EtOH nor tamoxifen incubation resulted in elevated cell death compared with non-treated wild-type embryos (Fig. S1B). Next, heterozygous Tg(*her3:KalTA4*) transgenic embryos with KalTA4 (a zebrafish-optimized Gal4-variant) expression in cells of the ventral spinal cord were injected at the one-cell stage (Babaryka et al., 2009) with a 5 $\times$ UAS:FyntagRFP-T-T2A-Caspase8ER^{T2} expression vector co-expressing the membrane-bound fluorescent protein FyntagRFP-T with Caspase8ER^{T2} linked by a self-cleaving T2A-peptide (Kim et al., 2011). At 24 hpf, these embryos showed transgene expression throughout the ventral spinal cord, allowing Acridine Orange (AO) staining for cell death analysis (Fig. S2A).

Elevated levels of cell death in cells along the ventral spinal cord could be observed in EtOH-treated control specimens, as indicated by AO staining (Fig. S2B), such elevation in cell death could not be found with FyntagRFP-T expression alone (Fig. S2B). Incubation of inducible caspase-expressing embryos with tamoxifen starting at 24 hpf strongly increased cell death after only 4 h (Fig. S2B), reaching highest level at about 8 h of incubation in tamoxifen (Fig. S2B). Image recording of these cells using a confocal laser scanning microscope revealed that cell death strictly localized to FyntagRFP-T/Caspase8ER^{T2}-co-expressing cells (Fig. S3A), with 25.3 $\pm$ 3.5% (s.d.) of transgene-expressing cells already entering cell death without tamoxifen treatment, which was clearly above background levels when compared with 5 $\times$ UAS:FyntagRFP-T-expressing control cells. Importantly, cell death was significantly increased [up to 89.1 $\pm$ 11.5 (s.d.)] in all transgene-expressing cells 8 h after initiation of tamoxifen treatment (Fig. S3B). This shows that the activity of the Caspase8ER^{T2}-fusion protein is leaky under transient transgenic conditions, but that its activity can be strongly induced by tamoxifen treatment, resulting in death of the vast majority of all transgene-expressing cells within a time-window of 8 h.

To show that other cells apart from ventral spinal cord cells can be driven into apoptotic cell death (Fig. S4A), immunostaining against activated Caspase 3 was performed in 5 $\times$ UAS:FynVenus-T2A-Caspase8ER^{T2}-injected embryos expressing KalTA4 either in rhombomeres 3 and 5 [Et(*shhb:KalTA4,UAS-E1b:mCherry*)

hzm6Et] (Fig. S4B) or in skeletal muscle [Et(*shhb:KalTA4,UAS-E1b:mCherry*)hzm8Et] (Fig. S4C). Again, although in EtOH-treated control fish elevated levels of apoptosis were detected (Fig. S4), likely due to the leakiness of Caspase8ER^{T2} activity, apoptotic cell death was significantly induced upon 6 h of tamoxifen treatment (Fig. S4).

Establishment of stable transgenic PC-ATTACTM fish

To address whether the leakiness of tamoxifen-inducible Caspase8-ER^{T2} activity in the absence of tamoxifen (Fig. 1A) exists under stable transgenic conditions, we established a zebrafish strain co-expressing the membrane-bound fluorescent protein FyntagRFP together with Caspase8ER^{T2} linked by a self-cleaving T2A-peptide (Fig. 1B) (Kim et al., 2011). To achieve specific expression in differentiating cerebellar Purkinje cells (PCs), a regulatory element isolated from the upstream region of the zebrafish *carbonic anhydrase 8* (*ca8*) homolog was used (Matsui et al., 2014), resulting in the stable transgenic zebrafish strain Tg(*ca8:FMA-TagRFP-2A-casp8ERT2*)^{bz11Tg} (Fig. 1C) which we named PC-ATTACTM, according to the previously used nomenclature (Pajvani et al., 2005) but emphasizing its tamoxifen dependence.

Whole-mount mRNA *in situ* hybridization against *tagRFP* at 4 dpf confirmed transgene expression only in the differentiating cerebellum (Fig. S5). Next, PC-ATTACTM larvae of the F2 and F3 generations were analyzed *in vivo* using a confocal laser scanning microscope. First, red fluorescent cerebellar cells could be observed around 3 days postfertilization (Fig. S6A,B), which formed the wing-like structures characteristic of the two cerebellar hemispheres (Fig. S6B). PCs are GABAergic and the gene encoding pancreas transcription factor 1a (*ptf1a*) is transiently expressed in precursors of GABAergic neurons of the cerebellum. Therefore, its enhancer drives the expression of GFP in Purkinje cell progenitors and other cerebellar interneurons (Fig. S6A,B) (Lin et al., 2004; Hibi and Shimizu, 2012). When we crossed PC-ATTACTM carriers with the transgenic reporter line Tg(*ptf1a:egfp*), all red fluorescent cells analyzed were marked by cytoplasmic co-expression of green fluorescence (Fig. S6B), supporting their normal development from *ptf1a*-expressing embryonic progenitor cells. To confirm the specific expression of inducible Caspase 8 in PCs, we performed double immunohistochemistry staining using whole-mounts of 5 dpf transgenic larvae and antibodies against tagRFP and the PC-specific ZebrinII antigen (Lannoo et al., 1991). Indeed, we found that all red fluorescent cells co-expressed ZebrinII, proving their identity as PCs (Fig. 1D). Concomitant Caspase8ER^{T2}-expression was confirmed by western blot analysis (Fig. 1G) and immunohistochemistry, using an anti-Caspase 8 antibody and demonstrating that Caspase 8 expression is confined to FyntagRFP-expressing cells (Fig. 1E).

Because tagRFP is fused at its N-terminus with the myristoylation site of the Fyn kinase, the axons, somata and the prominent dendrites of PCs could be observed in detail (Kameda et al., 2008), including the long axonal projections of PCs that form the cerebello-octavolateralis tract to vestibular nuclei (Bae et al., 2009; Matsui et al., 2014; Biechl et al., 2016) (Fig. 1F, white arrowheads). These axonal projections did not show any signs of degeneration, fragmentation, abnormal projection or sprouting. Neither did homozygous F3 larvae, which are easily detectable by their brighter fluorescence, show any signs of altered PC morphology (not shown), suggesting that the inducible Caspase 8 was inactive in larvae of the PC-ATTACTM strain in the absence of tamoxifen.

