

Phylostratigraphic Profiles in Zebrafish Uncover Chordate Origins of the Vertebrate Brain

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Abstract

An elaborated tripartite brain is considered one of the important innovations of vertebrates. Other extant chordate groups have a more basic brain organization. For instance, cephalochordates possess a relatively simple brain possibly homologous to the vertebrate forebrain and hindbrain, whereas tunicates display the tripartite organization, but without the specialized brain centers. The difference in anatomical complexity is even more pronounced if one compares chordates with other deuterostomes that have only a diffuse nerve net or alternatively a rather simple central nervous system. To gain a new perspective on the evolutionary roots of the complex vertebrate brain, we made here a phylostratigraphic analysis of gene expression patterns in the developing zebrafish (*Danio rerio*). The recovered adaptive landscape revealed three important periods in the evolutionary history of the zebrafish brain. The oldest period corresponds to preadaptive events in the first metazoans and the emergence of the nervous system at the metazoan–eumetazoan transition. The origin of chordates marks the next phase, where we found the overall strongest adaptive imprint in almost all analyzed brain regions. This finding supports the idea that the vertebrate brain evolved independently of the brains within the protostome lineage. Finally, at the origin of vertebrates we detected a pronounced signal coming from the dorsal telencephalon, in agreement with classical theories that consider this part of the cerebrum a genuine vertebrate innovation. Taken together, these results reveal a stepwise adaptive history of the vertebrate brain where most of its extant organization was already present in the chordate ancestor.

Key words: brain evolution, vertebrates, phylostratigraphy, zebrafish, gene expression, development.

Introduction

The central nervous system (CNS) is the part of nervous system responsible for processing sensory information, integrating it and responding to environmental stimuli (Schmidt-Rhaesa 2007). The majority of bilaterian animals possess some form of centralized nervous system, even though there is a great structural diversity of the CNS in different phyla (Schmidt-Rhaesa 2007; Northcutt 2012). The CNS in chordates is positioned dorsally and consists of an anterior brain and a posterior spinal cord. Vertebrates have the most complex brain within chordates (Butler and Hodos 2005; Kaas 2009). The vertebrate brain is composed of a forebrain, a midbrain, and a hindbrain, therefore having a tripartite organization (fig. 1). The forebrain can be further divided into a caudal diencephalon and a rostral telencephalon with a well-developed pallium involved in higher order processing and behavioral control (Butler 2009; Huesa et al. 2009; Vargas et al. 2009; Wullimann and Vernier 2009a, 2009b). The midbrain is composed of the optic tectum, which is the primary visual center in fishes organized in layers that map environmental space, and the tegmentum, which innervates the hindbrain and spinal cord motor structures and is involved in rapid “fight or flight” behavior (Butler and Hodos 2005; Fritzsche et al. 2009; Saidel 2009; Winn 2009). The hindbrain influences such basic autonomic functions as heartbeat and breathing

rates as well as influencing body movements and processing sensory information from the head (Butler and Hodos 2005; Fritzsche et al. 2009; Wullimann and Vernier 2009a). All vertebrates, including even the earliest diverging cyclostomes, possess the same basic brain centers (Northcutt 2002; Butler and Hodos 2005). However, size and complexity of the cytoarchitecture of specific brain regions vary greatly within each particular vertebrate class (Van Dongen 1998).

Although the CNS in cephalochordates, which are the earliest diverging chordates (Delsuc et al. 2008), is relatively simple, it is similar to the vertebrate brain (Wicht and Lacalli 2005). For instance, the most frontal part of the CNS is expanded into the cerebral vesicle, whose frontal part is thought to be homologous with the vertebrate forebrain. Cephalochordates also express a part of the conserved gene network that characterizes important developmental organizers involved in patterning of vertebrate brain during embryogenesis, the midbrain/hindbrain boundary (MHB), and the zona limitans intrathalamica (Shimeld and Holland 2005; Irimia et al. 2010; Holland et al. 2013). Tunicates are the closest vertebrate relatives (Delsuc et al. 2006, 2008). The swimming larva of sessile ascidian tunicates and both larval and adult stages of free-swimming appendicularians possess a tripartite brain whose parts are traditionally considered to be homologous to the vertebrate forebrain, midbrain

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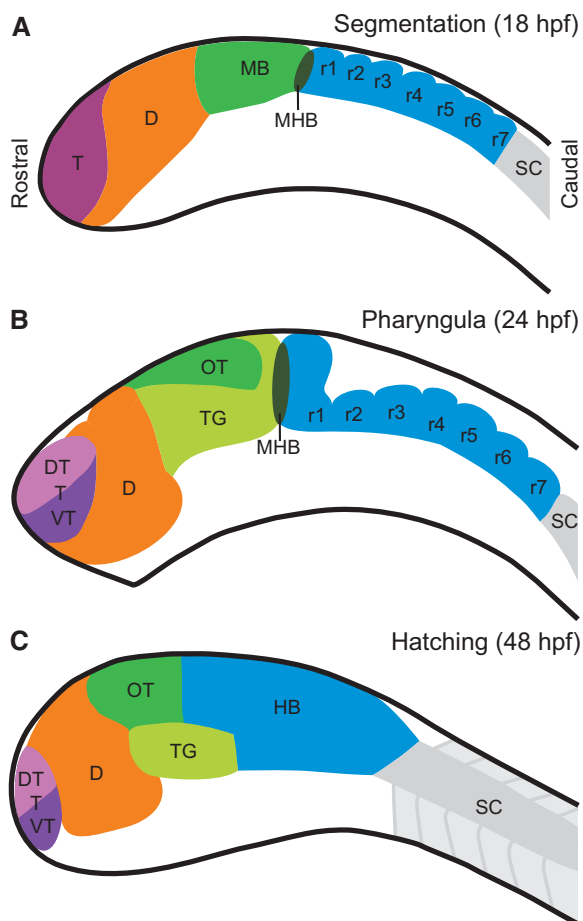


Fig. 1. Early brain development in zebrafish. A schematic side view of zebrafish embryonic head. Anterior (rostral) is to the left and dorsal at the top. Approximate anatomical positions of individual brain regions are reconstructed from photos of *in situ* hybridizations retrieved from ZFIN database (Bradford et al. 2011). Embryo during (A) segmentation stages (18 h postfertilization) and (B) pharyngula stages (24 h postfertilization). (C) Larva at the start of hatching (48 h postfertilization). Distinct parts of the CNS are marked by different colors: forebrain violet and orange, midbrain green, and hindbrain blue. D, diencephalon; DT, dorsal telencephalon; HB, hindbrain; MB, midbrain; OT, optic tectum; r1–r7, rhombomeres 1–7; SC, spinal cord; T, telencephalon; TG, tegmentum; VT, ventral telencephalon.

(alternatively only MHB), and hindbrain (Butler 2000; Sorrentino et al. 2000; Meinertzhagen and Okamura 2001; Meinertzhagen et al. 2004; Dufour et al. 2006; Lacalli 2008; Nishida 2008). However, the tunicate brain has a quite simple cytoarchitecture without specialized brain centers, possibly a secondary reduction related to the passively filter-feeding lifestyle of most adult tunicates or, in the case of appendicularians, an extremely short life cycle (Burighel and Cloney 1997; Dehal et al. 2002; Lemaire et al. 2008; Nishida 2008; Mallatt 2009). In addition, despite these parallels among chordate brains, the degree of brain complexity in the ancestor of chordates remains quite obscure (Wicht and Lacalli 2005). For instance, some authors think that the brain of cephalochordates is largely degenerate (Pani et al. 2012). The closest outgroups to chordates, hemichordates and echinoderms, have very simple, or alternatively simplified, nervous system

that does not help much in understanding of the prechordate brain properties (Pani et al. 2012; Holland et al. 2013).

