Chapter 6
Alcohol and Embryology

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Overview of Embryological Development

No discussion on the effects of alcohol during early embryological development would be complete without at least a brief description of the key developmental events occurring during these critical periods of early life. While this section could not possibly provide a complete education on embryology, it will detail enough for a basic understanding of early developmental events that are impacted by prenatal alcohol exposure. It is fortunate that many aspects of development discussed here are conserved across many vertebrates; so we are able to use mouse and zebrafish models of fetal alcohol spectrum disorders (FASD) to rather accurately predict what might occur in humans.

In any discussion comparing embryological development across species, it is important to recognize that clinicians and developmental biologists often count the beginning of development differently. In rodents and fish, it is possible to know the exact timing of fertilization, down to the exact day, or even in some cases, to the exact hour, particularly in fish, which are fertilized externally to the mother. In humans, outside of in vitro fertilization cases, it is often difficult to know exactly when fertilization occurred, and clinicians usually count from the first day of the

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women's last menstrual period. This typically adds 2 weeks to the actual stage of human development. For the sake of comparison, we will count development from the day/hour of fertilization.

Gastrulation

Most animal species are triploblastic with adult tissues arising from three germ layers: ectoderm, mesoderm, and endoderm. The ectoderm is the outermost germ layer and will generate structures such as the epidermis, central nervous system, cranial placodes, and the neural crest. Endoderm is the innermost layer and generates the gastrointestinal tract and internal organs including the lungs, liver, pancreas, thyroid, and parathyroid. The middle layer, mesoderm, generates muscle, skeletal elements of the body and parts of the head, and blood vessels.

These germ layers arise through the process of gastrulation. While there are differences between species, the evolutionarily conserved process of gastrulation requires cell rearrangements to position the mesoderm between the ectoderm and endoderm. Generally, in vertebrate species, following fertilization and the subsequent series of cell division, the developing embryo forms a bilaminar disk, the dorsal epiblast and ventral hypoblast that will begin to undergo the process of gastrulation. Starting roughly in the middle of the cranial (rostral)-caudal axis of the embryo, the epiblast cells will proliferate and migrate ventrally through the midline-situated primitive node as the primitive node progresses toward the caudal end of the embryo creating the primitive streak. The first wave of migrating cells will join the cells of the hypoblast to form the endoderm. The next wave of cells to migrate through the node and subsequent primitive streak will form the mesoderm, the middle layer of cells in the embryo. The rest of the epiblast cells that did not migrate into one of the other layers will remain dorsal to form the ectoderm. These three layers of cells will make up all of the structures in our body. The endoderm will become the lining of the gastrointestinal system and parts of the liver and respiratory system. The mesoderm will form the majority of the musculoskeletal system, while the caudal part of the ectoderm will form the spinal cord and skin. The cranial portion of the ectoderm will proliferate and generate ectodermal tissue that will form the cranial portion of the neural plate and will develop into the brain and neural crest cells that go on to form many structures of the head, face, and neck.

Neurulation

The vertebrate central nervous system (CNS) is a hollow structure with the central canal of the spinal cord and the ventricles of the brain filled with cerebrospinal fluid that supports the function of the CNS. Neurulation is the process by which ectoderm is shaped into this hollow structure. There are two general types of neurulation. In

primary neurulation, a flat sheet of cells is folded into a hollow tube while in secondary neurulation a solid rod of cells undergoes cavitation to hollow out. Individual species may use both types of neurulation, frequently with just the most posterior region of the embryo using secondary neurulation. For this reason, here we focus on primary neurulation.

In vertebrate species, neural ectoderm is generated in the dorsal midline of the embryo and is induced by signals from adjacent tissues. Following its induction, the neural ectoderm is distinctive as a thickening, along its apical/basal (outside/inside) axis, relative to the adjacent non-neural ectoderm and is termed the neural plate. Cells within the neural plate are highly proliferative and will form all of the cells within the CNS, as well as cell types that emigrate away from the CNS, such as the neural crest. As the neural plate proliferates, cell shape changes in the midline of the neural plate cause the right and left halves to bend dorsally to form the neural folds. Similar shape changes in the lateral aspect of these neural folds cause them to begin to bend back toward the midline and approximate each other. Cell adhesion differences between cells in the neural ectoderm versus the non-neural ectoderm enable fusion of the two sides closing the neural tube and separating it from the overlying surface ectoderm.