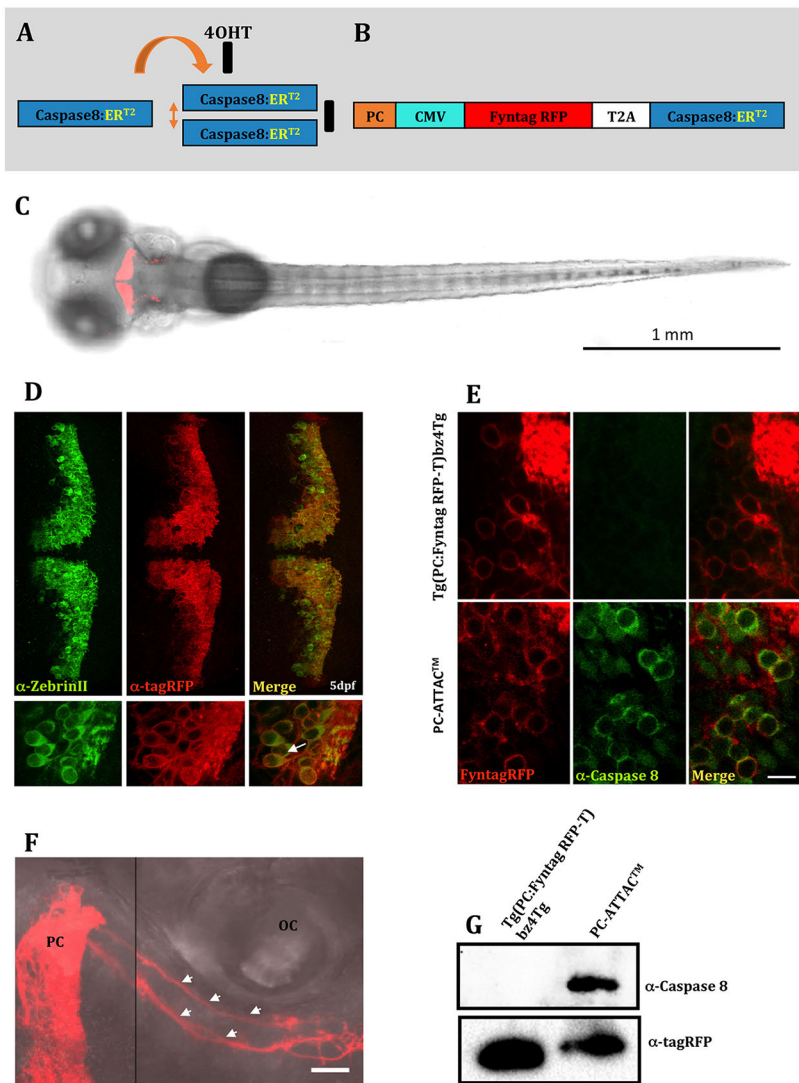


Fig. 1. Purkinje cell-specific expression of a tamoxifen-inducible caspase. (A) Schematic diagram of the Caspase 8 activation. Caspase 8 fused to the mutant estrogen receptor ligand-binding domain (ER^{T2}) dimerizes upon addition of tamoxifen (4OHT; orange double-headed arrow). Subsequent proteolytic self-activation of Caspase 8 leads to activation of endogenous Caspase 3 and cell apoptosis. (B) Construct design for the generation of stable transgenic zebrafish PC-ATTACTM strain. Membrane-bound fluorescent FyntagRFP together with Caspase8ER^{T2} linked by a self-cleaving T2A-peptide is co-expressed under the control of a Purkinje cell-specific regulatory element (PC) from carbonic anhydrase 8, which is combined with a CMV basal promoter (CMV). (C) F2 transgenic larvae were analyzed at 5 dpf by confocal microscopy to verify transgene encoding FyntagRFP expression exclusively in the cerebellum. (D,E) Purkinje cell (PC)-specific expression of the transgene was confirmed by double-immunostaining against (D) FyntagRFP (red) and ZebrinII (green), and (E) Caspase 8 (green, combined with FyntagRFP fluorescence), demonstrating that Caspase8ER^{T2} expression is confined to FyntagRFP-fluorescent ZebrinII-expressing PCs. (F) Owing to membrane targeting of FyntagRFP axons, somata and dendrites of PCs, including the long axonal projections forming the cerebello-octavolateralis tract to vestibular nuclei (white arrowheads), could be monitored. (G) Concomitant Caspase8ER^{T2} and FyntagRFP-T or FyntagRFP expression was verified by western blot analysis. FyntagRFP-T (bz4Tg, Matsui et al., 2014) alone was used as a control. PC, Purkinje cell; OC, otic vesicle. Scale bars: 10 μm (E) and 20 μm (F).

Tamoxifen-induced PC death in PC-ATTACTM fish

In order to test the PC death-inducing properties of the Caspase 8, heterozygous PC-ATTACTM larvae with a red fluorescent PC layer were treated with the active form of tamoxifen, 4-hydroxy-tamoxifen (4OHT), diluted in 0.5% EtOH starting at 4 dpf (Goetz et al., 2008). A concentration of 5×10^{-6} M 4OHT, which lies in the range of other 4OHT-mediated application protocols in zebrafish (Hans et al., 2009; Kroehne et al., 2011; Mosimann et al., 2011), induced widespread ablation of PCs. Cell ablation was evident by the formation of massive PC debris visible after 16 h (Fig. 2A). Strikingly, the extensive dendritic layer of PCs was completely disrupted (Fig. 2B, white arrowheads), somata were replaced by round cell debris (Fig. 2B, insets) and the projections forming the cerebello-octavolateralis tract were fragmented (Fig. 2A, yellow square). These findings show that the caspase-mediated PC ablation achieved by 4OHT incubation for 16 h at a concentration of 5 μM is both effective and highly efficient *in vivo*.

