BASIC SCIENCES REVIEW

Organization of the neuronal circuits in the central nervous system during development

H Lagercrantz and T Ringstedt
Karolinska Institute, Astrid Lindgren Children’s Hospital, Stockholm, Sweden


The human brain is a product of genetic instructions, cellular interactions and influences of innate activity and external stimulation. The formation of the neural tube and the patterning of the brain are determined by homeotic genes. After a prosencephalic phase with the formation of the hemispheres, the neurons proliferate to number about 100 billion halfway through gestation. They also migrate to their final positions in an inside–outside fashion with the newly formed neurons at the outer layer of the cortex, followed by synaptogenesis, programmed cell death and organization of the neuronal circuits. This phase is probably determined not only by genes but also by innate activity, which for example has been detected in the foetal retina: “Cells that fire together wire together while those which don’t won’t”.

Conclusion: Development of the neuronal circuits in the CNS can be viewed as epigenetic, i.e. many different components must come together at the right time and place.

Key words: Axonal guidance, homeotic genes, neural tube, neuron proliferation and migration, synaptogenesis

H Lagercrantz, Astrid Lindgren Children’s Hospital, Neonatal Programme, Karolinska Hospital H1: 02, SE-171 76 Stockholm, Sweden (Tel. +46 8 5177 4700, e-mail. Hugo.Lagercrantz@ks.se)

The development of the CNS proceeds through a series of milestones, from induction of the neuroectoderm to formation of the neural tube, cephalic folding, proliferation, migration, synaptogenesis and wiring (Table 1). The first steps are probably strictly genetically controlled. However, it is difficult to understand how about 30000 genes in the human can control the organization of about 100 billion neurons and trillions of synapses. Changeux (1) pointed out that there is a striking parsimony of genetic information to code for brain complexity in the human compared with, for example, nematodes which have only 302 neurons but nearly 20000 genes. One possible solution is multiple combinations of gene activity in time and space (2). Another possibility is that the genes are just involved in the scaffolding of the brain and only impose certain genetic constraints (3). Environmental mechanisms should then be responsible for the more detailed wiring. Alternatively, there is a redundancy of neuronal circuits in the immature brain: a jungle, which is successively organized owing to functional requirements by the group selection mechanism or neuronal Darwinism (3).

Patterning of the foetal human brain

The development of the human brain cannot be understood without considering that it has derived from evolutionary tinkering with ancestral brains (1). Numerous genes involved in the early formation of the brain have been retained during evolution, from 600 million years ago, when the insects and vertebrates started to develop along separate branches. These genes are called homeotic genes and are involved in controlling the Cartesian coordinates of the embryo, the segmentation of the body and identity of its segments. They may be used and reused in different ways during both phylogenesis and ontogenesis (Fig. 1).

The brain originates from the neuroectoderm, which may comprise about half of the whole ectoderm (4, 5). The neuroectoderm is developed by a default pathway, i.e. the formation of non-neuronal ectoderm on each side is achieved by blocking signals at either side (6).

Table 1. Milestones in the development of the central nervous system.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>3–4 wk</td>
<td>Formation of the neural tube</td>
</tr>
<tr>
<td>5–10 wk</td>
<td>Prosencephalic phase, formation of the hemispheres</td>
</tr>
<tr>
<td>8–18 wk</td>
<td>Neuronal proliferation</td>
</tr>
<tr>
<td>12–24 wk</td>
<td>Migration</td>
</tr>
<tr>
<td>25+ wk</td>
<td>Wiring of the brain, arborization of the neurons, synaptogenesis, apoptosis</td>
</tr>
<tr>
<td>40+ wk</td>
<td>Myelination</td>
</tr>
</tbody>
</table>
The neural groove is made by bending the neural plate and by the action of the notochord. To form the rostro-caudal axis in the embryo the HOX genes (clustered homeobox-containing genes) are important. They are homologous to the homeotic gene complex (HOM-C) in the fruit fly (Fig. 1). The Hox gene clusters (A–D) differentiate the segments. According to the Hox code, cells expressing many Hox proteins develop posterior structures, while those with lower levels form the anterior part of the axis. Knocking out these genes results in the replacement of more posterior segments (7). Excess doses of retinoic acid, which affects the expression of more posterior Hox genes, disrupt the order of rhombomeres. Genes from the Engrailed (En) family are required for the formation of the tectum (dorsal midbrain) and the cerebellum. EMX and OTX are expressed in the forebrain and midbrain region. Mutation of the EMX gene leads to severe malformation of the cortex (schizencephaly).

