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Hippocampal Pruning as a New Theory of Schizophrenia Etiopathogenesis

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Abstract Pruning in neurons has been suggested to be strongly involved in Schizophrenia's (SKZ) etiopathogenesis in recent biological, imaging, and genetic studies. We investigated the impact of protein-coding genes known to be involved in pruning, collected by a systematic literature research, in shaping the risk for SKZ in a case–control sample of 9,490 subjects (Psychiatric Genomics Consortium). Moreover, their modifications through evolution (humans, chimpanzees, and rats) and subcellular localization (as indicative of their biological function) were also investigated. We also performed a biological pathways (Gene Ontology) analysis. Genetics analyses found four genes (*DLG1*, *NOS1*, *THBS4*, and *FADS1*) and 17 pathways strongly involved in pruning and SKZ in previous literature findings to be significantly associated with the sample under analysis. The analysis of the subcellular localization found that secreted genes, and so regulatory ones, are the least conserved through evolution and also the most associated with SKZ. Their cell line and regional brain expression analysis found that their areas of primary expression are neuropil and the hippocampus, respectively. At the best of our knowledge, for the first time, we were able to describe the SKZ neurodevelopmental hypothesis starting from a single biological process. We can also hypothesize how alterations in pruning fine regulation and orchestration, strongly related with the evolutionary newest (and so more sensitive) secreted proteins, may be of particular relevance in the hippocampus. This early alteration may lead to a misstructuration of neural connectivity, resulting in the different brain alteration that characterizes SKZ patients.

Keywords Schizophrenia · Etiopathogenesis · Pruning · Genetics · Molecular pathways · Comparative genomics · Gene set · Enrichment analysis · Evolutive genetics

Introduction

Schizophrenia (SKZ) is a common disorder, with a lifetime prevalence of approximately 1 % [1]. It typically begins in adolescence or early adulthood and is characterized by unusual beliefs and experiences (namely, delusions and hallucinations), social withdrawal, flat affect, and cognitive impairment, notably in executive functions [1]. The ability to abstract thinking is a typical adolescence emerging ability which allows advanced reasoning during social and interpersonal interactions. These cognitive abilities are critically impaired during SKZ. This observation led Feinberg to propose, for the first time, a relationship between late adolescence onset and changes that occur during adolescent brain development [2]. In fact, SKZ patients (also neuroleptic naive) have a prominent reduction in the level of membrane phospholipid precursors and white matter changes in the prefrontal cortex [3, 4] and limbic region [5, 6]. These observations are consistent with an exaggeration of the changes that occur during the typical development of the central nervous system (CNS) [7]. So, starting from Feinberg hypothesis, an increasing number of researchers speculated that SKZ may be a consequence

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of an exaggeration of the typical synaptic elimination that takes place during adolescence (reviewed in Refs. [8, 9]): a mechanism called "pruning." Pruning is the process that shapes synapses and neurons to create fully functional neuronal nets, through a reduction in the CNS synapse density [10]. Even if its complete molecular orchestration is yet to be fully described, several studies reported the potential relevance of pruning to neurodevelopmental disorders, particularly SKZ [8, 11, 12]. In this regard, a critical issue is that pruning is primarily active during adolescence: the period of highest susceptibility for mental health disorders and especially SKZ [13, 14]. In fact, even though some psychiatric syndromes appear later in life, it is often possible to detect the presence of prodromic symptoms of SKZ during adolescence or late childhood [15, 16]. Moreover, the development of cognitive regulation of affective behavior would be related to adolescent changes in different brain regions, prefrontal cortex, and limbic system particularly [17]. For what pathogenesis is concerned, current knowledge on SKZ is based on dopamine and glutamate abnormalities leading to a cortical hypofunction and limbic hyperfunction that are responsible for negative and positive symptoms, respectively [18]. These are also the bases on which all current effective antipsychotic drugs have been developed [19, 20]. Recent findings have highlighted that these different neurotransmitter alterations probably arise from a general dysfunction of GABAergic interneurons responsible for a general misfunctioning of neural connectivity and regulation [21–23], leading to the current neurodevelopmental interpretation of SKZ [24]. This interpretation disclosed how SKZ is more likely to be related to a general misdevelopment of brain connectivity rather than a cumulation of different single neurotransmitter alterations [25, 26]. In this regard, a single mechanism responsible for the regulation and development of brain connectivity, such as pruning, may be the missing central step to understand these alterations establishment.

Several imaging lines of evidence also supported this hypothesis [27–29]. Particularly, new observations that structural changes in SKZ patients are revealable not only during prodromic phases [30] but also in their relatives at high genetic risk for SKZ [31] create a pathological continuum from adolescent relatives to most severe SKZ cases, supporting the etiopathogenic abnormal pruning role in SKZ. Consistently with pruning errors, direct evidence of a decreased number of synapses and other neural elements in SKZ comes from post-mortem studies that indicated a decreased density of synaptic spines [32] and a reduction in neuropil [33]. Neuropil is the neuronal area, where synaptic connections are formed between branches of axons and dendrites [34], of primary importance for neural connections. SKZ structural changes have been revealed to be possibly due to a primary neuropil reduction in these areas [35, 36], so the subcellular localization and cell line expression of the altered pruning proteins may be relevant in this sense.

Starting from all these lines of evidence, we reviewed, extended, and completed the description of the proteins and their genes involved in dendritic pruning and tested their relevance in SKZ from different perspectives including rate of amino acid conservation between species, subcellular localization, and genetic association with SKZ. This process may also offer new information to help pharmacological development in the field [9, 37]. In this regard, the Genome Wide Association Studies' (GWAS) lacking biological informative power has been recently stressed in literature [38, 39]. Single SNP's association in complex tract diseases (such as SKZ) fails in clarifying the pathological mechanisms underlying the disorder [39, 40], resulting in no useful information for diagnosis, prevention, or treatment progression. In order to go beyond this ceiling effect at which single SNP's association has come, new approaches with much higher biological informative power [e.g., gene set enrichment analysis (GSEA)] have been applied at genetic association studies [41, 42]. So, we tried to improve the present biological knowledge by investigating the molecular basis of this event in three different species: testing where evolution played more in adapting this neuronal remodeling mechanism at complexer brain architectures until human one. Moreover, we analyzed the subcellular localization of the proteins as predictor of their function [43–45], obtaining indications about the biological functions in which the pruning mechanisms found differences through species evolution. We finally tested if these differences are specific of the different cellular compartments and whether or not they are related to SKZ, testing each gene's SNP association score in a SKZ case–control sample of 9,490 (4,486 cases and 5,004 controls) subjects and operating a modern biological pathway analysis.

