Human Neural Tube Defects: Developmental Biology, Epidemiology, and Genetics

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Birth defects (congenital anomalies) are the leading cause of death in babies under 1 year of age. Neural tube defects (NTD), with a birth incidence of approximately 1/1000 in American Caucasians, are the second most common type of birth defect after congenital heart defects. In humans, the most common NTD are anencephaly and myelomeningocele. Anencephaly results from a failed closure of the rostral end of the neural tube and is characterized by a total or partial absence of the cranial vault and cerebral hemisphere. Myelomeningocele is a defective closure of the neural tube in the vertebral column. Depending on the size and the location of the defect, the patient can suffer either no physical handicap or lifelong disabilities [86]. These common birth defects vary in frequency depending on the geographical localization. They occur at frequencies ranging from 0.9 in Canada to 7.7 in the United Arab Emirates and 0.7 in central France to 11.7 in South America per 10,000 births, for anencephaly and spina bifida respectively [86].

The mortality rate for children with spina bifida is increased over the general population risk in the first year of life. The cost of providing for medical care for a child with myelomeningocele has been estimated to be over $70,000 (adjusted to 2001 dollars) annually for the first 20 years of life, including costs associated with an average of 5 surgeries per year [94] in the first five years of life (20 year lifetime cost is $1.4 million/case).

The phenotypes of the open NTDs include myelomeningocele (spina bifida cystica, open spina bifida) and anencephaly. Anencephaly, an incomplete formation of the brain and skull, is uniformly lethal. The most common form of NTD, myelomeningocele, is an open lesion in the caudal spine and contains dysplastic spinal cord, often resulting in a lack of neural function below the level of the defect. Affected patients usually have reduced ability to walk, or need the use of a wheelchair, have little or no bowel and/or bladder control, and require frequent surgical interventions to minimize the effects of hydrocephalus. The most common presentations, spina bifida and anencephaly, can occur within the same family, raising the question as to whether these phenotypes are related and due to a common underlying gene [29,29,31,33,38,65,65,77,77].

Defining the phenotype in affected patients is paramount to the evaluation of human neural tube defects. Phenotypic parameters include: location and level of the defect, whether the defect crosses CNS segmental boundaries, and cataloguing the variety of anomalies in a patient or family. Open defects such as anencephaly, cranioraschisis, myelomeningocele, and myeloschisis are defined based upon the location and level and are descriptive in nature.

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Associated anomalies, Chiari II, hydrocephalus, syringomyelia, polymicrogyria, cortical heterotopias, agenesis of the corpus callosum further add to and can confuse the phenotypic definitions.

NTDs in humans result from the combined effects of genetic and environmental influences, and as such are a classic example of a multifactorial disorder. Identifying the genetic factors is critical for characterizing the interactions between genes and the environment, and understanding these interactions will provide the basis for designing novel preventive strategies and for offering accurate reproductive risks to couples. The genetic factors will likely involve aberrant variations in genes key for the normal closure of the neural tube. Neural tube closure is a complex, early developmental process, informed not only by nascent studies in human embryos, but by the plethora of investigations in a variety of experimental systems including but not restricted to mouse, zebrafish, and chick.

Formation of the human neural tube

Neurulation, which is the formation of the neural tube, is an important morphogenetic event in human development. The neural tube gives rise to the brain and the spinal cord to form the central nervous system. Neurulation in mammalian embryos occurs in two phases: primary and secondary neurulation [68]. These two phases occur in distinct areas along the rostro-caudal axis of the embryo. Secondary neurulation is limited to the tail bud, which lies beyond the caudal neuropore. In contrast to primary neurulation, described in detail below, secondary neurulation occurs by proliferation of stem cells[8], which form a rod-like condensation that subsequently cavitates. The cavitation transforms the rod into a tube, and the lumen of this tube comes into continuity with the lumen of the tube formed during primary neurulation. In tailless humans, the tail bud does not develop as in tailed animals, and secondary neurulation does not appear to be responsible for open neural tube defects. For this reason, we will focus on primary neurulation.

