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Converging evidence for brain stem injury in autism

PATRICIA M. RODIER
University of Rochester

Abstract
The hypothesis that brain stem injury plays a role in the autism spectrum disorders was suggested by evidence that exposure to thalidomide during the earliest stages of brain development increases the risk of autism spectrum disorders. The implications for the embryological origin of autism first led to studies of neuroanatomy in a human case and an animal model and then to examinations of minor craniofacial features in autism. But the general hypothesis had much broader implications. It has now generated studies of the behavioral and neurological symptoms of human patients, of human molecular genetics and population genetics, and of animal behavioral teratology and molecular pharmacology. The collection of this range of data was made possible by adding experts from many fields to the research team. They worked both independently and collaboratively to try to unravel the etiology of autism.

So much of modern clinical assessment and treatment is carried out by teams of people representing several different backgrounds that most clinical studies might qualify as multidisciplinary in personnel, even when they have a single hypothesis and a single dependent variable. For example, a genetic study of a developmental disability might involve the clinicians who diagnose the cases, the molecular geneticists who test their genotypes, and the population geneticists who analyze the data. A study of a pharmacologic treatment for a developmental psychopathology is likely to involve at least the psychologists or psychiatrists who diagnose the patients, the pharmacologist who designs the treatment, and the psychologists who test the patients for changes in symptoms. In many clinical settings, teams to carry out studies like these are already in place, working together.

Most of us use the term *multidisciplinary* to describe another kind of research, in which scientists from different backgrounds collect multiple measures related to a single problem. Unfortunately, this second kind of research is more difficult to establish. An example is a psychologist testing the behavior of an animal model of a disorder and then passing the animals to a neurophysiologist, who then studies them and divides the tissue between an anatomist and a biochemist. This study requires four testable hypotheses. The problem is that good hypotheses arise as new ideas and new

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Address correspondence and reprint requests to: Patricia M. Rodier, PhD, Box 668, OB/GYN, University of Rochester, 601 Elmwood Avenue, Rochester, NY 14642; E-mail: patricia_rodier@URMC.rochester.edu.

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methods become available. It is unlikely that scientists in four disciplines would have inspiration strike simultaneously with regard to the same disorder, unless there is some general hypothesis that generates test hypotheses in many fields. Investigators cannot create this kind of multidisciplinary effort just by willing it to occur. Further, in this second model, recruiting personnel is a major problem, because the model requires multiple independent investigators willing to work together. Employers and funding agencies demand that investigators be independent entrepreneurs. Scientists who are successful in the system are understandably reluctant to join a group effort unless it supports their own research agenda.

Successful multidisciplinary projects demonstrate a variety of different approaches. Hubel and Wiesel's studies of the development of the visual system (e.g., Hubel, 1982) are the perfect example of a scientific partnership. These investigators, a physiologist and an anatomist, shared the same interests. They developed hypotheses together and then applied their different skills to test them. Another model has scientists working separately but taking results from one field and testing their implications in another. An example is Smithells' test of the value of folic acid supplementation to reduce neural tube defects (NTDs) in women planning a second child after the birth of a child with an NTD (Smithells, Sheppard, Schorah, Seller, Nevin, Harris, Read, & Fielding, 1980). The hypothesis that folic acid might be important came from studies by teratologists that showed that NTDs resulted when animals were treated to disrupt the folic acid pathways (Nelson, Wright, Asling, & Evans, 1955). The inspiration to test women with evidence of a genetic susceptibility came from an obstetrician. The result was the demonstration that early supplementation reduced the number of NTDs dramatically. A third model is the research that leads an investigator from one field into another, necessitating the recruitment of collaborators with different expertise. Rutter's research on the inheritance of autism (e.g., Folstein & Rutter, 1977) led to the development of a large team, including many scientists with backgrounds outside of psychiatry. Clinical psychologists, geneticists, and neurobiologists have all contributed to the effort. All these examples have two things in common. First, they were driven by hypotheses. The goal was not to do a multidisciplinary project, but to test a general hypothesis that happened to create test hypotheses in more than one discipline. Second, they involved experts who saw an opportunity, rather than scientists compelled to work on a project outside their interests.

The General Hypothesis

Our studies of the etiology of autism are based on a general hypothesis that early injury to the brain stem is a cause of the autism spectrum of disorders. With the support of converging evidence from many different kinds of studies, this idea has generated a number of testable hypotheses that range over many levels of analysis. The result is projects in several different fields of science. Some involve collaborations on a single project (e.g., two sites collecting and testing patients for a cluster study), others have people trained at one site who evaluate a clinical population at another, and there is an investigator who travels among sites to do specialized testing. Several of the projects share animal models or genetic material. There is no question that this is multidisciplinary research, but it evolved so naturally from the demands of the hypothesis that we only think of it that way occasionally, typically when one of us cannot understand the language of another and has to request explanation. If the reader has trouble jumping from experiment to experiment, s/he is not alone. No one is an expert in all these fields. My job is to translate as well as I can.

Our general hypothesis comes from a study of the thalidomide-exposed population of Sweden. Two pediatric ophthalmologists set out to examine all the victims who were compensated for their injuries after in utero exposure to this drug. It had been noted as early as the 1960s that thalidomide exposure caused abnormalities of craniofacial innervation (d'Avignon & Barr, 1964), and Miller and Strömland were interested in the causes of strabismus. They ex-

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amined 86 patients, recording all their physical and neurological anomalies. The sample was rich in disorders of eye movement, just as the investigators had anticipated. To their surprise, four cases had severe psychiatric problems. Christopher Gillberg was asked to examine those patients, whom he identified as having autism spectrum disorders (ASDs). Because the somatic defects caused by thalidomide have been studied extensively in thousands of cases, the time table for the production of different defects is well known. Using that information, it is possible to determine the stage of development when a particular injury occurred. In the case of the patients with autism, the presence of ear anomalies and absence of limb anomalies pointed to injury between days 20 and 24 after conception (Miller, 1991; Strömland, Nordin, Miller, Akerstrom, & Gillberg, 1994). Only 15 of the subjects had this pattern of somatic defects, making the rate of autism about 30% among people injured during the critical period.

This period is the time when the neural tube of the embryo is closing and the first neurons are being created. They are ones destined to innervate the muscles of the eyes, face, jaw, tongue, and throat. The patients with autism had dysfunctions of some of these muscles, confirming that the exposure to thalidomide affected the development of craniofacial innervation, as well as causing autism.

Why is this study so important? First, it is a very fine study. The authors collected an enormous amount of data on every patient available for testing, even though that required some excursions to the northern parts of Sweden in winter weather. Because both investigators are among the world's top experts on the development of the eye and the evaluation of its form and function, their methods of assessment were the best available. By carrying out the study in people exposed to thalidomide, they were able to draw on a large empirical data base to relate their results to the underlying embryology of craniofacial development. Second, although autism was not the impetus for the investigation, the results address directly the issue of when autism is initiated. Prior to this study, that issue usually had been addressed theoretically, basing speculation about timing on more speculation about what might be wrong in the brain. In contrast, the thalidomide study does not appeal to theory in any way. The somatic malformations of the cases tell us the critical period when they were injured by thalidomide, and their neurological dysfunctions confirm the critical period by indicating injury to the very neurons known to form at that time.

I was fortunate to hear Marilyn Miller describe the thalidomide results before they were published and to have the opportunity to offer my congratulations and express the conviction that she had made an important breakthrough. She was already starting another team study in Sweden, this time looking at people with Moebius syndrome, who were thought to have a high rate of autism (Gillberg & Steffenberg, 1989).

