Marine Origin of the Arachnid Brain Reveals Early Divergence of Chelicerata

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Cambrian preservation of fossilized tissues provides crucial information about divergent cerebral arrangements amongst stem arthropods. One such genus is *Mollisonia*, whose clustered appendages beneath a frontal carapace suggest an early chelicerate. Here we apply neuroanatomical, genetic, and developmental analysis to curated fossil data of *Mollisonia* to demonstrate that instead of a linear organization of seriate parts like in other Cambrian arthropods, the *Mollisonia* brain is folded back over segmental ganglia of the prosoma, an organization characterizing the brains of all modern arachnids. The unexpectedly early acquisition of this apomorphic character state demonstrates the divergence of marine Arachnida represented by *Mollisonia* from Merostomata (horseshoe crabs) and its sister group Pycnogonida (sea spiders), both of which have linearly organized cerebra. While fossil evidence supports a marine origin of total group Chelicerata, we show Mollisoniidae defining the base of the arachnid tree of life that excludes Merostomata and Pycnogonida.

INTRODUCTION

Clarifying the organization of the arthropod head and brain, both in extant and extinct genera, has been called "the endless pursuit (*1*)" owing to the striking morphological diversity and the assumption that partitions of the brain are equivalent to segments of the trunk (*2-4*). However, developmental gene expression provides no support for cephalic segmentation. Precisely timed non-cyclic combinatorial gene expression patterns determine the partitioned asegmental brains of euarthropods (*5*). This differs markedly from the cyclic expression of the segmentation genes that determine their trunks (*6*), where each segment is a 'repeated unit along the anterior-posterior (a-p) axis having the same a-p polarity' (*7*). In contrast to the trunk's segmental ganglia,

the cerebrum comprises three unique neural domains denoted as ce1-ce3 (*8*). Each domain is specified by the unique combinatorial activity of a subset of homeobox genes that are highly conserved across genera (*8-12*), thus indicating their origin in deep time (Fig. 1A). Each domain is further defined by its unique neuropil and sensory traits (Fig. 1A-G), which align with identifiable fossilized soft tissue interpreted as neural (*8, 10*). For example, the most anterior domain ce1 (the prosocerebrum) is defined by combinatorial FoxQ2, Six3, and hbn activity, anterior to the rostral limit of Pax6 expression (*8, 10, 11*). Its suite of sensory and neurological traits identifiable in fossils includes the paired labra, principal eyes of arachnids, ocelli and nauplius eyes of pancrustaceans, and their cognate neuropils (*13-15*). The second domain ce2 (the protocerebrum) is likewise specified by unique combinatorial gene expression patterns, as is the most posterior domain ce3 (the deutocerebrum) (*8-12*).

Exceptional "Burgess Shale-like preservation" (BST) of the Cambrian biota occasionally results in the preservation of soft tissue, including nervous system (*13, 16)*. This provides a singular window into the evolution of the arthropod brain and the corresponding evolution of sensory organs and appendages (*13, 15)*, traits recognized in upper stem euarthropods that anticipate organization of descendant crown genera. For example, the identification of fossilized nervous system in the megacheiran *Alalcomenaeus* reveals a brain and contiguous ventral ganglia that closely match those in the larval horseshoe crab *Limulus* (Fig. 1E, F), a member of Xiphosura (*14)*. The appendicular chelicerae of *Alalcomenaeus* and *Limulus* are supplied by nerves extending from the ce3 domain (*14, 17),* and the dioptric organization of the *Alalcomenaeus* eye corresponds to the layer of inward protruding corneal cones of *Limulus* lateral eyes (*18*.) Thus, traits defining cerebral domains and their innervated head appendages of living euarthropods appear to correspond to traits resolved in early Cambrian fossils, as already exemplified by observations of the cerebrum and segmented trunk nervous system of the armored lobopodian *Cardiodictyon catenulum* (*8, 10*). Together these findings predict a highly constrained ground pattern conserved across living euarthropods: linearly organized cerebra that are defined by corresponding expression domains of homeobox genes that likely have existed since at least the early Cambrian (*8, 10*). Here we test this hypothesis on a mid-Cambrian fossil proposed to be a basal representative of Chelicerata (*19*), the second most species-rich clade of segmented ecdysozoans.

