Homeoprotein Signaling in Development, Health, and Disease: A Shaking of Dogmas Offers Challenges and Promises from Bench to Bed

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Abstract—Homeoproteins constitute a major class of transcription factors active throughout development and in adulthood. Their membrane transduction properties were discovered over 20 years ago, opening an original field of research in the domain of vector peptides and signal transduction. In early development, homeoprotein transfer participates in tissue patterning, cell/axon guidance, and migration. In the axon guidance model, homeoproteins exert their non–cell autonomous activity through the regulation of translation, in particular, that of nuclear-transcribed mitochondrial mRNAs. An important aspect of these studies on patterning and migration is that homeoproteins sensitize the cells to the action of other growth factors, thus cooperating with established signaling pathways. The role of homeoprotein signaling at later developmental stages is also of interest. In particular, the transfer of homeoprotein Otx2 into parvalbumin-expressing inhibitory neurons (PV-cells) in the visual cortex regulates cortical plasticity. The molecular deciphering of the interaction of Otx2 with binding sites at the surface of PV-cells has allowed the development of a specific Otx2 antagonist that reopens plasticity in the adult cortex and cures mice from experimental amblyopia, a neurodevelopmental disease. Finally, the use of homeoproteins as therapeutic proteins in mouse models of glaucoma and Parkinson disease is reviewed. In the latter case, engrailed homeoproteins protect mesencephalic dopaminergic neurons by increasing the local translation of complex I mitochondrial mRNAs. In conclusion, this review synthesizes 20 years of work on the fundamental and potentially translational aspects of homeoprotein signaling.

I. Introduction: The History of Homeoprotein Transduction

Homeoprotein (HP) transduction is defined by the ability of proteins from this family of transcription factors to pass from cell to cell by nonconventional mechanisms, allowing direct access to the cytosol and nucleus of receiving cells (Prochiantz and Theodore, 1995; Prochiantz, 2000; Prochiantz and Joliot, 2003; Brunet et al., 2007). Although protein (including HP)
transduction is recognized in plants, where the traveling entities use the intercellular corridors termed plasmodesmata (Lucas et al., 1995; Crawford and Zambrisky, 2001; Kim et al., 2002; Ruiz-Medrano et al., 2004; Bolduc et al., 2008), such recognition has been long to establish itself for metazoans.

The first experiments that led to the demonstration that HPs signal between cells were aimed at understanding whether they could define neural shape as they define the shape of organs. Because the DNA-binding domain (the homeodomain) of many HPs is very highly conserved, we decided to inject that of Antennapedia (AntpHD), a drosophila HP, into nerve cells, with the aim of displacing endogenous HPs from their genomic cognate binding sites and to follow the anticipated shape modifications (Joliot et al., 1991). To that end, we used the scrape loading procedure consisting of the loading of exogenous molecules into the cytoplasm of adherent cells via a mechanical perturbation of the plasma membrane.

The results were encouraging, but because the scrape loading procedures leaves most of the protein outside, we performed a control experiment consisting of adding the protein outside without disturbing the cells. We were disappointed to observe the same changes obtained in the scrape loading experiment. However, before giving up with this approach, we tagged the homeodomain (HD) with FITC (a fluorescein derivative), and this is how we observed its accumulation in the cell cytoplasm and nucleus of cells in culture (Joliot et al., 1991). Using several HD mutants, we were then able to demonstrate that HD internalization regulates neuronal morphology through a specific competition, at the genome level, with endogenous HPs from their genomic cognate binding sites and to follow the anticipated shape modifications (Joliot et al., 1991). To that end, we used the scrape loading procedure consisting of the loading of exogenous molecules into the cytoplasm of adherent cells via a mechanical perturbation of the plasma membrane.

This led to the discovery that full-length HPs are internalized and secreted and to the identification of the sequences responsible for the two steps of intercellular transfer: secretion and internalization (Chatelin et al., 1996; Joliot et al., 1998; Maizel et al., 1999; Joliot and Prochiantz, 2004). This is how penetratin, the first in a long series of cell-penetrating peptides (CPPs) corresponding to the third helix of the AntpHD, was characterized and later used to internalize hydrophilic cargoes (Derossi et al., 1994, 1996; Joliot and Prochiantz, 2004). This is how penetratin adopts the structure of an amphipatic helix in position 48 of the HD (Fig. 2, C and D) and to design several penetratin variants also able to translocate across the cell membrane in absence of endocytosis (Derossi et al., 1994, 1996; Joliot and Prochiantz, 2004).

However, before giving up with this approach, we tagged the homeodomain (HD) with FITC (a fluorescein derivative), and this is how we observed its accumulation in the cell cytoplasm and nucleus of cells in culture (Joliot et al., 1991). Using several HD mutants, we were then able to demonstrate that HD internalization regulates neuronal morphology through a specific competition, at the genome level, with endogenous HPs from their genomic cognate binding sites and to follow the anticipated shape modifications (Joliot et al., 1991). To that end, we used the scrape loading procedure consisting of the loading of exogenous molecules into the cytoplasm of adherent cells via a mechanical perturbation of the plasma membrane.

The latter attribution of the internalization properties to a short sequence allowed us to undertake a chemical approach to identify several key amino acids (in particular, the highly conserved tryptophan residue in position 48 of the HD) and to design several penetratin variants also able to translocate across the cell membrane in absence of endocytosis (Derossi et al., 1994, 1996). It also allowed us to demonstrate that penetratin adopts the structure of an amphipatic helix in a hydrophobic environment but that this helical structure is not necessary for internalization, as shown by the introduction of 1–3 proline residues (to break the helix) within the sequence (Derossi et al., 1994, 1996).