To the best of our knowledge, for the first time, this work attempts to identify the pruning involved genes role in SKZ, based on modern altered prefrontal cortex and *meso*-limbic region and neuropil reduction hypothesis (all consistent with the neurodevelopmental hypothesis), investigating the biological influence of these gene products in a large sample of 9,490 SKZ case–controls.

Methods and Materials

Identification of Pruning Involved Genes

Proteins associated with pruning were first identified by interrogating Pubmed dataset on published articles that focused on genetics experimental study on pruning molecular mechanism in different species. In order to consider the whole literature knowledge on the argument, we covered almost 20 years of publications (1995–2013). Pubmed database was interrogated using the following subject headings: {(dendritic pruning) AND genetics} AND ("1995" [Date — Publication]: "2013" [Date — Publication]) and {(axon pruning) AND genetics}

AND ("1995" [Date — Publication]: "2013" [Date — Publication]). We then manually selected only studies focused on gene related with pruning mechanism. The 83 articles that survived the selection for the present review are listed in Table 1, supplementary materials. Then we extrapolated the proteins and their genes (or the respective hortolog in humans) reported in the articles and those that resulted from the interrogation of the international databases to complete a list of proteins and their genes that may play a role during pruning. To consider all the possible pruning-involved genes, the authors utilized Cytoscape [46] and its GeneMania [47] plug in that identifies the most related genes to a query gene set using a guilt-by-association approach. GeneMania uses a large database of functional interaction networks from multiple organisms, and each related gene is traceable to the source network used to make the prediction [47]. This plug in also identifies the biological pathway in which these genes play a role based on the Gene Ontology international pathway database [48]. So, we studied the biological function related to pruning by analyzing the functions harbored by the identified genes involved in pruning. Then we examined which of these function was also significantly associated with SKZ in our 9,490 case–control sample using the GSEA methodologies described below. At the end of the process, we were able to identify 139 human genes in 261 biological pathways (Gene Ontology [48]) playing a role in pruning events. Fig. 1 represents the biological functions that play a central role in pruning and are also significantly associated with SKZ, according to our results.

Comparative Evolutionary Genomic

The aminoacidic sequences for the products of genes involved in pruning were identified by the international database UniprotKB [49] in all of the three species under analysis (humans, *Rattus norvegicus*, and chimpanzees). We selected these different evolutive step mammals because of their large utilization and validity in comparative genomics studies [10, 50] and because other studies already compared evolutive characteristics in these three species [51]. CLUSTAL W served for calculating the rate of local and global aminoacidic conservation rate [52]. We selected this software due to its large utilization and proven validity [53–57]. The aminoacidic conservation rate is the result of the sequence alignment of the amino acids of two proteins that are more or less conserved (changes in the amino acid sequence) in different species. The more the sequences are similar, the higher the amino acid conservation rate is. The global conservation rate is an index that results from the confrontation of the complete sequences of the proteins under analysis; it can result even negative when the sequences are very differently aligned. The local conservation rate is an index that results from the confrontation of parts of the sequences of the proteins under analysis, corrected for sequence lengths. Both indexes are reported in this paper as results of the alignment function from the Biostrings [58] library in R environment. Specifically, it is the "$score" result of the function. To maximize the analysis precision, extreme values [±(group mean+group SD)] of aminoacidic conservation rate were excluded from the analysis. The rate of their products' conservation and the characteristics of each gene are listed in Table 2, supplementary materials. To avoid possible bias because more studied proteins may result in higher knowledge of their sequence and so influence the conservation rate, we compared the number of studies (as Pubmed number of results with the protein name as subject heading) with conservation scores by linear regressions. A number of studies for each protein are reported in Table 2, supplementary materials, and statistics of the analyses are reported in Table 1.

Table 1 Conservation rate by number of studies for each protein under analysis (linear model regression)

	Estimate	Std. error	*T* value	*p* Value
Global human vs. chimpanzees	−0.05	0.06	−0.9	0.35
Local human vs. chimpanzees	−0.07	0.05	−1.3	0.19
Global human vs. rats	−0.06	0.04	−1.3	0.19
Local human vs. rats	−0.08	0.04	−2.04	0.04

Genetic Product's Subcellular Function and Localization

We compared the UniprotKB reported subcellular localization (as indicative of their biological function) of the 139 analyzed proteins (mitochondrion, nuclear, cytoplasmic, cellular membrane, and secreted) with their aminoacidic conservation rate between the three species under analysis (global/local human vs. rat and global/local human vs. chimpanzees). Analysis of variance (ANOVA) test was served for the analysis. For the statistics of the analysis, please refer to Fig. 2 and Table 2. To find out the rate of association of pruning involved proteins with SKZ by their subcellular localization, we also analyzed the odds ratio (OR) for SKZ association ($p<0.05$) of SNPs harbored by these genes and their products subcellular localization [in the case–control sample under analysis (*n* tot=9,490)]. Fisher's exact test was the test of choice. Table 3 reports the statistics of the analysis, and Fig. 3 shows the OR for each group.

Case–Control Sample Under Investigation

The sample under investigation was retrieved from the Psychiatric Genomics Consortium (PGC) (http://www.med.unc.edu/pgc/). In this sample, we analyzed 5,221 males and 4,269 females divided into 4,486 cases and 5,004 controls (*n* tot=