Krox-20 is necessary for the development of the cranial nerves in the hindbrain. The Sonic Hedgehog gene serves several functions dependent on timing and spacing. The hedgehog genes encode for proteins that determine, for example, which neurons should develop into motor neurons controlling muscle movements in the spinal cord. If this gene is knocked out all neurons will become sensory neurons by a default pathway. Furthermore, the phenotype will develop a cyclopic eye. Mutation of this gene in the human results in holoprosencephaly.

Another important family of genes comprises the Pax genes. The name is derived from the paired box first identified in the fruit fly. They encode for transcription factors and are important for the formation of the dorso-ventral axis. Mutations of PAX3 in the human result in Wardenburg’s syndrome and of PAX6 in Peter’s anomaly (aniridia).

How do these early immature nerve cells know how to differentiate? The concentration of the inducing substance seems to be important. For example, high concentrations of the Sonic Hedgehog protein induce the formation of most of the ventral cells of the neural plate, while lower levels specifically induce motor neurons. Thus, if undifferentiated cells are exposed to a high concentration of the substance they will become different types of cells than if they had been exposed to lower levels (5).

Proliferation of neurons

After formation of the neural tube and prosencephali, proliferation of new neurons takes place. New neurons originate from the pseudostratified ventricular epithelium (PVE). Cells in the pseudostratified epithelia become neurons first by elongation during the G phase and enter the S phase maximally elongated with the nucleus in the other half of the epithelium. The cells proliferate asynchronously, i.e. after mitotic cell divisions, one will migrate and not proliferate further, while the other returns to the PVE and undergoes a new cell cycle. The pace of neuron production is dependent on the exit rate of cells from the ventricular epithelium (8).
Young, newly formed neurons migrate and form the neocortex.

To determine the rate of proliferation, the cells in the epithelium are labelled by the thymidine analogue BrdU, which will be incorporated into DNA. By using this cumulative labelling parameters such as the duration of the cell cycle or neurogenetic interval can be calculated. In the mouse there are 11 cell cycles over a 6 d period.

About 200,000 new neurons are formed every minute between the 8th and 18th wk of gestation. The fact that most nerve cells are formed after the 8th and before the 18th gestational week was first established by Dobbing and Sands (9), who analysed DNA in aborted foetuses. This was also learned in a tragic way: foetuses that were exposed to the first atomic bombs in Hiroshima and Nagasaki during this period of pregnancy became microcephalic, a condition which did not affect foetuses who were younger or older at the time of these events (10).

Based on a series of very careful studies in primates, Rakic postulated that there is no neurogenesis after birth in the human (11, 12). Other animals such as male canarian birds generate new nerve cells in the singing centre during the mating season. The number of syllables that they perform seems to be directly related to the number of neurons (13). Recent studies indicate that some new neurons can be formed even in the human adult. Terminally ill cancer patients were given radioactively labelled thymidine, and after their deaths the nuclei of about a few hundred hippocampal neurons were found to be labelled, i.e. they were newly formed neurons (14). Whether these neurons are of functional significance remains to be elucidated.

**Migration**

During formation of the neocortex the first postmitotic neurons, born in the ventricular zone, will migrate radially to form the primitive plexiform zone or the preplate. Cells born later migrate into the preplate and split it into an outer marginal zone or future layer I, and an inner subplate. These neurons migrate along a fan-like scaffold of glial threads. Newly born cells will migrate past those cells that arrived earlier. The neocortex therefore has an “inside–out” pattern, with the latest born cells in layer II and the first born (excepting those in the subplate and the marginal zone) in layer VI. Migration occurs mainly between embryonic day 12 and the first postnatal days in the rat and mouse, and between the 12th and 24th wk of gestation in the human foetus.