II. Penetratin, Homeodomain, and Homeoprotein Internalization

Because of its FITC tag, HD internalization could be followed without fixation, and it was very striking to observe its diffusion into the cytoplasm and nucleus, with a somewhat homogenous distribution but no or few endocytotic structures (Fig. 2, A and B). This led us to propose that classic endocytosis is not the mode or sole mode of internalization, a hypothesis supported by the finding that internalization takes place at 4°C. However, to eliminate the possibility of an artifact, it was necessary to identify the sequences involved and, if possible, the mechanisms. Thanks to a site-directed mutagenesis approach, it was rapidly verified that within the 60 amino acids of the AntpHD structure, its third helix (16 amino acids) was necessary and sufficient to allow HD internalization (Fig. 2, C and D) (Derossi et al., 1994, 1996; Joliot and Prochiantz, 2004).

ABBREVIATIONS: 6-OHDA, 6-hydroxy-dopamine; CPP, cell-penetrating peptide; DA, dopamine; DPP, decapentaplegic; eIF4E, eukaryote initiation factor 4E; FITC, fluorescein isothiocyanate; GABA, ?-amino butyric acid; HD, homeodomain; HP, homeoprotein; MAD, mother against DPP; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NLS, nuclear localization signal; OPC, oligodendrocyte precursor cell; PV, parvalbumin; RGC, retinal ganglion cell; scAb, single-chain antibody.
1998). Table 1 summarizes the main peptides designed, with their translocation properties.

It was extremely surprising and very much against the view of the plasma membrane as a Berlin Wall that a peptide could be internalized without endocytosis. Some of us (E.D. and A.J.) are still examining the internalization mechanisms, but to date, we favor (from several biophysical studies, including phosphorus NMR studies) a two-step process involving electrostatic and hydrophobic interactions. A first step is the binding of the peptide to the negative charges of the membrane (lipids and sugars). This was demonstrated by the requirement of cell-surface carbohydrates and negatively charged lipids for the interaction of penetratin with live cells and artificial vesicles, respectively (Christiaens et al., 2002; Lensink et al., 2005; Jiao et al., 2009). A second step consists in a transient destabilization of the membrane induced by the insertion of W48 into the acyl core of the bilayer, which in some situations, can lead to the formation of inverted micelles identified by 31P nuclear magnetic resonance (Berlose et al., 1996; Christiaens et al., 2002; Dupont et al., 2007). Whether the peptide is within the inverted micelles or traverses the membrane because of its destabilization is unknown at this point.

Because of numerous biophysical studies, the understanding of penetratin internalization is much more advanced than that of full-length HPs. However, it is likely that parts of the mechanism allowing penetratin internalization are also valid for HPs. The reasons for this statement are that HP internalization can take place at 4°C and that sequence changes that hinder penetratin uptake (e.g., that of Trp48 into a phenylalanine) also hinder HD and HP internalization (Brunet et al., 2005; Torero Ibad et al., 2011). However, there must be differences, and work is in progress to understand them. Finally, with regard to internalization, we insist that it is the ability to address hydrophilic reagents to the cell cytosol and nucleus, thus requiring the crossing of a membrane bilayer, that has led to the definition of the CPP family, even though all CPPs may not enter the cell using the same mechanism (Lindgren

![Fig. 1. Homeoprotein secretion and internalization signals. (A) Homeodomain sequence alignments for 10 HPs verified for intercellular transportation. The internalization sequence corresponds to the third helix of the HD, and the secretion sequence includes the end of helix 2, the beginning of helix 3, and the β-turn between the two helices. The three helices are indicated by bold dark lines. For internalization, the consensus sequence is I/V-X-I/V-W-F-X-N-R/K-R-X-K/R-X-R-X-K/X. For secretion, the consensus sequence is X-X-L/I-X-L-X-E-I/V-X-I/V. Note the conservation of hydrophobic and basic residues in the internalization sequence and that of hydrophobic residues in the secretion one with characteristics of a nuclear export sequence. (B) Schematic representation of the internalization and secretion sequences and of their functions in intercellular transportation.](image-url)
In the case of penetratin, the uptake of a large spectrum of cargoes, ranging from small compounds (including small interfering RNAs, oligonucleotides, peptide nucleic acids, short peptides, and phosphopeptides) to full-length fusion proteins and viral particles has been successful (Derossi et al., 1998; Joliot and Prochiantz, 2004; Mae and Langel, 2006).

Finally, most mechanistic studies were done in vitro on cells and lipid vesicles. In these models, no saturation was observed for peptide, HD, and HP internalization. In vivo, the situation might be different because of the presence of HP binding sites (Beurdeley et al., 2012) that can trap the proteins and regulate their internalization. From this point of view, of note, whenever the quantification was done, ~5% of the HPs were at the cell surface, compared with 95% in the intracellular compartments (Wizenmann et al., 2009; Di Lullo et al., 2011).

**III. Homeoprotein Secretion**

Similarly to internalization, secretion is not canonical as can be anticipated from the absence of a classic signal peptide for secretion (Joliot and Prochiantz, 2004; Dupont et al., 2007). Most of the work on secretion has been done on Engrailed2 through a site-directed mutagenesis approach (Maizel et al., 1999). Of most importance, the signal for secretion is internal to the protein and spans the second and the third helix of the HD (thus, within the HD as is the internalization domain) (Fig. 1). A second point of interest is that this domain overlaps with a nuclear export sequence, suggesting a link between secretion and nuclear export.