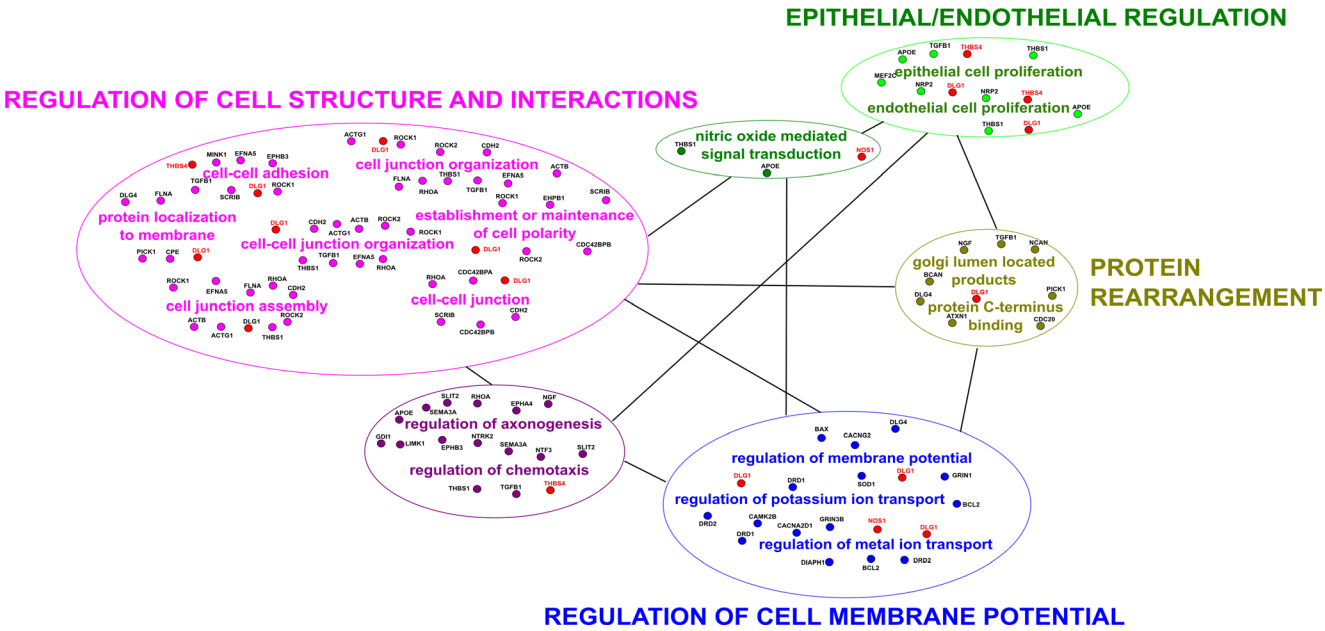

Fig. 1 The image represents the biological function harbored by the genes involved in pruning that also showed a significant association with SKZ in the sample under analysis. Node names are gene names' abbreviation, full names and characteristics for each gene are reported in Table 2, supplementary materials, as biological pathways, and the genes involved in each one are reported in Table 5, supplementary materials. *Red nodes* are the single genes significantly associated with SKZ in the sample under analysis

9,490). The characteristics of the studies (number of case and controls for each study, chip served for genotyping, etc.) are reported in Table 3, supplementary materials.

Study of Population Stratification Factors

To maximize the power of the study and eliminate the effect of possible linkage clustering of individuals, the genetic admixtures were investigated as covariate. PLINK [59] tools clustered the 9,490 subjects in six groups (n tot=1,136, 1,132, 652, 5,334, 619, and 617, respectively). We also considered the sex of our subjects as covariate (5,221 males and 4,269 females). For further information, please consult the PLINK website (http://pngu.mgh.harvard.edu/~purcell/plink/strat.shtml).

Power of the Study

We had sufficient power (0.80) to detect a small effect size (0.02) between two allelic frequencies each one represented by at least 4,745 subjects [R-cran (R Foundation for Statistical Computing 2012) pwr package [60] served for the analysis].

Imputation

Imputation was run for the genes under analysis in order to decrease the computational effort. The CEU HapMap 1000 genomes [61] served for the analysis. The imputation was undertaken using Impute2 software [62]. In short, Impute2 uses a fine-scale recombination map and a densely genotyped reference panel to "fill in" missing genotypes in a study dataset, which might consist of cases and controls typed on a commercial SNP chip. By estimating the genotypes of SNPs that were not in the original study data, imputation allows a much larger set of SNPs to be tested for association. This can increase both the power to detect association signals and the signal resolution near a causal or associated variant. For further information, please refer to the website: https://mathgen.stats.ox.ac.uk/impute/impute_v2.html. We obtained 14,198 SNPs harbored by analyzed genes that passed the imputation quality control (info>0.9) and the pruning (r^2>0.5). Pruning was undertaken after imputation. Table 4, supplementary materials, reports the characteristic of each SNP.

Statistical Methods

Covariated linear regression was the statistical model for the single SNP's association analysis. PLINK software was used

Table 2 Mean rate of amino-acidic conservation between species by subcellular localization of their genetic products (ANOVA)

	Groups	Mean	SD	*n tot*	*F* value	*df*	*p* Value
Global human vs. chimpanzees	Secreted	1,840.38	1,528.92	9	1.91	4	0.11
	Cell membrane	2,869.83	1,444.01	39	–	–	–
	Cytoplasm	2,095.97	1,054.89	24	–	–	–
	Mitochondrion	2,333.52	2,259.71	2	–	–	–
	Nucleus	2,192.30	1,304.95	11	–	–	–
Local human vs. chimpanzees	Secreted	1,390.08	714.18	9	2.66	4	0.03
	Cell membrane	2,782.51	1,381.44	42	–	–	–
	Cytoplasm	2,219.84	996.25	25	–	–	–
	Mitochondrion	2,415.63	2,143.58	2	–	–	–
	Nucleus	2,108.36	1,538.81	11	–	–	–
Global human vs. rats	Secreted	1,200.98	1,315.46	13	5.16	4	0.0007
	Cell membrane	2,501.97	1,315.66	48	–	–	–
	Cytoplasm	1,577.47	895.35	31	–	–	–
	Mitochondrion	1,395.79	2,076.17	3	–	–	–
	Nucleus	1,512.04	980.08	12	–	–	–
Local human vs. rats	Secreted	1,440.15	1,275.44	14	5.01	4	0.0009
	Cell membrane	2,538.03	1,273.74	51	–	–	–
	Cytoplasm	1,635.86	826.05	33	–	–	–
	Mitochondrion	2,230.78	2,106.61	2	–	–	–
	Nucleus	1,601.52	895.63	13	–	–	–

for the analysis (http://pngu.mgh.harvard.edu/%7Epurcell/plink/). As previously discussed in other studies [63, 64], we analyzed the genetics results organized as pathways and as genes comparing the distribution of the p values<0.05 (of association with the phenotype under analysis) between each pathway/gene subset of SNPs and an equal number of SNP's association p values randomly chosen from the genome under analysis. Fisher exact test was the statistical method for the analysis. We then permuted these p values randomly reassigning the SNPs in the two groups 100,000 times for each

