The involvement of retinoic acid in the development of the vertebrate central nervous system

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Summary

We discuss here both previously published data and our current experiments which suggest that the vitamin A derivative, retinoic acid (RA), may play a role in the development of the vertebrate central nervous system (CNS). This evidence comes from the following: both an excess and a deficiency of vitamin A causes embryonic defects of the CNS; RA has been detected endogenously in the CNS; RA stimulates neurite outgrowth; the retinoic acid receptors have been detected with interesting distributions in the CNS; the binding protein for retinol, namely cellular retinol binding protein (CRBP) is found in the radial glia of the ventral floor plate; the binding protein for RA, namely, cellular retinoic acid binding protein (CRABP) is found in particular sets of axons in the developing spinal cord, in particular rhombomeres in the developing hindbrain and in the neural crest. Some hypotheses for the possible role of RA in various aspects of CNS development are discussed.

Key words: retinoic acid, retinol, cellular retinoic acid binding protein, cellular retinol binding protein, neurites, rhombomeres.

Introduction

Retinoic acid (RA), a vitamin A metabolite is a molecule that has recently excited interest as a morphogenetically active compound in the developing and regenerating limb. When absorbed onto a bead and grafted into the anterior margin of the chick wing bud, RA acts as a developmental organiser and stimulates the outgrowth of a mirror-imaged additional limb (Tickle et al. 1982). In the regenerating amphibian limb each of the three cardinal axes are respecified by RA rather than just the one as in the chick limb bud. In the proximodistal axis two limbs can be produced in tandem (Maden, 1982), in the anteroposterior axis mirror-imaged limbs result (Maden, 1983; Kim and Stocum, 1986) and the dorsoventral axis can also be respecified after suitable doses of RA (Ludolph et al. 1990). These results clearly have a bearing on the current debate as to whether RA is the endogenous morphogen which organises the anteroposterior axis of the chick limb bud (Slack, 1987; Brockes, 1991). Theory tells us (Wolpert, 1969) that positional signalling molecules may be utilised repeatedly in different developing fields of the embryo. We might therefore reasonably ask whether this applies to RA and here we consider the evidence that the developing CNS is another embryonic system in which RA is involved in the generation of pattern.

Evidence from teratology

The discovery of vitamin A as a vital component of the diet lead to many nutritional studies on the effects of excess and deficiency of this compound on the developing embryos of a variety of mammals including pig, cattle, sheep, rats and rabbits (Kalter and Warkany, 1959). The effect of a deficiency of vitamin A in the maternal diet is to produce a range of congenital malformations - eye defects (anophthalmia, microphthalmia and retina defects), hydrocephalus, cleft palate, ear defects (accessory ears, otocleisis), malformed hind legs, urino-genital malformations (renal hypoplasia, pseudo-hermaphroditism) and cardiovascular malformations (aortic arch defects, incomplete septation of the heart). Surprisingly, the effects of excess vitamin A are similar – eye defects (anophthalmia, microphthalmia, retina defects), hydrocephalus, cleft palate, brachygnathia, short maxilla, spina bifida and limb defects. Sadly, the same spectrum of abnormalities can also be seen in the human embryo after exposure to excess RA (Lammer et al. 1985).

At first glance, only some of these defects are obviously associated with the CNS (spina bifida, anencephaly, hydrocephalus, eye defects) whereas others are not (ear defects, cleft palate, cardiovascular defects, limb defects, urino-genital malformations). However, in the light of modern studies on the widespread contribution that the neural crest makes to the structures of the head and neck (Noden, 1983), it transpires that the majority of these malformations can be attributed to effects on tissues derived from the neural plate. For example, neural crest from the level of somites 1-3 contributes to the thymus, thyroid,
parathyroids and the heart (Kirby et al. 1983; Bockman and Kirby, 1984). In the heart the crest forms the cardiac ganglia, aorticopulmonary septum and the tunica media of the aorta and pulmonary trunk and its removal results in incomplete septation and transposition of the great vessels, precisely those defects described above. Ear defects, jaw defects and cleft palate are also seen when the migration of cranial neural crest is inhibited (Webster et al. 1986).

