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Krüpple-like factors 7 and 6a mRNA Expression in Adult Zebrafish Central Nervous System

Sunil Bhattarai, **Alicja Sochacka-Marlowe**, **Gerald Crutchfield**, **Ramisha Khan**, **Richard Londraville**, and **Qin Liu***

Department of Biology and Integrated Bioscience Program, University of Akron, Akron, OH 44325

Abstract

Krüpple-like factors (KLFs) are transcription factors with zinc finger DNA binding domains known to play important roles in brain development and central nervous system (CNS) regeneration. There is little information on KLFs expression in adult vertebrate CNS. In this study, we used in situ hybridization to examine Klf7 mRNA (*klf7*) and Klf6a mRNA (*klf6a*) expression in adult zebrafish CNS. Both *klfs* exhibit wide and similar expression in the zebrafish CNS. Brain areas containing strongly labeled cells include the ventricular regions of the dorsomedial telencephalon, the ventromedial telencephalon, periventricular regions of the thalamus and hypothalamus, torus longitudinalis, stratum periventriculare of the optic tectum, granular regions of the cerebellar body and valvula, and superficial layers of the facial and vagal lobes. In the spinal cord, *klf7*- and *klf6a*-expressing cells are found in both the dorsal and ventral horns. Numerous sensory structures (e.g. auditory, lateral line, olfactory and visual) and several motor nuclei (e.g. oculomotor, trigeminal, and vagal motor nuclei) contain *klf7*- and/or *klf6a*-expressing cells. Our results may provide useful information for determining these Klfs in maintenance and/or function in adult CNS.

Graphical abstract

* To whom correspondence should be addressed. Phone: 330-972-7558; Fax: 330-972-8445; qliu@uakron.edu.

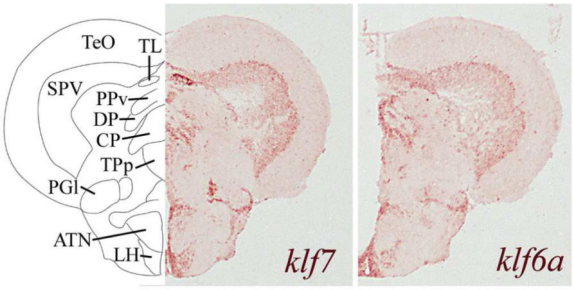
Authors' contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. QL designed and supervised the study.

SB, AMS, GC, RK and QL conducted the experiments. SB, AMS, GC, RLL and QL analyzed the data and wrote the manuscript.

Conflict of interest statement

There is no known conflict of interest to declare from all the authors.



Keywords

zinc-finger binding transcription factors; brain; spinal cord; visual structures; auditory nuclei

1. Introduction

Krüpple-like factors (KLFs) are a family of transcription factors containing highly conserved three zinc-finger DNA binding domains near their carboxyl ends that bind to GC/CT-rich regions in the promoter of their target genes (reviewed by Bieker, 2001; McConnell and Yang, 2010; Moore et al., 2011; Knoedler and Denver, 2014). Most KLFs can be assigned to three groups based mainly on their similarities in their N-terminal domains (Bieker, 2001; Moore et al., 2011). KLF members in group 1 (e.g. KLF3, 8 and 12) contain a PVALS/T N-terminal consensus sequence known to interact with co-repressors of the C-terminal binding proteins. All members in group 2 have shared acidic activation and inhibitory domains (e.g. KLF1, 2 and 4), or shared acidic activation and hydrophobic S-rich domains (e.g. KLF6 and 7) in their N-termini. KLFs in group 3 (KLF9, 10, 11, 13, 14 and 16) share a consensus Sin3a-interacting domain located near their N-terminal ends. The remaining KLFs (e.g. KLF5, 15 and 17) are not classified into any of the above groups because they lack any identifiable consensus motifs other than the three zinc-finger binding domains (Moore et al., 2011).

KLFs are expressed in almost all major tissues and organs including both CNS and peripheral nervous system (reviewed by McConnell and Yang, 2010). Although most KLFs have wide expression, each KLF has strong expression in limited tissues and organs. For example, KLF3 (also called basic KLF or erythroid KLF) is widely expressed, but is strongly expressed in erythroid cells (Crossley et al., 1996). At the cellular level, KLFs are regulators of cell differentiation, proliferation and apoptosis (reviewed by McConnell and Yang, 2010). KLFs play roles in numerous developmental and physiological processes including adipocyte differentiation, angiogenesis, B- and T-cell development, bone development, cardiac development, intestinal epithelial homeostasis, lung development, neurogenesis and regeneration, regulation of uterine receptivity to pregnancy, and somatic cell reprograming (Bieker, 2001; McConnell and Yang, 2010; Moore et al., 2011; Knoedler and Denver, 2014).

KLF7 is a member of the group 2 KLFs (see above). Its mRNA (*Klf7*/*klf7*) is strongly expressed in developing mouse (Laub et al., 2001a) and zebrafish CNS (Thisse and Thisse, 2004; Li et al., 2010; Xue et al., 2015). *Klf7* mutant mice exhibit defects in several brain

structures including olfactory bulb, cerebral cortex, hippocampus and retinal axons (Laub et al., 2005). Veldman and colleagues (2007) showed that zebrafish *klf7* (called *klf7a* in the article, but since reassigned to *klf7b* or ubiquitous *klf7*) is greatly up-regulated in the retinal ganglion cells (RGCs) following optic nerve crush in adult zebrafish. Moreover, Klf7, working together with Klf6a, promotes retinal axon regeneration *in vitro* (Veldman et al., 2007). A recent *in vitro* study in rats also demonstrates that KLF7 enhances axon regeneration in corticospinal tracts (Blackmore et al., 2012). Like KLF7, KLF6 belongs to the group 2 KLFs (see above). *Klf6*/*klf6a* mRNA is also expressed in the CNS of embryonic mice and zebrafish, but its expression domains are more limited compared to *Klf7*/*klf7* (Laub et al., 2001b; Thisse et al., 2001; Xue et al., 2015). There is a wealth of information on KLF6/Klf6a roles in tumor suppression and pathogenesis of several cancers (reviewed by Andreoli et al., 2010; Moore et al., 2011), and erythroid maturation (Xue et al., 2015), but there is little published data on KLF6/Klf6a involvement in neural development and function. Also similar to *klf7*, *klf6a* expression is greatly increased in the regenerating RGCs in adult zebrafish, and Klf6a likely promotes successful optic nerve regeneration (Veldman et al., 2007).

Most studies on KLFs/Klfs have been carried out in non-nervous tissues in mammals (Moore et al., 2011). There is little information on KLFs/*Klfs* expression and function in CNS of nonmammals, and no published reports, to the best of our knowledge, on *klf7* and *klf6a* expression in adult zebrafish CNS. As the first step in determining these two Klfs functional roles in normal and/or regenerating CNS of adult zebrafish, a model organism for studying neural development, adult neurogenesis, regeneration and human disorder (reviewed by Santana et al., 2012; Schmidt et al., 2013; Stewart et al., 2014), we examined *klf7* and *klf6a* expression in the adult zebrafish CNS.

2. RESULTS

Staining pattern and intensity for each *klf* cRNA probe were similar for all twelve brains. *klf7* appeared to have slightly wider and/or stronger staining in most brain regions, schematic drawings for helping localization of their distributions in the brain were made using selective tissue sections processed for *klf7* staining as templates. We therefore first describe *klf7* expression, followed by *klf6a* expression, proceeding from anterior to posterior brain regions. Figure 1 shows a schematic drawing of a lateral view of an adult zebrafish brain indicating levels and angles of cross sections in all subsequent figures.

Telencephalon

The zebrafish telencephalon is similar to that of most other vertebrates in that it consists of a pair of anteroventrally situated olfactory bulbs and two posterodorsally located telencephalic hemispheres (Wullimann et al., 1996). *klf7* expression was detected in both structures (Fig. 2). In the olfactory bulb, *klf7*-expressing cells were mainly concentrated in the central region, the internal cellular layer (ICL, Fig. 2E). A few labeled cells were seen scattered in the peripheral regions of the olfactory bulb: the external cellular (ECL containing mitral cells) and glomerular layers (GL, Fig. 2E). In the anterodorsal telencephalon (i.e. anterior pallium), *klf7*-expressing cells appeared to be evenly distributed throughout the region (Fig.

2A middle panel, and C), with ventricular cells, especially in the medial areas, showing stronger labeling than deeper cells. Careful observation of the *klf* expression in this region revealed that there was a border between the stronger and weaker labeled areas in the ventricular layer (vertical arrowhead in Fig. 2C). Interestingly, a difference in *klf7* staining (less obvious than that in the ventricular layer) also appeared to exist in the deeper part of the dorsal telencephalon (horizontal arrowheads and diagonal arrow in Fig. 2A middle panel, diagonal arrow and horizontal arrowhead in C). In the precommissural telencephalon (Fig. 2B and G), there was more *klf7* expression in the dorsomedial region (Dm, medial to the sulcus ypsiloniformis, SY) than the remaining dorsal telencephalon (i.e. central and lateral zones, Dc and Dl, respectively). Like the dorsal telencephalon at the more anterior level, the ventricular cells were more strongly labeled than deeper cells (Fig. 2G), and the ventricular cells medial to SY had stronger staining than those lateral to SY. In the ventral telencephalon (i.e. subpallium), *klf7* expression was detected in the dorsal and ventral nuclei of the ventral telencephalic area (Vd and Vv respectively), and the central nucleus of the telencephalic area (Vc) (Fig. 2B middle panel, and I).

klf6a expression in the olfactory bulb (Fig. 2F) and the anterodorsal telencephalon (Fig. 2A right panel, and D) was similar to *klf7*, showing stronger staining in ventricular cells in the medial region (vertical arrowhead in Fig. 2D) and a difference in *klf6a* expression in deeper telencephalon (horizontal arrowheads and diagonal arrow in Fig. 2A right panel, and horizontal arrowhead in D). *klf6a* expression in the precommissural telencephalon (Fig. 2B right panel, H and J) was also similar to *klf7* expression in this region, except that there was a less obvious difference in *klf6a* staining between the Dm and Dc in both the ventricular and deeper brain areas (Fig. 2H).

In the telencephalon at a level immediately post-anterior commissure (Fig. 3), *klf7* expression in the lateral zone of the dorsal telencephalic area (Dl) became increased (Fig. 3A middle panel, and C), and ventricular cells adjacent to the sulcus ypsiloniformis were more strongly labeled than most cells in the dorsal telencephalon (Fig. 3C). In the ventral telencephalon, *klf7* expression was found in the postcommissural nucleus of ventral telencephalic area (Vp, Fig. 3A middle panel), and the entopeduncular nucleus (EN, Fig. 3E). At a more caudal level of the telencephalon, *klf7* expression was stronger in the ventricular regions and the posterior zone of the dorsal telencephalic area (Dp, Fig. 3B middle panel, and G). *klf6a* expression in these brain regions (Fig. 3A right panel, D, B right panel, and H) was almost identical to the *klf7* expression.

