Embryological Origin for Autism: Developmental Anomalies of the Cranial Nerve Motor Nuclei

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ABSTRACT
The underlying brain injury that leads to autism has been difficult to identify. The diagnostic criteria of the disease are not readily associated with any brain region or system, nor are they mimicked by vascular accidents, tumors, or degenerative neurological diseases occurring in adults. Fortuitously, a recent report of autism induced by thalidomide exposure provides evidence that the disease originates by an injury at the time of closure of the neural tube. The human data suggest that the initiating lesion includes the motor cranial nerve nuclei. To test this hypothesis, we first examined motor nuclei in the brainstem of a human autistic case. The autopsy brain exhibited near-complete absence of the facial nucleus and superior olive along with shortening of the brainstem between the trapezoid body and the inferior olive. A similar deficit has been reported in Hoxa-1 gene knockout mice in which pattern formation of the hindbrain is disrupted during neurulation. Alternatively, exposure to antimotic agents just after neural tube closure could produce the observed pattern of deficits. Thus, the lesions observed in the autopsy case appear to match those predicted by the thalidomide cases in both time of origin and central nervous system (CNS) location. To produce similar brain lesions experimentally, we exposed rat embryos to valproic acid, a second teratogen newly linked to autism. Dams received 350 mg/kg of valproic acid (VPA) on day 11.5 (the day of neural tube closure), day 12, or day 12.5 of gestation. Each treatment significantly reduced the number of motor neurons counted in matched sections of the earliest-forming motor nuclei (V, XII), and progressively later exposures affected the VIth and IIIrd cranial nerve nuclei. All treatments spared the facial nucleus, which forms still later. Counts from the mesencephalic nucleus of trigeminal, the dorsal motor nucleus of the vagus, and the locus ceruleus were not affected by exposure to VPA, even though these nuclei form during the period when exposure occurred. Despite its effects on the motor nuclei, valproic acid exposure did not alter the further development of the brain in any obvious way. Treated animals were robust and had no external malformations. The autopsy data and experimental data from rats confirm that CNS injuries occurring during or just after neural tube closure can lead to a selective loss of neurons derived from the basal plate of the rhombencephalon. The results add two new lines of evidence that place the initiating injury for autism around the time of neural tube closure.

Autism is a behaviorally defined syndrome characterized by impairment of social interaction, deficiency or abnormality of speech development, and limited activities and interests (American Psychiatric Association, 1994). The last category includes such abnormal behaviors as fascination with spinning objects, repetitive stereotypic movements, obsessive interests, and abnormal aversion to change in the environment. Symptoms are present by 30 months of age. The prevalence rate in recent Canadian studies using total ascertainment is over 1/1,000 (Bryson et al., 1988).

Attempts to identify the cause of the disease have been difficult, in part, because the symptoms do not suggest a brain region or system where injury would result in the diagnostic set of behaviors. Further, the nature of the behaviors included in the criteria preclude an animal model of the diagnostic symptoms and make it difficult to relate
much of the experimental literature on brain injuries to the symptoms of autism.

**Anatomical studies of the defect underlying autism**

Several quantitative changes have been observed in autistic brains at autopsy. An elevation of about 100 g in brain weight has been reported (Bauman and Kemper, 1985). While attempts to find anatomical changes in the cerebral cortex have been unsuccessful (Williams et al., 1980; Coleman et al., 1985), several brains have been found to have elevated neuron packing density in structures of the limbic system (Bauman and Kemper, 1985), including the amygdala, hippocampus, septal nuclei and mammillary body. Multiple cases in multiple labs have been found to have abnormalities of the cerebellum. A deficiency of Purkinje cell and granule cell number, as well as reduced cell counts in the deep nuclei of the cerebellum and neuron shrinkage in the inferior olive, have been reported (Bauman and Kemper, 1985, 1986, 1994; Ritvo et al., 1986; Kemper and Bauman, 1992).

Imaging studies have allowed examination of some anatomical characteristics in living autistic patients, providing larger samples than those available for histologic evaluation. In general, these confirm that the size of the brain in autistic individuals is not reduced and that most regions are also normal in size (Piven et al., 1992b). Reports of size reductions in the brainstem have been inconsistent (Gaffney et al., 1988; Hsu et al., 1991), but a new, larger study suggests that the midbrain, pons, and medulla are smaller in autistic cases than in controls (Hashimoto et al., 1995). In light of the histological effects reported for the cerebellum, it is interesting that the one region repeatedly identified as abnormal in imaging studies is the neocerebellar vermis (lobules VI and VII; Gaffney et al., 1987; Courchesne et al., 1988; Hashimoto et al., 1995). Not all comparisons have found a difference in neocerebellar size (Piven et al., 1992b; Kleiman et al., 1992), but a recent reevaluation of positive and negative studies (Courchesne et al., 1994) indicates that a few autistic cases have hyperplasia of the neocerebellar vermis, while many have hypoplasia. Small samples of this heterogeneous population could explain disparate results regarding the size of the neocerebellum in autism. The proposal that the cerebellum in autistic cases can be either large or small is reasonable from an embryological standpoint, because injuries to the developing brain are sometimes followed by rebounds of neurogenesis (e.g., Andreoli et al., 1973; Bohn and Lauder, 1978; Bohn, 1980), and it is possible that such rebounds could overshoot the normal cell number. Further, because increased cell density has been observed in the limbic system, the cerebellum is not the only brain region in which some form of overgrowth might account for the neuroanatomy of autistic cases. It may well be that some autism-inducing injuries occur just prior to a period of rapid growth for the cerebellar lobules in question or the limbic system, leading to excess growth, while other injuries continue to be damaging during the period of rapid growth, leading to hypoplasia. However, the hypothesis that autism occurs with both hypoplastic and hyperplastic cerebella calls into question whether cerebellar anomalies play a major role in autistic symptoms.

A particularly instructive result has appeared in an MRI study on the cerebral cortex (Piven et al., 1992a). Of a small sample of autistic cases, the majority showed gyral anomalies (e.g., patches of pachygria). However, the abnormal areas were not located in the same regions from case to case. That is, while the functional symptoms were similar in all the subjects, the brain damage observed was not. The investigators argue convincingly that the cortical anomalies were not responsible for the functional abnormalities. This is a central problem in all attempts to screen for pathology in living patients or in autopsy cases. While abnormalities may be present, it is not necessarily true that they are related to the symptoms of autism.