Recent interest in the involvement of RA in CNS development was stimulated by the experiments of Durston et al. (1989) on Xenopus embryos. After treating embryos with RA between late blastula and early neurula stages, anterior neural structures (forebrain, midbrain, eyes) failed to develop. Measurements of the volume of CNS tissue showed that treated and normal embryos were the same, which lead to the conclusion that the anterior neural structures had been respecified to form posterior neural structures. In addition, anterior-specific genes such as XCG-1, XAG-1, XA-1 and engrailed were repressed by RA whereas the expression of an anterior gene XIF3 and a posterior gene Xihbox 6 was increased (Sive et al. 1990).

The suggestion that RA can posteriorize the neuroepithelium accords with its posteriorizing action in the chick limb bud. Even though RA is acting on the mesoderm in the chick limb bud and the epithelium in the CNS, it is conceivable that RA is a posteriorizing agent. However, a recent re-evaluation of this phenomenon in Xenopus embryos has failed to provide any evidence for the concept of respecification. Rather than an increased hindbrain size, as would be expected from posterior respecification, anterior hindbrain units (rhombomeres) were reduced and one of the two stripes of expression of the segmental marker Krox-20 was absent (Papalopulu et al. 1991; Krumlauf et al. 1991; Hunt et al. this volume). Furthermore, the position of the cranial nerve roots, central motor nuclei and sensory tracts in the posterior hindbrain rhombomeres (r5–8) was normal whereas the location of the cranial nerves and their root projections was abnormal for rhombomeres 1–4 and their central motor nuclei and sensory tracts were compressed in this region. Since loss of anterior hindbrain structures is also a feature of mammalian embryos (Morriss-Kay, 1991) and zebrafish embryos (see below) treated with RA, this suggests that its primary effect in the CNS is on the posterior midbrain–anterior hindbrain region. The loss of forebrain and anterior midbrain in Xenopus may be an additional feature brought about by a separate mechanism.

The highly localised effect of RA on the zebrafish embryo

Our recent studies on the effects of RA on the zebrafish embryo have shown just how precisely RA can act (Holder and Hill, 1991). Fig. 1A shows a whole-mounted zebrafish embryo at 24 h stained with an antibody to the engrailed protein (Patel et al. 1989). This antibody highlights two regions of the embryo – a stripe in the CNS at the midbrain–hindbrain border and a small group of muscle cells in each segment. When embryos are treated at mid-gastra stages with $10^{-7}$ M RA for 30 min the resulting embryo at 24 h is remarkably normal except for the absence of the engrailed stripe at the midbrain–hindbrain border (Fig. 1B). The repeating somitic staining is unaffected and the embryo has an apparently normal forebrain and anterior midbrain. Thus RA has selectively repressed the neural expression of the engrailed gene while having no effect on its mesodermal expression. The only additional effect of RA, as mentioned above, is to compress the anterior hindbrain rhombomeres.

Endogenous RA in the CNS

Teratological experiments such as these do not, of necessity, imply a role for endogenous RA in the development of the CNS. What is needed is a direct demonstration of its presence in the CNS and we have sought to provide this. Unfortunately, the minute size of the developing CNS precludes a high pressure liquid chromatography (HPLC) analysis as has been done on the chick limb bud (Thaller and Eichele, 1987). To circumvent these problems we have turned to the CNS of the axolotl, Ambystoma mexicanum, an amphibian which is a continuously growing larva that adds neurons at postembryonic stages, thereby retaining its embryonic characteristics (Holder et al. 1991). This allows us to collect large enough amounts of tissue for HPLC analysis.

Following organic extraction by the same methodology used for the chick limb bud (Thaller and Eichele, 1987), various retinoids could be detected in the spinal cord of the axolotl (Hunter et al. 1991). Fig. 2 is an example of a reverse phase chromatogram showing peaks of all-trans-RA, all-trans-retinol and 13-cis-RA. These were identified by various criteria – coelution with internal radioactive standards, coelution with external cold standards and further chromatography of these peaks using normal phase. The amount of RA in the spinal cord was estimated to be 1–2 pg pg$^{-1}$ DNA, a similar figure to that found in the chick limb bud.

Despite the difficulty of using embryonic CNS for HPLC analyses, there is evidence to suggest that RA is an endogenous component of the chick embryo neural tube. This tissue can synthesise RA and another biologically active analogue, 3,4-didehydroretinoic acid, when incubated in vitro with retinol (Wagner et al. 1990).

