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Early Neural Specification by the Planar Signal in *Xenopus laevis* Development

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ABSTRACT—Neural differentiation in amphibian embryos had been thought to start after gastrulation because of vertical neural induction from the underlying dorsal involuting marginal zone (DIMZ). However, recent studies show that another mode of induction, namely the planar induction, also directs neural differentiation in *Xenopus laevis*. Here, we attempted to specify when the planar induction occurs. From middle blastula to early gastrula stages, explants that included animal cap (AC) and dorsal noninvoluting marginal zone (DNIMZ), but not DIMZ were prepared. Neural differentiation was detected in such explants (named AC-DNIMZ explants) from the early gastrula (St. 10+) but not from the late blastula (St. 9) or earlier embryos, indicating that the planar induction occurs at about St. 10+. To assess the further effect of planar induction on neural differentiation, Keller sandwiches were prepared from St. 10+ embryos, and cultured for 2~6 hr *in vitro*, after which the explants were separated into AC-DNIMZ and DIMZ parts. AC-DNIMZ part differentiated a little larger neural tissues, suggesting that the planar neural induction during the gastrula stages promote neural differentiation. Neural tissues in these explants could be detected by histology and immunohistochemistry using a monoclonal antibody specific for neural tissues, as opposed to earlier studies that indicated the planar induction to be insufficent for inducing morphological neural structures.

INTRODUCTION

Neural induction in amphibian development has been vigorously studied for years since the era of Hans Spemann [7]. In *Xenopus laevis*, many studies have suggested that the first step of neural differentiation is directed by at least two inductive signals, namely the vertical and the planar inductions. Planar induction can induce the neural ectoderm to express not only pan-neural genes like NCAM, but also position-specific genes such as *XIF-3, En-2, Krox-20, XIHboxes 1* and *6, Xdll-3* and *Xotx2* in correct antero-posterior order under experimental conditions to exclude vertical induction, such as in Keller explants [3–6, 17], or in exogastrulae [18]. Several experiments, however, have failed to detect planar induction [19, 23]. Xlim-1 protein was not detected in the ectodermal part of the Keller sandwich [20]. Furthermore, ectoderm induced by planar induction expresses many neural genes as mentioned above, but did not show histological differentiation [4, 17, 18]. Therefore, the importance of planar induction in amphibian neurogenesis has not been clearly settled.

There have been a few attempts to specify when the planar induction occurs. For instance, neural ectoderm loses an inhibitory effect on muscle differentiation of the lateral marginal zone by the late gastrula stage of St. 13, apparently due to planar induction [11]. The presumptive neural cells of the gastrula embryos become progressively nonepidermal between Stages 10.5 and 12 [1, 22]. More directly, the ectodermal part isolated from the Keller sandwich at later than the middle gastrula stage of St. 11 manifested neural-type convergence and extension morphogenesis, suggesting that the planar induction have occurred at this stage [21]. The present work was planned to obtain more information on when the presumptive neural cells get the neural differentiation potency. The presumptive neural cells were excised from the middle, late blastula or early gastrula embryos, and were cultured alone. Neural differentiation of the explant was observed histologically and immunohistochemically. We observed serial sections of the explants carefully and excluded cases when any notochordal, muscular or other mesodermal fragments were found. The results indicate that the neural differentiation potency emerges at the very early gastrula stage of St. 10+, and that the potency is strengthened by the planar signal during the gastrula stages.

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MATERIALS AND METHODS

Embryos

Pairs of *Xenopus laevis* were injected with 600 IU of human chorionic gonadotropin (hCG). Fertilized eggs were dejellied with 4% cysteine in Steinberg's solution (pH 7.8) and cultured in agar-coated dish containing sterile modified Danilchik's medium (MDM; 13, 24), supplemented with 5 mM Hepes and 100 $\mu g/l$ kanamycin sulfate until desired stages at 20°C. Staging of embryos was according to Nieuwkoop and Faber [16], and more precisely on gastrula embryos, according to Keller [12].

