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Mini Review

Molecular genetic determinants of human brain size

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Abstract

Cognitive skills such as tool use, syntactical languages, and self-awareness differentiate humans from other primates. The underlying basis for this cognitive difference has been widely associated with a high encephalization quotient and an anatomically distinct, exceptionally large cerebral cortex. Investigations on congenital microcephaly had revealed several genes that affect mammalian brain size when mutated. At least four of these, microcephalin (MCPHI), abnormal spindle-like microcephaly-associated (ASPM), cyclin-dependent kinase 5 regulatory associated protein 2 (CDK5RAP2), and centromere-associated protein J (CENPJ) are known to have undergone significant positive selection in the great apes and human lineages during primate evolution. MCPHI and ASPM both have very young single nucleotide polymorphism haplotypes associated with modern humans, and these genes are presumably still evolving in Homo sapiens. Microcephalin has a role in DNA damage response and regulation of cell cycle checkpoints. The other known microcephaly-associated genes encode microtubule-associated centrosomal proteins that might regulate neural progenitor cell division and cell number. Recent reports have also unveiled a previously unknown function of ephrins and Eph in the regulation of neural progenitor cell death with a consequential effect on brain size. Understanding the mechanism for developmental control of brain organogenesis by these genes, and others such as FOXP2, shall provide fresh perspectives on the evolution of human intelligence.

Keywords: APSM; Brain; Eph; Ephrin; Microcephalin; Neural progenitors

The human brain, constituting about two percent of body mass, is strikingly larger than that of other primates. Humans have an encephalization quotient of 7.4–7.8, more than threefold that of our genetically closest neighbors in the great ape clade [1]. Such an increase mostly resulted from a rather disproportionate, allometric growth of the cortex during development. In the course of evolution of *Homo sapiens*, its cortical surface area has increased by three orders of magnitude relative to body size, and as such is a thousand times larger than that of mouse, the popular animal model [2,3]. Such a large cortical surface area and the projected exponential increase in the number of neural connections have made sheer brain size one strong correlative explanation for the evolution of cognitive intelligence in the *Homo* genus.

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Development of the brain cortex follows roughly similar sequences in mammals. Progenitor cells formed at a narrow region around the telecephalic ventricle, where they divide symmetrically and give rise to more progenitor cells. Each then undergoes asymmetric cell division, generating another progenitor cell and a neuron. Neurons migrate from the ventricles to more distant positions, along radial glia tracks, to form the cortical plate [4]. It is conceivable, and has been proposed, that changes in the relative numbers of the two modes of cell divisions would result in changes in cortical surface area [5]. Since each round of symmetric cell division would double the number of progenitors, prolonging this mode of cell division (or a delay in the onset of asymmetric division) could theoretically lead to an exponential growth in the cortical area. Humans indeed have the largest number of cortical neurons [1]. There appears to be some experimental evidence for the above notion. Transgenic mice over-expressing the signaling molecule β-catenin in neural progenitors developed

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enlarged brains with increased cerebral cortical surface area, as well as surface folds resembling the sulci and gyri of higher mammals [6]. This is apparently due to a greater proportion of progenitors reentering the cell cycle after mitosis. A diametrically opposite way to control brain size is the regulation of progenitor cell death. In this regard, both caspase 3 and caspase 9 knockout mice exhibited excessive and deformative cerebral hyperplasia [7,8].

Lately, our understanding of genetic determination of brain size has been greatly illuminated by studies on genes mutated in primary microcephaly, a Mandelian developmental disorder manifested by a marked reduction in brain size [9]. A microcephalic brain is about a third the size of a normal human brain and is roughly comparable to brains of early hominids. The disorder is interesting because in spite of the reduction in brain size and a clearly simplified gyral pattern, there is no significant neurological dysfunction and only a moderate loss in cognitive ability. An interesting notion is therefore that microcephalic conditions may recapitulate what the brain and cognitive ability of our early ancestors were like. On the other hand, new data have emerged implicating a previously unknown role for ephrins and Eph receptors in regulating neural progenitor cell proliferation and death. I discuss below these new findings pertaining to their importance in our understanding of genetic control of brain size, in relation to that of cognitive intelligence.