Surprising results are always interesting, and this one was surprising because autism is known to be mainly genetic in origin. Twin studies had demonstrated this conclusively in the 1970s (Folstein & Rutter, 1977), and subsequent results have added support to the genetic hypothesis (Bailey, LeCouteur, Gottesman, Bolton, Simonoff, Yuzda, & Rutter, 1995). The few exogenous factors that already had been associated with ASDs were ones like rubella infection in the first trimester (Chess, 1977), which is known to cause widespread brain damage. The genetic disorders associated with autism (e.g., phenylketonuria, tuberous sclerosis) were also causes of widespread damage. Thus, they did not tell us much about which parts of the brain might be affected in autism. In contrast, the thalidomide study turned our attention to a small group of early forming neurons. There is nothing about the study that excludes additional developmental brain abnormalities that might occur as sequelae of the early injury, nor does it rule out the malfunction of other regions due to distorted input from the brain stem. However, it gives strong support to the hypothesis that the ASDs can arise from an insult during neural tube closure, when most of the structures that neuroscientists prefer to study have not begun to form.

What makes the hypothesis that brain stem injury leads to autism valuable? Here the is-

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sue is not the quality of the evidence on which the hypothesis is based, but whether its implications are testable. We started with the most obvious ones.

Test hypothesis 1

The thalidomide cases of autism are unique; they differ from idiopathic cases. Therefore, their physical and neurologic features are not characteristic of other cases. This hypothesis is definitely false. A review of the literature showed that other autism researchers had noted minor ear and facial anomalies (Steg & Rapoport, 1975; Walker, 1977) and dysfunctions of eye movement (Rosenhall, Johansson, & Gillberg, 1988; Scharre & Creedon, 1992), to say nothing of the lack of facial expression, which is part of the diagnostic criteria. At a conference at the NIH, I met Susan Bryson, a clinical psychologist who had carried out a now famous study of autism prevalence in Nova Scotia (Bryson, Clark, & Smith, 1988). She had collected data on minor malformations but had not yet analyzed them. Those data provided more evidence for ear anomalies being significantly more common in people with autism than in their siblings or in people with mental retardation (Rodier, Bryson, & Welch, 1997).

Test hypothesis 2

The brain stem injuries predicted by the thalidomide cases can be detected in the histology of brains of people with autism of unknown cause. This hypothesis was supported by an examination of the brain of a well-documented case of autism compared to the brain of a control (Rodier, Ingram, Tisdale, Nelson, & Romano, 1996). The number of facial nucleus motor neurons was reduced by more than 90%; the superior olive (an auditory relay nucleus) was absent. Some neurons of the hypoglossal nucleus appeared to have failed to differentiate. Details of structure indicated that the affected regions had never developed, rather than being lost later. For example, the boundaries of the facial nucleus and superior olive are normally outlined by passing fibers that skirt the nuclei, but in the brain of the person with autism, fibers passed through the places where the nuclei should have been, suggesting that the space had never been occupied. Further, measurements of the position of more caudal brain stem nuclei, such as the inferior olive, indicated that they were shifted orally, as though a band of tissue had failed to form in the region of the facial nucleus (See Figure 1; Rodier et al., 1996). This kind of change in the anatomy of the hindbrain could not occur after the earliest stages of central nervous system (CNS) development.

Test hypothesis 3

If thalidomide disturbs the development of the brain stem as the neural tube is closing, it should be possible to recreate that injury in an animal model. Here there was a technical problem, as thalidomide is not a proximal teratogen (In other words, thalidomide itself is not teratogenic. It is only after metabolism of the parent compound that teratogenic products are produced.) The metabolite of the drug that does the damage has not been identified, but it is not manufactured in rodents (Schumacher, Terapane, Jordan, & Wilson, 1968), nor is its mechanism of action known. We first tried an agent that interferes with cell proliferation, methylazoxymethanol (MAM), which we knew interfered with the formation of the neurons of the hypothalamus that produce luteinizing hormone releasing hormone. These neurons form at the same time as the neurons of the motor nuclei of cranial nerves (Gavin, Kates, Hoffman, & Rodier, 1994), yet MAM exposure did not affect postnatal cell numbers in the motor nuclei of cranial nerves. In retrospect, we recognize that this is probably because those neurons are formed in double the needed quantity and later die off to appropriate numbers. Agents that interfere specifically with proliferation are probably not the ones most likely to reduce the numbers of neurons with this pattern of excess production followed by cell death. Fortunately, the same issue of *Developmental Medicine and Child Neurology* in which the thalidomide paper was published contained an article with case reports linking another teratogen to autism (Christianson, Chesler, & Kromberg, 1994).

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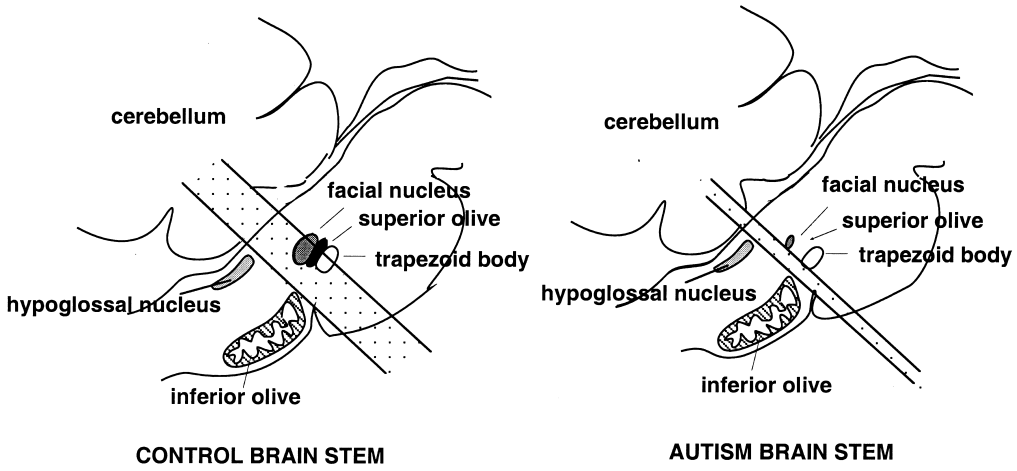


Figure 1. A sagittal view shortening of the brain stem observed in the brain of a person with autism. When the brain stem of a person with autism was studied in serial sections, it differed from that of a control in several ways. The diagrams show the reduction of neuron numbers in the facial nucleus, the absence of the superior olive, and the shortening of the brain stem observed in this case.

This was valproic acid (VPA), an antiseizure medication earlier demonstrated to cause high rates of developmental delays and neurological dysfunctions in offspring (Ardinger, Atkin, Blackston, Elsas, Clarren, Livingston, Flannery, Pollock, Farrod, & Lammer, 1988), as well as limb and craniofacial anomalies that resemble those of thalidomide. This was a teratogen already known to cause similar deficits in rodents (Binkerd, Rowland, Nau, & Hendrickx, 1988). Subsequently, a larger study of children exposed to anticonvulsants in utero showed that of 46 exposed to VPA alone or in combination with other drugs, 5 were diagnosed with ASDs (Moore, Turnpenny, Quinn, Glover, Lloyd, Montgomery, & Dean, 2000).

Teratogenic doses of VPA did reduce the number of neurons in the cranial nerve motor nuclei (Rodier et al., 1996), supporting the hypothesis that the morphologic effects seen in humans could be reproduced in animals. Further, valproate exposure shortened the brain stem, the same effect we saw in the human case.

Having been trained as an experimental psychologist, I had spent a lot of time thinking about how to test an animal model for behavioral parallelism to autism, but I could not think of any way to translate the diagnostic behaviors into something applicable to animals (Rodier, Bryson, & Welch, 1997). Susan Bryson was working on simpler human behaviors that might discriminate autism, but it was likely to take years to finish the human studies and more years to develop and test an animal version. I discussed this problem with Mark Stanton, an expert on how teratologic brain damage is reflected in animal behavior. We were speaking at a conference in the United Kingdom, and had several days to mull over how animal models might be assessed. He did not have an immediate solution, but he was excited by the brain stem hypothesis and interested in working on the problem.