The neurons find their way by climbing the radial glial cells. These glial guides correspond to a protomap present in the germinative zone and the cortical areas (11). Integrin receptors play a critical role. About 20% of the new neurons migrate horizontally without the support of radial glia. Some of the early-born neurons in the marginal zone, the Cajal-Retzius cells, secrete reelin. This substance is essential for correct cortical lamination. It may act by inhibiting the migrating neurons, instructing them to leave the radial glia and obtain their final position. Thus, reelin would act as a stop signal for the successive waves of cortical neurons.

Neuronal migration can be affected by glutamate. N-Methyl-D-aspartate (NMDA) antagonists were found to retard migration or result in the formation of heterotopias and the arrest of migrating neurons (15). Neurotrophic factors such as neurotrophin-4 (NT-4) and brain-derived neurotrophic factor (BDNF) can also affect neuronal migration.

Severe disturbance of migration can be seen in the Zellweger cerebro-hepato-renal syndrome, which is a peroxisomal disease. A migration disorder is also involved in schizencephaly (16).

**Axonal guidance**

The crucial question is how the neurons find their way to their targets. Navigating the interstates from New York to San Francisco is relatively easy compared to what the neurons of the developing nervous system must do in order to reach their goals, as it was expressed in Science (17).

In 1892 the Spanish anatomist Ramon y Cajal discovered that the axons have special growth cones on their tips. These growth cones are like immune cells sniffing out chemical scents released by different tissues. This has now been confirmed. The target seems to release a diffusible substance that promotes its own innervation: attractant molecules. Given that the distance between the target and the origin of the axon can be quite long, mechanisms other than simple attraction are also involved. By analogy with the traveller crossing continental USA, the axon divides its path into several steps, manoeuvring between choice points along more or less established routes. The signals that guide a growing axon along its way can, based on their function, be subdivided into four categories: chemoattractive, chemorepellent, contact attractive and contact repellent. While the chemotactic and chemorepellent signals are diffusible molecules that act over longer distances, the contact-attractive and -repellent molecules are bound to cell membranes or to the extracellular matrix. Repulsive signals seem to be particularly important in axonal pathfinding, because they can be used to outline a permissive path for the growing axons. The complicated task of navigating long distances through the CNS is eased by following routes established by pioneer axons, which reached their targets during early development, when the brain was smaller and its structure considerably less complex. Axons that grow along these routes are bundled together in fascicles. At the various choice points they have to de-fasciculate in order to
change route. Therefore, molecules that modulate fasciculation are important in axonal guidance.

Certain structures in the brain are of particular importance to navigating axons. An example of this is the floor plate, an area with mainly non-neuronal cells in the ventral part of the developing spinal cord. The floor plate seems to express several guidance cues. Among these is netrin, which attracts commissural neurons in the upper part of the spinal cord. Netrin is evolutionary conserved and is also found in the fruit fly (Drosophila). The midline of the fly’s nervous system is, similarly to the spinal cord floor plate, an important landmark for axons. Axons are attracted towards the midline by netrin. The midline also expresses the diffusible ligand Slit, which repulses axonal growth cones carrying the Slit receptor (Robo), thereby preventing these from entering the midline (Robo is an abbreviation of roundabout, as found along British roads). For axons destined to cross over the midline the situation becomes very complicated, since once they have entered the midline they cannot leave it (on the other side) unless repulsed by Slit. This paradox is solved by the expression of another gene, Comm, in the midline. Comm downregulates Robo expression on the neurons growing towards the midline, thus enabling them to enter despite the presence of Slit. After they have entered the midline, the axons lose their sensitivity to Comm, Robo is upregulated and they are forced to leave the midline by the repellant activity of Slit. The combined activities of Netrin, Slit and their receptors with Comm thus guide the axons across the midline and ensure that they do not recross it (18). Like Netrin, Slit and Robo are also found in mammals (including humans).

Several other ligand and receptor families are known in addition to those mentioned above. Among these are the semaphorins with over 30 members, some of which are secreted, while others are membrane bound. Semaphorins can signal both attraction and repulsion and, at least in some cases, the same ligand can serve both functions. The semaphorin ligands also seem to have different biological specificities. Classes of membrane-bound molecules that mediate contact inhibition and attraction include the immunoglobulin cell adhesion molecules (Ig CAMs), the cadherins, and the Eph receptors (19). The Ephs constitute the largest subgroup within the receptor tyrosine kinases. Their ligands, the ephrins, are also quite numerous, and the receptor-ligand interaction is quite promiscuous. Like their receptors, the ephrins are membrane bound and it is possible that they may also have a receptor function.

