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Inhibition of Epibranchial Placode-Derived Ganglia in the Developing Rat by Bisdiamine

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ABSTRACT Although bisdiamine has been shown to affect the development of mammals, its effect on the nervous system has gone largely unrecognized. In the present study, rats were given bisdiamine by gavage on days 9 and 10 of pregnancy. They were sacrificed at intervals and the fetuses were prepared for study of serial sections stained with hematoxylin and eosin, or by immunohistochemical reaction with HNK-1 monoclonal antibody. HNK-1 reacted strongly with the nervous system, allowing precise analysis of the components and their relationships. Controls receiving no bisdiamine were prepared and studied in parallel with the experimental fetuses. Administration of bisdiamine inhibited development of the petrosal and nodose ganglia, altered associations of the glossopharyngeal, vagus, and hypoglossal nerves, and inhibited contributions of vagal nerve fibers to the developing enteric system. The proximal ganglia of the glossopharyngeal and vagus nerves developed normally. It is concluded that bisdiamine affects, directly or indirectly, the differentiation of nervous components derived from the epibranchial placodes. It seems likely that these placode-derived components serve as pioneer neurons in establishing the pathway for the posteriorly extending trunks of the glossopharyngeal and vagus nerves. The early changes in congenital conditions such as the DiGeorge syndrome may not be limited to alterations in neural crest derivatives. It may be worthwhile to investigate more closely whether there are alterations in the nervous system associated with these syndromes.

There have been several studies in which bisdiamine has been administered during the early stages of pregnancy (Taleporos et al., 1978; Oster et al., 1983; Chon et al., 1984; Ikeda et al., 1984; Binder, 1985), because this treatment produces a cluster of defects similar to those encountered in congenital conditions in humans, including the DiGeorge syndrome, the fetal alcohol syndrome, and retinoic acid embryopathy (Amman et al., 1982; Lammer et al., 1985). It has been suggested that the defects in experimental animals as well as in the human syndromes are caused by the effect of agents or genetic factors on inhibited contributions by derivatives of the neural crest (Couly et al., 1983; Kirby and Bockman, 1984).

Usually these studies have concentrated on defects in the cardiovascular system, thymus, parathyroids, and facies. The peripheral nervous system has been largely ignored, or defects have not been observed and reported.

The present study, utilizing fetuses of rats given bisdiamine on the 9th and 10th days of gestation, concentrated on the developing nervous system. Profound changes in the peripheral nervous system in the pharyngeal region were discovered. The nodose and petrosal ganglia, derivatives of epibranchial placodes, were affected, as were the extensions of the vagus and glossopharyngeal nerves. Thus, the early vagal contributions to the heart and enteric system were affected. It appears that the effects of bisdiamine in the pharyngeal region are not limited to those on neural crest. Furthermore, the results suggest that the nervous system should be evaluated more carefully in both experimentally induced anomalies and in the human syndromes.

MATERIALS AND METHODS

The Sprague-Dawley rats used in this study were kept in a controlled environment with food and water constantly available. The day sperm was found was taken as day 0 of pregnancy. Bisdiamine (Fertilysin™, Aldrich Chemical Co., Milwaukee, Wisconsin; N,N′-bis-(dichloroacetyl)-1,8-octamethylenediamine; Win 18,446) was administered by gastric tube under ether anesthesia on gestation days 9 and 10. Bisdiamine was suspended in 1% aqueous gum tragacanth. One ml was administered on each of the two days. The dosage for each administration was 100 mg (n = 28) or 50 mg (n = 6). Controls (n = 20) received 1 ml of 1% gum tragacanth on each of the two days.

On gestation day 11, 12, 13, 14, 15, or 20, the animals were anesthetized with ether and the fetuses were re-