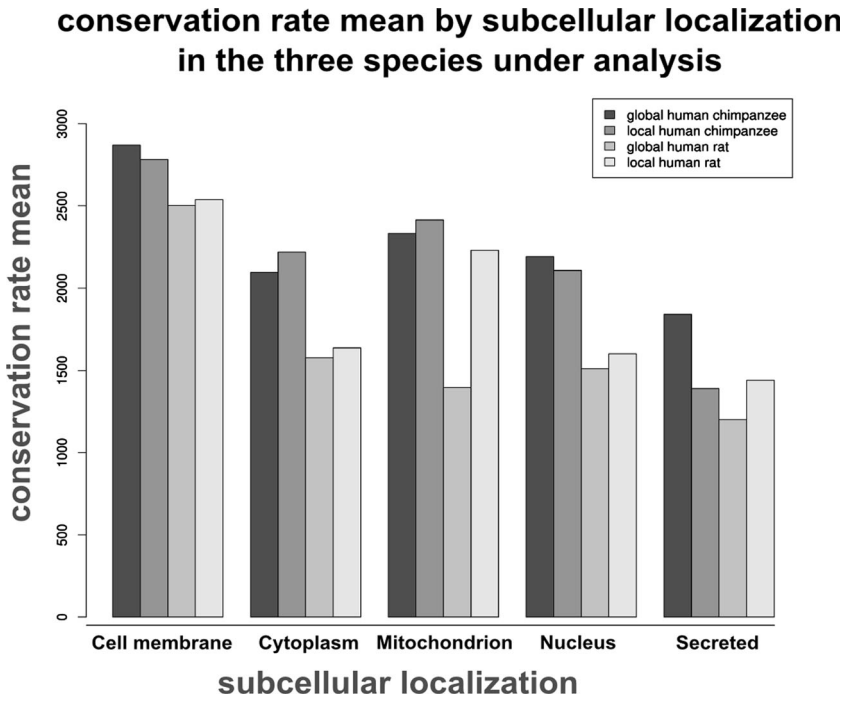

Fig. 2 Means of conservation rate by subcellular localization of the genetic products for the pruning involved genes (n tot=139)

Table 3 Fisher's exact test statistics for the SNP's association with SKZ ($p<0.05$) in the sample under analysis (n tot=9,490) and their genetic products subcellular localization

	n tot	n $p<0.05$	Percentage	95 % Confidence interval lower	95 % Confidence interval upper	Group odds ratio	p Value
Secreted	2,014	164	8.2 %	1.13	1.62	1.36	0.0008
Cell membrane	7,541	494	6.5 %	0.91	1.20	1.05	0.45
Cytoplasm	3,495	188	5.3 %	0.66	0.93	0.78	0.004
Nucleus	784	48	6.1 %	0.68	1.28	0.95	0.82
Mitochondrion	364	15	4.1 %	0.34	1.04	0.62	0.08

pathway/gene. The resulting permuted p for each pathway/gene is an association p resulting from the frequency of this random groups (with the same number of SNPs of the pathway/gene), reaching a significance level (number of SNPs associated with the phenotype) equal or stronger than the pathway/gene under analysis. With this method, it is possible to consider the effect of both the overall pathway association (considering the overall rate of association of the SNPs within the pathway) with the phenotype versus other identical numerous SNPs groups in the same genetic database with the same phenotype (not a priori established) and the numerosity of the SNPs within the pathway (because Fisher's exact test generates a higher and lower p value for the group with smaller and bigger number of observations, respectively, if the same rate of observation satisfying the condition are observed). These are important characteristics to define gene set's association, characteristics that the analysis based on the smaller single SNP's p value (the basis of the other method used in gene set enrichment analysis) [65] within the pathway cannot reveal, neglecting also the possible relationship within the genes in the pathway [66]. In fact, methods based on Fisher's exact test resulted to be the one with highest power in such analysis [67]. Statistics and characteristics of the GSEA pathways are reported in Tables 4 and 5 and Table 5, supplementary materials.

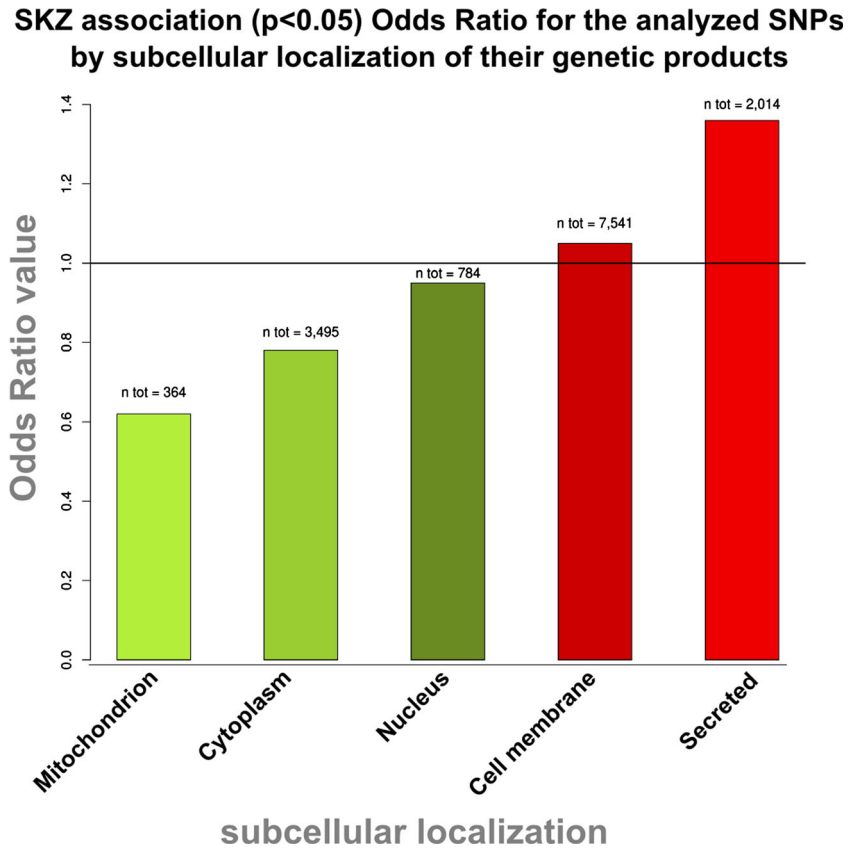

Fig. 3 SKZ odds ratio for genetic association (rate of single SNPs, $p<0.05$) by subcellular localization of their genetic products

Table 4 Characteristics and statistics of the five most associated single genes with the phenotype under analysis (n tot=9,490)