Preoptic region and hypothalamus

In the preoptic region, *klf7-expressing* cells were found in both the anterior part (Fig. 3E and I) and posterior part (Fig. 4C) of the parvocellular preoptic nucleus (PPa and PPp, respectively). The suprachiasmatic nucleus (SC) of the preoptic region also contained *klf7*-expressing cells (Fig. 4C). Most *klf7*-expressing cells in the hypothalamus (Hy) were found in periventricular regions including the ventral zone of the periventricular nucleus (Hv, Figs. 4E, 4I, 5D, and 6B), anterior tuberal nucleus (ATN, Figs. 4I and 5D), lateral hypothalamus (LH, Fig. 5D), and caudal zone of the periventricular hypothalamus (Hc, Fig. 6F). The dorsal zone of the periventricular hypothalamus (Hd), located in more lateral hypothalamic

regions, also contained *klf7*-expressing cells (Fig. 6B, F, and I), while scattered *klf7*-expressing cells were detected in laterally situated torus lateralis (TLa, Fig. 6F) and the diffuse nucleus of the inferior lobe (DIL, located in lateral and posterior regions) of the hypothalamus (Figs. 5E, 6F, 6I and 6K). *klf6a* expression in the preoptic (Figs. 3F, 3J, 4D) and hypothalamic regions (Figs. 4F, 4J, 5E, 6D, 6H, 6J and 6L) was in general similar to *klf7* expression, except that the *klf6a*-expressing domain in SC (Fig. 4D) was smaller than that of *klf7* (Fig. 4C).

Diencephalon

klf7 was expressed in the bed nucleus of the stria medullaris (BNSM, the former ventral part of the entopeduncular nucleus), one of the most anterior structures of the zebrafish diencephalon (Muller and Guo, 2009). The anterior portion of BNSM contained a few *klf7*-expressing cells (Fig. 3A middle panel and E, near the anterior commissure level), but its expression was stronger at a level near the optic chiasm, outlining the lateral half of the lateral forebrain bundle (LFB, Fig. 3B middle panel, and I). In the epithalamus, *klf7* expression was observed in both the dorsal part (Had) and ventral part (Hav) of the habenula (Ha, Fig. 4B middle panel, C and G), while there was no obvious *klf7* expression in the dorsal saccus (data not shown). In the dorsal thalamus, *klf7* expression was found in the anterior thalamic nucleus (A, Fig. 4C and G), dorsal posterior thalamic nucleus (DP), intercalated nucleus (IC), and central posterior thalamic nucleus (CP) (Fig. 5B). *klf7* expression in the ventral thalamus was mainly found in the ventromedial nucleus (VM), while there was weak labeling in the ventrolateral nucleus (VL) (Fig. 4C and G). In the posterior tuberculum, *klf7*-expressing cells were found in the periventricular nucleus of the posterior tuberculum (TPp, Fig. 4G and I), posterior tuberal nucleus (PTN, Figs. 5D and 6B), anterior, lateral and medial preglomerular nuclei (PGa, PGl and PGm, respectively, Figs. 4I, 5D and 6B), and corpus mamillare (CM, Fig. 6F, I and K). The *klf7* staining in CM was consistently stronger than that in most other brain regions. *klf6a* had similar expression as *klf7* in Ha (Fig. 4D and H), DP, IC and CP (Fig. 5C), VM and VL (Fig. 4D), TPp (Fig. 4H), PTN (Figs. 5E and 6D), and the preglomerular nuclei (Figs. 4J, 5E, 6D). *klf6a* was also expressed in CM (Fig. 6H, J and L), but unlike *klf7*, there was no apparent difference in *klf6a* staining intensity between CM and other brain regions.

In the pretectum, *klf7* expression was detected in several pretectal nuclei including the magnocellular superficial pretectal nucleus (PSm, 4C), central pretectal nucleus (CPN, Fig. 4G), posterior pretectal nucleus (PO, Fig. 4G), ventral part of the periventricular nucleus (PPv, Fig. 5B). However, *klf7* expression in CPN, PSm and PO was limited to a few cells that partially outlined these structures. The nucleus of the medial longitudinal fascicle (NMLF), derived from the prosomere one in zebrafish (Wullimann and Puelles, 1999; Wullimann and Mueller, 2004), contained a few scattered *klf7*-expressing cells (Fig. 6E). *klf6a* expression was also detected in all of these pretectal structures (Figs. 4D, 4H, 5C) and NMLF (Fig. 6G).

Mesencephalon

Like most other nonmammalian vertebrates, the zebrafish mesencephalon consists of dorsally localized optic tectum and torus longitudinalis, and ventrally situated torus

semicircularis and tegmentum (Wullimann et al., 1996). According to Vanegas (1983), the teleost optic tectum (TeO) can be divided into six layers (from surface toward the tectal ventricle): stratum marginale (SM), stratum opticum (SO), stratum fibrosum et griseum superficiale (SFGS), stratum griseum centrale (SGC), stratum album centrale (SAC) and stratum periventriculare (SPV). *klf7* expression was mainly confined to SPV where most cells appeared to contain *klf7* (Fig. 7A middle panel, and E). There were only a few labeled cells scattered in the superficial layers such as SO and SAC (Fig. 7E). Most cells in the torus longitudinalis (TL) were strongly labeled with the *klf7* cRNA probes (Fig. 7C). The staining intensity of these *klf7*-expressing cells in TL was stronger than that in most other regions of the adult zebrafish brain (Figs. 6 and 7). *klf7* expression was detected in both the central nucleus (TSc) and ventrolateral nucleus (TSvl) of the torus semicircularis (TS, Fig. 7A, B middle panels, and I, Fig. 8A middle panel, and D). *klf7* expression was found mainly in the cortex areas of TS where most cell bodies reside, with clusters of labeled cells scattered within these two nuclei (Figs. 7I, 8D). In the tegmentum, structures that contained *klf7* expression included the dorsal tegmental nucleus (DTN) and oculomotor nucleus (NIII) (Fig. 7G). The zebrafish DTN contains many densely packed cells and most of them were *klf7*-expressing.

klf6a expression in the mesencephalon (Fig. 7A right panel, and F, H and J, Fig. 8A right panel, and E) was almost identical to *klf7* expression, except in TL where *klf6a* staining intensity was similar to that in SPV (Fig. 7D). In contrast, *klf7* staining in TL was much more intense than that in SPV (Fig. 7C).

Isthmus, rhombencephalon and spinal cord

Several structures in the isthmus contained *klf7*-expressing cells (Figs. 7G, 7I, 8B and 8D). These structures included the nucleus of the lateral lemniscus (NLL, Fig. 7G), perilemniscal nucleus (PL, Fig. 7I), griseum centrale (GC, Figs. 8B and 9C), locus coeruleus (LC, Fig. 8B), superior raphe (SR, Fig. 8B), lateral valvular nucleus (NLV Figs. 7I and 8D), and nucleus isthmi (NI, Fig. 8B and D). *klf6a*-expressing cells were found in all of these isthmus nuclei (Figs. 7H, 7J, 8C, 8E, and 9D).

The zebrafish cerebellum has three major parts: the cerebellar valvula consisting of the medial part (Vam) and lateral part (Val), the cerebellar body (CB), and the vestibulolateral lobe containing the medial caudal lobe (LCa) and the paired lateral granular eminence (EG) (Wullimann et al., 1996). *klf7* expression was found in all these three cerebellar structures, but its expression was confined to the granula regions (Figs. 7B middle panel, and 7G, 8A middle panel, and 8D, 9A and 9B middle panels, and M 9C). Moreover, it appeared that the vast majority of the cells in the granula regions contained *klf7*. *klf6a* had similar expression in the cerebellum (Figs. 7B right panel, 7H, 8A right panel, 8E, 9A and 9B right panels, and 9D).

In the medulla, nuclei of several cranial nerves expressed *klf7*. Both the sensory and motor nuclei of the trigeminal nerve (NVs and NVm, respectively, Fig. 9C), and nucleus of the descending trigeminal root (NDV, Fig. 9G) contained numerous *klf7*-expressing cells. Most cells in the cortex regions of the facial lobe (LVII, facial sensory nerve terminal site) were *klf7*-expressing (Fig. 11A middle panel, and B). The magnocellular octaval nucleus (MaON)

and descending octaval nucleus (DON), two of the cranial nerve VIII sensory nuclei, also contained a few *klf7*-expressing cells (Figs. 9G and 10B). Most cortex cells in the vagal lobe (LX, sensory terminal site of the X cranial nerve) were *klf7*-expressing (Fig. 11B). *klf7* expression was also observed in the vagal motor nucleus (NXm, Fig. 11B). The medial octavolateralis nucleus (MON), one of the sensory nuclei of the lateral line nerves in zebrafish, had numerous *klf7*-expressing cells (Figs. 9G and 10B). *klf6a*-expressing cells were detected in all the above mentioned medulla structures (Figs. 9D, 9H, 10C, and 11C).

Additional structures in the medulla that contained *klf7*-expressing cells included the secondary octaval population (Sop, Fig. 9G), medial funicular nucleus (MFN, Fig. 12B), commissural nucleus of Cajal (NC, Fig. 12B). There were only a few scattered *klf7*-expressing cells in NC but there were more labeled cells located in border regions between NC and MFN (Fig. 12B). *klf7*-expressing cells were also found surrounding the central canal (C) in the posterior medulla (Fig. 12D). Numerous *klf7*-expressing cells were detected in the reticular formation at all levels examined (Figs. 9E, 9I, 10D, 11D, and 12D). *klf7* staining patterns (e.g. clustered or scattered) and intensities (weak or strong) varied greatly among this complex and large structure. Finally, *klf7* expression in the spinal cord was observed in the dorsal horn (Dh, near the midline), ventral horn (Vh) and regions surrounding the central canal (Fig. 12F). *klf6a*-expressing cells were also detected in these medulla regions (Figs. 9F, 9H, 9J, 10E, 11E, 12C, 12E) and spinal cord (Fig. 12G), although its labeling patterns in the reticular formation varied at different levels, and were different from that of *klf7* (e.g. Fig. 9I and 9J).

3. Discussion

3.1. Comparison of klf7 and klf6a expression in adult zebrafish CNS

It is interesting and surprising that expression patterns of these two *klfs* show such a high degree of similarity in most regions of the adult zebrafish CNS. It is surprising because *klf7* and *klf6a* expression patterns are distinct in the embryonic stages: *klf6a* expression is more confined than *klf7* in the brain (see Introduction and below), and it is not expressed in the retinal ganglion cell layer (gcl) in two and three days old embryos (*klf7* is expressed in gcl at these stages, Veldman et al., 2007). To confirm their expression patterns in these tissues using our *klf* cRNA probes, we performed whole mount in situ hybridization and in situ hybridization on one to three days old embryonic zebrafish tissue sections, and on adult zebrafish eye sections. Our results (data not shown) confirmed those published findings. For example, our *klf7* cRNA probe labeled gcl of embryonic fish eyes, but not the adult retina, while our *klf6a* cRNA probe did not label either the embryonic or adult retinal tissues. The overall similar expression of *klf7* and *klf6a* in the adult zebrafish CNS suggests that these two Klfs may play a similar and/or redundant role in the function and maintenance of those CNS structures that contain both *klfs*. This is supported by the findings of Veldman et al. (2007) demonstrating that blocking Klf7 or Klf6a alone had no detectable effect on outgrowth of adult zebrafish retinal explants, while simultaneously blocking both Klfs significantly reduced the outgrowth of the retinal explants. The apparent difference in the *klf7* and *klf6a* expression in the reticular formation observed in this study may reflect an actual difference in their expression in this large and complex structure. Alternatively, it may

have resulted from using different (adjacent) tissue sections processed for each *klf* staining, because we noticed that in adult zebrafish brain, staining patterns for even the same cRNA probe varied substantially in the reticular formation of adjacent sections (16-32 μm apart), while similar expression of the same mRNA in dorsal structures (e.g. cerebellum) is often observed in these sections (Liu et al., unpublished observations).

