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Transcriptomic changes in brain development

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Abstract

The transcriptome changes hugely during development of the brain. Whole genes, alternate exons and single base pair changes related to RNA editing all show differences between embryonic and mature brain. Collectively, these changes control proteomic diversity as the brain develops. Additionally, there are many changes in non-coding RNAs (miRNA and lncRNA) that interact with mRNA to influence the overall transcriptional landscape. Here we will discuss what is known about such changes in brain development, particularly focussing on high throughput approaches and how those can be used to infer mechanisms by which gene expression is controlled in the brain as it matures.

Keywords

Adult; Editing; Embryo; Gene expression; RNA-Seq; Splicing

Introduction

The cellular and molecular complexity of the mature adult brain is influenced both by processes in development and by the experience-dependent formation of neuronal circuits (Innocenti & Price, 2005; Sur & Rubenstein, 2005). Brain development occurs throughout embryonic growth and, in most species, continues after birth with a wide variety of developmental programs between species (Borrell & Calegari, 2014). To form the mature brain, many different cell types need to differentiate from basal progenitors, migrate to their anatomical positions and, for neurons, form synapses. As such, brain development is a highly regulated process.

Part of the regulation of brain development includes the coordinated expression of many different genes in a spatially and temporally appropriate context. Virtually all levels of gene expression, from whole genes to splicing and RNA editing, show evidence of regulation during brain development. The purpose of this review is to discuss all levels of gene expression in the development of the brain with a particular emphasis on genome-wide techniques that have allowed for an overall view of the generality of expression changes in this organ.

Gene expression

A number of studies have employed microarray technology in an attempt to study the molecular changes occurring during brain development including many reports at a genome

wide scale. Although these could be organized by species or brain region, here we will use the order of publication as, in general, the depth of coverage in genomewide techniques has increased over time.

In 2001, two papers were published using arrays to look at development in the mouse hippocampus (Mody et al., 2001) and cerebellum(Kagami & Furuichi, 2001) using affymetrix arrays. These two brain regions have slightly different trajectories of development in the mouse and the two studies used slightly different choices for developmental time point. Mody et al., examined the hippocampus at embryonic day 16 (E16) and postnatal days 1 (P1), P7, P16, and P30 whereas E18, P7, P14, P21, and P56 were used in the cerebellum by Kagami and Furuichi. Despite the differences between the two regions and slight differences in the choices of time points, there are some substantial overlap in the genes and, more importantly, types of genes identified in both studies. In both studies there was a decrement in expression of gene related to neuronal proliferation or cell division from embryonic to postnatal stages(Kagami & Furuichi, 2001; Mody et al., 2001). This presumably represents the maturation from dividing neuronal precursor cells to mature, post-mitotic neurons. One example of gene expression that tended to increase with brain development noted by Mody et al was the upregulation of genes involved in glycolysis, consistent with a shift from ketone to glucose metabolism with brain maturation (Mody et al., 2001). Similarly, gene expression profiles consistent with synaptic maturation and associated signal transduction were clearly seen in both studies(Kagami & Furuichi, 2001; Mody et al., 2001). A summary of some of the key types of genes that are developmentally regulated in different brain regions are shown in Figure 1.

A similar study of the developing cerebellum focused particularly on granule cells which largely develop during the postnatal period in the mouse (Díaz et al., 2002). As well as examining expression patterns in the cerebellum, Diaz et al used the same technology to look at cultured granule cells developing *in vitro*, mutant mice where granule cells are lost in the postnatal period, as well as the pontine nucleus to which the granule cells project. Although the data series are therefore complex to interpret, they indicate that gene expression in development involves both cell autonomous and non-cell autonomous regulation.