Two contrasting theories concern the evolution of CNS in bilaterian animals (Holland 2003; Northcutt 2012). According to one view, centralized nervous systems evolved independently in protostome and deuterostome lineages from a diffuse nerve net of the bilaterian ancestor (Moroz 2009; Northcutt 2010). This scenario says that in the chordate lineage the dorsal CNS emerged by *de novo* centralization of hemichordate-like diffuse nerve nets (Lowe et al. 2003, 2006), and it demands that nervous system centralization was independently acquired in chordates and other bilaterian lineages (Northcutt 2012). An alternative view holds that the chordate CNS is homologous to CNS of other bilaterians and that nervous system centralization characterized the first bilaterians (Arendt and Nubler-Jung 1999; Hirth et al. 2003; Lichtneckert and Reichert 2005; Denes et al. 2007; Hirth 2010; Holland et al. 2013). For example, some morphological studies find a *bona fide* CNS in hemichordates, which would render the idea of *de novo* centralization in chordates less probable (Benito-Gutiérrez and Arendt 2009; Nomaksteinsky et al. 2009; Kaul and Stach 2010; Stach et al. 2012; Miyamoto and Wada 2013). Some similarities in anatomy, functions, and conserved gene toolkits led some authors to propose deep homology between the part of the vertebrate basal ganglia and the arthropod central complex (Strausfeld and Hirth 2013a, 2013b) as well as between the vertebrate pallium (dorsal telencephalon) and the arthropod mushroom bodies (Tomer et al. 2010). However, a phylogenetic character reconstruction suggests that the bilaterian ancestor most likely lacked a CNS because most of the approximately 30 bilaterian phyla do not possess a CNS (Northcutt 2010, 2012).

The tripartite brain of vertebrates is widely assumed to have evolved through enlargement of the rostral part of the spinal cord. It has been viewed as a key innovation leading to the evolution of a predatory or highly active lifestyle in the ancestor of vertebrates (Gans and Northcutt 1983; Northcutt and Gans 1983; Northcutt 2005). However, fossils of the oldest known vertebrates indicate that they were not predators but most probably fast-swimming filter feeders, that is, *Haikouichthys* and *Metaspriggina* (Shu 2003; Shu et al. 2003; Mallatt 2009; Conway Morris and Caron 2014). These findings suggest that selection for detecting predators, not prey, drove vertebrate brain evolution. Although Northcutt and Gans (1983) suggested that vertebrate brain regions emerged simultaneously in the vertebrate ancestor, other researchers recognized that building of the vertebrate brain could have proceeded in a more gradual way. For instance, Butler (2000, 2006) proposed that the forebrain evolved in connection with the emergence of the camera eye and complex visual system. Likewise, our recent phylostratigraphic study in zebrafish suggested that the vertebrate visual system evolved first among head sensory structures in the ancestor of chordates (Šestak et al. 2013). Interestingly, *Haikouella*, a fossil said to represent sister group to vertebrates, was reported to have paired eyes and an enlarged brain with forebrain (diencephalon) and hindbrain (Mallatt and Chen 2003; Chen 2008, 2011). However, the uncertain taxonomic position of these fossils

and controversy over whether the tiny eyes and large brains really exist in the specimens preclude reliable conclusions about the succession of brain evolution during the chordate–vertebrate transition (Shu 2003; Shu et al. 2010).

To gain a fresh perspective on the evolutionary origin of the vertebrate brain, we applied here the phylostratigraphic approach (Domazet-Lošo et al. 2007; Domazet-Lošo and Tautz 2010a; Carvunis et al. 2012; Quint et al. 2012; Šestak et al. 2013) based on the in situ hybridization data from the developing zebrafish (Bradford et al. 2011). Our results indicate that most of the vertebrate brain regions were already present in the first chordates. An exception is the dorsal telencephalon, homologous to the cerebral cortex of mammals, which seems to have evolved later, at the base of the vertebrate lineage.

Results

Adaptive Patterns in the Zebrafish CNS

We started the phylostratigraphic procedure for zebrafish by defining a framework of 14 phylogenetic levels (phylostrata) (fig. 2) (Domazet-Lošo et al. 2007; Domazet-Lošo and Tautz 2008, 2010b). This consensus phylogeny covers a time span from the origin of the first cell to the terminal lineage, that is, zebrafish, and follows the currently best supported topology (Blair and Hedges 2005; Delsuc et al. 2006, 2008; Putnam et al. 2008; Philippe et al. 2009; Heimberg et al. 2010; Edgecombe et al. 2011; Philippe et al. 2011). We mapped 20,378 zebrafish genes into corresponding phylostrata (fig. 2 and supplementary tables S1 and S2, Supplementary Material online), and then used these phylostratigraphic maps for the statistical