The latter point is confirmed by the study of plant HPs. Indeed, the intercellular traffic of several proteins, including HPs, can take place in plants because of specialized corridor structures called plasmodesmata (Crawford and Zambrayski, 2001). This is well illustrated with Knotted-1 (KN1), a HP expressed in the shoot meristem of higher plants. In this case, it was demonstrated that the transfer of KN1 is dependent on its HD only and is abolished after mutation of the nuclear localization sequence (NLS) present within the HD (Bolduc et al., 2008).

Of interest, we demonstrated that the HD of KN1 is transported between animal cells and, thus, in absence of plasmodesmata (Tassetto et al., 2005). Moreover, mutating the KN1 NLS delocalizes the protein toward the cytosol and prevents its secretion, whereas adding the SV40 NLS to the mutated HD restores both nuclear accumulation and transfer. These experiments suggest that HPs acquire the competence for secretion within the nuclear compartment and, in addition, may use similar mechanisms for intercellular transport in the two phyla.

The latter point is important because it places HP signaling upstream of the separation between metazoa and metazoans and, thus, very early in the evolution of signal transduction and the invention of multi-cellularity. It might thus be that HPs came before many of the classic signaling pathways. If so,

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**Table 1**

Main peptides derived from the Antennapedia third helix and their properties

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Internalized</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>43-58:</td>
<td>RQIKIFQQRKMKWKK</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>48-43:</td>
<td>KKWMRQRNFWIKIIQR</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Asp43-58</td>
<td>RQIKIFQQRKMKWKK</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Pro43-58</td>
<td>RQIKIFQQRKMKWKK</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>3Pro43-58</td>
<td>RQIKIFQQRKMKWKK</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Phe48,56</td>
<td>RQIKIFQQRKMKWKK</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>7Arg</td>
<td>RQIRIFQQRKMKWKK</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Trp-Arg</td>
<td>RRWRRWRRRWR</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>
it is possible, as discussed below, that the latter pathways were recruited later and cooperate with HP signaling, possibly allowing a greater developmental and physiologic robustness.

In addition to exiting the nucleus, secretion necessitates exit from the cells and crossing the plasma membrane. Contrary to what may have been anticipated from internalization studies, HPs do not seem to use the same mechanisms for entering and exiting (Joliot and Prochiantz, 2004; Dupont et al., 2007; Joliot et al., 1997). A first hint that this is so was the sensitivity of secretion to low temperature (as opposed to internalization). In fact, we observed an association of Engrailed2 with caveolae-like vesicles (enriched in cholesterol and glycosphingolipids), with a small fraction of the protein present within the lumen of the vesicles and protected against proteinase K (Joliot et al., 1997). A consequence of such an association might be a basolateral to apical (thus axonal) transportation of the protein, as shown for Engrailed2, which, in contrast with Krox 24, is translocated to the axons of transfected neurons in vivo (Fig. 3, A and B).

This difference between secretion and internalization and the basolateral to apical transportation is further supported by the transportation across a tight junction epithelium of a chimeric peptide encompassing the secretion and internalization sequences (Sec-Pen peptide) (Dupont et al., 2007). Sec-Pen loaded on the apical or the basolateral side can only travel from basolateral to apical (Fig. 3C). We also observed that Sec-Pen added at the basolateral side at 4°C is internalized but not secreted at the apical side, unless a 4–37°C temperature shift is made after internalization (Fig. 3D). In addition to revealing the mechanism, this experiment demonstrates that Sec-Pen and, possibly HPs, can travel across a tight-junction epithelium, provided that peptide presentation occurs at the basolateral side. With regard to the nervous system, this suggests that a preferential route of Sec-Pen (plus cargo) or HP entry from blood into the brain is the choroid plexus (Emerich et al., 2005). Indeed, the choroid plexus endothelium, in contrast with that of brain capillaries, has its basolateral side oriented toward the blood flow.

**Fig. 3.** Intracellular localization of two transcription factors. (A) Engrailed expressed by electroporation in neurons from the rat cortex (E18) travels into the axon. (B) Krox 24 expressed in the same conditions as Engrailed is confined to the nucleus and cell body. (C) A compound peptide with the penetratin internalization peptide linked to the secretion peptide (see Fig. 1) presented to a tight junction epithelium is internalized at the basolateral or apical side but is only secreted at the apical side. (D) The same peptide is internalized at 4°C because of the penetratin sequence (no endocytosis required), but secretion needs a shift to 37°C and passage into a vesicular compartment. A and B correspond to maximum projections of confocal section stacks.
Finally, the issue of the regulation of secretion must be addressed. On the basis of the brief summary of our present knowledge presented above, it appears that regulation can occur at several levels, for example, nuclear entry, nuclear export, association with vesicles, transportation to secretion sites, secretion per se (after vesicular fusion with the plasma membrane or through another mechanism, although this is not yet clear). It is clear that Engrailed2 phosphorylation at a serine-rich domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002). Because Engrailed2 is a phospho-domain outside the HD can regulate its secretion (Maizel et al., 2002).

IV. Non–Cell Autonomous Homeoprotein Activity in Pattern Formation

Although HP intercellular transfer is now well accepted, the physiologic interest of this unexpected signaling mechanism has remained challenged until recently. This is quite understandable for a hypothesis that bruises so many dogmas at the cell biologic level and, more profoundly, at the level of the general view of signal transduction, whereby transcription factors regulate the expression of morphogens and receptors, but do not travel from cell to cell, thus bypassing many other signaling pathways.

