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REVIEW

Transplants in annelids, nemerteans and planarians: a tool for embryology, immunology, endocrinology and regeneration research

EE Zattara

National Museum of Natural History, Smithsonian Institution, Department of Biology, Indiana University, USA

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Abstract

While transplantation procedures are often associated with biomedical applications, they are also an invaluable tool for basic research. This review focuses on how transplantation techniques have been used to understand the biology of three large lophotrochozoan phyla: Annelida, Nemertea and Platyhelmintha. I describe how transplantation paradigms have uncovered fundamental principles regarding the embryology, immunology, endocrinology and regeneration biology of representative species within these three groups. In particular, embryologists have used blastomere transplantations to show that both mosaic and regulative development occurs in animals within the phyla. Immunologists have used transplantation techniques to demonstrate that these invertebrates mount a variety of innate immune responses, some of which include surprising features that classically characterize adaptive immunity. Endocrinologists have used transplantation experiments to uncover hormonal requirements for sexual development and maturation. Meanwhile, regeneration biologists continue to address fundamental questions regarding tissue polarity, post-embryonic patterning, stem cell physiology, and the role of the nervous system in regeneration. Along with recent technical and conceptual advances, transplantation remains a powerful tool for invertebrate research.

Key Words: transplantation; Annelida; Nemertea; Platyhelmintha; immunity; regeneration

Introduction

Tissue transplantation can be defined as the removal of a piece of tissue from a donor organism, followed by grafting into a recipient or host. Based upon the relationship between the tissue donor and host, transplants are classified into three basic categories: autografts, allografts, and xenografts (Fig. 1). Autografts are transplants between a donor and host who are the same individual (Fig. 1A). Allografts are transplants between different individuals of the same species (Fig. 1B). Xenografts are transplants between separate species (Fig. 1C). Additionally, the anatomical origin of the graft and its placement in the host further delineates transplantations into two subtypes: homotopic and heterotopic (Fig. 1). A homotopic transplant involves tissue grafted into the identical anatomical location in the host compared to where it was excised from the donor. Heterotopic transplants involve tissue grafted into a new anatomical location in the host. The amount of tissue transferred may range from a single cell transplant to a graft of two complete organisms which freely exchange internal body fluids (also known as a parabiotic graft, Fig. 1D). Within these transplantation categories lies a continuum of variants, each determined by the nature of the question addressed by a given transplantation experiment. However, for simplicity's sake, this review will not consider transplantations to include ectopic contacts made between tissues after selective ablation of cells or anatomy, such as in the generation of out of register blast cells by teloblastic bandlet slippage in leeches (Shankland, 1984).

Tissue transplantation has been successfully used to interrogate many types of developmental and physiological interactions between tissues and cells. The first reported grafts of different animal pieces were made by Trembley (1744) in *Hydra* (Cnidaria: Hydrozoa). The first report of successful grafts on worms is that of Morren (1829) in earthworms. Since then, transplantation has been used with great success to address questions in the fields of embryology, immunology, endocrinology, and regeneration biology. While many such questions

Corresponding author:
Eduardo E Zattara
National Museum of Natural History
Smithsonian Institution
Department of Biology
Indiana University
915 E. Third Street, Myers Hall 150
Bloomington, IN 47405-7107, USA
E-mail: ezattara@gmail.com

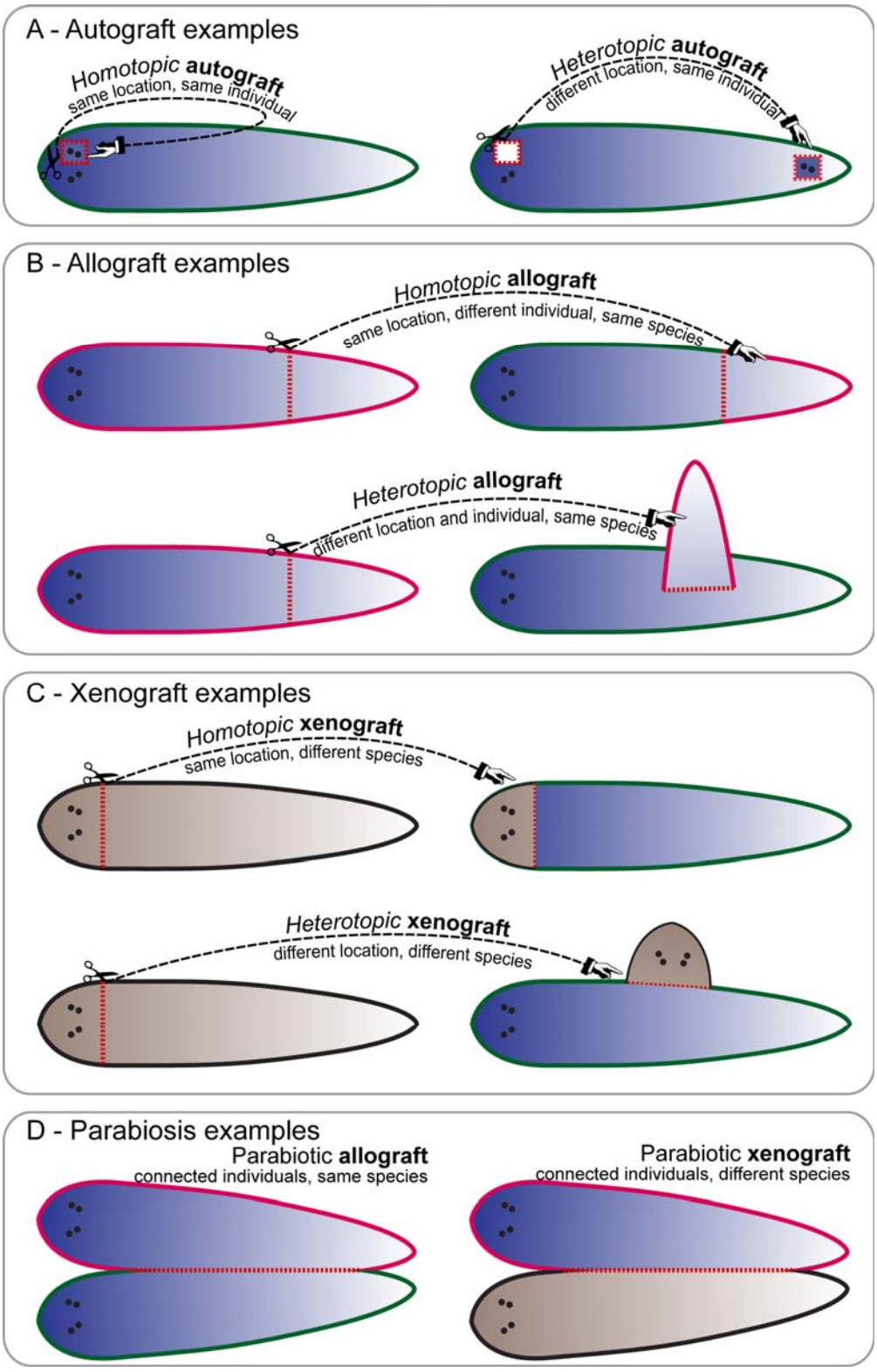

Fig. 1 Generalized examples of transplantation types. Color fill represents species, outline represents individuals, dark-to-light gradient represents anterior-to-posterior axis, and dashed lines represent planes of transection. A) Autografts, in which tissue is grafted back to its donor individuals, and is transplanted into the same location (homotopic, left) or a different location (heterotopic, right). B) Allografts, in which tissue excised from a donor is transplanted into the same location (homotopic, upper) or a different location (heterotopic, lower) of another individual from the same species. C) Xenografts, in which tissue excised from a donor is transplanted into the same location (homotopic, upper) or a different location (heterotopic, lower) of another individual from a different species. D) Parabiotic transplants, in which two individuals of the same species (allograft, left) or different species (xenograft, right) are conjoined and can freely exchange body fluids and diffusible molecules.

are biologically closely related and benefit from integrative approaches, they will be treated here separately by discipline and topic to reflect their historical segregation.

