

On the trail of the 'new head' in Les Treilles

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The vertebrate brain develops in association with neighboring tissues: neural crest, placodes, mesoderm and endoderm. The molecular and evolutionary relationships between the forming nervous system and the other craniofacial structures were at the focus of a recent meeting at the Fondation des Treilles in France. Entitled 'Relationships between Craniofacial and Neural Development', the meeting brought together researchers working on diverse species, the findings of whom provide clues as to the origin and diversity of the brain and facial regions that are involved in forming the 'new head' of vertebrates.

Introduction

The vertebrate brain develops in close association with neighboring tissues, including cells from the neural crest, mesoderm and endoderm, such that their development is interlinked and interdependent. Together, these tissues form the central and peripheral nervous systems and the craniofacial skeleton of the vertebrate head. A recent meeting at the Fondation des Treilles in Provence, organized by Sophie Creuzet (Institute of Neurobiology, Gif-Sur-Yvette, France), Joy Richman (University of British Columbia, Vancouver, Canada) and Filippo Rijli (Friedrich Miescher Institute, Basel, Switzerland), focused on understanding and highlighting the tissue, cellular, molecular and evolutionary relationships between the forming nervous system and other craniofacial structures.

The 'new head', as proposed by Gans and Northcutt (Gans and Northcutt, 1983), is a defining feature of vertebrates, distinguishing them from invertebrate chordates that have a central nervous system (CNS) but lack a complex brain, peripheral ganglia and craniofacial skeleton (see Fig. 1). During development, the vertebrate brain forms from the neural plate, a specialized region of the ectoderm that invaginates to form the neural tube. The edge or 'border' of the neural plate contains presumptive neural crest cells that migrate away from the CNS to form peripheral ganglia, craniofacial bone and muscle, pigment cells, and numerous other derivatives. These cells travel through mesodermal tissue and interact with endoderm within the branchial (pharyngeal) arches; these arches, in vertebrates, give rise to jaws, among other structures. Immediately lateral to this tissue is the preplacodal domain, which contains precursors to the 'ectodermal placodes'. Placodal ectoderm thickens and gives rise to cells that ingress and contribute to neurons of the cranial sensory ganglia. Together with the neural crest, these placodes form the peripheral nervous system of the head. The focus of this meeting was to understand the developmental and evolutionary relationships between these tissues in the building of the new head of vertebrates.

Neural crest and placodes

Both neural crest and placodes form at the border between neural and non-neural ectoderm. Thus, the establishment of this neural plate border region is an essential first step in the formation of these defining vertebrate cell types. Under the influence of various growth factors, including FGFs, Wnts and BMPs, and of a set of transcription factors, such as *Msx*, *Zic1* and *Pax3/7*, the border region is specified at an early step in neural crest formation. Recent advances include the definition of gene regulatory interactions that contribute to the formation of these border regions.

As Anne-Hélène Monsoro-Burq (Institut Curie, Orsay, France) has shown in *Xenopus*, the transcription factors *Pax3*, *Msx* and *Zic1* cooperate to specify the neural plate border (Monsoro-Burq et al., 2005). To expand the cascade of known players, she has identified new factors involved in regulating neural crest formation. As she discussed at the meeting, she found that another transcriptional regulator, *Hairy2*, is expressed early at the neural plate border. Her group's loss-of-function experiments indicate that, by regulating *Msx*, *Hairy2* mediates FGF and BMP signaling at the border. Thus, *Hairy2* is an essential mediator of neural crest induction, functioning at a proximal position in the neural crest gene regulatory network.

Further analysis of this network was presented by Marianne Bronner-Fraser (CalTech, CA, USA), whose laboratory is dissecting the direct interactions of neural crest specifier genes, such as *Sox10*. Using experimental manipulations and morpholino antisense oligonucleotides in both chick and the basal vertebrate lamprey, her colleagues have found that neural plate border genes are essential for regulating neural crest specifier genes, as well as for cross-regulating one another. Dissection of this neural crest gene regulatory network (NC-GRN) reveals that it is largely conserved in the base of vertebrates, suggesting that this is an ancient gene battery that has been tightly conserved for over 350 million years (Sauka-Spengler et al., 2007). Homologues of most genes involved in neural crest formation are also present in the basal chordate amphioxus, but most of the NC-GRN members involved in the specification of the vertebrate neural crest do not localize to the neural plate border (Yu et al., 2008).