Dissection

(a) AC-DNIMZ sandwich: From St. 8 to 10+ embryos, animal cap-dorsal noninvoluting marginal zonal (AC-DNIMZ) sandwiches

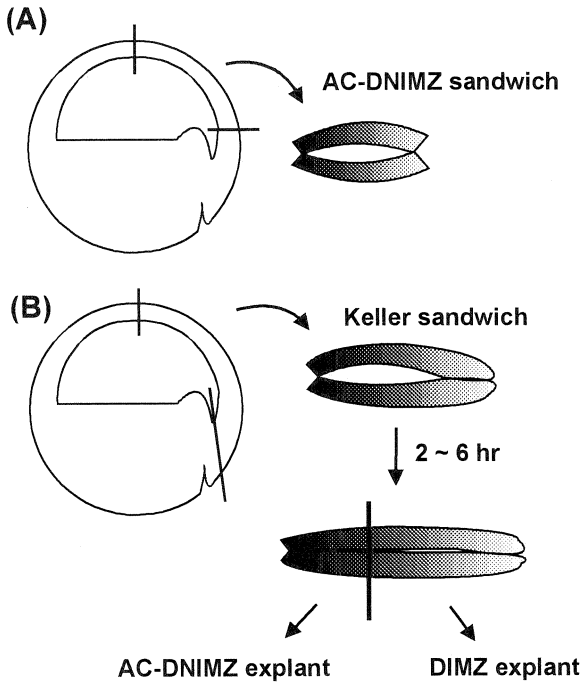

FIG. 1. Illustration of the isolation of explants. (A) AC-DNIMZ part was isolated and sandwiched from embryos of Stage 8, 9 and 10+. (B) Keller sandwich was made from Stage 10+ embryos, and was cultured for 2~6 hr in an agar-coated dish, after which the AC-DNIMZ part was isolated and further cultured for 3 days (see also Fig. 2).

were made (Fig. 1A). Dorsal animal region, from the animal pole to the dorsal equator was excised with a tungsten needle. The explant was rectangular and its size from St. 10+ embryo was 0.57±0.09 mm long in animal-vegetal direction, and 0.79±0.11 mm wide, corresponding to 72.6±9.8 degrees wide (mean±SD of 5 examples), as measured on a TV monitor of the image under the binocular microscope. Dorso-ventral polarity of the embryo was judged by the pigmentation pattern of the animal hemisphere in wild type embryos. When using albino embryos, dorsal region was marked by powder of nile blue sulfate at the 4- to 8-cell stage. Two such explants were sandwiched with the blastocoelic surface contacting inside and each edge matching. About 30 min later when the sandwich became healed, explants were transferred to a new agar-coated dish containing MDM, and cultured for three days at 20°C.

(b) Keller sandwich: From St. 10+ embryos, Keller sandwiches were made (Fig. 1B). Embryos were cut at the animal pole and the dorsal blastoporal lip. The size of a rectangular Keller explant was 1.08 mm±0.17 mm long in animal-vegetal direction and 0.79±0.13 mm wide, corresponding to 72.7±11.8 degrees wide (mean±SD of 27 examples). Two such Keller explants were sandwiched and cultured in the agar-coated dish. The sandwich was covered with a coverglass to promote convergent extension. Vegetal part of the explants started convergent extension soon after the formation of the sandwich. After 2~3 hr, or 5~6 hr, the sandwich was bisected into AC-DNIMZ part and DIMZ part, at the interface between the narrow-and-thick vegetal part and the wide-and-flat animal part (Figs. 1B and 2). The AC-DNIMZ part was further cultured in MDM at 20°C for 3 days.