A new test hypothesis requires collaborators

The results in the human brain and animal model studies suggested another hypothesis. The neuroanatomy we were studying bore a striking resemblance to that of a knockout mouse (Carpenter, Goddard, Chisaka, & Manley, 1993; Mark, Lufkin, Vonesche, Ruberte, Olivo, Dolle, Gorry, Lumsden, & Chambon, 1993). The knocked out gene, *Hoxa1,* was one that is expressed only around the time of neural tube closure, and the expression occurs in the developing hindbrain (Murphy & Hill,

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Table 1. *Comparison of human histology to the phenotype of the Hoxa1 knock-out mouse*

	Hoxa1 Knockout	Human Autism
Absence of		
Posterior part of facial nucleus	Yes	Yes
Superior olive	Yes	Yes
Abducens nucleus	Yes	NA
Maldevelopment of		
Inner, middle, and external ear	Yes	NA
Cranial sensory ganglia	Yes	NA
Hypoglossal nucleus	NR	Yes
Shortening of brain stem at fifth rhombomere	Yes	Yes

Note: NR, not reported; NA, not available.

1991). It codes for a transcription factor that controls the expression of other genes. It is the first of the *Hox* family of genes to be expressed in the embryo, and it initiates a cascade in which members of the family responsible for the development of more and more caudal structures turn on in sequence. These genes establish the body axis, and the early ones are critical to development of the brain stem. They are among the most highly conserved of all genes across species (Means & Gudas, 1995).

At the time the *Hoxa1* idea arose, it was believed that the whole family of *Hox* genes (39 in all) was immutable. No one had ever found a naturally occurring polymorphism of any of the *Hox* genes in any mammal. The best guess was that any change in these critical early developmental genes must be lethal. The *Hoxa1* knockout mice (homozygous for the damaged gene) supported that idea by dying soon after birth, but heterozygotes survived. If a variant form of the gene that was compatible with survival existed, we would have to find it. We would be starting from ground zero. Still, we were convinced that the correspondence between effects reported in *Hoxa1* knockout mice and those seen in our human brain study was too specific to be an accident (see Table 1).

The implications of this are both obvious and exciting. If there is a gene that controls the development of the parts of the brain altered in human histology, might that gene be one of those involved in the genetic susceptibility to autism? The studies we had carried out had given support to the general hypothesis that a brain stem injury was involved in autism, but they did not put us much closer to preventing such injuries because most cases could not have been exposed to thalidomide or valproate. There were probably other teratogens that could cause similar changes in the developing brain, but the evidence that autism is genetic was overwhelming. Groups all over the world had been searching for the genes for years without success.

The evidence said *HOXA1* should be studied, but we were ill equipped to mount the effort. We had no training or experience in genetics. Furthermore, we had no population of people with autism to study. A meeting with Denise Figlewicz solved the first problem. Not only was molecular genetics her specialty, but she had years of experience in detecting mutations and testing candidate genes. Her interest was in degenerative diseases of the nervous system, but her neuroscience background made our project interesting to her. She would teach us how to do it. The second problem resolved just as easily a few months later. Susan Hyman, a developmental pediatrician with extensive research experience, moved to Rochester determined to reduce her clinical load and devote more time to research. Her knowledge of developmental disabilities was impressive. She said she would be happy to recruit families for the study. At this point, we were ready to begin a multidisciplinary study to test the hypothesis that *HOXA1* was

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involved in susceptibility to autism. The hypothesis was such an obvious corollary of the previous work that the multidisciplinary part did not seem too foreign. Figlewicz could handle the molecular requirements and Hyman could supply the clinical sample.

As I continued to think about the genetic studies, I met Lorraine Gudas at a National Institute of Environmental Health Sciences (NIEHS) conference on CNS development. Here was one of the people who first sequenced *Hoxa1* in the mouse, and she was full of new ideas and new techniques for animal studies. In many ways her interests were close to mine, but her molecular approach to developmental biology was beyond my comprehension. Fortunately, she is also a good teacher, and we agreed to develop more animal studies to compliment the human studies.

About this time we were funded by the EPA and the National Institute of Child Health and Human Development (NICHD) to extend the studies of the VPA animal model. We got a small local pilot grant to start the mutation detection studies. Susan Hyman collected DNA from several patients, including two affected brothers. Denise Figlewicz patiently introduced my graduate student, Jennifer Ingram (a toxicology graduate student with an undergraduate specialization in inorganic chemistry), to the intricacies of molecular genetics. Jennifer was soon producing sequencing reactions and reading the nucleic acid code of *HOXA1* from the DNA of people with autism. The work was tedious, but the gels had the same mystic beauty that morphology has for me. Because I did not really understand how a series of reactions could put a DNA sequence on a gel, I could only think of the whole process as magic.

Jennifer and Denise continued to produce sequences and read them for several months. Many thousands of bases were checked, but all matched the published sequence. Then, Jennifer spotted something. One of the affected brothers had a single base substitution in one of his copies of *HOXA1*. It was in an area near the beginning of the coding region, where the sequence was often compressed and hard to read. She did new reactions and designed runs to spread out the region. Now we could see that the same substitution was present in both brothers and in one of their parents. This was no fluke. The substitution was being inherited, and it appeared in the DNA of several other people with autism. Even better, the substitution changed the code for a histidine to that for an arginine. The change interrupted a string of histidine repeats considered critical to the function of the gene product. In fact, Lorraine Gudas had already suggested that this region was an important one to investigate.

The substitution could be detected by a faster method. If we amplified the first exon of *HOXA1* by polymerase chain reaction (PCR) and digested the product with a restriction enzyme that recognizes the normal sequence, we would get fragments of different lengths in people with and without the substitution. Tests of Dr. Hyman's cases ran about 50% positive for the variant sequence. Dr. Figlewicz was able to offer a set of DNA samples of people ascertained through probands with other disorders. We hoped that none would have the substitution. Unfortunately, some did, but the rate was much lower than in the small sample of individuals with autism. We felt that we were pursuing something promising. A postdoc trained in toxicology–pharmacology, Christopher Stodgell, arrived. Along with Jennifer Ingram, he would soon be at home in the land of molecular genetics. A NICHD-funded RO1 for the genetic studies allowed us to add a full-time molecular technician and part-time help from a clinical psychologist and a speech pathologist.

I have taken the route of describing the sequence of events in 1993–1997 because I know no other way to explain how we came to apply for a program project grant. In essence, we had already started a multidisciplinary effort by accident. Making it official could only make it easier. The combination of a general hypothesis with many implications and the interest of many experts in those implications allowed us to construct hypotheses that made sense to reviewers. In the next section, I describe some of the results generated thus far and some of the new approaches we hope to apply in studying the etiology of autism. First, I want to point out the back-

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grounds of the investigators on the program project grant.

My interest is in injuries to the developing nervous system. Starting as an experimental psychologist and doing a postdoc in embryology–teratology broadened my skills, but when my department needed gross anatomy teachers, I really started to learn another discipline. Because I had taught both the embryology and the adult innervation of the head and neck for many years, I had the right background to appreciate the thalidomide study. I suspect that it is no accident that several of the key people in the autism project have crossed disciplinary lines during their careers. Susan E. Bryson (Toronto) is an experimental clinical psychologist who was once a nurse. Her first big grant was for an epidemiological study of autism. Why did she recruit a dysmorphologist for her team? I think her medical background suggested it. Mark E. Stanton (Research Triangle Park) has always been an experimental psychologist, but he has done both animal and human studies and is an experienced toxicologist, having worked at the EPA for many years. Lorraine J. Gudas (New York City) has her PhD in biochemistry but is the chairman of a pharmacology department. Her interest is in the role of early developmental genes in cancer and in the endogenous controls of those genes, especially retinoic acid. Denise A. Figlewicz followed her interest in neurodegenerative disorders through graduate school in biochemistry into postdocs in neuroscience and molecular genetics. Susan L. Hyman is a developmental pediatrician who did a fellowship in neuroscience research, studying neurotransmitters in mice, and then proceeded to do clinical research on several developmental disabilities. Marilyn T. Miller (Chicago) is the pediatric ophthalmologist who initiated the thalidomide study. She did graduate work in genetics before she went to medical school. Unlike some of us, Hyman, Bryson, and Miller had experience with various kinds of clinical teamwork in both patient care and research. Early in her career, Miller had collaborated with Samuel Pruzansky, an expert on congenital disorders who often involved investigators from several fields in his studies. Her studies in Sweden were carrying on that tradition. Not only were all these people unusually broad in their research experience, but they shared an interest in the development of the nervous system that went well beyond the focus of their own research. It is unclear whether they were willing to work on the general hypothesis because of their backgrounds or because of the appeal of the hypothesis itself, but both factors were probably important. All were active, successful investigators. Far from being dragooned into service because of proximity, they were willing to collaborate despite being scattered around the United States and Canada.