Neural tube closure initiates in the middle of the embryo around the region that will eventually become the junction between the brainstem and spinal cord. After this initial fusion event, the neural folds fuse together in a "zipper-like fashion" progressing both rostrally and caudally simultaneously. The anterior neuropore closes first in the region of the neural tube that will eventually become forebrain tissue. The posterior neuropore closes shortly thereafter. This basic process of forming the neural tube from the neural plate is very highly conserved across vertebrate species, although it should be noted that some of the basic cellular mechanisms involved in neurulation and the relative utilization of primary versus secondary neurulation may vary.

As the neural tube is closing, it is already beginning to segment itself into vesicles, with the neural tube first developing three vesicles, represented by the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). Prior to closure of the anterior neural tube, the optic cups are already beginning to evaginate out of the neural folds that will form the prosencephalon. Following the formation of the prosencephalic vesicle, it is already possible to distinguish between the caudal part of the prosencephalon that will form the diencephalon and the more rostral telencephalon. Similarly, the rhombencephalon subdivides into the metencephalon and the myelencephalon. Following the formation of this five-vesicle brain massive waves of neurogenesis begin in earnest to form the mature brain. The myelencephalon will generate the medulla oblongata and the metencephalon will generate the cerebellum. The majority of the diencephalon will form the thalamus and associated structures. The ventral telencephalon will form the hypothalamus (from which the optic primordia are evaginating) and the dorsal telencephalon will form two additional vesicles that will be the cerebral cortices.

Neural Crest Cells

As mentioned previously, after the ectoderm is formed during gastrulation, the midline portion begins to differentiate into neuroectoderm that will form the neural plate, while the lateral edge of the ectoderm will differentiate into surface ectoderm. At the border of the neural plate ectoderm and surface ectoderm, cells will differentiate into neural crest cells (NCCs; Fig. 6.1). NCCs are a vertebrate-specific cell type that are migratory, multi-potent cells and will form diverse structures within the embryo. Distinct subpopulations of NCCs are distributed along the anterior-posterior axis of the embryo. In posterior regions of the embryo, trunk NCCs will generate cell types such as melanocytes, sensory neurons of the dorsal root ganglia, and sympathetic neurons. Vagal and sacral NCCs flank the trunk NCCs, anteriorly and posteriorly, respectively. These NCCs will generate structures including enteric ganglia lining the gastrointestinal track. Cardiac NCCs reside toward the anterior end of the embryo and will contribute to the outflow tract of the heart. Cranial NCCs reside most anteriorly in the embryo and generate the vast majority of the craniofacial skeleton and its associated connective tissue among other cell types. Because of their lineage, cranial NCCs are particularly important in the genesis of FASD.