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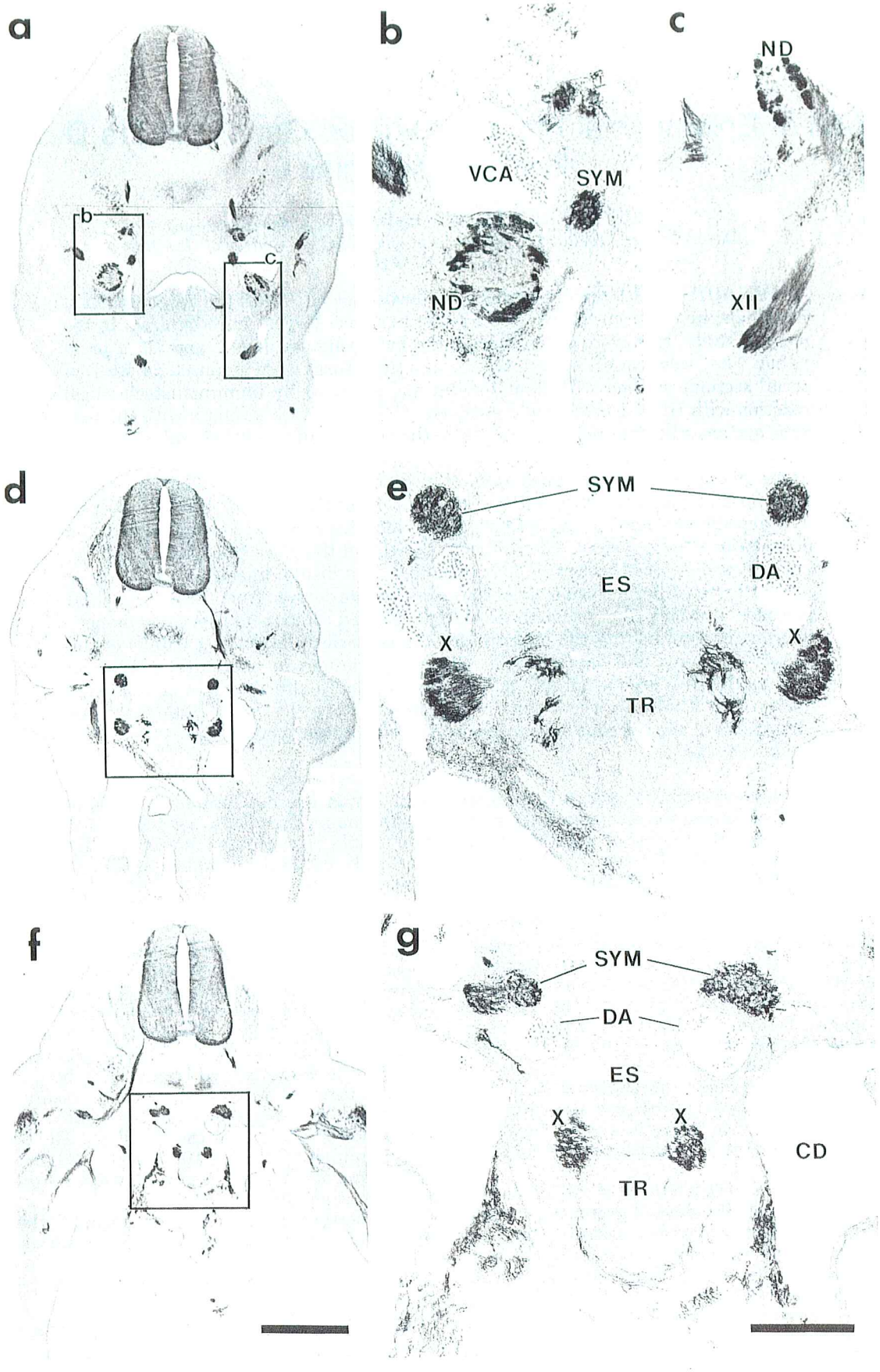

Fig. 1.

moved for study. Fetuses were prepared routinely for study of paraffin sections. Both cross-sections and longitudinal sections were prepared and studied. Those fixed in Bouin's fixative were stained with H&E. Those fixed in Carnoy's fixative were stained with the monoclonal antibody HNK-1 in order to allow precise identification and tracing of neural elements. This antibody recognizes the carbohydrate epitope on a number of cell adhesion molecules (Chou et al., 1986; Shashoua et al., 1986). In the rat fetuses, it reacted with the central nervous system and strongly with the peripheral nervous system.

For HNK-1 immunostaining, the sections were deparaffinized, incubated in 1% aqueous periodic acid for 10 minutes, washed in phosphate-buffered saline containing 0.3% Triton X-100 (PBST), and incubated with HNK-1 diluted 1:20 in PBST containing 0.2% bovine serum albumin (PBST-BSA) for 2 hours at room temperature. After washing in PBST, the sections were incubated with the secondary antibody, goat anti-mouse IgM conjugated with horseradish peroxidase, diluted 1:20 in PBST-BSA, for 30 minutes at room temperature. After washing again in PBST, the peroxidase reaction was performed in a solution made by adding 2 mg of 3,3′-diaminobenzidine and 50 μl of 30% hydrogen peroxide to 500 ml of Tris-HCl buffer. The reaction was allowed to continue at room temperature for 45 minutes. Some of the sections were counterstained with hematoxylin. Sections from 15 fetuses from mothers subjected to the 100 mg dose of bisdiamine were analyzed by HNK-1 antibody; two each on gestation days 11, 12, 14, and 15, and seven on gestation day 13. One control was analyzed by HNK-1 antibody on each of days 11 through 14.