3.2. Comparison of klf7 and klf6a expression in zebrafish CNS with their expression in other vertebrate CNS

In the vertebrate CNS, *Klf7*/*klf7* expression was examined in embryonic zebrafish (Thisse and Thisse, 2004; Li et al., 2010), developing and adult mice (Laub et al., 2001a; Lei et al., 2001). During mouse embryogenesis, *Klf7* expression becomes detectable in restricted regions in dorsal forebrain, midbrain, hindbrain and spinal cord in E9.5-E10.5 mice. *Klf7*-expressing domains and staining intensities become greatly increased in E11.5 mice CNS (Laub et al., 2001a). In addition, *Klf7* is also expressed by the developing retina. In the embryonic mouse brain, *Klf7*-expressing cells are mainly found in the mantle zone (lateral regions) where post-mitotic neurons reside. In the adult mouse CNS, *Klf7* expression is mainly restricted to granule cells in the cerebellum, although weak expression (based on staining intensity) was seen in the cortex, hippocampus and spinal cord (Laub et al., 2001a; Lei et al., 2001). This expression pattern is similar to that in zebrafish CNS in that *klf7* expression begins in restricted embryonic brain regions and its expression increases as development proceeds (Thisse and Thisse, 2004; Li et al., 2010); and *klf7* is strongly expressed in the granular regions of the adult cerebellum (this study). Moreover, similar regions (e.g. spinal cord ventral horn) and topographically corresponding brain regions including the cortex (central zone of the dorsal telencephalon) and hippocampus (dorsolateral zone of the telencephalon) in both species contain *klf7*-expressing cells. An obvious difference between mice and zebrafish in *klf7* expression in the adult CNS is that strong *klf7* expression, based on staining intensity, is maintained throughout the zebrafish brain and spinal cord, whereas strong *Klf7* expression is restricted mainly to the cerebellum in the adult mice. This difference may indicate conserved function of KLF7/Klf7 in the maintenance and function of the adult vertebrate cerebellum, while KLF7/Klf7 function in other adult brain areas may be more species specific.

Similar to *Klf7*/*klf7*, studies of *Klf6*/*klf6a* or KLF6 distribution in the CNS were conducted only in embryonic zebrafish (Thisse et al., 2001; Xue et al., 2015), developing (Laub et al., 2001b) and adult mice (Jeong et al., 2009), with KLF6 expression in the adult mouse brain limited to the forebrain. In the embryonic and neonatal mouse CNS (E11.5-P1), *Klf6* expression became apparent first in the caudal hindbrain (dorsal regions) and neural tube (ventral horn) (Laub et al., 2001b). As development proceeds, obvious *Klf6* is detected mainly in the cortical plate and hypothalamic regions, with low expression (judging by staining intensity) throughout the forebrain and midbrain (Laub et al., 2001b). Using immunohistochemical method, Jeong and colleagues (2009) showed that KLF6 is ubiquitously expressed in the adult mouse forebrain. Forebrain structures containing strongly labeled cells include the olfactory bulb, cerebral cortex, medial septal nucleus, hippocampus, amygdala, numerous thalamic regions (e.g. habenula) and hypothalamus (e.g. paraventricular nucleus). A somewhat similar *klf6a* expression pattern is observed in

developing (Thisse et al., 2001; Zhao et al., 2010; Xue et al., 2015) and adult zebrafish CNS (this study). Apparent *klf6a* expression is mainly found in the hindbrain (dorsal region) and spinal cord of young zebrafish embryos (e.g. 24 hours post fertilization, hpf), and its expression (weak and/or scattered) is observed also in other CNS regions including the fore- and midbrain in older embryos (30-72 hpf). In the forebrain of adult zebrafish, *klf6a* also exhibits a wide expression, especially in the olfactory bulb, periventricular regions of hypothalamus, and topographically corresponding brain regions such as the central zone of the dorsal telencephalon (cortex), dorsolateral zone of the telencephalon (hippocampus). This similar KLF6/*Klf6*/*klf6a* expression in the CNS of zebrafish and mice suggest a conserved role of this transcription factor in CNS development and maintenance in adult organisms. Moreover, most of these forebrain regions in adult mice also contain *Klf7* (see above), it will be interesting to perform a comparative study of *Klf7* and *Klf6* in the entire adult mouse CNS to see if these two *Klfs* have extensive overlapping expressing domains as their orthologs in zebrafish.

Our findings about differential *klf7* and *klf6a* expression by the ventricular cells (e.g. medial vs. lateral) and deep cells in the dorsal telencephalon (Figs. 2 and 3) provide additional support for the existence of distinct anatomical and/or function subdivisions in this zebrafish brain region (Wullimann et al., 1996; Mueller et al., 2011).

3.3. klf7 and klf6a expression in sensory systems

Our results show that both *klf7* and *klf6a* are expressed in structures of multiple sensory systems in the adult zebrafish CNS. Both *klfs* are found in the olfactory bulbs and in telencephalic regions including the dorsal and ventral nuclei of the ventral telencephalic area (Vd and Vv), and dorsoposterior zone of the telencephalon (Dp), that receive heavy olfactory inputs (Wullimann et al., 1996). In the visual system, the major brain targets of visual inputs, the pretectum and optic tectum, contain both *klf7*- and *klf6a*-expressing cells. *klf7* and *klf6a* are also detected in the nucleus isthmi that is reciprocally connected with the optic tectum and involved in processing visual information (Northmore and Gallagher, 2003). In addition, *klf7*- and *klf6a*-expressing cells are also present in most structures that comprise two ascending visual pathways in teleost fish: 1) retina- anterior thalamic nucleus-telencephalon, and 2) retina-optic tectumdorsal posterior thalamic nucleus -telencephalon. These two pathways resemble the geniculate and extrageniculate visual systems of mammals (Braford and Northcutt, 1983; Wullimann et al., 1996). There is no obvious *klf7* or *klf6a* expression in retinal ganglion cells (RGCs) of adult zebrafish (Veldman et al., 2007), but their expression becomes greatly increased twelve hours after optic nerve crush, and the increased *klf7* and *klf6a* expression in the RGCs is maintained in the regenerating retina until three weeks after the optic nerve injury when the regenerating retinal axon terminals cover the entire tectal lobe (Veldman et al., 2007). These results are consistent with the demonstrated roles of these KLFs/Klfs in retinal axon outgrowth and pathfinding in developing mice (Laub et al., 2005; Steketee et al., 2014), and in promoting retinal axon regeneration in adult zebrafish (Veldman et al., 2007) and mice (Moore et al., 2009).

In the auditory system, *klf7*-and *klf6a*-expressing cells are found in structures that belong to several auditory pathways including octaval nerve-cerebellar granular eminence (EG) and

octaval nerve-the magnocellular octaval nucleus (MaON) and descending octaval nucleus (DON) (Wullimann et al., 1996). DON projects to the secondary octaval population (Sop) and the central nucleus of the torus semicircularis (TSc), which in turn send axons to the central posterior thalamic nucleus (CP) (McCormick and Hernandez, 1996; Wullimann et al., 1996). *klf7* and *klf6a* expression is also detected in the lateral line circuit that mediate mechanoreception: medial octavolateralis nucleus (MON and CON)-ventrolateral nucleus of the torus semicircularis (TSvl)-lateral preglomerular nucleus (PGl)-dorsal telencephalon (Dm and Dl) (Wullimann et al., 1996). In addition, several structures involved in processing gustatory information, such as the facial (LVII) and vagal lobes (LX), medial funicular nucleus (MFN), and dorsomedial telencephalon (Dm) (Morita et al., 1980; Finger, 1983; Kanwal and Caprio, 1987), contain numerous *klf7*- and *klf6a*-expressing cells (Table 1).

3.4. klf7 and klf6a expression in the motor and premotor systems

Motor nuclei of several cranial nerves, including nucleus of the medial longitudinal fascicle (NMLF), oculomotor nucleus (NIII), trigeminal motor nucleus (NVm), and the vagal motor nucleus (NXm), contain *klf7*- and *klf6a*-expressing cells. Other *klf7*- and *klf6a*-expressing structures implicated in the control of movement include the superior raphe nucleus (SR), nucleus of the lateral lemniscus (NLL), reticular formation and motor neurons in the spinal cord (Wullimann et al., 1996). Finally, *klf7*- and *klf6a*-expressing cells are found in structures belonging to a premotor network containing the telencephalon, pretectum, torus longitudinalis (TL), optic tectum, dorsal tegmental nucleus (DTN), lateral valvular nucleus (NLV), cerebellum and spinal cord (Wullimann, 1994; Ikenaga et al., 2002; Folgueira et al., 2007).

3.5. Conclusions

This study presents a comprehensive and detailed expression of mRNAs of Klf7 and Klf6a, two transcription factors with zinc finger DNA binding domains, in the CNS of adult zebrafish. The wide and similar expression of *klf7* and *klf6*a in many structures of multiple sensory and motor systems in the adult fish brain suggests that they have similar roles in the maintenance and/or normal function of these systems. Findings from this study will set the stage for investigating Klf7 and Klf6a roles in the adult CNS.

4. EXPERIMENTAL PROCEDURE

4.1. Animals

Adult zebrafish (*Danio rerio*) of 12-18 months old were raised from embryos obtained from in house breeding of wild type adult zebrafish. Fish were kept in 10-gallon tanks at 28°C on a 12-h light/12-h dark cycle and maintained according to procedures described in the Zebrafish Book (Westerfield, 2007). All animal related procedures comply with NIH standards and were approved by the University of Akron Committees on Use and Care of Animals in Research.

4.2. Tissue preparation

Twelve adult zebrafish of both sexes were anesthetized in tricaine methanesulfonate (0.02%, Sigma) and killed by cervical transection. The fish brains and segments of spinal cord from

the thoracic region were quickly removed and placed in 4% paraformaldehyde (Fisher Scientifics, Waltham, MA) in 0.1 M phosphate buffered saline (PBS, pH=7.4) at 4°C overnight. Following the fixation, the tissues were washed three times, 10 min. each in PBS at room temperature. The tissues were placed in a 20% sucrose solution (in PBS) at 4°C overnight. The next day the tissues were embedded in a mixture (1:1) of 20% sucrose solution and OCT compound (Sakura, Netherlands) as described (Barthel and Raymond, 1990). The embedded tissues were stored at −80°C until cryosectioned. The tissue sections (16 μm) were collected on Fisher superfrost glass slides (Fisher Scientifics), air-dried for one hour at room temperature before stored at −20°C. Two sets of alternate-cross sections from each brain or spinal cord were collected, each set for staining one of the *klf* cRNA probes (see below).