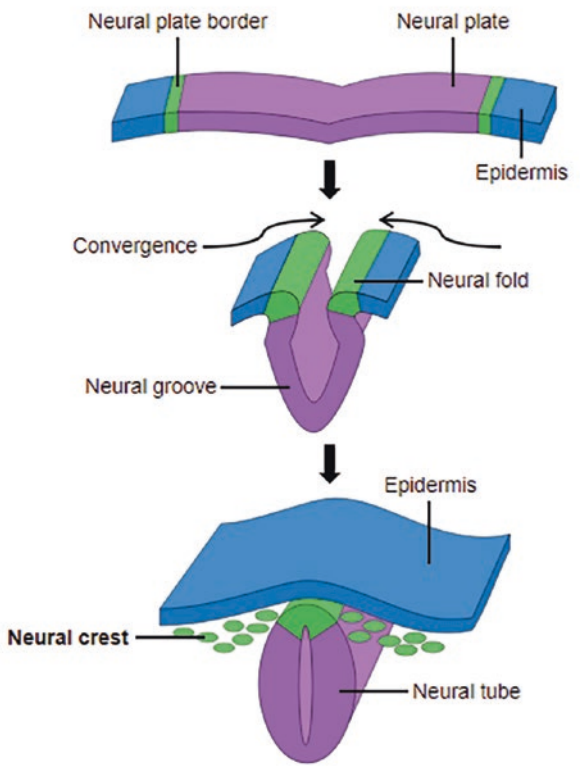

Fig. 6.1 Generation of neural crest cells from the dorsal neural tube

In all vertebrate species, cranial NCCs migrate away from the neural tube in three migratory streams. Cells within these streams will populate transient reiterated embryonic structures termed pharyngeal (or branchial) arches. The total number of pharyngeal arches varies by species (e.g., 4 in human and mouse or 7 in zebrafish). Across species, the first and second neural crest streams populate the first and second pharyngeal arches, respectively. The third stream populates all of the remaining arches. Ectoderm and pharyngeal endoderm bound the NCCs and provide signals that support their survival, proliferation, and differentiation, as well as the subsequent shaping of the resultant skeletal elements.

The first pharyngeal arch is of particular importance to our understanding of FASD as it generates the facial skeleton. As NCCs proliferate within the first pharyngeal arch three swellings (prominences) form that will generate the skeletal elements of distinct regions of the face. The mandibular prominence is located adjacent to the floor of the oral ectoderm and will generate the lower jaw. The maxillary prominence rests on the roof of the oral ectoderm and generates the upper jaw and the secondary palate. The frontonasal prominence is medial to the maxillary prominence spanning the midline of the embryo. It will generate the midfacial skeleton, including the primary palate. Interestingly, fate mapping experiments have demonstrated a correlation between the location of a NCC along the anterior-posterior axis and the skeletal elements that it will populate [1, 2]. For example, NCCs from the most anterior part of the neural tube will develop into the midface, while NCCs that arise from hindbrain neural tube will go on to form part of the lower jaw. This information can be extremely useful in identifying to where NCCs will migrate, as well as the future potential abnormalities that may develop following an insult to a specific part of the dorsal neural tube. Thus, as NCCs arise from the dorsal neural tube, they migrate to form structures in a pattern consistent with where they originated from in the neural tube.

Comparative Developmental Timing

While the processes of gastrulation, neurulation and initial neural crest formation are complex, involve substantial cell proliferation and movement, and are vital to embryonic development, the actual timing of these events is quite short relative to the rest of development. In humans, from the beginning of gastrulation to the end of neural tube closure is just over 10 days, beginning about embryonic day 16 (E16) and ending about E28. In mice these events occur in just over 3 days from about E6.5 to E10 and in zebrafish it is less than a day, beginning about 6 h post-fertilization (HPF) to about 20 HPF. Gastrulation and neurulation are often described as sequential events and indeed, within a given part of the embryo, they are. However, it must be recognized that within the entire embryo, one part may be undergoing gastrulation while another part of the neural plate is beginning to fold. For example, in humans, gastrulation occurs from about E16–E20, while neurulation spans

E18–E28. This information is critical in modeling and comparing specific developmental events among various species.

Methods for Studying the Effects of Prenatal Alcohol Exposure on Embryologic Development

Vertebrate embryologic development has been studied in many different species including the frog, chicken, zebrafish, and mouse. The use of these varied species to model human diseases is facilitated by the fact that, as mentioned previously, much of embryological development is fairly similar across vertebrate species. The same is true of early prenatal alcohol exposure. The effects of alcohol on gastrulation, neurulation, and neural crest cell development have been studied in the chicken, zebrafish, and mouse. Each species has its advantages and disadvantages. Zebrafish and chicken have the advantage that developmental events can be imaged in real time. This is especially true of the zebrafish which has a clear chorion surrounding the embryo. Chickens are a bit more limited as a window can be cut into the shell, but only in certain locations. Mice have the advantage that like humans they are placental mammals with a maternal-fetal interface and fully subject to maternal physiological changes, which could be an important factor in examining the effects of environmental agents. However, in utero development largely precludes real time imaging, particularly of younger embryos, which are too small for even the most advanced in vivo imaging options, such as high-resolution MRI or ultrasound. Mouse embryos can be cultured during some of these early stages, which can be very useful, but only for a short amount of time, and this takes away the maternal component, which is one of the strengths of the mouse model.