Converging Evidence From a Multidisciplinary Program

Brain stem injury and the cerebellum

Jennifer Ingram had done the first studies of the anatomy of the valproate-exposed rat model, demonstrating that the treatment resulted in reduced numbers of motor neurons in several brain stem motor nuclei (Rodier et al., 1996). The literature of histology of human cases of autism suggested that Purkinje cell numbers were consistently reduced (Bailey, Luthert, Dean, Harding, Janota, Montgomery, Rutter, & Lantos, 1998; Bauman & Kemper, 1985; Ritvo, Freeman, Scheibel, Duong, Robinson, Guthrie, & Ritvo, 1986). Some magnetic resonance imaging (MRI) studies indicated that, compared to controls, people with autism had volume reductions in the cerebellar vermis, especially its posterior part (Courchesne, Townsend, & Saitch, 1994; Hashimoto, Tayama, Murakawa, Yoshimoto, Miyazaki, Harada, & Kuroda, 1995), whereas others failed to find any difference (Kleiman, Neff, & Rosman, 1992; Piven, Nehme, Simon, Barta, Pearlson, & Folstein, 1992). We had designed the initial studies of the brain stem after valproate exposure to injure the developing brain during the critical period indicated by the thalidomide study. The treatment preceded the first production of Purkinje cells by several days. The plan was to then test the cerebellum to see whether it was altered as a result of the disruption of earlier forming structures. When Jennifer completed a stereological evaluation of the cerebellum in rats exposed to VPA be-

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fore the first Purkinje cells form, she found significant reductions in both Purkinje cell numbers and the volume of the cerebellum (Ingram, Peckham, Tisdale, & Rodier, 2000). The effect was greatest in the posterior part of the vermis, as predicted by human studies. The structures we had found to be damaged by the valproate exposure were not ones likely to affect the development of the cerebellum directly. However, the inferior olive forms at the time of the exposure and is an excellent candidate to impact the developing cerebellum. Several investigators found the inferior olive to be altered in cases of autism (Bailey et al., 1998; Bauman & Kemper, 1994). Both the volume of the nucleus and the number of neurons of the inferior olive are significantly reduced in the valproate model.

Eyeblink conditioning and visual orientation

Mark Stanton and Susan Bryson explored an essential problem that we discussed from the beginning: what behavioral measures can be used to test for parallelism to autism in animal models? This is hardly an academic issue. With the valproate model already established anatomically and the prospect of genetically engineered models to come, better behavioral tests of animal models are an urgent requirement. Stanton found a human study that indicated very specific deficits in eyeblink conditioning in people with autism (Sears, Finn, & Steinmetz, 1994). The effects were unusual compared to most demonstrations of eyeblink conditioning abnormalities after brain damage. The acquisition of the conditional response was accelerated and there were differences in its timing. Here was a paradigm that could be applied to animals in a task virtually identical to that used in human subjects. Further, the CNS circuits contributing to the rate of conditioning and the timing of the response are well known. These aspects of conditioning are mediated exclusively by loops between the brain stem and cerebellum (Lavond, Kim, & Thompson, 1993). The task can be adjusted to test the integrity of the hippocampus, because conditioning with the introduction of a delay in the procedure (trace conditioning) is known to be disturbed by hippocampal lesions (Schmajuk & DiCarlo, 1991; Woodruff–Pak, 1993).

As both groups received repeated sessions of conditioning trials, Dr. Stanton established that valproate-exposed rats did not differ from controls in their response to the tone that would serve as the conditional stimulus, nor in their response to the tiny shock that would provoke a reflexive blink. Just as in the human studies (in which the shock is replaced by a puff of air), the brain-damaged animals acquired the conditional response more rapidly than the controls. In addition, they demonstrated a difference in timing (Stanton, Erwin, Rush, Robinette, & Rodier, 2001). This confirms that the brain stem injuries of the model are sufficient to affect behavior, and the results parallel those reported in people with autism (Sears et al., 1994). When the paradigm was changed to trace conditioning, there was no evidence of hippocampal dysfunction in the valproate-exposed rats.

Preliminary data have now been collected on two maze tasks often used to assess frontal lobe injury. Treated animals did not differ from controls on delayed alternation but were significantly impaired on a reversal task (Stanton, 2000). These measures will next be collected from genetically engineered mice provided by Lorraine Gudas. Dr. Stanton has trained a postdoc and a technician to carry out eyeblink conditioning studies in human children. They have now moved to Toronto, where they are comparing children with autism to several other groups of children at Dr. Bryson's lab.

Many of the same children have already been tested on a simple visual orienting task being used by Dr. Bryson and her students (Landry & Bryson, 2001). Like eyeblink conditioning, the orienting task requires no instructions and is not related to IQ. It can be performed on infants. We hope it eventually can be translated for animal testing. The subject faces three screens where stimuli are presented, and a videocamera records the subject's eye movements. On some trials, flashing lights appear on the middle screen, prompting a reflexive movement of the eyes to that screen. The screen then goes blank and the same stimulus appears on a side screen, prompting the

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eyes to move to that screen. On other trials the sequence of events is the same except for one variation: the stimulus on the first screen stays on as a second stimulus appears on an adjacent screen. The shift from one screen to the other required by the first kind of trial is automatic for all children, but the requirements of the second kind of trial are different. The child must disengage from the first stimulus before shifting to the second. In children with autism this more complex process often fails to occur at all. Instead, on about a third of the trials requiring disengagement, children with autism get "stuck" on the first stimulus and never reorient to the second.

Notice that the eye movements required by the two conditions are identical. It is some higher order processing in the nervous system that differs. Bryson and her students studied the development of the ability to disengage and shift attention (McConnell & Bryson, 2001). It appears in the third or fourth month of life, long after shifting is seen in younger infants. Among the behaviors used to diagnose autism, none is exclusive to autism and none is seen in every case. It is only by the coexistence of many of the diagnostic behaviors in one individual that we can discriminate people who have the disorder from people who do not. In contrast, the visual orienting task, alone, discriminates autism from typical development and from other developmental disabilities with an accuracy of essentially 100% in the cases tested thus far (see Figure 2).

Conditions behaviorally related to autism

One of the goals we set for a clinical study being carried out jointly between Toronto and Rochester was to examine a host of biological and behavioral variables in children with diagnoses of autism or any of several behaviorally related disorders: Asperger syndrome, Moebius syndrome, and semantic–pragmatic language disorder. The result will be a cluster analysis to determine whether all these disorders belong on the autism spectrum or whether they are different from autism. Many alternative results could be obtained. Perhaps Asperger syndrome is related to semantic–pragmatic language disorder, and autism is more related to Moebius syndrome than to Asperger syndrome. That study will not be complete for some time, but I want to discuss Moebius syndrome here because a substantial number of these subjects have now been tested on the visual orienting task.