Lophotrochozoan worms: annelids, nemerteans and platyhelminths

This article reviews the use of transplantation experiments to study embryology, immunology, endocrinology and regenerative biology within three phyla of invertebrate "worms." This includes Annelida, the segmented worms; Nemertea, the ribbon worms; and Platyhelmintha, the flatworms. These phyla belong to the Lophotrochozoa, a main branch of the bilaterian tree, which has historically been understudied relative to the Ecdysozoa (arthropods, nematodes and related groups) and the Deuterostomia (vertebrates and other chordates, echinoderms and related groups) (Simakov *et al.*, 2013; Henry, 2014). The Mollusca, the fourth main lophotrochozoan phylum, are not included in this review because of their significantly derived body plan and antero-posterior axis, which makes comparisons difficult with members of worm-like taxa.

Annelida, the segmented worms, is a large phylum of over 17,000 species of marine, freshwater and terrestrial worms (Zhang, 2013; Bely *et al.*, 2014). It comprises two main clades, the Errantia and the Sedentaria (Fig. 2) (Struck *et al.*, 2011; Weigert *et al.*, 2014). Most Errantia are active benthic and pelagic marine worms. The Sedentaria constitute several families of benthic burrowing worms, in addition to a large clade of freshwater and terrestrial annelids, the Clitellata, which includes naidids (water nymph and sludge worms), crassiclitellates (earthworms), enchytraeids (potworms), lumbriculids (blackworms) and hirudines (leeches). Annelids have a body plan composed of segmentally iterated celomic compartments and organs intercalated between two non-segmental terminal regions. They possess a body wall that, in larger groups, includes thick muscle layers (Stephenson, 1930; Brusca and Brusca, 1990). Given this body plan, transplantation procedures usually imply grafting pieces of the body wall, ventral nerve cord or brain in larger animals like earthworms, leeches and ragworms (Errantia:Nereididae). In smaller animals like sludge worms, water nymph worms, blackworms and many meiofaunal groups, grafting is technically challenging, due to small size, fragility, and a tendency to autotomize (self-amputate) injured segments.

Nemertea, the ribbon worms, is a smaller phylum of around 1,300 species. They are mostly marine, although a few freshwater and terrestrial representatives are known (Andrade *et al.*, 2012; Zhang, 2013). It comprises three main clades: Palaeonemertea, Pilidiophora and Hoplonemertea (Fig. 2) (Andrade *et al*., 2014). All members have an unsegmented body plan characterized by a unique proboscis apparatus used for prey capture and kept within the only body cavity. Most organs are contained within a muscular body wall (Gibson, 1972; Brusca and Brusca, 1990). Ribbon worms are, by far, the least studied of the three phyla. However, they have unique developmental and physiological properties, including very high tolerance to injury and robust wound healing. This has made ribbon worms particularly amenable for complex grafting experiments (Bierne, 1990).

Platyhelminthes, the flatworms, is a large phylum comprising nearly 30,000 free-living and parasitic species that inhabit oceans, freshwater and land (Zhang, 2013). They comprise two main clades, Catenulida and Rhabditophora (Fig. 2). Most species are found in the latter clade, including better known taxa like Polycladida, Tricladida and the obligately parasitic Neodermata (tapeworms and flukes) (Laumer *et al.*, 2015). Flatworms have a relatively simple acoelomate body plan lacking a circulatory system. Most possess a blind gut with only one opening that serves as both the mouth and the anus of the animal. Some species do not even possess a gut (Laumer *et al.*, 2015). Interestingly, the flatworm body plan is hypothesized to be similar to that of the Urbilateria (starred in Fig. 2), the last common ancestor of bilaterian animals (De Robertis and Sasai, 1996). Free-living flatworms, and likely some of the parasitic lineages as well, have a remarkable system of cellular homeostasis in which mitotic divisions are restricted to a population of mesenchymal stem cells which gives rise to all differentiated cells. Within this phylum, the tricladid freshwater planarians have received particular attention, mostly due to the amazing regenerative capabilities of some species in this group (Bely *et al.*, 2014).

Transplantation as a tool to understand embryonic development

Since the earliest days of experimental embryology, transplantation of embryonic tissues has been a crucial tool to test models of signaling and interaction between the components of developing embryos (see examples in Hörstadius, 1928, 1937, 1975; Gilbert, 2006; Sweet *et al.*, 2004). Arguably, the most famous example is a series of xenografts between urodele embryos that led to the discovery of the Spemann-Mangold organizer (Spemann and Mangold, 1924). Furthermore, many insights into the processes that control early development were gleaned from tissue grafting studies and transplantation of blastomeres, the latter being pioneered by Hörstadius between the 1920s and the 1960s, and later continued by several research groups (reviewed by Sweet *et al.*, 2004).

However, while these approaches were widely used to study deuterostomes including echinoderms, hemichordates, cephalochordates, tunicates and vertebrates, reports of transplantations experiments in annelid, nemertean and flatworm embryos are extremely rare (Hörstadius, 1937; Novikoff, 1938; Nakamoto *et al*., 2011; Shimizu and Nakamoto, 2014). Likely reasons for such dearth are the small embryonic size in many species and the common presence of extraembryonic layers that difficult accessing the embryos and keeping them alive after extraction long enough to perform experimental manipulations. Among annelids, egg diameters range from 40 - 350 µm in marine species to 50 - 1000 µm in terrestrial direct developing species; nemertean eggs are ~90 - 120 µm in diameter; while flatworm