RESULTS

Together with *Alalcomenaeus*, two other genera have been considered the earliest chelicerate: the mid-Cambrian *Sanctacaris uncata* (*20*), and *Mollisoniida* represented by the morphologically similar coeval species, *Mollisonia plenovenatrix* (*21*) and *M. symmetrica* (*19*). All three are considered chelicerates because of their tagmosis (*20*): a frontal carapace under which extend appendages is claimed as the prosoma contiguous with an opisthosoma represented by a seven-segment trunk followed by a three-segment telson (*19, 21*). Here we examine curated raw data of a specimen of *M. symmetrica* (specimen MCZ 1811, Fig. 2A; see also Acknowledgements) which although missing its appendages, is nearly identical to *Mollisonia plenovenatrix*. In both genera, paired ovoid-shaped eyes each house two immediately adjacent areas that are each interpreted as fossilized photoreceptor layers with their associated first optic neuropil (inset to Fig. 2A, B, C) (*21*). In both mollisoniid species, one of these adjacent areas subtends a forward quadrant of the eye's perimeter of crystalline cones; the other area subtends the lateral quadrant (Fig. 2B). This arrangement corresponds to the confluent double retinal neuropils seen in late embryonic stages of the extant wandering spider *Cupiennius salei* (*22*) before the segregation of the lateral secondary eyes from the forwards-gazing principal eyes. Prior descriptions of the cephalon of both *Mollisonia* species point to extreme rostral traces of preserved optic nerves (*19*). We confirm the divergence of two stout nerves, one from each of the retinal neuropils of *M. plenovenatrix* (Fig. 2B, see also *21*). Descriptions of *M. symmetrica* reveal an association of 2-3 small swollen volumes nested along the trajectory of tracts from the lateral retinal neuropil. These volumes have been previously suggested as belonging to a fused cerebrum or "synganglion" close to the anterior margin of the prosoma (*19*).

Unlike specimens of *M. plenovenatrix*, neural traces in *M. symmetrica* are extremely well defined. Obscured on the left in Fig. 2A but prominently visible on the right where some of the capping of the prosomal carapace appears to be missing (dashed box and enlarged in Figures 2E, F), three discrete fossilized cerebral domains and their associated nerve tracts occupy a substantial area of the prosoma. We interpret the overall laterality of these domains and their associated nerve tracts to be a consequence of the dissociation of the brain's two halves along the midline, the likely structural weakness during burial. The spatial interrelationships of the visible

residues are clear and unbroken, however, as are the fossilized tracts associated with them. We identify three contiguous yet individually distinct cerebral domains. Two nerve tracts extend to them from the eyes. The lateral retinal neuropil extends a tract decorated by possibly three small neuropils (Fig. 2C), as previously suggested (*19*), to the second of the three cerebral domains (Fig. 2F), which we designate the ce2 domain. The eye's medial retinal neuropil, here named the principal eye retina for reasons that will become apparent, extends a tract (pet in Fig. 2D, F) that curves around the perimeter of the three domains to merge with the most posterior domain (ce1).

The observed arrangements thus depart from all previously described Cambrian brains (*8, 10, 16*), as do the fossilized tracts from the most anterior domain that extend to the front of the head shield where they continue into the small-paired chelicerae (Fig. 2A inset, E). To further investigate these differences, we compared *Mollisonia* to other euarthropod cerebral arrangements. Comparisons of fossils and crown taxa are enabled by aligning their identifiable junction between the fore- and midgut, known as the endomesodermal interface (EMI; ref. *8*) as shown in Figure. 1C-F. In *Mollisonia* we identified the EMI as situated anterior to its most rostral cerebral domain (Fig. 2F). This contrasts with other stem euarthropods where the EMI is located at or immediately caudal to the first trunk segment, T1 (Fig. 1A-F; also ref. *8*). To rule out that we may have mistakenly identified neural traces as serially arranged gut diverticuli, we superimposed elemental calcium carbonate scanning of *M. symmetrica* (specimen MCZ1811 (*19*); see Acknowledgements). This identified gut diverticuli positioned between deposits interpreted as neuropils and the lateral wall of the midgut (Fig. 2E).

These data identify three neural domains in *Mollisonia* (Fig. 2E, F, G), as expected of the arthropod cerebrum, and resolved in *C. catenulum* (*8*). But their relationships with the retinal neuropils and chelicera are in the reverse order compared to the linear brain ground pattern shown in Figure 1. In *Mollisonia*, a nerve tract from the medial member (principal eye) of the two adjacent retinal neuropils extends to the most caudal of the three cerebral domains (pet in Fig. 2D). This arrangement is homologous to fossilized tracts in the linear brains of *Alalcomenaeus* and to histologically demonstrable tracts in extant mandibulates, here exemplified by Hexapoda (Fig. 1D), in which the genetic specification of simple eyes (ocelli) and their connections to the cerebrum is part of the combinatorial code defining the