Gene	Gene full name	Statistics ($p<0.05$); expected 5 %		SNPs (n tot)	Permuted p
		True	False		
DLG1	Discs, large homolog 1 (*Drosophila*)	68 (51 %)	63 (49 %)	131	<0.00001
Gene function	*DLG1* is a gene found to have possible role in shaping SKZ susceptibility [115–117] This gene is found in neurons soma, postsynaptic densities and other tissues and plays role in adherens junction assembly, signal transduction, cell proliferation and synaptogenesis [118–121], functions clearly consistent with pruning activity. This gene's product when expressed in astrocytes is able to influence the glutamate response of astrocytes [122]. By this way the neurons' environment is shaped by the activity of DLG1 through the activation of astrocytes after the glutamatergic system.				
NOS1	Nitric oxide synthase 1	38 (28 %)	94 (72 %)	132	<0.00001
Gene function	*NOS1* codes for the enzyme that produces nitric oxide (NO), which is a messenger molecule with various functions regulating endothelial permeability and structure throughout the body, that in the brain displays many properties of a neurotransmitter [123–126]. These functions are consistent with neuron environment modifications and pruning regulation [127]. In fact, this gene was found to be associated with brain structural alterations and SKZ [128–131]. There is evidence that *NOS1*'s activity exerts a paracrine effect in the nervous system [132].				
THBS4	Thrombospondin 4	21 (42 %)	28 (57 %)	49	<0.00001
Gene function	*THBS4* gene codes for an adhesive glycoprotein pertaining to a family of protein (thrombospondins) that mediates cell-to-cell and cell-to-matrix interactions and is involved in various processes including cellular proliferation, migration, adhesion and attachment, inflammatory response to CNS injury, and regulation of vascular inflammation [133, 134]. This protein can bind to various extracellular matrix components fibrinogen, fibronectin, laminin, and type V collagen [135, 136], whose importance in SKZ and pruning have been strongly reported [137] and have also been involved in shaping SKZ susceptibility [138]. The gene's product is involved in neuronal development in animals, where it plays a role as an extracellular protein [102].				
FADS1	Fatty acid desaturase 1	16 (72 %)	6 (27 %)	22	<0.00001
Gene function	*FADS1* product catalyzes biosynthesis of highly unsaturated fatty acids, playing a central role in fatty acid metabolism [139, 140], necessary to maintain the correct membrane structure [141, 142]. Clearly, such function has been strongly related with pruning process [143] and SKZ [144, 145]. It has also become an interesting possible SKZ treatment target [146–148]. There is evidence that this protein has a role in extracellular events, whose disruption may lead to a degenerative disease that involves the retina [149].				

True/false in statistics column are referred at the number (and percentage) of SNPs with an association $p<0.05$ in the considered subset. Expected number (due to chance) is 5 % of total

Correction for Multitesting

The p value for a significant single SNPs result was set at 0.05/14,198=0.000003 (Bonferroni correction). The p value for a significant single gene result was set at 0.05/139=0.0003 (Bonferroni correction). The p value for a significant single pathway result was set at 0.05/261=0.0001 (Bonferroni correction).

Cell Line and Brain Area Genetic Expression

The analysis of the cell line and brain region that primarily expressed secreted pruning-involved proteins (as it resulted to be the most associated group in all the operated analyses) was aimed at obtaining indications about where in human brains (microscopically and macroscopically) pruning errors may be of particular relevance for future development of SKZ. With this aim, Human Protein Atlas [68] database (http://www.proteinatlas.org) was used to obtain information about the cell line that mostly expressed the genes that resulted significantly associated with SKZ (secreted, n=18). This database contains the protein expression profiles based on immunohistochemistry for a large number of human tissues, cancers and cell lines, and their transcript expression [69]. The cell line of expression was considered as the one that showed the highest rate in central nervous tissues, as indicated in the "Tissue atlas" "Antibody staining overview" tool for each protein. In case two tissues had the same highest rate, both were recorded as primary. Table 6 and Fig. 4 report the characteristics of the analysis. In order to investigate their regional brain expression, we utilized the Brain Architecture Project [70] (http://brainarchitecture.org) that combines informatics and experimental approaches including analysis of gene expression patterns and experimental efforts to comprehensively determine the mesoscopic connectivity patterns in the adult mouse brain (not enough information are disposable for humans in public databases, so we choose this animal) [70]. For further information about these databases and their data, please consult the websites. Tables 6 and 7 and Fig. 5 report the statistics of the analysis and the characteristics of each group.

Results

Comparative Genomics and SKZ Association by Biological Function and Subcellular Localization

Our analyses showed how secreted pruning-involved proteins are the lowest conserved ones through evolution. ANOVA

Table 5 Biological process pathways significantly associated with SKZ case–control sample under analysis (*n* tot=9,490)

Pathway	Pathway name	Statistics ($p<0.05$); expected=5 %		SNPs (*n* tot)	Permuted *p*	Compartmental function
		True	False			
GO:0001935	Endothelial cell proliferation	89 (28 %)	236 (72 %)	325	<0.00001	Endothelial and epithelial regulation
GO:0005796	Golgi lumen	25 (15 %)	151 (85 %)	176	<0.00001	Regulation of proteins rearrangement
GO:0007263	Nitric oxide mediated signal transduction	38 (23 %)	129 (77 %)	167	<0.00001	Endothelial and epithelial regulation
GO:0008022	Protein C-terminus binding	109 (19 %)	470 (81 %)	579	<0.00001	Regulation of proteins rearrangement
GO:0010959	Regulation of metal ion transport	152 (15 %)	871 (85 %)	1,023	<0.00001	Regulation of cell membrane potential
GO:0016337	Cell-cell adhesion	121 (22 %)	447 (78 %)	568	<0.00001	Regulation of cell structure and interactions
GO:0034329	Cell junction assembly	111 (16 %)	606 (84 %)	717	<0.00001	Regulation of cell structure and interactions
GO:0034330	Cell junction organization	111 (15 %)	636 (85 %)	747	<0.00001	Regulation of cell structure and interactions
GO:0042391	Regulation of membrane potential	98 (19 %)	420 (81 %)	518	<0.00001	Regulation of cell membrane potential
GO:0043266	Regulation of potassium ion transport	78 (32 %)	166 (68 %)	244	<0.00001	Regulation of cell membrane potential
GO:0050673	Epithelial cell proliferation	92 (21 %)	350 (79 %)	442	<0.00001	Endothelial and epithelial regulation
GO:0072657	Protein localization to membrane	68 (25 %)	213 (75 %)	281	<0.00001	Regulation of cell structure and interactions
GO:0005911	Cell-cell junction	91 (16 %)	505 (84 %)	596	0.00009	Regulation of cell structure and interactions
GO:0045216	Cell-cell junction organization	111 (15 %)	636 (85 %)	747	0.00002	Regulation of cell structure and interactions
GO:0050770	Regulation of axonogenesis	100 (12 %)	803 (88 %)	903	0.00004	Regulation of cell structure and interactions
GO:0050920	Regulation of chemotaxis	69 (14 %)	451 (86 %)	520	0.0001	Regulation of cell structure and interactions
GO:0007163	Establishment or maintenance of cell polarity	85 (13 %)	598 (87 %)	683	0.0001	Regulation of cell structure and interactions

True/false in statistics column are referred at the number (and percentage) of SNPs with an association $p<0.05$ in the considered subset. Expected number (due to chance) is 5 % of total

analysis found a significant association between the conservation rate and the subcellular localization of our protein in all the analysis operated (local human vs. chimpanzee: $F=2.66$, $p=0.03$; global human vs. rat: $F=5.16$, $p=0.0007$; and local human vs. rat: $F=5.01$, $p=0.0009$) except the global human vs. chimpanzee one, where a trend was found anyway ($F=1.91$, $p=0.11$). As clearly shown in Fig. 2 and Table 2, the secreted group is always the lowest conserved. The number of studies showed no influence their conservation score (Table 1), only a trend was found for local human vs. rats comparison ($p=0.04$). As shown in Fig. 3, the Fisher's exact test for the distribution of associated single SNPs found the maximum OR for the secreted groups (OR=1.36, $p=0.0008$). It found a significant result for cytoplasm-located ones, but their OR resulted protective ($p=0.004$, OR=0.78). Nucleus-, cell membrane, and mitochondrion-located SNPs did not show any significant association ($p=0.82$, $p=0.45$, and $p=0.08$, respectively), and among them, only cell membrane located OR would have been of increased risk (OR=1.05). Statistics of each analysis are reported in Table 3.