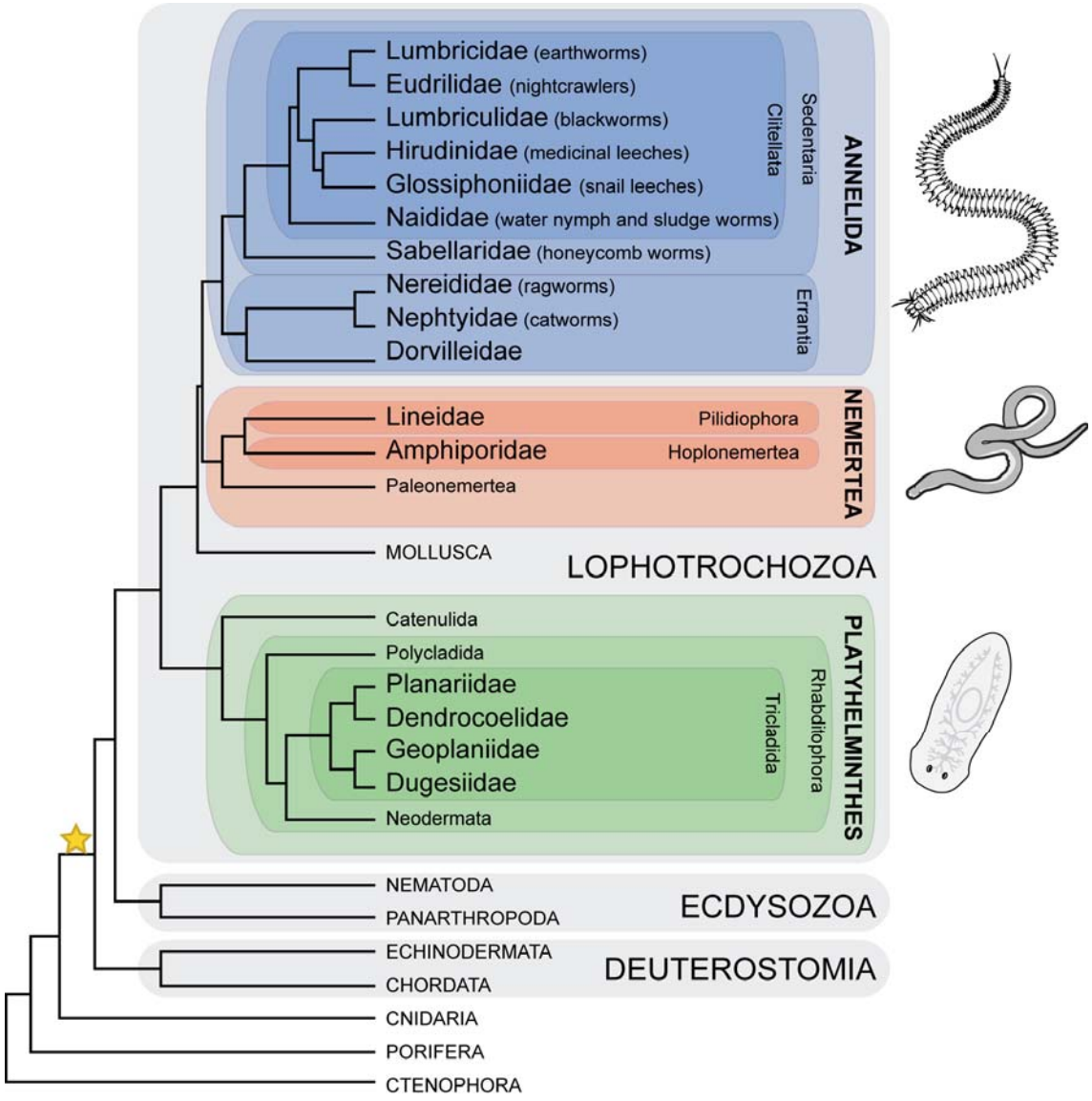

Fig. 2 Lophotrochozoan worms compose three of four major phyla: Annelida, Nemertea and Platyhelminthes (Weigert *et al.*, 2014; Andrade *et al.*, 2014, 2015; Laumer *et al.*, 2015a; Laumer *et al.*, 2015b). Phylogenetic tree showing relationships among themselves, and relationships to the fourth major phylum, the Mollusca, and other metazoan phyla. Families mentioned in this review are shown along with some major groups for each of Annelida (blue shaded boxes), Nemertea (red shaded boxes) and Platyhelminthes (green shaded boxes), and other major metazoan phyla (small font, all caps). The three main branches of Bilateria are indicated by a grey box. The star indicates the location of the Urbilateria, the hypothetical last common ancestor of all bilaterian animals.

eggs go from 130 - 150 µm in marine species to the large 550 - 1000 µm eggs of terrestrial tricladids (Novikoff, 1938; Anderson, 1973; Fernández and Stent, 1982; Henry and Martindale, 1994, 1998; Dohle, 1999; Alvarado, 2003; Maslakova *et al*, 2004; Pernet and Jaeckle, 2004; Arenas-Mena, 2007; Martín-Durán and Egger, 2012). In contrast, *Xenopus laevis* frogs lay 1000 µm eggs, while the *Triturus* newts used to discover the Spemann-Mangold organizer are 2000 µm in diameter. Even in recent years, most experimental manipulations of annelid, nemertean and flatworm embryos have used cell ablation, lineage tracing and molecular interference of gene function rather than grafts (see for example Shankland, 1984; Martindale and Shankland, 1988; Henry and Martindale, 1998; Shimizu and Nakamoto, 2014), attesting to the technical challenges of transplantation in smaller embryos.

Another reason for the scarcity of transplant studies amongst these three animal groups is the general assumption that most spiralians display mosaic development. This means that their blastomeres operate on fixed developmental programs and lack interaction with other cells during ontogeny. In contrast, numerous deuterostomes

studied during the late 19th and early 20th centuries display robust cellular interactions during regulative development, a type of embryogenesis in which cells alter their fates to compensate for environmental perturbations. In fact, up until World War II, much of the field of experimental embryology focused on questions pertaining to regulative development, predominantly in sea urchins (Driesch, 1892; Hörstadius, 1928, 1950) and amphibians (Lewis, 1904; McClendon, 1910; Harrison, 1918; Huxley and Beer, 2015). For these reasons, the use of transplantation studies in annelids, nemerteans, and flatworms has historically attracted far less attention than in their deuterostome counterparts.

Ironically, the few transplant studies that were performed in these phyla helped uncover the existence of mosaic development amongst many spiralians, and subsequently reduced researchers' interest in these animals. Hörstadius (1937) grafted together pieces of embryos older than the 4-cell stage from the nemertean ribbon worm *Cerebratulus lacteus* and reported that they differentiated as they would have before the transplant. Novikoff (1938) developed a technique to isolate blastomeres and polar lobes (cellular extrusions used to asymmetrically segregate embryonic determinants) from the 60 µm early embryo of the honeycomb worm *Sabellaria vulgaris* (Annelida: Sabellaridae), and fused them together in novel combinations. As in nemerteans, he found that in all cases both donor and host cells continued their original developmental programs with no evidence of the inductive interactions found in many deuterostome embryos.

It was not until more than half a century later that several transplantation studies in the sludge worm *Tubifex tubifex* (Annelida: Naididae) finally uncovered the existence of inductive interactions in spiralian embryos (Nakamoto *et al*., 2011; Shimizu and Nakamoto, 2014). In the relatively large *Tubifex* embryos (~500 µm), two D quadrant micromeres, 2d and 4d, are necessary for embryonic axis formation, and their ablation results in a cell mass surrounded by epithelium. When isolated 2d and 4d micromeres were heterotopically grafted onto an intact embryo, the embryo developed a secondary axis, including terminal anterior or posterior structures. Cell lineage tracing showed that while ectodermal and mesodermal structures in the secondary axis were derived from the donor tissues, the endoderm originated from the host. Thus, the graft induced host endoderm to form a new axis. This work, along with studies of teloblastic bandlet slippage in leech embryos (Shankland, 1984; Martindale and Shankland, 1988; Wedeen and Shankland, 1997), shows that after gastrulation, annelid embryonic development presents significant inductive interactions between cells and tissue layers.

Transplantation studies to understand embryonic development in annelids, nemerteans and flatworms are challenging. But new technologies developed for stem cell biology and *in-vitro* fertilization to allow manipulation of 50 - 100 µm mammalian eggs are opening the spectrum of tools available to perform grafts, even in very small embryos (Sweet *et al*., 2004). Combined with an ever-increasing toolkit to label cells, modify gene expression levels, alter genetic information, and perform high-resolution live imaging, transplantation experiments have a very promising future. With technologies enabling isolation, labeling and even genome editing of blastomeres, their grafting back into the original embryo or a new host will undoubtedly provide profound insights into the mechanisms of embryonic development in these animal groups.

Transplantation as a tool to understand invertebrate immunity

A central question of immunology is how animals respond to invasion by foreign elements. This includes asking whether and how organisms achieve self vs. non-self recognition, what mechanisms they use to fight off foreign elements, and whether there are mechanisms to mount stronger responses upon repeated exposure to specific elements. Transplantation experiments have proven to be a powerful approach in beginning to address these questions.

Most, if not all, multicellular organisms have the ability to distinguish self from non-self, and present some degree of reaction against foreign cells or substances (Parish, 1977; Coombe *et al.*, 1984; Bayne, 1990; Tsutsui, 2004). This ability is known as innate immunity, and is characterized by a generic response to most foreign elements. In contrast, vertebrates also possess adaptive immunity, characterized by the production of immunoglobulins, specialized cell-surface receptors and a system of clonal cell selection and expansion that together provide memory and specificity to the immune response. While such adaptive immunity has been classically considered to be unique to vertebrates, the innate immune response is shared by vertebrates and non-vertebrates. However, a number of studies have shown that non-vertebrates also have immune-related molecules that confer a certain levels of immunological memory and specificity (Kvell *et al.*, 2007).