**New data from a group of cases caused by thalidomide exposure**

Until recently, there has been nothing to link the many associations reported for autism to the disease in any way that is causal. In contrast, new data from a report in which prenatal thalidomide exposure was shown to lead to autism (Miller and Strömland, 1993; Strömland et al., 1994) can be interpreted to suggest a specific hypothesis regarding the relationship between autism and brainstem injury. What makes the thalidomide cases so informative is that the external stigmata of thalidomide teratogenesis allow accurate dating of the stages of development when damaging levels of the drug were present (Miller, 1991). In these cases, unlike the others in the literature, we have a known cause, a set of physical, neurological, and psychiatric symptoms, and a firm identification of the stage of development when the insult occurred.

Thalidomide exposure in early pregnancy is best known for its teratogenic effects on the limbs (Lenz, 1962), but cranial nerve deficits were actually reported soon after the initial findings (d’Avignon and Barr, 1964). While studying eye motility in thalidomide victims, Miller and Strömland (1993) and Strömland et al., (1994) recognized that 4 of the 86 cases available for study in Sweden had autism, a highly significant increase over the 1/1,000 rate of cases in that country (Gillberg et al., 1991). Among the 100 living thalidomide cases in the Swedish registry, only 15 had indications of exposure on days 20–24 (Miller, 1991). Of the 86 cases available for study in Sweden had autism, a highly significant increase over the 1/1,000 rate of cases in that country (Gillberg et al., 1991). Among the 100 living thalidomide cases in the Swedish registry, only 15 had indications of exposure on days 20–24 (Miller, 1991). The malformations of each of the four autistic cases pointed to exposure on those days. A fifth case previously diagnosed as autistic was not available for study, but her malformation pattern indicated the same exposure period. Thus, the five cases of autism represent a rate of 5/15, or 30%, when thalidomide exposure occurred on days 20–24. Conversely, the rate of autism was 0% at every other exposure time.

The fact that autism resulted only when exposure occurred between the 20–24th days of gestation severely restricts the candidates for what parts of the nervous system could have been injured directly by thalidomide, because so little of the central nervous system (CNS) is present at that age (Lemire et al., 1975). Autoradiographic studies of cell birthdays in the mammalian and avian brainstem demonstrate that only the basal plate derivatives of the brainstem form so early (Taber Pierce, 1973; Sohal and Holt, 1977; Nornes and Morita, 1979; Altman and Bayer, 1980). The neurological anomalies of the autistic patients indicate that neuron populations from the basal plate were severely damaged, as described below. Since there is no reason to suspect that each of five thalidomide cases was first injured in a way that caused cranial nerve anomalies and then injured in a second accident that caused autism, the exposure data indicate to us that the brainstem injury which produced the neurological defects and that which produced the autism must be one and the same.
The neurological abnormalities observed in the five thalidomide autistic cases included the following: three patients had Duane syndrome, a failure of the VIth cranial nerve (abducens) to innervate the lateral rectus muscle of the eye with subsequent reinnervation of the muscle by the IIIrd cranial nerve (oculomotor; Hotchkiss et al., 1980); one had gaze paresis (oculomotor palsy); four had Möbius syndrome, a failure of the VIIth cranial nerve (facial) to innervate the facial muscles, often associated with other cranial nerve symptoms (May, 1986); two had abnormal lacrimation, a failure of the neurons of the superior salivatory nucleus (cranial nerve VII) to innervate the lacrimal apparatus with subsequent misinnervation of the structure by neurons which normally supply the submandibular glands (Ramsey and Taylor, 1980). Each patient had ear malformations and hearing deficits. The last symptom does not necessarily indicate an injury to cranial nerve VIII, which carries auditory information, because the damage could be located in the external, middle, or inner ear or at other stations along the auditory pathways.

Both the cranial nerve motor symptoms and the ear malformations are consonant with the hypothesis that thalidomide interfered with pattern formation for the rhombomeres from which the brainstem nuclei arise and/or neuron production for the cranial nerve motor nuclei. The period when the otic placode appears (O'Rahilly, 1963), the presumptive neural tissue is divided into rhombomeres (Streeter, 1948), and the first neurons appear (Bayer et al., 1993) is the fourth week of gestation, when the neural tube is closing.

External ear malformations are the most common physical abnormality associated with autism of unknown cause and the one which distinguishes best between autistic cases and mentally retarded cases (Walker, 1977). Thus, the stage of development when injury occurred in the autistic thalidomide-exposed cases cannot be viewed as unique to thalidomide; it appears to be characteristic of many cases. Most strikingly, the first report of a case of autism after exposure to valproic acid appeared simultaneously with the publication of the thalidomide data (Christianson et al., 1994). The anticonvulsant has long been known to cause developmental delay and mental retardation, with expressive language behaviors being the most affected (Ardinger et al., 1988). Like thalidomide, its mechanism of action is not known, but it also produces external malformations in a sequence related to the time of peak exposure (Collins et al., 1991; Ehlers et al., 1992). Not surprisingly, the valproic acid autistic case had malformed ears without malformations of the hands, indicating exposure at the same period of development as the thalidomide cases.

Data pointing to an underlying injury which occurs so early in development are informative in two ways. First, they tell us the location of the injury, because we know which neurons were present to be injured. Second, they tell us which neurons were not injured, a much longer list, which includes virtually the whole brain, aside from the motor nuclei of the cranial nerves. The conclusion that the initial injury is restricted to this relatively small set of neurons, of course, does not suggest that no other parts of the nervous system were altered in the long run. The early injury could disturb development of any or all structures that form subsequently by altering the environment in which they develop, or it could disturb the anlage of later-forming structures directly, leading to abnormalities as development proceeds. Thus, the hypothesis that the initial injury is focused on the brainstem is compatible with the CNS anomalies already reported in the limbic systems and the cerebella of autistic cases.