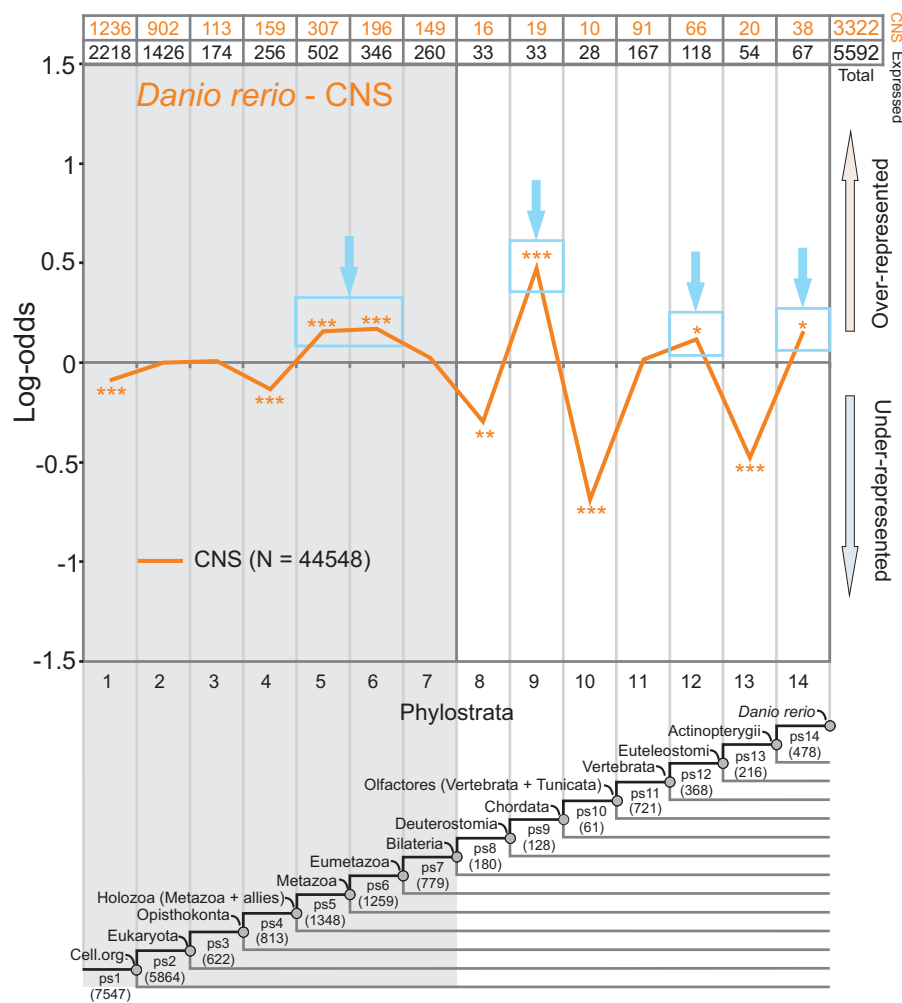


Fig. 2. Phylostratigraphic analysis of the entire CNS in zebrafish. The consensus phylogeny spanning from the origin of the first cell to *Danio rerio* is depicted in the lower panel. Numbers in parentheses denote the total number of genes per phylostratum. The rows of blocks at the top show distributions of all zebrafish genes with spatially restricted expression (black) and a subset of genes with expression in the CNS of zebrafish (orange). The horizontal x axis and the phylogeny in the lower panel depict 14 phylostrata (“1–14” = “ps1–14”). Shaded left half (gray) denotes the prebilaterian part of the phylogeny. In every phylostratum, the frequency of expression domains in an analyzed trait is compared with the frequency in the complete sample, and deviations are shown by log-odds (y axis). The total number of analyzed expression domains in the zebrafish CNS is 44,548 (in parenthesis). The blue frames with arrows denote the most important overrepresentation peaks. A log-odd of zero means that the frequency of expression domains in a phylostratum equals the expected frequency estimated from the total number of expressions. Deviations from the expected frequencies were tested by a two-tailed hypergeometric test controlled for multiple comparisons by FDR at the 0.05 level (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, little-circle symbols denote deviations that were significant before the FDR correction).

analysis of 141,257 zebrafish expression domains (Bradford et al. 2011) (supplementary table S3, Supplementary Material online).

To reveal global patterns, we first analyzed all of the 3,322 genes that are expressed in the zebrafish CNS. The first adaptive peaks are placed during the metazoan–eumetazoan transition, which is defined by sponges (ps5) and cnidarians (ps6) in our phylogeny (fig. 2). The adaptive peak at the metazoan ancestor (ps5) correlates with the finding that core synaptic signaling components were present already at the base of animal kingdom, defined by sponges here, and were later co-opted in building a functional synapse in eumetazoans (Sakarya et al. 2007; Emes et al. 2008; Kosik 2009; Renard et al. 2009; Nickel 2010; Conaco et al. 2012). Similarly, the adaptive peak at ps6, defined by cnidarians, correlates with a common view that the first nervous system evolved in the eumetazoan ancestor (Koizumi 2007; Schmidt-Rhaesa 2007; Galliot et al. 2009; Watanabe et al. 2009). Further adaptive peaks are located at the chordate ancestor (ps9, represented by the cephalochordate *Branchiostoma floridae*), euteleostome ancestor (ps12, common ancestor of tetrapods and bony fish; represented

by 27 tetrapods), and in zebrafish (ps14) (fig. 2 and supplementary table S1, Supplementary Material online). Among these signals the strongest one is at the chordate ancestor (ps9) indicating a special importance of this stage for the evolution of the vertebrate CNS. It is interesting to note that here in the total CNS we do not recover any signal at the origin of bilaterians (ps7, represented by 26 protostome animals) where some theories predict centralization of the nervous system (see more below) (Arendt and Nubler-Jung 1999; Hirth et al. 2003; Lichtneckert and Reichert 2005; Denes et al. 2007; Hirth 2010; Holland et al. 2013).

Adaptive Patterns in the Zebrafish Brain

To gain better resolution of the history of adaptations in the zebrafish CNS, we further focused on the zebrafish brain and its specific regions (fig. 1). Figure 3 shows the adaptive profile for the complete brain and separate profiles for the forebrain, midbrain, and hindbrain. Similar to the CNS, patterns of the complete brain showed adaptive signal at the metazoan–eumetazoan transition (ps5–6) and at the ancestor of chordates (ps9). Such signal is also seen at the vertebrate ancestor (ps11, represented by cyclostome lamprey

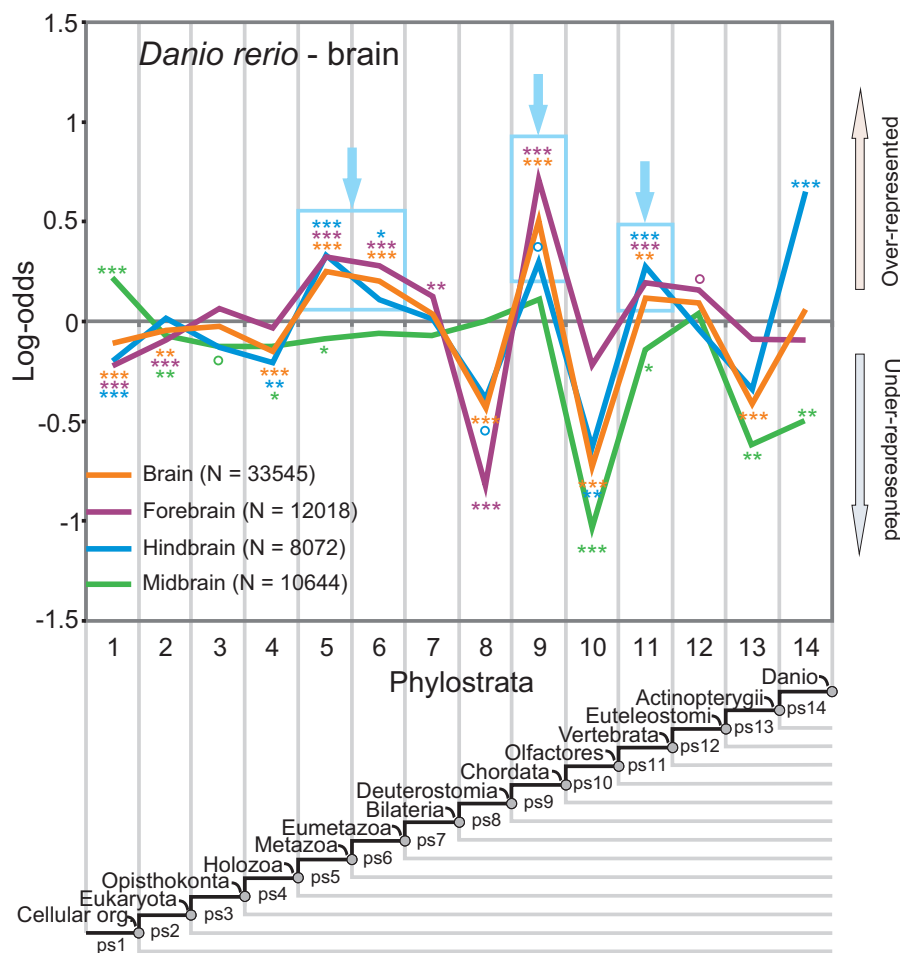


FIG. 3. Phylostratigraphic analysis of the zebrafish brain. Phylostratigraphic profiles of the total brain (orange), forebrain (violet), midbrain (green), and hindbrain (blue) are shown. Note peaks at Chordata (ps9) and Vertebrata (ps11). Only the midbrain shows no significant overrepresentations at any stage after the origin of cellular organisms (ps1). Refer to figure 2 for details on the annotation and statistics.

and Chondrychthyes genomes). However, partitioning of the brain profile into its components revealed some nonuniformity among major brain parts (fig. 3). The forebrain and hindbrain were very similar to the total brain profile except that the forebrain showed an additional adaptive signal at the bilaterian ancestor (ps7), and the hindbrain showed a signal that marks a zebrafish specific innovation (ps14). Intriguingly, the midbrain had a very different pattern from the other brain regions, with only one significantly positive signal, at the ancestor of all cellular organisms (ps1) (fig. 3). It is also notable that the midbrain is missing signals at the metazoan and eumetazoan transitions (ps5-ps6), which are otherwise present in all CNS tissues we analyzed here.