Tissue grafting facilitates the measurement of an organism's capacity to recognize self versus non-self, and to explore the mechanisms of immunity (Cooper, 1970; Tettamanti *et al*., 2003; Söderhäll, 2011). Immune responses, both innate and adaptive, are usually based on cellular and humoral reactions (Coombe *et al.*, 1984; Cooper and Roch, 2003; Salzet *et al.*, 2006). Cellular responses include phagocytocis, encapsulation of foreign elements too large to be engulfed by host cells, nodulation and wound healing. Humoral responses are most commonly mediated by soluble factors produced by the circulating cells. These soluble factors are usually able to identify non-self elements, activate a signaling response and neutralize those elements (e.g. antimicrobial peptides, reactive oxygen species, melanization cascade, coagulation cascade) (Coombe *et al.*, 1984; Loker *et al.*, 2004; Tsutsui, 2004).

Both cellular and humoral innate immunity mechanisms have been reported for most metazoans studied. However, the presence of adaptive-like immunity in non-vertebrates, defined

as the ability of the organism to show an enhanced response after repeated exposure to the same non-self elements, is still hotly debated. Adaptive immunity has two main hallmarks: memory (the ability to retain information about past contacts with foreign elements) and specificity (the ability to mount a differential response against different non-self elements). The presence of adaptive immunity is a hypothesis with straightforward predictions for transplantations experiments. Most organisms have some level of innate immunity, and are expected to reject tissue grafts recognized as non-self. However, if adaptive immune memory is present, a second graft from the same donor will elicit a stronger rejection response. If adaptive immune specificity is present, a second graft from a different donor species not previously encountered will fail to elicit a stronger response.

Transplantation paradigms have been used to study the ability of annelids and nemerteans to detect and react to non-self tissues (Fig. 3A). Amongst annelids, studies on ragworms (Errantia: Nereididae) have revealed that these worms can indeed distinguish self from non-self and mount a cytotoxic response to allografts and xenografts, leading to graft rejection (Porchet-Henneré *et al.*, 1987). In almost all cases, the likelihood of graft rejection was found to be lower for xenografts of closely related species than for more distant ones (Clark and Clark, 1959; Boilly-Marer, 1974, 1976; Cuvillier-Hot *et al.*, 2014), suggesting that these organisms use an evolving and divergent set of molecules in order to distinguish self versus non-self.

Likewise, in the 1960s, Duprat (1964) and Cooper (1968) independently reported that earthworms (Clitellata: Crassiclitellata) are capable of rejecting body wall grafts. The speed of rejection correlated with the phylogenetic distance between donor and host. This led to a number of studies testing whether transplantation sensitized the host to repeated challenges from the same donor. Work on *Lumbricus terrestris, Eisenia fetida, Aporrectodea trapezoides, Dendrobaena veneta* (Lumbricidae) and *Eudrilus eugeniae* (Eudrilidae) revealed that most autografts surviving the procedure were accepted. In contrast, allografts were usually rejected, and the chance of rejection apparently correlated with the inferred genetic distance between the conspecific individuals. Furthermore, xenografts were always rejected. Rejection times showed a rough inverse correlation to phylogenetic distance (Valembois, 1963; Duprat, 1964, 1967; Cooper, 1968, 1969, 1970; Chateaureynaud-Duprat, 1970; Bailey *et al*., 1971; Dales, 1978; Parry, 1978). Along with the ragworm data, these results support that self-recognition and histocompatibility are likely mediated by genetically encoded markers that diverge between lineages over time.

Tests for adaptive immunity in earthworms have given more ambiguous results (Fig. 3B). Some results showed that a first graft from a given donor species could alter the speed of rejection of subsequent grafts from the same species, but had no influence on rejection of grafts from a different donor species. This suggested the presence of memory and specificity (Hostetter and Cooper, 1973; Cooper, 1975). This claim has been disputed, since results show both strengthening and weakening of subsequent graft rejection, and some results failed to be independently replicated (Dales, 1978; Parry, 1978).

More convincing support for the existence of immune memory and specificity in annelids came from transplantation studies in leeches (Tettamanti *et al.*, 2003). Body wall autografts in the medicinal leech *Hirudo medicinalis* (Clitellata: Hirudinidae) caused only an inflammatory and angiogenetic response. In contrast, allografts and xenografts from the broad snail leech *Glossiphonia complanata* (Clitellata: Glossiphoniidae) were rejected in the span of 7 days. Rejection consisted in a characteristic sequence of events resulting in graft destruction. A second allograft or xenograft resulted in a similar series of events; however, if the second graft came from the same donor as the first graft, rejection was markedly accelerated, taking place within 3 - 4 days rather than 7 days. This stronger response was verified both in the short term (3 to 7 days between first and second graft) and long term (1 or 4 months between first and second graft). Immunolabeling using antibodies against human cell surface markers revealed the presence of different cell types between second grafts from same versus different donor, hinting that this putative immune memory in annelids might be cell-mediated. Surprisingly, there were no differences in rejection time between allografts and xenografts from a distantly related species, suggesting that self-recognition does not depend on species-specific markers in leeches. There are numerous known cellular and molecular effectors in annelid immune response (Cooper and Roch, 2003; Salzet *et al*., 2006; Cuvillier-Hot *et al.*, 2014) but the key players in self versus non-self recognition are still mostly unknown, and are a prime target for study using transplantation experiments (Coombe *et al*., 1984; Loker *et al.*, 2004).

Transplantation experiments in ribbon worms of the genus *Lineus* (Nemertea: Lineidae) have also found support for immunological memory and specificity outside the vertebrates (Langlet and Bierne, 1973, 1982, 1984; Bierne and Langlet, 1974; Bierne, 1985). Thanks to a plastic body plan and robust wound healing, species in this genus are highly tolerant to grafting and can survive surgeries where partial or full pieces from the same or different worms are spliced together into multiparental chimeras (Bierne, 1985, 1990). The resulting chimeric worms can be homo- or heterospecific and combine grafts of the same or different sexes. Despite being genetic mosaic individuals with up to 16 parents, many chimeras can survive for several years. This potential has opened the possibility of using transplantations to address several questions regarding phylogenetic and cellular aspects of graft rejection and immunity in nemerteans.

Bierne and Langlet explored rejection of reciprocal grafts in six *Lineus* species: *L. ruber*, *L. viridis*, *L. longissimus*, *L*. (=*Ramphogordius*) *lacteus*, *L*. (=*Ramphogordius*) *pseudolacteus* and *L*. (=*Ramphogordius*) *sanguineus* (Langlet and Bierne,

1973; Bierne and Langlet, 1974). Autografts and allografts were never rejected, and became integrated with the donor. Xenografts produced various reactions ranging from integration to rejection. As in annelids, likelihood and speed of rejection were correlated with phylogenetic distance between donor and host (Langlet and Bierne, 1982; Zattara *et al*., 2015).

Using a combination of three *Lineus* species, Langlet and Bierne (1982) found that initial xenografts from *L. sanguineus* onto *L. ruber* where rejected after more than 15 days. A subsequent xenograft from the same donor species (same or different individual donors) was rejected in less than 9 days. In contrast, rejection of a subsequent xenograft from a third species still took over 15 days. By changing the time between the initial and subsequent grafts, they showed that this memory-like effect is present up to 80 days but has faded by 120 days. These observations support the hypothesis that the immune response to xenografts in *Lineus* has species-specific mid-term memory, but that such memory does not last longer than 4 months.