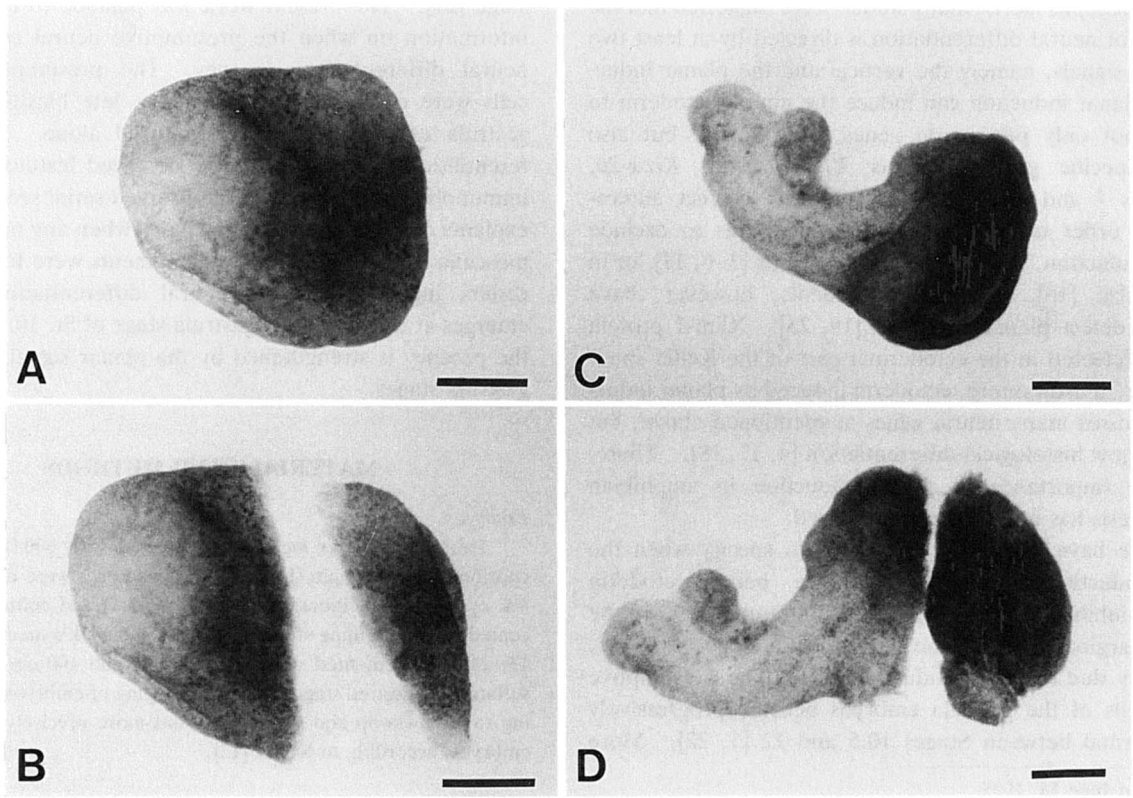

FIG. 2. Cutting of the Keller explants. Keller sandwich was prepared from St. 10+ embryos and was cultured for 2~3 hr (A), or 5~6 hr (C). DIMZ part is seen on the left, and darker AC-DNIMZ part is on the right. The sandwiches were separated into AC-DNINZ part and DIMZ part, respectively (B and D). Bar=250 μm.

Histological procedures

Explants were fixed in Bouin's fluid for 2 hr and embedded in Histoprep (Wako chemicals, Japan), serially sectioned at 5 μm, and stained with hematoxylin and eosin. For visualization of neural differentiation in the whole-mount sample, explants were made from albino embryos, and were fixed in methanol and stored at -20°C. Stored explants were stained with NEU-1 monoclonal antibody [9] as described [8] except that all blocking and antibody solutions contained 2% skim milk.

RESULTS

Culture and cutting of the Keller explants

When a sign of invagination first appeared on the straight blastopore pigment line in the dorsal vegetal part of the embryo (St. 10+), Keller explants were prepared as shown in Figure 1. These explants did not survive and differentiate well in Steinberg's solution when cultured in isolation for 3 days. Instead, MDM was employed for the culture medium that turned out far better for Keller explants. On the other hand, MDM was not suited for whole embryos. Embryos cultured in MDM often became incomplete exogastrulae, and/or the neural plates did not close completely. Therefore, whole embryos were cultured in Steinberg's solution to monitor normal time table of development.