Single SNP's Association Analysis

Investigating the 14,198 SNPs within our pruning-involved genes that passed the selection process and operating the association analysis on the sample of 9,490 SKZ case–controls, we observed interesting results for ten of the analyzed SNPs (*p* values between 0.00001 and 0.0001). No one survived the high specific Bonferroni correction. The *p* values and characteristics of these ten best associated SNPs are reported in Table 8, and the characteristics and distribution of all analyzed SNPs are reported in Table 4, supplementary materials.

Single Genes Association Analysis

The analysis of single genes revealed significant associations (Bonferroni $p<0.0003$) for 4 of the 139 human analyzed

Table 6 Cell line and brain region of primary expression for each of the secreted pruning-related genes

Gene	Primary cell line expression	Expression rate for each brain area											
		Cortex	Olfactory	Hippocampal	Retro-hippocampal	Striatum	Pallidum	Thalamus	Hypothalamus	Midbrain	Pons	Medulla	Cerebellum
NGF	NA	1.01	1.34	5.94	0.97	0.14	0.29	0.63	0.48	0.10	0.48	2.16	0.52
APOE	neuropil	0.91	1.10	1.38	1.25	1.15	1.28	0.73	1.47	0.98	0.81	1.07	1.14
BDNF	neuropil/neuronal	1.66	1.06	5.23	1.47	0.24	0.31	0.45	0.37	0.12	0.09	0.58	0.17
TGFB1	NA	–	–	–	–	–	–	–	–	–	–	–	–
SEMA3F	Glial	0.09	1.00	0.01	0.03	0.06	0.02	0.56	0.21	9.29	0.18	0.10	0.85
BCAN	Neuropil	0.58	1.00	1.37	1.04	.061	0.94	0.55	0.88	1.06	2.02	1.86	1.88
TNC	Neuropil	0.45	0.87	0.24	0.03	0.14	0.04	3.65	0.02	0.15	0.09	0.17	5.01
TNR	Neuropil	1.89	1.19	3.88	1.06	0.41	0.37	0.54	0.25	0.24	0.13	0.15	0.11
NCAN	Neuropil	–	–	–	–	–	–	–	–	–	–	–	–
SEMA3A	Endothelial/neuron	0.54	2.40	0.01	3.35	0.63	0.65	0.001	0.07	0.02	0.58	0.70	2.96
NTF3	Neuropil/glial/neuronal	–	–	–	–	–	–	–	–	–	–	–	–
SLIT2	Neuron	1.46	1.10	3.30	2.05	0.52	0.14	0.30	1.38	0.23	0.31	1.44	0.08
SLIT3	Neuron	1.22	0.93	7.87	1.69	1.32	0.07	0.27	0.04	0.02	0.13	0.11	0.04
SLIT1	NA	0.55	0.62	12.91	1.94	0.37	0.37	0.29	1.28	0.51	0.19	0.04	0.01
C1QA	Endothelial	1.42	1.65	1.39	1.89	0.64	0.65	0.41	0.37	0.39	0.89	1.41	0.28
C1QB	Endothelial	–	–	–	–	–	–	–	–	–	–	–	–
C1QC	Endothelial	1.58	1.58	1.41	1.19	0.90	0.91	0.59	0.40	0.72	0.18	0.90	0.18
PLAT	Glial/neuronal	–	–	–	–	–	–	–	–	–	–	–	–
	Mean	1.02	1.21	3.45	1.38	0.50	0.46	0.69	0.55	1.06	0.46	0.82	1.01

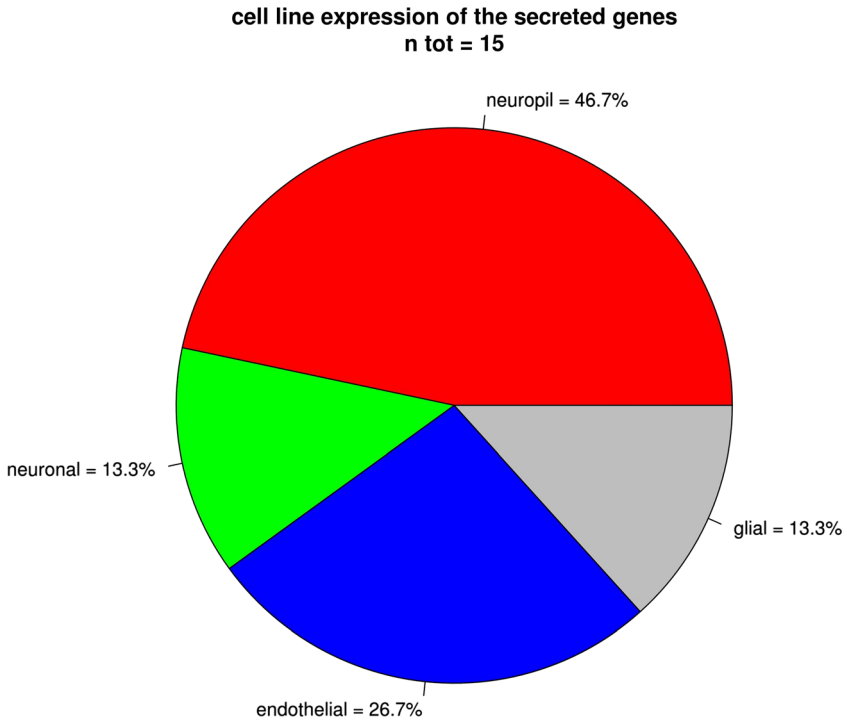

Fig. 4 Distribution of cell line expression for the secreted proteins

genes: *DISCS LARGE DROSOPHILA HOMOLOG 1* (*DLG1*, $p<0.00001$), *NITRIC OXIDE SYNTHASE 1* (*NOS1*, $p<0.00001$), *THROMBOSPONDIN 4* (*THBS4*, $p<0.00001$), and *FATTY ACID DESATURASE 1* (*FADS1*, $p=0.0001$). Table 4 reports the statistics and the characteristics for each of the significant associated gene.