The hypothesis that autism is initiated at the time when the cranial nerve motor nuclei are forming cannot be tested from the existing anatomical literature. We prepared and examined serial sections from the brainstem of an autistic patient for evidence of abnormalities in cranial nerve nuclei. A theoretical embryological argument against a brainstem injury as the initiating event in autism is that injuries as early as the neural tube stage might disrupt development so completely as to be incompatible with survival. However, there is strong evidence from two kinds of embryological anomalies that this is not always the case. Defects of closure of the neural tube, whether induced by genetic defects, exogenous agents, or a combination of the two, arise in the period under discussion, and many are compatible with normal brain development as judged by function. It is true that some Hox gene knockout mice with hindbrain injuries visible before neural tube closure are not viable after birth because of failure to breathe (Lufkin et al., 1992) or other brainstem malfunctions (Chisaka et al., 1992). However, the gross form of the brains of some such animals at term is surprisingly normal (Chisaka et al., 1992). The kreisler mouse, with a deficiency that appears to represent the absence of two rhombomeres, is normal on external inspection and can survive to adulthood (McKay et al., 1994). Thus, it was our hypothesis that if we exposed rats to a teratogen during motor neuron production, it should be possible to reduce the number of motor neurons but allow the rest of CNS development to proceed normally enough to produce a brain of grossly normal form.

We did not attempt this experiment with thalidomide, because its effects in rodents are different from its effects in humans (Schumacher et al., 1972). The anticonvulsant, valproic acid, on the other hand, is an effective teratogen in rodents, producing many of the same external defects seen after human exposure (Binkerd et al., 1988; Ehlers et al., 1992; Collins et al., 1992), and the malformations produced are similar to those observed in humans exposed to thalidomide. Behavioral studies after chronic prenatal dosing of rats with valproic acid have confirmed its brain-damaging effects (Vorhees, 1987). Morphologic studies 48 hours after acute exposure of the early mouse embryo have demonstrated failures of closure of the cranial neural tube and disorganization of the neuroepithelium (Turner et al., 1990). No investigations have evaluated the consequences of prenatal exposure to valproic acid on the details of postnatal anatomy of the CNS.

In the rat, the neural tube closes on day 11, and neuron production for the motor nucleus of trigeminal, the trochlear, abducens, and hypoglossal nuclei is completed during the twelfth day of gestation (counting the day of finding a vaginal plug as day 1). Neurons for the oculomotor nucleus are produced on days 12 and 13 in about equal numbers, while most facial neurons form on day 13 (Altman and Bayer, 1980). All these nuclei except the trochlear were sampled for neuron numbers after exposure to valproic acid (VPA) on day 11.5, 12, or 12.5 of gestation. Only a few other parts of the brainstem form as early as the motor nuclei. These include the mesencephalic nucleus of trigeminal, the locus ceruleus, and the dorsal motor nucleus of the vagus (Altman and Bayer, 1980). These were sampled in the same animals to provide information on whether any injuries observed were selective only on the basis of which neurons...
were undergoing their final mitoses during exposure or whether they were selective on some other basis. Controls and treated animals from the day 12.5 treatment group were held to adulthood to test the permanence of any changes in cell numbers observed in young animals.

MATERIALS AND METHODS

Study 1: Autopsy case

The patient had been followed at this hospital from the age of five, when she was first described as having a psychiatric illness "of psychotic proportions." A few months later, she was admitted for 6 months, and the diagnosis was "childhood psychosis, autistic type." While the patient under discussion died two decades ago, one of the authors (Dr. Romano) observed her from her first admission to her death and preserved her brain for study because of his interest in her case. His records include extensive interviews with parents and other relatives carried out prior to and after the patient's death, records of conferences with her caregivers regarding her treatment, and correspondence with other experts about her symptoms. The records of her hospitalization are unusually rich in detail. They include notes on 26 individual play therapy sessions at the age of six and the notes of two speech therapists who worked with the patient at age seven, in addition to daily notations. The criteria for diagnosis have changed over the years since the original diagnosis was made, and thus we have reevaluated the patient from her records, using modern standards. She meets the DSM-IV criteria for autism.

The child was born two years after the delivery of a normal sister. Her mother was a chronic abuser of alcohol and Dexedrine during the patient's infancy, but she could not remember whether the addiction problems arose before or after the birth. It is certain that the patient was not exposed to thalidomide, because she was born before it was introduced or after the birth. It is certain that the patient was not exposed to thalidomide, because she was born before it was marketed, or to valproic acid, because her mother was not treated for seizures. The mother was hospitalized repeatedly for psychiatric illness, with many different diagnoses, including hysterical disorder, depressive neurosis, chronic anxiety neurosis, and drug addiction to barbiturates and amphetamines.

Descriptions of the patient in infancy include that she had no smiling response, did not reach out when approached or look into the eyes of others. She "was too good," "did not cry, "was a bit distant." In childhood, her speech development consisted of a few words and phrases, often spoken in imitation. For example, she often answered a question by repeating it. At seven years, her speech was rated as being appropriate for a three-year-old. Her speech did not progress far beyond that described at the early evaluations, although she continued to imitate, as in learning songs from the radio. She covered her ears in response to some sounds but showed interest in others (music, the vacuum cleaner). Repetitive movements included rocking, hitting her head with her hand, and flicking her fingers in front of her eyes or at the side of her head. An example of her play was to take two pieces of doll furniture and try to attach them to each other. She then pressed the pieces into clay and studied the resulting holes. She sometimes examined people's hands and showed interest in brightly colored objects, such as buttons on other's clothes. She had hyperactive, aggressive episodes. While there is no formal test of intelligence in the record, one physician described her as "below average" and another as "average."

The only record of a neurological exam is in the context of a physical exam at age six. Cranial nerves II–XI were checked as normal. Gait, balance, and reflexes were judged to be normal, while cutaneous sensation, coordination, and visual field testing were noted as untestable due to poor cooperation. The statement, "She had no facial expression" appears in the initial evaluation and many others and agrees with the author's observations. However, there are reports of the patient's bizarre grimaces when excited. Photographs of the patient in childhood show no nasolabial folds and a sagging lower lip and jaw, but active corrugator muscles. Two EEGs while the patient was taking barbiturates were inconclusive; she was treated for seizures.

Over 15 years of institutional care, her medications included antipsychotics, stimulants, anticonvulsants, and benzodiazepines, but neither drug therapies nor psychotherapies had any significant effect on her condition. In the last few months of her life, her hyperactivity and aggression became a severe, leading to the frequent use of restraint. At the age of 21, she was found comatose, in shock, with a high fever, five hours after a normal check. She died three days later. The cause of death was not clear but was listed as "sepsis of unknown origin."