Further studies extended these observations using whole brains using additional embryonic stages(Matsuki, Hori, & Furuichi, 2005), focusing on the prefrontal region of the cerebral cortex in postnatal development (Semeralul et al., 2006), or examining the cortex from embryonic to postnatal development (Pramparo et al., 2011). In general, these studies confirmed earlier results in that there were consistent decreases in cell division proteins and acquisition of genes that encode for synaptic proteins. Another interesting observations across several of these experiments are the sheer numbers of genes that show some evidence of regulation during brain development. For example, Matsuki et al reported that 1413 genes (~11%) showed altered expression in the prenatal period while Semeralul et al. reported 366 differentially expressed probe sets in the postnatal period. Although these two studies are not strictly comparable, they suggest that effects on gene expression are greater in the prenatal compared to postnatal period.

A similar picture of gene expression during development emerges from studies of the human prefontal cortex (Colantuoni et al., 2011). The absolute number of genes that show differential expression in prenatal development is higher than those in postnatal development, which is higher still than in aging. Furthermore, the magnitude of changes is higher in fetal development than later stages. Also consistent with mouse data were the types of genes that showed changes during development with, for example, diminishment of cell cycle genes and increases in synaptic components. One complication with human gene expression studies is the genetic diversity of humans compared to inbred mouse strains where genetic variation is minimal. Because gene expression is under genetic control, including in the brain(Gibbs et al., 2010), it is potentially important to dissect out genotypic and developmental effects on gene expression. However, single nucleotide polymorphisms (SNPs) that influence gene expression appear to influence overall expression levels rather than rates of change, that is to say that most genes that are developmentally regulated retain that regulation irrespective of genotype(Colantuoni et al., 2011).

One of the attractions of using genomewide approaches is that they might be mined to look for unexpected associations that, in turn, might be used to predict mechanisms. One useful approach is to generate self-organizing clusters of genes with similar trajectories of change with development. For example, several transcription factors (SMBP2, FE65 and Sox-M) show correlated expression with genes that increase in the post-natal time period in the hippocampus (Mody et al., 2001). As specific transcription factors are involved in neuronal specification (Thompson & Ziman, 2011), the increase in transcription factor expression in the postnatal period suggests that such proteins might also be important in the maintenance of neuronal phenotype through adulthood.

Genomewide approaches can further be used to understand how specific transcription factors work during development. For example, knockout of the transcription factor aristaless-related homeobox (*Arx*) results in altered migration of interneurons and abnormal neuronal differentiation. Using Affymetrix arrays in a conditional knockout where *Arx* was depleted in the subpallium, Fulp et al were able to reconstruct a genetic network where *Arx* normally represses additional transcription factors including *Lmo1*, *Ebf3* and *Shox2* (Fulp et al., 2008).

As another example of the use of mutant mice, Prampano *et al.*, compared gene expression in several different mutations that are associated with deficits in neuronal migrations (*Lis1*, *Dcx* and *Ywhae*) and found alterations in classes of genes expressed (Pramparo et al., 2011). Specifically, disruption in neuronal migration genes caused alterations in cell cycle and cytoskeleton categories, but for some mutants there were also differences in genes encoding synaptic proteins. These observations suggest that there is a dependency of later gene expression events on earlier ones, i.e., that neuronal migration is a required step for synaptic maturation.

The above datasets were generated using microarrays, but there are many other ways to look at gene expression. In situ hybridization (ISH) has been applied at a genomewide level to both mouse(Liscovitch & Chechik, 2013) and human(Miller et al., 2014) brain development. The principle advantage of ISH over arrays is that gene expression can be addressed at the

level of brain regions, layers and even single cells. Such analyses have generally found similar categories of genes as in the array studies but indicate that there are specific genes that are developmentally regulated in germinal zones, for example(Miller et al., 2014).

