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Gene expression profiles of nucleus accumbens, prefrontal cortex and hippocampus in an animal model of schizophrenia: proposed candidate genes

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Introduction: It has been suggested that schizophrenia may be induced by "accidents" or injuries that occur during early brain development and result in a reduction of the neural connections in different regions. In this study, we evaluated differences in the expression of brain genes using a recognized experimental prototype of schizophrenia: the animal model of ventral hippocampal lesion in neonate rats (VHLN) compared to control animals.

Methods: Using microarray technology, we obtained gene expression profiles of three brain areas (nucleus accumbens, prefrontal cortex and hippocampus) of juvenile (45 days) and adult (90 days) Wistar male rats that underwent either VHLN or sham VHLN.

Results: Based on three criteria: 1) expression in more than one brain area, 2) participation in cellular pathways relevant to the central nervous system (CNS), 3) Z-score values >2 (overexpression) and <-2 (underexpression), we found overexpression of the *ppp3cb*, *dctn1*, *jag1, ide, limk2* and *cpz* genes and underexpression of *chrna4* and *sod1*.

Conclusions: Two of the genes proposed in this paper, *limk2* and *cpz*, have not been previously associated with schizophrenia, so future studies will be necessary to understand their possible role in the pathogenesis of this disease.

Keywords: Schizophrenia, Animal model, Microarrays, Expression, Candidate genes

Actas Esp Psiquiatr 2013;41(3):154-63

Perfiles de expresión génica de núcleo accumbens, corteza prefrontal e hipocampo en un modelo animal de Esquizofrenia: una propuesta de genes candidatos

Introducción: Se ha sugerido que la Esquizofrenia puede ser inducida por "accidentes" o lesiones durante el desarrollo temprano del cerebro del individuo, que conllevan a una reducción en las conexiones neuronales de diferentes regiones. En este trabajo, hemos evaluado las diferencias en la expresión de genes cerebrales, usando un reconocido prototipo experimental para Esquizofrenia: el modelo animal de lesión en hipocampo ventral en ratas neonatas (LHVN), respecto a animales control.

Metodología: Mediante la técnica de chips de ADN, se obtuvieron los perfiles de expresión génica de tres áreas cerebrales (núcleo accumbens, corteza prefrontal e hipocampo) de ratas macho Wistar juveniles (45 días) y adultas (90 días) sometidas o no a LHVN.

Resultados: Con base a tres criterios: 1) expresión en más de un área cerebral, 2) participación en rutas celulares relevantes para el sistema nervioso central (SNC), 3) valores de Z-score >2 (sobre-expresión) y <-2 (sub-expresión); se encontraron sobre-expresados los genes: *ppp3cb*, *dctn1*, *jag1, ide, limk2* y *cpz*, y sub-expresados: *chrna4* y *sod1*.

Conclusiones: Dos de los genes propuestos en este trabajo: *limk2* y *cpz*, no han sido relacionados previamente con Esquizofrenia, por lo que se hará necesario realizar estudios futuros para dilucidar sus respectivas contribuciones en la etiopatogenia de esta enfermedad.

Palabras clave: Esquizofrenia, Modelo animal, Microarreglos, Expresión, Genes candidatos

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INTRODUCTION

Schizophrenia is a serious mental disorder that affects 1 in 100 people worldwide and is characterized by distorted thoughts and perceptions that are probably due to alterations in different neurotransmitter systems[1,2]. Various hypotheses have been proposed to explain the pathogenesis of this disease, the genetic component being the most important[3]. However, due to the unavailability of brain tissues from patients and healthy controls for this type of studies, experimental animal models have been used[4,5].

The implementation and use of animal models in psychiatry has clear limitations because we are attempting to reproduce complex human behaviors in a healthy animal[4-6]. Nonetheless, animal models of psychiatric disorders have made it possible to explore the therapeutic potential of specific drugs for treating these disorders, and to obtain relevant data about the mechanisms of action of these drugs; models are also valuable tools for determining the neurobiological substrates of psychiatric disorders[7]. For the study of schizophrenia, several animal models, pharmacological models (using phencyclidines [PCP] and ketamine)[8-10], genetic models (induced by genetic mutations or deletions)[11-13] and neurodevelopmental models (induced by physical or neurotoxic lesions and environmental factors during neural development) have been proposed[14-16].

It has been suggested that schizophrenia may be due to "injuries" that occur during the early brain development of the individual and reduce neural connections in different limbic regions and the prefrontal cortex[14].

The ventral hippocampal lesion in neonate rats (VHLN) model has been widely tested. In this model, a small excitotoxic lesion is made in the hippocampus of the immature brain of neonate rats and the rats are then allowed to mature further. It has been observed that the injured animals, in the adult stage, exhibit conduct related to the positive and negative symptoms of schizophrenia, such as hyperlocomotion, reduction in prepulse inhibition, memory deficits and decreased social interaction, among other[15,16].

DNA microarrays are a valuable genomic tool for studying complex diseases, facilitating the global evaluation of expression of a large number of genes in various tissues and/or physiological conditions[17]. Using this methodology, we obtained gene expression profiles at 45 and 90 days for three different brain areas (hippocampus, prefrontal cortex and nucleus accumbens) of male Wistar rats with a neonatal lesion in ventral hippocampus (VHLN). These profiles were compared with those of animals with sham lesions, considered negative controls of the disease, in order to detect significant changes in gene expression that could be related to the schizophrenia phenotype in both juvenile and adult rats.

METHODOLOGY

Biological material

The study began with twenty male Wistar rats from litters of rats inseminated in the laboratory and kept in individual isolation cages under inverted 12-hour light-dark cycle conditions. The total sample was divided into four groups: a) 5 VHLN-juvenile rats, b) 5 sham injured juvenile rats, c) 5 VHLN-adult rats, d) 5 sham injured adult rats. All experiments were performed according to the regulations established by the Mexican official standard for the use and care of laboratory animals "NOM-062-ZOO-1999" and the regulations of the ethics committee of the International Association for the Study of Pain[18].