Keller explants, whether isolated alone as an open-face explant, or combined as a sandwich explant, went to morphogenesis in a few hours (Fig. 2A, C). The animal side including AC and DNIMZ, became flat and extended in lateral directions. On the other band, the vegetal side composed of DIMZ, converged laterally, and extended in animal-vegetal directions. Thus, convergent extension morphogenesis characteristic for the two respective regions occurred, as described previously [14, 15]. Sandwich explants survived and differentiated tissues better than open-face explants. Therefore, sandwich explants were used in subsequent experiments. Sandwich explants were cultured in MDM for 2~3 hr until control embryos reached the midgastrula stage (St. 11.5), or for 5~6 hr until control embryos reached the late gastrula stage (St. 12.5). The explants were then cut at various positions horizontally to isolate the AC-DNIMZ part. The AC-DNIMZ part was further cultured for 3 days, and was observed histologically.

When cut on the limit of pigmented area, most of the AC-DNIMZ explants differentiated not only ectodermal tissues like epidermis and neural tissue but also various mesodermal tissues such as notochord, muscle, mesenchyme, coelomic epithelium and blood cells. These explants must have included DIMZ or lateral marginal zone (LMZ). Such explants were classified as type B, which means that the explants were composed of mixtures of ectodermal and mesodermal tissues. The explants did not necessarily keep their planar conformations throughout the culture period. Therefore, vertical signaling from the mesodermal cells to the ectoderm might occur in the type B explants. When the explant was cut at 1/3~1/4 of the length from the edge of the animal side, many AC-DNIMZ explants did not contain a trace of mesodermal tissues. These explants were classified as type A. Type A explants also included explants that contained only atypical epidermis and cement gland. In these cases, it is possible that the AC-DNIMZ part missed

TABLE 1. Differentiation of the AC-DNIMZ explant

Stage	AC-DNIMZ part isolated from embryo at			AC-DNIMZ part separated from Keller sandwich at	
	St. 8	St. 9	St. 10+	St. 11.5[a]	St. 12.5[b]
Type A explant (n=)	20	35	28	36	39
Neural tissue not specified[c]	0	0	10 (36%)	10 (28%)	13 (33%)
Neural tissue w/ melanophore[d,e]	0	0	3 (11%)	3 (8%)	5 (13%)
Neural tissue w/ brain character[d,f]	0	0	3 (11%)	4 (11%)	8 (21%)
Neural tissue total	0	0	13 (46%)	13 (36%)	18 (46%)
Diameter of neural tissue (μm)[g]	0	0	115±27	139±58	190±66
No neural tissue	20 (100%)	35 (100%)	15 (54%)	19 (53%)	21 (54%)
Type B explant (n=)	23	18	40	25	22
Neural tissue w/ mesoderm	15 (65%)	11 (61%)	20 (50%)	16 (64%)	19 (86%)
Epidermis w/ mesoderm	8 (35%)	7 (39%)	20 (50%)	9 (36%)	3 (14%)

[a,b] Corresponding stages of control embryos to 2~3 hr and 5~6 hr after St. 10+, respectively. [c] Neural tissue without the spinal cord- or brain-like morphology. [d] "w/" means "with". [e] Melanophores usually accompanied a few number of mesenchymal cells. [f] neural tissue with retinal pigment epithelium was classified in this group. [g] mean±standard deviation.

Note that all explants contained atypical epidermis and cement gland. These tissues were omitted in this table.