prosocerebral ce1 domain (*10*). We identify the most caudal of the cerebral domains of *Mollisonia* as the ce1 domain because of its connection to the principal eye retinal neuropil. Further, in all upper stem mandibulates examined to date, and in *Alalcomenaeus*, *Limulus* and its sister taxon Pycnogonida (*14, 17, 23*), the most posterior of the three domains, ce3 provides nerves to the paired deutocerebral appendages: antennal in mandibulates, cheliceral in *Alalcomenaeus* and *Limulus* (Fig. 1C-F). In *Mollisonia*, however, neural traces innervate its chelicerae from the most rostral domain of the fossilized cerebrum (Fig. 2F, G), hence our identification of it as corresponding to the deutocerebral ce3 domain (Fig. 3C). The passage of the principal eye tract (pet, Fig. 2D-F) that bypasses the ce3 and then the ce2 domain *en route* to the caudal ce1 domain (Fig. 2F) typifies the passage of axons and relays from the principal eyes to the ce1 domain arcuate body of crown araneans as evidenced by silver-stained brains of spiders (Fig. 2H, I; compare to D-F). This organization is especially prominent in sparassid and salticid spiders, both errant predators with prominent principal eyes (Figs. 2J-L, 3F) but is also present across most aranean lineages (*24-26*) that possess principal eyes. Together these findings identify a cerebral organization of *Mollisonia* that is essentially indistinguishable from extant arachnopulmonates.

DISCUSSION

The present study examined curated material of the mid-Cambrian fossil *Mollisonia*, which has been proposed to be a basal representative of Chelicerata (*19*). Our comparative analysis has identified a brain that comprises three unique neural domains (ce1-ce3) and tracts typifying the euarthropod cerebrum, but in reverse order. That the reflected cerebral organization of *Mollisonia* diverges from the linear cerebral organization of other upper stem euarthopods, such as *Alalcomeneaus* (Fig. 3A-E), has implications for understanding the earliest appearance of euarthropod classes recognized today, as well as the evolution and diversification of Chelicerata discussed below.

Our findings differ from prior descriptions that assume a linear mollisoniid brain (*19*, *21*) and propose seven nerve roots extending from a cerebral 'syncerebrum' of *Mollisonia* (*19*). However, this would mark a striking exception to all linearly organized crown and stem euarthropod cerebra as these are invariably endowed with just two pairs of uniramous

appendages, the ce1 labral and the ce3 deutocerebral appendages (Fig. 1). Six pairs of prosomal appendages described for *M. plenovenatrix* (*21*) are not visible in the *Mollisonia* specimen MCZ 1811 but are assumed to lie deeper, obscured by the matrix. The ventral disposition of segmental ganglia in the opisthosomal trunk has, however, been shown in another much smaller specimen of *M. symmetrica* (USNM 305093). An image of this specimen (Fig. 3a,b of *19*) offers a ventral view of the prosoma and opisthosma whose segments in the latter are each equipped with an identical ganglion, except for segments 9 and 10 in which ganglia appear fused (Fig. 3c of *19*). This image of USNM 305093 also demonstrates a single elongated prosomal neuropil from which paired ventral nerve cords extend posteriorly, linking the widely spaced trunk ganglia. The rostral neuropil has been interpretated as a ventral brain from which it is suggested seven pairs of nerves extended laterally to the presumed locations of as many pairs of appendages (*19*). We interpret this organization differently, suggesting the elongated deposit comprises six fused trunk segments T1-T6, the first of which in extant arachnids would innervate the paired pedipalps, with the remaining others associated with the walking legs, as proposed in Fig. 2G, L and Fig. 3E.

The observations discussed above indicate a completely novel variation of the euarthropod cerebrum. It is reflected, meaning that it is doubled back over the prosoma's appendicular ganglia. This organization of the cerebrum in *Mollisonia* also explains the location of its endomesodermal interface which at face value appears to be an exception to the rule. In crown and stem euarthropods, the EMI is located at or immediately caudal to the first trunk segment, T1 (Fig. 1C-F; see also ref. *8*). In *Mollisonia*, however, its EMI is seen located anterior to its deutocerebral domain ce3 as it is in extant arachnids (Fig. 2F; Fig. 3D–F). Nevertheless, if the reflected cerebrum of *Mollisonia* would be unfolded, the location of the EMI is perfectly aligned with those in linear brains, immediately in front of the first trunk segment T1 as in *Alalcomenaeus* (Fig. 3B, C). In living araneans, reflection of the brain is a result of morphogenetic movements during late embryogenesis when the opisthosoma straightens and condenses its trunk segments, whereas the curvature of the embryo around the spherical yolk persists rostrally (*27*), resulting in the deutocerebrum becoming located most anteriorly with reference to the body axis (Fig. 3D). The observed similarities thus identify the 'reflected brain' as a shared derived character of both *Mollisonia* and extant arachnids (Fig. 2L; 3D-F), strongly suggesting they belong to the same lineage.