This is why deciphering functions for HP signaling has been very high on our agenda since the beginning of this line of research in 1990. Our intent was to produce an animal model in which the sequences for transportation would have been mutated, leaving only the cell autonomous functions intact. Unfortunately, as shown above, the transfer sequences are in the HD, and mutating them also interferes with cell autonomous functions (Joliot and Prochiantz, 2004). This is why other strategies were developed, in particular the cloning of mini-genes encoding single chain antibodies (scAbs) directed against specific HPs. Indeed, the expression of such antibodies and their addressing to the extracellular space by the addition of a classic secretion signal ensure that they only interfere with the nonautonomous activities of HPs.

The scAb strategy was tested in the early development of the zebrafish eye and drosophila wing disc (Lesaffre et al., 2007; Layalle et al., 2011). On the basis of the idea that a territory marked by an HP in the early neuroepithelium depends on the initial induction of this HP followed by the extension of its territory through its serial passage between cells and non–cell autonomous induction (Brunet et al., 2007; Holcman et al., 2007; Kasatkine et al., 2008) (Fig. 4A), we expressed a secreted anti-Pax6 scAb in the developing zebrafish and found that this led to a reduced eye field (Lesaffre et al., 2007). In parallel experiments, to ascertain that this effect is really nonautonomous, a classic anti-Pax6 antibody was injected in the extracellular space at the blastula stage, giving the same small eye phenotype (Lesaffre et al., 2007). Figure 4B also shows the hypothesis that HP non–cell autonomous activity may participate in the refinement of boundary formation. Indeed, in the early neuroepithelium, HPs with self-activating and reciprocal inhibitory activities (thus, morphogens in Turing’s definition (Turing, 1952)) are often localized on either side of the future boundary. Consequently, decreasing the synthesis of one HP gives an advantage to the other one, resulting in a boundary shift. Such a phenomenon has been modeled (Holcman et al., 2007) and observed in experimental paradigms (Bishop et al., 2000; Schwarz et al., 2000; Simeone, 2000; Toresson et al., 2000; Yun et al., 2001; Brodski et al., 2003; O’Leary et al., 2007).

A second model demonstrating a participation of HP transfer in early pattern formation is the fly wing disc. We demonstrated that the secretion of Engrailed by cells that line the anterior/posterior boundary of the drosophila wing disk (Patched expression territory) is necessary for the formation of the anterior cross-vein (Fig. 5A). This result was obtained as the result of the region-specific expression of secreted scAb and Engrailed RNAi using the UAS/Gal4 induction system (Layalle et al., 2011). In the latter experiments, of note, extracellular Engrailed acted in synergy with DPP (TGFβ-ortholog) signaling (Fig. 5B). This cooperation between HP signaling and more classic modes of signal transduction will be expanded.

V. Non–Cell Autonomous Homeoprotein Activity in Axon Guidance and Cell Migration

Axon guidance has been the first model allowing a clear demonstration of HP non–cell autonomous activity in metazoans. The patterning of the retinal...
ganglion cell (RGC) axons on the optic tectum (chick) and superior colliculus (mouse) reflects the topographic order of RGCs at the level of retina (Fig. 6) (Flanagan and Vanderhaeghen, 1998). One aspect of this topographic map is the projection of the nasal-temporal axis of the retina onto the posterior-anterior axis of the tectum. Classically, this conservation is explained by the graded anterior (low)/posterior (high) expression of EphrinA5 in the tectum and the graded temporal (high)/nasal (low) expression of the EphA2 ephrin receptor in the retina (Braisted et al., 1997; Ciossek et al., 1998; Frisen et al., 1998; Brown et al., 2000; McLaughlin and O’Leary, 2005). Indeed, because of the collapsing activity of high Ephrin/Eph signaling, temporal axons collapse when they reach the posterior region of the tectum, where only nasal axons can navigate.

However, Engrailed1 (En1) and Engrailed2 (En2; globally Engrailed or En1/2) also show a graded expression in the tectum, and the two proteins, because of their ability to travel between cells, could not be ignored as candidate retina-tectal patterning cues (Friedman and O’Leary, 1996; Logan et al., 1996). Starting from this hypothesis, we demonstrated that En1/2 has a graded extracellular expression in the tectum and that blocking extracellular neutralization of Engrailed (Sec scFV for secreted single chain antibodies). In contrast, ACV formation is antagonized by extracellular Engrailed neutralization when the antibody is secreted by cells of the Dpp domain (where Engrailed is not expressed). In addition, extracellular Engrailed synergizes with Dpp signaling (enhances MAD phosphorylation).

**Fig. 5.** Extracellular Engrailed is required for the anterior cross vein formation in the fly wing disk. (A) Wild-type adult wing veins consist of 5 lateral veins (L1 to L5) and 2 cross-veins: one posterior (PCV, in dashed) and one anterior (ACV, in full). The ACV extends beyond the Engrailed-expression domain indicated in light gray. (B) PCV formation requires cell autonomous Engrailed activity, because it is blocked by RNAi expression in the posterior domain but not by extracellular neutralization of Engrailed (Sec scFV for secreted single chain antibodies). In contrast, ACV formation is antagonized by extracellular Engrailed neutralization when the antibody is secreted by cells of the Dpp domain (where Engrailed is not expressed). In addition, extracellular Engrailed synergizes with Dpp signaling (enhances MAD phosphorylation).