PC-ablated specimens returned to 4OHT-free rearing medium at this time point were reanalyzed 24, 48 and 72 h after 4OHT treatment (8, 32 and 56 h after removal of 4OHT, respectively). Red fluorescent PC debris could still be detected, although to a lesser extent, being widely distributed throughout the larval cerebellum (Fig. 2C). This shows that PCs were not able to recover quickly but

had been irreversibly removed and were unable to escape cell death. This indicates that 4OHT treatment resulted in the fast induction of PC death through the activation of Caspase 8 and was therefore likely to be mediated by apoptosis.

Lower concentrations of 4OHT (10^{-6} M) induced only mosaic cell death or 'dendritic damage', which was associated with locally confined dot-like disruptions of the otherwise smooth texture within dendritic compartments of PCs, as well as debris nearby somatic membranes (not shown). Treatments with such a low concentration of 4OHT might be of interest if neuronal cell damage, rather than the consequences of cell death, are the focus of interest, e.g. when studying the plasticity or the regrowth and remodeling of PC dendrites. Larvae incubated in tamoxifen-free medium that contained only the diluent EtOH showed no signs of damage to the PC layer (Fig. 2A,B), further confirming that caspase activity in PC-ATTACTM larvae is not leaky but can be well controlled.

Potentially, Caspase 8 could be active at low levels in the absence of tamoxifen or Caspase 8 activity could be induced by endogenous estrogen signaling. Both would result in slow but continuous PC death accumulating over time. As there is currently no evidence for Purkinje cell progenitor proliferation in adult zebrafish (Kaslin et al., 2013), such leaky Caspase 8 activity would result in progressive gaps in the PC layer of the adult zebrafish cerebellum.

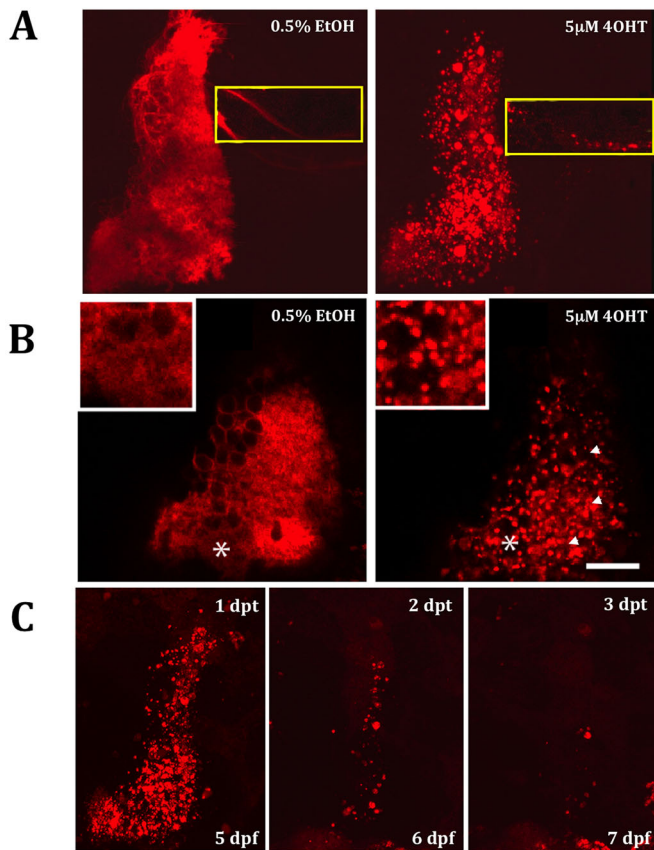


Fig. 2. Tamoxifen-induced PC death in PC-ATTAC™ fish. (A,B) Incubation of 4 dpf PC-ATTAC™ embryos ($n=6$) in 4OHT (5×10^{-6} M) for 16 h induces cell death of PCs in larvae at 5 dpf compared with controls treated with 0.5% EtOH. Cell ablation was visible by way of large amounts of PC debris (white arrowheads in B). The extensive dendritic layer was completely disrupted (the asterisks mark the region shown in the inset in B) and the projections of the cerebello-octavolateralis were fragmented (yellow square in A). (C) PC-ablated larvae re-analyzed 24, 48 and 72 h after tamoxifen treatment show decreasing amounts of red fluorescent PC debris and a lack of fast PC recovery. Scale bars: 20 μ m; dpt, days post-treatment.

Sagittal sections of the cerebellum from adult PC-ATTAC™ zebrafish revealed no such gaps but a continuous PC layer without signs of progressive PC degeneration, further excluding the possibility of leaky Caspase 8 activity (Fig. S7). These findings show that in the established PC-ATTAC™ strain Caspase8ER^{T2} is inactive but can be triggered reliably by tamoxifen to induce cell death specifically in cerebellar PCs.

Dynamics of PC ablation in PC-ATTAC™ larvae

To be able to score the success of our ablation system numerically, we counted the PCs that expressed tagRFP in PC-ATTAC™ larvae from confocal *z*-stacks of the right cerebellar hemisphere at 4 dpf, when ablation was usually induced in this study. We counted an average of 131.3 ± 22.4 (s.d., $n=11$) per hemisphere, indicating that the PC population encompasses roughly 260 cells at this developmental stage, which does not increase dramatically over subsequent days (Fig. S8) (Hamling et al., 2015).

Caspase 3 acts as an executing caspase downstream of Caspase 8. To clarify that PC death indeed occurs by apoptosis and to determine the time course of cell death, heterozygous PC-ATTAC™ larvae were treated at 4 dpf with 4OHT and processed at 2 h intervals for activated (cleaved) Caspase 3 immunohistochemistry. EtOH (0.5%)

control treatments for 0 and 8 h (Fig. 3F,G) showed background levels of cells with activated Caspase 3, similar to PC-ATTAC™ larvae at the onset of 4OHT treatment (0.6 cells on average at 0 h) (Fig. 3A).