Primary neurulation generates the entire neural tube rostral to the caudal neuropore. During this process, occurring during the third and fourth weeks of development (Carnegie stages (CS) 8 to 13) Fig 1, the flat layer of ectodermal cells overlying the notochord is transformed into a hollow tube.

Eighteen days after fertilization (CS 8), the midline dorsal ectoderm of the embryo thickens and forms the neural plate while cell shape changes. The neural plate first appears at the cranial end of the embryo and differentiates in the caudal direction. The edges of the plate thicken and begin to move upward forming the neural fold. The neural plate becomes narrower, longer, and is transformed from an elliptical to a key-hole shaped structure. This transformation occurs by polarized cell movements in the medial direction and cell intercalation in the midline. The mechanism of these movements, known as convergent extension, is not specific to neural tube formation. Convergent extension has been widely studied in animal models (mouse, Xenopus and Drosophila), where it depends on the highly conserved Wnt-frizzled signal transduction pathways (See Lawrence at al. 2003 [48] and Copp et al. 2003 [12] for reviews on convergent extension).

On day 19 (CS 8.5), the border of the neural plate becomes gradually more pronounced and elevated. The neural plate folds longitudinally along the midline of the plate from the head toward the tail to form the neural groove. The folds rise up dorsally, approach each other and ultimately merge together, forming a tube open at both ends by day 23 (CS 10.5) (Fig 1 A and B). As the neural folds fuse, the cells adjacent to the neural plate also fuse across the midline to become the overlying epidermis. The rostral and caudal openings are called neuropores and are best distinguished around day 23 when about 17−19 somites are visible (Figure 1C). The rostral and caudal neuropores close later, on the 26th (CS 12) and 28th (CS 13) days of gestation

respectively (Figure 1 D to E). We utilize the terminology suggested by O'Rahilly and Müller [60], who reserve the term "closure" for the closing of neuropores, while the term "fusion" is used to designate the merging of the neural folds and the formation of a tube.

Although there is general agreement on the morphogenetic movements of the first events of neural tube formation, the last event in neural tube formation, the fusion of neural folds, is subject to debate concerning the number of initiation sites of fusion and their location. Indeed, the fusion of the neural folds has originally been described in humans as a process initiated at a single site, and extending bi-directionally, rostrally and caudally, from this initiation site to the rostral and caudal neuropores [68]. However, over the past 20 years, a hypothesis of "multiple site of neural tube fusion" has been investigated in animal models and in humans. This hypothesis has been extensively studied in mice and rats [74]. According to Sakai, who wrote a comprehensive review of available data in mice and rat, rodent neural tube fusion occurs between day E8 to day E10 of gestation [74]. Four sites of neural tube fusion were identified. Site 1 initiates in the future cervical region between the third and fourth somites at the caudal part of the hindbrain, and progresses both caudally and rostrally. Caudally, it proceeds all the way down to the end of the neural groove until the caudal neuropore. The next two sites of initiation of fusion are located rostral to site 1. A second fusion initiates at the prosencephalon-mesencephalon boundary (Site 2) and extends both rostrally and caudally. This second fusion completely closes the roof of the telencephalon and the metencephalon. A third fusion site (site 3) progresses caudally, and closes the rostral end of the neural plate. Finally, the fourth fusion site (site 4) appears at the caudal end of the neural plate and extends rostrally to meet the fusion extending back from site 1.

Single site of neural fold fusion

Since the susceptibility to NTD's in human is known to vary among ethnic groups, one might hypothesize that heterogeneity of human neural tube defects could also originate from differences in fusion at site 2. This statement implies that the multiple sites of neural tube fusion occur in humans. In 1993, van Allen proposed multiple sites of fusion in human embryos, although a human site 2 had never been observed. She based her model on the observation of the type and the frequency of human tube defects. A model of a single site of fusion would predict that most human neural tube defects would be localized in the caudal and rostral ends of the tube where the neuropores close, which is not the case. van Allen's model predicted 5 sites of fusion and four neuropores. In addition to the rostral and caudal neuropores, she postulated the existence of a prosencephalic and a mesencephalic neuropore, resulting respectively from fusion of a second and a fourth closing site [88]. In the mid 90's, Seller [78,79] and Golden [30] arrived at similar conclusions from the study of human neural tube defects.