The brain weighed 1,380 g, above the average (1,252 g, S.D. = 125 g) for adult females in a recent report, but within two standard deviations of the mean (Ho et al., 1980). It had been studied previously for neuron numbers in cortical regions related to speech and hearing, and no differences were found (Coleman et al., 1985). The tissue remaining for study consisted of three blocks, one from the pons, one from the medulla, and one from the cerebellum. These samples had been cut free-hand from the brain during the autopsy, fixed in neutral buffered formalin and then embedded in paraffin with the cephalic surface as the face of each block. We cut the samples of the pons and medulla in 10-μm sections and stained them with cresyl violet and luxol fast blue. To be sure that no tissue was lost, we did not "even-up" the face of either block, but cut, saved, mounted, and stained serial sections of all the tissue present. At the outset, we had no way to know what part of the brainstem would be represented in the blocks. Only when all the sections had been prepared were we able to see that the two blocks were contiguous, with the missing tissue at the dorsal surface of the caudal part of the pontine block being present in the most oral sections from the medullary block. As shown in Figure 1, the sections cut from the pontine block revealed tissue from the oral pole of the facial nucleus to a level just oral to the hypoglossal nucleus, a distance of almost 6 mm (582 sections). The sample of the medulla began at the oral pole of the hypoglossal nucleus and ended about halfway through the medulla, a distance of 2.5 mm (246 sections). In a control brain, we attempted to prepare two blocks of the same region with the faces set at the same angles, dividing the brainstem just cephalic to the hypoglossal nucleus. Again, we cut, mounted, and stained every 10-μm section. The control was an 80-year-old male who died from coronary artery obstruction leading to a fatal arrhythmia. The control brain weight was 1,230 g.

On the left side of the autistic and control brains, every eighth section containing facial nerve motor neurons was counted. The numbers of neurons in the facial nuclei of the control and autistic brains were then calculated using the empirical correction of Coggeshall et al. (1990). That is, approximately 100 neurons in each case were reconstructed.
from serial sections to determine precisely the rate at which a single cell appears in more than one section, and counts were corrected for double counting.

Study 2: Experimental induction of injury to the cranial nerve motor nuclei

Sodium valproate (Sigma) was dissolved in saline, and the pH was adjusted to 7.3. The solutions were tested in the hospital clinical chemistry laboratory by an enzyme-multiplied immunooassay technique to confirm the concentration of the acid. Long Evans or Sprague-Dawley rats (Charles River, Portage, ME, and Hollister, CA) were mated and Baxter, 1954) could be seen in its normal location in the autistic case, the region is distinguished by the presence of myelinated fibers passing in all directions. In contrast, the patch of facial motor neurons in the autistic brain was less than 500 km long, with no neurons caudal to the cranial edge of the sample from the autistic case. Thus, we could not learn anything about the status of that nucleus or the more cranial nuclei of the V, IV, and III.

Figures 2 and 3 compare the facial nuclei of the two cases. The anterior part of the nucleus is seen at the level of the trapezoid body in both brains. In the sections shown, the control has 74 facial motor neurons and the autistic case has only one complete neuron. At the third level pictured, the control has 67 neurons and the autistic case was 345 on the left side of the brain. Using the same methods, the count was 9,356 neurons on the left side of the control case. Thus, we could not learn anything about the status of that nucleus or the more cranial nuclei of the V, IV, and III.

RESULTS

Study 1: Autopsy case

Many CNS regions of the autistic case contained pyknotic neurons, consistent with the patient’s condition over the last days of her life. Most cranial nerve motor neurons were intact, with occasional inclusions or shrinkage. The control brain had many more pyknotic motor nuclei, and virtually all of the facial motor neurons were pyknotic.

Only a few motor neurons from the caudal border of the abducens nucleus were present at the cranial edge of the sample from the autistic case. Thus, we could not learn anything about the status of that nucleus or the more cranial nuclei of the V, IV, and III.

At all levels where the facial nucleus should appear in the autistic case, the region is distinguished by the presence of myelinated fibers passing in all directions. In contrast, the nuclear region in the control is essentially free of fibers. The facial nerve (half motor fibers and half sensory; Olszewski and Baxter, 1954) could be seen in its normal location in both cases.
Fig. 2. Low power views (a and b) of sections just caudal to the abducens nucleus show typical landmarks of the caudal pontine tegmentum: the medial lemniscus (ML), the central tegmental tract (TT), and the trapezoid body (TB). Boxes outline areas seen as close-ups in Figure 3. At this level, the facial nucleus is small in both the control (a) and autistic (b) cases, while the superior olivary complex (arrow in a) is present in the control case and absent in the autistic case. Thirty sections (300 µm) caudal to the first level illustrated, a larger portion of the facial nucleus is seen in the control case (e), but only one motor neuron is present in the autistic case (d). Six-hundred microns caudal to c and d, the facial nucleus is growing smaller in the control (e) and still absent in the autistic brain (f). At this level, the capsule of the inferior olive (arrow in f) is present in the autistic case. In the control, this structure first appears more than a millimeter caudal to the level shown in e.
The superior olive should appear between the facial nucleus and the trapezoid body, as seen in the control case (Fig. 2a). Neither the spindle-shaped cells of this nuclear group nor the distinctive pattern of fibers that surround the cells were visible in the autistic case (Fig. 2b).

The distance from the first section with uninterrupted crossing of the trapezoid fibers to the last in the control was 1,100 μm. In the autistic brain the distance was 900 μm. The distance from this point to another landmark, the inferior olive, was 1,100 μm in the control and 200 μm in the autistic case. The distance from the oral pole of the inferior olive to the oral pole of the hypoglossal nucleus was 2,100 μm in the control and 2,400 μm in the autistic case. These measures suggest that the brainstem of the autistic case was shortened in the region between the trapezoid body and the inferior olive, the same region where the facial nucleus was absent (Fig. 5). The region which appeared to be shortened was in the middle of the “pontine” block. Thus, neither the shortening nor the absence of facial neurons could be due to any loss of tissue between blocks.