These findings are in line with just one of Charles Walcott's ascriptions of Burgess Shale fossils to extant clades. Famously assessed as "shoehorning"(*28*), in the present context this is serendipitously vindicated by early descriptions of the Burgess Shale *Sanctacaris*, whose tagmatal organization signifies the morphological ground pattern of Chelicerata (*20*). Two cladistic analyses based predominantly on non-neural traits of the mid-Cambrian Mollisoniida have proposed a deep origin of Chelicerata, placing the genus *Mollisonia* stemward of Megacheira (*19, 21*). Like *Sanctacaris* (*20*), the status of *Mollisonia* as a chelicerate relates in the first instance to its distinctive carapaced prosoma and elongated opisthosoma, its segments defined by prominent exopods proposed as precursors of book gills (*21*). The present finding that *Mollisonia* possessed a reflected cerebrum resembling those of arachnopulmonates raises the question whether this taxon can be viewed as the exclusive upper stem ancestor of total Chelicerata, as recently proposed (*19*), or as the exclusive upper stem ancestor of Arachnida, as proposed here (Fig. 4). The present findings also bring Megacheira, as represented by *Alalcomenaeus* (*14*), to the fore as denoting a divergent merostomatan branch of Chelicerata implied by its separate retinal arrangements (*18*) and its linear brain (Fig. 1E, F). Fossils of crown chelicerates are less informative regarding early divergence: the earliest fossils of terrestrial arachnopulmonates are from the lower Devonian (*29, 30*). The first limulus-like fossils are ascribed to the late Ordovician. However, a late Carboniferous specimen preserved with its entire brain outlined demonstrates the posterior location of its cheliceral 'ganglia' (*30*) indicating the linear organization of its cerebrum.

Molecular phylogenetics indicates that Arachnida likely colonized land well before fossil evidence indicates it (*31*). The reflected configuration of the arachnopulmonate cerebrum here ascribed to mollisoniids requires a divergence from the conventional view that *Limulus* is included within Arachnida. Pycnogonida, recognized as the sister group of Euchelicerata, also possess linear brains: although in many pycnogonid species the neuraxis is tilted upwards, their chelicerae are innervated by a deutocerebrum situated posterior to the protocerebrum (*32-34*), as in Xiphosura (*23, 33*). The character state 'linear brain' thus supports the proposition that megacheirans with linear cerebra comparable to the developing limuliid brain (*17*) are best viewed as upper stem euarthropods most closely related to a xiphosuran/pycnogonid grouping

with the exclusion of Arachnida (Fig. 4). This distinction of cerebral organization, linear in fossil *Alalcomenaeus* and extant Pycnogonida and Xiphosura, but reflected in fossil Mollisoniida and extant Arachnida, resonates with Ballesteros et al.'s recent analysis of over 500 high-quality genomes and transcriptomes sampling all living orders of Chelicerata (*35*). Their phylogenomic analyses highlight the ambiguous status of Merostomata, including Xiphosura, suggesting that they may constitute derived arachnids (*35*). Acknowledging those phylogenomic considerations, we nevertheless propose that fossil evidence of cerebral disparities between two stem euarthopods and their descendants indicate an early divergence of Arachnida and Merostomata that had already occurred by the mid-Cambrian (Fig. 4), with the linear cerebrum of the megacheiran *Alalcomenaeus* (*14*) identifying it as the stem taxon closest to Pycnogonida and Xiphosura; and *Mollisonia* as the stem taxon closest to extant Arachnida, supporting a marine origin of total group Chelicerata.

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Materials and Methods

Fossil provenance and analysis. Original raw high-definition TIFF files of the main specimen MCZ1811a and MCZ1811b, including elemental analyses, were kindly provided by Dr. Javier Ortega-Hernández, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, and the Museum of Comparative Zoology in which the curated specimens MCZ1811a and MCZ1811b are stored. Selected enlargements of the high-definition files, as shown in Figure 1A, C-E, were processed using Adobe Photoshop 24.6 employing camera raw filter functions for adjusting color parameters in preparation for conversion to greyscale mode.