A deeper view of gene expression changes in the developing mouse cerebral cortex has been achieved using transcriptome sequencing or RNA-Seq (Dillman et al., 2013; Han et al., 2009)(Dillman et al., 2013). Such techniques have been increasingly chosen for gene expression studies as it has been reported that RNA-Seq has greater linearity and reliability compared to microarrays (’t Hoen et al., 2008). Consistent with previous array studies, genes with higher expression in the embryonic brain included many genes involved in cell division, while those that were more highly expressed in the adult brain were related to neurotransmission and ion homeostasis (Dillman et al., 2013). However, probably due to the improved dynamic range of RNA-Seq, such approaches tend to nominate many more genes as being regulated during development compared to array-based studies. In our hands, from ~24,000 genes identified, about 4,000 were differentially expressed comparing E17 mice with adult (3–4 month old) females. There was also good quantitative agreement between RNA-Seq and qRT-PCR for a subset of genes chosen for validation, again supporting the idea that RNA-Seq reliably estimates fold differences between conditions. A similar estimate (4000/16000 detected genes) of differentially expressed genes was reported by Han et al., comparing E18 and P7 mouse cortex (Han et al., 2009). RNA-Seq data is also available for the human brain at various stages of development (e.g., http://www.brainspan.org.) Analysis of this dataset again shows a distinct developmental trajectory for expression of a large number of genes including many involved in synaptic function (Parikshak et al., 2013).

An important additional utility of RNA-Seq is that as well as estimating overall gene expression, we examine more complex aspects of gene expression, which will be discussed later in the review, namely splicing and RNA editing. However, it is perhaps interesting to discuss some genetic events that may underly some of the changes in gene expression in brain development.

DNA Sequence variation and epigenetic modification in brain development

A discussion of gene expression at the RNA level should also consider the architecture of DNA itself. Within a given species, DNA is highly polymorphic and some of that variation can manifest itself as differences in expression levels rather than coding sequence of genes. Mapping the relationship between DNA variation and gene expression levels identifies expression quantitative trait loci (eQTLs). Conceptually, eQTLs are regions of the genome where polymorphic variants are statistically associated with differences in mRNA expression levels. Studies in human (Gibbs et al., 2010) and mouse (van Nas et al., 2010) brain have identified a large number of such eQTLs for many genes. Such polymorphic loci might, therefore, influence brain development. In one study overlaying genetic data with gene expression across brain development, there were very few examples of genetic polymorphisms that altered the trajectory of gene expression changes throughout development (Colantuoni et al., 2011). Nonetheless, genetic background effects do need to be considered in gene expression profiling experiments, including brain development.

As discussed above, alterations in expression levels of transcription factors may be important in the control of gene expression during brain development. However, the underlying interaction between transcription factors and DNA is dynamic as DNA is subject to a number of regulatory modifications, including methylation. DNA methylation generally occurs at cytosine bases to form 5-methylcytosine in the promoter region of genes. Cytosine methylation generally represses gene expression (Tate & Bird, 1993), although it may also be a mechanism involved in generation of alternate splicing events (Zhou, Luo, Wise, & Lou, 2014).

Methylation seems to play a particularly important role in cell differentiation in the brain. For instance, astrocyte differentiation is dependent on the transcription factor STAT3 but expression levels alone are not enough to trigger differentiation as the promoter of the astrocyte marker GFAP is methylated to prevent STAT3 binding. Once this site is demethylated the cells can respond to the presence of STAT3 and differentiation can occur (Takizawa et al., 2001). In this way, transcription factors and DNA modifications work together to control gene expression.

DNA methylation can also be involved in genomic imprinting, in which a gene is expressed in a parent-of-origin-specific manner. A substantial proportion of imprinted genes are highly expressed in the brain with unique spatial and temporal expression. For example, *UBE3A* has maternally based expression in specific subpopulations of neurons in the hippocampus and cerebellum but is biallelically expressed in the rest of the brain and body (Albrecht et al., 1997; Rougeulle, Glatt, & Lalande, 1997). In chimeric mice embryos, duplicated maternal genomes contributed to the development of the hypothalamsic but not to the cerebral cortex, while a duplicated paternal genome contributed to cortical but not to hypothalamic structures indicating unique differential roles for parent of origin genomes (Keverne, Fundele, Narasimha, Barton, & Surani, 1996). An example of temporal regulation is the gene *Murr1,* which has biallelic expression in embryonic and neonatal mice but only the maternal allele is expressed in adult brain (Wang et al., 2004).