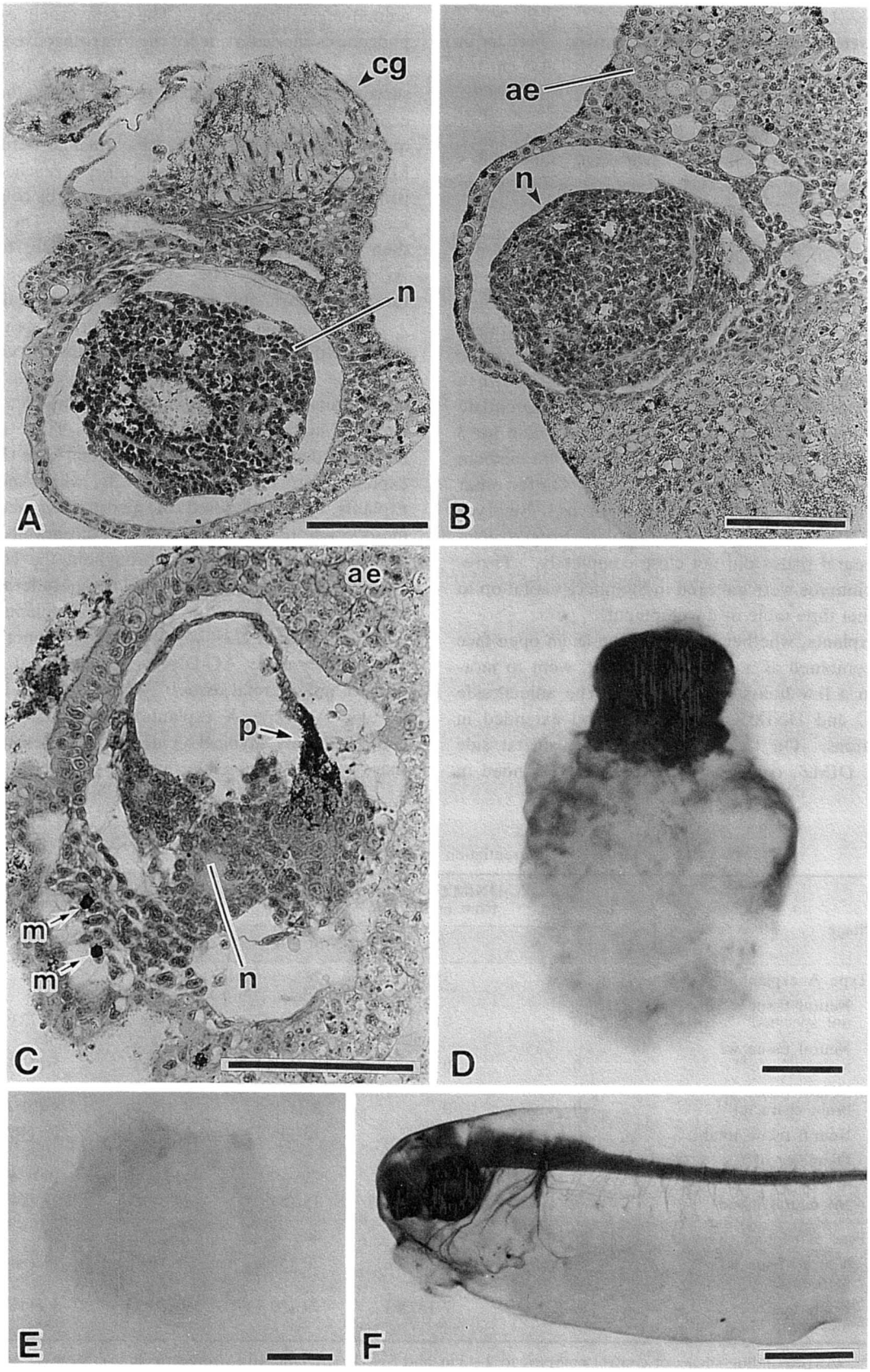

FIG. 3. Photographs showing the neural differentiation of AC-DNIMZ explant isolated at 5~6 hr (A), 2~3 hr (B) after St. 10+, and at St. 10 + (C). (D) An AC-DNIMZ explant isolated at 2~3 hr after St. 10+ was immunostained with NEU-1 monoclonal antibody. (E) An AC-DNIMZ explant isolated at St. 9 was not stained with NEU-1. This photograph is slightly overdeveloped to show the contour of the explant. (F) Control tadpole at stage 39 stained with NEU-1. Bar=100 μm in (A) to (D), 200 μm in (E), and 500 μm in (F). ae: a typical epidermis; cg: cement gland; n: neural tissue; p: retinal pigment epithelium; m: melanophore.

including sufficient amount of the presumptive neural region, since pure animal cap always generated these epidermal derivatives (data not shown). However, it is also possible that the explant had included the presumptive neural region, which, however, did not differentiate into histological neural structures. These two possibilities were indistinguishable in the present study. The position of the cutting was shown in Figures 2B and D.