Gillberg and Steffenberg (1989) group had reported several Moebius cases comorbid for autism. After the thalidomide study, Marilyn Miller, Kirsten Strömland, and Christopher Gillberg set out to examine all the cases of this rare congenital neurological disorder that they could identify. Each of the four thalidomide cases with autism had Moebius syndrome, which involves a lack of innervation to the muscles of facial expression and the lateral rectus muscle of the eye. The two motor nuclei responsible for these muscles, the facial and the abducens, lie in close proximity at the pontomedullary junction of the brain stem. Clearly, the thalidomide exposures that caused autism disturbed the development of these nuclei, so it seemed reasonable that Moebius cases from other causes might be associated with autism. The causes of Moebius are multiple, sometimes genetic, and sometimes teratologic, as in the thalidomide cases. Studies of extended families with several affected members suggest that a number of different genetic anomalies can produce the syndrome. Linkage to chromosome 13 has been demonstrated in some families (Slee, Smart, & Viljoen, 1991; Ziter, Wiser, & Robinson, 1977), and linkage to chromosome 3 (Kremer, Kuyt, van den Helm, van Reen, Leunissen, Hamel, Jansen, Mariman, Franto, & Padberg, 1996) or chromosome 10 (Verzijl, van den Helm, Hamel, Kuyt, Padberg, & Kremer, 1999) has been demonstrated in others. The results from Sweden showed that 7 of 25 cases of Moebius had ASDs (Miller & Strömland, 1999).

Toronto is an internationally known center for the surgery used to give Moebius patients the ability to smile (by moving a strip of innervated muscle into the position occupied by the poorly innervated risorius muscles). Miller and Bryson planned to recruit families coming for the procedure in order to build a substantial group of cases. Before they started, there was a stroke of luck. The biannual meeting of the International Moebius Society was

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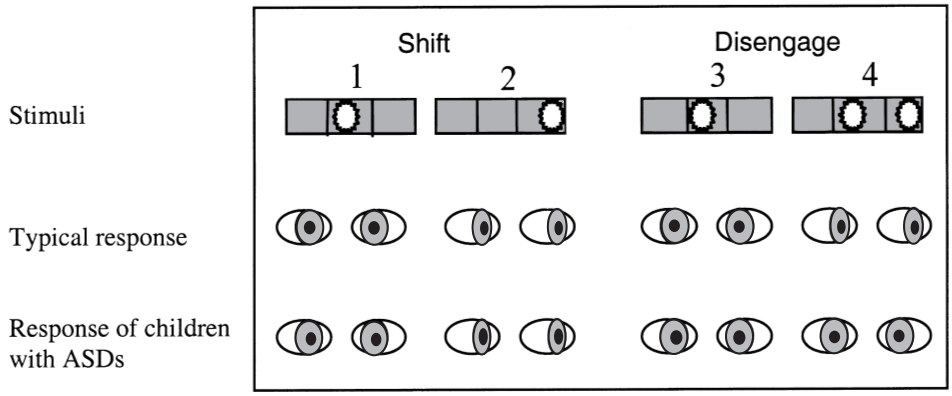


Figure 2. Reflexive eye movements elicited by two kinds of visual orienting trials. Children with ASDs perform like others when the trial requires shifting orientation, as in events 1 and 2; but when the first stimulus continues while the second one appears, as in 3 and 4, children with ASDs often fail to orient to the new stimulus.

set to be held in Toronto. The investigators rented several rooms at the convention hotel and set up a team. In one room, volunteers were examined by Dr. Miller; in another, they took an IQ test; in another they were tested for symptoms of autism by the Autism Diagnostic Observation Schedule (Lord, Rutter, Goode, Heemsbergen, Jordan, Mawhood, & Schopler, 1989); and in another, they performed the visual orienting task. Despite several long days, the investigators could not finish testing all the volunteers. They were overwhelmed by families who suspected that Moebius often means more than deficits of facial expression. A recent meeting of the same society in Boston yielded another set of volunteers. Thus, in a relatively short period, these intrepid researchers had recruited and tested the largest group of Moebius subjects ever examined.

The rate of autism in Bryson and Miller's sample is 35% (Bryson, Baron, Brian, Miller, Rombough, & Roberts, 2001). The method of ascertainment in Sweden focused on individuals who were institutionalized, and thus was biased toward those with mental retardation. The ascertainment in the North American study drew on cases whose families suspected behavioral problems and thus was biased toward cases with various behavioral anomalies. Both samples probably overstate the rate of autism in Moebius syndrome, however, the coincidence of the two disorders would be highly significant, even if it were only a fraction as frequent as these studies suggest. It is interesting that, although in the Swedish sample all the autism cases were at least mildly retarded, only about half the autism cases in the North American sample were retarded. Perhaps families of children without mental retardation are more likely to seek surgery or go to conferences. In any case, the new sample agrees with earlier chart reviews that MR is not present in the majority of Moebius cases (Holmes, Moser, Halldorsson, Mack, Pant, & Matzelivich, 1972).

When children with Moebius syndrome were tested on Bryson's visual orienting task, the investigators had no idea what to expect. What happens to reflexive eye movements in people lacking normal innervation of the eye muscles? The answer is that the reflex persists but is mediated through the neck muscles. The Moebius cases switched their orientation just like controls and about as quickly, but by moving their heads instead of their eyes. Were they able to disengage and shift? Those positive for autism could not do so reliably, but those negative for autism performed like controls (Bryson, Rombough, & McConnell, 2001).

The association of autism with Moebius syndrome is especially important for the general hypothesis that damage to the brain stem can lead to autism. Unlike the other genetic

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disorders associated with autism, Moebius results from what appears to be a relatively localized injury of the brain stem. Recalling that the facial nucleus and abducens nucleus are reduced or absent in the *Hoxa1* knockout mouse, that Moebius syndrome was present in thalidomide-induced autism cases, and that Moebius is often associated with ear malformations, the co-morbidity of idiopathic Moebius with autism gives strong support to the idea that there is something special about injuries around the time of neural tube closure that damage this region of the hindbrain.

We have seen that the timing of injuries can be inferred from the structure injured with thalidomide and many teratogens. Moebius syndrome offers an opportunity to ask the question, "Is the risk for autism a function of the timing of the injury or of the region that is injured?" The possibility of a test arises from the fact that Moebius syndrome can be caused in a way that is different from the thalidomide effect. We know it is different because it occurs at a different stage of development. Misopostol, an abortifacient in common use in South America, is a powerful prostaglandin. It works by inducing uterine contractions that expel the embryo. When it fails, the blood supply to the embryo can be compromised transiently, resulting in birth defects. This kind of teratogenic action affects structures that are already formed rather than those in the process of forming. Thus, when misoprostol abortions at about six weeks of gestation fail, the brainstem is sometimes affected. Moebius syndrome is one of the results. Marilyn Miller has been examining patients in Brazil, comparing children with idiopathic Moebius syndrome to those with the same disorder following misoprostol exposure. Preliminary results show that both groups have elevated rates of autism (Miller & Ventura, 2001). This suggests that it is the location of the injury that is critical.

More evidence supporting the importance of brain stem injury in autism comes from another rare genetic condition, Joubert syndrome (Joubert, Eisenring, Robb, & Andermann, 1969). This disorder appears to be inherited in a recessive pattern. Like Moebius syndrome, its genetic causes are heterogeneous, with some afflicted families showing linkage to a locus on chromosome 9 but others failing to do so (Saar, Al-Gazoli, Sztrika, Rueschendorf, Nur-E-Kamal, Reis, & Bayorioni, 1999). The victims have minor craniofacial anomalies, but their main problems are neurological. At birth, they exhibit breathing problems. Other brain stem dysfunctions include problems with eye movement, sucking, and control of the tongue (Maria, Boltshauser, Palmer, & Tran, 1999). MRI studies and examination of a few postmortem cases indicate agenesis of the cerebellar vermis and the motor nuclei of the brain stem tegmentum (Joubert et al., 1977; Yachnis & Rorke, 1999). The brain stem is shortened at the pontomesencephalic junction, and there is dysplasia of the caudal medulla (Yachnis & Rorke, 1999). This injury is larger than the one necessary to cause Moebius syndrome and probably occurs over a period longer than the critical period identified in the thalidomide study. When 11 cases were tested for autism, 4 met the diagnostic criteria and all had symptoms (Ozonoff, Williams, Gale, & Miller, 1999). The common interests of scientists studying Joubert syndrome and those studying autism are now so obvious that some of us are starting to collaborate.