**Fig. 6.** Role of secreted Engrailed in retinal ganglion cell guidance (A) Schematic representation of how the nasal-temporal axis of the retina projects onto the posterior-anterior axis of the optic tectum. (B) Temporal and nasal retinal ganglion cell axons placed in an Engrailed gradient are repelled and attracted respectively. A noninternalized Engrailed variant ENSR has no guiding activity.
a series of loss and gain of function studies, in vitro and in vivo, we confirmed the hypothesis that extracellular Pax6 regulates OPC migration (Fig. 7A) (Di Lullo et al., 2011). This guiding activity is lost after neutralization of an important signaling cue, Netrin (Fig. 7B). Netrin is an extracellular, laminin-related, protein that functions as chemotactic guidance cue for migrating cells and axons during neural development. In the embryonic spinal cord, it is described as a repulsive cue for migrating OPCs; this has been shown with the use of a chemotaxis assay. The requirement of Netrin signaling in the Pax6 mediated regulation of OPC migration again suggested a physiologic interaction between HP signaling and classic signaling pathways.

VI. Non–Cell Autonomous Homeoprotein Activity in the Regulation of Cortex Plasticity

It has been long recognized that developmental genes are expressed not only during development, but also at late postnatal stages and even throughout adulthood. We have been interested in understanding the functions of homeoproteins in adulthood with the idea that they are related to physiology and could thus shed some light on pathologies of still obscure etiology. Among the studies that were developed is the regulation of the critical period for ocular dominance (Katz and Crowley, 2002; Berardi et al., 2003; Wiesel and Hubel, 1965; Hensch, 2005b).

Critical periods correspond to periods when the cortex can respond through morphologic and physiologic modifications to the information coming from the environment. One classic example is binocular vision that develops from postnatal days 20 (shortly after eye opening) through 40 in the mouse. This period is marked by the maturation in the primary visual cortex of a specific subset of fast-spiking GABAergic inhibitory interneurons expressing parvalbumin (Fagioli et al., 2004; Hensch and Fagioli, 2005; Hensch, 2005a). For the latter reason, they are called PV-cells. During postnatal days 20–40, plasticity is progressively opened and closed as the inhibitory/excitatory balance shifts toward enhanced inhibition.

This plasticity is best demonstrated by eye closure protocols. If one eye of the mouse is closed during postnatal days 20–40, its representation at the level of the binocular visual cortex is reduced to the advantage of the open eye, thus leading to an enduring loss of visual acuity for the closed eye, a definition of amblyopia. This adaptation is limited to the critical period, meaning that closing one eye before postnatal day 20 or after postnatal day 40 has no or little effect on visual acuity (Fig. 8).

Several growth factors and protocols have been used to manipulate the timing of the critical period (Maffei et al., 1992; Gordon and Stryker, 1996; Hensch and Stryker, 1996; Hanover et al., 1999; Pizzorusso et al., 2002; Bartoletti et al., 2004; McGee et al., 2005; Thompson et al., 2008; Shatz, 2009; Maffei et al., 2010; Morishita et al., 2010; Southwell et al., 2010). For our part, we have described the progressive accumulation of Otx2 in PV-cells during their maturation and showed that Otx2 is both necessary and sufficient for the opening of the plastic period at postnatal day 20 and its closure at postnatal day 40 (Sugiyama et al., 2008) (Fig. 8). Of interest, Otx2 protein is found in PV-cells in which the Otx2 locus is not active. Thus, the protein originates from other sources, among which the best candidates are the eye and the choroid plexus.

The choroid plexus source is presently under exploration, but we have confirmed that Otx2 injected in the eye can travel through the thalamus and terminate in cortical PV-cells. If this pathway is physiologic, protein synthesized by bipolar cells is transferred to RGCs and from there to higher centers in the nervous system. This non-autonomous Otx2 activity that results from the specific capture of Otx2 by PV-cells requires that binding sites be expressed by these cells at the onset of the plastic period. This hypothesis was confirmed by the fact that Otx2 directly infused into the cortex accumulates preferentially into PV-cells, not only in the course of postnatal plasticity,
...but also in the adult (Sugiyama et al., 2008). A plausible scenario is that Otx2-binding sites start to be expressed at the opening of the plastic period in an experience-dependent manner and subsequently drive the permanent capture of Otx2 by PV-cells.

In agreement with the latter hypothesis, it was recently demonstrated that the specific accumulation of Otx2 into PV-cells results from the recognition of complex sugars of the chondroitin sulfate family present in the perineuronal nets (Fawcett, 2009) that surround this class of interneurons (Beurdeley et al., 2012; Miyata et al., 2012). A short sequence of the Otx2 protein starting just upstream of the homeodomain and including its first helix mediates the latter recognition and possesses molecular traits of a glycosaminoglycan-binding sequence. When a synthetic peptide (RK-peptide) corresponding to this sequence is infused into the adult visual cortex, the amount of Otx2 captured by PV-cells is decreased, resulting in a down-regulation of PV expression and a clear decrease in perineuronal net assembly, as if the cells had been driven back into a critical period state (Fig. 8B). This observation prompted us to verify whether and demonstrate that this immature phenotype was correlated with a reopening of physiologic plasticity in the adult. A dramatic consequence of this treatment was the possibility to induce amblyopia in the adult and, more strikingly, to restore binocular vision in amblyopic mice (Beurdeley et al., 2012). To our knowledge, this is the first demonstration that a neurodevelopmental cortical disease can be cured in an adult mouse.

VII. Homeoprotein Signal Transduction Pathways

HPs are well-established transcription factors, and at least some are translation regulators. Regulation of translation has been firmly established for Bicoid, in Drosophila (Rivera-Pomar et al., 1996; Niessing et al., 2002), but also for a handful of vertebrate HPs (Topisirovic and Borden, 2005). It was shown that this regulation requires a direct interaction with the translation initiation factor eIF4E through a sequence conserved in over 200 HPs, raising the possibility that translation regulation may be a near universal HP property (Nedelec et al., 2004; Topisirovic and Borden, 2005).