The first significant elevation of apoptosis in PC-ATTAC™ larvae could be observed after 6 h of 4OHT treatment in about 6.6%

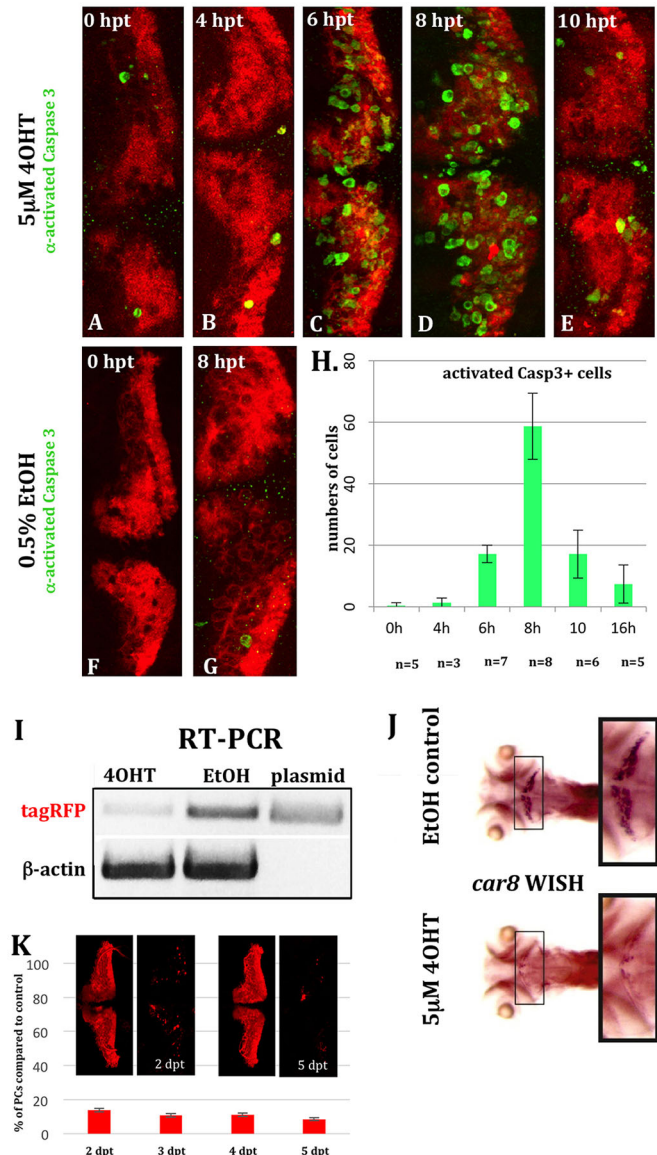


Fig. 3. Time course of apoptosis in PC-ATTAC™ larvae. (A–G) Whole-mounts of 5-dpf transgenic PC-ATTAC™ embryos were stained with an antibody against activated Caspase 3 (green) after treatment at 4 dpf with 5×10^{-6} M 4OHT (A=0 h; B=4 h; C=6 h; D=8 h; E=10 h) or 0.5% EtOH (F=0 h; G=8 h). (H) Significant elevation of apoptosis could be observed after 6 h, peaking after 8 h of 4OHT treatment based on PC counts at 4 dpf. (I–K) To score the extent of PC ablation over time, we performed 4OHT treatments with 4 dpf heterozygous larvae for 16 h (compared with 0.5% EtOH controls) and analyzed the cell death 24 h later. (I) RT-PCR revealed a strong reduction of *fyntagRFP* mRNA for 4OHT-treated larvae in contrast to EtOH controls with β -actin used as ubiquitously expressed control gene. (J) Although mRNA *in situ* hybridization ($n=5$) showed carbonic anhydrase 8 (*ca8*) expression was prominent in the cerebellum in control larvae, virtually no *ca8* expression was visible in PC-ablated specimens (black squares). (K) PC counts 2 and 5 days post-treatment (dpt) revealed a 90% reduction in PC number compared with EtOH controls (K). hpt, hours post-treatment.

of all FyntagRFP-expressing PCs (17.1/260, $n=7$ larvae; Fig. 3C,H) based on previously determined cell numbers of 260 PCs at 4 dpf. After 8 h of 4OHT treatment these levels increased to 22% (58.6/260, $n=8$ larvae) and subsequently declined to 6.6% (17.2/260, $n=6$ larvae) and 2.8% (7.4/260, $n=5$ larvae) after 10 and 16 h of 4OHT incubation, respectively (Fig. 3D,E,H). This shows that the induction of apoptosis is fast occurring synchronously within a few hours. The low percentage of PCs containing activated Caspase 3 compared with widespread fragmentation of the PC layer after 16 h of 4OHT treatment suggests that activated Caspase 3 staining is only transient in dying PCs. Activated Caspase 3 staining always colocalized with FyntagRFP expression, confirming that only PCs were driven into apoptosis.

To better score the extent of PC ablation, we performed 4OHT treatments for 16 h with heterozygous 4 dpf PC-ATTACTM larvae and waited for another 24 h after 4OHT washout to allow for completion of cell death. Subsequently, these embryos were subjected to both RT-PCR analysis to detect remaining *fynTagRFP* expression and to mRNA *in situ* hybridization against *carbonic anhydrase 8* (*ca8*), a Purkinje cell-specific marker in both adult and larval zebrafish (our unpublished results). RT-PCR analysis of isolated heads showed strongly reduced amounts of remaining *fynTagRFP* mRNA when compared with EtOH controls (Fig. 3I). In addition, although *ca8* expression was prominent across the cerebellum in EtOH controls, virtually no *ca8* expression was visible in PC-ablated specimens (Fig. 3J). Finally, the number of PCs 2 and 5 days post-4OHT treatment was about 90% fewer than in controls (Fig. 3K, $n=5$ larvae counted per time point). This value may even underestimate the efficiency of ablation, as PC progenitors that did not yet express Caspase 8 during 4OHT treatment and that differentiated into fluorescent PCs after 4OHT removal could counteract diminishing PC numbers. These findings demonstrate that 16 h of 4OHT treatment of heterozygous PC-ATTACTM larvae resulted in near-complete elimination of the entire cerebellar PC population. Thus, cell ablation in PC-ATTACTM larvae is not only fast but also efficient.