Collectively, these examples show that the transcriptome of the brain is regulated at multiple levels in a manner that depends on epigenetic modification. As might therefore be expected, these single examples likely generalize across the genome. It has been demonstrated recently that widespread DNA methylation changes occur in development in both the mouse and human brain (Lister et al., 2013; Numata et al., 2012). As seen with expression changes, the most dramatic differences in DNA methylation occur during prenatal development with a slowing of progression after birth and even more modest changes in aging (Numata et al., 2012). In some cases, there are DNA methylation events that reverse course after initial development, ie where a sequence may undergo demethylation before birth then becoming methylated after birth. This is generally consistent with previous data using smaller sets of methylation events that showed a general increase in methylation in the human brain with age that was also confirmed using isolated neurons (Siegmund et al., 2007).

Alternative splicing

Many of the above approaches, generally considered each ‘gene’ as a single unit. However, many tissues, including the brain, show a large number of splicing events with perhaps half of all genes showing some evidence of alternate exons being incorporated into mature mRNA (Lee & Irizarry, 2003).

As might therefore be expected, there are many examples of regulated alternative splicing in neuronal development. In mice, fetal *Mapt* has only minor incorporation exon 10 but by postnatal day 24 all tau contains this exon (McMillan et al., 2008). Interestingly, human *MAPT* retains exon 10 throughout adulthood (Liu & Gong, 2008), perhaps related to the larger size of human neurons compared to neurons leading to a higher requirement for axonal stability. The glutamate receptor gene *Gria2* has a pair of exons that can be spliced in or out leading to two different protein isoforms, flip and flop, that have different electrophysiological characteristics (Sommer et al., 1990). In rats, flip is expressed at stable levels throughout development while flop expression is low until postnatal day 8 (Monyer, Seeburg, & Wisden, 1991). Although not comprehensive, these examples show how alternate splicing in brain-expressed genes can be functionally important in different species.

Another level of regulation related to splicing is intron retention, where sequences that would normally be spliced out are included in the mature mRNA. In general, retention of introns is high in the brain than other tissues and is developmentally regulated, with levels of retention higher in the fetal brain than in the adult (Ameur et al., 2011). One example of intron retention during development is in the axon guidance molecule Robo3 (Colak, Ji, Porse, & Jaffrey, 2013). A Robo3 isoform containing an intronic sequence (Robo3.2) is expressed but translationally repressed and allows for neuronal attraction to the spinal cord midline. Once the axon crosses the spinal cord midline, it receives signals from the floor plate to translate Robo3.2 allowing nonsense-mediated decay to occur, causing repellence to the midline. Whether other examples of intron retention are similarly functionally important in brain development is not known, but given that intron retention is frequent in the embryonic brain(Ameur et al., 2011), it is likely that this is an important mechanism of gene regulation relevant for neuronal maturation.

The brain also has been found to have longer 3-′UTR regions (Miura, Shenker, Andreu-Agullo, Westholm, & Lai, 2013; Ramsköld, Wang, Burge, & Sandberg, 2009) than other tissues and this lengthening of UTRs occurs during development(Ji, Lee, Pan, Jiang, & Tian, 2009). This may be related to stability of mRNA transcripts, as 3′-UTR regions contain poly-adenylation signals that control the turnover of mRNA; interestingly, the brain has more alternate poly-adenylation than other tissues (Hu, Liu, & Yan, 2014). Alternate 3′-UTR signals may also be important in targeting mRNA to neuronal processes as there are signals that direct mRNAs to axons and dendrites (Mohr, 1999).

There have been several studies looking at alternative exon usage in brain development in different species including humans and other primates using genome-wide exon arrays (Johnson et al., 2009; Mazin et al., 2013). What is impressive about these studies is that, like measures of overall gene expression, they estimate that a large proportion of genes show

alternative splicing. In our own work using RNA-Seq in the mouse brain, we found almost 400 exons that were differentially expressed with examples where exon inclusion were higher or lower in the adult compared to embryonic brain and these included well characterized examples such as *Mapt*. We also found that there were many types of alternative exon usage, including 5′ and 3′-UTR sequences (Dillman et al., 2013), showing that some of the specific examples discussed above may generalize to many genes.