One of the advantages of animal model systems is that developmental staging can be quite precise. Because we can control the timing of fertilization and we know the relative rates of development we can study precise periods of alcohol exposure. For example, if we only wanted to expose the embryos to alcohol during gastrulation, we can simply administer the alcohol at the appropriate times. However, this highlights some of the methodological differences between species and some of their relative strengths and weaknesses. Pregnant mice can be administered alcohol via the drinking water, through a liquid diet containing alcohol, by intragastric (IG) intubation, by intraperitoneal (IP) injection, or even inhaled via vapor. Each has its advantages, with drinking being the most similar to human consumption, and IG and IP the most amenable to precise control of exposure timing. However, relying on mice to drink alcohol does not lend itself to accurate control of alcohol dosage or timing and is extremely variable among the many different mouse strains. Regardless of the manner of alcohol administration, in the end, mouse embryos still receive alcohol in a similar manner, i.e., placental transfer from maternal blood via the uterine arteries. In contrast to the myriad different ways to expose developing rodents to alcohol, developing zebrafish are simply immersed into their normal water

containing the pre-determined concentration of alcohol. The tissue levels of alcohol equilibrate within 5 min, with a majority of studies showing that these levels are roughly one fourth to one-third of the level in the water [3]. Alcohol is equally rapidly eliminated from the zebrafish embryo following wash out. This demonstrates one of the advantages of the zebrafish model in that both the beginning and ending of the alcohol exposure can be tightly controlled.

Findings on Timing and Pattern of Alcohol Exposure on Development

The effects of prenatal alcohol exposure vary greatly across individuals. This variability is due to many different factors including genetics, amount of alcohol exposure, nutrition, and the timing and pattern of alcohol exposure. For example, the characteristic craniofacial features of FAS are largely caused by alcohol exposure during the periods of gastrulation and early neurulation [4]. These periods, particularly gastrulation, are also particularly vulnerable to the most severe alcohol-induced brain defects, those within the holoprosencephaly (HPE) spectrum [5]. These developmental defects include varying amounts of growth delays and fusion of the cerebral cortices, as well as dysgenesis or agenesis of the corpus callosum as has often been observed in genetic holoprosencephaly cases. In the most severe cases, a complete absence of the cortices has been observed. Although less well characterized clinically, gastrulation-stage alcohol exposure can also induce defects of the ventral midline brain, particularly involving the septal region, striatum, and hypothalamus. Concomitant with these effects in the brain, a gastrulation-stage exposure can also significantly affect the face, especially the philtrum, upper lip, and eyes (reduced palpebral fissure length/microphthalmia). These brain and craniofacial effects lie within the HPE spectrum and are believed to be a result of alcohol-induced apoptotic cell death within the part of the mid-gastrulation-stage embryo that will form the anterior neural ridge [6]. In turn, the anterior neural ridge will give rise to much of the neural plate that will form the forebrain (cortex and anterior ventral midline subcortical regions), as well as the neural crest cells that will develop into midline structures of the face.