Microscopy. Images of the reduced silver-stained cerebrum of the huntsman spider *Delena cancerides* were taken using a JenaOptic Progress C3 digital camera fitted to a Leica Diaplan microscope equipped with plan neofluar oil immersion objectives.

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FIGURES and FIGURE LEGENDS

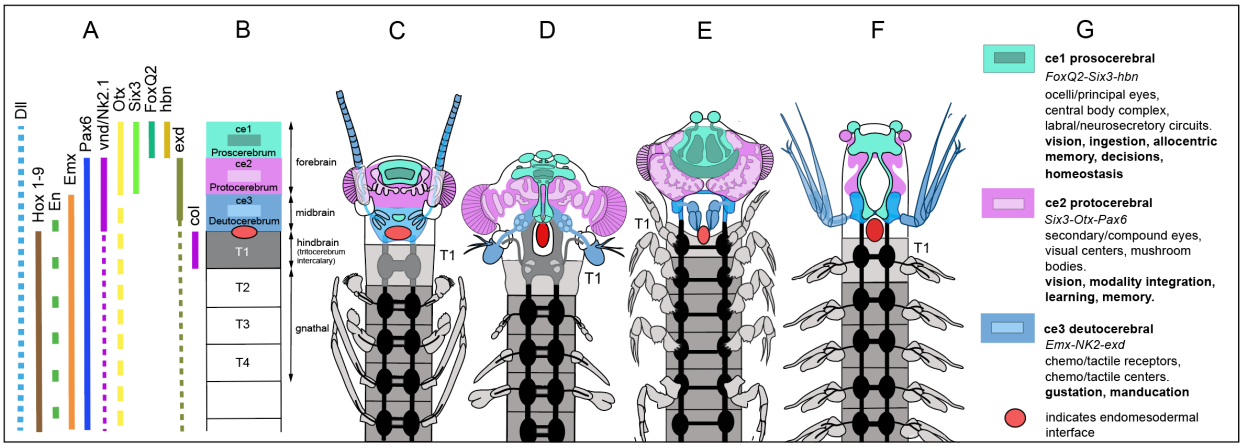

Figure 1. Conserved organization of the crown and stem euarthropod brain. **(A, B)** Combinatorial expression of homologous genes define three domains of the asegmental cerebrum across taxa: ce1 prosocerebrum; ce2 protocerebrum; ce3 deutocerebrum (refs. *8, 10* and references therein). The first true segment (T1; tritocerebrum or intercalary segment) defined by *collier* (*col;* ref. *36*) is part of the hindbrain as are gnathal segments T2-T4. The junction between asegmental brain and segmental trunk coincides with the endomesodermal interface (EMI) between the fore- and midgut (red ovoid; ref. *8*). **(C)** Schematized nervous system of the chilopod *Strigamia maritima* indicating domain ce1 including the central complex; ce2 denoted by mushroom bodies and compound eye neuropil; and ce3 characterized by its association with the mouth, antennules, and centers for chemoperception. **(D)** Schematized cerebral domains of a pancrustacean (Diptera) showing ocellar eyes ascribed to ce1, as is the central complex, labrum, and neurosecretory islets. **(E)** Schematized embryonic *Limulus*, showing linear arrangement of ce1, with central complex supplied by principal eyes; ce2 with centers associated with mushroom bodies supplied by lateral (compound) eyes; and ce3, which supplies a short nerve to ventral chelicerae in front of first trunk appendages. **(F)** Schematized cerebral domains of the megacheiran upper stem euarthropod *Alalcomenaeus* (*14*) (ventral view). ce1 defined by paired principal eyes; ce2 by lateral secondary eyes and protocerebrum; ce3 by nerves to cheliceral appendages. Labral ganglia connect to the ce1 prosocerebrum. **(G)** Genetic code of brain domains and their domain-specific neural centers applying across extant Euarthropoda and Onychophora (*8, 10*)**.**