As already mentioned (see section V), Engrailed attracts and repels nasal and temporal growth cones, respectively, through a mechanism dependent on local protein translation, with no transcription involved (Brunet et al., 2005). The strongest point of the demonstration for a translation-only mechanism, in addition to the use of specific inhibitors, was the attraction and repulsion of growth cones isolated from the cell bodies.

The next step to better understand the guidance mechanisms was to identify the mRNAs translated after Engrailed internalization. To that end, two protocols were developed consisting of internalizing Engrailed into purified growth cone preparations and identifying translated proteins either by identifying...
the mRNAs present on polysomes (microarray experiments) or separating and analyzing the neosynthesized proteins by mass spectrometry. The same protocols were applied to adult synaptoneurosomes isolated from the ventral midbrain for reasons that will become evident in section VIII on therapeutic proteins (Alvarez-Fischer et al., 2011; Stettler et al., 2012). Thus far, in agreement with a mechanism involving eIF4E, identified mRNAs are capped in 5′, but we cannot totally eliminate the possibility that Engrailed or other HPs also regulate the translation of noncapped mRNAs. In addition, we have no evidence that a HP will regulate the expression of a given gene at the two levels of transcription and translation.

With embryonic RGC growth cones or adult midbrain synaptoneurosomes, a striking result is that 60% of the proteins in which translation is altered after Engrailed internalization are mitochondrial proteins or proteins involved in mitochondrial physiology. Indeed, all but 13 mitochondrial proteins are encoded in the nucleus, and their mRNAs surround mitochondria (the mitochondrial cloud) and are translated locally. Among the upregulated mitochondrial proteins, those of complex I are particularly represented, among which are many of the Ndufs (NADH-ubiquinone oxidoreductase Fe-S) family.

This led us to demonstrate that ATP is synthesized and released by growth cones within less than one minute after the application of Engrailed (Stettler et al., 2012). This synthesis (and secretion) is protein synthesis dependent (blocked by protein synthesis inhibitors and not triggered by an Engrailed mutant protein not able to bind eIF4E) and corresponds to neo-synthesized ATP (blocked by oligomycin). This raises the issue of whether extracellular ATP participates in Engrailed signaling.

Before answering this question, we need to introduce another twist in Engrailed signaling. As already mentioned (see section V), it is well known that EphrinA5 is in part responsible for the avoidance of posterior tectum by temporal axons and provokes temporal growth cone collapse. Using two assays (EphrinA5 stripes and the collapse assay), we showed that EphrinA5 is only active at high concentrations and not at low subthreshold (perhaps, physiologic) ones (Wizenmann et al., 2009). However, adding Engrailed at nanomolar concentrations restores Ephrin-collapsing activity at subthreshold concentrations (Wizenmann et al., 2009). This means that Engrailed sensitizes temporal cones to low EphrinA5 concentrations.

Figure 9A summarizes our conclusions that Engrailed/EphrinA5 synergy requires ATP synthesis, secretion, and degradation into Adenosine that subsequently signals through the adenosine receptor A (AR1). As shown in Fig. 9B in the collapse experiment, the synergy is lost if one blocks ATP degradation or in absence of adenosine signaling (A1R antagonist), and in contrast, stimulating A1R with a specific agonist induces growth cone collapse at subthreshold EphrinA5 concentrations in the absence of Engrailed (Stettler et al., 2012). Figure 9B also shows that temporal growth cones can grow on posterior membranes (stripe assay) when A1R receptor activity is inhibited.

Figure 9C gives a summary of this “ménage à trois” signaling pathway and indicates that things are much more complicated than anticipated. Figure 9C also shows the possibility that HP signaling might only be useful at physiologic concentrations of its cofactors. In a loss of function situation, HP transfer is useless, which is also seen in a gain of function situation, when the concentration of the agonist is much above threshold and, very likely, nonphysiologic. We thus propose that it is only when the classic signaling entity is present at physiologic concentrations that HP signaling is detectable by its effects. Figure 9C proposes that, at physiologic concentrations of cofactors, minor variations in HP transfer may turn signaling on and off, thus acting as a sensitizing rheostat.

In any case, this cooperation between two classic signaling pathways and Engrailed signaling has the merit to reconcile our own findings with those of the guidance community. Of interest, HPs are endowed with positional information and could thus superpose classic signaling to positional information. For example, the same cell contacted by axons from glutamatergic neurons coming from different regions may respond differently to the two stimulating axons on the basis of their respective origins.

We have no hint of whether this synergy model can be generalized, but supporting the idea are the results on the formation of the anterior cross vein in Drosophila. Indeed, as summarized in Fig. 5, we have observed that extracellular Engrailed synergizes with DPP (Layalle et al., 2011). This is clearly demonstrated by the fact that the neutralization of extracellular Engrailed blocks the formation of the anterior cross vein and decreases the content of phosphorylated MAD, the main downstream effector in DPP signaling. In this model, we have not verified whether the interaction between the two pathways requires Engrailed-regulated ATP synthesis.

VIII. Therapeutic Homeoproteins

In a way, this chapter has already been initiated with the amblyopia story (Sugiyama et al., 2008; Beurdeley et al., 2012). In that chapter on Otx2 traveling into the cortex, one can anticipate the opening of the critical period for binocular vision through Otx2 infusion. Conversely, blocking Otx2 transfer in the adult reopens plasticity in the visual cortex, allowing restoration of binocular vision in ambylopic mice. Of interest, Otx2 (but not its mRNA) is present in PV-cells throughout the cortex, including regions...
associated with complex behaviors (e.g., the amygdala), suggesting that the regulation of plasticity by endogenous Otx2 might not be limited to the visual cortex. If so, manipulating Otx2 might have its own interest in diseases associated with defects in distinct critical periods, including psychiatric diseases (Lewis et al., 2005; Do et al., 2009; Insel, 2010; Uhlhaas et al., 2010). However, this generalization remains very speculative at the moment. We shall therefore concentrate on the possible therapeutic use of Otx2 and Engrailed in animal models of glaucoma and Parkinson’s disease.