Microcephaly genes and their adaptive evolution

Linkage mapping of consanguineous families has identified six gene loci for primary microcephaly (designated MCPH1-6) [9-12]. Two members of this group, namely microcephalin (MCPHI) and abnormal spindle-like microcephaly-associated (ASPM/MCPH5), have been extensively analyzed genetically and have interesting evolutionary tracings. Lahn's laboratory had compared polymorphism and divergence of a good number of brain-expressed genes to more ubiquitous genes. The authors used the McDonald-Kreitman test for positive selection during evolution, checking for each gene the ratio of non-synonymous substitution (Ka) to that of synonymous (Ks) (the Ka/Ks ratio), as to whether they are higher within the species or between species [13,14]. The general conclusion is that a good fraction of brain-specific genes displayed significantly higher rate of changes in protein primary sequence in primates than in rodents, with the increase being particularly prominent along the lineage leading to humans. Amongst the brain-expressed genes which showed such clear signs of positive selection, or adaptive evolution, are microcephalin and ASPM. On average, one presumably advantageous amino acid change in every 300,000-400,000 years was "fixed" in the ASPM gene since the human lineage diverged from chimpanzees some 5-6 million years ago [15]. For microcephalin, 45 advantageous amino acid changes might have been fixed during the 25-30 million years of evolution from early simian progenitors to modern

humans [16]. Other groups have also made similar findings with regard to the adaptive evolution of these genes [17–19]. Indeed, it was also recently shown that two other microcephaly-associated gene products, cyclin-dependent kinase 5 regulatory associated protein 2 (*CDK5RAP2/MCPH3*) and centromere-associated protein J (*CENPJ/MCPH6*), have significantly higher evolutionary rates in primates than rodents or carnivors, particularly in the great ape/human lineages [20].

Lahn's group went further, and asked whether some these genes are evolving within the *Homo sapiens* species since its appearance around 2-3 million years ago. They did this by sequencing DNA samples of 90 ethnically diverse individuals of these loci and then extended these to more individual samples for haplotyping. For both microcephalin and ASPM, a single haplotype (or single nucleotide polymorphism, SNP) was found to predominate and had increased in frequency too rapidly to be explained by neutral genetic drift [21,22]. These SNPs had apparently spread in the modern human population under strong positive selection, the exact nature of which is currently unknown. The genetic variant of *microcephalin* in modern humans arose approximately 37,000 years ago. That of ASPM in humans arose merely about 5800 years ago. These findings suggest that the amino acid changes associated with microphilin and ASPM genes may be influencing brain development in modern humans. Intriguingly, these genes may still be evolving.

It is, however, unclear at the moment what these changes meant, in functional terms, to the cellular and physiological roles of the proteins in question. In particular, it is unclear if these changes resulted could be directly correlated to significant changes in brain size. The feeling is that these are more likely to offer advantages to more subtle aspects of brain function, which would explain their positive selection [23]. What then are the actual functions of the *MCPH*s in brain development?

MCPH gene products and their cellular functions

The domain signatures and motifs within the four microcephaly mutant genes identified thus far have provided some clues as to their cellular function, and some of the predictions have received recent experimental evidence. Microcephalin is a large, 835 amino acid protein which is expressed in the developing cerebral cortex of the fetal brain [24]. The most prominent feature of its primary sequence is the presence of three BRCA1 C-terminal (BRCT) domains, which is known to mediate phosphorylation-dependent protein-protein interactions in cell-cycle checkpoint and DNA repair functions [25]. Indeed, microcephalin nonsense mutation causes premature chromosome condensation in the early G2 phase, a phenomenon which could be reproduced in vitro by siRNA-mediated microcephalin knockdown. Microcephalin-deficient cells also exhibit delayed chromosomal de-condensation after mitosis [26]. In another report, microcephalin depletion was

shown to impair ionizing radiation-induced cell cycle S and G2/M checkpoints, with a decrease in both protein and transcript levels of the checkpoint regulators BRCA1 and Chk1. Microcephalin is thus clearly involved in cellular DNA damage response [27,28]. Microcephalin has also been identified in a separate study as a negative regulator of telomerase activity [29]. Very recently, it has also been shown to localize to the centrosome [30], just as the other *MCPH* gene products discussed below are.

ASPM is an even larger protein (3477 amino acids) and appears to be the human orthologue of *Drosophila* abnormal spindle (Asp). It has an N-terminal microtubule-binding domain, two calponin homology domains (which are actin-binding domains), and a large number of isoleucine glutamine (IQ) repeats (which binds calmodulin) [31,32]. The N-terminal microtubule binding domain is homologous to that of cilia proteins and has led to a prediction of ciliary function for ASPM [33]. ASPM is widely expressed, but in the brain it is prominent in the neuroepithelium of the lateral ventricles [34], which is suggestive of a role in neurogenesis. ASPM localizes to the centrosome in interphase and to the spindle poles during mitosis [34,35], indicating that it might function in regulating the organization of cetrosomal processes and mitotic spindle organization during the cell cycle, as its Drosophila orthologue, Asp, does [36,37]. Like microcephalin, its role in cell cycle regulation may involve functional interactions with BRCA1, as downregulation of endogenous ASPM by siRNA also downregulated endogenous BRCA1 [35].