RESULTS

By comparing serial sections of experimental fetuses from mothers receiving bisdiamine with controls from mothers receiving the vehicle only, it was evident that the peripheral nervous system in the pharyngeal region had been altered. Preparations stained with hematoxylin and eosin revealed an absence of certain ganglia and an alteration of nerves in the region. Those reacted with HNK-1, however, most clearly revealed not only the missing ganglia and altered nerves, but also the absence of fine branches that normally distributed to developing organs in controls. The alterations observed through the developmental stages studied were consistent with an early inhibition that was maintained. There was no indication that development was temporarily delayed, then restored.

The normal arrangement and relationships of the nervous system in the pharyngeal region, as revealed by HNK-1, may be seen in Figure 1, which is from a control embryo at day 13 of gestation. Representative sections from three different levels demonstrate how the vagus nerves and sympathetic trunks are arranged. Figure 1a–c shows the size and location of the nodose ganglia of the vagus nerves. As the vagus nerves proceed posteriorly, they assume a position immediately ventral to the dorsal aortae (Fig. 1d,e), then come to lie in the lateral interval between the esophagus and the trachea (Fig. 1f,g). The sympathetic trunks are at first dorsomedial to the nodose ganglia (Fig. 1a,b), then assume and maintain through the region a position immediately dorsal to the dorsal aortae (Fig. 1d–g). The location and size of the hypoglossal nerve is evident in Figure 1a,c.

Nodose (distal X) ganglia were missing in experimental fetuses. In one case, there was a ganglion-like nodule on one side of the pharynx, but it did not have any connection with cervical sinus epithelium as was characteristic of control nodose ganglia at this stage of development. The petrosal (distal IX) ganglia were variable. They were present in less than half of the fetuses examined.

Figure 2 illustrates the lack of ganglia in an experimental embryo of 13 days gestation. The sections were selected to be comparable with the control embryo in Figure 1. The alterations of nerve trunks caused by the administration of bisdiamine also are evident. The sympathetic trunks were present bilaterally, and seemed comparable to controls except for being somewhat smaller in some of the more anterior sections. The vagus nerve, on the other hand, was missing in the pharyngeal region. The vagus nerve was absent from the interval between trachea and esophagus (Fig. 2c–f). Some small fibers were observed around the trachea (Fig. 2f).

The glossopharyngeal nerve did not pass through the pharyngeal region as a separate entity. The proximal ganglia of the vagus nerve and the glossopharyngeal nerve were normally developed in experimental fetuses (Fig. 3a,b). Fibers from the proximal ganglia, and from the petrosal ganglia when present, passed ventrally and joined with the hypoglossal nerve to form a compound nerve trunk (Fig. 2a,b; Fig. 3a,c) that passed into the primordium of the tongue.

More posteriorly, the vagus nerve normally arborizes to supply the developing enteric structures (Fig. 4a). After administration of bisdiamine, these arborizations were not present (Fig. 4b), indicating a situation that would be expected to lead to a defective enteric nervous system.

Inhibition of nervous elements by bisdiamine seemed to involve predominantly postotic structures. Graphic reconstruction of an HNK-1-stained specimen (Fig. 5) revealed that the trigeminal, facial, and acoustic nerves and ganglia developed normally, as did the proximal ganglia of the glossopharyngeal and vagus nerves. No differences were found in the dorsal root ganglia.