A growth cone is likely to carry receptors for several classes of axon guidance molecules. The inputs from these are integrated into a “decision” (20). One way that this could be achieved is by intracellular signalling mechanisms affecting the growth cone’s Ca$_{2+}$ level. It has recently been demonstrated that restricted elevation of [Ca$_{2+}$] on one side of the growth cone induces turning either towards that side (attraction) or away from it (repulsion), depending on the extracellular Ca$_{2+}$ level (21). Many axonal guidance molecules have also been shown to induce both attraction and repulsion, although, at least in some cases, this is dependent on the type of receptor involved (22).

Some ligands involved in axonal guidance, for example Slit, also affect cell migration. It has lately become increasingly clear that axonal guidance and cell migration have many mechanisms in common. The migrating cell sends out a leading process that trails ahead of the cell soma and probably orients towards the target like an axonal growth cone.

**Neurotrophic agents**

Nerve growth factor (NGF) was discovered by Rita Levi-Montalcini and Viktor Hamburger in 1951. They found that sensory and sympathetic ganglia grow better in the vicinity of sarcoma tumours and they postulated that this was due to a secreted agent. To characterize this factor they used snake venom to inactivate the postulated protein. To their surprise this resulted in better stimulation of nerve growth, since the salivary glands contain high amounts of NGF. Salivary glands were then used to isolate NGF. [See the fascinating autobiography by Rita Levi-Montalcini (23).]

Other nerve growth factors or neurotrophic agents have since been discovered (24). The neurotrophin family comprises NGF, BDNF, NT-3 and NT-4. They are diffusible peptide factors active in the form of homodimers. They act through two classes of receptors: the p75 neurotrophin receptor (p75 NTR) and the tropomyosin kinase receptors (trkA, trkB and trkC).

The role of the neurotrophins is better understood in the peripheral than in the central nervous system. In the peripheral nervous system they mainly act as target-derived neurotrophic agents ensuring the survival of axons that innervate the target correctly. However, they also seem to be important in the CNS, although studies on mice with neurotrophin ligands and receptor genes deleted, showed, contrary to expectations, that most of the cell populations in the CNS were retained, indicating that their function there is a different one (25).

As an example, embryonic overexpression of BDNF in the brain of transgenic mice resulted in a striking and unexpected phenotype with clustering of the Cajal-Retzius cells and a reduced expression of reelin. The normal inside–out laminar formation of the neocortex was disturbed. On the basis of these findings it is assumed that BDNF acts as an intrinsic determinant of cortical maturation (26).

**Neurotransmitters**

Although the development of the scaffold of the CNS is mainly determined by genes, the detailed wiring of the neuronal circuits is more self-generated, depending on
the action of neurotransmitters and neuromodulators. They can promote, amplify, block, inhibit or attenuate the microelectric signals which are passed on to them and through them, and thereby give rise to the signalling patterns between myriads of neuronal networks providing the physical networks of cerebral neurons. Catecholamines appear in the embryos of vertebrate and invertebrate animals even before neurons are differentiated. Possibly, they then function as morphogenetic or trophic factors. Some of the neuronal crest-derived neurons are noradrenergic during early development, but later become cholinergic through environmental influences.

A neuroactive agent might be abundantly expressed during certain stages of development, but later remain only in only a small proportion of the CNS synapses. This agent may play a transitory role during a critical window during development or remain mainly as an evolutionary residue with only minor functions, e.g. in mammals (27).

It is interesting to note that if the synthesis of some these neurotransmitters and modulators is blocked pharmacologically or knocked out by transgenic techniques, the apparent effect may be minimal. This illustrates the plasticity of the brain during early development. Other neuroactive agents seem to be able to take over.

Noradrenaline and acetylcholine are regarded as classical neurotransmitters and dominate in the peripheral nervous system. They appear at an early stage both during phylogenesis and ontogenesis. Many of the neuropeptides were first identified in the gastrointestinal tract and probably also appear early during development. They act slowly since they have to be synthesized and packaged in the cell soma and carried to the terminals before they can be released. The more developed sophisticated mammalian brain requires more fast-switching neurotransmitters acting directly on ion channels, such as excitatory amino acids which seem to dominate in the mature brain, whilst the monoamines and neuropeptides may act more as neuromodulators.