An obvious mechanism for alterations in splicing during development is that splicing factors might themselves be differentially expressed. There is some support for this from large-scale experiments, which have found age-dependent changes in expression of *PTBP1*, *PTBP2*, *hnRNPA1*, *hnRNPF*, *hnRNPH1*, and *hnRNPH3* in the developing human cerebral cortex (Mazin et al., 2013). Differential expression of RNA binding proteins also occurs in mouse development. One of the genes with the largest differences in gene expression in our own dataset (Dillman et al., 2013) was *Igf2bp1*, which is associated with translational repression of a subset of mRNA (Bell et al., 2013). Nova2, a neuron specific RNA binding protein, is required for the development of the spinal cord and brain stem. Using high throughput sequencing of RNA isolated by crosslinking, it was discovered that the binding of Nova2 affects alternative splicing (Licatalosi et al., 2008). These observations demonstrate that alternative splicing is therefore required for normal brain development.

It is likely that there are additional levels of complexity in transcript generation that would also be relevant to brain development. One of the limitations of that RNA-Seq we used is that sequences were limited to ~200bp, although there are technologies that allow for longer reads and hence to recover a greater depth of information about full length transcripts (Au et al., 2013). Applying a similar approach to the developing brain would be of particular interest in the future.

RNA Editing

An additional source of transcriptome diversity is generated at the single base level via RNA editing. Although there were some early claims of a huge diversity of RNA editing events in the mammalian genome(Li et al., 2011) many of the observed events were shown to be sequencing errors and other technical artifacts(Pickrell, Gilad, & Pritchard, 2012). Instead, it is generally accepted that in many species, RNA editing events are limited to Adenosine to Inosine and Cytosine to Uracil, both of which have a well defined enzymatic basis.

Adenosine to inosine substitutions in mammalian RNA are carried out by adenosine deaminases (ADARs), of which there are three isoforms. ADAR1 and ADAR2 are ubiquitously expressed, with expression levels are highest in the brain while ADAR3 is exclusively expressed in the brain(Hogg, Paro, Keegan, & O'Connell, 2011). ADARs act on double stranded RNA and may require dimerization to be enzymatically active(Cho et al., 2003; Gallo, Keegan, Ring, & O'Connell, 2003). ADARs are localized primarily in the nucleolus and are bound to ribosomal RNA(Sansam, Wells, & Emeson, 2003) but can translocate to the nucleus upon expression of specific ADAR substrates(Desterro et al., 2003).

Inosine is recognized as guanosine in translation and, as such, editing in the coding region of a gene can result in a change in the amino acid sequence(Sommer, Köhler, Sprengel, & Seeburg, 1991). Editing may be particularly important in the brain as there are multiple isoforms of neurotransmitter receptors that are targeted by ADARs(Seeburg, 2000). The majority of A-to-I editing sites are highly conserved across genetically divergent mouse strains(Danecek et al., 2012), supporting the idea that editing is biologically important. In mice, editing of Gria2 leads to a lower permeability of this glutamate channel to calcium ions. If only the unedited isoform of Gria2 is present, mice die within two weeks of birth due to seizures(Higuchi et al., 2000). In octopuses, RNA editing plays a role in the temperature adaptability of potassium channels(Garrett & Rosenthal, 2012), again showing that RNA editing may influence neuronal excitability. There are examples of genes that are both edited and that undergo alternative splicing during development (Barresi et al., 2014).

The other major editing enzyme in mammals, APOBEC1, deaminates cytidine to produce uracil (Koito & Ikeda, 2012). Genome-wide surveys suggest that C to U editing is far less numerous than A to I editing (Kleinman, Adoue, & Majewski, 2012). APOBEC1 is expressed in neurons and is thought primarily to have an antiviral role (Gee et al., 2011) and, perhaps because of this, at the time of this review any potential role in development has not been well studied.