In the chordate ancestor (ps9), we recovered the strongest adaptive signals for the complete brain and the forebrain along with the second highest signal for the hindbrain (fig. 3), suggesting that this stage was important for the assembly of the vertebrate brain. Discovered signals support the claim that the frontal vesicle in amphioxus (cephalochordates), the earliest diverging chordates, is homologous to the vertebrate forebrain and that the region directly posterior

to this vesicle is homologous to the hindbrain (Butler 2000; Wicht and Lacalli 2005; Lacalli 2008; Holland 2009). These signals also agree with the proposal that the fossil species *Haikouella* possessed these brain regions (Mallatt and Chen 2003; Chen 2008, 2011).

Adaptive Patterns in the Zebrafish Forebrain

To capture more details on adaptive patterns in the forebrain, we separately analyzed its two major parts. The vertebrate forebrain is composed of a diencephalon, positioned more caudally, and a telencephalon, positioned more rostrally (fig. 1). Similar to the total forebrain, the diencephalon and the telencephalon each showed their strongest adaptive peaks in the chordate ancestor (fig. 4, ps9), in agreement with a claim that frontal part of the cerebral vesicle of amphioxus (cephalochordates) is homologous to the vertebrate diencephalon (Butler 2000; Wicht and Lacalli 2005; Lacalli 2008; Holland 2009) and possibly to some parts of the vertebrate telencephalon (Wicht and Lacalli 2005; Lacalli 2008). Evidently, the adaptive signal in the whole forebrain that we detected in the bilaterian ancestor (fig. 3, ps7) actually comes from the diencephalon (fig. 4, ps7). Finally, we found

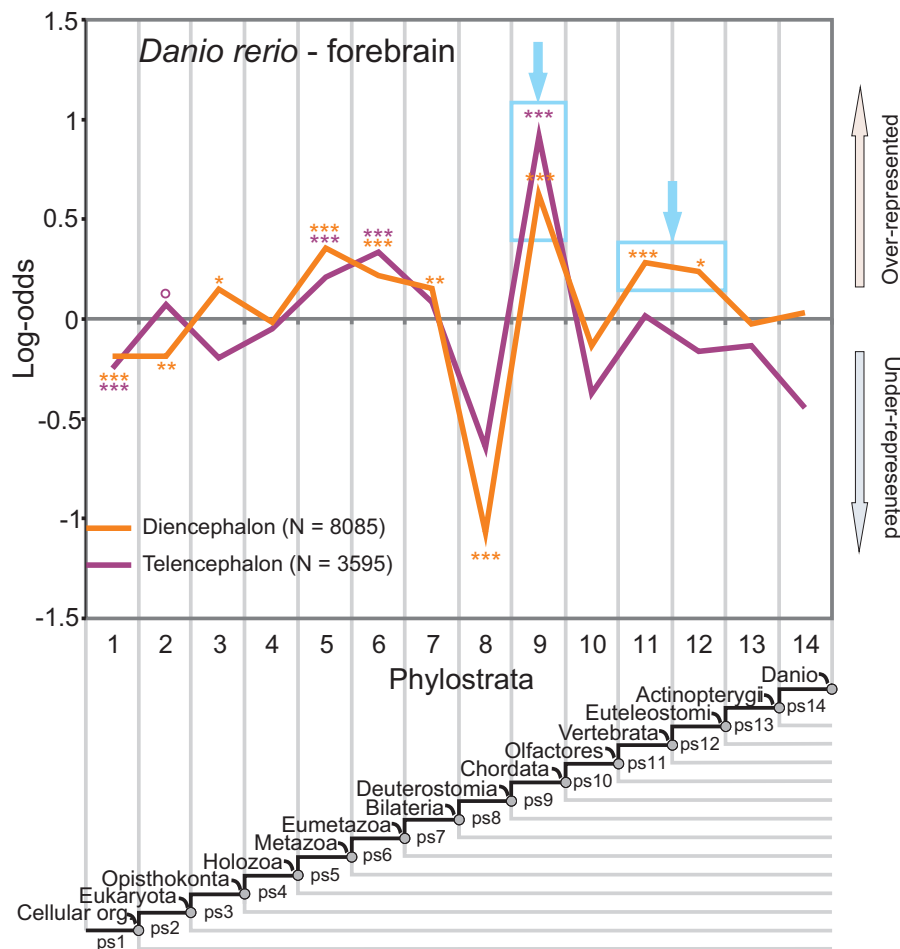


FIG. 4. Phylostratigraphic analysis of the zebrafish forebrain. Phylostratigraphic profiles of the diencephalon (orange) and the telencephalon (violet) are depicted. Note the peaks at Chordata (ps9), Vertebrata (ps11), and Euteleostomi (ps12) marked by blue frames and arrows. Refer to figure 2 for details on the annotation and statistics.

preadaptive signals for both the diencephalon and the telencephalon at the Metazoa stage (ps5, in the nerveless sponge) as well as the Eumetazoa stage (ps6, in cnidarians which lack a CNS).

The zebrafish telencephalon can be further divided into the ventral part (subpallium), homologous to the septum, striatum, and pallidum of vertebrates, and a dorsal part (pallium), which includes the cerebral cortex in mammals (Huesa et al. 2009; Vargas et al. 2009; Wullimann and Vernier 2009b; Perry et al. 2010). The ventral part of the telencephalon, which is involved in motor control, core affects (or basic emotions), and motivation (Wullimann and Vernier 2009b; O'Connell and Hofmann 2011; Panksepp et al. 2011; Ganz et al. 2012; LeDoux 2012; Solms and Panksepp 2012; Grillner et al. 2013) (fig. 1), showed a dominant adaptive signal at the chordate ancestor (fig. 5, ps9). In contrast, the dorsal telencephalon, with functions in memory (hippocampus), multisensory integration, associative behavior, spatial and emotional learning (Vargas et al. 2009; Wullimann and Vernier 2009b) (fig. 1), showed a major adaptive signal at the vertebrate ancestor (ps11) and an absence of any significant signal at the chordate ancestor (ps9, fig. 5). This profile supports the traditional view that the pallium emerged in the vertebrate ancestor (Northcutt 2002; Butler and Hodos 2005).

Adaptive Patterns in the Zebrafish Midbrain

The unusual and puzzling profile for the midbrain (fig. 3) prompted us to decompose the midbrain signal into its two major components: from the tectum and the tegmentum (fig. 6). The optic tectum, which is a highly developed part of the zebrafish brain, showed the same profile as the total midbrain; that is, a sole overrepresentation signal placed at the origin of cellular organisms (ps1). The tegmentum, on the other hand, showed a similar pattern to the rest of the brain (compare figs. 3 and 6), with signals at the chordate (ps9) and the vertebrate (ps11) stages, and marginally significant jumps at the metazoan (ps5) and the euteleostome ancestor (ps12). Accordingly, it is evident that the optic tectum alone accounts for the strange profile of the entire midbrain, with no increase in tectal gene numbers at any eukaryotic stage (ps2–ps14).

Another important component of the central part of the vertebrate brain is the MHB. This developmental organizer is involved in controlling the formation of the midbrain and the anterior hindbrain (Hirth et al. 2003; Holland 2009). We recovered a major adaptive signal for the MHB at the chordate ancestor (ps9) (fig. 6). This is in line with studies showing that amphioxus (cephalochordates) has most of the genes crucial for functioning of this organizer (Holland et al. 2013).