Fig. 1. Transverse sections of a gestation day 13 control embryo immunohistochemically stained with HNK-1. **a:** Section at the level of the nodose ganglion. **b:** Enlargement of the left box in a. The nodose ganglion (ND) is an accumulation of cells and HNK-1$^+$ neurites. SYM, sympathetic trunk; VCA, anterior cardinal vein. **c:** Enlargement of the right box in a. The hypoglossal nerve (XII) lies ventral to the nodose ganglion (ND). **d:** Section at the level of the truncus arteriosus. **e:** Enlargement of the box in d. Bilateral sympathetic trunks (SYM) and vagus nerves (X), occupying opposite sides of the dorsal aortae (DA), are HNK-1$^+$. ES, esophagus; TR, trachea. **f:** Section at the level of the sinus venosus. **g:** Enlargement of the box in f. The sympathetic trunks (SYM) have maintained their positions relative to the dorsal aortae (DA). The vagus nerves (X) lie in the interval between esophagus (ES) and trachea (TR). CD, duct of Cuvier. Bar = 0.5 mm for a, d, and f; 0.2 mm for b, c, e, and g.

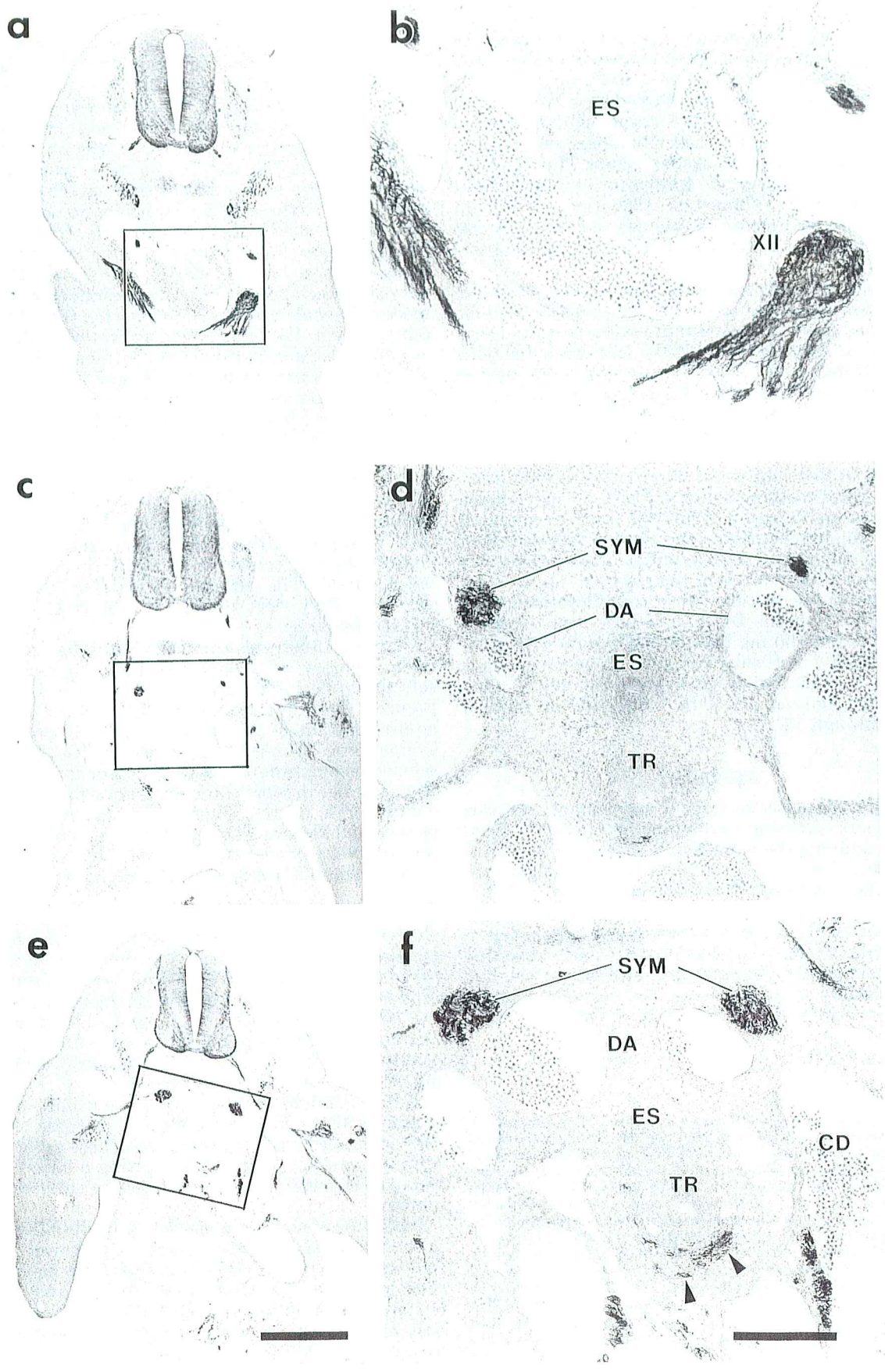

Fig. 2.