To identify the effector of the graft rejection response, Langlet and Bierne (1982, 1984) devised an ingenious experiment (Fig. 3C): anterior (antecerebral) ends of *L. sanguineus* were homotopically grafted onto individuals of *L. lacteus* or *L. ruber*, or onto chimeras composed of a *L. lacteus* middle portion and a *L. ruber* posterior (intestinal) portion or vice versa. As expected, non-chimeric *L. ruber* hosts rejected the grafts in less than 20 days, while most non-chimeric *L. lacteus* had not rejected the grafts after 90 days. Strikingly, when grafting onto chimeric hosts, the rejection response did not depend on the source species of tissues adjacent to the graft, but only upon the source species of the posterior (intestinal) portion. Histological analyses showed cell-mediated lysis of donor tissue. Since the strength of the rejection response depends on the donor species of the posterior (intestinal) portion, it is likely that the response effectors are cells located on the intestinal region that migrate anteriorly in response to signals coming from the anterior graft.

In contrast to annelids and nemerteans, very little is known about immunity in free-living flatworms. Reports of many grafting experiments done in tricladid planarians to address questions in regenerative biology (Morgan, 1901, 1906; Moretti, 1911; Rand and Browne, 1926; Miller, 1938) hint at a phylogenetic signal in immunity. Autografts and allografts are usually accepted by the host, while strength of rejection responses to xenografts somewhat reflects evolutionary distance between donor and host species. Cellular immunity is the most likely effector of graft rejection, as phagocytic cells accumulate after injury near a wound site (Morita and Best, 1974; Morita, 1991), and destroy or encapsulate the foreign elements. Humoral immunity is likely present, since many orthologues to genes involved in the immunity in vertebrates have been found in the genome of *Schmidtea mediterranea* (Tricladida: Dugesiidae) (Peiris *et al.*, 2014). On the surface, observations suggest that the immune system of free-living flatworms may have more “lenient” self-recognition compared to annelids and nemerteans, but this conclusion is still premature. None of the above studies aimed to characterize graft rejection or immunologic parameters. Experiments designed to systematically test for variability in average graft survival times are still pending in flatworms, and will be essential for reconstruction of immunity in the Urbilateria.

In summary, transplantation experiments in annelids, nemerteans and flatworms show that their immune systems share several common features. All three phyla are capable of healing transplanted tissue, recognizing self from non-self, and mounting a rejection response against grafts recognized as non-self. Similar capabilities have recently been reported in at least one representative of the fourth main lophotrochozoan phylum, the Mollusca (Yamaguchi *et al.*, 1999), so it is likely that they were present in stem group lophotrochozoans, and perhaps even the Urbilateria. The strength of the response tends to increase with the phylogenetic distance between donor and host, though the slope of this trend varies both between and within phyla. Several lines of evidence point at cellular rather than humoral response as the main effector of graft rejection. In some annelids and nemerteans, there is evidence for memory and specificity of the immune response, although not with the characteristics of vertebrate adaptive immunity.

While transplantation has given key insights into immunity in these lophotrochozoan groups, much more is still to be learned. What are the molecular players modulating self/non-self recognition and cellular responses to grafts? What roles do putative homologs of vertebrate immunity genes play in these groups? Are the mechanisms behind memory and specificity common among these groups and thus inherited from a common ancestor, or do they represent independent evolutionary innovations? With the development of increasingly powerful model systems in each of these phyla, many of these experiments can now be revisited to molecularly dissect the mechanisms of lophotrochozoan immunity.

Transplantation as a tool to understand invertebrate endocrinology

Transplantation is a common tool in studies of invertebrate endocrinology, as it can be used to remove and re-implant suspected sources of hormones, and to test interactions between endocrine organs and developmental stages. Differentiation and maturation of gonads and sexually dimorphic structures in species that cyclically develop and resorb their reproductive organs is an example of endocrine regulation that has been extensively studied in annelids, nemerteans and planarians using transplantation experiments.

In ragworms (Annelida: Nereididae), removal of the brain induces precocious gonadal maturation and development of secondary sexually dimorphic traits found in reproductive worms. Heterotopic autografts in which the brain is removed and reinserted in the coelom delay or inhibit this precocious sexual maturation (Hauenschild, 1960; Durchon, 1962). Interestingly, the brain, either intact