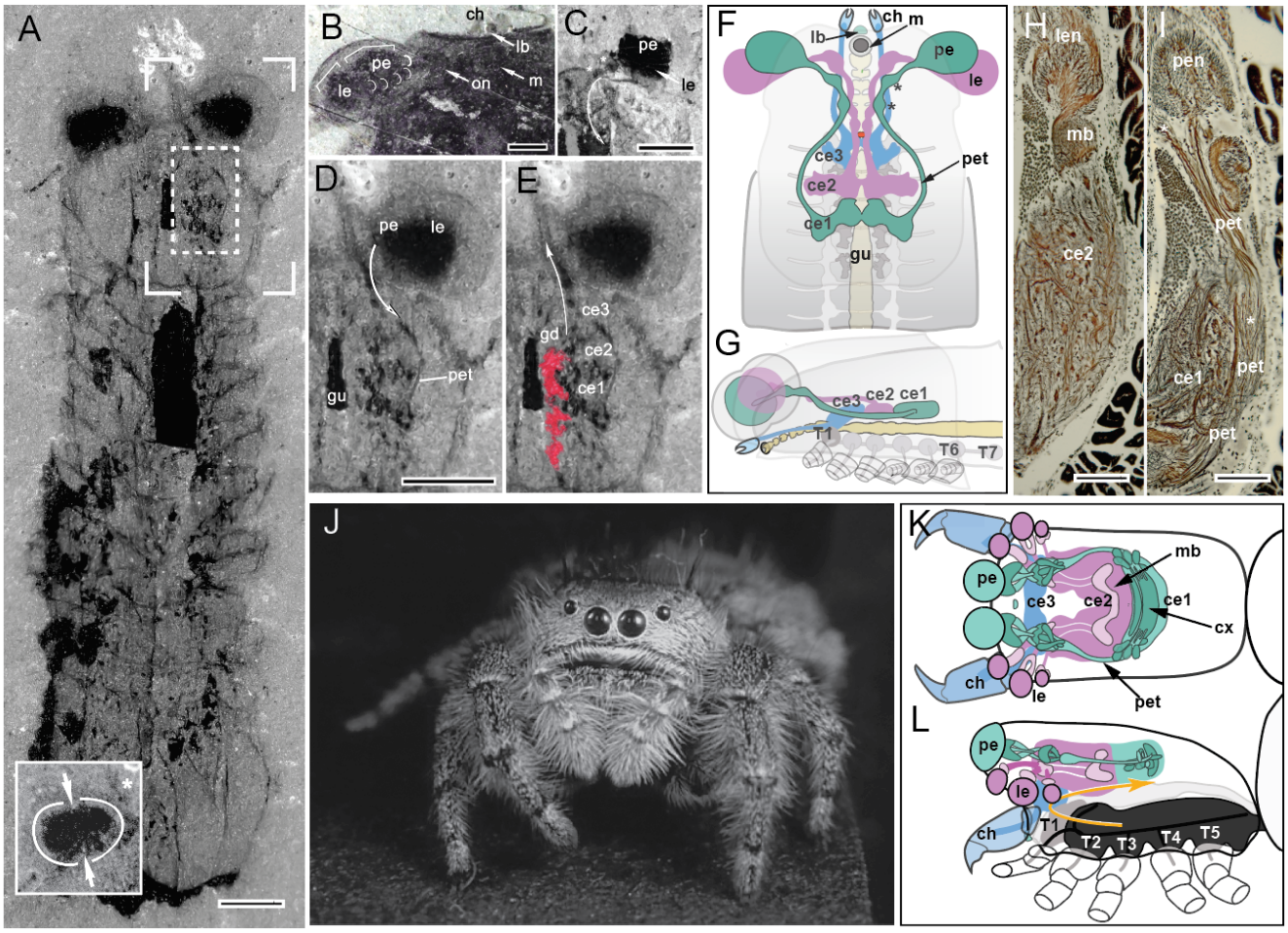

Figure 2. Arachnid-specific cerebrum of *Mollisonia*. (A) Specimen of *M. symmetrica* (MCZ1811, image courtesy of J. Ortega-Hernández, Museum of Comparative Zoology, Harvard University) one side of its prosoma bracketed with box indicating enlargements in panels D, E). The inset lower left shows a high contrast image of the left eye indicating the approximately midline division between the outer and medial (principal) retinas and their associated neuropil. The asterisk draws attention to the left chelicera. **(B)** Detail of specimen ROMIP 62978 *M. plenovenatrix* (with permission, *Springer Nature*) showing two retinae (bracketed) subtending one ovoid eye as indicated in ref. *21*. Thin serial rings indicate a nerve from the lateral eye retina (le) to the optic neuropil (on). The principal eye retina (pe) extends a separate tract (encircled by a thick ring). **(C)** One optic tract shows small ganglion-like swellings (asterisks) (backscatter electron microscopy (*19*), courtesy of J. Ortega-Hernández). **(D, E)** Contiguous carbonaceous residues interpreted as cerebral domains ce1-ce3. White arrow (panel D) indicates the principal eye tract (pet) bypassing ce3 and ce2, terminating laterally in ce1. Overlaid calcium signal [red:

elemental scan (*19*) courtesy of J. Ortega-Hernández] denotes gastric diverticula (gd) between ce1-ce3 and the gut (gu) wall. A nerve fiber (panel E) indicated by the white arrow extends forward from ce3 to a chelicera. **(F, G)** Interpretive drawings of a top-down (F) and side (G) view of ce1-ce3, folded back over a presumed cohesion of five trunk ganglia, T1-T6. Red spot indicates the endomesodermal interface. **(H, I)** Reduced silver stains of the brain of the brown huntsman spider *Delena cancerides* showing, at two levels of the right hemibrain, lateral eye connections to the ce2-specific mushroom body and the diagnostic optic nerve from the principal eye to the ce1-specific central complex. (**K, L**) Dorsal and side view of cerebrum of the salticid *Phiddipus* sp. (panel J) overlying the prosoma's fused trunk ganglia. The yellow arrow in L indicates the reflected neuraxis. Note the four pairs of walking legs (T2-T5) indicating the evolved loss of the ancestral mollisoniid T6. Abbreviations: ch, chelicera (*21*); ce, cerebral domain; gu, gut; lb, labrum; m, mouth (*21*); mb, mushroom body; pet, principal eye tract; len, lateral eye neuropil; pe, principal eye (and neuropil); Tx, trunk segment. Scales: A-D, 1 mm; H, I, 200 μm.

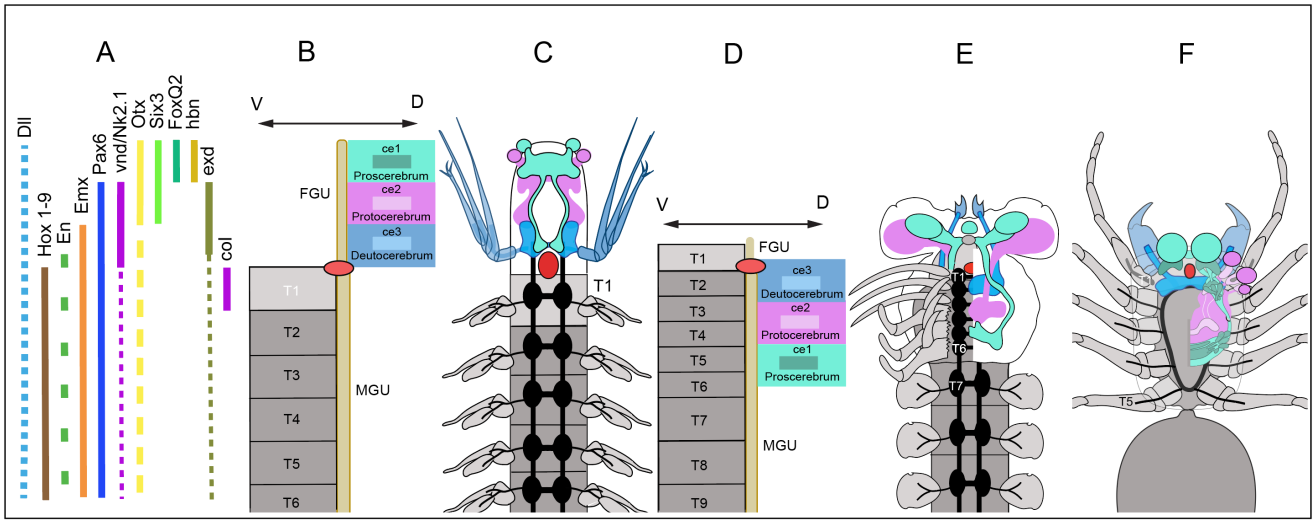

Figure 3. The linear cerebrum of *Alalcomenaeus* and reflected cerebrum of Arachnida. **(A)** Combinatorial expression of homologous genes across extant taxa defines the three domains of the asegmental cerebrum (ce1 prosocerebrum; ce2 protocerebrum; ce3 deutocerebrum (*8*, *10* and references therein) with first trunk segment T1 defined by *collier* (*36*). The junction between the asegmental cerebrum and the segmental trunk coincides with the endomesodermal interface (EMI, red ovoid; ref. *8*). (**B, C**) Linear brains exemplified here by the megacheiran *Alalcomenaeus*, align along the anterior-posterior axis with the cerebrum situated dorsally above the foregut (FGU) and subsequent trunk ganglia beneath the midgut (MGU), as schematically shown for the linear cerebrum of *Alalcomenaeus* whose deutocerebral nerves supplying the chelate appendages define the most caudal domain ce3. (**D**) Schematic of the reflected arachnid brain and segmental ganglia (grey, T1-T8 etc.). (**E**) Top-down view into the prosoma and first three opisthosomal segments (T7, etc.) of *Mollisonia*, with the carapace and left half of the brain removed to expose the underlying prosomal appendages and their ganglia (T1-T6). The evolution of a reflected cerebrum, typified by *Mollisonia* and defining extant Arachnida, brings the deutocerebrum (ce1 domain) forward as the most rostral domain (D) from where it supplies nerves to the paired chelicerae (E). (**F**) The reflected arachnid cerebrum is essentially unchanged in extant genera, here represented by the brain of a salticid aranean in which the ancestral prosomal appendages are further reduced to paired pedipalps and four pairs of walking legs. V-D, ventral–dorsal axis.