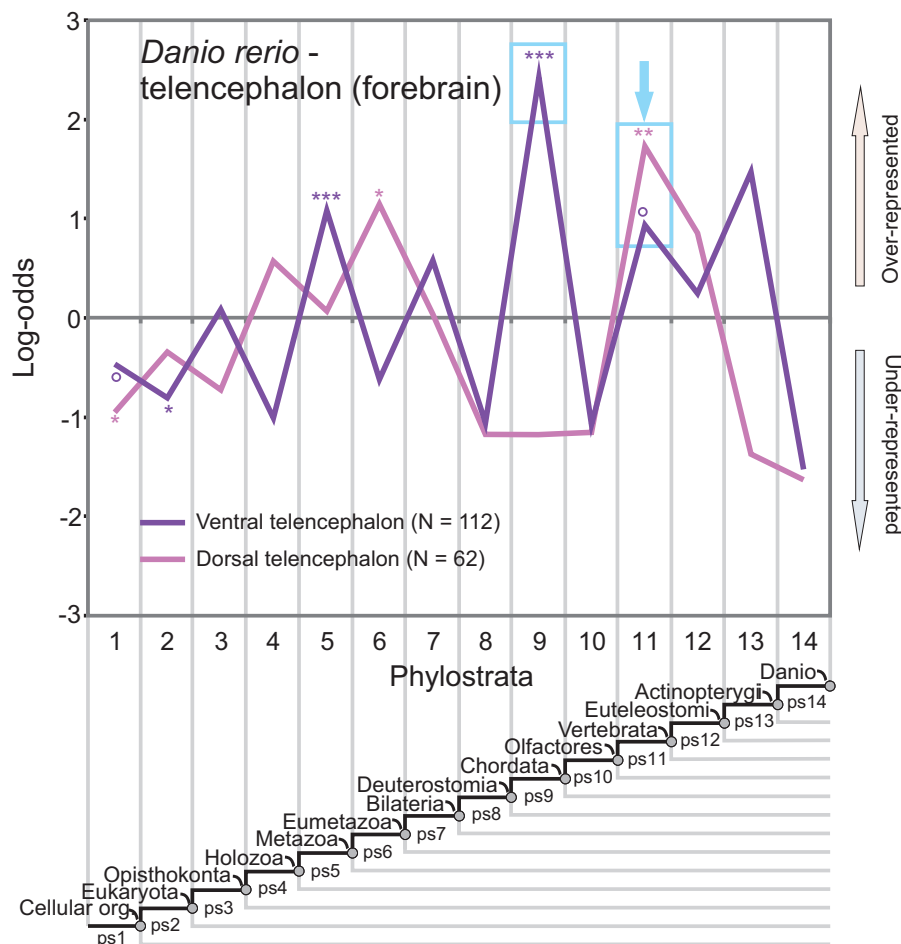


FIG. 5. Phylostratigraphic analysis of the zebrafish telencephalon. Phylostratigraphic profiles of the ventral (violet) and the dorsal telencephalon (pink) are shown. Note the peaks at Chordata (ps9) and Vertebrata (ps11) marked by blue frames and arrows. Refer to figure 2 for details on the annotation and statistics.

Additional adaptive signals in the MHB profile are seen at the metazoan, eumetazoan, and bilaterian ancestors (ps5–7).

Discussion

To obtain a global picture of the evolution of tripartite vertebrate brain, we condensed all the adaptive profiles from figures 2 to 6 into a set of simple vertical lines that show the statistically significant adaptive signals (fig. 7). This representation shows that there were three important phases of the new-gene evolution in the history of the vertebrate brain.

The first phase spans the ancestors of metazoans (ps5), eumetazoans (ps6), and bilaterians (ps7, fig. 7). We found signal for every brain structure in this phase, except for the optic tectum that dominates the midbrain signal. These increases might be expected, given that the first nervous system emerged in this evolutionary range (Koizumi 2007; Schmidt-Rhaesa 2007; Galliot et al. 2009; Watanabe et al. 2009). However, the first metazoan most likely had no nervous system (Moroz et al. 2014), so the signals at the first multicellular animal (ps5) probably mark a preadaptive event where genes that now play role in the CNS emerged initially in some other adaptive context.

The ongoing dispute over the phylogenetic position of Ctenophora has important implications on the origin of the nervous system within Metazoa, as ctenophorans possess nerve nets and share some of the neural genes with cnidarians and bilaterians (Ryan et al. 2013; Moroz et al. 2014). To test the dependence of our findings on the phylogenetic position of Ctenophora, we repeated our analyses by including the recently sequenced genomes of ctenophorans *Mnemiopsis leidyi* and *Pleurobrachia bachei* in the context of two alternative topologies, that is, Ctenophora at the Metazoa (ps5) or Ctenophora at the Eumetazoa internode (ps6) (Nosenko et al. 2013; Ryan et al. 2013; Moroz et al. 2014). This test revealed that our initially recovered patterns are quite stable regardless of where the ctenophores go on the consensus phylogeny (supplementary figs. S1 and S2, Supplementary Material online). This stability is especially evident for the signals within the deuterostome lineage (ps8–14) which are essentially unchanged (fig. 7 and supplementary figs. S1 and S2, Supplementary Material online).

It is also striking that so many adaptive signals are present at the origin of metazoans in all versions of the analyses (ps5, fig. 7 and supplementary figs. S1 and S2, Supplementary Material online), supporting the view that the preadaptive

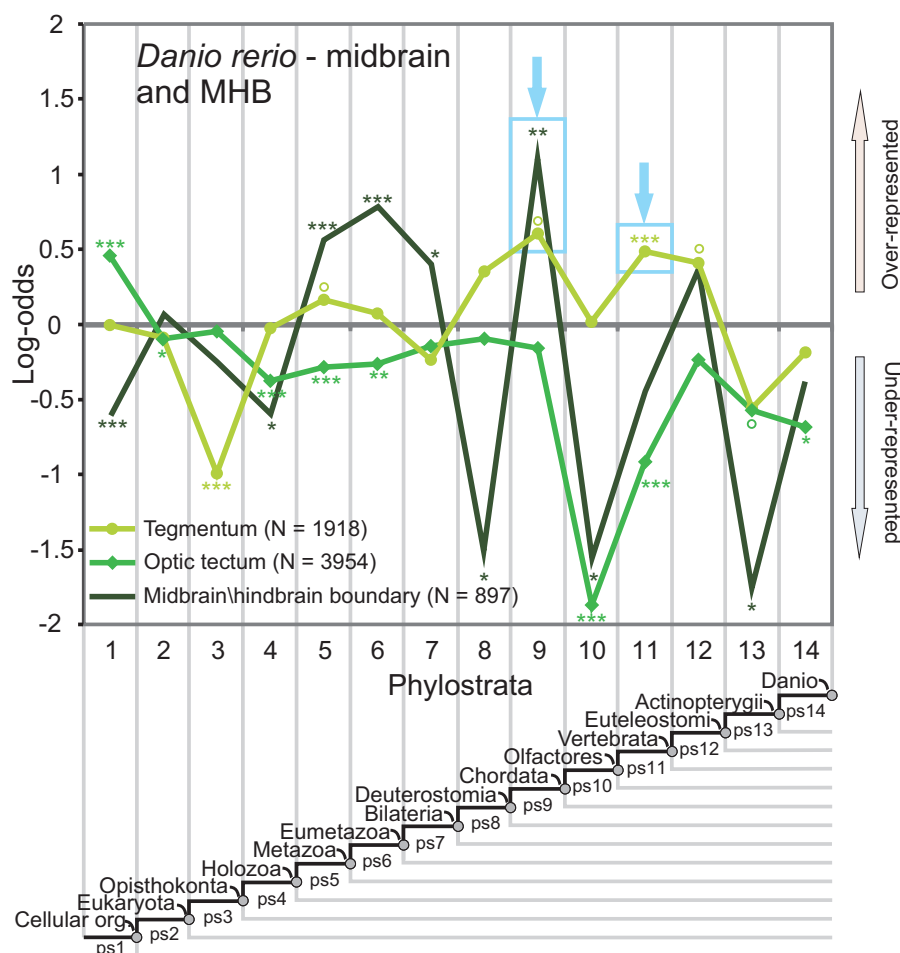


FIG. 6. Phylostratigraphic analysis of the zebrafish midbrain. Phylostratigraphic profiles of the tegmentum (light green), optic tectum (green), and MHB (dark green) are depicted. Note the peaks at Chordata (ps9) and Vertebrata (ps11) marked by blue frames and arrows. Refer to figure 2 for details on the annotation and statistics.