There have been attempts to look at editing in a genome-wide manner in brain development. For example, Wahlstedt et al. described examined 28 known A to I editing sites and found that many showed an increase in editing as the brain develops (Wahlstedt, Daniel, Enstero, & Ohman, 2009) In our own analysis of A-to-I editing, we discovered 176 sites in the mouse brain. Although some coding edits were found, the majority of sites were sin the 3′UTR of genes. We also confirmed that there was a tendency for increase in the proportion of edited transcripts with development (Dillman et al., 2013). The tendency of RNA to become more completely edited during development suggests that protein diversity is less tolerated in the mature CNS than during development.

The mechanism underlying increased completion of A-to-I editing may be partly related to increased expression of Adar enzymes through the postnatal period (Dillman et al., 2013). However, why there is variation in the level of editing between different sites, varying from less than 20% to nearly 100% edited as for *Gria2*, is not at all clear. One possible future experiment would be to examine RNA editing in mice lacking specific *Adar* isoenzymes, which might allow for estimation of the redundancy in editing between the different *Adar* genes.

Non-coding RNA

In recent years it has become clear that the genome contains many types of RNA distinct from protein coding mRNA species. Some small non-coding RNA (ncRNA), including micro-RNA (miRNA), have important roles in regulating stability and translation of mRNAs (Dogini et al., 2014). Others, such as long non-coding RNAs (lncRNA) influence epigenetic regulation by structural mechanisms (Peschansky & Wahlestedt, 2014) and are highly conserved across species (Chodroff et al., 2010). Human accelerated regions (HARs) are

noncoding regions of the genome that are conserved throughout vertebrate evolution but have significant substitution rates in humans. There is significant enrichment adjacent to genes known to play a role in neuronal development(17082449, 16915236). One particular HAR, HAR1, had the most genomic changes in humans with 18 substitutions compared to chimpanzees, while there were only 2 base differences comparing chimpanzees to chickens. HAR1 overlaps with two ncRNAs HAR1F and HAR1R. Interestingly HAR1F is specifically expressed in the fetal brain in Cajal-Retzius neurons along with reelin a gene critical in the specification of layering in the cortex (16915236). As might be expected, there are many single examples of changes in expression of non-coding RNAs as the brain develops (Barry, 2014; Iyengar et al., 2014; Nowak & Michlewski, 2013), including instances where a neuronal specific function is impacted by miRNA expression levels during development (Schratt et al., 2006).

Several studies have attempted to use genome-wide approaches to look at miRNA in brain development. In the developing rat forebrain from E2 to P5, about 20% of mature miRNA species were shown to have altered expression patterns using a custom array (Krichevsky, King, Donahue, Khrapko, & Kosik, 2003). All of the proposed differences were validated by Northern blots, suggesting that such changes are methodologically robust. Similarly, in studies using several different microarray platforms, a large proportion of miRNA were found to show changes in expression during development of the mouse brain (Miska et al., 2004; Sempere et al., 2004), in the pig cortex and cerebellum (Podolska et al., 2011) and in many regions of the human brain (Moreau, Bruse, Jornsten, Liu, & Brzustowicz, 2013). ncRNA can also be quantified by RNA-Seq, usually by making libraries that are enriched for small RNA species. As for conventional mRNA, sequencing tends to identify a greater number of genes than arrays in brain (Juhila et al., 2011). These types of methods have been applied to the developing pig hypothalamus and pituitary and again reported a large number of differences (Zhang et al., 2013). Collectively, these results show that many miRNA are regulated during development.