Although the model of multiple sites of fusion was attractive to explain such defects, experimental observation of human embryos clearly corroborates the hypothesis of a single site of fusion and a zipper-like process of neural tube closure. Using light microscopy and laser scanning electron microscopy to observe successive stages of development, Sulik and coworkers showed a zipper-like fusion of the human neural tube from a single initiation site located in the middle of the future hindbrain region [84]. This finding was later corroborated by two studies. Nakatsu and coworkers examined histological sections of human embryos at various stages of neural tube formation, and described three sites of apposition. Site 1 was the widely recognized site of true fusion located in the cervical region. From site 1, fusion extended both rostrally and caudally, reaching the caudal neuropore at the caudal end of the embryos. Site 2 was located at the boundary between mesencephalon and rhombencephalon, but was only an apposition site before being caught up by the rostralwards fusion. Site 3 corresponded to the rostral tip of the neural folds and is also an apposition, becoming fusion upon closure of

the anterior neuropore. [57]. Finally, a study by O'Rahilly found two regions of fusion in humans [60] as observed by Sulik and coworkers [84], extending bi-directionally from the rhombencephalic region. Caudally, the fusion extended until the caudal neuropore, while ending rostrally at the dorsal lip of the rostral neuropore, closing the neuropore rostrocaudally.

Relationship of human neural tube closure to mouse neural tube closure

Three initiation sites of fusion have been confirmed by several groups in rodent models [11, 30,42,43], while a fourth one has not been described elsewhere (see [25] for a comparison of these studies). The locations of sites 1 and 3 were uniform between studies, but the location of site 2 showed strain differences. Genetically determined, it is considered to modify the susceptibility of each strain to neural tube defects (NTD's) [12,43].

It seems clear that in mice, the multiple sites of fusion model can be applied, even if the exact location of each site varies between mouse strains. In contrast, there seems to be a single initiation site of fusion in humans. Apposition of the neural folds may occur at several sites, but fusion itself only occurs when the extension of fusion reaches the area where the neural folds were apposed. This difference between humans and rodents does not necessarily imply that the mechanisms of fusion and closure are different; the same genes are likely to be involved in both species. Understanding the processes, both environmental and genetic, that influence neural tube closure in humans is critical so that relevant, rational interventions and preventions can be designed; but because humans are non-experimental systems, it is equally important to understand the similarities and differences between the human system and experimental systems such as mouse.

Clues from observational data

Attempting to define the defects based upon the underlying embryopathy may be the most appropriate method for defining NTD phenotype. Shum et al. [80] demonstrated that at least three different modes of neural tube formation might exist along the rostrocaudal axis; therefore, regional differences in modes of neural tube closure may result in different types of open defects. Mode 1 occurs in the cervicothoracic region, where a distinct medial hinge point (MHP) forms without any clear morphological evidence of dorsolateral hinge points (DLHP) resulting in an ovoid neural tube and slit shaped central canal. Defective mode 1 has been proposed to cause craniorachischis by interfering with MHP formation resulting in normal but widely spaced neural folds preventing proper fusion. In the midbrain/hindbrain region, mode 2 has been described as generating both MHP and DLHP prior to fusion. After fusion, the neural tube has a diamond shaped configuration, perhaps foreshadowing the shape of the adult fourth ventricle. Defects of mode 2 results in exencephaly due to defective DLHP function.

Neural tube formation in the lumbosacral region, mode 3, is different in that there is only a suggestion of DLHP formation along with a well-developed MHP. The closed tube has a more oval shape with a large patent central canal. Where the driving force of neural tube closure in mode 1 appears to be extrinsic to the neural tube, the source of the force in mode 3 is less defined.