Reinnervation and misinnervation

One of our initial hypotheses was that early injury to the motor nuclei of the cranial nerves might disrupt the subsequent connection of axons to their targets (Rodier et al., 1996). The human clinical literature is replete with examples of altered innervation. The four thalidomide cases of autism included three with Duane syndrome, a neurological anomaly in which the normal abducens innervation of the lateral rectus muscle is absent or reduced and the later forming, neighboring oculomotor fibers innervate the muscle (at least, that is the theory). Because the medial rectus is also innervated by the oculomotor nerve, attempts to move the eye laterally pull it from both sides, making the globe retract. The eyelids then close, producing a squint.

In rats exposed to VPA in utero, we set out to determine whether animals with reduced

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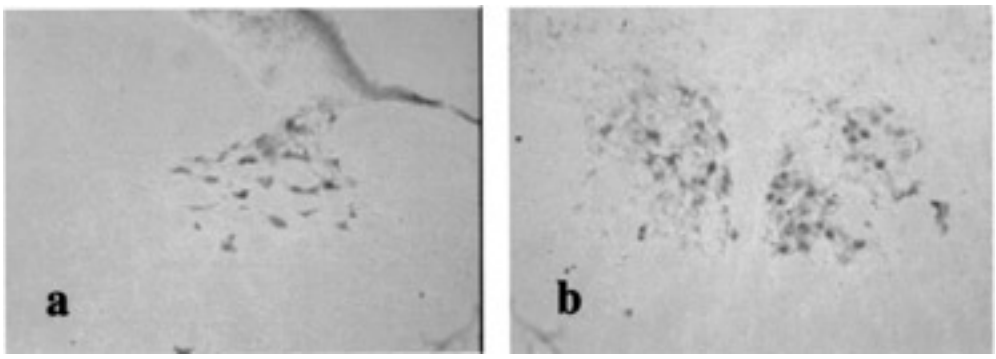


Figure 3. Innervation of the lateral rectus muscle of the eye in valproate-exposed rats. Photographs of coronal sections show HRP-filled neurons stained in (a) the abducens nucleus (b) the oculomotor nuclei of an animal treated with valproic acid on the 12th day of gestation. The projection from the oculomotor nucleus is not seen in the controls.

numbers of abducens neurons would reproduce the reinnervation characteristic of this human abnormality. Iontophoretic injection of the tracer horseradish peroxidase (HRP) into the lateral rectus muscle in controls produces immunocytochemically stainable HRP in the cell bodies of the abducens nucleus that peaks after 2 days. The oculomotor nucleus is nearer to the eye and exhibits peak HRP levels from the other eye muscles after 1 day. After many attempts to label both nuclei simultaneously, we found that 1.5 days allowed some staining in both nuclei from the appropriate muscles. When the lateral rectus was injected with HRP, controls showed filled cells only in the abducens nucleus. However, rats treated with VPA to reduce the number of abducens motor neurons showed staining in the oculomotor nucleus as well (Figure 3; Rodier, Ingram, & Tisdale, 2001). Judging from neurological exams, rewiring of this muscle is not a common feature of autism, but it does occur in some idiopathic cases, as it did in the thalidomide cases. The important point is that reinnervation can result from lack of normal innervation. If it occurs in this muscle, it may occur within the brain, resulting in projections to the wrong targets. Functionally, Duane syndrome demonstrates that this kind of rewiring in the periphery can be more disruptive than low levels of proper input would be, which is probably true in the brain as well.

It has always been our concern that deficits in the numbers of cranial nerve motor neurons could have a major impact on connections of sensory systems (Rodier et al., 1996). In general, motor neurons form prior to sensory neurons (Bayer, Altman, Russo, & Zhang, 1993). This means that the likely candidates for provision of axonal guidance for developing sensory systems are the motor neurons that are already in place. Although sensory abnormalities are not included in the diagnostic criteria of autism, they have been described by high-functioning people with autism as a major part of their disability (Cesaroni & Garber, 1991). Further, patients, parents, and clinicians often report sensory anomalies, including abnormal sensitivity to touch, hyperacusis, and synesthesias. Donna Williams described her own high threshold for sensation from her bladder, which makes it necessary to watch the clock, rather than wait for sensory signals (Williams, 1992).

Taste is a sensory system implicated as being affected in autism by the unusual dietary preferences of some people with the disorder. Many appear to select foods on the basis of texture, rather than taste (e.g., eating only foods that are crunchy or only foods that are creamy). Susan Hyman worried about this problem because of evidence that it predisposes children with autism to dietary limitations (Arnold, Hyman, Mooney, & Kirby, 2002). But if sensory systems are disturbed in autism, there are many other implications, especially for understanding how children with autism respond to sensations that act as

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critical reinforcers early in life. Thus, Dr. Hyman has begun to test taste and smell in children with autism, comparing them to their unaffected siblings and to unrelated controls. The result is that high-functioning children with an ASD diagnosis are significantly impaired in their ability to identify familiar foods and scents by taste or smell, as compared to either group (Hyman, 2000).

The development of taste buds in the tongue is known to be dependent on the arrival of sensory fibers from the cells of the geniculate ganglion (Morris–Wiman, Basco, & Du, 1999), which is located near the bones of the middle ear. In the future, we hope to study taste bud numbers in humans and in animal models of autism.

Genetic susceptibility to autism

Since the 1970s, researchers have known that autism is a complex genetic disorder (Folstein & Rutter, 1977). Twin studies and other family studies make this clear (Bailey, Le Couteur, Gottesman, Bolton, Simonoff, Yuzda, & Rutter, 1995). The pattern of inheritance of the ASDs is not Mendelian (Jorde, Fasstedt, Ritro, Mason–Brothers, Freeman, Pingree, McMahon, Peterson, Jenson, & Mo, 1991), and the genes involved are not always fully expressed. Estimates of the number of genes contributing have ranged from 3 to 15 or more (Pickles, Bolton, Macdonald, Bailey, Le Couteur, Sim, & Rutter, 1995; Risch, Spiker, Lotspeich, Nouri, Hinds, Hallmeyer, Kaladjieva, McCague, Dimiceli, Pitts, Nguyen, Yang, Harper, Thorpe, Vermeer, Young, Hebert, Lin, Ferguson, Chiotti, Wiese–Slater, Rogers, Salmon, Nicholas, & Myers, 1999). The extent to which environment factors are involved is not known. These patterns make identification of the genes quite difficult. For example, genome screens using sib pair analysis have failed to locate highly significant regions of sharing for study (Collaborative Linkage Study of Autism, 1999; International Molecular Genetic Study of Autism Consortium, 1998; Philippe, Martinez, Guilloud–Bataille, Gillberg, Rastam, Sponheim, Coleman, Zapella, Aschauer, Van Maldergem, Penet, Feingold, Brice, & Leboyer, 1999; Risch et al., 1999). The minor effects obtained in the screens thus far may indicate important loci, but they are not consistent from one study to another and the expense of examining each minor peak is prohibitive. More evidence that a given region is promising is needed to begin finer analysis. Such evidence exists for a few regions. For example, a locus related to speech and language is known to exist on the long arm of chromosome 7 (Fisher, Vargha–Khadem, Watkins, Monaco, & Pembrey, 1998), and a peak has appeared in the same general region on several of the genome screens. Therefore, many groups are combing this area for genes that might contribute to autism (e.g., Vincent, Herbrick, Gurling, Bolton, Roberts, & Scherer, 2000). A second promising region has been identified because chromosomal anomalies in the region appear to be an uncommon cause of autism. This area is at chromosome 15q11–15q13. A recent case with a small deletion and autism has added a second region of interest at 15q22–15q23 (Smith, Filipek, Wu, Bocian, Hakim, Modahl, & Spence, 2000), which duplicates many motifs in the first region; however, the critical loci are still not known.