4.3. In situ hybridization

cDNAs corresponding to the open reading frames of zebrafish *klf7* (GenBank Accession No. BC124329) and *klf6a* (GenBank Accession No. NM_201461), kindly provided by Dr. Daniel Goldman, were used as templates to generate respective antisense cRNA probes. Detailed information on the cloning (from 3-day post-optic nerve crush adult zebrafish retinal RNA) of these *klfs* cDNAs was described previously (Veldman et al., 2010). Procedures used for the generation of antisense digoxigenin (DIG)-labeled RNA probes, and in situ hybridization (ISH) were described in detail in Liu et al. (1999). Briefly for ISH, the tissue sections were rehydrated in decreasing concentrations of ethanol followed by treating with proteinase K (Roche, Indianapolis, IN). The tissue sections were incubated in 0.1 M triethanolamine (Sigma, St. Louis, MO), rinsed in 0.1 M triethanolamine with 0.25% acetic anhydride (Fisher Scientific), dehydrated in increasing concentrations of ethanol and air-dried. 75 μl hybridization solution (2 μg/ml of *klf7* or *klf6a* cRNA probes) was applied to the tissue sections on each slide, and the tissue sections were hybridized overnight at 58°C. Immunocytochmical detection of the DIG-labeled cRNA probes was performed by incubating the sections in a maleate buffer solution containing an alkaline phosphatase coupled anti-DIG Fab fragment antibody (Roche, 1:5,000). Visualization of the labeling was achieved by incubating the tissue sections in a solution made from dissolving a 4-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) tablet (Roche) in 10 ml of distilled water. Satisfactory labeling was achieved after overnight incubation at room temperature in dark.

4.4. Data Analysis

Processed tissue sections were observed under an Olympus BX51 microscope equipped with Normarski optics, and digital images were obtained with a SPOT Insight digital camera (Diagnostic Instrument Inc., Sterling Heights, MI) attached to the microscope. The images were adjusted for contrast and sharpness using Adobe Photoshop 6.0 software (San Jose, CA). Most Images from tissue sections show cross sections with dorsal side up. Neuroanatomy of the Zebrafish Brain by Wullimann et al. (1996) and more recent works by Rink and Wullimann (2001), Mueller et al. (2004), Castro et al. (2006a, b) and Mueller and Guo (2009) were used as the reference sources for identification of brain regions and terminology.

Acknowledgment

We would like to thank Dr. Daniel Goldman (University of Michigan) for providing the zebrafish *klf7* and *klf6a* cDNAs. This research was supported by NIH EY13879 (Q. Liu). S. Bhattarai, A. Sochacka, and Gerald Crutchfield were supported by teaching assistantships from the University of Akron.

4.5. Abbreviations

A	anterior thalamic nucleus
APN	accessory pretectal nucleus
ATN	anterior tuberal nucleus
BNSM	the bed nucleus of the stria medullaris
C	central canal
CC	cerebellar crest
CB	corpus cerebellar body
CBg	granular layer of CB
CBm	molecular layer of CB
CM	corpus mamillare
CP	central posterior thalamic nucleus
Cpop	postoptic commissure
Cpost	posterior commissure
CPN	central pretectal nucleus
Cven	ventral rhombencephalic commissure
D	dorsal telencephalic area
Dc	central zone of dorsal telencephalic area
Dh	dorsal horn of the spinal cord
DIL	diffuse nucleus of the inferior lobe
DiV	diencephalic ventricle
Dl	lateral zone of dorsal telencephalic area
Dm	medial zone of dorsal telencephalic area
DON	descending octaval nucleus
DOT	dorsomedial optic tract
DTN	dorsal tegmental nucleus

DP	dorsal posterior thalamic nucleus
Dp	posterior zone of dorsal telencephalic area
ECL	external cellular layer of olfactory bulb
EG	granular eminence
Fr	fasciculus retroflexus
GC	griseum centrale
GL	glomerular layer of olfactory bulb
Ha	Habenula
Had	dorsal habenular nucleus
Hav	ventral habenular nucleus
Hc	caudal zone of periventricular hypothalamus
Hd	dorsal zone of periventricular hypothalamus
Hv	ventral zone of periventricular hypothalamus
Hy	hypothalamus
IC	intercalated nucleus
ICL	internal cellular layer of olfactory bulb
IMRF	intermediate reticular formation
IRF	inferior reticular formation
LC	locus coeruleus
LCa	posterior cerebellar lobe
LCag	granular layer of LCa
LCam	molecular layer of LCa
LFB	lateral forebrain bundle
LH	lateral hypothalamic nucleus
LVII	facial lobe
LX	vagal lobe
Ma	Mauthner axon
MaON	magnocellular octaval nucleus
MFN	medial funicular nucleus

MLF	medial longitudinal fascicle
MON	medial octavolateralis nucleus
NC	commissural nucleus of Cajal
NDV	nucleus of the descending trigeminal root
NIII	oculomotor nucleus
NI	nucleus isthmi
NIn	interpeduncular nucleus
NLL	nucleus of the lateral lemniscus
NLV	lateral valvular nucleus
NMLF	nucleus of medial longitudinal fascicle
NVmd	trigeminal motor nucleus, dorsal part
NVs	primary sensory trigeminal nucleus
NXm	vagal motor nucleus
OB	olfactory bulb
ON	optic nerve
PGa	anterior preglomerular nucleus
PGl	lateral preglomerular nucleus
PGm	medial preglomerular nucleus
PL	perilemniscal nucleus
PPd	periventricular pretectal nucleus, dorsal part
PPa	parvocellular preoptic nucleus, anterior part
PPp	parvocellular preoptic nucleus, posterior part
PPv	periventricular pretectal nucleus, ventral part
PO	posterior pretectal nucleus
PS	superficial pretectal nucleus
PSm	magnocellular superficial pretectal nucleus
PSp	parvocellular superficial pretectal nucleus
PTN	posterior tuberal nucleus
RV	rhombencephalic ventricle

SAC	stratum album centrale
SC	suprachiasmatic nucleus
SFGS	stratum fibrosum et griseum superficiale
SGC	stratum griseum centrale
SM	stratum marginale
SO	stratum opticum
Sop	secondary octaval population
SPV	stratum periventriculare
SR	superior raphe
SRF	superior reticular formation
SY	sulcus ypsiloniformis
Tel	telencephalon
TeO	optic tectum
TeV	tectal ventricle
TL	torus longitudinalis
TLa	torus lateralis
TPp	periventricular nucleus of posterior tuberculum
TS	torus semicircularis
TSc	central nucleus of torus semicircularis
TSvl	ventrolateral nucleus of torus semicircularis
Va	valvula cerebelli
Val	lateral division of the valvula cerebelli
Vam	medial division of the valvula cerebelli
Vamg	granular layer of Vam
Vamm	molecular layer of Vam
Vc	central nucleus of ventral telencephalic area
Vd	dorsal nucleus of ventral telencephalic area
Vh	ventral horn of the spinal cord
VL	ventrolateral thalamic nucleus

VM	ventromedial thalamic nucleus
Vv	ventral nucleus of ventral telencephalic area
VIII	octaval nerve
X	vagal nerve

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Highlights of this study

1) Expression patterns of krüpple like factor 6a and 7 mRNAs in the entire brain and thoracic spinal cord of adult zebrafish were examined using in situ hybridization.

2) Both *klfs* exhibited wide and overlapping expression in most regions of the adult zebrafish CNS.

3) *klf6a* and *klf7* were detected in numerous structures in several sensory and motor systems.

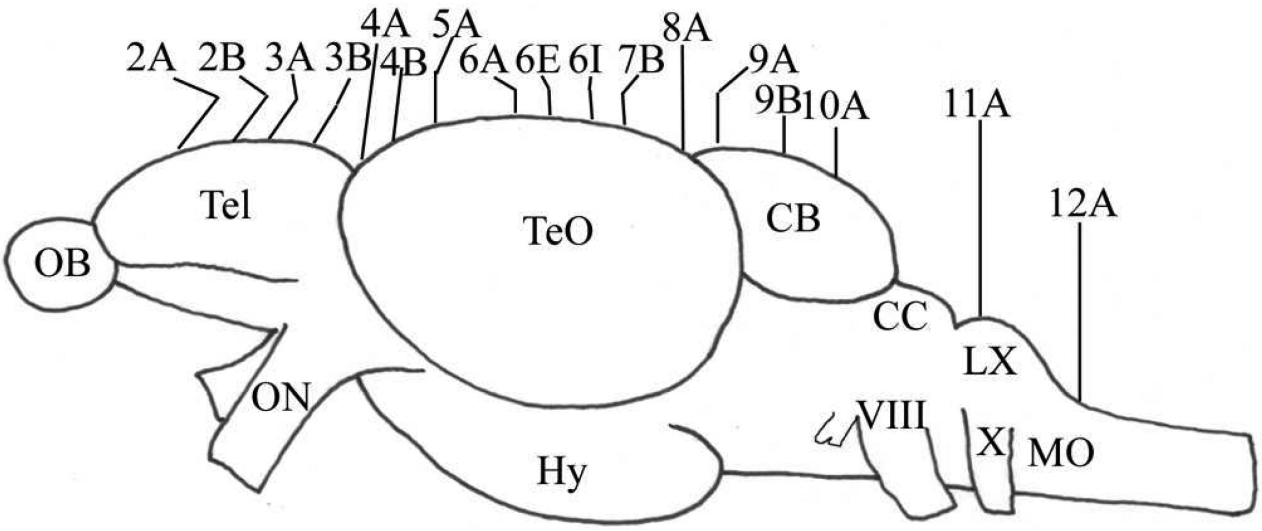

Figure 1.

Schematic drawing of an adult zebrafish brain (lateral view, anterior to the left and dorsal up) showing levels and angles of cross sections. Numbers and letters represent respective figure and panel numbers. See list for abbreviations.