The last embryopathic mechanism proposes that a properly neurulated neural tube can be reopened. The only spontaneous mutant in which this mechanism occurs is the *curtailed* mouse in which increased cerebrospinal fluid pressure is thought to rupture a thinned roof plate and dermis in the absence of competent dorsal bony vertebrae [64]. Although the *curtailed* mutant may indeed have a reopening of a previously closed neural tube, this mechanism is not thought to be a likely cause of human NTD.

Evidence for a genetic factor in human neural tube defects

Several lines of evidence suggest a genetic component to NTDs. First, NTDs are associated with known genetic syndromes including Meckel syndrome, anterior sacral meningomyelocele and anal stenosis, and the Mohr syndrome in addition to others. NTDs are frequently associated with trisomies 13 and 18 and various chromosome rearrangements. Secondly, in NTDs occurring without other syndromes, the recurrence risk for siblings is approximately 2−5% (giving a λ_s value [70,71] between 20−50), which represents up to a fifty-fold increase over that observed in the general population. Khoury et al. [47] have shown that for a recurrence risk to be this high, an environmental teratogen would have to increase the risk at least 100 fold to exhibit the same degree of familial aggregation, making a genetic component essentially required. Such potent teratogens are extraordinarily rare; however, one example of a teratogen exerting such a high relative risk is thalidomide.

Evidence of a genetic factor is further strengthened by the presence of a family history in a number of those affected. While family history of NTDs has been reported in 8.5% of one group of families studied [66], inspection of these multiplex NTD families shows that affected parent-child pairs are rare; most affected relative pairs are related at either the second or third degree, thus suggesting oligogenic inheritance. More data on parent-child transmission will be available over the next two decades, as children born with NTDs now receive sufficiently sophisticated medical care that they can live to maturity and reproduce. Segregation analysis studies demonstrating evidence of a major gene have been performed in series of NTD families, one demonstrating evidence for a major dominant gene and another for a major gene with recessive effect [16,24]. These studies are admittedly small and suffer from common problems of ascertainment. Twin studies for the NTDs are anecdotal in nature, comparing concordance in like-sex vs. unlike-sex twins instead of the more formal comparison between dizygotic and monozygotic twins. The limited available data are based on very small sample sizes, but range from 3.7% − 18% [20].

Chromosome abnormalities, specifically aneuploidy, are found in 5−17% of cases with NTDs [37,46,67]. NTDs are frequently associated with trisomies 13 and 18. A study by Kennedy et al. [46] suggests a frequency of chromosomal anomalies in 6.5% (13/212) neural tube defect patients. A gene or genes in the region of 13q33−34 associated with a 13q deletion syndrome has been shown to cause NTDs [51]. These cytogenetic rearrangements can be key positional clues to candidate genes and have been recently summarized [53].

If neural tube defects are genetic, how do they present in families?

One of the longest running controversies, as yet undecided, is whether NTDs at different levels represent different defects. In other words, are rostral level defects (e.g., anencephaly) different in some fundamental way than caudal defects (e.g., myelomeningocele)? Additionally, are lesions that include both rostral and caudal levels (e.g. cranioraschis) altogether variant embryopathies? If the etiology of upper and lower lesions are different, then it would be expected that recurrences in families would breed true: affected individuals in an upper lesion family would all have upper lesions and vice versa for lower lesions. NTDs tend to breed true within families; in other words, recurrences in families in which the case is affected with spina bifida tend to be spina bifida, and recurrences in families in which the case is anencephaly tend to be anencephaly [18,26,28,33,87]. However, between 30−40% of recurrences involve an NTD phenotype that is different from the case phenotype. This intra-family heterogeneity may represent the pleiotropic effect of a common underlying gene or may suggest that families with different phenotypic presentations may result from different underlying genes. Alternatively, these dramatic phenotypic differences within families may suggest slight differences in timing to key environmental exposures in susceptible pregnancies, or may suggest that the underlying

genes are different. Or, these differences may represent the variable outcomes following different environmental exposures at key developmental times, or even just the result of random chance. While studies to date have provided conflicting and inconclusive results, the availability of such families will be vital to understanding the genetic and environmental influences to NTDs.