Like neural crest, the ectodermal placodes originate from the neural plate border region, from a common 'preplacodal domain' marked by *Six1* and *Eya1*. Using gain- and loss-of-function experiments in *Xenopus*, Gerhard Schlosser (Brain Research Institute, University of Bremen, Germany) reported that these transcription factors synergistically regulate placodal neurogenesis (Schlosser et al., 2008). By producing a fate map within the preplacodal ectoderm, his group has determined the region of origin of different placodes within the pre-placodal ectoderm. Competence to respond to placode induction is confined to non-neural ectoderm, whereas neural crest is induced from neural ectoderm. This raises the intriguing hypothesis that placodes and neural crest have independent evolutionary and developmental origins from different sides of the neural plate border.

After neural crest cells emerge from the neural tube, they migrate along stereotypic and precise pathways to form multiple distinct derivatives. By performing clonal analysis to assess the developmental potential of avian neural crest cells, Elizabeth Dupin (Institute of Neurobiology, Gif-Sur-Yvette, France) showed that the cranial neural crest comprises progenitors that are multipotent, with the capacity to form both neural and skeletogenic derivatives. She reported that the treatment of trunk neural crest with *Shh* increases

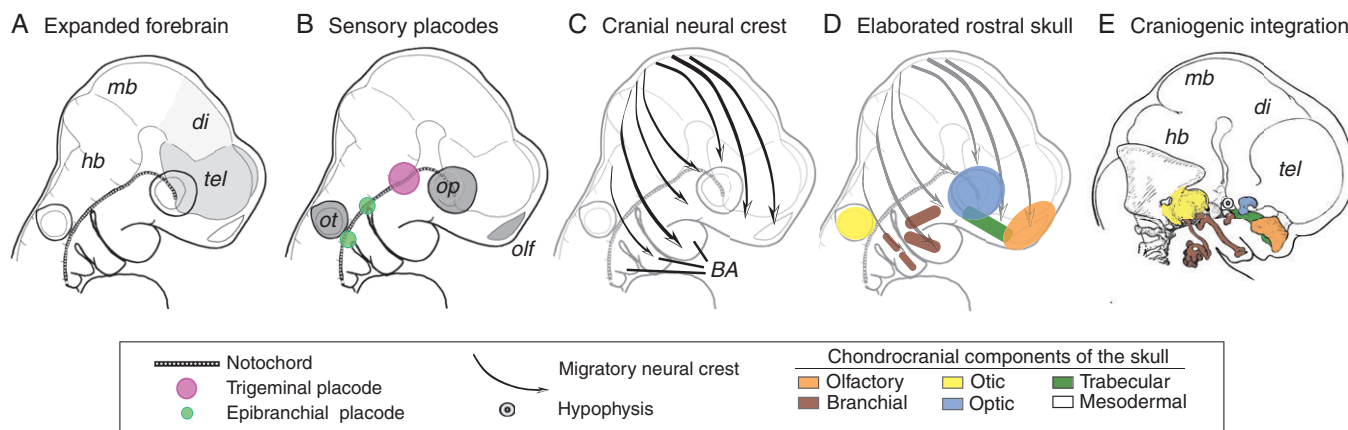


Fig. 1. Elements of cranial organization at the core of the 'New Head Hypothesis'. The New Head Hypothesis (NHH) of Carl Gans and Glenn Northcutt (Gans and Northcutt, 1983), as schematized in mouse (A-D) and human (E) embryos. (A-D) Schematics of embryonic day 9.5 (E9.5) mouse embryos, highlighting specific major tissue components of the NHH. The NHH posits that vertebrates exhibit unique characteristics, such as (A) an expanded alar plate of the forebrain; (B) formation of neurogenic sensory placodes from the surface cephalic ectoderm; (C) a migratory mesenchymal population of cranial neural crest cells that invade the branchial arches (BA) and surround the expanded forebrain and rostral primary sensory placodes; and (D) an expanded skeletal system to support the new rostrally expanded forebrain, rostral sensory capsules and BAs. (E) The hypothesized shift from a passive suction feeding protovertebrate to a predatory vertebrate with jaws made from modified BA elements necessitated a high degree of craniogenic developmental and functional integration. Abbreviations: BA, branchial arches; di, diencephalon; hb, hindbrain; mb, midbrain; olf, olfactory; op, optic; ot, otic; tel, telencephalon. (A-D) Modified, with permission, from Depew and Olsson (Depew and Olsson, 2008).

their ability to become skeletogenic (Calloni et al., 2007). These results show that neural crest cells at all axial levels are multipotent and that discrete growth factors can influence their differentiation.