The hypoglossal nucleus was not obviously reduced in cell number but contained an unusual bilateral structure shown in Figure 6. One of the globular clusters of cells characteristic of the nucleus was ringed by fine myelinated fibers which could be followed into the medial longitudinal fasciculus (MLF). In the embryo, projections like the one pictured connect the somatic efferent column neurons to the MLF, but they should persist only for the neurons controlling eye muscles, being effete for the muscles of the tongue, pharynx and larynx. Both the ring and the connection between the hypoglossal and the MLF have been reported in the fetal human brain (Bruce, 1892) but not the mature brain.

Within the fibrous ring of the autistic case, the cells were pyknotic and much smaller than any pyknotic motor neurons outside the ring. No similar fiber pathways were observed in the control case.

Nuclei of sensory cranial nerves, such as the vestibular nuclei, the cochlear nuclei, and the nucleus of the spinal tract of the trigeminal, appeared normal in size and cellularity. The pontine nuclei were also normal in appearance, as was the dorsal motor nucleus of the vagus. This is not to say that these nuclei were normal anatomically or functionally. Neither proposition could be proven from any autopsy case. We describe their appearance only because it contrasts to the obvious abnormalities of the facial nucleus, superior olive, and hypoglossal nucleus.

Study 2: Experimental induction of injury to the cranial nerve motor nuclei

All brains were grossly normal. Each treatment reduced both brain and body weight significantly, with the latest treatment having the largest effect. This was true of adult animals as well as young animals. In all cases, brain weight as a percentage of body weight did not differ significantly between controls and treated animals. Table 1 shows brain and body weights and brain weight as a percent of body weight for animals treated on day 12.5 and their controls.

Results of cell counts in the five motor nuclei studied are shown in Table 2. Cell counts in the earliest-forming nuclei, the trigeminal and hypoglossal, were significantly reduced by exposure to VPA on the afternoon of the eleventh day of gestation. Treatment 12 hours later affected these nuclei and the abducens.
Fig. 3. The areas containing the facial nucleus are outlined in Figure 2 and shown here at higher magnification. All were photographed with a green filter to emphasize the violet-stained neurons. a, c, and e are three levels of the control facial nucleus. Most facial motor neurons in this brain were pyknotic, but they were numerous at every level. The actual counts from the sections pictured were 74, 102, and 67 motor neurons. b, d, and f are from the autistic autopsy brain. In this case most of the motor neurons were intact and thus not so dark in appearance. A cluster of 22 facial neurons was present at the most oral level shown (b), but more caudal sections are remarkable for the lack of facial motor neurons. Notice that the facial nucleus of the control is distinguished almost as much by the absence of myelinated fibers as by the presence of motor neurons. In contrast, the region in the autistic case not only lacks motor neurons but has many fibers passing in all directions. This suggests that the motor neurons were lost before the architecture of the region was established.

12.5, the later-forming oculomotor nucleus was significantly reduced, along with the first three nuclei. The facial, which forms principally on the thirteenth day, was unaffected in each group.

Results for the three cell groups counted for comparison to the somatic motor nuclei are shown in Table 3. None of these was reduced significantly by VPA exposure. When the motor nuclei were reexamined in adult rats exposed to VPA
Fig. 4. Still higher magnifications from the regions shown in Figure 3b, d, and f confirm that facial neurons were well-stained in the autistic brain (a and b). An arrow indicates the lone motor neuron at level b. In c, where no motor neurons were present, small interneurons and giant reticular neurons (arrows) were well-stained.

Fig. 5. The distances between structures of the tegmentum are represented for control and autistic brains. The scale is in millimeters. Note the gap between the facial nucleus and the inferior olive in the autistic brain, and the short distance between the trapezoid body and the inferior olive in the same brain as compared to the control. The region in which the autistic brain was shortened lay in the middle of one of the blocks of tissue under study (compare Fig. 1). Any loss of tissue between the two blocks would make it appear that the hypoglossal (from the second block) was moved orally relative to the inferior olive. Instead, the hypoglossal-inferior olive relationship was normal and both structures were moved orally to about the same degree.

controls was 155 neurons on each side, with a standard error of 9.8. The mean number of neurons on each side in eight treated animals was 92 with a standard error of 16.4. The difference in means of total cell counts was significant at $P < .001$ (df = $\infty$; $F = 13.9$). The average reduction in abducens neuron numbers in rats exposed to valproic acid on day 12 was 41%, and each animal had cell counts lower than the mean of controls. The correlation between abducens neuron counts from the left and right sides of the brain of each animal was 0.82 in controls and 0.92 in the VPA-exposed animals, indicating that the number of neurons in the two nuclei was a characteristic of each animal, so that the animal with the smallest left nucleus had the smallest right nucleus, etc. There was a range of effects in the treated animals, but within an animal the effect of treatment was consistent.

It is worth noting that the female animal with near-total destruction of the abducens nucleus had a brain weight of 0.98 g, while the mean of all female controls was 1.01 g. That is, the brain size of the most affected brain was not grossly reduced. In addition, comparison between groups of the number of histological sections on which each nucleus appeared (an estimate of the length of the nucleus) did not differ. Thus, in nuclei with reduced cell numbers, a smaller number of cells was spread over the same area taken up by larger numbers of cells in controls.

**DISCUSSION**

**Discussion of the autopsy case**

The injuries observed in the autopsy case could account for some of the patient's symptoms, such as her lack of facial expression, and are consonant with the symptoms of
The hypoglossal nucleus of the autistic case contained a region surrounded by fine myelinated axons, with fibers connecting to the medial longitudinal fasciculus (MLF). The cells within the fibrous ring do not appear to have developed into normal motor neurons.

**TABLE 1.** Mean Brain Weight (g), Body Weight (g), and Brain/Body Ratio (%) for Treated (VPA on Day 12.5) and Control Animals Sacrificed at PN 10 or PN 60

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<td>Young controls (n = 8m, 7f)</td>
<td>1.18 (.02) 19.2 (.51) 6.01 (.20)</td>
<td>1.09 (.00) 17.6 (.10) 6.25 (.22)</td>
</tr>
<tr>
<td>Young treated rats (n = 7m, 6f)</td>
<td>0.97** (.03) 16.3** (.56) 6.14 (.12)</td>
<td>0.94** (.03) 15.19 (.10) 6.24 (.29)</td>
</tr>
<tr>
<td>Adult controls (n = 17m, 13f)</td>
<td>2.01 (.04) 40.8 (.73) 8.39 (.00)</td>
<td>1.97 (.05) 25.6 (.94) 8.26 (.04)</td>
</tr>
<tr>
<td>Adult treated rats (n = 19m, 10f)</td>
<td>1.89** (.07) 34.0** (.53) 6.55 (.09)</td>
<td>1.64** (.11) 20.6* (.6) 7.87 (.05)</td>
</tr>
</tbody>
</table>

*PN. postnatal day.
**Different from controls at P < .01, by analysis of variance. Comparison of brain/body ratio of adult males is borderline (P < .059), with treated animals having higher ratios than controls.