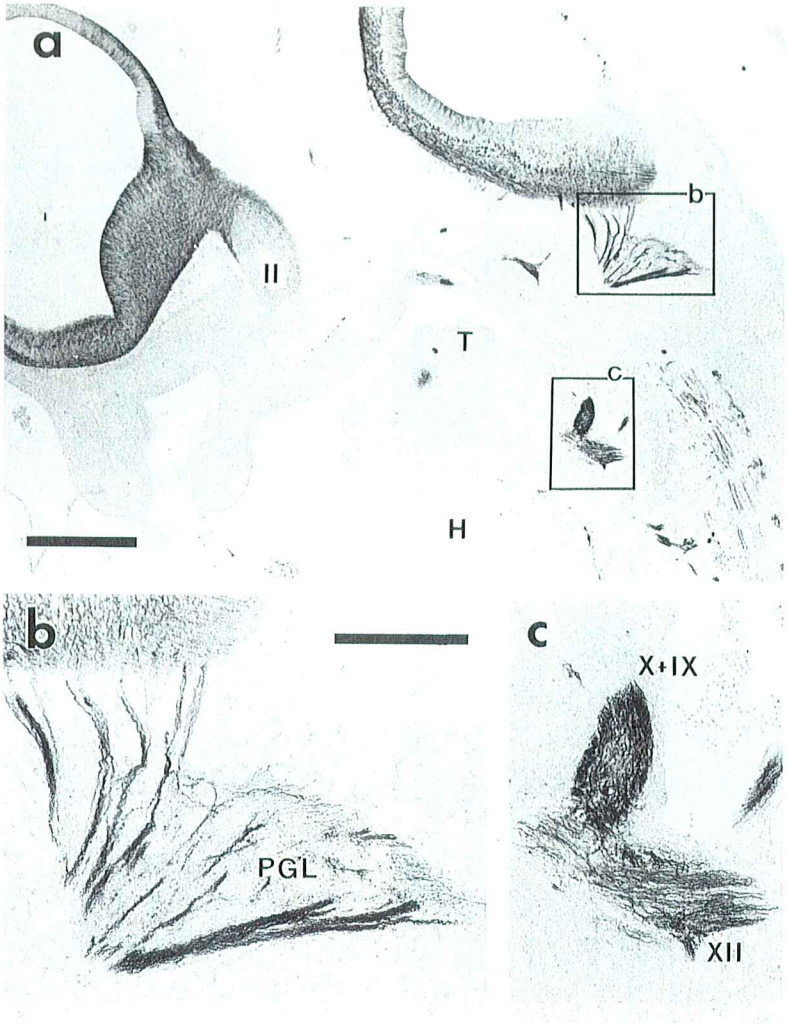

Fig. 3. **a:** Saggital section of a day 14 experimental embryo. The tongue primordium (T), heart (H), and optic nerve (II) are obvious in this section. **b:** Enlargement of the upper box in a. The proximal ganglion (PGL) of the glossopharyngeal and vagus nerves is comparable to controls. **c:** Enlargement of the lower box in a shows the connection of the hypoglossal nerve (XII) and the common nerve trunk of the glossopharyngeal and vagus nerves (X and IX). Bar = 0.5 mm for a; 0.2 mm for b and c.

DISCUSSION

This study has used a combination of immunohistochemistry and graphic reconstruction to reveal profound alterations in the developing nervous system that were produced as a result of administration of bisdiamine. HNK-1 immunoreactivity of the nerves made it possible to compare, with precision, their normal development in the primitive pharyngeal region as compared with the impaired development found in experimental fetuses. These bisdiamine-induced defects in nervous system development have been revealed for the first time by this study.

The major alterations produced included consistent absence of the nodose (distal X) ganglia, partial absence of the petrosal (distal IX) ganglia, absence of distal branches of the vagus and glossopharyngeal nerves, and fusion of the fibers of the vagus and glossopharyngeal trunks with the hypoglossal nerve. These alterations occurred in fetuses with normally formed proximal ganglia of the vagus and glossopharyngeal nerves. They also had normal trigeminal, facial, and acoustic nerves. Thus it appears that postotic structures were affected. The otocyst developed normally.