Acute Caspase 8-mediated apoptosis does not spread to PC connecting neurons

An interesting issue is whether PC apoptosis will affect the immediate health and survival of afferents or efferents, e.g. due to the loss in physiological connectivity with PCs. The most prominent and numerous direct afferents of PCs are cerebellar granule cells (GCs), whereas eurydendroid cells (ECs) are the only efferents of PCs (besides the cerebello-octavolateralis tract that projects to vestibular nuclei) (Fig. 4A). GCs are marked by EGFP fluorescence in the line Tg(*gata1:egfp*) (strain 781), (Long et al., 1997; Volkmann et al., 2008), whereas ECs express EGFP in the Tg(*olig2:egfp*) strain (Park et al., 2002). Thus, PC-ATTACTM carriers were crossed with carriers of these different transgenic backgrounds and the double transgenic larvae were subjected to 16 h of 4OHT treatment at 4 dpf to induce PC ablation.

gata1:EGFP-expressing GCs, as well as their axonal projections (the parallel fibers), did not show any signs of fragmentation or cell death in EtOH controls (Fig. 4B) or in 4OHT-treated specimens (Fig. 4C). Similarly, the somata of *olig2:EGFP*-expressing eurydendroid cells were arranged in their expected pattern without signs of cell death (Fig. 4D), which was also not observed subsequently during the removal of PC debris (Fig. S9).

Taken together, these data show that direct PC afferents or efferents are not obviously affected in their morphology, health or survival shortly after 4OHT-induced PC ablation. However, as these

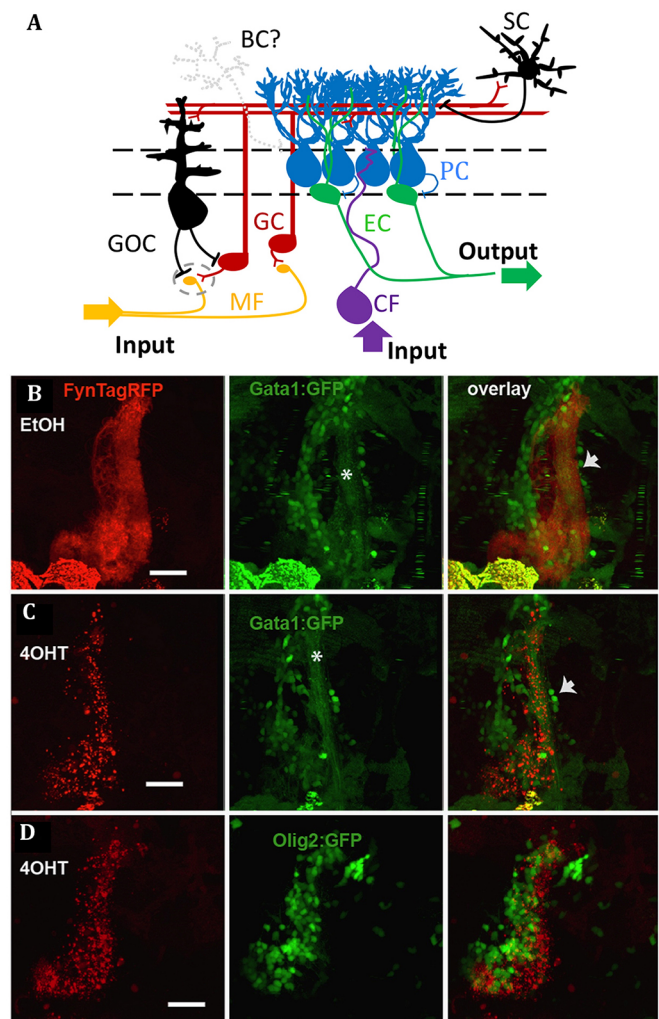


Fig. 4. Caspase8ER^{T2}-mediated apoptosis elicits no acute adverse effects on granule and eurydendroid cells. (A) Schematic of zebrafish cerebellar neurons and circuits. (B–D) Heterozygous PC-ATTACTM fish were crossed with carriers of the Tg(*gata1:egfp*) strain marking granule cells by green fluorescence (B,C) or with Tg(*olig2:egfp*) carriers displaying green fluorescent eurydendroid cells (D). Double transgenic 4 dpf larvae were treated with EtOH (B) or 4OHT (C,D) for 16 h to induce PC ablation followed by confocal microscopy image recording. (C) *gata1:egfp*-expressing GCs, somata (arrows) and their axonal projections – the parallel fibers (asterisks) – showed no signs of cell death, in contrast to fragmented PCs after 4OHT treatment. (D) The same was true for *olig2:egfp*-expressing eurydendroid cells. Scale bars: 20 μ m. BC, basket cell; CF, climbing fiber; EC, eurydendroid cell; GC, granule cell; GOC, Golgi cell; MF, mossy fiber; PC, Purkinje cell; SC, stellate cell. Scale bars: 40 μ m.

cerebellar neurons are integrated into circuits containing PCs, the electrophysiological input or output of these PC afferents and efferents is lost upon PC apoptosis. Whether such interruption of cerebellar connectivities – where PCs take a central role – and the loss of electrophysiological coupling will result in later-occurring secondary cell death, could reveal essential interdependencies of cerebellar neurons and should open interesting routes for future research.

Clearance of apoptotic bodies involves microglial phagocytosis

After treatment with 4OHT for 16 h at 4 dpf, we observed an accumulation of apoptotic bodies from dying or dead PCs. When

larvae were analyzed again 24, 48 and 72 h later by *in vivo* confocal microscopy, the amount of debris derived from dying PCs was markedly reduced based on the wider spacing of red fluorescent debris and fewer particles in the PC layer (Fig. 2C). This decrease in fluorescent cell debris could be attributed to microglial cells, which are the major phagocytosing cells of the brain. However, their number is low in the cerebellum and increases only slightly – likely because of continued brain growth – from 4 dpf to 5 dpf to about

one microglial cell per cerebellar hemisphere (Fig. 5D, see number of microglia in EtOH and 4OHT control specimens). Microglia are involved in developmental apoptosis in the larval zebrafish brain (Cole and Ross, 2001), which still occurs in the optic tectum at 3 dpf, whereas it has ceased in the cerebellum (Peri and Nüsslein-Volhard, 2008; Sieger and Peri, 2013; Svahn et al., 2013). To address the potential involvement of microglia in the clearance of apoptotic debris, we induced the ablation of PCs at 4 dpf in