One of the genome screens implicated the short arm of chromosome 7, where the *HOXA* cluster is located (Risch et al., 1999). In addition, over the period when we were collecting probands, several other *Hox* genes were shown to have polymorphic forms related to birth defects. First, a mutation of *HOXD13* was determined to cause synpolydactyly (Muragaki, Mundlos, Upton, & Olsen, 1996). Then, a variant of *HOXA13* was identified as the cause of hand–foot–genital syndrome (Mortlock & Innis, 1997). More recently, a mutation of *HOXA11* was associated with radioulnar synostosis and amegakaryocytic thrombocytopenia (Thompson & Nguyen, 2000). Now it is common knowledge that variations in the sequence of the *Hox* genes are not always fatal and that they can cause birth defects.

Given the complexity of genetic susceptibility to autism, we had to plan our study to optimize the chance of detecting a relationship of the candidate gene to the ASDs, if such a relationship existed. Being wary of the possibility of teratologic causes, we wanted to

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choose probands with some evidence of genetic causation. Sibling pairs meet this standard but would also be likely to be exposed to similar environmental influences. The best cases are cousin pairs, and we found quite a few. There were also families in which a parent reported having symptoms. Some parents had symptoms that qualified them for a diagnosis on the autism spectrum; others had milder symptoms, such as language delay. Still other families had an affected aunt or uncle. We included a few families in which the proband exhibited strong evidence of craniofacial features consistent with *Hox* gene effects (e.g., anotia, severe strabismus). Almost half of the cases selected as being familial had minor craniofacial anomalies (e.g., simple ears, hypertelorism) as well.

We noted that even though the A to G substitution is not rare, people with two copies of it are quite unusual. In fact, of the first 500 people we tested (mostly various kinds of controls), there were only two homozygotes. Rarity of homozygotes is often interpreted to mean that homozygosity interferes with viability. This, in turn, suggests that the infrequent allele in question is functionally different from the more common allele. In our sample of families ascertained through a child with an ASD diagnosis, there was no question that the frequency of homozygotes for the allele with an A to G substitution was significantly less than expectation (Ingram, Stodgell, Hyman, Figlewicz, Weitkamp, & Rodier, 2000).

As we collected additional cases, we continued to find a higher rate of the single base substitution in people with ASDs than in other populations. However, we gradually realized that this kind of comparison was subject to too many assumptions about the ethnic background of the controls. In fact, modern genetic methods have largely discarded this kind of comparison. Instead, it has become standard to compare the probands to a group of people who are demonstrably similar in ethnic background—their parents (Spielman & Ewens, 1996). Dr. Lowell Weitkamp, an experienced population geneticist, has been indispensable in helping us analyze data and plan experiments. If the genotypes of the parents are known, it is possible to calculate the expected frequencies of all the genotypes that might occur in their offspring; in our case, we could predict how often children should be homozygous for the common sequence, heterozygous for the newly discovered substitution, or homozygous for the substitution allele. If the variant allele of *HOXA1* is related to the disorder for which the children were selected, we should see more heterozygotes than expected. Another way to say this is that there will be selective transmission "into the disorder." Of course, there is no force that makes transmission selective—it is random. The appearance of distorted rates of transmission (transmission disequilibrium) is created by the process of selecting families through an affected child. In contrast, the unaffected children in the families should have the genotype frequencies predicted by Mendelian expectation.

Offspring with ASD diagnoses were heterozygous for the new allele at a rate significantly greater than expectation, whereas their unaffected siblings matched expectation precisely (Ingram, Stodgell, et al., 2000). This suggests that the A to G substitution is, indeed, related to autism. Another major effect in these data was that the increase in heterozygosity in affected offspring was modest in children whose fathers had the substitution but striking in those whose mothers had the substitution. We know that the parental source of genetic factors plays a role in several other developmental disabilities (e.g., Angelman syndrome and Prader–Wiley syndrome; Nicholls, 1994). Some of the chromosomal anomalies that cause autism are only expressed when inherited from the maternal side (Cook, Lindgren, Leventhal, Courchesne, Lincoln, Schulman, Lord, & Courchesne, 1997), and some family studies have suggested that maternal inheritance is especially important in autism (Szatmari, Jones, Fisman, Tuff, Bartolucci, Mahoney, & Bryson, 1995). Thus, our result agrees with several earlier findings.

Many more studies are needed to confirm the role of *HOXA1* polymorphisms in susceptibility to autism and to understand the genetic and environmental factors that determine whether a change in *HOXA1* function will be expressed as symptoms of ASDs. As the genes involved in autism are identified,

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we should be able to see how they interact to create the range of symptoms from minor to severe. If different combinations lead to slightly different phenotypes, that will make it much easier to identify more genes, because we will be able to examine samples stratified by phenotype. By engineering mice to have the genetic anomalies discovered in human ASDs, we should be able to study the neurobiology of autism in depth. In addition, once genetically susceptible animals are created, they will be the appropriate animals in which to investigate suspected environmental factors.

The molecular biology of teratologic induction of autism

Hox genes make transcription factors, proteins that bind to DNA to control gene expression. Thus, if *HOXA1* is involved in autism, all the genes it controls are candidates to play a role. Lorraine Gudas's lab has used cDNA subtractive hybridization and differential hybridization screening to begin to identify those downstream genes (Shen, Wu, & Gudas, 2000). She has also created special genetically engineered animals and cell lines in which the function of normal or altered versions of *HOXA1* can be studied. I want to mention some of these to give the reader a sense of the amazing possibilities they offer. For example, she has created a cell line in which *HOXA1* is turned on or off in response to tetracycline levels in the media. The expression of other genes in response to *HOXA1* can be studied in these cells by comparing their expression when *HOXA1* is off to their expression when it is on. Two versions of the cell line are now being prepared using genetic material from the human genetic study. One contains the typical human allele, and the other contains the substitution variant. This will allow a direct test of how the variant allele's protein product functions as a transcription factor. Mice transgenic for a *LacZ* marker of *Hoxa1* expression have been engineered with and without the retinoic acid responsive element through which retinoids act to initiate *Hoxa1* expression. In these animals, staining the embryo for the marker reveals not only the level of expression of the gene but also the spatial distribution of that expression. These animals will allow Dr. Gudas to determine whether environmental factors that influence *Hoxa1* expression act through the retinoic acid responsive element or some other mechanism.

Why do the phenotypes of cases of autism induced by drugs so closely resemble the phenotypes of cases with genetic causation? Might it be that drugs like VPA and thalidomide affect the expression of *Hox* genes? The idea seems too simplistic to be true. Yet this is exactly the mechanism of retinoic acid's teratogenicity (e.g., Kessel, 1993; Marshall, Nonchev, Sham, Muchamore, Lumsden, & Krumlauf, 1992). Ingram tested the hypothesis by exposing rat embryos to VPA, retinoic acid, or saline and then measuring the expression of *Hoxa1*. She used reverse transcriptase PCR to quantify the messenger RNA produced by the gene at various time points following exposure. The results indicated that a teratogenic dose of VPA drives expression as much as, or more than, a teratogenic dose of retinoic acid (Ingram & Rodier, 1998). The technique is only semiquantitative and we needed more accuracy to answer some important questions about this effect. The generous gift of a real-time PCR machine made it possible to quantify expression precisely. Dr. Stodgell has now repeated and extended Dr. Ingram's studies with this improved method (Stodgell, Ingram, Faeth, Gnall, Tisdale, & Rodier, 2001). Figure 4 shows the fold change in *Hoxa1* expression after exposure to VPA, retinoic acid, or saline. Each point on the graph represents at least 20 embryos. The increase in *Hoxa1* expression in retinoic acid exposed embryos was significant after 6 hr, but the increase in VPA-exposed embryos was significant at $p < .01$ or less at every sacrifice time.