Crucial for understanding events in craniofacial formation is the ability to see where cells are at various stages of development and to follow their progress as a function of time. Scott Fraser (CalTech, CA, USA) discussed the most recent techniques for imaging cellular and morphogenetic events in development (Ruffins et al., 2007). These include advanced tools for microMRI, digital dissection, laser scanning microscopy and optical tomography, all of which make it possible to visualize events in real time and/or at higher resolution than previously possible. These tools offer new insights into developmental mechanism and allow the key interactions between signaling and responding tissues to be followed with unprecedented accuracy. An ultimate goal is to assemble these insights into interactive three-dimensional atlases (see, for example, the atlas of mouse development at <http://mouseatlas.caltech.edu/>) for various species.

Factors involved in brain patterning

Hox genes are patterned in their expression along the rostrocaudal axis of the developing vertebrate nervous system, co-linear with their order on the chromosome. The most anterior vertebrate Hox gene is expressed in the hindbrain at the border between rhombomeres 1 and 2. Hox-positive neural crest cells migrate from the hindbrain into branchial (pharyngeal) arches 2 and 3, where they contribute to the formation of the bones of face. As they migrate, they express Hox genes in a similar manner to the rhombomeres from which they emanate.

Nicole LeDouarin (Académie des Sciences, Paris, France) posed the question 'would it be a head without the neural crest?'. LeDouarin and Sophie Creuzet have found that ablation of the neural crest from the anterior, Hox-negative domain, causes loss of the dorsal brain, the source of the modern 'intelligent' brain (Creuzet et al., 2006). The

resultant animals have no facial skeleton and an open brain. Interestingly, this phenotype can be rescued by grafting back a small piece of neural fold from anywhere within the Hox-negative neural folds. Thus, the Hox-negative domain appears to be an equivalence group with equal ability to rescue. By contrast, the Hox-positive caudal hindbrain region is unable to rescue the phenotype. This indicates that important regional differences exist in the neural crest that depend on Hox gene expression (Le Douarin et al., 2007).

Further evidence for the interdependence of facial and brain development was provided by Sophie Creuzet. She showed that ablation of the neural crest causes severe loss of facial structures and defects in the brain. This effect appears to be mediated by the loss of signals from the neural crest cells (Creuzet et al., 2006). In support of this, she found that the neural crest cells are an important source of the BMP antagonist gremlin. Exogenous addition of gremlin causes the expansion of the telencephalon and maintains expression of FGF8. After ablation of the neural crest, gene expression patterns characteristic of the dorsal midline of the neural tube are lost; however, the implantation of an FGF8-soaked bead restores this gene expression. These results suggest that FGF8 controls telencephalon development and limits Shh, which specifies the basal fate. These data provide a molecular explanation for the interactions that underlie the influence of the neural crest on the developing brain.

Factors involved in face patterning

In vertebrates, face and throat structures, such as the jaw, hyoid and thyroid cartilages, develop from a rostrocaudal metamer series of branchial (pharyngeal) arches, colonised by cranial neural crest cells, and represent a conserved feature of all vertebrate embryos. Anthony Graham (MRC Centre for Developmental Neurobiology, King's College London, UK) and colleagues (Blentic et al., 2008) have been exploring the interactions that occur between the neural crest and the pharyngeal epithelia. As Graham discussed, they have

found that FGF signalling plays an important role in promoting ectomesenchymal fate. If neural crest cells are rendered insensitive to FGF signaling, they enter the pharyngeal arches but fail to assume an ectomesenchymal fate.