Cyclin-dependent kinase 5 regulatory associated protein 2 (CDK5RAP2) [38] is first isolated as an inhibitory interacting partner of a regulator of CDK5RI, a regulator of CDK5 (a kinase with multiple regulatory roles in the brain, reviewed extensively in [39,40]). CDK5RAP2 has a Drosophila orthologue, centrosomin (Cnn), which is required for centrosome assembly [41]. Loss of zygotic Cnn expression in flies perturbs development of the gut and the nervous system [42]. The centromere-associated protein J (CENPJ) [43] was first identified as a centrosomal protein that interacts with the 135-kDa isoform of 4.1R (termed 4.1R-135). The latter is a microtubule-associated protein involved in microtubule aster assembly and mitotic regulation [44]. CENPJ itself carries a microtubule-destabilizing motif that could inhibit microtubule nucleation from the centrosome, as well as depolymerizing taxol-stabilized microtubules [45]. Both CDK5RAP2 and CENPJ are widely distributed in the developing embryo, but with the highest expression in the developing central nervous system (CNS), and are centrosomally localized [46].

It appears that all mutations associated with microcephaly identified thus far are associated with gene products that might function in a similar aspect of cell cycle regulation, namely the cytoskeletal control of the mitotic apparatus. An interesting point to note is that all of these genes are rather widely expressed and would be expected to play similar roles in several cell types. The non-lethal phenotype associated with their nonsense mutations could be

explained by the presence of paralogues with possibly redundant functions. For instance, myomegalin is a homologue of CDK5RAP2 and the nuclear mitotic apparatus (NuMa) [47] protein has been proposed to be able to cover for ASPM function in its absence [12]. It is however. unclear why these nonsense mutations affect brain size rather specifically and not that of other organs. Given that these are all expressed in the neuroepithelium during development and have undergone adaptive evolution in the human lineage, they may have all evolved a specialized and non-redundant function in brain neurogenesis. As discussed earlier, it is conceivable that this function may involve some temporal order of developmental decisions during neurogenesis, for example to switch from symmetric to asymmetric cell division. It would be interesting to see if the other two yet unidentified MCPH loci encode gene products that are similarly involved in cell cycle regulation and CNS neurogenesis.

An important aspect of brain architectural organization during neurogenesis has to do, however, with the modulation of progenitor cell death. In this regard, it is worth noting that caspases 3 and 9 are amongst the genes noticed by Lahn's group [13] that had shown signs of positive selection during primate evolution. In fact, as recently reported and discussed below, control of progenitor cell death during neurogenesis may involve other molecular players not previously associated with a programmed cell death function.

Ephrin/Eph signaling and neural progenitor cell death

Ephrin and Eph receptors have been extensively implicated in various aspects of development of multiple organ systems [48,49], as well as pathogenesis of malignancies [50]. However, perhaps their best known function is in the control of axonal guidance, neural circuit formation, topography, and plasticity [51–53]. Recent reports now indicate that ephrin-A/EphA signaling in different parts of the CNS regulates neural progenitor cell proliferation and neurogenesis, and may control brain size by modulating programmed cell death of progenitors.

Holmberg et al. [54] analyzed the expression pattern of all A-type ephrins and EphAs in the developing mouse brain, and noted prominent expression and rather interesting expression patterns of some of these in the ventricular zone, a neural stem cell niche [55]. In the adult mouse brain, Eph7 and ephrin-A2 are expressed in a mutually exclusive pattern in the different cell types found at the lateral ventricular wall. Infusion of recombinant Fc-fusion proteins of ephrin-A2 and EphA7 both increased proliferation in the neural stem cell niche (as indicated by an increase in bromodeoxyuridine (BrdU) labeling). Both ephrin-A2 and EphA7 null mice have an increase in BrdU labeled cells at the ventricle wall, and in the case of ephrin- $A2^{-/-}$ mice, this is paralleled by an increase in the number of newborn neurons at the olfactory bulb. Progenitor cells in these mice have an apparently shorter cell cycle and generated higher numbers of secondary neurospheres

when cultured in vitro. Absence of ephrin-A2 resulted in proliferation increase in cells normally expressing ephrin-A2 but not EphA7. Transgenic over-expression of a dominant-negative truncated form of EphA7 (EphA7-T1, lacking the kinase domain) in wild type and EphA7^{-/-}, but not ephrin-A2^{-/-} neurospheres, significantly decreased proliferation. These results suggest that forward signaling through Eph7A is probably not involved in the proliferative effect, and that ephrin-A2's presence is required for Eph7-T1 to exert its growth inhibitory effect. Interestingly, therefore, the apparently negative regulation of neural progenitor cell proliferation by ephrin-A2 and EphA7 appears to be mediated by reverse signaling through ephrin-A2, but not forward signaling from EphA7.