Results of the histological observation of the AC-DNIMZ explants were shown in Table 1. In the type B explants, massive neural tissues were always observed in notochord-containing explants, irrespective of when the AC-DNIMZ part was isolated (n=5 for St. 10+ 5~6 hr explants, and n=6 for St. 10+2~3 hr explants). All these explants contained brain-like large neural tissues accompanied with retina as identified by the presence of pigment-rich neural epithelium near by. Melanophores and mesenchymal cells were also observed in every case. In explants that contained muscle and/or ventral mesodermal tissues, differentiation of the neural tissue was very variable from explant to explant. Some explants did not contain neural tissue, and others had big neural tissues with many melanophores and well-developed mesenchymal cells.

In type A explants, neural tissues were observed in 40% to 50% of the explants. Histologically, these neural tissues were characterized by round aggregates of tightly packed cells with narrow ellipsoid nuclei that were darkly stained with hematoxylin (Figs. 3A and B). Often, these aggregates contained neural fiber-like structure well stained by eosin. However, most of these neural tissues were not organized like a brain or a spinal cord. Melanophores and a few number of accompanying mesenchymal cells were observed in a few cases in explants isolated both at 2~3 hr and 5~6 hr after St. 10+ (Table 1). Also, a sheet of epithelial cells with abundant melanin granules characteristic of the retinal pigment epithelium was observed in these explants at a low ratio (Table 1), suggesting that some induced neural structure had brain-like character.

Appearance of potency of neural differentiation

From the results of the previous section, it was indicated that histologically identifiable neural differentiation occurred independently of mesodermal cells in AC-DNIMZ explants isolated at 2~3 hr after St. 10+. To assess when the neural differentiation potency emerges, we next made AC-DNIMZ sandwich from St. 10+ or earlier embryos and cultured them for 3 days. Explants were classified as type A or B, similarly with the previous section. Results of the histological examination were shown in Table 1.

All type A explants of AC-DNIMZ sandwiches from blastulae of either St. 8 or 9 formed atypical epidermis only (n =20 and 35, respectively). Therefore, it was indicated that neural differentiation in AC-DNIMZ part has not yet been committed at St. 9. In contrast, type A explants of AC-DNIMZ explants from St. 10+ embryos differentiated neural tissues at a frequency of 46% (n=28). They had similar histological features to the AC-DNIMZ part isolated from Keller sandwiches 2~6 hr after St. 10+. Melanophores and the retinal pigment epithelium were also observed in a few cases (Fig. 3C, Table 1). However, the size of the neural tissue was small. The diameter of the neural mass was statistically smaller than those of the AC-DNIMZ part isolated at 2~3 hr or 5~6 hr after St. 10+ ($p<5\%$ by the Student's t test).

Immunohistochemistry using NEU-1

To investigate whether the nervous tissue in AC-DNIMZ sandwich expresses a neural marker or not, whole-mount AC-DNIMZ explant was stained with NEU-1 monoclonal antibody. A patch of cells in an AC-DNIMZ explants isolated 2~3 hr after St. 10+ were stained positively (in 5 out of 5 cases; Fig. 3D) which were devoid of mesodermal tissues (data not shown). In contrast, AC-DNIMZ explants devoid of mesodermal tissues isolated from St. 9 embryos were not stained (in 7 out of 7 cases; Fig. 3E). In the control experiment, this antibody stained most neural tissues, including whole brain, spinal cord and cranial/spinal nerves of the tailbud embryos (Fig. 3F).