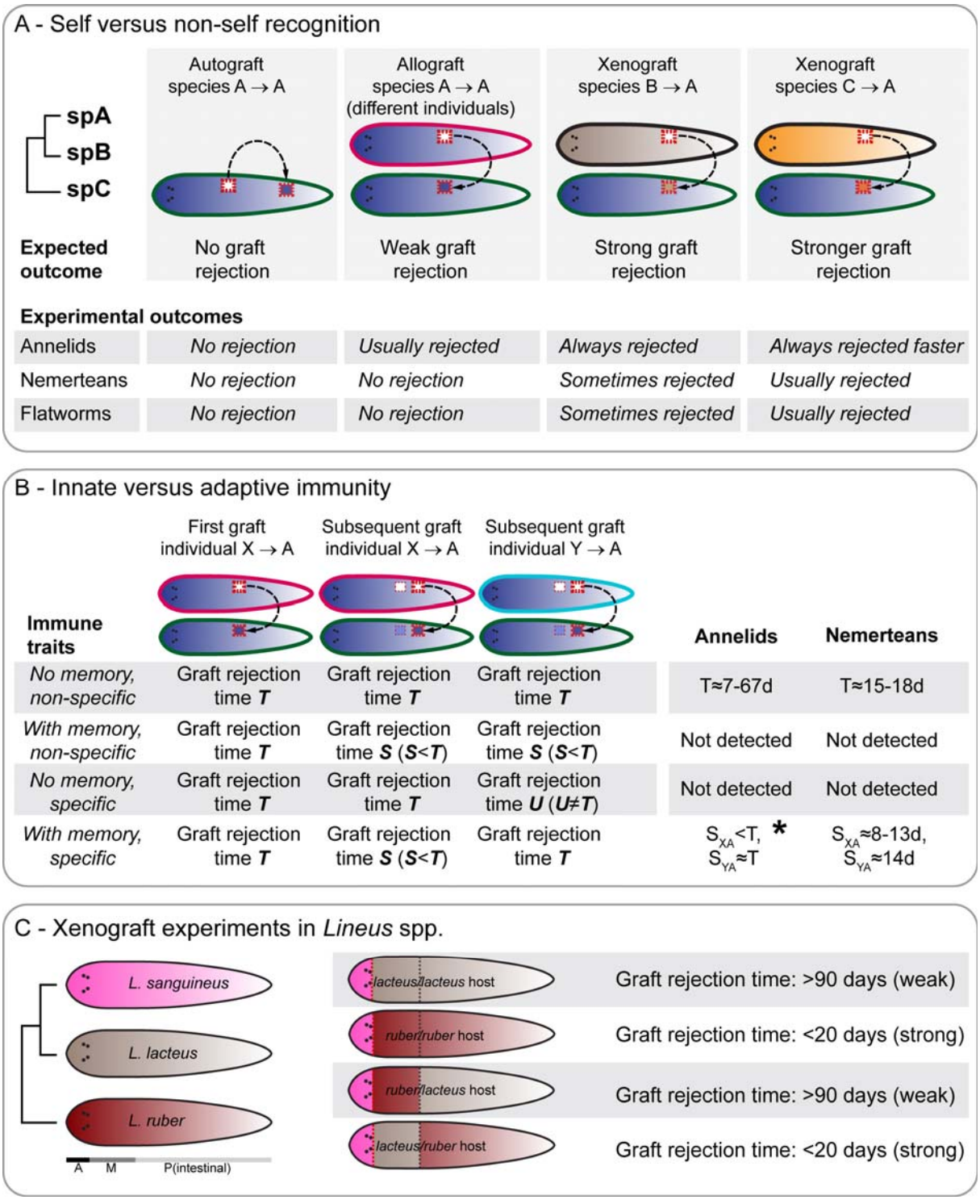

Fig. 3 Transplantation paradigms to test for self versus non-self recognition and innate versus adaptive immunity. A) In the presence of a self-recognition system that is genetically encoded, increasing the genetic distance between donor and host is expected to increase the strength of the graft rejection response. Experimental evidence suggests that annelids have stronger self versus non-self discrimination than nemerteans and flatworms. B) Experimental paradigm to test for memory and specificity in immune responses. Expected changes in mean rejection times depending on traits of the immune system (left) and data from tests in annelids and nemerteans (right). The "*" highlights disputed results. C) Xenograft experiments to determine the effector mechanism of nemertean immune response. Grafting the anterocerebral portion of *L. sanguineus* to the medium region of its sister species *L. lacteus* elicits a weak response (graft rejection time over 90 days); if the host is the more distantly related species *L. ruber*, the response is stronger (graft rejection time is less than 20 days). When *L. sanguineus* antecerebral regions are grafted to homo and heterospecific chimeras made from the medium and posterior (intestinal) portions, the response is determined by the species of the posterior (intestinal) portion and not by the species of the medial portion adjacent to the donor tissues.

or grafted into the coelom, is also necessary for regeneration of posterior segments (Clark and Bonney, 1960; Hauenschild, 1960; Clark and Evans, 1961; Golding, 1967b, 1974). Brain homotopic transplants and heterosexual chimeras obtained by grafting two individuals of different sexes have been used to explore regulation of gonadal maturation and secondary sexual character re-development in ragworms. Such experiments have shown that some sexually dimorphic traits (male and female swellings and male crenellations of the parapodial cirri) are determined by the genetic sex of the source tissue. In contrast, another dimorphic trait (male pygidial papillae) is not sex-specific, but is inhibited by female hormones (Durchon, 1962; Boilly-Marer, 1974, 1976).

A similar neuroendocrine control of sexual maturation has been described in nemerteans (Bierne and Rué, 1979; Sivaradjam and Bierne, 1981; Vernet and Bierne, 1988). In several species, removal and grafting of the brain has demonstrated that a gonad-inhibiting hormone is secreted from this organ (Bierne, 1966; Bierne and Rué, 1979). However, while the ragworm brain hormone controls also growth and restorative regeneration (see above), the nemertean hormone affects exclusively sexual maturation (Vernet and Bierne, 1988). Experiments with heterosexual chimeras made by allografting lateral halves of a male and female individuals of two species of *Lineus* have shown that sexual maturation occurs in two phases: initial formation of new gonads, that develop according to the sex of each half, resulting in immature gynandromorphs; and unilateral sex reversal of one of the halves by a putative diffusible factor coming from the other half (Bierne, 1975; Sivaradjam and Bierne, 1981). The dominant sex is species specific: *L. sanguineus* chimeras became all female, but *L. ruber* chimeras become all male.

Although studies of flatworm endocrine abilities are scarce (Basch, 1986; Pincus *et al.*, 2013), similar transplantation experiments on the control of sexual maturation have been done in *Dugesia tigrina* and *D. gonocephala* (Kenk, 1941; Okugawa, 1957). Allografts of anterior or posterior pieces from sexually mature donors onto the complementary piece of an asexual host eventually induced gonad maturation and development of secondary sexual traits in the host. While these studies were not able to differentiate between hormonal induction and migration of stem cells from the sexual donor tissue into the asexual host, recent work supports the role of a diffusible factor in controlling sexual maturation (Kobayashi *et al.*, 2002; Maezawa *et al.*, 2014).

Transplantation as a tool to understand regenerative biology

Regenerative biology is probably the field that has been making use of transplantation experiments for the longest time (Morgan, 1901). The regeneration of missing body parts is a fascinating process that implies a re-deployment of developmental trajectories into a post-embryonic context, and thus combines basic questions of embryology like cell and tissue differentiation, with unique problems of the adult context, like sources of stem cells, short and long range signaling pathways used to initiate regeneration after wound healing, restoration of correct body proportions and functional integration of the regenerated parts. Transplantation experiments covered in this last section have been used to test hypotheses exploring three major regeneration topics: axial polarity and spatial information, roles of the nervous system and stem cell biology.

The ability found within annelids, nemerteans and flatworms to re-develop missing body parts and regulate their morphology to adapt to changes in body condition has made them superb models to study developmental and physiological mechanisms of regeneration (Bely *et al.,* 2014). Both regenerative ability and research efforts are unequally distributed across these three phyla. Anterior (including complete head) and posterior regeneration ability is widespread in Annelida (Bely, 2006), but most transplantation experiments are restricted to larger species, like earthworms and errant polychaetes. Within Nemertea, only a few species show complete head regeneration; when present however, regeneration is fast and robust (Bely *et al.*, 2014; Zattara *et al.*, 2015), and can be studied using multiparental chimeras created by multiple grafting (Bierne, 1990). Distribution of regenerative ability is also patchy in Plathyhelmintha, but it is particularly strong in a number of tricladid planarians (Egger *et al*., 2006; Bely *et al*., 2014). These planarians have been a long time favorite of regeneration research (Elliott and Sánchez Alvarado, 2013) which have endured too large a number of transplantation experiments to be fully covered in this paper. Thus, while brief mention of some key studies are made in the following paragraphs (Morgan, 1901, 1906; Moretti, 1911; Rand and Browne, 1926; Santos, 1931; Miller, 1938; Chandebois, 1985; Kato *et al.,* 1999, 2001; Kobayashi *et al*., 1999), the interested reviewer is referred to several excellent articles specifically reviewing past and present planarian regeneration research (Salo and Baguñà, 2002; Reddien and Alvarado, 2004; Baguñà, 2012; Elliott and Sánchez Alvarado, 2013).

The parsing of positional information into axial polarity is a crucial step of early embryonic development, but also a fundamental requisite for adequate regeneration of lost body parts. Is axial polarity an intrinsic property of tissues, or does it result from context-dependent interactions? Most amputation experiments have shown that antero-posterior polarity is usually retained by the stump tissue, so that anterior wound surfaces regenerate anterior ends, and posterior wound surfaces regenerate posterior ends (Fig. 4A, left). Heteromorphic regeneration, where a stump regenerates a “wrong” body part, can very infrequently result in two-tailed or two-headed worms (Fig. 4A, center). Heteromorphic regeneration of annelids and flatworms is rare and usually seen only in very short fragments, or due to physical (Morgan, 1901, 1902; Gates, 1950; Moment, 1951; Kawamoto *et al.*, 2005), pharmacological (Fitzharris and Lesh, 1969) or molecular interference (Gurley *et al.*, 2008; Adell *et al.*, 2009; Petersen and Reddien, 2009). Heteromorphic regeneration is unreported in

nemerteans, even in very small fragments (Coe, 1929, 1934).

At the end of the 19th century, it was shown that heterotopic grafts in earthworms could result in the ectopic regeneration of body parts (Morgan, 1897, 1901; Joest, 1895, 1897; Korschelt, 1897, 1898). Is then tissue polarity an intrinsic quality or is it the result of induction by specific structures nearby? Grafting together posterior pieces of earthworms by their anterior surfaces, followed by amputation on one of the pieces near the suture site still results in regeneration of a posterior end (Fig. 4A, right), proving that antero-posterior polarity is not reversed by influence of the larger, uncut fragment (Morgan, 1901). More recent observations made after coincidental grafting of amputated fragments of the polychaete *Parougia bermudensis* (Errantia: Dorvilleidae) confirmed that grafted annelid fragments do not switch polarity; interestingly, this study also reported that fusion of contralateral regenerating connectives from the nerve cord results in development of a brain, irrespective of whether the connectives originate from the same or separate nerve cords (Müller, 2004). Nemerteans are even more resistant to polarity switching: transversely cut fragments split longitudinally and grafted together in opposite direction retain their individual polarity, and a head blastema forms at anterior end of each piece (Coe, 1934). In contrast, posterior fragments of planarians grafted by their anterior surfaces and then amputated close to the suture line occasionally regenerated a head from a posterior surface (Morgan, 1906), suggesting that tissue polarity in flatworms is more labile than in annelids or nemerteans.