Glaucoma results from the specific death of RGCs in response to or associated with elevated intraocular pressure (in general) (Stamer and Acott, 2012). One of the main models to induce RGC cell death in the retina is the injection of NMDA, which induces RGC apoptosis and necrosis through the secretion of TNF by Mueller Glia cells (Barnett et al., 2009; Lebrun-Julien et al., 2009). In a recent study, we demonstrated that Otx2 injected in the eye at nanomolar concentrations fully antagonizes RGC cell death induced by NMDA and preserves visual acuity (Torero Ibad et al., 2011) (Fig. 10A). This protective activity of Otx2 is long lasting (at least three weeks) and requires that the protein be internalized, because it was not observed with an internalization deficient mutated protein.

Fig. 9. Engrailed activity on retinal growth cones involves a synergy with EphrinA5 and purinergic signaling pathways. (A) The procollapse non–cell autonomous signaling pathway in the temporal retinal ganglion cell growth cones (green). Blocked effects of En1/2 on growth cone collapse (red). En1/2 enters the growth cone and, within a minute, stimulates the synthesis and release of ATP. This is blocked by protein translation inhibitors, such as anisomycin (Aniso) or ATP synthase inhibition by oligomycin (Oligo). Outside the growth cone, ATP is hydrolyzed to adenosine, which acts at the A1 receptor to synergize the effects of EphrinA5. Adyrase hydrolyzes ATP and increases En1/2-mediated growth cone collapse. Inhibition of ATP hydrolysis by β,γ-metATP blocks En1/2-mediated collapse. The A1R antagonist DPCPX blocks En1/2-mediated collapse, and the A1R agonist CCPA can replace En1/2 in enhancing EphrinA5 collapse. (B, top) In the presence of low EphrinA5 (LoA5), En1/2 induces the collapse of growth cones. When ATP hydrolysis is inhibited by β,γ-metATP, collapse is blocked. The pan-Adenosine antagonist CGS15943 (CGS) blocks En1/2-mediated collapse. The A1R-specific antagonist DPCPX inhibits En1/2-induced collapse, and the A1R agonist CCPA can replace En1/2 in enhancing EphrinA5-mediated collapse. (B, bottom) When RGC axons from temporal retina grow on a substrate of alternating stripes of membranes from posterior (P, red) and anterior (A, black), the axons prefer to grow on the anterior stripes and avoid the posterior stripes. When the A1 receptor is blocked (DPCPX), temporal axons are no longer inhibited by guidance cues in posterior membrane stripes. (C) Ménage à trois interaction between Engrailed, Adenosine, and EphrinA5 signaling. (D) This general scheme illustrates the hypothesis that HPs are regulators of signaling at physiologic concentrations of the classic agonist. In loss and gain of function situations, they are useless or unnecessary, respectively. In contrast, in physiologic situations, they might be necessary to rapidly shift from an off to an on situation (and vice-versa).
Of note, although RGCs contain the Otx2 protein, the Otx2 locus is inactive in these cells (Sugiyama et al., 2008), suggesting that they import the protein, probably from bipolar cells with which they are in contact. This raises the intriguing possibility that this non–cell autonomous Otx2 activity allows RGC survival throughout life and that, in this system, Otx2 acts like an authentic trophic factor or as a cofactor for more classic trophic factor(s). We are now exploring the latter possibility.

The hypothesis that Engrailed could serve as a survival factor for mesencephalic dopaminergic neurons (mDA) and be in the Parkinson disease (PD) pathway came from the observation that Engrailed1 and Engrailed2 are strongly expressed in this cell type throughout life (Alberi et al., 2004; Sonnier et al., 2007). Moreover, mDA survival was found to depend on the level of expression of Engrailed. In our laboratory, we observed that mice heterozygous for Engrailed1 in the Engrailed2 wild-type context (En1+/−) show progressive death of mDA neurons. Death starts at six weeks of age, reaching 40% in the substantia nigra and 20% in the ventral tegmental area at one year (Sonnier et al., 2007). This progressive death and the higher susceptibility of substantia nigra, compared with ventral tegmental area, are very reminiscent of the PD phenotype. In addition, the mice display many of the motor and nonmotors phenotypes associated with the disease.

Of interest, in this mutant model, the infusion of Engrailed fully arrested mDA cell death, raising the possibility that Engrailed could act as a therapeutic protein in other PD models (Sonnier et al., 2007). To investigate this hypothesis, we developed three PD models (MPTP, 6-OHDA, and injection in the SN of the A30P form of α-synuclein, [linked to penetratin for internalization]) and found that En1 infusion saves the mDA neurons in all three models (Alvarez-Fischer et al., 2011). All rescue experiments were done in male mice, but we have no indication of a sex bias in protection by Engrailed.

As mentioned above, Engrailed (En1 or En2) regulates the synthesis of several mitochondrial complex I proteins and enhances complex I activity. We thus verified the expression of Ndufs1 and Ndufs3, two En1/2 translation targets, in the SN of En1+/− mice and found that their expression was reduced by 30% in the SN but remained unchanged in other mesencephalic neurons (oculomotor nucleus), where Engrailed is not expressed (Alvarez-Fischer et al., 2011). This suggested that complex I regulation by Engrailed might be at the basis of Engrailed rescuing activity. This point was confirmed by the demonstration that the latter rescuing activity was fully lost after the infusion of a cell-permeable siRNA directed against Ndufs1 (Alvarez-Fischer et al., 2011).