The facial nucleus and the superior olive form at the same stage of development (Altman and Bayer, 1980), and they also derive from the same rhombomeres (Chisaka et al., 1992). Thus, the pattern of abnormalities in the autopsy brain could be explained by an inherited or exogenously caused disruption of pattern formation for the rhombomeres, as well as by direct interference with neuron production. The shortening of the hindbrain we observed is best explained by loss of a horizontal band of developing tissue. In addition, the normal appearance of the inferior olive, which forms from the rhombic lip (Harkmark, 1954) at the same time that the facial nucleus and superior olive are forming (Altman and Bayer, 1980) in the fourth and fifth rhombomeres, favors the idea that the injury was related to rhombomeric patterns rather than cell production time.

**Hoxa-1** transgenic knockout mice lack the facial nucleus and superior olive and have malformations of the inner, middle, and external ear (Chisaka et al., 1992). These defects have been interpreted to represent a complete failure of formation of the fifth rhombomere, with structures normally associated with the more caudal rhombomeres shifted orally in the shortened hindbrain (Chisaka et al., 1992; Carpenter et al., 1993). A second **Hoxa-1** knockout has slightly different characteristics, including the reduction or absence of the abducens nucleus along with the facial (Lufkin et al., 1992). This mouse is thought to have a major reduction of the fourth rhombomere, absence
of the fifth, and possibly some reduction of the sixth (Mark et al., 1993). Again, the caudal elements of the brainstem appear shifted toward the motor nucleus of trigeminal. Both mutants have abnormalities of neural crest derivatives, such as the sensory ganglia of cranial nerves VII, VIII, IX, and X, and abnormal development of the ears. Related abnormalities of patterning of rhombomeres have been produced experimentally by exposure of mice to teratogens just before closure of the neural tube. For example, disruption of expression of Hoxb-1 and c-1 by retinoic acid affects the third rhombomere, leading to reduction of numbers of neurons and misdirection of axons from the motor nucleus of trigeminal (Kessel, 1993).

It should be noted, as well, that motor neurons can be lost or damaged selectively at times other than the period when they proliferate. For example, disturbances of blood pressure have been shown to cause selective degeneration of cranial nerve motor neurons long after their formation (Gilles, 1969). Some cases of Moebius syndrome are thought to occur in this way during later stages of gestation (Lipson et al., 1989). While there has not been a report connecting this mechanism to autism, it is certainly possible that degeneration of neurons, as well as failure of their production, could lead to autistic symptoms. Degeneration of previously formed neural elements must be the mechanism of injury in the rare instances in which the symptoms of autism have been observed to follow encephalopathies (DeLong et al., 1981; Gilberg, 1986). Thus, we do not mean to suggest that the absence of motor neurons alone implies an injury during their production. However, the invasion of myelinated fibers into the space normally occupied by the nucleus is a feature of the autistic brain which suggests that the neurons were already absent when the architecture of the area was established. Further, the combination of neuron loss and shortening of the fifth rhombomere region of the hindbrain in the autopsy case cannot be reconciled with later loss of previously formed elements. Only a failure of formation of elements could leave a brainstem shortened in this way.

On the basis of a single case, one cannot predict what the anatomy of the pons and medulla in other autistic cases should be. However, adding other cases would not provide any further information about when this case was injured. The data indicate that the patient’s brain was injured during or soon after closure of the neural tube. When these data are combined with those from the thalidomide autistic cases, in which an external defect marks the time of exposure at day 20–24 and the neurological symptoms indicate damage to the cranial nerve motor nuclei, the obvious conclusion is that an early insult to the basal plate can cause brain development to deviate in a way that leads to autism.

**Discussion of the animal studies**

The rat data demonstrate that the kind of brain damage shown in our autopsy case and predicted by the thalidomide cases can result from early exposure to a teratogen. It is possible to damage the basal plate derivatives of the neural tube without obvious derangement of the development of later-forming structures. Further, it is possible to create this damage without gross somatic malformations or changes in viability.

At the exposure times investigated, VPA apparently works as an antimitotic agent, eliminating cells forming at the time of exposure and sparing others. This pattern of effects, in which the outcome differs sharply with even minor changes in exposure time, is characteristic of many teratogens (e.g., Wilson, 1959; Rodier, 1986). However, the lack of effect observed in cell groups other than those innervating somatic muscle suggests that VPA is selective in its effects on some early-forming cell groups recover more than others. We do not know the basis for this selectivity, but it may be related to the similar pattern of effects observed in the human case, in which there appeared to be sparing of some early-forming nuclei, such as the dorsal motor nucleus of the vagus. We are now investigating exposure to VPA before neural tube closure, to determine whether it has the potential to disrupt the development of the rhombomeres.

The possibility of modeling the underlying defect of autism in animals offers many valuable research opportunities. For example, we intend to study the limbic system and the cerebellum in animals with motor neuron deficits, to determine whether this early injury leads to changes in the development of later-forming parts of the brain. In addition, both the thalidomide data and our own human data suggest that early motor neuron loss disrupts connections in the brainstem. This has been observed already in teratogenic (Kessel, 1993) and genetic (Mark et al., 1993) abnormalities of the hindbrain and may represent a general principle of failure of axonal guidance under conditions in which important landmarks are absent or misplaced. We can now investigate connections in rats exposed to valproic acid, examining both the projections of injured motor nuclei and the projections of axons from the sensory nuclei, which grow past the motor nuclei during development. It is our hypothesis that both motor and sensory pathways develop abnormally when many motor neurons are lost early in CNS development.