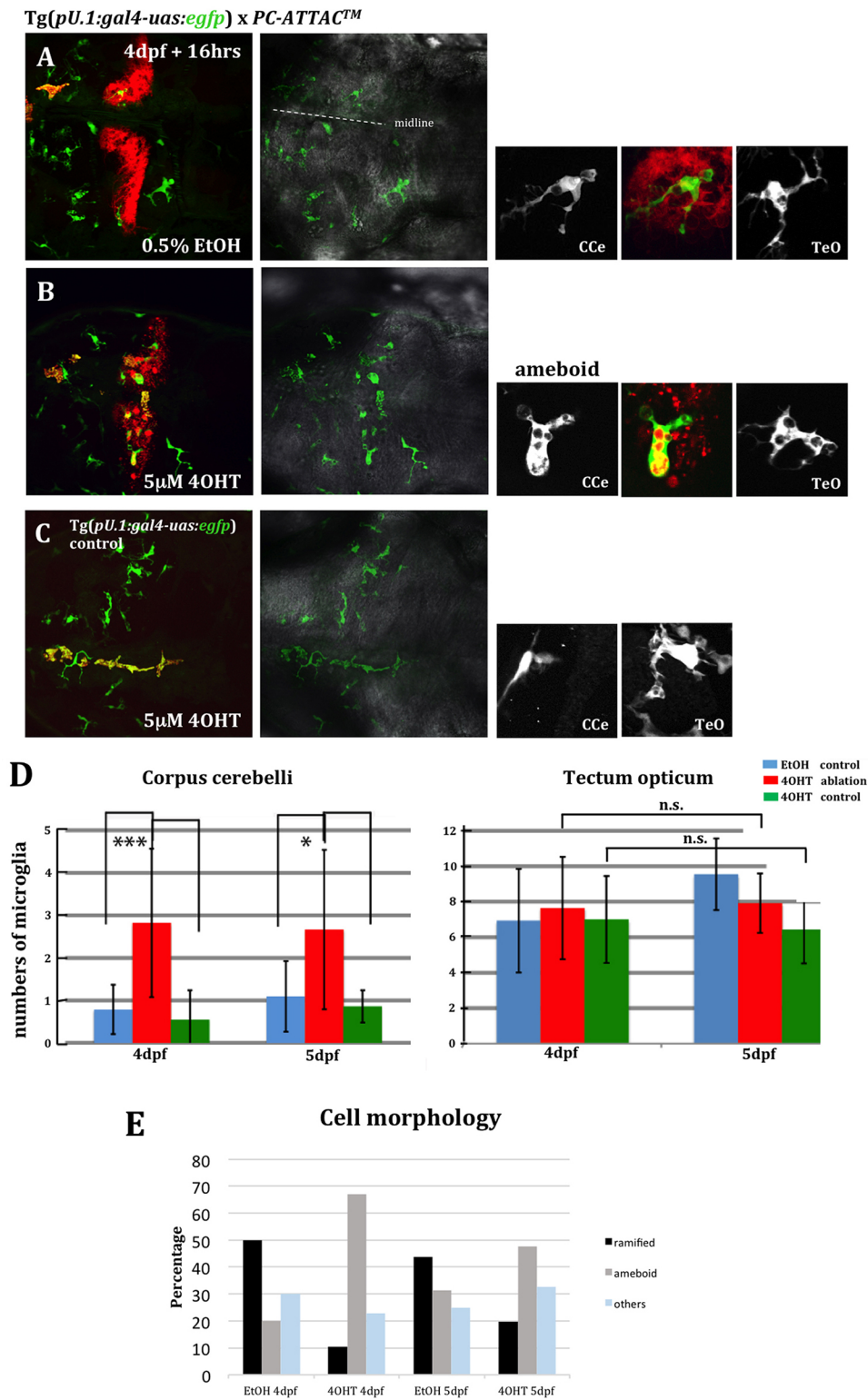


Fig. 5. Phagocytosis of apoptotic bodies by microglia. (A-C) Double transgenic PC-ATTACTM/Tg(pU.1:gal4-uas:egfp) larvae with green fluorescent microglial cells in the zebrafish brain. PC ablation (16 h of 4OHT treatment) caused an increase in the numbers of microglial cells specifically in the cerebellum (B,D), accompanied by microglial activation based on ramified to ameboid morphology changes (E). (C) 4OHT treatment of Tg(pU.1:gal4-uas:egfp) control larvae had no effect on microglial cells. CCe, corpus cerebelli; TeO, tectum opticum. **P*<0.05, ****P*<0.001 (Student's *t*-test); n.s., not significant.

PC-ATTACTM larvae in the *Tg(pU.1:gal4-uas:egfp)* background, where microglia are highlighted by their expression of EGFP (Fig. 5) (Peri and Nüsslein-Volhard, 2008).

Strikingly, the number of microglia in the cerebellum significantly increased in response to PC ablation (Fig. 5B,D, 2.82 microglia per hemisphere, $n=28$ hemispheres) alongside a change in their morphology from ramified to amoeboid (Fig. 5E, 66.9% of microglia compared with 20.0% in EtOH control specimens) containing several vacuoles, which indicates their activation and their involvement in engulfing and phagocytosing PC debris. Indeed, EGFP-expressing cerebellar microglia with internalized red fluorescent PC debris could be observed by confocal microscopy sectioning (Fig. 5B). Such an increase in microglia number or a change in microglia morphology could not be observed in either EtOH controls (Fig. 5A,D,F, 0.79 microglia per hemisphere, $n=14$ hemispheres) or in *Tg(pU.1:gal4-uas:egfp)* transgenic larvae treated with 4OHT (Fig. 5C,D, 0.5 microglia per hemisphere, $n=11$ hemispheres). Furthermore, microglia numbers were not significantly affected in the nearby optic tectum [Fig. 5E, 7.6 (4OHT/PC-ATTACTM), 6.9 (EtOH/PC-ATTACTM) and 7.0 (4OHT/*pU.1:gal4-uas:egfp*) microglia with 28, 14 and 11 analyzed tectal areas, respectively], neither increasing nor decreasing to support microglia in the cerebellum. This is consistent with the finding that microglia avoid crossing tissue boundaries such as the MHB or the midline (Sieger et al., 2012) and indicates that the induced acute PC apoptosis results in the increase in microglia number in the cerebellum likely for removing PC debris. As there are only three microglia per cerebellar hemisphere, this could explain why complete removal of debris lasts for about 2–3 days (Fig. 2C). Recent elegant studies have demonstrated that upon cell death in the developing brain, invasion of macrophages into the zebrafish larval brain occurs in areas where these macrophages differentiate into microglia (van Ham et al., 2014; Casano et al., 2016). These findings provide an explanation for the increase in microglia numbers that we observed in the cerebellum of 4OHT-treated PC-ATTACTM larvae.