DISCUSSION

From the results of the present study, it was indicated that the neural differentiation potency emerges at an early gastrula stage of St. 10+. This is an early phenomenon, considering that many studies on the vertical or planar neural inductions use gastrula ectoderm of St. 10+ embryos as a responding tissue, to investigate inductive events that take place after this stage. NCAM protein expression was studied in the isolated animal cap explants [10]. It was revealed that NCAM protein emerged in the explant that was isolated later than Stage 10.75. In another experiment, morphogenetic movement of AC-DNIMZ part isolated at various stages from the Keller sandwich was studied [21]. The results indicated that neural morphogenetic movement emerges in the explant isolated before St. 11. Thus, it has been indicated that commitment for neural differentiation has begun around Stage 11. This stage is about 2 hr after St. 10 + in the normal developing rate at 20°C. These differences are probably due to the difference in the position of the explants. We excised the AC-DNIMZ as close as possible to DIMZ, and therefore, detected neural differentiation in explants isolated at St. 10+. However, in our experiments, only about half of the explants differentiated neural tissue. We presume that the neural differentiation potency emerges in so narrow area next to the DIMZ, that it was frequently missed in the explant, or was isolated together with the mesodermal cells.

This emergence of neural differentiation potency can be attributed either to some endogenous cytoplasmic factor(s), or to the planar induction from the DIMZ. It is shown that the origin of the dorsal-animal region of the blastula to gastrula ectoderm gradually changes as time passes, due to

the spreading morphogenesis of the animal cap [2]. The dorsal-animal region of the St. 9 embryo is derived mainly from the tire-1 and -2 dorsal blastomeres of 32-cell embryos, whereas the same region of the St. 10 embryo is mainly derived from the tire-1 ventral and tire-1 dorsal blastomeres of 32-cell embryos. In short, cytoplasmic components of the dorsal-animal region of the ectoderm becomes more and more "animal" in nature as development proceeds. Therefore, it is unlikely that the DNIMZ cells taken from the St. 10+ embryo had some special cytoplasmic components for neural differentiation that were not present in the same explant taken from St. 9 or earlier embryos. Then, the neural differentiation of the explant should be attributed to the planar induction. However it should be noted that in a recent literature, it is suggested that vertical signalling is already occurring in the deep blastoporal region in St. 10+ embryos [3].

In the AC-DNIMZ part isolated at 2~6 hr after St. 10+, the size of the neural tissue became bigger than that isolated at St. 10+. This is also apparently due to the planar induction. Neural tissues formed in these explants could be identified histologically. This is contradictory to the previous reports that indicated that planar neural induction does not evoke morphological differentiation [5, 6, 18]. The reason is not very clear, but we experienced that Keller sandwiches did not develop well in Steinberg's solution. The morphological neural differentiation takes place one or two days after the expression of position-specific neural genes. Therefore, it may be that the difference came from the culture conditions or the period.

In the AC-DNIMZ explant, retinal pigment epithelial cells were observed at a low rate. This suggests that the neural tissues in these explants contained brain-like cells. It is not so surprising because many position-specific genes of the hindbrain, midbrain and even the forebrain origin are expressed in the Keller explants and in exogastrulae, as mentioned previously. However, lens and organized retina were not observed in the AC-DNIMZ explant. It is coincident with the previous results that exogastrulae never have eyes [18].

In the present study, it is indicated that the planar signal from the DIMZ has positive effect on the neural differentiation of the AC-DNIMZ. Nevertheless, the volume of neural tissue was much less than the fate map predicted, and the differentiation was incomplete, too. On the other hand, explants that contained even a tiny fragment of notochord had huge neural tissues including retina, pigment epithelium, brain-like and spinal cord-like neural tube and many melanophores. Thus, presumptive notochordal cells have evidently strong neural inducing activity. Therefore, it is concluded that in the normal development, the vertical induction strongly direct neural differentiation during the gastrula stages, before which a small number of cells in the presumptive neural region are first committed to the neural pathway by the planar induction.

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