The pharyngeal endoderm provides positional cues for the neural crest and is involved in the induction of a number of components of the pharyngeal arches. Thus, endoderm plays a role in facial patterning. Accordingly, Le Douarin and colleagues have shown that removal of the endoderm that underlies the first branchial arch causes a loss of Meckel's cartilage derived from neural crest. In the reciprocal experiment, Jose Brito (Institute of Neurobiology, Gif-Sur-Yvette, France) and LeDouarin and colleagues showed that grafting anterior endoderm laterally results in the production of a supernumerary lower beak (Brito et al., 2008). Shh is expressed in the endoderm and is responsible for this effect because, as Brito discussed, an antibody against Shh caused cell death in the branchial arch and a concomitant loss of the lower beak (Brito et al., 2006). Jill Helms (Stanford University, CA, USA) also discussed the role of Shh in shaping the face. Her studies show that modulation of Shh signaling can alter the shape of the face and can account for species-specific differences in facial features, such as differences in the spacing of the nostrils between chick and mouse. Her group's working hypothesis is that a gradient of Shh may be responsible for this scaling effect: low Shh levels cause fusion at the midline, whereas high levels cause expansion of the midline (Brugmann et al., 2006; Cordero et al., 2004).

The co-linear Hox gene expression patterns described above underlie arch-specific morphologies, with the exception of the first (mandibular) arch, which is devoid of any Hox gene activity. Filippo Rijli presented evidence that the simultaneous inactivation of all Hoxa cluster genes in the neural crest cells of mice leads to multiple jaw and first arch-like structures, partially replacing second, third and fourth arch derivatives, suggesting that rostral and caudal arches share the same mandibular arch-like default patterning program. This severe phenotype was not enhanced by concomitantly deleting the Hoxd cluster. Moreover, he showed that *Hoxa2* and *Hoxa3* act synergistically to pattern third and fourth arch derivatives. These results provide insights into how facial and throat structures are assembled during development, and have implications for our understanding of how the pharyngeal region of the vertebrate head has evolved.

Although jaws are principally derived from the first branchial arch, there is also a contribution from the frontonasal prominences. Joy Richman presented her group's fate map analyses in chick and mouse, from which they have found no evidence that large scale movements of cells occur between the mandibular and maxillary regions of the face. Moreover, their microarray gene expression studies reveal that gene expression patterns in the mandibular and maxillary regions are very similar. Other studies from her laboratory suggest that the signals that are involved in tooth development are conserved among animals, as they have found that Shh is involved in tooth production in the python, as it is in birds and mammals.

Michael Depew (Department of Craniofacial Development, King's College London, UK) has previously shown that Dlx genes are required for the construction of the jaws, such that loss of *Dlx5/6* function causes homeotic transformation around the jaw hinge (Depew et al., 2002), and also that FGF8 is required for jaw development in the oral ectoderm (Trumpp et al., 1999). At the meeting, Depew reported that the cell population expressing the transcription factor *Satb2* in the developing jaw primordia of mice represents a developmentally and evolutionarily significant jaw module (Britanova et al., 2006). Furthermore, a repertoire of

transcription factors, including Pax6, Foxg1 and Dlx5, which are expressed both in the developing CNS and the surface cephalic ectoderm, interact to establish ectodermal competence to generate a signaling center called the lambdaoidal junction that is essential for the patterning of the upper jaws.

Cell and molecular mechanisms of brain development

The CNS initially forms from a cylindrical neural tube that subsequently balloons into three primary vesicles: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). After neural induction, 'secondary organizers' refine the anteroposterior specification of the brain. Salvador Martinez (Institute of Neurosciences, Alicante, Spain) discussed the nature and function of several secondary organizers: the anterior neural ridge (ANR) at the anterior end of the neural plate/tube, the zona limitans intrathalamica in the middle of the diencephalon and the isthmus organizer (IsO) at the mid-hindbrain boundary. FGFs activate signals from IsO and ANR organizer regions, and are present in a gradient-like distribution in the extracellular compartment. This gradient, acting through FGF receptors, activates intracellular transduction pathways that are required for the cell-autonomous control of *Fgf8* expression and for activating the expression of multiple developmental genes that regulate cell fate decisions, axial polarity and cell survival. In addition, the morphogenetic activity of these secondary organizers controls the polarity and generation of neural subregions within the forebrain, midbrain and hindbrain. Thus, secondary organizers appear to confer positional identity by secreting a graded concentration of a signal, which in turn triggers concentration-specific genetic cascades.