Fig. 2. Transverse sections of a gestation day 13 experimental embryo, immunohistochemically stained with HNK-1. The sections have been cut at approximately the same levels as those in Figure 1. **a:** At the level of the hypoglossal nerve. **b:** Enlargement of the box in a. There is no nodose ganglion at this level. None was found at higher or lower levels. The hypoglossal nerve (XII) appears thicker than in controls because fibers of the vagus and glossopharyngeal nerve join it. ES, esophagus **c:** Section slightly below the level of the truncus arteriosus. **d:** Enlargement of the box in c. Note the absence of the nodose ganglia or the vagus nerves (Compare with Fig. 1e). The sympathetic trunks (SYM) are in the proper location; one seems smaller than controls. DA, dorsal aortae; ES, esophagus; TR, trachea. **e:** Section at the level of the sinus venosus. **f:** Enlargement of the box in e. The vagus nerves are missing from the interval between esophagus and trachea. HNK-1$^+$ fibers are associated with the trachea (arrowheads), and the sympathetic trunks seem normal. Compare with Fig. 1g. CD, duct of Cuvier. Bar = 0.5 mm for a, c, and e; 0.2 mm for b, d, and f.

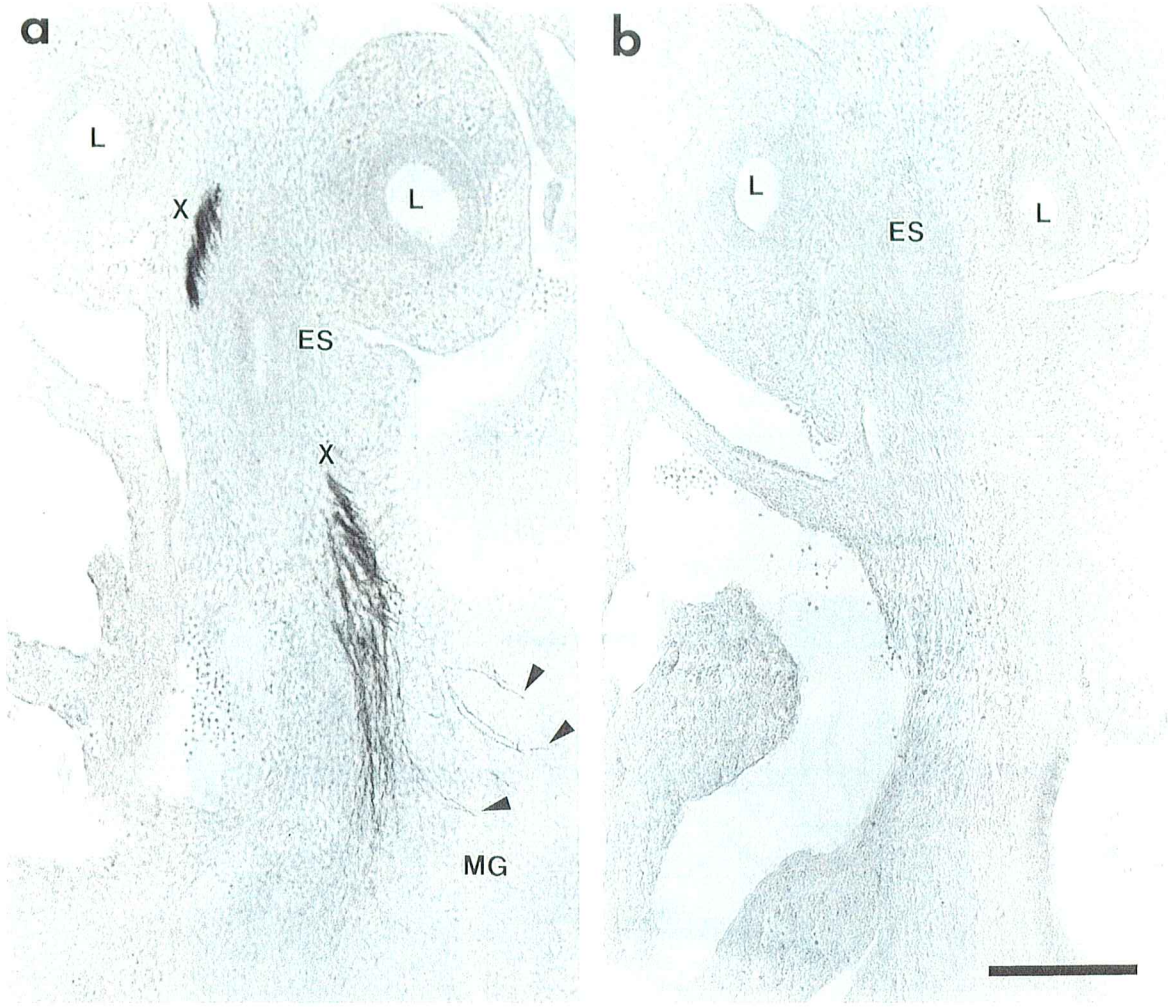

Fig. 4. Sections of day 13 fetuses at the level of the lung buds (L) and transverse septum. **a:** Control embryo. The HNK-1$^+$ vagus nerves flank the esophagus (ES). One arborizes distally (arrowheads) to supply the stomach (MG). **b:** In an experimental embryo, at a comparable level, no vagus nerves are present. Bar = 0.2 mm for both a and b.

Because the observations were all made on fetuses, it is not possible to state with certainty that the alterations were permanent. It would be necessary to study postnatal specimens in order to establish that. It is difficult to believe, however, that the distinct alterations in ganglia and nerves that were observed consistently through succeeding stages could be restored to a normal state by later development of a nodose ganglion, extension of nerve processes, and restoring of cranial nerve branches.