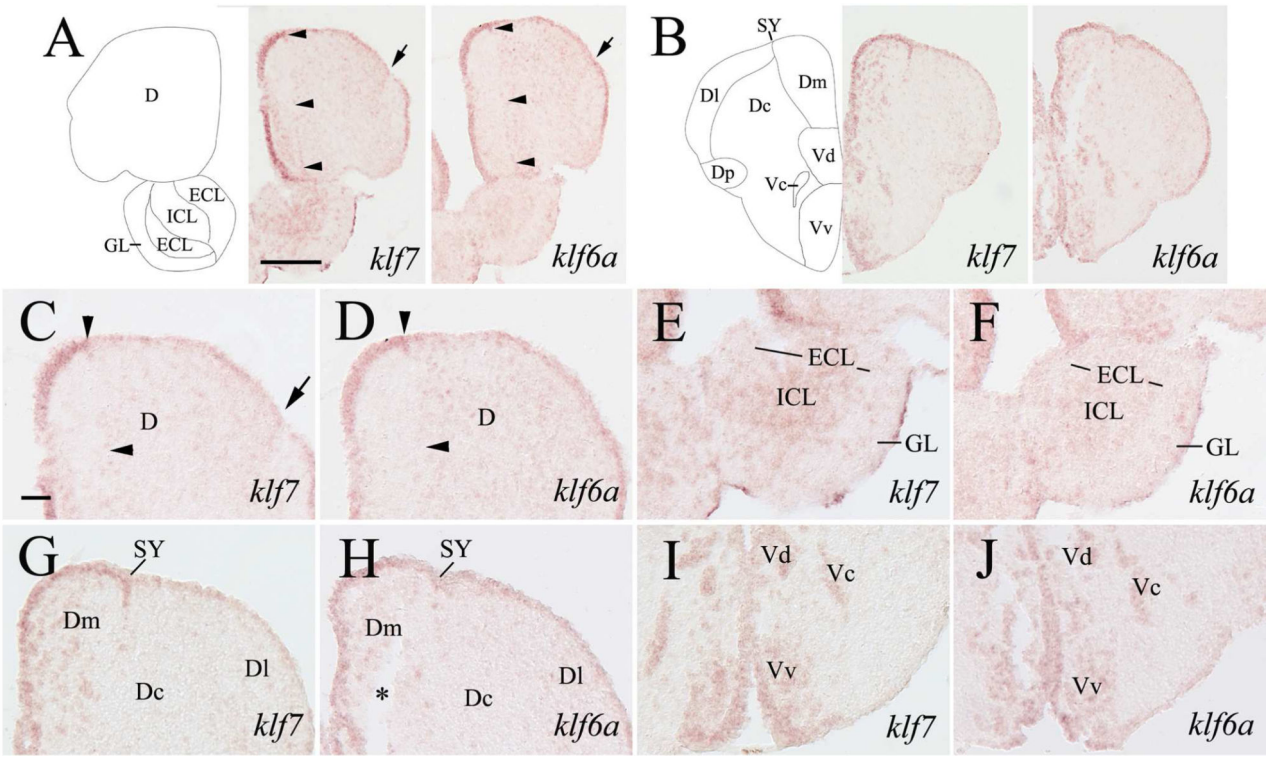

Fig. 2.

Expression of *klf7* and *klf6a* in the anterior telencephalon and olfactory bulb of adult zebrafish brain. In the top panels, a schematic drawing of half of the brain section (dorsal side up) processed for *klf7* staining is used to indicate major brain structures at this level, which is accompanied by low magnifications of half of the brain sections. Lower panels show higher magnifications of selective brain regions (same orientation) from their respective lower magnified views in the top panels. Similar arrangement is made for most of the subsequent figures. Panels C, D, G and H are higher magnifications of the dorsal halves of their corresponding images in the top panels, while panels E, F, I and J are magnified views of ventral portions of their corresponding images in the top panels. The diagonal arrow in panels A and C indicates a presumptive border region separating two brain regions with different *klf7* staining. Horizontal arrowheads in panels A, C and D point to a presumptive border region in deeper brain areas with different *klfs* expression on either side, while vertical arrowheads in panels C and D indicate a border region in the ventricular layer with different *klf* staining on either side. The asterisk in panel H indicates an artificial tissue crack. See list for abbreviations. The scale bar (200 μm) in panel A applies to all images in the top panels, while the scale bar (50 μm) in panel C is for all higher magnified images.

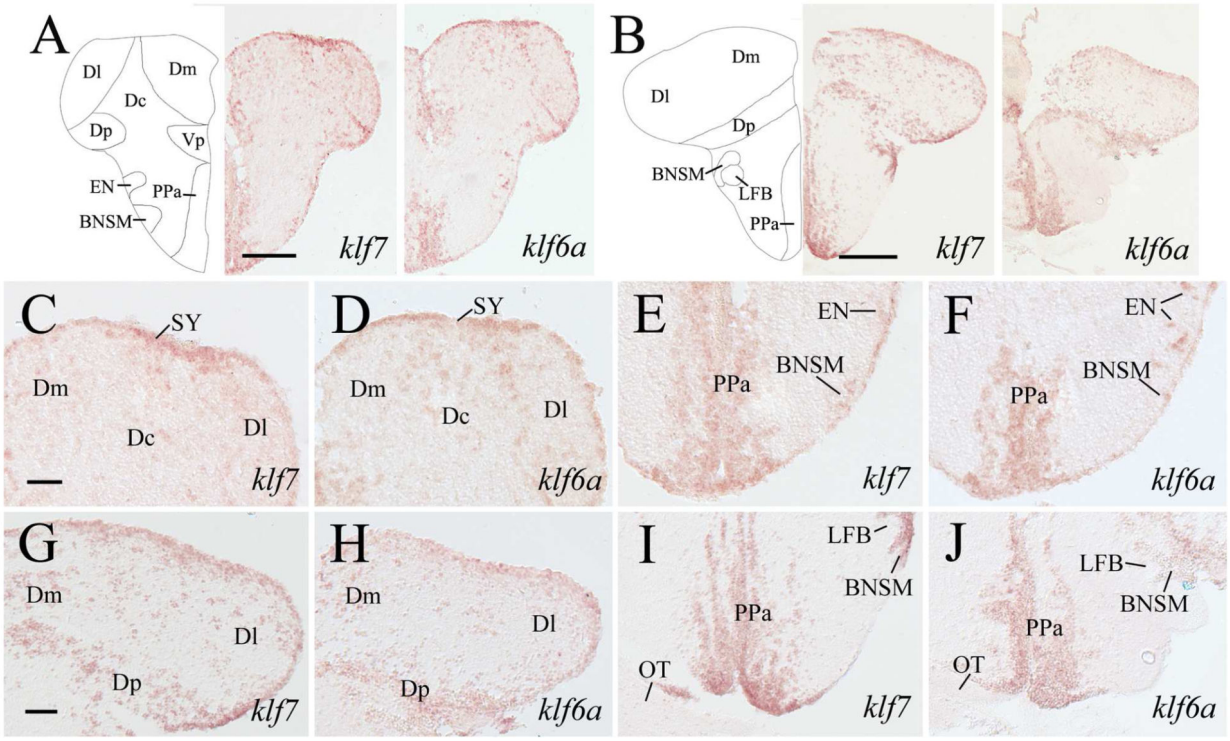

Figure 3.

Expression of *klf7* and *klf6a* in telencephalon at post anterior commissure levels. Top panels show low magnified views of halves of the brain sections, while the remaining panels show higher magnifications of the dorsal (panels C, D, G and H) or ventral (panels E, F, I and J) portions of their respective images in the top panels. See list for abbreviations. Scale bars = 200 μm for top panels, and 50 μm for the remaining panels. Images in the middle and bottom rows have the same magnifications, respectively.

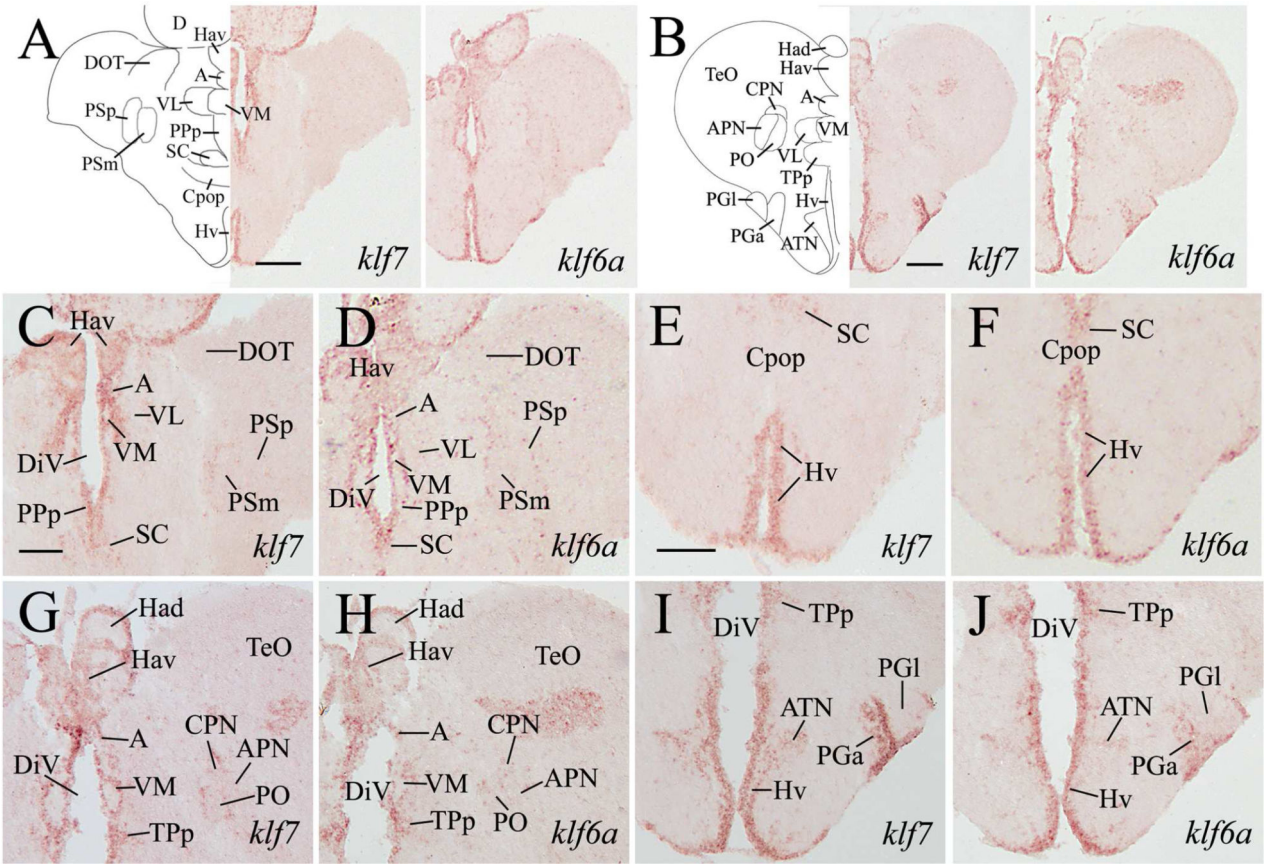

Figure 4.

Expression of *klf7* and *klf6a* in the preoptic, habenular, pretectal regions and diencephalon of the adult zebrafish brain. Images in the top panels are low magnified views of halves of brain sections from levels shown in Figure 1. Panels C, D, G and H (same magnification) are higher magnifications of the dorsomedial portion of their respective images in the top panels, while panels E, F, I and J (same magnification) are ventral halves of their corresponding images in the top panels. See list for abbreviations. Scale bar = 200 μm for the top panels, 50 μm for the remaining panels.