As the brain differentiates, there is extensive migration of neurons to proper sites. Filippo Rijli showed that *Hoxa2* genes control the long-distance migration of pontine neurons along the rostrocaudal axis in the developing brainstem. These neurons are essential for coordinated motor activity and provide the principal input to the cerebellum. He showed that *Robo2* is a direct target gene of *Hoxa2* and interacts with its ligand Slit2, which is secreted by the facial motor nucleus, preventing the premature attraction of neurons towards the brainstem ventral midline (Geisen et al., 2008).

Another aspect of neuronal maturation is the acquisition of neurotransmitter phenotype. Philippe Vernier (Institute of Neurobiology, Gif-Sur-Yvette, France) discussed the evolution of the dopamine system and neuromodulation in chordates. Many unrelated systems depend upon dopamine and control different brain functions, such as sensory discrimination in the olfactory bulb, sensorimotor programming in the basal ganglia, or body temperature and feeding behaviors. Tyrosine hydroxylase (TH) is the key enzyme of dopamine biosynthesis. Interestingly, genome-wide duplication in vertebrates resulted in two TH genes in early gnathostomes, one of which (*Th2*) was lost in mammals. Despite this gene loss, the dopamine systems are essentially conserved across vertebrates. By contrast, there is little homology between the dopamine systems of invertebrate chordates, such as amphioxus and Ciona, to each other or to those of vertebrates. Interestingly, ascidians lack dopamine receptors and transporters but appear to use other monoamine receptors and the serotonin uptake system instead. Thus, most dopamine systems are true vertebrate novelties.

Evolution of the new head

Cephalochordates and urochordates have body plans that are similar to those of vertebrates, with a nerve cord, gill slits, notochord, segmented body and post-anal tail. The simple brain of amphioxus

holds clues to the origin of all chordates as many of its gene expression patterns are homologous to those of vertebrates. However, amphioxus lacks both migratory neural crest cells and ectodermal placodes. Linda Holland (Scripps Institution of Oceanography, CA, USA) discussed the evolution of head segmentation in amphioxus and reported that the head mesoderm of vertebrates appears to be related to the anterior somites of amphioxus. She provided an update on sequencing of the amphioxus genome, which is now complete and shows interesting synteny with the human genome. The data suggest that a twofold genome-wide duplication occurred between cephalochordates and mammals. Interestingly, the overall gene numbers are not very different between amphioxus and higher vertebrates. However, transcription factors and signaling molecules appear to have been preferentially retained in vertebrates (Putnam et al., 2008; Holland et al., 2008).

By comparing the developmental patterns of jawless versus jawed vertebrates, Shigeru Kuratani (Centre for Developmental Biology, Riken, Japan) focused on differences that occur during jaw evolution. He showed that tissue interactions involving *Bmp2/4*, *Fgf8/17*, *Dlx* and *Msx* genes are important for the specification of the oral domain. Interestingly, he also showed that, in cyclostomes, the oral domain was primarily decoupled from mandibular arch patterning, suggesting this may represent the ancestral state (Shigetani et al., 2002). However, the oral domain apparently became coupled with mandibular arch patterning in jawed vertebrates. Kuratani also reported on the ability to obtain embryos from hagfish for the first time in 100 years (Ota et al., 2007). Properly fixed embryos revealed that the hagfish had migrating neural crest cells that express *Sox9*, indicating that their molecular program is similar to that of other vertebrates and that their morphology is similar to that of lamprey.

The gnathostome (jawed vertebrate) skull has two general traits: (1) fidelity of bauplan; and (2) elaboration of design. Michael Depew presented a model of jaw development and evolution ('Hinge and Caps') in which he operationally defined jaws as two appositional hinged cranial units characterized by polarity and potential modularity. The hinge and cap model provides a mechanism that explains how the jaws are kept in functional registration over evolutionary time (Depew and Compagnucci, 2008). The hinge is a coordinated region involving the ectoderm at the mandibular-maxillary junction, plus the first pharyngeal plate (where the cleft ectoderm meets the pouch endoderm). The position of the hinge is driven by factors common to the junction of maxillary and mandibular process of the first branchial arch and the first pharyngeal plate (where the cleft meets the pouch). These two caps form the distal midline of the mandible for the lower jaw and the lambdoidal junction for the upper jaw. The lower jaw cap is driven by factors at the distal-most region of the first branchial arch, whereas the upper jaw cap is patterned by the above-mentioned lambdoidal junction. The integration of hinge and cap signals during development thus coordinates jaw development. Notably, the model indicates that both the upper and lower caps share a developmental history among themselves, as well as with the anterior neural plate.