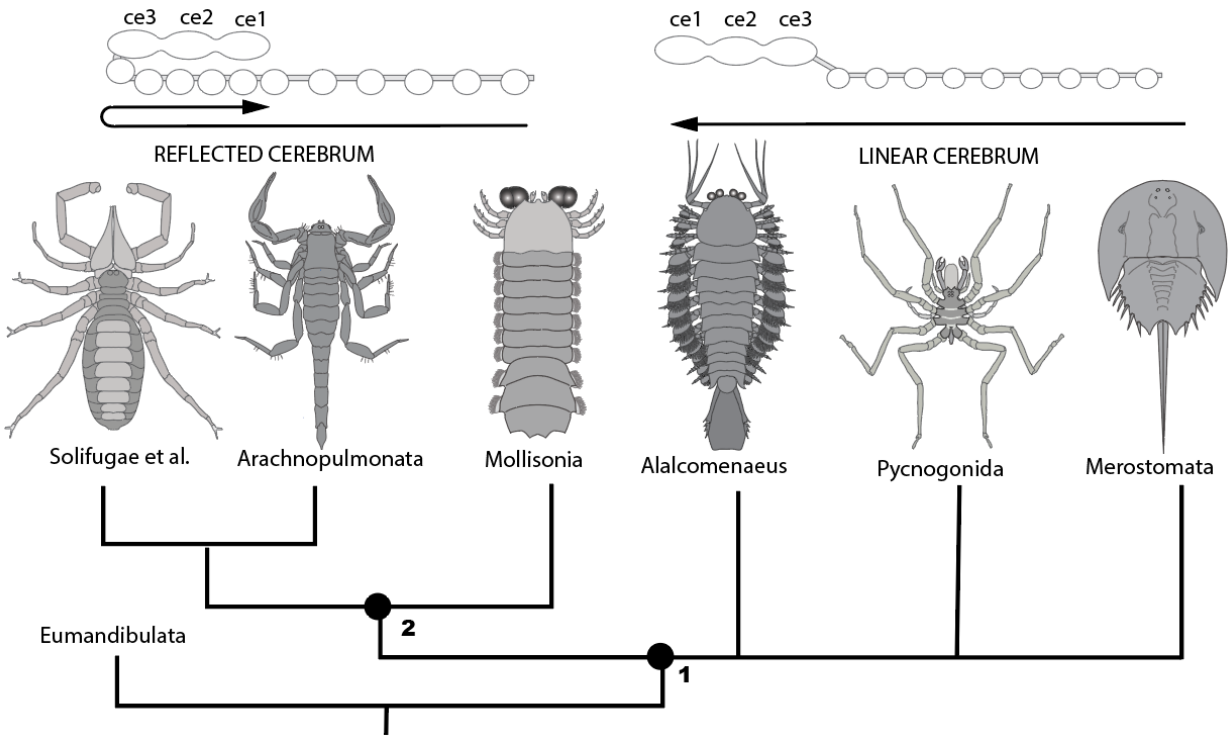

Figure 4. Divergent evolution of Chelicerata *sensu lato*. Reconstruction of *Mollisonia symmetrica* here incorporates rostral walking limbs recognized in related Cambrian species (*19-21, 37*). Two fossil total-group chelicerates, *Mollisonia* and *Alalcomenaeus,* exemplify divergent ground pattern organizations of the cerebrum (ce1-ce3); namely, linear or reflected, where the lineages *Alalcomenaeus* (*14*), limulids (*17*) and pycnogonids (*23*) participate in a four-way polytomy (node 1) with the clade total-group Arachnida at node 2. These phylogenetic relationships resonate with recent phylogenomic analyses of all living chelicerate orders (*35*); they identify the megacheiran *Alalcomenaeus* (*14*) as the stem taxon closest to Pycnogonida and Xiphosura/Merostomata, and *Mollisonia* as the stem taxon closest to extant Arachnida, thus revealing a marine origin of Arachnida and total group Chelicerata.