Alterations in the expression levels of miRNA are an additional mechanism that might contribute to some of the changes in mRNA expression and splicing discussed above. Because miRNA generally bind multiple mature mRNA species it has been predicted that they might be important for co-ordinated control of gene expression in multiple species (Favre, Banta Lavenex, & Lavenex, 2012). It has been suggested that miRNA:mRNA interactions are particularly important in allowing for maintaining the overall stability in gene expression levels while still allowing for fine-tuning in response to developmental stimuli (Follert, Cremer, & Béclin, 2014). The lncRNA Evf2 binds to intergenic regions and influences expression of proximal genes *Dlx5/6* and *Gad1* (Bond et al., 2009). There is some evidence that a relationship between expression of lncRNAs and nearby protein coding genes generalizes across many examples (Mercer, Dinger, Sunkin, Mehler, & Mattick, 2008). Small RNAs may also contribute to the generation of transcript diversity. For example, a miRNA expressed selectively in the nervous system can influence splicing via the factors PTBP1 and PTBP2 (Makeyev, Zhang, Carrasco, & Maniatis, 2007).

Summary

The examples above show that the brain transcriptome undergoes a number of significant changes throughout development. Importantly, there are many levels of regulation including at the levels of whole gene, single exons and single base pairs in the case of RNA editing sites. The mechanism(s) underlying all of these changes are not always understood, but many are likely to be important in the functional specification of the brain. Future challenges include developing additional ways to look at the whole transcriptome in an unbiased manner.

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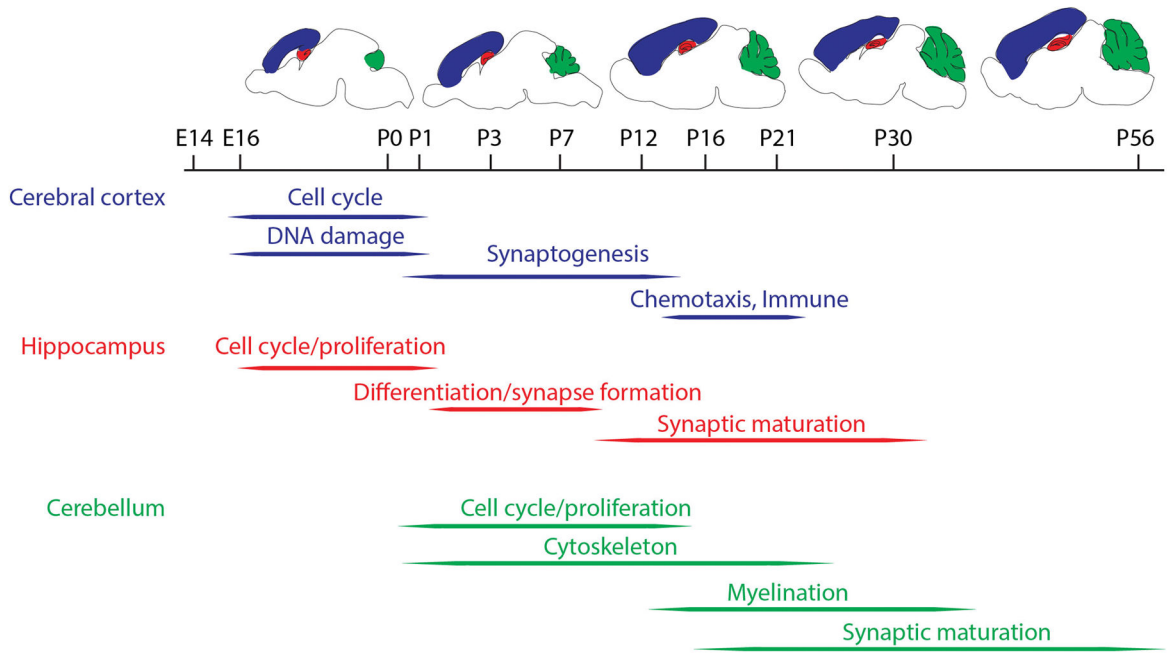

Figure 1.

Categories of gene expression in the developing mouse brain. The timeline from embryonic (E) to postnatal (P) gene expression is given in days and above the timeline are schematics of the brain approximately equivalent to their positions. Three brain regions where gene expression has been studied are colored in blue (cerebral cortex), red (hippocampus) and green (cerebellum). Below the time line are groups of genes that are prominently expressed at given developmental ranges in each region.