The distal cranial sensory ganglia are derived from ectodermal thickenings, the epibranchial placodes (D'Amico-Martel and Noden, 1983). These ganglia appear in the branchiomeric series of cranial nerves and are quite different from the neural crest-derived ganglia in their developmental pattern. They do not migrate as extensively as crest-derived cells. They behave differently in the presence of nerve growth factor. Their neuronal and cytological phenotypes during development are different from those of crest-derived neurons (reviewed by Lindsay, 1988). In the present study, the placode-derived neurons seemed to be more affected by bisdiamine than those derived from neural crest. The sympathetic trunks, the proximal ganglia of the cranial nerves, and the dorsal root ganglia were affected less or not at all. All of the latter neurons have been shown, in the chick, to derive from neural crest (Le Douarin, 1982; D'Amico-Martel and Noden, 1983). Most of the studies that have delineated the contributions from placode versus neural crest have been conducted in the chick. It is possible that the derivation of the relevant nervous components in mammals does not correspond directly to the chick. It seems reasonable, however, to assume that the situations are similar until there is evidence to the contrary. If this proves not to be the case, an alternate explanation would need to be sought for the observation that the affected ganglia correspond to the placode-derived ganglia in the chick.

Absence of the distal ganglia apparently altered the pathway and connections made by the vagus and glos-

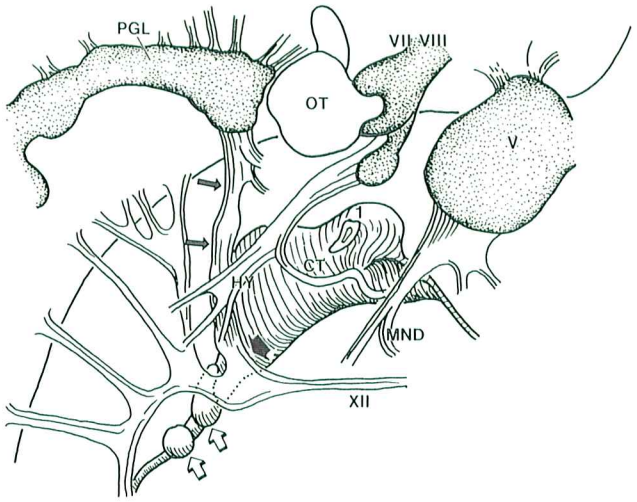

Fig. 5. Graphic reconstruction of a day 14 experimental embryo. Lateral view. The trigeminal (V) and acousticofacialis (VII and VIII) nerves are comparable to controls, while the glossoparyngeal and vagus nerves lack their distal ganglia and form a single nerve trunk (small arrows) that joins with the hypoglossal nerve (XII) at the large arrow. The combined trunks pass into the tongue. The proximal ganglion (PGL) of these nerves is comparable to controls. CT, chorda tympani of the facial nerve; HY, hyoid branch of the facial nerve; MND, mandibular branch of the trigeminal nerve; OT otocyst; 1, first pharyngeal slit; open arrows, outgrowths from posterior pharyngeal region.