DISCUSSION

Using cell type-specific expression of a tamoxifen-inducible initiator Caspase 8, we have established a stable transgenic strain named PC-ATTACTM as a genetic model for cerebellar Purkinje cell ablation. The co-expression of a fluorescent protein with Caspase8ER^{T2} not only helps to monitor and verify the course and extent of apoptotic PC death, but also allows for observing cell interactions such as the activity of microglia with dying PCs.

Our data demonstrate the engagement of microglia in the uptake and probably the removal of dying or dead PCs after caspase-mediated apoptotic cell ablation in PC-ATTACTM zebrafish larvae, similar to the normal role of microglia during developmental apoptosis. This fast response of microglia may indeed reflect the young age of the larvae, because in adult brains of other non-mammalian vertebrates a detectable increase in the numbers of microglia at the site of neuronal ablation has been shown to take up to 3 days, after which their increased numbers remain stable at the site of lesion for several weeks (Kirkham et al., 2011). Our findings could reflect the importance of a microglia response to cell ablation in the brain for subsequent restructuring and regeneration processes. Importantly, this endogenous neuroinflammatory response should be left unaltered by 4OHT, as this compound does not significantly influence innate immunity. This provides an opportunity to reveal the significance of neuroinflammation in mediating the response of the brain to the acute loss of specific neuronal populations. In

addition, ablation and removal of nearly all PCs will not allow plasticity to compensate for a loss of cerebellum function but requires regeneration for cerebellum recovery. Thus, after removal of tamoxifen, fluorescent protein expression will make the dynamics of potential PC regeneration accessible by bio-imaging approaches with membrane-targeted fluorescence, thus facilitating the observation of regenerative axon and dendrite outgrowth.

Based on our results in transient transgenics, other cell types should be equally accessible for the generation of ATTACTM zebrafish with tamoxifen-inducible apoptosis. However, the time course of differentiation specific for each cell type must be taken into account. If ablation is induced too early, progenitor cells not yet expressing the inducible caspase may escape the induction of apoptosis. To distinguish between such late-differentiating progenitor cells and non-ablated mature cells, the use of fluorescent proteins with a time-dependent change in the color of fluorescence emission (so-called timer fluorescent proteins) could be used as co-expressed reporters (Terskikh et al., 2000; Tsuboi et al., 2010). Although mature cells that escaped apoptosis will appear in the late-emission state of the fluorescent timer protein, cells that escaped ablation (owing to their late birth) will have only started to express the fluorescent protein and will thus appear in the early-emission state.

Because ligand-induced activity is also dependent on the cellular expression level of Caspase8ER^{T2}, which is determined by the promoter strength that drives such expression, the optimal conditions for maximal ablation will have to be tested empirically when implementing new promoter/enhancer elements into ATTACTM constructs. We recommend starting with a tamoxifen concentration of 5×10^{-6} M diluted in EtOH at a maximum concentration of 0.5% EtOH and treatment overnight.

Transgenic cell type-specific ATTACTM zebrafish complement the existing NTR-mediated inducible cell ablation method. First, the ATTACTM approach may broaden the range of cell types that could be targeted by inducible cell death. In addition, unlike metronidazole, tamoxifen exerts neither antibiotic nor prominent anti-inflammatory activity. Thus, when cell ablation and its consequences are to be studied in the context of a naturally occurring inflammatory responses, such as the behaviors of microglia, macrophages or neutrophils and their contribution to post-ablation processes, ATTACTM zebrafish may serve as an informative tool.

In summary, the choice of ablation approach – mechanically, genetically, chemically or light-induced – will influence subsequent events such as regeneration or remodeling of neuronal connections, and these issues have to be evaluated by the investigator. Here, we introduce a useful supplement to the steadily growing genetic zebrafish toolbox in form of a fast, efficient and inducible cell ablation method, thereby increasing the number of technical possibilities from which to choose.

MATERIALS AND METHODS

Animal husbandry

Zebrafish were maintained under 14 h light/10 h dark cycles at 28°C. Juvenile and adult fish were treated according to the Declaration of Helsinki for the care and use of animals, and in accordance with legal regulations (EU-Directive 201_63). Phenylthiourea (PTU, 0.15 mM) to reduce pigmentation was used only where indicated. In addition, established transgenic lines expressing KalTA4 in the ventral neural tube (hzm9; Babaryka et al., 2009), in rhombomeres 3 and 5 (hzm6Et), and in skeletal muscles (hzm8Et; Distel et al., 2009) were used as well as *Tg(ca8-ADV. E1b:TagRFP)bz4Tg*, also named PC-FyntagRFP-T, as a control (Matsui et al., 2014).

Cloning of constructs

A pB-Tol2 construct was used as a backbone into which a PC-specific enhancer (Matsui et al., 2014)/CMV basal promoter was inserted followed by FyntagRFP (a membrane-targeted red fluorescent protein tagRFP) fused to a T2A-peptide (myrCasp8ER^{T2}; Chu et al., 2008), the chicken β -globin intron and SV40 polyA sequences (GlpA). This resulted in the final construct pBS_Tol2-Sall-PC-EcoRI:FP-SpeI-T2A-myrCasp8ER^{T2}-XbaI-GlpA-NotI (Fig. 1B). The cell type-specific promoter and the FP can be exchanged using unique Sall/EcoRI and EcoRI/SpeI sites, respectively.

For transient transgenic assays, transgene cassettes encoding FyntagRFP-T, FyntagRFP-T-T2A-CaspaseER^{T2} or FynVenus-T2A-CaspaseER^{T2} flanked by 5×UAS-E1b and globin intron-SV40 polyA (Distel et al., 2009), were cloned into pDon122, allowing for Tol1-mediated genome integrations (Koga et al., 2008).