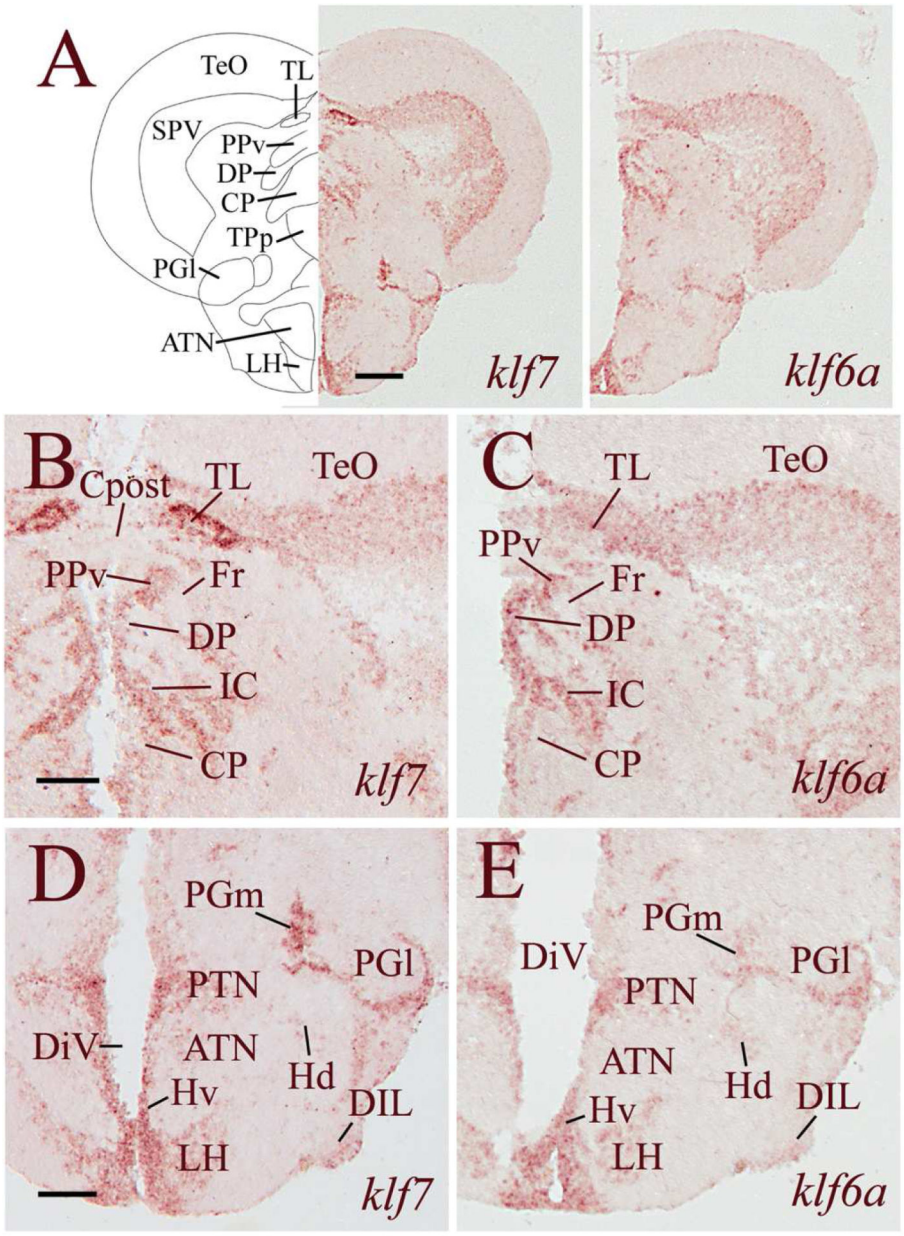

Figure 5.

Expression of *klf7* and *klf6a* in the diencephalon of the adult zebrafish brain. Top panels show low magnified views of halves of brain sections from a level shown in Figure 1. Middle and bottom panels show higher magnifications of parts of dorsomedial and ventral diencephalon, respectively, of their corresponding images in the top panels. See list for abbreviations. Scale bar = 200 μm for the top panels, 50 μm for the remaining panels.

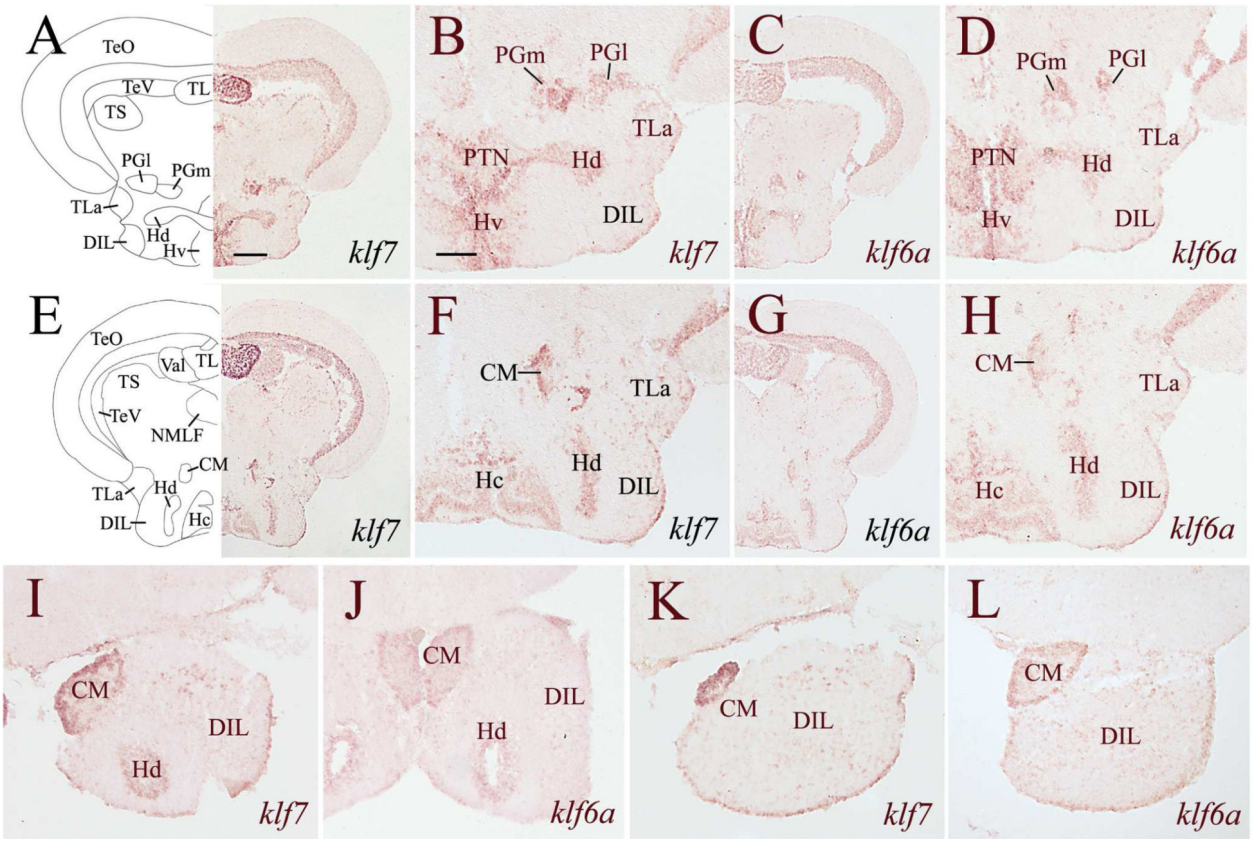

Figure 6.

Expression of *klf7*and *klf6a* in preglomerular nuclei and hypothalamus of the adult zebrafish brain. Panels A, C, E and G show low magnifications of halves of brain sections from levels shown in Figure 1. Panels B, D, F and H are higher magnifications of the preglomerular complex and hypothalamus of their respective lower magnified views on the left. Panels I to L show higher magnified views of more posterior hypothalamus. See list for abbreviations. All low magnified images have the same magnifications (Scale bar = 200 μm), and all higher magnified images have the same magnifications (Scale bar = 100 μm).

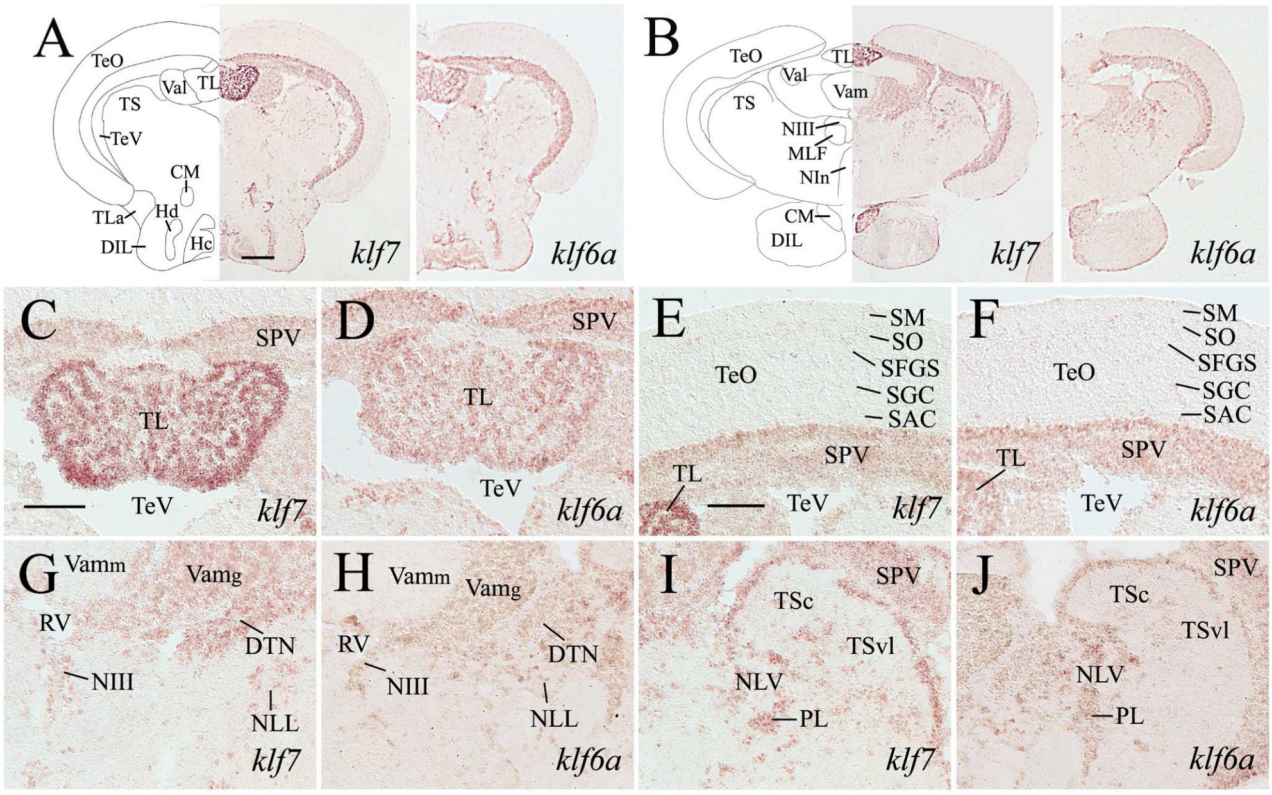

Figure 7.