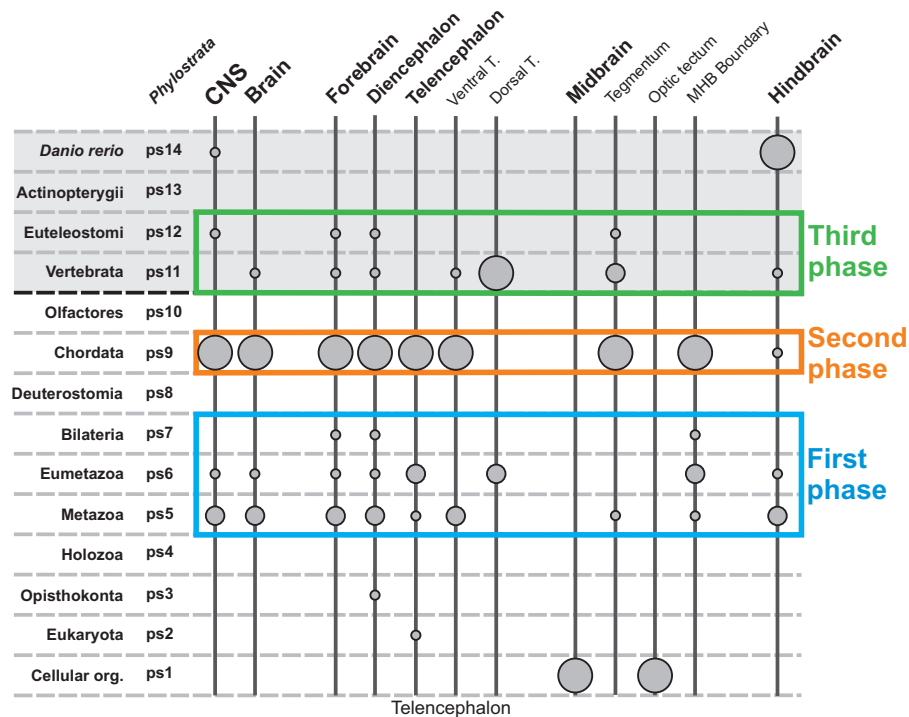


Fig. 7. Summary of the adaptive landscape of the zebrafish CNS. At left are shown the phylogenetic levels (phylostrata) from the ancestor of the cellular organisms (ps1) to the present day zebrafish (ps14). The gray-shaded area at top marks the vertebrate section of the phylogeny. A simplified version of every phylostratigraphic profile from figures 2 to 6 is shown by the vertical lines and the corresponding circles of various sizes. An adaptive signal with the strongest amplitude is represented by the largest circle, the second highest signal is marked by the medium size circle, and all other overrepresentation signals are marked by the circles of the smallest size. Only statistically significant signals are shown. The three phases in the evolution of zebrafish brain are labeled by rectangles of different colors (first phase—blue, second phase—orange, and third phase—green).

emergence of neural genes in the first metazoan was important for the subsequent origins of neurons, which could have evolved independently in eumetazoans and ctenophores (Moroz et al. 2014). This preadaptive scenario is also in line with studies that found neural genes but not the nerve cells in sponges (Emes et al. 2008; Richards et al. 2008; Kosik 2009; Renard et al. 2009; Ryan and Grant 2009; Conaco et al. 2012; Moroz et al. 2014). Nevertheless, the stable signals at the origin of eumetazoans (ps6) (fig. 7 and supplementary figs. S1 and S2, Supplementary Material online) support the classical view of the importance of this stage in the emergence of the eumetazoan nervous system (Koizumi 2007; Schmidt-Rhaesa 2007; Galliot et al. 2009; Watanabe et al. 2009).

Turning to the bilaterian ancestor, the signal in the forebrain region (diencephalon) at this stage (fig. 7, ps7) gives some support to the idea that a centralized nervous system is an ancestral bilaterian trait (Arendt et al. 2008; Tomer et al. 2010; Holland et al. 2013). However, the question of the centralization of the nervous system remains open because only the forebrain (diencephalon) and the MHB show signals at the bilaterian ancestor (fig. 7, ps7). It should also be noted that the signal in the forebrain (ps7) comes from the diencephalon and not the telencephalon, which is the brain region that was suggested to have roots in the bilaterian ancestor (Tomer et al. 2010). Thus, together with the previous phylogeny-based work (Northcutt 2010, 2012) our finding is inconsistent with the idea that the pallium, part of the vertebrate

telencephalon, is homologous to the annelid mushroom bodies (Tomer et al. 2010). Similarly, Strausfeld and Hirth (2013a, 2013b) proposed homology between the vertebrate basal ganglia of the telencephalic and midbrain origin and the arthropod central complex. However, although the basal ganglia of the diencephalic origin also exist in vertebrates these authors could not find a homologous brain structure in arthropods. Therefore, a preadaptive event is a more plausible explanation for the forebrain signatures in our work, especially if one takes into account the rather low intensity of the signal for a diencephalon at the bilaterian ancestor (fig. 7, ps7).

The second prominent phase in the evolution of zebrafish brain is the origin of chordates (fig. 7, ps9). Here, we found significant phylostratigraphic signals for almost all parts of the CNS including the total brain, forebrain, tegmentum, and MHB that show their strongest peaks at this phylostratum (fig. 7). This finding supports the view that the substantial part of the vertebrate brain was in place already in the first chordate (Northcutt 2002; Butler 2012). It also supports the idea that the vertebrate brain evolved independently of the brains within the protostome lineage (Northcutt 2010, 2012). Finally, even though we recovered some signals, of lower magnitude, at the origin of vertebrates (fig. 7, ps11), our results contrast with traditional predictions that strictly link the vertebrate brain with the origin of the vertebrates (Gans and Northcutt 1983; Northcutt and Gans 1983; Northcutt 2005; Moroz 2009). Butler's Serial Transformation Hypothesis,

which envisages a stepwise evolution of the vertebrate innovations following an early chordate origin of forebrain and hindbrain, is the scenario that probably agrees best with our profiles (Butler 2000, 2006). Remarkably, the strongest adaptive signal in the MHB at ps9 supports the hypothesis that this organizer was present and becoming pronounced in the chordate ancestor (Holland et al. 2013).