Biological Pathway Association Analysis

The analysis of the Gene Ontology biological pathways related to our pruning-involved genes subset revealed 17 pathways playing a role in four biological compartmental functions that survived the Bonferroni correction (p values between 0.00001 and 0.0001). The significant pathways and their statistics are reported in Table 5.

Table 7 ANOVA test on the different mean of expression in the brain regions analyzed for secreted located proteins (n tot=18)

Group	Mean	SD	F value	df	p value
Cortex	1.02	0.55	4.04	10	0.00007
Olfactory	1.21	0.45	–	–	–
Hippocampal	3.45	3.75	–	–	–
Retro-hippocampal	1.38	0.87	–	–	–
Striatum	0.50	0.41	–	–	–
Pallidum	0.46	0.39	–	–	–
Thalamus	0.69	0.90	–	–	–
Hypothalamus	0.55	0.52	–	–	–
Midbrain	1.06	2.49	–	–	–
Pons	0.46	0.53	–	–	–
Medulla	0.82	0.72	–	–	–
Cerebellum	1.01	1.48	–	–	–

Cell Line and Brain Regional Expression of the Secreted Genes

Analyzing the cell line and regional expression of the secreted proteins involved in pruning (as they resulted to be the most associated in all the performed analysis), we found clear evidence that the primary localization is in the neuropil (46.7 %, n tot=15; please refer to Table 6 and Fig. 4). Analysis of brain region expression found significant differences in the expression of these genes (ANOVA: $F=4.04$, $p=0.00007$). The one with the highest expression mean resulted to be the hippocampal area of the limbic system (mean=3.45). For the statistics of the analysis, please refer to Table 7 and Fig. 5.

Discussion

SKZ etiopathogenesis could be related to abnormalities or errors occurring during adolescence pruning process of neuron branches. Alterations in this early brain remodeling mechanism may be the basis for the altered neural connectivity and functioning that, later in life, results in SKZ diagnosis. In this regard, the function of the proteins involved in this process, their modification through evolution, and their rate of association with our SKZ case–control sample may reveal about the

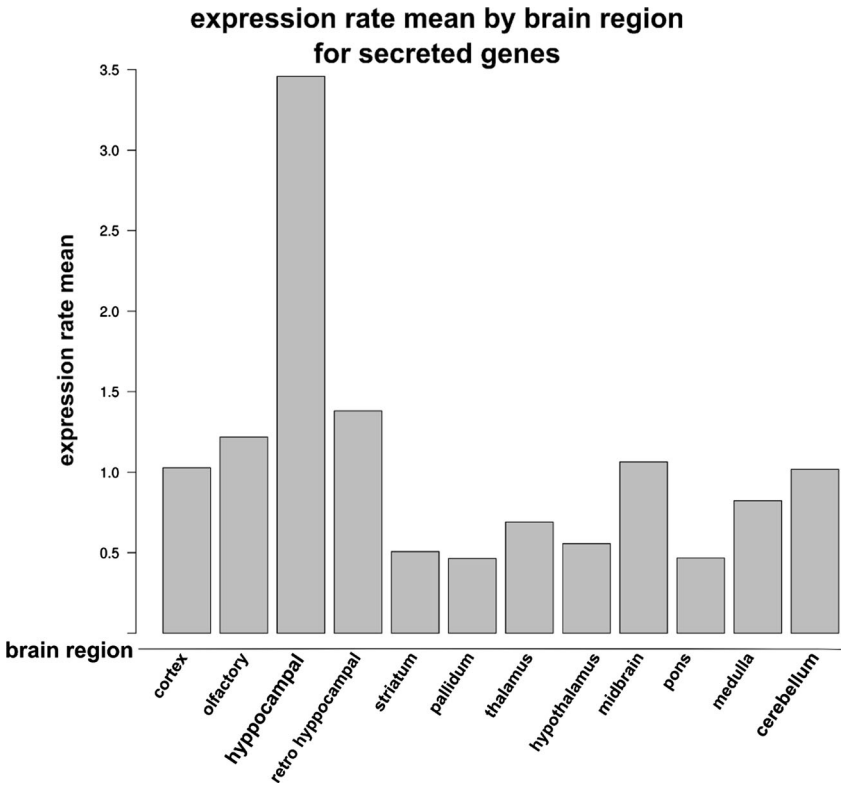

Fig. 5 The secreted proteins mean expression rate for each of the brain regions under analysis

pruning role in neurodevelopmental hypothesis for SKZ and future treatment perspectives.

Secreted Proteins-Based Pruning Regulation as Evolutionary Flash Point

The analysis of the subcellular localization of our proteins was aimed to figure out in which compartment and function rely the biggest differences of pruning between more and less evolute organisms. That could be of primary importance to understand the evolutionary adaptation of this mechanism at the higher complexity of human brain, which may underlie its higher susceptibility for such kind of complex neurodevelopmental disorder [71–73]. Considering the final subcellular localization as indicative about proteins function, it is known that secreted proteins have generally signaling and regulatory roles [74]. Cell membrane located ones act both as receptor for these signals and play crucial roles in establishment of cell polarity, of primary importance for cell migration and interaction with the surrounding cells/environment and dendritic stability or removal [75–77]. Cytoplasm-located proteins play roles especially in signal transmission both in centripetal and centrifugal ways [78] or even in direct receptor activity, as in the case of steroid hormones [79]. In cytoplasm, we also found the transcription apparatus from RNA to aminoacidic chains of the eucaryote cells, organized in ribosomes [80, 81] and the Golgi apparatus, of primary importance for the assembly and final remodeling of secreted proteins [82]. Finally, we know the role of mitochondrions as primary energy producers for the cell [83, 84] and nucleus in regulating genetic expression and cell life cycle and reproduction [85, 86]. Our results clearly show how pruning-related secreted proteins are the evolutionary newest (least conserved) and also the best associated with SKZ. So, we can deduce how regulation of this mechanism represents its evolutionary newest part and also the most associated with SKZ. Consistently, secreted signaling proteins are already known to be generally less conserved through evolution, and this fact has been involved in several diseases [87–89]. This subcellular located group also represents the most capable to interact