In VPA, we are fortunate to have a compound that was studied extensively by teratologists. Because the drug has a low risk of causing NTDs in humans, investigators studied its effects on the expression of a variety of genes important in early development. In Bennett et al.'s studies (Bennett, Wlodarczyk, Calvin, Craig, & Finnell, 2000) expres-

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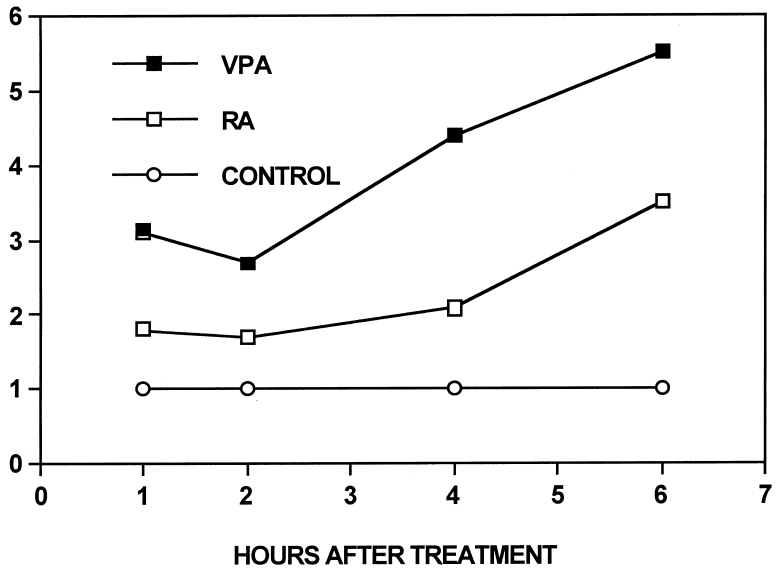


Figure 4. The fold change in expression of *Hoxa1* compared to controls after exposure to valproic acid or retinoic acid. (Retinoic acid drives the expression of *Hoxa1* in rat embryos.) Six hours after dams were injected with a teratogenic dose, expression had more than tripled. The effect of valproic acid was greater. Expression was significantly increased at every time point sampled, approaching a sixfold increase over the control value at 6 hr.

sion of a number of genes is reported to change after valproate exposure during neural tube closure. In a collaborative study, Faiella and others (Faiella, Wernig, Consaliz, Hostick, Hofmann, Hustert, Boncinelli, Balling, & Nadeau, 2000) described the effects of the drug on the homeotic transformation of rhombomeres, effects which are similar to those of retinoic acid. In addition, they found changes in the expression of an assortment of genes that are active in the embryo. The effects on the expression of other genes reported in these studies were not as strong as the effect we observed on the expression of *Hoxa1*. Thus, we believe that the large effect exerted on this gene is unusual.

Heinz Nau has worked to try to find compounds related to VPA that share its beneficial pharmacological properties but lack its teratogenicity (e.g., Nau, Hauck, & Ehlers, 1991). With Dr. Nau's assistance, we were able to compare the effects of two of these compounds on *Hoxa1* expression. Both have the antiseizure effects of valproate, but one is highly teratogenic and the other is not. Only the teratogenic analog of the parent compound drives the gene, suggesting that the teratogenicity of VPA in the early embryo is mediated by the alteration of *Hox* gene expression (Stodgell et al., 2001).

This study completes a circle of findings. Starting with the discoveries that teratogens increased the risk of autism, we studied a human brain, created an animal model of the initiating events, and developed ideas for candidate genes. We then studied the model for cerebellar effects, behavioral parallelism, and miswiring. We began to study more basic behaviors in children with ASDs, using a simple visual orienting task and taste and smell discriminations. We investigated another disorder with brain stem abnormalities, Moebius syndrome, and found that the rate of autism was high in both idiopathic and teratologic cases. We detected a polymorphism of *HOXA1* in human cases and tested its relationship to ASDs in human populations. We investigated whether valproate might have its teratologic effects on the embryo by disturbing the expression of *Hoxa1*. This brings us back to the

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teratologic research that provided our general hypothesis.

Conclusion

Contributors to this special issue were asked to discuss the approaches they used to combine data from different levels of analysis. We have not really faced this problem because our results converge, rather than tracking one another. Our greatest problems were those faced by everyone trying to characterize people with developmental disabilities. Do any of our behavioral measures mean the same thing in children of different ages or in children of the same chronological age but different mental ages? How do you recruit typically developing controls? There are issues special to the ASDs also. How do we categorize a child who had a firm Autism Diagnostic Interview/Autism Diagnostic Observation Schedule (ADOS) diagnosis at age 4 but whose ADOS score is much better at age 6, after 2 years of intensive intervention?

Even though our group has now been working together for several years, it is not easy to describe any rules about how we interact. We set up our projects so that some of us are working on more than one of them, so we do communicate frequently. We would prefer more face to face contact for the whole team, but that is expensive and our schedules are overloaded. When we all meet once a year, that is a special event. Scientists have many opportunities to talk with others in their own fields, but the chance to talk to someone with a completely different background and a high tolerance for your ignorance is exhilarating. It has been a surprise to me that the basic scientists and clinicians do not like to separate into two groups. In fact, the clinicians prefer to listen to the basic scientists and vice versa. This is partly because all of us seem to be anxious to learn new things. It is even more exciting to witness good ideas being tested by others that you cannot test. If you asked Marilyn Miller which of these studies is her favorite, I think she might say the animal model of Duane syndrome or the eyeblink conditioning studies in rats. Why? Because she does not work with animals but has had the chance to be involved through the labs of her collaborators. I might pick Hyman's taste study, Miller's misoprostol study, or Bryson's visual orientation. I have no skills or legal authority to see patients, so I could never have participated in these studies directly. Yet I feel intellectually connected to them through my association with these investigators. We all enjoy being involved in something much more extensive than anything we could have done alone.

Perhaps all scientists have many things in common in their histories and their aspirations, but in our group, that is especially true. Two of us performed with rock bands as teens; another is still performing in a chorus. Three of us did graduate work at schools with great basketball teams and fell in love with the sport. Stanton and Miller are experts on Chicago blues clubs. We all love the theater. We read the same books and pick the same restaurants. We felt like old friends from the start. We never have enough time to catch up on everyone's families and discuss all our common interests.

Bryson and I can meet Gudas in New York City for a single day because of good flight schedules. Figlewicz and Stodgell have been to New York, too, and Gudas has come to Rochester several times. Hyman, Bryson, and I have met halfway between Toronto and Rochester twice. Stanton, Miller, Stodgell, and I always attend the yearly Teratology Society Meeting, and Stanton and Bryson meet at the American Psychological Association and Society for Research on Child Development. Miller visits both Rochester and Toronto to examine patients. I have met with Stanton often, because of business at the EPA or NIEHS. When anyone asks us to do a symposium, we take that as an opportunity to gather. This has worked out to our advantage several times. Also, meeting organizers occasionally have invited more than one of us to speak, with no idea that we were working together. All of us communicate by e-mail, and most of us are late night telephoners.

When I first prepared a diagram of this program for a grant, I drew the overlapping projects as pieces of a pie with one administrative office at the center. Reviewers found

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this idea absurd and asked me to produce a hierarchical diagram with people reporting to other people. The reviewers were right; that is the best plan for most group efforts. However, this multidisciplinary program has played out as it was first described. It is our good fortune to have found a hypothesis that lets us work toward common goals, expanding our own research ambitions through collaboration.

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