Clues to genes involved in human neural tube defects from mouse models

The folding of the plate results from a number of region-specific mechanisms, as suggested by the regional localization of neural tube defects observed in humans and in mutant mice. More than 80 mutations in a variety of genes have been identified and linked to a variety of rodent NTDs, implicating more than 100 genes directly or indirectly in neural tube formation. These genes have recently been comprehensively reviewed [12,34,35,95]. Unlike the majority of human cases, many of these mutants show autosomal recessive inheritance and, in addition to NTDs, these mice present other associated anomalies. Moreover, the penetrance and expression of many of these mutations are affected by the genetic background, which can increase the susceptibility to teratogen-causing NTDs, consistent with multifactorial inheritance. The mechanisms by which NTD arise in these murine models are generally unclear, even when the mutated gene has been identified. The most relevant animal model of human NTDs are the SELH mice, where the liability to exencephaly is genetic and best fits a multifactorial threshold model of inheritance involving 2 or 3 loci [41].

The best model for caudal spinal NTD, the most common presentation in humans, is the curly tail mouse, that naturally develops a lumbosacral myelomeningocele and is a phenocopy of nonsyndromic multifactorial human neural tube defects [59]. Recently, a mouse homologue of the Drosophila grainyhead transcription factor, Grhl-3, was shown to be responsible for this phenotype [85]. At the tissue level, mutant curly tail mouse embryos exhibit a cell-type-specific abnormality of cell proliferation that affects the gut endoderm and notochord but not the neuroepithelium [13]. The reduced rate of ventral embryonic cell proliferation results in a growth imbalance between ventral gut primordia and the dorsal neural elements. The result is a delay in posterior neuropore closure because of abnormal caudal flexion, resulting in spinal neural tube defects [10].

Mutations in the Macs gene in mouse lead to exencephaly and other midline NTDs; its human homologue MACS has been localized to 6q21−22.2 [4,50,83]. Most mouse models for NTD lead to exencephaly, the mouse counterpart for anencephaly, the less common but most severe NTD manifestation in humans. Murine models with hindbrain exencephaly, such as the Pax-3-splotch mutant, are noted to have defective DLHP formation in the region of the hindbrain [17,21−23]. Of relevance to the human condition, the *Pax3* gene has been reported to be defective in Waardenburgs syndrome patients with a subset having spinal neural tube defects [2]. It is not known how mutant *Pax3* causes neural tube defects; increased apoptosis [5,62], faulty pyrimidine synthesis or alterations in cell migration [19,52] have been proposed.

In four mouse mutants with cranioraschisis, *disheveled* [3,40], *loop-tail* [45], *circletail* [56], and *crash* [14], the underlying cellular mechanism has been attributed to abnormal neural plate development as a consequence of disturbed convergent extension. Disturbing convergent extension yields a shortened and broad neural plate, thus a widened and misshapen MHP. The planar-polarity gene-Wnt signaling pathways [91] are thought to be the responsible molecular substrate.

No mutations identified in mouse have yet been shown to represent major genes for NTD in humans. Mimicking the genetic complexity seen in humans will be difficult, since it is likely to be caused by a cumulative effect of several interchangeable loci, not a major gene with modifiers. Nonetheless, since humans are a non-experimental system, understanding the

relationship between humans and a model system such as mouse will be key to eventually considering interventions based on genetic and environmental risk.

Environmental factors associated with neural tube defects

Myriad exogenous causes for NTDs have been postulated and investigated (see [20,32] for review). Factors for which no significant association with NTDs has been found to date include maternal and paternal age effects, maternal periconceptual infections, number of prior "successful" pregnancies, recreational drug use, caffeine intake, smoking, and alcohol use. Hyperthermia (fever and/or hot tub use) has been investigated, though most of these studies are subject to extreme recall bias and have yielded inconsistent results. However, increased risk for NTDs is definitively associated with maternal diabetes and maternal obesity (both associated with glucose metabolism), and maternal use of anti-convulsant medications (for the treatment of epilepsy). For example, anti-epileptic drugs administered to pregnant mothers induce congenital malformations, the incidence rising from 3% without drug to 9% with drug administration [44]. These numbers can rise up to 28% when 3 or more antiepileptic drugs were given to the epileptic mother [36]. The well-known anti-epileptic drug, valproic acid, is teratogenic when given to pregnant women, and its administration results in 1 to 2% incidence of spina bifida [49,58]. Moreover, recent data suggests that this agent also induces mental retardation in children with no physical manifestation.