Another report by Depage et al. [56] examined the effect of ephrin-A/EphA signaling in the mouse cerebral cortex. EphA7 is preferentially expressed in cortical progenitors and ephrin-A5 in the telecephalon. Perinatal expression of Eph7 and ephrin-A5 is non-overlapping and complementary to each other. In order to ectopically express ephrin-A5 in the cortex during development, the authors generated a transgenic mouse line (TGA7A5) in which a Cre-recombination inducible ephrin-A5 transgene is driven by the EphA7 promoter. Crossing this with a Cre-recombinase line generated progenies which died at birth. These fetuses show a severe reduction in cerebral hemisphere size, but with the rest of the brain appearing normal. Nestin staining revealed no decrease in density of the cortical progenitors, and BrdU labeling indicates that proliferation and cell cycle regulation were unimpaired compared to wild type. The cortical progenitors have however a large increase in terminal deoxynucleotidyltransferase-mediated dUTP nick end labeled (TUNEL) positive cells and are immunoreactive for activated caspase 3. Ectopic ephrin-A5 expression had therefore apparently generated a transient, developmental stage specific wave of progenitor cell apoptosis. In vitro, ephrin treatment of cultured E12-E13 cortical progenitors resulted in rapid induction of apoptosis that is reversible by caspase inhibitors. The authors also found that $EphA7^{-/-}$ mice had an approximately twofold decrease in cortical progenitor death at the peak period of cortical neurogenesis compared to wild type. These mice also have a significantly larger $(\sim 20\%)$ cortical size than wild type, and exencephalic overgrowth is observed in a small proportion of the embryos. It appears therefore that ephrin/Eph signaling may directly regulate progenitor cell death during cortical development, and with consequential impact on brain size.

The reports discussed above raised several interesting questions. The first is that fundamentally different phenomena were observed in the two reports. While Depaepe et al. [56] clearly documented cortical progenitor apoptosis, Holmberg et al. [54] documented no apparent increase in cell death in the ventricular wall progenitor population. This is almost certainly because the studies focused on different neural progenitor populations. It is at the moment unclear how physiologically significant is ephrin/Eph

interaction in mediating progenitor cell number during development in different parts of the brain, as the experiments involved infusion of proteins and ectopic expression of transgenes. Granted that Eph7A has a physiological function in regulating neural progenitor cell proliferation and death, it remains to be determined which ephrins serve as its endogenous ligand in different parts of the brain. Another interesting aspect of the results discussed above that is unclear is whether ephrin-A reverse signaling is involved in both the phenomena observed, i.e., antiproliferation of ventricular zone progenitors and apoptosis of cortical progenitors. Ephrin-As are linked to the membrane by a glycosylphosphatidylinositol (GPI) linker and do not span the membrane. Reverse signaling must therefore involve the engagement of transmembrane co-receptors. This engagement and downstream signaling events are not understood in detail, particularly in neural progenitor cells, and would be an area of great interest in the near future. Further, it would of course be interesting to see if genes encoding EphA7 and ephrins have evolved more rapidly along the primate lineage.

Concluding remarks

It would appear that recent developments suggest that we are inching closer to an understanding of how brain size is determined by neuroprogenitor cell division and death, regulated by a complex interplay of molecular determinants. Those proteins whose function had a direct impact on brain size could have all undergone positive selections as modern human evolved from their forefathers, and some of these may indeed be still evolving.

Another question that might follow is whether we are any nearer to understanding how cognitive intelligence, a trait we believe is unique to humans and tells us apart from higher primates like chimpanzees, is determined. It is important to bear in mind that brain size is but one anatomical correlate for intelligence, and that other genes which do not affect brain size may nonetheless be essential for cognitive functions of the modern human brain. Comparative gene profiling analyses revealed that some brain genes are up-regulated in humans relative to non-primates [57]. Interestingly, among the gene set with a human-specific increase in expression revealed in a more recent analysis, there is an excess of transcription factors [58]. The forkhead domain-containing transcription factor FOXP2 is interesting in this regard as human mutations of the FOXP2 gene are known to cause severe impairment in articulation and speech [59]. The gene shows positive evolutionary selection compared to other species but unusually low sequence diversity amongst humans, which suggest its evolutionary selection occurred very recently and fixed only in modern humans [60]. Another gene which has distinct features of evolutionary selection along the human lineage is the brain-enriched AHII. Mutation of AHII causes Joubert syndrome, an autosomal recessive disorder with motor deficiencies and cognitive and behavioral disturbances such

as autistic behaviors [61,62]. Figuring out exactly how FOXP2, AHI1, and other gene products function in intelligence traits such as language capacities would indeed be interesting pursuits in the coming years of human cultural evolution.

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