Among the lophotrochozoan worms, planarians present the most amenable body plan for grafting, robust regenerative abilities and a dynamical patterning system (Elliott and Sánchez Alvarado, 2013). These traits have made them a popular organism to study the dynamics of morphogenetic gradients, being used to test concepts like Child's gradients of physiological dominance (Child, 1929), Turing morphogenetic fields (Turing, 1952; Erneux *et al*., 1978), Gierer-Meinhardt systems (Gierer and Meinhardt, 1972) and positional information (Wolpert, 1969). Juxtaposition of tissues from different axial regions induces ectopic anatomy (possibly indicating the induction of a new body axis) in many grafting experiments. For instance, cutting out a circular plug of tissue and grafting it back but inverting its dorsoventral orientation induces formation of blastema-like structures at the graft boundaries that produce what appears to be an ectopic body axis (Santos, 1929, 1931; Kato *et al.*, 1999). Heterotopic autografts from anterior regions of the animal to posterior ones and vice versa also induces the formation of ectopic anatomy in host tissues (Santos, 1931; Kato *et al*., 1999; Kobayashi *et al*., 1999). With an expanding functional toolkit, grafting experiments could be very useful in testing hypotheses about the molecular underpinnings of dynamic regulation of scale and proportion, and the mechanisms by which positional information is reset and re-interpreted after injury.

A second major topic in regeneration biology is the role of the nervous system in inducing or inhibiting the regeneration process (Kumar and Brockes, 2012). Transplantation experiments have been widely used to explore this problem (Fig. 4B). Working with earthworms, Avel (1930) made heterotopic autografts of ventral body wall from posterior segments onto the dorsal side of the anterior region. When such grafts included the ventral nerve cord, small ectopic heads regenerated at the point where the cord contacted the anterior suture of the graft; however, denervated grafts simply wound healed. A somewhat similar result was obtained by Sayles (1939) after heterotopic autografts of nerve cord placed into the dorsal body wall of *Clymenella torquata* (Annelida: Sedentaria). Numerous experiments on several species of planarian have shown that grafting of anterior tissues, including brain ganglia, can inhibit regeneration of heads from adjacent anterior wound surfaces, and induce the formation of ectopic structures, depending on the anterior level at which the graft is placed (Moretti, 1911; Steinmann, 1925; Gebhardt, 1926; Goetsch, 1926, 1929; Rand and Browne, 1926; Santos, 1929, 1931, 1931; Miller, 1938).

In contrast, it has been found that extirpation of the brain greatly inhibits posterior regeneration (Fig. 4B, center and right) in earthworms (Clitellata: Lumbricidae), ragworms (Errantia: Nereididae) and catworms (Errantia: Nepthydae) (Kropp, 1933; Hubl, 1956; Clark and Clark, 1959; Hauenschild, 1960; Clark and Evans, 1961; Golding, 1967a, b). In earthworms, autografts of whole or minced brains onto the anterior end of decapitated worms fails to rescue posterior regeneration (Kropp, 1933). On the other hand, transplantation of minced brain macerates does partially rescue regeneration on a fraction of decerebrated *Platynereis dumerilii* and *Hediste diversicolor* ragworms (Hauenschild, 1960; Clark and Evans, 1961), suggesting that some secretion from the brain is necessary for posterior regeneration. Generation of parabiotic chimeric individuals by grafting posterior fragments into intact or decerebrated hosts in different combinations indicate that such a secretion is a permissive factor, but that growth rates of the regenerate are autonomously regulated (Golding, 1967b, c). No such inhibition of posterior regeneration by removal of the cerebral ganglia has been observed on nemerteans (Vernet and Bierne, 1988) or planarians, save for one anecdotal account (Moretti, 1911).

A third topic of regeneration biology addressed by transplantation experiments is the neoblast hypothesis of regeneration (Baguñà, 2012). Neoblasts are proposed to be a population of reserve stem cells that migrate towards the wound site after amputation and serve as a source of new tissues in the regenerate. The term *neoblast* was originally coined after observations on annelid regeneration revealed the presence of certain spindle shaped cells with unusual staining properties and large nuclear-to-cytoplasm ratios, which seemed to migrate along the ventral nerve cord and accumulate at the wound site (Randolph