RA stimulates neurite outgrowth

What, then, is the function of this RA in the spinal cord? We have tested the role of RA in one aspect of neural development, neurite outgrowth, using explants of the axolotl spinal cord (Hunter et al. 1991). Small pieces (2 mm$^{3}$) of spinal cord from 4–8 cm axolotls were
various retinoids in the spinal cord.

Fig. 2. High pressure liquid chromatography (HPLC) reverse-phase chromatogram of a methyl acetate/ethyl acetate extract of axolotl spinal cords. —, absorbance at 350 nm. ----, elution profile of a \(^{3}H\)RA internal standard determined by scintillation counting. Arrows mark the elution positions of: 1, all-trans-RA; 2, all-trans-retinol; 3, 13-cis-RA. These coincidences confirm the presence of various retinoids in the spinal cord.

explanted into a semi-solid collagen gel environment adjacent to a piece of nitrocellulose paper which had been previously soaked in various concentrations of RA. Control cultures, with the nitrocellulose paper soaked in ethanol, showed no neurite outgrowth or migration of cells from the explant. In the presence of RA, however, neurite outgrowth was promoted (Fig. 3A, B), their identification as neurites being confirmed by anti-neurofilament immunostaining. When the effect of concentration was investigated, a clear threshold was apparent at a soaking concentration of 0.1 jUM (Fig. 3C) above which the length of neurites that grew out was stimulated 4- to 5-fold.

Interestingly, when similar cultures were exposed to retinol, the majority of cultures responded not by producing neurites but by showing extensive cell migration. These cells were immunoreactive with antibodies characteristic of glial cells.

Thus retinol and RA seem to induce activity in different cell types. Retinol acts primarily on glial cells whereas RA acts primarily on neurons, a specificity reflected by the expression of the cytoplasmic binding proteins for each of these retinoids, as we will now describe.

Complementary distribution of CRBP and CRABP in glial cells and neurons

Cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) are cytoplasmic proteins of approximately \(15 \times 10^3 M\), which specifically bind retinol and retinoic acid respectively. They are found in almost all organs of the adult rat at varying concentrations (Ong et al. 1982), but in the embryo show a highly specific distribution, particularly in the CNS.

The other class of protein inside cells with which RA (but not retinol) interacts is the retinoic acid receptors (RARs and RXRs), which are located in the nucleus. There are three different RARs, RAR-alpha, RAR-beta and RAR-gamma (Ruberte et al. 1991a) and one RXR (Mangelsdorf et al. 1990) and these are the components by which RA causes changes in the pattern of gene activity. The RARs are also found with interesting distributions in the developing CNS (Ruberte et al. 1991b and see later), as is RXR (Rowe et al. 1991), providing further evidence for a role for RA, but we will concern ourselves here only with the cytoplasmic binding proteins.

We have investigated the distribution of CRBP and CRABP in the spinal cord of the axolotl, being the system on which RA was shown to stimulate neurite outgrowth. CRBP localises to an extremely precise region of the cord, the ventral floor plate (Fig. 4A) which consists of radial glia. CRABP, on the other hand is found in the axons of the white matter rather than the glial cells of the grey matter (Fig. 4B). CRABP was also evident in the commissural axons running through the ventral floor plate. The specificity of these anti-peptide antibodies in axolotl tissue was confirmed on immuno-bLOTS (Hunter et al. 1991), both recognising different proteins of approximately \(15 \times 10^3 M\).

We have previously described a virtually identical distribution of CRBP and CRABP in the developing chick spinal cord (Maden et al. 1989a,b). CRBP is localised to the ventral floor plate glia (Fig. 4C). CRABP is found in individual neuroepithelial cells in the mantle layer of the neural tube (Fig. 4D) which subsequently become commissural neurons in the spinal cord (Fig. 4E). The presence of CRABP is first detected very early in the development of the CNS, a few hours after neural tube closure, in single neuroepithelial cells at the rostrocaudal level of somite 1–3, that is, in the future hindbrain region. The induction of CRABP protein in single neuroepithelial cells spreads caudally as the neural tube develops, remaining at a level 6–8 somites behind the last formed somite (Maden et al. 1991). Later in development, these CRABP positive neurons send axons ventrally towards the floor plate and, as in the axolotl, become commissurals.