The period of neurulation is longer than that of gastrulation, and the effects of alcohol are more subtle, but still quite impactful. During this stage, alcohol typically has much less impact on craniofacial development, but in both mice and fish is capable of inducing major brain and ocular abnormalities [2, 7–10]. While both types of defects are typically less severe during this developmental period relative to those occurring following a gastrulation-stage exposure, they are still quite dysmorphic. In addition to microphthalmia, a neurulation stage exposure has been shown to induce defects in midline subcortical brain regions, the cerebellum, and midbrain areas in mice, as well as midbrain/hindbrain junction abnormalities in zebrafish. Finally, a consistent finding, particularly in mice across the different days

of neurulation, is that of hydrocephalus, or enlarged ventricles, although it is unclear if this is due to an obstructive hydrocephalus, versus a general trend toward microencephaly following alcohol exposure at this stage. Finally, as neurulation nears completion, the effects of alcohol on gross ocular dysmorphology wanes, although research at later periods of development has clearly shown that the retina remains sensitive to alcohol's effects for quite some time [11, 12]. Similarly, while not discussed here, as the gross dysmorphic effects on the brain seem to diminish as the end of neurulation is reached, it should be stressed that the brain remains sensitive to alcohol throughout the rest of development, including the rest of gestation and through adolescence [13, 14]. Together, these studies demonstrate that even small differences in developmental timing during early embryogenesis gradually alter different aspects of the brain and face generally progressing from anterior to posterior as development proceeds.

While this short summary of alcohol's stage-dependent effects has focused largely on early development, it demonstrates the basic idea that the effects of alcohol on the developing embryo and fetus can vary largely depending on the timing of exposure. This factor is one of many that explains the large variability present in FASD populations. Of course, adding to the complexity of FASD presentations is that some children are exposed nearly continuously throughout pregnancy. These children are likely to be most severely affected. However, all other elements being equal, embryos exposed to binge alcohol sessions at different periods of time will likely manifest with different phenotypes, particularly those involving the brain and face. Of course, as discussed in the next section, there are numerous other factors to consider, not the least of which is genetic variation.

Historical and Future Trends

In the nearly 50 years since FAS was first diagnosed, the field has changed dramatically. Over the years, we have come to realize that FAS is just the tip of the iceberg, and that alcohol can disrupt many developmental processes without inducing the distinctive craniofacial characteristics of FAS. This led to the development of the umbrella term FASD to encompass the wider range of effects of prenatal alcohol exposure, including individuals without the effects on the face, but still possessing significant neurobehavioral and cognitive issues. Over the years we have also discovered many of the confounding variables that can alter susceptibility to prenatal alcohol exposure. For example, preclinical work has demonstrated that peak blood alcohol concentration (BAC) is a critical factor that can determine the amount and extent of alcohol-induced damage at any given point in development [15, 16]. Likewise, nutritional factors have also been shown to dramatically influence how alcohol affects development. We have discovered that many drugs, legal and non-legal, prescription and non-prescription, can interact with alcohol to disrupt normal development [17–21]. Finally, there are genetic factors that can also modify susceptibility to prenatal alcohol exposure.

It has long been recognized that there is a genetic component to FAS and FASD. Almost 30 years ago, it was observed that monozygotic twins had a significantly higher concordance of being diagnosed with FAS as compared to dizygotic twins, despite the same intrauterine environment [22]. Not surprisingly, it has also been noted that allelic variations in alcohol metabolism genes alter susceptibility to prenatal alcohol exposure [23]. Likewise, several genes related to the Sonic hedgehog (Shh) pathway and other growth factors have been identified to be either protective or associated with greater susceptibility to prenatal alcohol exposure in various animal model systems of FASD [24–26]. Human and animal studies investigating the genetics of FASD have been discussed in great detail elsewhere [27]. More recently, it has been demonstrated that numerous genes involved in apoptosis, reactive oxygen species (ROS) homeostasis, and immune signaling can also alter susceptibility to prenatal alcohol exposure, with some gene variants making an individual more susceptible to alcohol while other variants confer resistance [26, 28–32]. While only a small percentage of the total number of genes have been explored in terms of their interaction with prenatal alcohol exposure, this area of the FASD field has improved our understanding of the pathogenesis involved in alcohol's mechanisms of action during development. This has remained one of the most elusive areas of research as we still know very little about the mechanisms as to how alcohol affects the developing brain. Understanding even the basic pathogenic mechanisms has the significant potential to enhance future therapeutic and/or preventative studies designed to lessen the burden of FASD. Our pace of knowledge regarding all of these variables that contribute to FASD continues to grow; however, there are still many gaps in our knowledge that require extensive further investigation.