We have not evaluated any aspect of behavior in animals with brainstem lesions, because we have no tests for rodents that address the functions used as diagnostic criteria of autism. The problem with finding animal equivalents for behaviors involving speech is obvious, but the problems with other communicative and social behaviors are almost equally difficult. For example, because virtually any brain injury alters social behavior, it should be easy to prove that animals like the ones we have described have abnormalities of social interaction, but such a demonstration would be meaningless in regard to separating behaviors specific to autism from behaviors resulting from brain damage in general. For many behaviors, it is difficult to produce evidence that they are the same in different species. Are the repetitive movements so common in mice related to the repetitive movements of autism? Are ultrasonic vocalizations of infant rodents related to human speech? Creating animal versions of the present diagnostic behaviors would carry a very high risk of false positive effects. Several more specific measures have been reported to be abnormal in autism (e.g., vestibular reflexes: Ornitz, 1983, auditory reflexes: Hayes and Gordon, 1977) and these might be useful in rodents. However, since these measures are not “diagnostic,” occurring in only a subset of autistic cases, positive results would only support the idea that the subjects were brain-damaged. The development of a useful animal model of the behaviors characteristic of the disease awaits identification of behavioral effects in humans that are discriminating and that could be tested very specifically in other species. However, an animal model of the developmental anomalies underlying autism can be created without the same problems of transferring measures from one
species to another, because early anatomical development is so similar, and so well-known, from one mammalian species to another.

**General discussion**

How do the present findings fit with the literature on autism? We believe that they are in agreement with all that is known about the disease. For example, Piven and Folstein (1994) have summarized the large literature on genetic factors in autism as indicating an increased risk for autism in siblings of autistic cases and an elevated incidence of social, language, and cognitive deficits among parents and siblings of autistic individuals. The data suggest that what is inherited is not autism but a “genetic liability” for the disease. This pattern is similar to that seen in many birth defects. For example, intensive study has revealed that there is a major genetic component in human heart defects (Boughman et al., 1993), even though we also know that specific teratogens delivered at critical periods of development can induce the same defects. In heart defects, as in autism, we do not know exactly how the genotype makes the defect more likely, but it is easy to imagine how such associations might occur. For example, variation in levels of specific growth factors at critical embryonic stages might either increase susceptibility to injury or decrease an embryo’s ability to recover from an insult. This pattern has been demonstrated for neural tube defects in mice. The Pax-3 gene deletion of Splotch mice leads to high rates of spontaneous neural tube defects in homozygotes, while heterozygotes are more susceptible than controls to neural tube defects induced by exposure to toxic agents (Dempsey and Trasler, 1983). Given the similarity of the lesions reported in Hoxa-1 knockout mice to those observed in the autopsy case, the anteriorly expressed Hox genes are excellent candidates for genes that might be defective in families with high rates of autism.

The behavioral symptoms of autism can be caused by a number of brain-damaging genetic defects, such as phenylketonuria, fragile X, tuberous sclerosis, and Rett syndrome (Reiss et al., 1986). The brain damage in these conditions is not selective. It is so widespread and/or variable that little information on the origin of autism is likely to come from these cases. The association of autism with Joubert’s syndrome (Joubert et al., 1969; Holroyd et al., 1991) is more informative, in that the eye motility problems, hypoglossal weakness, and cerebellar defects of that genetic syndrome point to an injury centered in the brainstem.

One interesting feature of the histological observations in autistic cases is that some of the abnormalities appear to change over time. For example, in the youngest cases, neurons of the nucleus of the diagonal band of Broca, the deep nuclei of the cerebellum, and the inferior olive appear swollen but normal in number, while in older cases the cells of the same structures are small, and cell numbers in the nucleus of the diagonal band and the deep nuclei are reduced (Kemper and Bauman, 1992). The progressive changes may signify that these regions are responding to abnormalities in their target cells or to abnormalities in input. The relationships of all these structures are complex, and the relationships among the injuries are difficult to interpret. For example, Bauman and Kemper (1994) point out that the most affected deep nuclei are those reciprocally related to the unaffected accessory nuclei of the olivary complex, while the least affected nucleus, the dentate, is intimately related to the most severely damaged regions of the cerebellum and to the clearly affected principal nucleus of the inferior olive. It is possible that these effects are secondary to a primary injury of the cerebellar cortex, but it is also possible that the effects on the olive, the deep nuclei, and the cerebellar cortex are all secondary to still earlier injuries.

The cerebellar histology and brainstem MRI studies are of special interest in relation to the present studies, for the first neurons destined to populate the cerebellum appear soon after the production of the motor cranial nerve nuclei. In the rat, most neurons of the deep nuclei of the cerebellum are born on embryonic day 14, with a few produced on days 13 or 15. More than 90% of Purkinje cells form on days 13 or 15. At an early age of 7 days, 72% of Purkinje cells were normal in number, while 28% were reduced (Kemper and Bauman, 1992). The progressive reduction of neurons in the deep nuclei of the cerebellum, and the inferior olive appear to be 350 mg/kg VPA day 12. The rates of autism are correlated with changes in the deep nuclei of the cerebellum, and the inferior olive appear to be 350 mg/kg VPA day 12. The rates of autism are correlated with changes in the deep nuclei of the cerebellum, and the inferior olive appear to be 350 mg/kg VPA day 12.

<table>
<thead>
<tr>
<th>Neuron Groups in Young Animals</th>
<th>Facial</th>
<th>Oculo-motor</th>
<th>Abducens</th>
<th>Hypoglossal</th>
<th>Trigeminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Evans controls (n = 19)</td>
<td>44.5 (2.6)</td>
<td>38.7 (1.4)</td>
<td>24.2 (1.6)</td>
<td>26.5 (2.0)</td>
<td>67.5 (5.0)</td>
</tr>
<tr>
<td>Sprague-Dawley controls (n = 12)</td>
<td>53.0 (3.4)</td>
<td>36.3 (1.3)</td>
<td>24.2 (1.8)</td>
<td>29.3 (2.1)</td>
<td>85.4 (5.1)</td>
</tr>
<tr>
<td>350 mg/kg VPA day 12 (n = 8)</td>
<td>33.9 (2.8)</td>
<td>30.4 (3.4)</td>
<td>19.1 (1.9)</td>
<td>23.3 (1.9)</td>
<td>72.8 (6.1)</td>
</tr>
<tr>
<td>350 mg/kg VPA day 15 (n = 8)</td>
<td>29.2 (2.6)</td>
<td>30.4 (1.1)</td>
<td>13.3 (1.1)</td>
<td>22.6 (0.8)</td>
<td>72.5 (7.7)</td>
</tr>
</tbody>
</table>