This distribution of CRBP in the floor plate and CRABP in the commissural neurons takes on a greater significance when we consider what the role of the floor plate is in neural development. It is known, largely from the work of Jessell and colleagues, that the floor plate produces a chemoattractant which attracts the axons of the commissurals towards it (Dodd and Jessell, 1988; Tessier-Lavigne et al. 1988; Placzek et al. 1990). The axons then make abrupt right-angled turns to project in the rostrocaudal axis along the lateral surface of the floor plate. The nature of the chemoattractant is currently unknown and one obvious candidate in the light of the binding protein distribution is RA.

We can, therefore, propose the following scheme (Fig. 5): since the floor plate is rich in CRBP it could
take up retinol from adjacent blood vessels, metabolise it to RA and release it into the surrounding neuroepithelium. RA will thus diffuse dorsally within the neuroepithelium and establish a gradient. Since commissural fibres possess CRABP, only they are able to respond to the gradient of RA and they do so by growing up the gradient and thus arrive at the floor plate.

Three pieces of evidence support this idea. Firstly, as described above RA does indeed induce axon outgrowth (Fig. 3A). Secondly, experiments by Wagner et al. (1990) have shown that grafts of chick floor plate...

Fig. 3. RA stimulates neurite outgrowth in the explanted axolotl spinal cord. (A) Phase contrast photograph of a RA treated explant (ex) after 18 h. Neurite bundles (white arrows) are seen emanating from the explant. The nitrocellulose paper (nc) had been soaked in a 3 μg ml⁻¹ solution of RA. Many individual cells are also seen leaving the explant. Bar=0.5 mm. (B) Phase contrast photograph of an explant (ex) treated with retinol. In contrast to RA, retinol does not stimulate neurite outgrowth, only cells can be seen migrating from the explant. Bar=0.5 mm. (C) Summary of the results showing the stimulation of neurite outgrowth (the maximum length of neurites in each culture) measured in mm at each concentration in which nitrocellulose paper was soaked. Each point represents data from 7-27 cultures. A threshold concentration of 0.1 μM is apparent above which a 4- to 5-fold stimulation of neurite length occurs.

Fig. 4. (A) Transverse section through the rostral neural tube-caudal hindbrain region of a 12 mm axolotl larva stained with an anti-CRBP antibody. The only immunoreactive area is the radial glia of the ventral floor plate (arrowhead). Note that the same region stains in the chick spinal cord (Fig. 4C). Bar=50 μm. (B) Transverse section through the same neural tube as in A, but stained with an anti-CRABP antibody. Here the axons on the periphery of the cord stain (arrows). Bar=50 μm. (C) Transverse section through the neural tube of a stage 24 chick embryo stained with an anti-CRBP antibody to reveal immunoreactivity only in the radial glia of the ventral floor plate (arrowhead) as in the axolotl in Fig. 4A. Bar=50 μm. (D) Transverse section through a stage 16 chick neural tube stained with an anti-CRABP antibody. CRABP is present in individual neuroepithelial cells in the mantle layer of the tube. Bar, 50 μm. (E) Transverse section through a stage 22 chick neural tube to show the later distribution of CRABP which now is localised to the commissural neurons. Bar=100 μm.
Fig. 1. (A) Lateral view of a zebrafish embryo at 24 h post-fertilisation wholemount stained with an antibody to the engrailed protein (4D9) which reveals a band of cells at the midbrain–hindbrain junction (arrowhead). Also stained are groups of muscle cells in each myotome (arrows). The cerebellar fold is located at the arrowhead. (B) Lateral view of a 24 h embryo treated with $10^{-7}$ M RA at 50% epiboly similarly stained with the engrailed antibody. The cranial band of staining is absent whereas the segmental myotomal cell groups are still present (arrows). Bar=75 $\mu$m.
Fig. 6. Mouse (A, C, D) and chick (B) embryos stained with an antibody to CRABP. (A) Horizontal section through a day 9 embryo showing the rhombomeres of the hindbrain numbered 1–7. R2 is lightly immunoreactive whereas r4–6 are strongly immunoreactive and r1, 3 and 7 show very little immunoreactivity. Also seen in this section are branchial arches 1–3 marked a1, a2 and a3. The neural crest derived mesenchyme in these arches is also lightly immunoreactive (arch 1) or strongly immunoreactive (arches 2 and 3). Bar=100 µm. (B) Sagittal section through the developing somites of a stage 19 chick embryo showing CRABP positive cells in the anterior half (A) of each somite, but not the posterior half (P). These cells are thus likely to be neural crest. Bar=25 µm. (C) Similar section to B in a day 9 mouse embryo showing the same phenomenon of CRABP positive cells in the anterior halves of the somites. Bar=50 µm. (D) Sagittal section through a day 9 mouse embryo showing CRABP positive neural crest cells streaming ventrally from the neuroepithelium at the top of the section (arrowheads) into the second branchial arch. a2 marks the position of the 2nd arch although it is not in this section. a1 shows the first arch. Bar=50 µm.
region inserted into the anterior margin of the chick hindbrain result in limb duplications exactly as one would obtain after grafting RA soaked beads. Other areas of the neural tube do not show this activity and thus in this biological assay system for RA, the floor plate does seem to contain significant levels of this molecule. Thirdly, a gradient of a radiolabelled RA analogue has been shown to be established after the implantation of a local source into the chick limb bud (Eichele and Thaller, 1987). Thus gradients of RA can be generated within small areas of embryonic tissue. Of course, there is still much that remains untested in this hypothesis. For example, we need to directly demonstrate that only axons that contain CRABP can respond to RA and we do not yet know whether RA is a chemoattractant or a neurotrophic molecule.