The optic tectum of the midbrain has the most enigmatic profile of all the brain regions. It is large and essential for visual processing in all nonmammalian vertebrates (including lampreys) where it controls complex visual reflexes, builds maps of the visual field, integrates vision with other kinds of sensory information (touch, sound), and uses this information to trigger motor behavior (Feinberg and Mallatt 2013). But the tectum seems strictly confined to vertebrates among the chordates. For instance, microanatomical studies in a late cephalochordate larva revealed a CNS region roughly equivalent to the midbrain tegmentum, but none to the tectum (Wicht and Lacalli 2005; Lacalli 2008), in agreement with our results that show signals only for the tegmentum at the origin of chordates (fig. 6, ps9). Similarly, many studies agree that tunicates do not possess a midbrain but do possess an MHB (Butler 2000; Sorrentino et al. 2000; Meinertzhagen and Okamura 2001; Meinertzhagen et al. 2004; Wicht and Lacalli 2005; Dufour et al. 2006; Lacalli 2008; Nishida 2008). This means the tectum probably evolved in the earliest vertebrates (ps11). Yet, entirely against expectation, we found no evidence that the tectum added genes at this stage (ps11, figs. 6 and 7). This is a complete conundrum that led us to hypothesize that the tectum was built in the ancestral vertebrates by using only existing genes.

To test this, we examined 17 genes known to be important for the embryonic development of the optic tectum in vertebrates and looked up their evolutionary stages of appearance. For example, *Otx2*, *Fgf8*, *En1/2*, and *Wnt1* are important for early differentiation of the midbrain, whereas *Pax3* and *Pax7* specify the optic tectum (Wurst and Bally-Cuif 2001; Agoston et al. 2012; Miyake and Itoh 2013; Dyer et al. 2014). We found that all 17 genes evolved at much earlier stages (ps2, ps5, and ps6), thus supporting our hypothesis of tectal evolution through co-option of old genes (supplementary fig. S3 and table S3, Supplementary Material online).

The origin of vertebrates (ps11) and the transition to euteleostome vertebrates (ps12, common ancestor of tetrapods and bony fish) mark the third and most recent phase in the vertebrate brain evolution (fig. 7). The only structure that showed a dominant adaptive peak at the emergence of vertebrates (ps11) is the dorsal telencephalon (i.e., pallium, including the cerebral cortex in mammals). This result suggests that this structure is a genuine vertebrate innovation and indeed, amphioxus and tunicate brains have no anatomical trace of a pallium (Wicht and Lacalli 2005; Dufour et al. 2006; Lacalli 2008) in line with the traditional view that argues for an entirely vertebrate character of the pallium (Butler and Hodos 2005; Wicht and Lacalli 2005; Kaas 2009). This contrasts, at least at the level of novel genes, with proposed homologies between the vertebrate pallium and annelid mushroom bodies (Tomer et al. 2010). It should also be noted that

genes that were used to establish this homology (Tomer et al. 2010) are not shared derived characters, but instead are conserved regulatory genes, such as *bf-1*, *lhx2*, and *svp*, that are placed in our analysis before the origin of bilaterians (ps7) (supplementary fig. S3 and table S3, Supplementary Material online). Similar is also true for genes used to establish deep homology between the vertebrate basal ganglia and the arthropod central cortex (Strausfeld and Hirth 2013a) (supplementary fig. S3 and table S3, Supplementary Material online).

We did not find any adaptive signals in the CNS at the origin of Olfactores (ps10), which is represented here by the tunicate genomes (fig. 7). This is not surprising as tunicates have very reduced nervous systems and, in fact, have lost quite a few genes and gene families (Dehal et al. 2002; Lemaire et al. 2008; Nishida 2008; Putnam et al. 2008; Mallatt 2009). Therefore, it is possible that some of the genes that appear to have arisen in the ancestor of vertebrates (ps11) actually originated at the base of the Olfactores (ps10) and were later lost in tunicates. However, in our previous work we found adaptive signals at the origin of Olfactores in the olfactory, lateral line, and auditory sensory system (Šestak et al. 2013). Therefore, it is unlikely that tunicates retained adaptive signals in the sensory systems and completely lost all adaptive signals in the CNS that receives, integrates, and responds to the sensory information. The implication is that tunicates represent a stage that had evolved the peripheral senses but not a lot of extensive processing of the sensory information by the brain.

In contrast to the results in zebrafish reported here, we previously demonstrated an absence of adaptive signals in CNS along the arthropod lineage leading to fruit fly *Drosophila melanogaster* (Domazet-Lošo et al. 2007). However, due to the differences in database content and phylogenetic resolution, the CNS profiles of the zebrafish and fruit fly were not directly comparable. To allow reasonable comparison between these two bilaterian lineages, we repeated the *Drosophila* analysis here using an updated phylogeny and the same database that was used for the zebrafish comparison. These updates did not yield any significant changes in the *Drosophila* CNS profile; that is, the fruit fly data set showed absence of any adaptive signals after the split between protostome and deuterostome animals (after ps7) except for ps14 (origin of *D. melanogaster*) (supplementary fig. S4, Supplementary Material online). These differences in adaptive dynamics of the CNS at the level of novel genes between zebrafish and fruit fly point to different mechanisms that were shaping assembly of the complex brains in protostomes and deuterostomes. However, before any solid conclusion can be made, adaptive patterns must be checked in other protostome lineages that do not show such a derived development and did not undergo substantial gene loss as had *Drosophila* (Tomancak et al. 2002; Clark et al. 2007; Tomer et al. 2010). Additionally, the *Drosophila* gene expressions that have been documented, in contrast to those of zebrafish, are limited to embryogenesis and do not cover later stages of ontogeny where the nervous system is fully developed.

While considering such caveats, we must stress that phylostratigraphic approach relies solely on novel-protein emergence to detect adaptively important macroevolutionary phases (Domazet-Lošo and Tautz 2010b; Domazet-Lošo et al. 2014). However, it has been argued repeatedly that morphological evolution could occur not only through a protein-coding change but also through gene-regulatory change (Hoekstra and Coyne 2007; Carroll 2008; Jones et al. 2012). Consequently, the picture that we recovered here on the evolution of the vertebrate brain is inevitably incomplete because we missed the adaptations caused by changes in gene regulation. For instance, the absence of the adaptive signals at the level of novel genes in the zebrafish midbrain and optic tectum after ps1 (figs. 3, 6, and 7) might also point to the predominantly regulatory evolution of this brain region. It is also important to note that morphological evolution could proceed through both mechanisms simultaneously. For instance, Yu and colleagues showed that after duplication of the ancestral *FoxD* gene in the ancestor of vertebrates, one duplicate (*FoxD3*) acquired new regulatory sequences and a new protein domain, both of which are essential for the role of *FoxD3* in the evolution of neural crest (Yu 2010; Ono et al. 2014).

From the paleontological perspective, the picture of the brain evolution is rather incomplete because traces of nervous systems are rarely preserved in the fossil record (Sansom et al. 2010, 2011). Nevertheless, early arthropods that possessed a complex brain are documented already from the early Cambrian (Ma et al. 2012), whereas the situation within early Cambrian chordates is more obscure (Mallatt and Chen 2003; Chen 2008, 2011; Shu 2003; Shu et al. 2010) leaving the question open on when the vertebrate brain originated (Shu 2003). Yet, early Cambrian fish called *Haikouichthys* and *Metaspriggina* had vertebrate eyes, olfactory capsules, and the ear capsules suggesting that they had the full tripartite brain (Shu et al. 1999, 2003; Shu 2003; Conway Morris and Caron 2014). In this regard, our phylostratigraphic signals for a forebrain and hindbrain in the chordate ancestor give some support to the interpretation that the “prevertebrate” fossil *Haikouella* possessed a complex brain (Mallatt and Chen 2003; Chen 2008, 2011). Together with previous results on the origin of complex visual system (Šestak et al. 2013) this finding portrays the first chordate as fully equipped, brain-wise, to participate in the evolutionary arms race with the other phyla in the early Cambrian ocean (Marshall 2006; Plotnick et al. 2010).