Paternal exposure to Agent Orange in Vietnam veterans has been implicated, as has water chlorination by-products [39] and maternal exposure to solvents through house cleaning occupation [7]. Exposure to fumonisins, a fungal metabolite commonly found in maize, has also been implicated and *in vivo* and *in vitro* studies have demonstrated an association of exposure with neural tube defects [73]. Prenatal exposure of mice to cadmium has shown that the metal is localized in the developing neural tube and can result in NTDs [15,92]. These known environmental associations, however, are insufficient to explain the degree of familial aggregation observed in NTDs.

Several studies have demonstrated that maternal periconceptional supplementation with folic acid reduces the recurrence risk for NTDs (e.g.,[54]) by $50 - 70\%$, implicating genes involved in the metabolism of folate. Yet the recurrence risk is not entirely eliminated (e.g., above and [9], suggesting that additional, genetic factors are responsible for the development of NTDs and these non-folate responsive cases may represent highly genetic cases of NTDs [76]. The mechanism for how folic acid works to reduce the risk is unclear and likely mediated by genetic effects. Folate acts as a cofactor for an enzyme involved in DNA and RNA biosynthesis, and is also a supplier of methyl groups to the methylation cycles [75]. Folate deficiency leads to up-regulation of folate receptors, which are ubiquitous and mediate folate uptake at physiological level [1]. A recent study by Rothenberg et al. [72] showed that some mothers with a pregnancy complicated by a NTD produced autoantibodies that bind to folate receptors on the placental membrane and therefore blocked the binding of folic acid.. The authors further suggest that the periconceptual administration of folate would bypass the autoantibodies that mediate a placental folate receptor blockage. Indeed, folate has a high affinity for its receptor and might displace the autoantibody when administered at high doses.

Identifying those women whose risk for NTD is minimized by folic acid supplementation would allow genotype-directed pharmacogenetic interventions. Researchers are looking at a number of different genes involved in folic acid metabolism, including those encoding folate receptors, 5,10-methylenetetrahydrofolate reductase (MTHFR), and cystathionine (beta)-synthase. Recent studies have implicated homozygosity for the C677T thermolabile variant of the MTHFR gene as a risk factor for NTDs ([27,61,93] among many others), and others have suggested that the effect may be dependent on level of lesion [90]. A recent meta-analysis

[6] found a pooled odds ratio for infants homozygous at C677T of 1.7 (95% CI 1.4 − 2.2), with a pooled attributable fraction of 6% for homozygosity. While the paternal effect was non-significant, the odds ratios for maternal genotype, either homozygous or heterozygous for the T allele, were consistent with a trend for MTHFR involvement (OR for homozygosity was 2.1 [95% CI 1.5 − 2.9] and for heterozygosity was 1.2 [95% CI 0.9 − 1.5]).

In addition, other mutations in the MTHFR gene have been investigated, including A1298C, and other genes, such as cystathionine β-synthase, that when in combination with the C677T allele may increase the risk for NTDs [81,89]. Several reports[63,69,82] have failed to demonstrate the association seen with the C677T MTHFR allele and NTDs. Additional data suggesting that MTHFR is not a major risk factor comes from a report by Molloy [55] confirming that homozygosity for the "risk" allele fails to influence maternal folate levels, which are known to predict NTD risk.