sopharyngeal nerves (Kuratani, 1990; Kuratani and Tanaka, 1990). Distal neurites from the proximal ganglia joined and ran with those of the hypoglossal nerve into the tongue primordium. This abnormal incursion of neurites may well have produced nonfunctional neuron-target connections. The failure of distal neurites from both nerves to follow the normal course of the vagus and glossopharyngeal nerves also implies that the epibranchial placode-derived neurons may play a role like the "pioneer neurons" described by Keshishian (1980). The fibers from the proximal ganglia may be unable to grow along their normal pathway without the guidance of the placode-derived neurons. Hamburger (1961) has shown, with respect to the trigeminal nerve, that ablation of the placode leads to the lack or abnormal development of the sensory branches in the face. Noden (1980a,b) had concluded that, in the trigeminal nerve, the placode-derived neurons are necessary for sensory branch formation, in which neurons derived from neural crest and from placodes are involved. In a previous study, we have shown that even after removal of the cardiac neural crest, the placodes seem to produce the usual branches in the pharyngeal region (Kuratani et al., 1991). Interestingly, in the absence of crest-derived cells, the neurites from the proximal ganglia of the vagus and glossopharyngeal nerves tend to join with the hypoglossal nerve (Kuratani et al., 1991).

It is possible that the vagal innervation of the heart and the gut must be initiated by placode-derived neurons. With the absence of placode-derived nerves, the vagal branches supplying the anterior of the primitive gut were missing. This would seem to have the possibility of affecting the development of the enteric nervous system. The interactions of fibers from the vagus nerve with intrinsic neurons of the enteric nervous system necessary for proper development are not known. It would seem worthwhile to determine what they are. There is evidence that precursor cells for the enteric nervous system originate in the neural crest and migrate along the pathway that will later be followed by the vagus nerve (Baetge et al., 1990a,b). The migrating crest-derived cells appear eventually to be overtaken by the fibers of the nerve. The possibility of alterations being caused by interfering with interactions between these elements remains to be determined.

Absence of vagal fibers to the developing heart also has the potential of affecting the cardiac nervous system and control of cardiac contraction. This effect could be through an imbalance of autonomic nervous control, or possibly through an indirect effect on cardiac muscle. Microsurgical removal of the nodose placode has been shown to be capable of altering cardiac contraction, characterized by prolongation of the QT interval (Christiansen and Kirby, 1990; Mulroy et al., 1990).

The nervous elements associated with the otic placodes, and anterior to them, seemed unaffected by bisdiamine administration as carried out in the present investigation. The trigeminal nerve possessed sensory branches of the face as well as the mandibular branch. The facial nerve possessed the chorda tympani and the hyoid branch. The development of the chorda tympani strongly suggests the normal development of the geniculate placode (epibranchial placode of the first pharyngeal pouch), from which the ganglion develops (Yntema, 1944; D'Amico-Martel and Noden, 1983). The normal development of the trigeminal branches also is dependent on the contribution of the placode-derived neurons (Hamburger, 1961; Noden, 1980a,b). Inhibition of postotic structures, with regular development of preotic structures, may be the result of the timing of administration of bisdiamine, since the pharyngeal system develops rostrocaudally. The appearance of the petrosal ganglion in some of the experimental animals could indicate that it was differentiated beyond the inhibitory capabilities of the bisdiamine by the time it was administered. This question can be answered by experiments altering the timing of administration. It is also possible that preotic structures have a different susceptibility to bisdiamine than postotic structures.

A number of congenital anomalies, such as the DiGeorge syndrome, the fetal alcohol syndrome, and retinoic acid embryopathy, have a spectrum of defects of the cardiovascular system, thymus, and parathyroids (Amman et al., 1982; Lammer et al., 1985). It has been suggested that these syndromes result from insufficient contributions from neural crest (Couly et al., 1983; Kirby and Bockman, 1984). Sulik et al. (1986, 1988) have suggested that the effects of ethanol, retinoic acid, and other environmental agents may cause increased cell death and affect the epibranchial placodes if administered at the appropriate time. The range of defects present in these syndromes is similar to those produced by the administration of bisdiamine in experimental animals. In the reports of studies of patients with these anomalies, there does not appear to have been a careful evaluation of the components of the peripheral nervous system that were found to be deficient in the present study. It is suggested that in future

cases, it would be worthwhile to investigate the presence and extent of the nodose and petrosal ganglia, the size and course of associated nerve trunks, the contributions to cardiac nerves, and the association of the vagus nerve with the enteric nervous system.

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