In the immature brain, synaptic transmission is weak, extremely plastic and mediated to a large extent by NMDA receptors. The z-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are more or less silent at resting membrane potentials (28). During maturation many NMDA receptors are substituted by AMPA receptors. Dark rearing or blocking the activity with tetrodotoxin results in preservation of the NMDA receptors. Dark rearing also preserves the immature form of the NMDA receptors containing NR2B and the expression of NR2A is delayed. This subunit switch is essential for rapid synaptic transmission. Thus, NMDA receptors are important for the experience-dependent synaptic traffic.
Another amino acid, \( \gamma \)-aminobutyric acid (GABA), is known as an inhibitory neurotransmitter in the mature brain. However, it is excitatory in the developing brain. This is due to the Cl concentration being high in immature nerve cells. When GABA opens the Cl channels a depolarization occurs (i.e. excitation). During maturation the Cl concentration decreases intracellularly, resulting in an opposite effect of GABA, i.e. Cl ions are pumped out and the cells become hyperpolarized (29). In this way GABA switches from being an excitatory neurotransmitter in the foetus to an inhibitory one after birth.

Synaptogenesis

Five “waves” of synaptogenesis have been identified in the primary visual cortex of the macaque monkey (30) (Fig. 2). Based on studies of the human occipital cortex (31) a tentative timetable can be applied for humans.

- Phase 1 begins around 6–8 wk of gestation, at the same time as the onset of neuron proliferation. Synaptogenesis is limited to lower structures such as the subplate.
- Phase 2 begins after 12–17 wk and is also relatively sparse. It occurs in the cortical plate. These early synapses form contacts on the neuronal dendritic shafts of the neurons.
- Phase 3 is much more rapid and is assumed to start around midgestation (20–24 wk) and persist up to 8 mo after birth. The rate of this synaptogenesis has been estimated as 40000 new synapses every second in each striate cortex of the macaque. It occurs simultaneously as the arborization of axons and dendrites.
- Phase 4 lasts until puberty and occurs also at a very high rate.
- Phase 5: synaptogenesis continues up to the age of 70 y, but there are also considerable losses during this phase.

The first two phases are not affected at all by a lack of sensory stimulation, while the third phase may be partially dependent on sensory input. This has been demonstrated by visual stimulation or ablation in the macaque. Synaptogenesis during the third phase is partially intrinsic and partially dependent on sensory stimulation. Thus, it coincides with the critical periods. Many of the sensory, motor and cognitive skills function very early after birth when the synaptoarchitecture is still being laid down.

Synaptogenesis during the fourth phase is even more dependent on experience. During this phase there is a reorganization and fine tuning of neuronal circuits. When this phase has ended during puberty there seems to be a “freezing” of personality and the end of several basic learning capacities such as learning to talk a new language without an accent.

There is some evidence that learning induces the formation of new synapses. However, learning during life would then result in continuous brain growth. Recently, it has been demonstrated that associative learning does not increase the number of synapses in the hippocampus (32).

Wiring the brain

The wiring of the precise neural circuits seems to be dependent on neuronal activity, which could be stimulated by either sensory input or endogenously driven activity. Redundant numbers of neuronal pathways and circuits are formed in the foetal brain. About half of the neurons disappear before birth by apoptotic processes. This was found in the 1930s by Hamburger, who observed that the number of neurons innervating the chicken wing decreased during maturation (33).

The importance of sensory stimulation was discovered by Hubel and Wiesel, who found in the 1960s that closing one eye in kittens or young monkeys resulted in blindness in that eye and disruption of the ocular dominance columns in the visual cortex (34). This occurred between 4 wk and 4 mo in the kitten, while the sensitive period began earlier in the monkey.

This process also occurs before birth (35). Penn and Shatz studied the lateral geniculate bodies in foetuses of the ferret. The optic nerves from the two eyes grow into the geniculate and spread out through all layers. During maturation these structures become organized and layers are formed. This process is dependent on spon-

Fig. 3. Neurons which fire together wire together, neurons which don’t won’t (35). (Figure by S Söderlind.)
taneous firing in the retina. If it is blocked with tetrodotoxin, the segregation into layers is disturbed.