As mentioned above, Engrailed is heavily expressed in mDA neurons from the adult SN, and it is unlikely that maintaining cells alive is its only function. In fact, another function of En1 is to increase the striatal DA content. This was revealed by dopamine measurements in the striatum of animals treated or not with

![Fig. 10. Therapeutic HPs in animal models of glaucoma and Parkinson disease. (Top) RGCs die selectively in response to an acute NMDA injection, with full protection by Otx2. Protection is also seen at the level of visual acuity (optomotor test). (Bottom) Engrailed protects mesencephalic dopaminergic neurons from MPTP. Because of the unilateral infusion of the HP, amphetamine (amphe) induces a rotation behavior contralateral to the infused side.](image-url)
MPTP or En1. This is probably of high interest, because it unravels an adult physiologic function of this protein in the nigro-striatal pathway. However we ignore the mechanisms (transcription, translation, or else) involved in the latter unsuspected activity.

**IX. Conclusion: The Future of Homeoprotein Transduction**

Homeoprotein transduction is thus involved in a large number of developmental and physiologic events, including eye field specification (Lesaffre et al., 2007), axon guidance (Brunet et al., 2005; Wizenmann et al., 2009; Stettler et al., 2012), axon maintenance (Yoon et al., 2012), Drosophila wing disk morphogenesis (Layalle et al., 2011), and the regulation of physiologic plasticity (Sugiyama et al., 2008; Beurdeley et al., 2012). These different activities imply transcriptional and nontranscriptional activities, including the regulation of protein synthesis. The latter observations and their biotechnological applications have established homeoprotein transduction as an important and original mode of signal transduction. However, in addition, they raise a large number of unresolved issues summarized in Fig. 11. First, we need to understand the extent to which the findings for a relatively limited number of homeoproteins may be extended to other homeoproteins that comprise a family of ~300 members. Although nothing is certain, we are fairly confident that most homeoproteins have the signaling properties observed for ten and four of them in vitro and in vivo, respectively. This opinion is based on the high conservation of the internalization and secretion sequences (Joliot and Prochiantz, 2004). However, even if transfer is a general property, its regulation remains to be deciphered and may differ among homeoproteins.

Another priority is the identification of non–cell autonomous transcription, translation, and epigenetic targets. Indeed, homeoproteins are both transcription factors and translation regulators. Regulation of translation (not limited to non–cell autonomous activities) was demonstrated for several homeoproteins and found to involve a direct interaction with the translation initiation factor eIF4E through a domain conserved in ~200 members of the family (Topisirovic and Borden, 2005). Moreover, a direct interaction can be observed in the absence of this conserved domain, as shown for Engrailed-1, in which punctual mutations in another domain abolish its ability to bind eIF4E and to regulate mRNA translation (Nedelec et al., 2004; Stettler et al., 2012). Finally, a regulation of the chromatin structure is possible, based on our unpublished observation that some effects of Otx2 and Engrailed can last for several weeks and even months in the models of pathologies that we have developed (Alvarez-Fischer et al., 2011; Torero Ibad et al., 2011).

An interesting future line of research takes its origin from the fact that up to 60% of the translation targets are nuclear-encoded mitochondrial mRNAs. This was shown for En1/2 and Otx2 (our unpublished results). It would be speculative to generalize this, because in contrast with internalization, secretion, and eIF4E-binding sequences, there are no means (to date) to predict which mRNAs are under homeoprotein control for their translation. However this is of real importance when it comes to the nervous system, because mitochondria are often located far away from the cell body, particularly in dendrites, where a local regulation of energy metabolism is an imperative necessity. A recent demonstration that En1 helps to maintain synapse integrity and the increasing recognition that many neurologic and psychiatric diseases may have a metabolic origin further underscore the interest of understanding how homeoproteins regulate local mitochondrial physiology and energy production (Yoon et al., 2012).

Because of the finding that, in growth cones, En1-induced ATP formation leads to purinergic signaling and enhances the response to low EphrinA5 levels (Stettler et al., 2012), a link may exist between mitochondrial activity (possibly homeoprotein-driven) and that of classic growth factors or mediators. Even independently of a systematic mitochondrial connection, it is striking that Engrailed signaling interacts with other signaling pathways in vertebrates and in the fly (Di Lulio et al., 2011; Layalle et al., 2011). This means that the identification of such interactions should be a high priority for additional study.

Finally (temporarily so), the studies on Otx2 recognition by specific sugars expressed by PV-cells and the
fact that, in contrast, En1 is not preferentially captured by these cells raise the issue of the presence of surface binding sites for homeoproteins (Beurdeley et al., 2012). It is indeed tempting, because of the extreme diversity that complex sugars of the glycosaminoglycan family can generate, to propose the novel concept that there exists a sugar code for homeoprotein recognition.

Taken together, a possible conclusion is that the studies devoted to homeoprotein signaling in the past twenty years may represent only the tip of the iceberg and that there is still a lot to do to better understand the developmental and physiologic functions of this novel and important signaling pathway, possibly involving as many as 30% factors working alone or in synergy.

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Authorship contributions

Conducted experiments: Spatazza, Di Lullo, Dupont.
Wrote or contributed to the writing of the manuscript: Joliot, Moya, Prochiantz.

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Bartoletti A, Medini P, Berardi N, and Maffei L (2004) Environmental enrichment and that there is still a lot to do to better understand the concept that there exists a sugar code for homeoprotein recognition.

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