**Different from controls at P < .01, by analysis of variance with post hoc Dunnett tests.**

TABLE 3. Mean (S.E.M.) Number of Neurons in Other Early Forming Neuron Groups in Young Animals

<table>
<thead>
<tr>
<th>Neuron Groups in Young Animals</th>
<th>Facial</th>
<th>Oculo-motor</th>
<th>Abducens</th>
<th>Hypoglossal</th>
<th>Trigeminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Evans controls (n = 19)</td>
<td>21.2 (2.4)</td>
<td>57.4 (4.6)</td>
<td>31.9 (2.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley controls (n = 12)</td>
<td>17.5 (1.1)</td>
<td>51.2 (3.3)</td>
<td>28.7 (1.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>350 mg/kg VPA day 11.5 (n = 8)</td>
<td>25.4 (4.1)</td>
<td>60.3 (4.8)</td>
<td>30.6 (2.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>350 mg/kg VPA day 12 (n = 8)</td>
<td>15.7 (2.9)</td>
<td>65.3 (4.4)</td>
<td>31.6 (2.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>350 mg/kg VPA day 12.5 (n = 11)</td>
<td>18.1 (1.0)</td>
<td>57.0 (3.4)</td>
<td>28.9 (1.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 7. These photographs contrast the abducens nuclei of a control rat (a,b), pictured at the level of the genu (g) of the facial nerve, and the same level of the nucleus in a rat treated with valproic acid on the twelfth day of gestation (c,d). Arrows indicate examples of the large motor neurons of the abducens nucleus. Counts of serial sections from the treated brain showed no motor neurons on the left side and only a few on the right, while numerous motor neurons are seen in all control brains.

TABLE 4. Mean (S.E.M.) Number of Motor Neurons in Matched Sections from Five Motor Nuclei in Adult Animals

<table>
<thead>
<tr>
<th></th>
<th>Trigeminal</th>
<th>Hypoglossal</th>
<th>Abducens</th>
<th>Oculomotor</th>
<th>Facial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control (n = 12)</td>
<td>26.1 (1.8)</td>
<td>16.4 (1.2)</td>
<td>11.7 (0.9)</td>
<td>16.0 (1.9)</td>
<td>46.7 (2.0)</td>
</tr>
<tr>
<td>350 mg/kg VPA day 12.5 (n = 10)</td>
<td>22.2 (3.0)</td>
<td>10.3 (0.8)**</td>
<td>9.2 (0.9)</td>
<td>10.9 (0.8)*</td>
<td>44.6 (2.5)</td>
</tr>
</tbody>
</table>

**Different from controls at P < .01, P < .05 by analysis of variance. The abducens difference is borderline (P < .055).

1992). Similar oddities of nystagmus were reported in about half of a sample of cases with autism or “autistic-like conditions” (Rosenhall et al., 1988). Ornitz (1983) has reported abnormalities of postrotatory nystagmus and secondary nystagmus in autism and speculated that these are best explained by abnormal function of connections from the vestibular nuclei to the thalamus. Such symptoms as aversion to touch, sensitivity to food texture, and hyperacusis, as well as self-reports of synesthesia (Cesaroni and Garber, 1991), could be explained by miswiring of brainstem connections. Brainstem auditory response studies of autistic cases were reviewed recently by Klin (1994), who concluded that the results were too variable to support the hypothesis of a brainstem abnormality of the auditory system. However, even though most studies involved preliminary screening to exclude patients with hearing deficits, the recordings of the brainstem auditory response revealed peripheral auditory impairment in one-third to a half of the autistic subjects. There have been no assessments of the rate of facial diplegia in autism, but patients with Moebius syndrome have a particularly high rate of autism, between 30% and 50% in one study (Gillberg and Steffenberg, 1989). In summary, the idea that injuries of lower centers of the nervous system are often associated with autism is not new. What is new is that the discovery of the connection between thalidomide exposure and autism puts these neurological symptoms into a new context, relating them to the initiating injury of the disease.

Much of the discussion of autism in recent years has focused on the disease as a cognitive problem. In light of the new information provided by the thalidomide studies and the present ones, it is possible to interpret some symptoms, lack of eye contact, lack of facial social responses, delayed development of speech, as having simpler explanations at the brainstem level, but this does not mean that the cognitive aspects of the disease are not important. Clearly, many common symptoms of autism are best explained by alterations of higher levels of brain function. Obsessive interests, echolalia and other expressive language abnormalities, and the occasional ability to remember great detail are examples of symptoms which might involve functional
changes in the forebrain. We submit that such functional changes could arise from a normal forebrain with abnormal input from the brainstem or from abnormal development of the forebrain secondary to an early brainstem injury. In either case, the abnormalities which lead to cognitive dysfunction need not be initiated by direct injury to the late-forming parts of the brain.

Is the abnormal anatomy we have described the cause of the functional syndrome called autism? This is a question that can never be answered from human anatomical studies. Correlations between the disease and cerebellar size, cerebellar histology, limbic system histology, or histology of motor nuclei all suffer from the same problem: correlations do not address the issue of causality. Even if future studies were to show that some of these features characterize every case of autism, there would always be the possibility that the known pathology is irrelevant to the symptoms and that the symptoms are actually mediated by an abnormality that no one has recognized.

In summary, we cannot yet describe the abnormalities of the CNS which underlie the symptoms of autism. Our data and those of the cases induced by thalidomide and valproic acid make a strong case that the abnormalities of at least some cases include an injury to cranial nerve motor neurons and are initiated by an insult to the developing neural tube. It remains to be determined what developmental sequelae follow such an injury and how many other structures can be injured directly during the formation of the motor nuclei. We suspect that the sequelae and related injuries are many, that they include some or all of those reported by other laboratories, and that still other abnormalities associated with autism are yet to be described. The contribution of these studies is not that they identify the pathology underlying autism but that they identify the kind of injury from which that pathology takes its origin.

ACKNOWLEDGMENTS

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LITERATURE CITED