Examining jaw evolution in teleosts, Bill Jeffery (University of Maryland, MD, USA) reported on the evolution of the blind cavefish compared with its close relative, the 'wild-type' surface fish (Yamatoto et al., 2003; Yamatoto et al., 2004). The presence or absence of the eye causes differences in the pattern of supra-orbital bones, the size of the nasal bones, and ossification and size of the sclera. However, jaw size, which is larger in cavefish, was changed in a different manner. The size of the jaws, as well as the number of taste buds, which is increased in cavefish, are related to the extent of

Shh signaling along an expanded embryonic midline. Downregulation of *Shh* expression decreases jaw span and taste-bud number, whereas *Shh* upregulation increases them. Behavioral experiments show that increased jaw size and associated changes in feeding posture are adaptive in cavefish. Thus, the increase in jaw size and taste bud number may have been driven by natural selection, with an indirect effect on eye degeneration. Another neural crest-related modification is the loss of melanin pigment cells in cavefish. Genetic studies have shown that the absence of melanin in cavefish is controlled by a single recessive gene: *Oca2*.

Meredith Moya Smith (Dental Institute, King's College London, UK) discussed how teeth evolved from jawless to jawed vertebrates (Fraser et al., 2004; Fraser et al., 2006). New fossil evidence shows the presence of internal denticles covering all the pharyngeal arches in an ancient agnathan. In addition, developmental data suggest a pharyngeal origin for the patterning mechanism that regulates sets of teeth. The initial teeth are formed from foregut endoderm interacting with cranial neural crest. These sets of thelodont denticles in the pharynx provide evidence that they are spatiotemporally programmed to make sequential denticles of similar polarity and shape. This suggests that this developmental mechanism could have been co-opted to become teeth arranged in similar tooth whorls at the mouth margins as in extant sharks. Smith presented evidence from three species that *Shh* is essential for tooth initiation of the primary and secondary teeth. In each, the dentition pattern develops from the first pioneer tooth initiated on each dentate bone in the oral cavity.

Teeth, feathers, hair and scales are all ectoderm derived and are types of skin appendages that in many animals undergo regeneration throughout life, suggesting the presence of a stem cell population. Chen-Ming Chuong (University of Southern California, CA, USA) discussed the evolution of ectodermal appendages, focusing on the feather. Feather stem cells were found by identifying a group of cycling cells at the base of follicles that retain BrdU label for a long time. Using quail/chick transplants and DiI labeling, they find that a torus in the 'collar bulge' appears to contain stem cells (Yue et al., 2005). Analysis of ancient feathers suggests that the ancestral feather was radially symmetric. Chuong finds that altering the *Wnt3a* gradient changes feather form. A number of other growth factors also affect feather patterning (Yue et al., 2006). *FGF10* yields a big collar type structure and *BMP* influences the size of the rachis. In reptiles, β -catenin is important for scale development and its overexpression can convert a scale into a feather. Thus, there may be a molecular code for the formation of skin appendages that is very malleable and that has led to an evolutionary drift in form.

Conclusions

This meeting brought together a unique mixture of participants working on different aspects of either facial or nervous system structures. By combining developmental and evolutionary perspectives, the talks created a framework for new ideas about interrelationships among cranial tissues. Clearly, key growth factors such as *Shh*, *FGFs*, *BMPs* and *Wnts* play crucial roles in patterning. This meeting stressed the concept that 'no tissue is an island'. When considering a developmental process involved in one aspect of head development, it is crucial to integrate across all tissues, as they interact with and influence one another. Evolutionarily, adjacent tissues are crucial for altering patterns of the brain and vice versa. By comparative analysis across diverse species, it is possible to obtain clues to the origin and diversity of brain and facial regions. By examining and integrating data from diverse chordate systems,

we will continue to come to a better understanding of how vertebrates acquired a 'new head' that facilitated predation and higher thinking.

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