**CRABP in the rhombomeres**

Another indication of the involvement of RA in CNS development comes from studies of the distribution of the RARs and CRABP in the rhombomeres. Rhombomeres (r) are periodic swellings present in the hindbrain of vertebrates and are considered to be the visible manifestation of the segmental nature of the CNS in this location (reviewed by Lumsden, 1990). This concept is supported by the observations that there is a segmental pattern of cell division, cells of adjoining rhombomeres do not mix, boundaries are recreated after grafting certain rhombomeres to abnormal positions (Guthrie and Lumsden, 1991) and that the morphological pattern corresponds with a segmental pattern of cellular and molecular differentiation. A remarkable pattern of molecular differentiation is expressed by the vertebrate homologues of genes involved in the establishment of segment phenotype in *Drosophila*, the Antennapedia-like homeobox genes, and another gene encoding the zinc finger protein, Krox-20 (reviewed by Wilkinson and Krumlauf, 1990). In mouse embryos Krox-20 is expressed on r3 and r5 and the boundaries of expression of the Hox-2 genes occur at two segment intervals. Furthermore, there is a colinearity between the chromosomal location of these genes and their domains of action – increasingly 3' genes are expressed in progressively more rostral regions of the hindbrain (Graham et al. 1989).

Both the RARs and CRABP are also expressed in precise domains within the rhombomeres. In the mouse embryo RAR-alpha is expressed in the neural tube and has a rostral limit of expression at the r3/4 boundary and RAR-beta has a rostral limit of expression at the r6/7 boundary (Ruberte et al. 1991b). In the chick, RAR-beta has a rostral expression boundary at r5/6 (Smith and Eichele, 1991).

We have recently studied the appearance of CRABP protein in the hindbrain of the chick embryo (Maden et al. 1991). Immunoreactive neuroepithelial cells first appeared in the hindbrain region soon after neural tube closure (at stage 9) and when the rhombomeres were recognisable at stage 11, these positive cells were located in r4 and r6 and continued into the anterior spinal cord. A dynamic pattern of CRABP immunoreactivity is apparent over the next few stages of development: while r4 and caudal to r6 remain labelled, positive cells appear in the anterior mesencephalon, then in r2 and r1 and finally immunoreactivity appears in the axons of the mantle layer throughout the hindbrain.

In the mouse we have observed a surprisingly similar distribution of CRABP (Horton, Maden and Eriksson, unpublished data). Immunoreactivity first appears as a single stripe throughout the full thickness of the neuroepithelium in the presumptive hindbrain region of a day 8.5 embryo, before neural tube closure. One day later CRABP is present in two stripes within the hindbrain – it is absent from r1, present at a low level in r2, absent from r3, present at high levels in r4, 5 and 6 and then fades out in r7 (Fig. 6A).