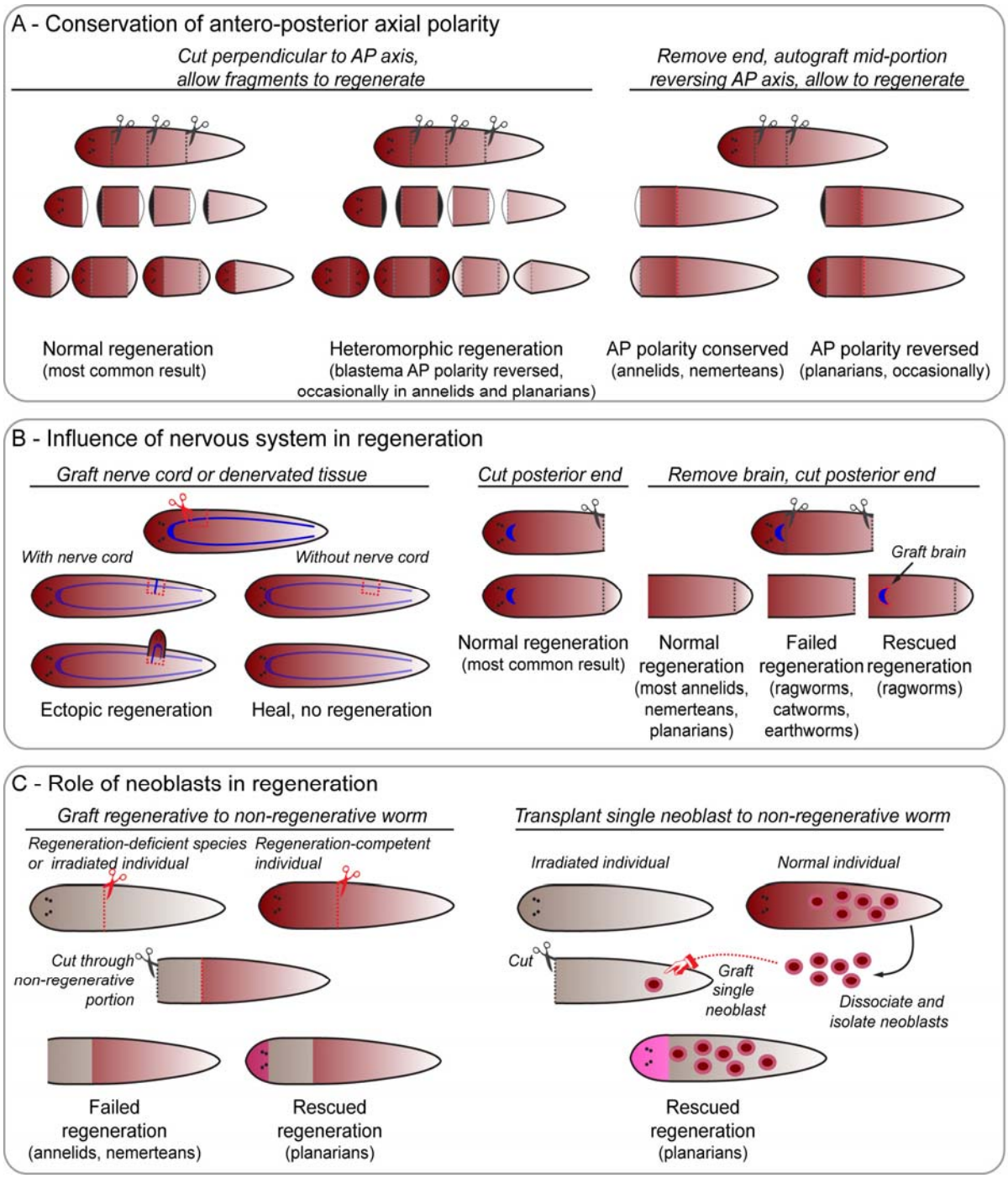

Fig. 4 Transplantation paradigms to study polarity conservation, influence of nervous system and the role of neoblast in regeneration. A) Transverse amputation (left) normally results in fragments that conserve their antero-posterior (AP) polarity, so that anterior wound surfaces form anterior blastemas (black) while posterior wound surfaces form posterior blastemas (white). In rare occasions in annelids and planarians, blastemas with reversed polarity appear, leading to heteromorphic regeneration. A middle portion autografted with a reversed AP axis and cut through (right) normally retains its polarity, except occasionally in planarians where its polarity is reversed. B) Grafting or deviation of a nerve cord can induce the regeneration of ectopic structures (left). Conversely, removal of the brain in some annelid groups (right) can inhibit posterior regeneration, which can be sometimes rescued by grafting a brain. C) The role of neoblasts (migratory stem cells) in regeneration can be tested by grafting together a fragment of a regeneration-competent worm with a fragment of an irradiated worm or an individual from a regeneration-deficient species (left), followed by amputation through the latter: neoblasts are expected to migrate to the wound site and rescue regeneration, a result usually seen in planarians. The ultimate test for this model is transplantation of a single neoblast into a regeneration-deficient host (right): a regeneration-competent worm is dissociated, its neoblasts are isolated, and a single neoblast is transplanted into the host. If neoblasts are present, they are expected to rescue regeneration.

1891, 1892). Finding similar cells in tricladid planarians led to adoption of the term neoblast by flatworm researchers (Elliott and Sánchez Alvarado, 2013). With the discovery that X-ray irradiation could abrogate regenerative powers in vertebrates, researchers of worm regeneration combined this technique with transplantation experiments to test whether grafting from an intact, regeneration-capable donor could rescue an irradiated, regeneration-disabled host (Fig. 4C, left).

Within annelids, such use of transplantation was very limited, since the larger earthworms are rather poor regenerators compared to other groups of smaller size like water nymph worms (Clitellata: Naididae), pot worms (Clitellata: Enchytraeidae) and blackworms (Clitellata: Lumbriculidae). Despite the difficulty and low survival of such transplants, Zhinkin (1934) showed that irradiated posterior ends of the blackworm *Rhynchelmis limosella* grafted onto a posteriorly amputated non-irradiated host were capable of regenerating, albeit with a delayed timing.

In nemerteans, neoblasts have also been proposed as key to regenerative abilities in some species of *Lineus* (Coe, 1930, 1934), but irradiation has not been used to test this hypothesis. However, experiments using bi-specific chimeras made from xenografts from species with different regenerative abilities have shown that the results of amputation depends only on the origin of the injured tissue (Bierne, 1967); in other words, grafting a fragment from a species with great regenerative ability cannot rescue regeneration in a poorly regenerating species host. These experiments indicate that migratory neoblasts are not crucial in nemertean regeneration.

In contrast to the ambiguous and inconsistent support for the role of neoblasts in annelid and nemertean regeneration, their role in planarian regeneration, already well supported by classical and recent experiments in which grafts of healthy donors can rescue regeneration of irradiated hosts (Wolff and Dubois, 1947; Guedelhoefer and Alvarado, 2012) has been spectacularly demonstrated in the planarian *Schmidtea mediterranea* (Tricladida: Dugesiidae) by a combination of classical techniques and recent molecular and cytological tools. By transplanting into a fully irradiated host neoblasts from a donor with a distinctive genotypic signature (Fig. 4C, right), it was confirmed that just a single neoblast is sufficient to rescue regenerative ability (Wagner *et al.*, 2011). Because donor and host cells were chosen to have different karyotypes, it was also possible to show that eventually all of the somatic tissues of the host eventually became replaced by the clonal progeny of that single neoblast, proving that in these flatworms, neoblasts are fundamental not only for regeneration, but for normal body-wide tissue turnover.

Neoblasts are necessary for planarian regeneration, but not sufficient. Several species of dendrocoelid planarians (Tricladida: Dendrocoelidae) are incapable of regenerating a head if cut at a level posterior to the pharynx (Morgan, 1904; Egger *et al*., 2006). Stéphan-Dubois used a clever set of reciprocal transplantations between anterior and posterior regions, and between intact and irradiated worms, to find whether lack of neoblasts or improper activation was the cause for such regeneration-deficiency in *Dendrocoelum lacteum* (Stephan-dubois and Kolmayer, 1959; Stéphan-dubois and Gilgenkrantz, 1961). Her data showed that neoblasts are present, divide and migrate to an anterior wound to initiate a blastema at all levels of the body, but the posterior regions were not competent to induce the correct antero-posterior polarity in the blastema, causing an arrest of the process. A series of recent studies used next-generation sequencing of mRNA to discover aberrant upregulation of the *Wnt* pathway in the posterior regions of *Dendrocoelum lacteum*, *Phagocata kawakatsui* and *Procotyla fluviatilis* (Liu *et al*., 2013; Sikes and Newmark, 2013; Umesono *et al.*, 2013). Notably, these researchers also demonstrated that regeneration deficiency can be rescued by downregulating canonical *Wnt* signaling using RNA interference against β-*catenin*.

Transplantation has been a crucial tool for research on regenerative processes in annelid, nemerteans and flatworms. It allowed elaborating mechanistic hypotheses that are now being investigated using an ever-increasing array of molecular techniques. With the advent of inexpensive massive parallel sequencing of DNA and RNA, and functional tools that can be used on adult animals of non-model species, the doors are open to take advantage of the wide range of regenerative abilities across the Metazoa to help us crack the riddle of regeneration.

Concluding remarks

Since Trembley's experiments on *Hydra* over 270 years ago, the use of transplantation as an experimental tool to study several aspects of invertebrate biology has resulted in important advances in the fields of embryology, immunology, endocrinology, and regeneration biology. However, the use of this tool is not limited to these four disciplines, and has also been used in other areas, like neurobiology (Drewes *et al*., 1988) and even systematics (Bierne *et al*., 1993). The main limitations to the use of transplants (beyond the imagination of the scientific mind) are the difficulty posed by low survival rates in animals that are highly intolerant of surgical procedures and the skill of the hands that make surgeries usually at a microscopic scale. With the advent of new technologies, these limitations might soon be lifted. It is worth noting that many of the newer tools, like high precision electronic micromanipulators, have been developed for use in areas more closely related to strongly funded biomedical research, placing them out of reach of invertebrate zoologists with more modest resources. However, this should not be seen as a constraint, but as an incentive to develop collaborations among research groups. Furthermore, the increasing number of molecular tools available for organisms outside the select club of traditional model systems and the development of techniques allowing the tracking of fate of the grafted tissues (Abdulreda *et al*., 2011; Yamazaki *et al.*, 2012), are opening the doors to new and

exciting ways to use transplantation experiments to address old questions with novel approaches. Planarians have led the way, and annelids and nemerteans are ready to follow suite.

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