Generation of transgenic zebrafish

Constructs were injected into fertilized eggs at the one-cell stage of wild-type embryos together with Tol2 transposase mRNA (Kawakami, 2007). Embryos displaying PC-specific fluorescence at 4 dpf were raised to adulthood. Transgene inheritance was followed to the F3 generation. Initially, three independent lines were established, two of which displayed efficient PC ablation. One of these was subsequently maintained and named Tg(*ca8:FMA-TagRFP-2A-casp8ERT2*)^{bz11Tg} or PC-ATTACTM.

Cell ablation

For transient transgenic cell ablation, pDon122 Gal4-dependent Caspase8ER^{T2} constructs were injected into Gal4 expression lines together with *tol1* mRNA (25 ng/μl each) at the one-cell stage. Dechorionated (1 mg/ml pronase) 24 hpf embryos were treated with either 0.5% EtOH or 5 μM 4-hydroxy-tamoxifen (4OHT, Sigma Aldrich) or 3 μM staurosporine (Sigma Aldrich) in 30% Danieau medium. Apoptotic cells were detected by staining for 1 h in 10 μg/ml Acridine Orange (Sigma Aldrich)/30% Danieau and observed under a confocal microscope.

For stable transgenic ablation in 24-well plates, up to six heterozygous PC-ATTACTM larvae were kept in 1 ml 30% Danieau/PTU supplemented with 4OHT at a final concentration of 5×10⁻⁶ M from stock (10⁻³ M in EtOH) that was stored at -20°C and protected from light. Incubation was performed at 28°C in the dark for up to 16 h followed by transfer into fresh 30% Danieau/PTU. Each experiment was paralleled by controls with embryos treated in 0.5% EtOH.

Imaging

Larvae were anesthetized in 0.04 mg/ml tricaine (MS-222, Sigma) and embedded in 1% ultra low melting agarose dissolved in 30% Danieau/PTU. Images were recorded with a confocal microscope (LSM510, Zeiss and SP8 Leica) using a 40×ApoChromat water immersion objective (NA 1.2) and subsequently processed with respective microscope software, ImageJ (NIH), Photoshop and Illustrator (Adobe).

Purkinje cells were counted manually from confocal z-stacks throughout the complete right cerebellar hemisphere. Microglia were counted in the right cerebellar hemisphere and compared with part of the optic tectum of similar size (55×80 μm).

RT-PCR

After treatment with 4OHT or EtOH, total RNA was extracted from pools of five embryo heads using peqGOLD (Peqlab). Total RNA (2 μg) was reverse transcribed with 50 μM oligo(dT) primers, 2.5 mM dNTPs and 10 U AMV reverse transcriptase (Promega) for 1 h at 42°C. RT-PCR experiments (*n*=3) were performed with GoTaq (Promega). β -Actin mRNA expression was used to normalize the expression of RFP. The following primers were used: β -actin fp, 5'-TCTGTTGGCTTTGGGATTC-3'; β -actin rp, 5'-TCTGTTGGCTTTGGGATTC-3'; tagRFP fp, 5'-ATGGTGTCTAAGGGCGAAGAGCTGAT-3'; tagRFP rf, 5'-TCAATTAAGTTGTGCCCCAGTTTGC-3'.

Expression analysis

For whole-mount *in situ* hybridization, larvae were fixed in 4%PFA/PBS overnight and processed according to an established protocol (Volkman et al., 2010).

To detect Venus cleaved Caspase 3 by immunohistochemistry, embryos at 1 dpf were fixed in 4%PFA/PBS 6 h after treatment with EtOH or 4OHT. Larvae at 4 or 5 dpf were fixed after 4OHT treatment in 4%PFA/PBS for 3 h or overnight. For sectioning, brains from adults older than 15 months were fixed with 4%PFA/PBS overnight at 4°C followed by cryoprotection in 20% sucrose/PBS. After embedding in Tissue-Tek (OCT), cryotome sections were sectioned at 20 μm.

The following primary antibodies were used: polyclonal rabbit IgG cleaved Caspase 3 (1:2000, Abcam, ab13847), polyclonal chicken IgY GFP (1:1000, Aves labs, GFP-1020), monoclonal mouse ZebrinII (1:300, a kind gift from Richard Hawkes, ACHR, University of Calgary), polyclonal rabbit IgG tRFP (1:2000, Evrogen, AB233) and monoclonal rabbit Caspase 8 IgG (EPR17366) (1:2000, Abcam, ab181580). The following secondary antibodies were used: donkey anti-chick IgY FITC (1:1000, Jackson ImmunoResearch, 703-545-155), donkey anti-rabbit IgG Alexa647 (1:1000, Thermo Fisher Scientific, A-31573), donkey anti-rabbit IgG Alexa546 (1:500, Thermo Fisher Scientific, A-10040) and donkey anti-mouse IgG Alexa488 (1:500, Thermo Fisher Scientific, A-21202).

Western blot analysis

For western blot analysis, dissected adult cerebellar tissues were homogenized with a hand pestle in 80 μl of NP40 lysis buffer (20 mM Tris-HCl, 180 mM NaCl, 1 mM EDTA and 0.5% NP40) and further lyzed by sonication (Bioruptor UCD-300 TM ultrasound sonicator, Diagenode). 12.5 μl of the cleared lysate per lane was subjected to 10% SDS polyacrylamide gel electrophoresis, and transferred to a PVDF membrane (Membrane Roti-Fluoro PVDF, Carl Roth). The following primary antibodies were used: polyclonal rabbit tRFP antibody (1:2000, Evrogen, AB233) and monoclonal rabbit caspase 8 (EPR17366) (1:1000, Abcam, ab181580). As a secondary antibody HRP-conjugated goat anti-rabbit IgG antibody (1:10,000, Jackson ImmunoResearch, 111-035-144) was used and visualized by chemiluminescence (SERVALight Eos CL HRP WB Substrate Kit, Serva Electrophoresis).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.W., K.N., B.W., K.M.-B. and R.W.K. designed the experiments and analyzed data. T.W., K.N., B.W. and K.M.-B. performed experiments. All authors wrote the manuscript.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.122721.supplemental>

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