Table 8 Characteristics of the ten best associated SNPs

rsid	Gene	SNP region	Association *p* value
rs4894814	TNIK	Intron	0.00001
rs17269688	NGF	Intron	0.00004
rs6537860	NGF	Intron	0.00005
rs7535026	NGF	Intron	0.00006
rs10938796	SLIT2	Intron	0.00007
rs10776798	NGF	Intron	0.00007
rs561712	NOS1	Intron	0.00008
rs816293	NOS1	Intron	0.00009
rs7523654	NGF	Intron	0.00009
rs7530686	NGF	Intron	0.0001

and alter all the neuron surrounding environment. This observation is coherent with the generalized alterations characterizing SKZ patients' brain. In fact, CNS system abnormalities have been revealed not only in neurons but also, and maybe predominantly, in glial and connective tissues, in which these secreted proteins interact [90–92]. This is also consistent with the neurodevelopmental theory foundation that SKZ is a consequence of global alterations in brain connectivity and transmission regulation [93, 94]. Intriguingly, various secreted and secretion-related proteins already showed significant role in shaping SKZ risk [95–97]. As secreted proteins appear to be the evolutionarily newest, we can suppose that evolution through mammals, to adapt this biological process at the higher complexity of brain, worked more on a fine orchestration and harmonization of its parts with the increasing number of simultaneous nervous process carried out, rather than modifications in the dendritic cut realization itself. That makes the regulation of this mechanism its evolutionary newest part, the less tested, and therefore the most susceptible to errors. Consequently, even a little malfunction, in a so crucial moment of neural remodeling, may later result in a connectivity larger deficit. Consistently, studies are also showing how regulation of pruning is the part probably related to its major abnormalities [98, 99]. In this sense, the role of secreted proteins in regulation of neuron interactions and connectivity (among them and with the environment) may be the base for these connectivity alterations. Moreover, evidences that these alterations characterize not only SKZ patients but all the subjects at high genetic risk for the disease and their offspring have been reported by hereditary and imaging studies [31, 100, 101]. Considering that pruning takes place in every individual during adolescence, these lines of evidence create a pathological continuum that may be related with different stages of adolescent connectivity remodeling errors, due to pruning mis-regulation.

Genetic Confirmation of Secreted Proteins Role

Genetic analyses of pruning-involved genes in 9,490 PGC case–control sample revealed the importance of these genes in central steps of brain development, and consistently, they were already found to be associated with SKZ (please refer to Table 4). In fact, we recognize how *DLG1* and *THBS4* basically define the neurons' interactions and connectivity with other cells and with the environment. In particular, there is evidence that the THBS4's product is involved in neuronal events such as migration in early postnatal and adult brain in animals [102]. Neuronal migration is a cellular event with impact on pruning [103]. For example, a deficit in the semaphorin system, which is strongly related to neuronal migration, also affects pruning [104]. *NOS1* is also acting as signaling molecule through its ability of regulating endothelial permeability. In fact, it encodes for an enzyme-producing NO, a messenger that is able to exit the cell and regulate the signaling for endothelial cells, neuron signaling, etc. [105] with its different isoforms. At the same manner, *FADS1* is a central step in a metabolic pathway involved in cellular membrane stability and permeability regulation, a function which is clearly essential to allow cells playing harmoniously with signals from the environment. We can therefore suppose that alterations in these genes impair the cellular capacity of interacting and relating with the environment. This impaired ability may create problems, especially during pruning process. Indeed, the misinterpretation of its fine and complex human regulation, based on extracellular signals, could lead to an over/under/wrong branch removal that appears to be strongly associated with SKZ alterations. Pathway analysis confirmed these perspectives. In fact, it revealed consistent associations between altered neuron connectivity and interactions biological function and SKZ. In this regard, we noticed that the most associated biological function group appeared to be the "regulation of cell structure and interactions," the core of pruning mechanism. At the same manner, environment regulatory functions resulted significantly associated "endothelial and epithelial (nervous surrounding environment) regulation" and "regulation of cell membrane potential" [10, 106, 107]. Moreover, consistently with the hypothesis that secreted proteins are the cellular compartment most susceptible group for SKZ, regulation of secreted proteins rearrangement resulted as strongly associated. In particular, Golgi apparatus (GO:0005796) is well known to be the cellular site where proteins destined to be secreted are rearranged and selected, and Protein C-terminus binding (GO:0008022) is clearly important in this protein's rearranging function. These data support the previously cited evidence that altered pruning regulation (both in terms of signaling that in terms of their interpretation) may be the base to understand these connectivity alterations developed by all SKZ patients and high genetic risk subjects during adolescence, as seen in imaging studies and previously cited.

Hippocampal and Neuropil Primary Role in SKZ-Pruning Relationship

Investigating the cell line and regional brain expression of secreted proteins we found that they are expressed primary in neuropil, which has been postulated to be the microscopic base of white matter reductions observed in SKZ [35, 36]. The analysis also showed that the brain region with the highest expression rate of these secreted genes is the hippocampal area (Tables 6 and 7). Notably, the hippocampus is known to be the brain area where, earliest in life, notable differences between a normal subject, a high genetic risk one, and a patient that will be later diagnosed as SKZ [108, 109] can be seen. Alterations of this area development and its connectivity can also be one of the bases of neurodevelopmental hypothesis for SKZ.

These two results bring indications that pruning may be particularly relevant in this region, as other studies already reported [110–112]. This developmental alterations in hippocampal neuropil, due to particularly altered pruning regulation signaling (based on secreted proteins), may be the driven mechanism that lead to the final typical SKZ brain abnormalities. These findings need further confirmation through a range of experimental studies which could also lead to innovative treatments.

Final Conclusion

All these lines of evidence are consistent with a possible genetically driven mispruning that by removing branches that should have not been removed or overremoving/underremoving them brings to these connectivity alterations, revealed both in biological and imaging studies characterizing with different severity SKZ patients, subjects at high genetic risk and their offspring. These regulations may be particularly susceptible to errors due to its newest evolutionary appearance. Alterations in the regulation of this mechanism would also be consistent with the observation that SKZ neurotransmitters' abnormalities are different in different brain regions (e.g., dopamine prefrontal hypo- and *meso*-limbic hyperfunctioning), and so, this global alteration probably becomes from an error in the development of the nervous connectivity and transmission regulation rather than a cumulation of independent single alterations identical in each patient [113, 114]. The limbic system, and especially the hippocampus, may be the most susceptible structure at these pruning regulation errors resulting earlier in a particularly altered structure (as found by previous studies [108, 109]) that, with time, ends in the generalized altered neural connectivity revealed in SKZ patients. Pruning errors may, in fact, be the starting point to understand the widespread alterations in interneuron functioning that has been suggested as the basis for dopamine prefrontal hypo- and *meso*-limbic hyperfunctioning. Thus, widening our knowledge about this mechanism regulation and its altered role in the disease may be the starting point to deal with SKZ typical lack of knowledge in etiopathogenesis and pharmacological treatment strategies.

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