Expression of *klf7* and *klf6a* in the torus longitudinalis, optic tectum, dorsal tegmentum, isthmus, torus semicircularis and anterior medulla of the adult zebrafish brain. Images in the top panels are low magnified (same magnification, scale bar = 200 μm) views of halves of brain sections from levels shown in Figure 1 (panel A is the same as Figure 6E). Middle and bottom panels are higher magnified views (C and D have the same magnification, while the remaining panels have the same magnification, scale bar = 100 μm) of portions of their respective images in the top panels. Panels C and D, E and F are torus longitudinalis and optic tectum, respectively. Panels G and H, I and J show dorsomedial tegmentum and torus semicircularis, respectively. See list for abbreviations.

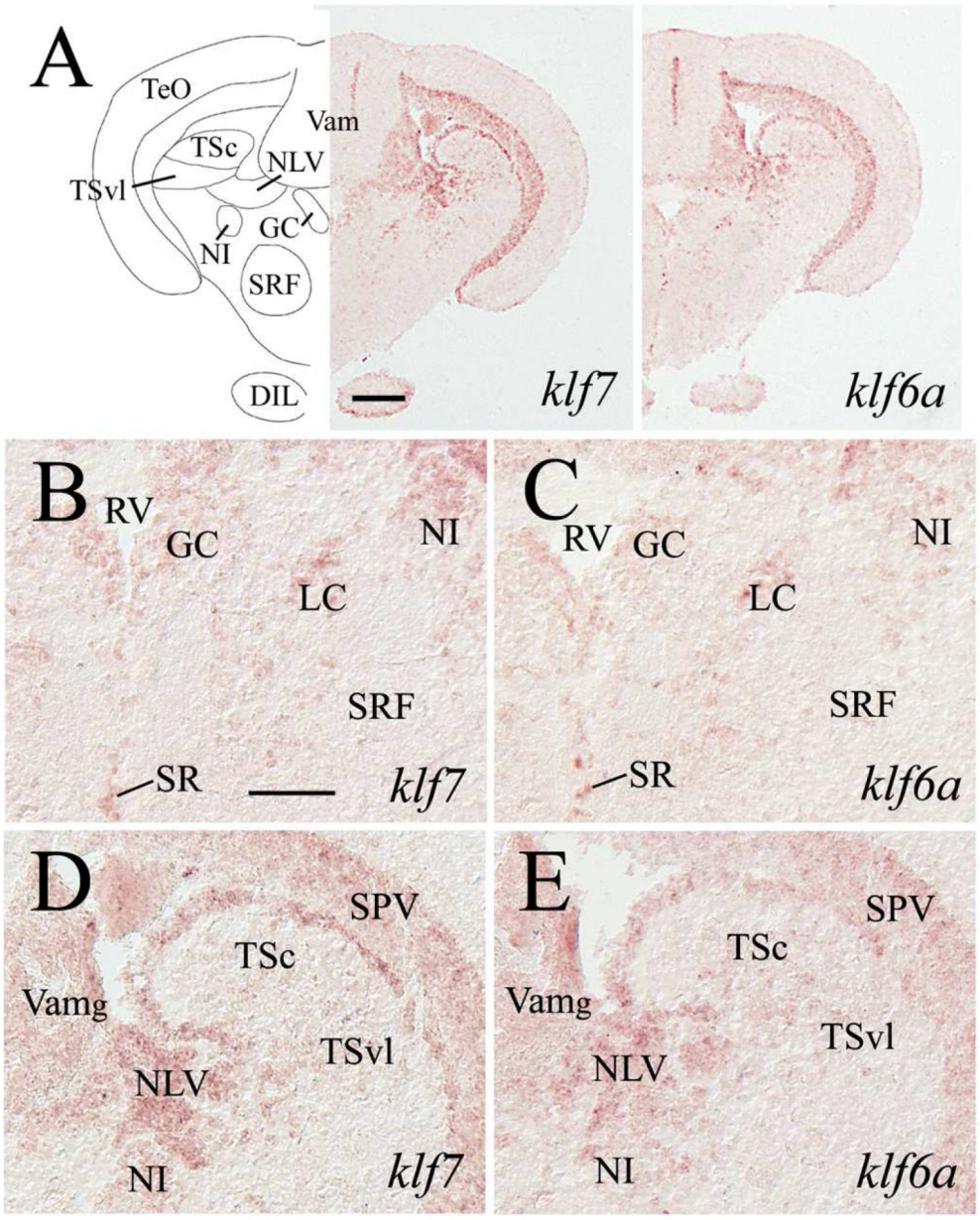

Figure 8.

Expression of *klf7* and *klf6a* in the dorsal tegmentum, isthmus, torus semicircularis and anterior medulla of the adult zebrafish brain. Top panels show lower magnified images (scale bar = 200 μm) of halves of brain sections from a level shown in Figure 1. Middle and bottom panels show higher magnified views (scale bar = 100 μm) of dorsomedial tegmentum and torus semicircularis, respectively. See list for abbreviations.

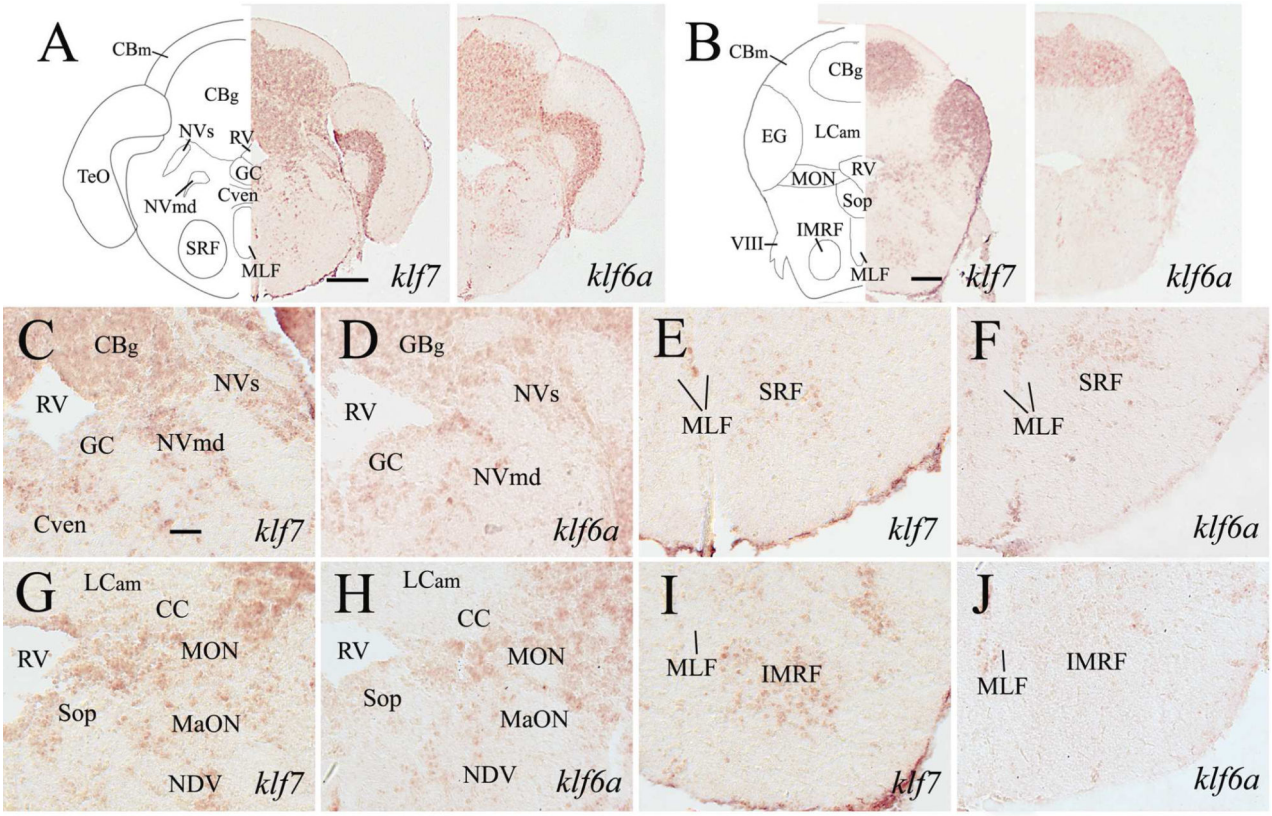

Figure 9

. Expression of *klf7* and *klf6a* in the cerebellum and medulla of the adult zebrafish brain. Top panels show lower magnified views of halves of the brain sections from levels indicated in Figure 1. The remaining panels are higher magnifications of the dorsomedial (C, D, G and H) or ventral portions (E, F, I and J) of the medulla of their corresponding images in the top. See list for abbreviations. Scale bar = 200 μm for the top panels, 50 μm for the remaining panels.