What do these patterns of CRABP expression mean and what is their relevance to the hindbrain? Firstly, it is possible that the distribution of CRABP reflects the distribution of RA in the hindbrain, because RA is the endogenous ligand for CRABP (Saari et al. 1982). In support of this, when a radiolabelled RA analogue was injected into pregnant mice the label became localised in the embryo in precisely those regions where CRABP was located (Dencker et al. 1990). Secondly, the striking patterns of CRABP expression and of Hox-2 expression may be related. Indeed *Hox*-2.9 expression in the chick both at the RNA level (Maden et al. 1991) and at the protein level (Sundin and Eichele, 1990) shows a very similar pattern of expression to CRABP at early stages. Furthermore RA activates the expression of the *Hox*-2 cluster in a precise temporal and concentration-dependent sequence which is related to the chromo-
somal location of these genes (Simeone et al. 1990, 1991; Papalopulu et al. 1990). It is thus likely that endogenous RA in the rhombomeres is playing a role in either generating the pattern of rhombomeres, that is, generating boundaries, or establishing segment identity in the rhombomeres once they have formed.

CRABP in the neural crest

A final aspect of RA in the nervous system worthy of mention is the detection of CRABP in the neural crest. This is clearly seen in chick (Maden et al. 1991), mouse (Maden et al. 1990) and rat embryos (unpublished data). CRABP may serve as a useful marker for neural crest cells in the mouse where other markers such as HNK-1 cannot be used. For example, one of the most characteristic features of the neural crest is that cell migration is restricted to the rostral half of each somite (Rickmann et al. 1985). This same feature can be seen in CRABP positive cells in the somites (Fig. 6B, C) suggesting that they are neural crest cells. Furthermore, streams of cells can be seen migrating out from the neuroepithelium, the ends of the streams being located in the branchial arches (Fig. 6D). These groups of CRABP-positive cells in the arches can also be seen in Fig. 6A where the weakly stained r2 gives rise to weakly stained mesenchyme in arch 1 and intensely stained r4 and 6 give rise to intensely stained mesenchyme in arches 3 and 4. Apart from the mesenchyme of the branchial arches, other neural crest derivatives are also CRABP positive such as the sympathetic ganglia, dorsal root ganglia, Schwann cells and sensory axons.

These observations clearly imply a role for RA in the normal function of the neural crest. Too little vitamin A in the maternal diet of mammals causes teratological effects on the neural crest and its derivatives (see above). Too much vitamin A, or RA in particular, does so as well and a direct effect on the migration of neural crest cells both in vivo (Thorogood et al. 1982) and in vitro (Webster et al. 1986; Pratt et al. 1987) has been observed. As described above, teratological doses of RA will result in RA localising to regions where CRABP is present (Dencker et al. 1990), one of those being the crest. Thus it is possible that because crest contains high levels of CRABP, exogenously administered RA will localise there, alter the pattern of gene expression (via the RARs) and result in an inhibition of migration.

Conclusions

The early development of the nervous system consists of several phases, two of which, neuronal specification and neurite outgrowth, appear to involve RA.

RA administration to the early embryo has striking effects on very precise parts of the CNS resulting in deletions and loss of segmentation of the rostral hindbrain. No precise respecification of the CNS has been demonstrated, despite earlier suggestions (Durston et al. 1989), but alterations in gene activity have been observed.

We have also demonstrated that neurite outgrowth is stimulated by RA and on the basis of this and the observations of CRBP and CRABP localisation we have developed a specific model for the role of RA in the directed outgrowth of commissural axons (Fig. 5). In embryonal carcinoma cells it has been known for several years that RA induces their differentiation, resulting in neurite outgrowth (Jones-Villeneuve et al. 1982), although it is not clear whether this is a secondary effect resulting from differentiation or a direct effect on neurite outgrowth. Both our results in vivo and more recent studies on the induction of one of the neurofilament proteins, MAP2 (Dinsmore and Solomon, 1991), suggest that RA has a direct effect on neurite outgrowth via the induction of specific gene activity.

At a later stage in neuronal development RA can also alter the neurotransmitter phenotype of embryonic rat spinal cord (Wuarin and Sidell, 1991) thus this vitamin A metabolite seems to be of major importance in many aspects of CNS development.

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