The spontaneous activity begins at some focus of the retina and spreads out in waves. This could be visualized by a fluorescence imagining technique in whole-mounted retinas from newborn ferrets. It seems to be generated by cholinergic amacrine cells, as it could be reversibly blocked by a nicotinic acetylcholine receptor antagonist (36).

Each wave lasts for several seconds, followed by 1 min interval. Neighbouring cells seem to fire in synchrony and this local retinal synchrony forms the basis for the layering of the geniculate bodies and the ocular dominance columns in the cortex. Shatz coined the expression: “Cells that fire together wire together while those which don’t won’t” (Fig. 3).

However, recent studies by Crowley and Katz (37) have shown that ocular dominance columns can be formed without electrophysiological stimulation and are thus genetically determined. They admit that visual stimulation is important for the refinement of the ocular dominance columns and thus for vision.

Organization of the brain (Fig. 4)

Changeux wrote in Neuronal man (2) that, “Recognizing the power of the genes in no way forces us to submit to their supreme authority”. The claim that complex behaviours are mainly genetically determined has been strongly refuted by Rose (38), who is one of the strongest critics of “neurogenetic determinism”. The discovery of a number of genes determining diseases and human behaviour may result in some social dezeitism, according to Rose (38). The genome is relatively simple, while the brain (in particular the human brain) is very complex. Genetic determinism assumes that genes directly control development, morphology and behaviour. Development, in this view, would be purely a process of programmed maturation, contaminated perhaps occasionally by a certain amount of noise (3). Development is rather epigenetic, i.e. many different components have to come together at the right time and in the right place. The idea that the genes provide the “blueprint” could probably be rejected. Genes certainly matter, but development is more a form
of jazz with improvisation than a fixed musical score (39).

Environmental instructionism cannot explain the formation of the neuronal circuits. The view of the newborn brain as a tabula rasa has been abandoned and it is now well established that the human baby is born with the ability to recognize the human face and voice, etc. The idea of there being an innate grammar is controversial, but the consensus seems to be that some kind of language ability is innate (40).

Edelman (3) has proposed the idea of neuronal Darwinism or the theory of neuronal group selection (TNGS) to explain how the brain is constructed with regard to the limited number of genes that encode the formation of the human brain. The TNGS consists of three tenets. The first is that dynamic primary processes lead to the formation of the neuroanatomy. This anatomy possesses enormous variation due to the “stochastic fluctuation of cell movement, cell process extension and cell death during development”. The entire process is a selectional one based on topobiological competition. The second tenet states that a variety of functioning circuits is carved out, i.e. strengthening of the synapses. In the third tenet physiology and psychology are combined. Maps are formed in the brain by sensory impressions and new impressions reinforce the neuronal wiring of certain maps. Neurotransmitters such as acetylcholine and dopamine have been proposed to be involved in this selection mechanism.

According to Edelman, the developing brain is a jungle. It is not a question of creating novel connections, but rather eliminating pre-existing ones (2).

Neuronal Darwinism or the TNGS has been challenged by Purves et al. (41). He claims that there is no selective elimination of an initial excess of dendrites and synapses. Although there is essentially a very small increase in the number of neurons after birth, the existing neurons grow and develop synapses throughout life. Growing neurons gain synapses rather than lose them. Spontaneous activity stimulates the formation of synapses and elaborates the synaptoarchitectonic organization. Losses of neurons, axons and synapses are merely epiphenomena (41).

According to Changeux, Purves favours a kind of Lamarckian constructivism: “the function would create the organ”. One example in favour of Purves is how well the tongue and the thumb are represented in the human brain, corresponding to the importance of these organs for humans. Conversely, associative learning does not seem to increase the total number of synapses and stimulate brain growth, which speaks against the idea of constructivism (42).

Acknowledgement.—This review is modified from a prior review entitled: Epigenetic and functional organization of the neuronal circuits in the CNS during development published in Fetal and Neonatal neurology and neurosurgery (eds M Levene, FA Chervenak, M Whittle) Harcourt Publ. London 2001.

References

17. Travis J. Wiring the nervous system (News). Science 1994; 266: 568–70

ACTA PÆDIATR 90 (2001)

Received Mar. 14, 2000; accepted Feb. 27, 2001