Glossary

Agenesis Complete absence of an organ or structure due to a failure to form.

Anterior neural ridge The most anterior (cranial) portion of the neural plate that will go on the form a significant portion of the telencephalon.

Anterior neuropore The most anterior (cranial) closure point of the neural tube.

Caudal Direction toward the tail.

Cranial placodes Specialized regions of the embryonic ectoderm that will give rise to structures in the head and neck such as sensory ganglia. They also contribute to many of the special sensory organs.

Cranial Direction toward the head.

Diencephalon Posterior portion of the prosencephalon which will generate the thalamus, subthalamus, epithalamus, and in some literature the hypothalamus.

Dysgenesis Abnormal formation of an organ.

Epiblast Primitive ectoderm in the inner cell mass. During gastrulation, the epiblast will proliferate and give rise to more ectodermal cells, and cells of the mesoderm and endoderm.

Forebrain (prosencephalon) Most cranial (rostral) primary vesicle of the developing neural tube. Will develop into the telencephalon and diencephalon.

Frontonasal prominence Region of the developing embryo that will generate the midface, the primary palate, and anterior skull.

Gastrulation Early developmental process in which the three germ layers (endoderm, mesoderm, and ectoderm) are generated and organized relative to one another.

Hindbrain (rhombencephalon) Most caudal of the three primary brain vesicles. Will develop into the metencephalon and myelencephalon.

Holoprosencephaly Failure of the two cerebral cortices to separate from each other forming one continuous structure with a continuous ventricle. The resulting defect in the brain also causes defects in the midline of the face and skull.

Hypoblast Layer of cells in the inner cell mass that sits beneath the epiblast. In mammals and birds, the hypoblast contributes to the chorion, while in fish it also contributes to the endoderm and mesoderm.

Mandibular prominence Region of the developing head that will form the lower jaw and associated structures.

Maxillary prominence Region of the developing head that will form the upper jaw, the secondary palate, and sides of the face.

Metencephalon Brain vesicle that will form the pons and cerebellum.

Microphthalmia Abnormally small eye.

Midbrain (mesencephalon) Middle primary vesicle of the brain.

Myelencephalon Brain vesicle that will generate the medulla oblongata.

Neural crest Cells derived from the dorsal neural tube that will migrate to develop into a wide variety of cell types including most of the craniofacial skeleton.

Neural plate Neuroectoderm that will proliferate and bend into the neural tube during neurulation.

Neurulation The process of generating a hollow neural tube that will go on to form the entire central nervous system.

Optic cups Evaginations of the prosencephalon that will form most of the future eye.

Pharyngeal (branchial) arches Transient, serially reiterated embryonic structures made up of endoderm, mesoderm, ectoderm, and neural crest cells that will generate hard and soft tissues in the head and neck. In fish, they also go on to form the gills.

Posterior neuropore Most posterior (caudal) point of closure of the neural tube. In normal development, this is the last part of the neural tube to close and becomes the most caudal part of the spinal cord. Failure to close the posterior neuropore is one of the most common neural tube closure defects and causes spina bifida.

Primitive node Gastrulation organizing center through which proliferating epiblast cells will migrate ventrally to form the mesoderm and endoderm. As gastrulation progresses, the node migrates caudally.

Primitive streak Midline streak formed from the caudal progression of the primitive node. Both the primitive node and subsequent primitive streak will help set up bilateral (left-right) asymmetry.

Rostral During embryogenesis, the direction toward the most anterior aspect of the neural tube. Later in development, it is defined as toward the nose, or the most anterior aspect of the frontal lobe.

Telencephalon Cerebral hemispheres and basal ganglia. Some literature also includes the hypothalamus.

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