Synthesizing the data

Current technology for approaching complex diseases continues to be developed at a phenomenal rate. Novel approaches from the molecular, expression, and statistical realms promise enhance ability to identify genetic influences, understand the interactions between genes, and characterize the relationship of environmental risk factors to genetic susceptibilities. Integrating these approaches will facilitate progress. Any insight into one or more genes predisposing to the development of neural tube defects will lend useful information towards more accurate genetic counseling for families and prevention of these frequent birth defects.

Acknowledgments

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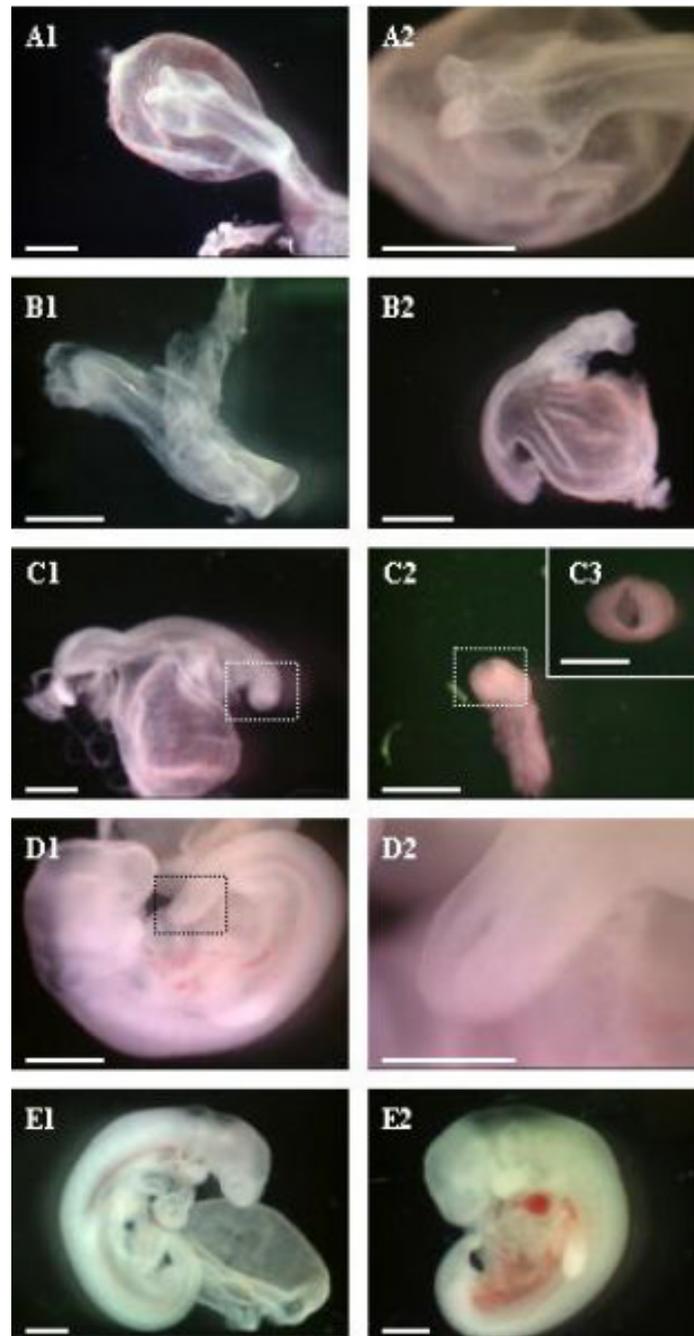
Figure 1.
Human embryonic developmental stages during which the neural tube forms. A1&2: Carnegie stage 9 (CS9 − 20 days) the neural groove is open and anterior neural fold is visible. B1&2: CS10 (22 days). The neural folds fuses centrally leaving an open tube in the rostral and caudal region. C1, 2 & 3: CS11 (24days) The neural tube is closed except for the rostral (C2&3) and caudal neuropores. D1&2: CS12 (26 days) the caudal neuropore is closing (C2). E1&2: CS13 (28 days) The neuropores are closed. E1 corresponds to early CS13 and E2 to a late CS13. The scale bars represent 1 mm in all photographs except C3 and D2 where they represent 0.5 mm.