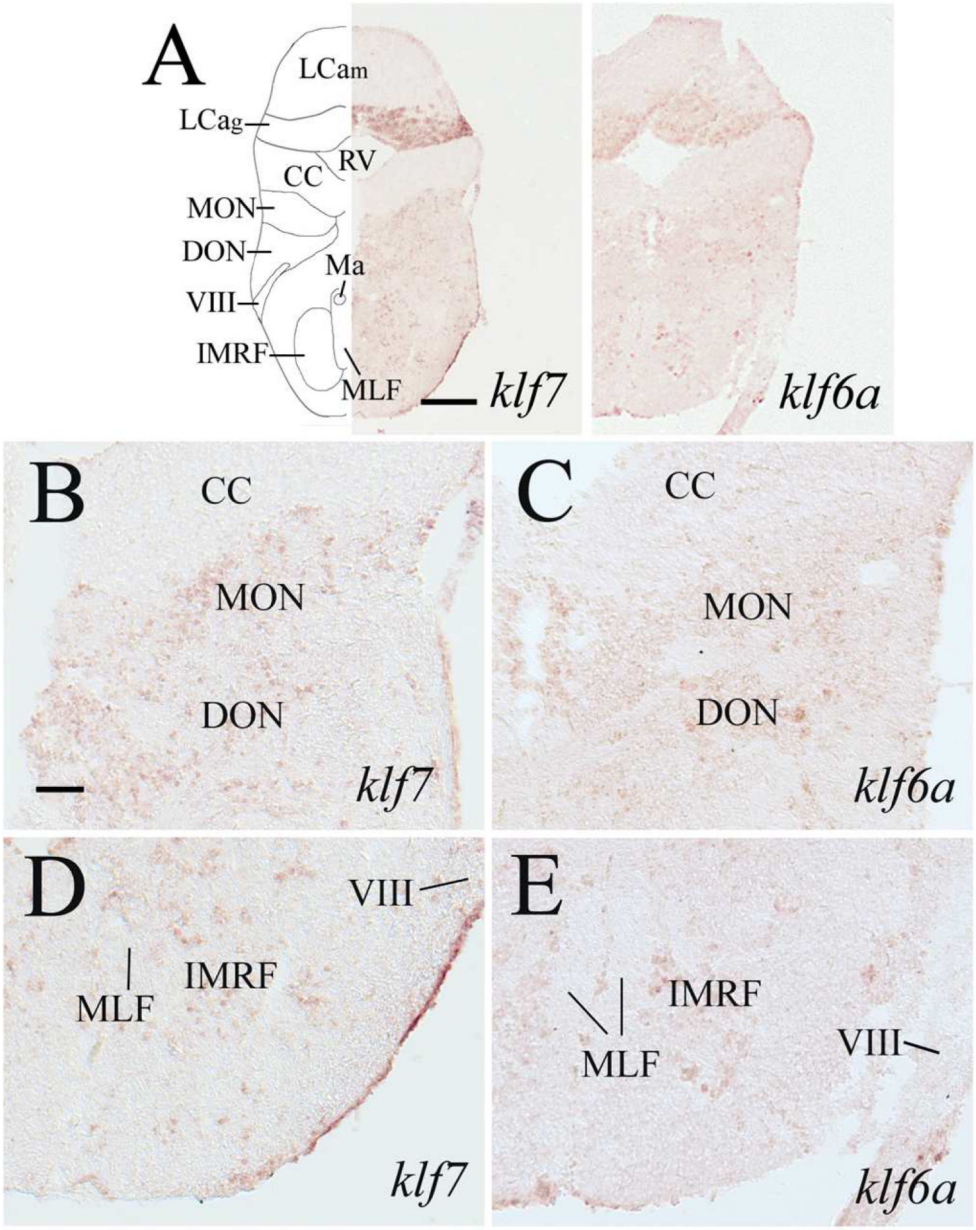

Figure 10.

Expression of *klf7* and *klf6a* in the posterior cerebellum and middle level medulla of the adult zebrafish brain. Top panels show lower magnified images (scale bar = 200 μm) of halves of brain sections from a level shown in Figure 1. Middle and bottom panels show higher magnified views (scale bar = 50 μm) of dorsal and ventral portions, respectively, of their corresponding images in the top panels. See list for abbreviations.

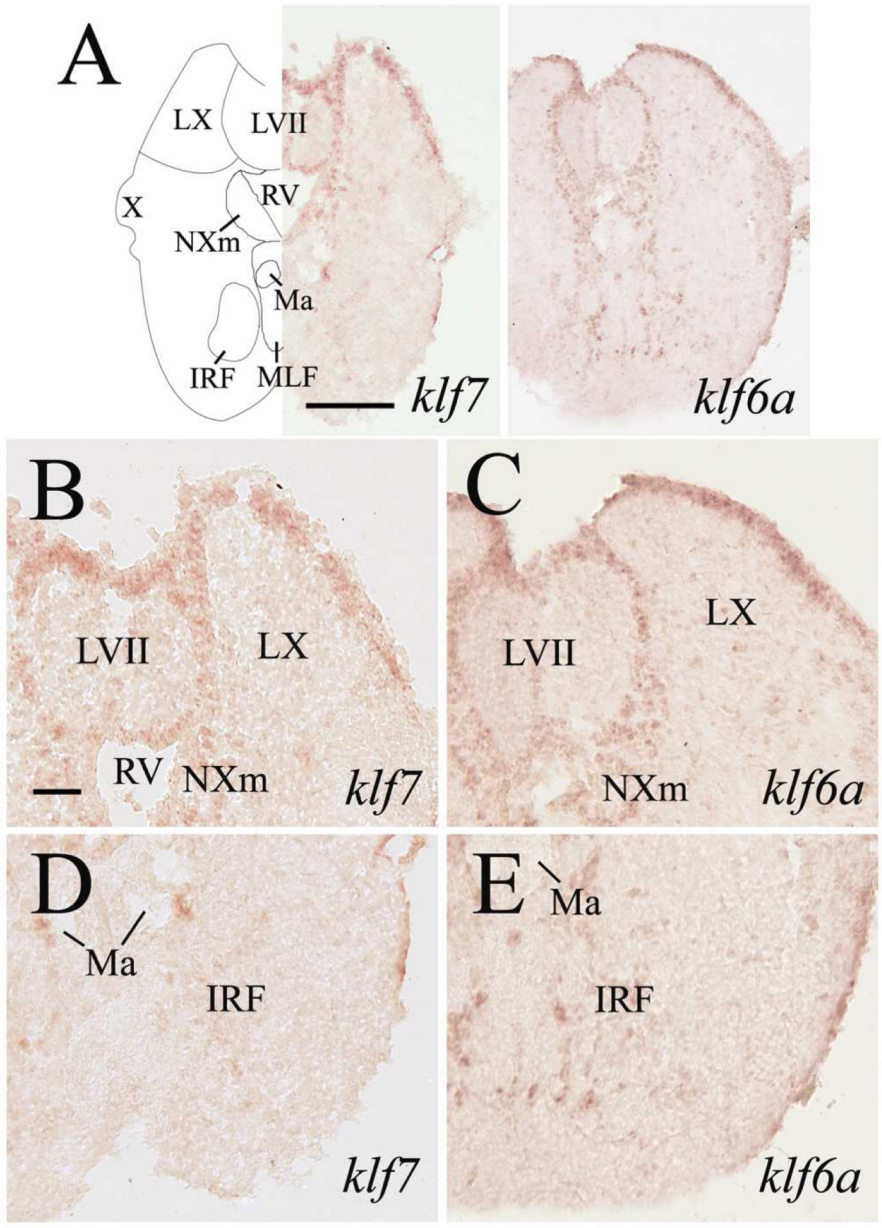

Figure 11.

Expression of *klf7* and *klf6a* in the facial and vagal lobes, and the medulla of adult zebrafish. Top panels show lower magnified images (scale bar = 200 μm) of halves of brain sections from a level shown in Figure 1. Middle and bottom panels show higher magnified views (scale bar = 50 μm) of the dorsal and ventral halves, respectively, of their corresponding images in the top panels.

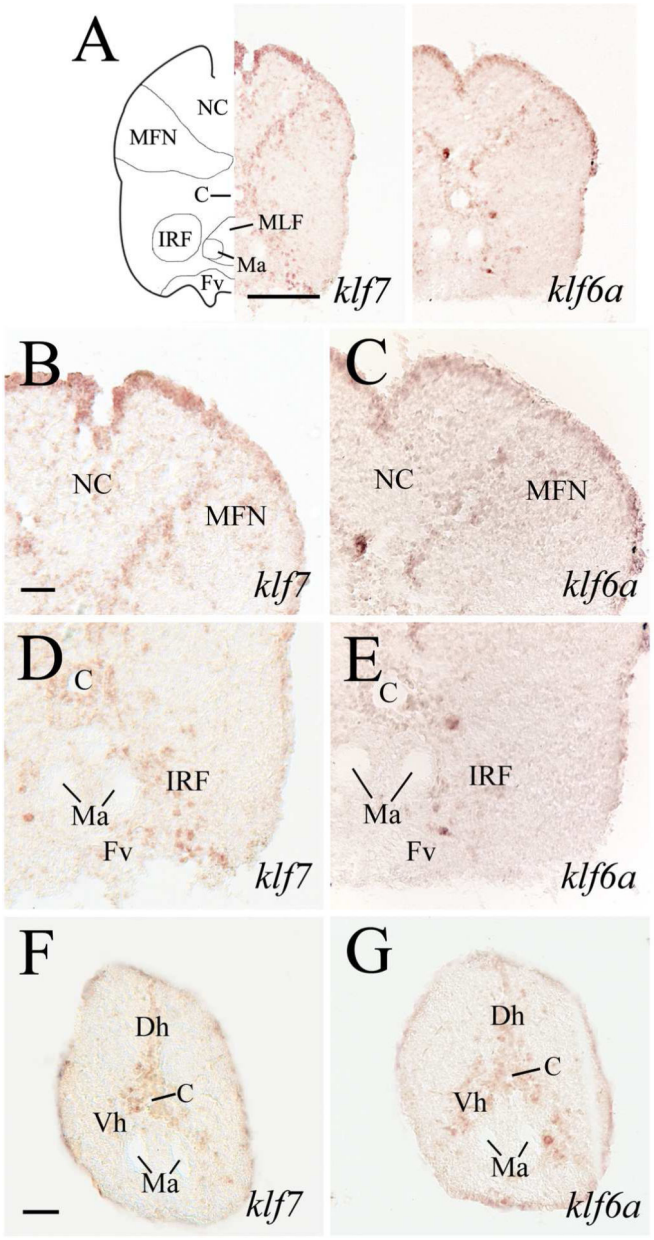

Figure 12.

Expression of *klf7* and *klf6a* in the posterior medulla and spinal cord of adult zebrafish. Top panels show lower magnified images (scale bar = 200 μm) of halves of brain sections from a level shown in Figure 1. Panels B and C, D and E are higher magnifications (Scale bar = 50 μm) of the dorsal and ventral halves, respectively, of their corresponding images in the top panels. Panels F and G are spinal cord sections from the middle body trunk level (Scale bar = 50 μm). See list for abbreviations.

TABLE 1

klf7 and klf6a Expression in Sensory Structures of Adult Zebrafish CNS

Olfactory system
Posterior zone of dorsal telencephalic area (Dp), olfactory bulb (Ob), parvocellular preoptic nucleus, anterior part (PPa), posterior tuberal nucleus (PTN), central nucleus of ventral telecephalic area (Vc), dorsal nucleus of ventral telecephalic area (Vd), ventral nucleus of ventral telecephalic area (Vv)
Visual system
Anterior thalamic nucleus (A), accessory pretectal nucleus (APN), central pretectal nucleus (CPN), lateral zone of dorsal telencephalic area (Dl), dorsal posterior thalamic nucleus (DP), nucleus isthmi (NI), parvocellular preoptic nucleus, posterior part (PPp), suprachiasmatic nucleus (SC), optic tectum (TeO)
Auditory and vestibular system
Central posterior thalamic nucleus (CP), descending octaval nucleus (DON), granular eminence (EG), magnocellular octaval nucleus (MaON), secondary octaval population (Sop), central nucleus of torus semicircularis (TSc)
Lateral line system
Central zone of dorsal telencephalic area (Dc), lateral zone of dorsal telencephalic area (Dl), medial zone of dorsal telencephalic area (Dm), granular eminence (EG), medial octavolateralis nucleus (MON), lateral preglomerular nucleus (PGl), ventrolateral nucleus of torus semicircularis (TSvl)
General visceral and gustatory systems
Medial zone of dorsal telencephalic area (Dm), facial lobe (LVII), vagal lobe (LX), medial funicular nucleus (MFN), commissural nucleus of Cajal (NC)
Somatosensory system
Dorsal posterior thalamic nucleus (DP), medial funicular nucleus (MFN), nucleus of the descending trigeminal root (NDV), primary sensory trigeminal nucleus (NVs), ventromedial thalamic nucleus (VM)