The basal position of cephalochordates (amphioxus) in the phylogeny of extant chordates makes this group essential for understanding the ancestral chordate brain. Yet, it is uncertain whether the weakly differentiated amphioxus brain reflects the anatomical situation in early chordates, or rather represents a secondarily reduced feature as suggested by a recent study (Pani et al. 2012). Ultrastructural work suggests that the brain in cephalochordates is much simpler than the vertebrate brain but it retains the same basic anatomical organization as in vertebrates (Wicht and Lacalli 2005). In addition, most of the genes that pattern major brain regions in vertebrates also seem to be present in amphioxus

(Holland 2009; Irimia et al. 2010; Holland et al. 2013). Our findings of adaptive signals at ps9 (which is defined by amphioxus) in the almost all brain regions further support the view that the amphioxus brain, although very simple in organization, shares many molecular signatures with the vertebrate brain. This contrasts with the view that the amphioxus brain has become so altered, both anatomically and genetically, that is not an appropriate model for studying evolution of the vertebrate brain (Pani et al. 2012).

Taken together, phylostratigraphic profiles recovered here reveal a stepwise adaptive history of the vertebrate brain where most of its extant organization was already present in the chordate ancestor. However, the pallium seems to be a genuine vertebrate innovation that allowed the shift in this lineage to a highly active lifestyle and the evolution of sophisticated behaviors in complex and diverse environments.

Materials and Methods

Phylostratigraphic Analysis of the Zebrafish

The theoretical foundations of genomic phylostratigraphy and details of the phylostratigraphic procedure were described previously (Domazet-Lošo et al. 2007; Domazet-Lošo and Tautz 2008, 2010a, 2010b). In short, we retrieved 20,378 *Danio rerio* (zebrafish) protein sequences from the ZFIN database (Bradford et al. 2011) and compared these sequences against a nonredundant (nr) database (NCBI) with the BLASTP algorithm (Altschul et al. 1997) at *E* value cutoff of 1e-03. Prior to performing the sequence similarity searches, we removed from the database all sequences of viral or unknown taxonomic origin, as well as those from metazoan taxa with a currently unreliable phylogenetic position (e.g., Chaetognatha, Placozoa) (Edgecombe et al. 2011; Nosenko et al. 2013). Following this cleanup procedure, we filled up the nr database with more sequenced genomes that were not present in the NCBI nr database but were available in other public repositories. [Supplementary table S1, Supplementary Material online](#), shows the number of sequences in the nr database and the list of genomes used in the BLAST analysis. The final database contained a total of 6,310,858 protein sequences from all taxa ([supplementary table S1, Supplementary Material online](#)).

Using the obtained BLAST output we mapped zebrafish genes onto a consensus phylogeny that spans 14 evolutionary levels (phylostrata) starting from the origin of cellular organisms (ps1) and ending at the origin of zebrafish (ps14) (fig. 2 and [supplementary table S2, Supplementary Material online](#)). We used the phylogenetically most-distant BLAST match above the significance threshold (BLAST *E* value < 1e-03) as a criterion to assign the stage of evolutionary origin to a gene. This is a quite conservative method of sorting genes aiming to catch a novelty in a protein sequence space (Domazet-Lošo and Tautz 2003, 2010b; Domazet-Lošo et al. 2007; Tautz and Domazet-Lošo 2011). The number and choice of internodes in the phylogeny are the result of balancing between the availability of the sequence data for sequence similarity searches, the importance of evolutionary transitions, and

the robustness of the internodes established in phylogenetic studies (Blair and Hedges 2005; Delsuc et al. 2006, 2008; Telford et al. 2008; Budd and Telford 2009; Philippe et al. 2009; Heimberg et al. 2010; Nosenko et al. 2013; Ryan et al. 2013) (supplementary fig. S1 and tables S1 and S2, Supplementary Material online).

Expression Data and Statistics

Among all vertebrates, the zebrafish in situ hybridization data set covers the largest number of genes and the largest span of ontogenetic stages (Bradford et al. 2011; Sestak et al. 2013). Therefore, we retrieved from ZFIN database the in situ hybridization expression data for 5,592 zebrafish genes that show tissue-specific expression during ontogeny (Bradford et al. 2011). In total, this set of genes contributes to 141,257 expression domains expressed over multiple tissues and the different stages of the ontogeny (supplementary table S3, Supplementary Material online).

We divided the zebrafish expression data set into subsets corresponding to the specific CNS regions (Bradford et al. 2011). For every CNS trait of interest, we performed an overrepresentation analysis by comparing a frequency of expression domains in a phylostratum with a frequency in the total data set (expected frequency) (Domazet-Lošo et al. 2007; Domazet-Lošo and Tautz 2010b). Obtained deviations, that is, more or less expression than expected, were depicted in the figures by log-odds ratios and their significance was tested by two-tailed hypergeometric tests (Rivals et al. 2007) controlled for multiple comparisons through a false discovery rate (FDR) at the 0.05 level (Benjamini and Hochberg 1995). For the purpose of cross-profile comparison between the individual phylostratigraphic maps of the different brain regions, we created a cumulative diagram where only significant overrepresentations are depicted and ranked by signal strength to show the phylogenetic stage with the highest amplitude of the signal (fig. 7).

Duplicated genes that retained the same expression patterns could potentially influence our analysis by inflating overrepresentation signals. To estimate the presence of duplicated genes within our data set, we clustered 5,592 zebrafish genes using the BLASTCLUST software (NCBI BLAST toolkit) (Altschul et al. 1997). We independently clustered genes within each phylostratum by requiring that clustered sequences overlap more than 80% of their length (-L 0, -b T) with the minimal sequence identity threshold of 80% (-S 80). This is a balanced cutoff that should identify paralogs and not just genes with similar domains. We found that only 365 genes (6.5% of the total) are paralogs, which are distributed over 155 clusters (supplementary table S3, Supplementary Material online). We tested the overlap in expression patterns within these paralog clusters and found that only genes in 16 clusters have identical expression patterns. This makes 0.6% of genes in our data set (33 out of 5,592). To further test the effect of these 33 genes on the results of our analysis, we excluded them from the data set and found that all phylostratigraphic profiles remained unchanged. Therefore, we decided to keep all available genes in the phylostratigraphic analysis independent of their duplication status.

Reanalysis of *Drosophila*

A phylostratigraphic reanalysis of the *D. melanogaster* CNS data (after Domazet-Lošo et al. 2007) was performed in a way similar to the zebrafish procedure. The specific goal here was to find how adaptive signals in the line leading to arthropods compare with the adaptive signals in the zebrafish line. We retrieved 13,389 fruit fly protein sequences from the FlyBase database (Tweedie et al. 2009) and compared these sequences against the nr database (NCBI) by the BLASTP algorithm (Altschul et al. 1997) at *E* value cutoff of 1e-03. Supplementary table S4, Supplementary Material online, shows the number of sequences and the list of organisms with genomes present in the updated BLAST database. We used the obtained BLAST output to map fruit fly genes onto our updated consensus phylogeny (supplementary table S5, Supplementary Material online) that spans 14 evolutionary levels (phylostrata) starting from the origin of cellular organisms (ps1) and ending at the origin of *D. melanogaster* (ps14). The *Drosophila* in situ expression data of embryonic stages that encompassed 1,964 fruit fly genes (14,024 expression domains) were retrieved from the Berkeley *Drosophila* Genome Project (Tomancak et al. 2002) (supplementary table S6, Supplementary Material online). Mapping and statistical analysis of genes expressed in the fruit fly CNS were performed as described above for zebrafish (supplementary table S6, Supplementary Material online).

Supplementary Material

Supplementary tables S1–S6 and figures S1–S4 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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