Newborn rats (10-13 g weight) were injured between days 5 and 7: the pups were randomly assigned to injury with ibotenic acid or sham injury with PBS. The animals were anesthetized using hypothermia by placing them on ice for 10-15 minutes. In order to perform bilateral lesions, the animals were immobilized on a Kopf stereotaxic fixed platform: an incision was made in the scalp and either ibotenic acid (0.15 µl/min for 2 min) (Sigma) or PBS (sham lesion) was administered through a needle according to the following coordinates: anteroposterior (AP) -2.5, mediolateral ±2.5 and dorsoventral (DV) -3.3 in relation to Bregma[19].

The animals were sacrificed by decapitation at 45 days (juvenile group: VHLN and sham) and 90 days (adult group: VHLN and sham). The hippocampus, prefrontal cortex and nucleus accumbens were dissected according to previously standardized procedures[20] and frozen at -80°C until RNA extraction.

RNA extraction

RNA was extracted from the 3 previously dissected brain areas using TRIZOL (Life Technologies), according to the manufacturer's instructions.

Microarray design, reading and standardization:

Five thousand *Rattus norvegicus* oligonucleotides from the Oligosets Operon (http://www.operon.com/arrays/oligosets_overview.php) library were used. The microarray design and manufacture was carried out at the Cellular Physiology Unit of the National University of Mexico (UNAM)[21].

Microarray hybridization started with 10 µg of total RNA, from which dUTP-Cy3 or dUTP-Cy5-labeled cDNA was generated with the CyScribe First-Strand cDNA kit (Amersham). Fluorophore uptake was confirmed by reading the absorbance at 555 nm for Cy3 and at 655 nm for Cy5[21].

Table 1 The number of genes that did not change expression is shown in the first part. The number of genes that significantly changed expression after normalization is shown in the lower part. (Alma D. Genis-Mendoza)

Juveniles		Adults	
Unchanged genes	%	Unchanged genes	%
4348	86.95	4331	86.61

	JUVENILES					ADULTS				
	Down	%	Up	%	Total (%)	Down	%	Up	%	Total (%)
N. ACCUMBENS	326	6.52	317	6.34	12.86	351	7.02	291	5.82	12.83
PREFRONTAL CORTEX	362	7.24	302	6.04	13.28	372	7.44	310	6.20	13.64
HIPPOCAMPUS	321	6.42	329	6.58	13.00	351	7.02	333	6.66	13.68

Signal and image acquisition and quantification was carried out with ScanArray 4000 equipment and ScanArray 4000 (Packard Biochip) software. All images were captured using 65% PMT gain, 70-75% laser power and 10-micron resolution at a 50% sweep rate. For each point labeled with Cy3 or Cy5, we calculated the average density and mean background value with Array Pro Analyzer (Cybernetics) software[21]. Gene expression was analyzed with the genArise statistical program developed at the Computer Unit of the Cellular Physiology Unit, UNAM (http://www.ifc.unam.mx/genarise/) to evaluate the degree of variation in gene expression (z-score). According to this criterion, elements with a z-score value greater or less than two standard deviations are differentially expressed genes[21].

RESULTS

After standardization, our analysis identified 652 genes that significantly changed their expression, of which 316 were found to be overexpressed (OE) and 336 were underexpressed (UE). The percentage of OE and UE genes for young rats was observed to be almost the same (about 6.5%), whereas the UE gene percentage (7%) was slightly higher than the OE gene percentage (6%) for adult rats. However, the overall percentage of genes that modulated their expression for each tissue remained at 13% ± 0.38 (Table 1). Genes were sorted into 3 groups according to the z-score (>1 and <-1, >2 and <-2, and >3 and <-3). Subsequently, to identify the metabolic and signaling pathways involved in each of the proteins encoded by these genes, we accessed the KEGG metabolic pathways database (http://www.genome.jp/kegg/kegg2.html), which is a genomic mapping tool.

Gene group with z-score value >1 and <-1

At this stage, general information was obtained for both overexpressed and underexpressed genes, and a list of all the metabolic pathways for each brain area and the number of genes involved was prepared. We then selected and plotted the pathways in which a higher number of genes (more than 5 genes) were modulated. Among the most compromised pathways were neuroactive ligand-receptor interaction (>10 genes), calcium signaling (>7 genes) and cancer (>7 genes). It is noteworthy that there were genes whose modulation was significant for two or more pathways.

Group of genes with z-score value >2 and <-2

We worked with genes with a z-score value greater than 2 and less than -2 for each tissue for a more robust analysis. Eighteen pathways associated with mental disorders were selected. For each tissue, the metabolic pathways of interest and participating genes were plotted against z-scores (Figure 1).

Because the same gene could be expressed in two or more of the brain areas evaluated (nucleus accumbens, hippocampus and prefrontal cortex), we looked for genes that were expressed in more than one area of interest in order to limit our analysis. As can be seen in Figure 2, in both the juvenile and adult groups, the expression of certain genes is regulated coincidentally in more than one tissue. We found a higher number of underexpressed genes than overexpressed genes, the group of adult rats having the highest number of underexpressed genes. Interestingly, among the overexpressed genes, *ppp3cb* and *dctn1* modulated their expression in all three areas of the brain and in both stages (juvenile and adult).

Group of genes with z-score values >3 and <-3

Lastly, the genes that presented larger changes in expression compared to their control group were analyzed. Similarly, genes that modulated their expression in more than one tissue were detected. Of all of these, *ppp3cb* varied

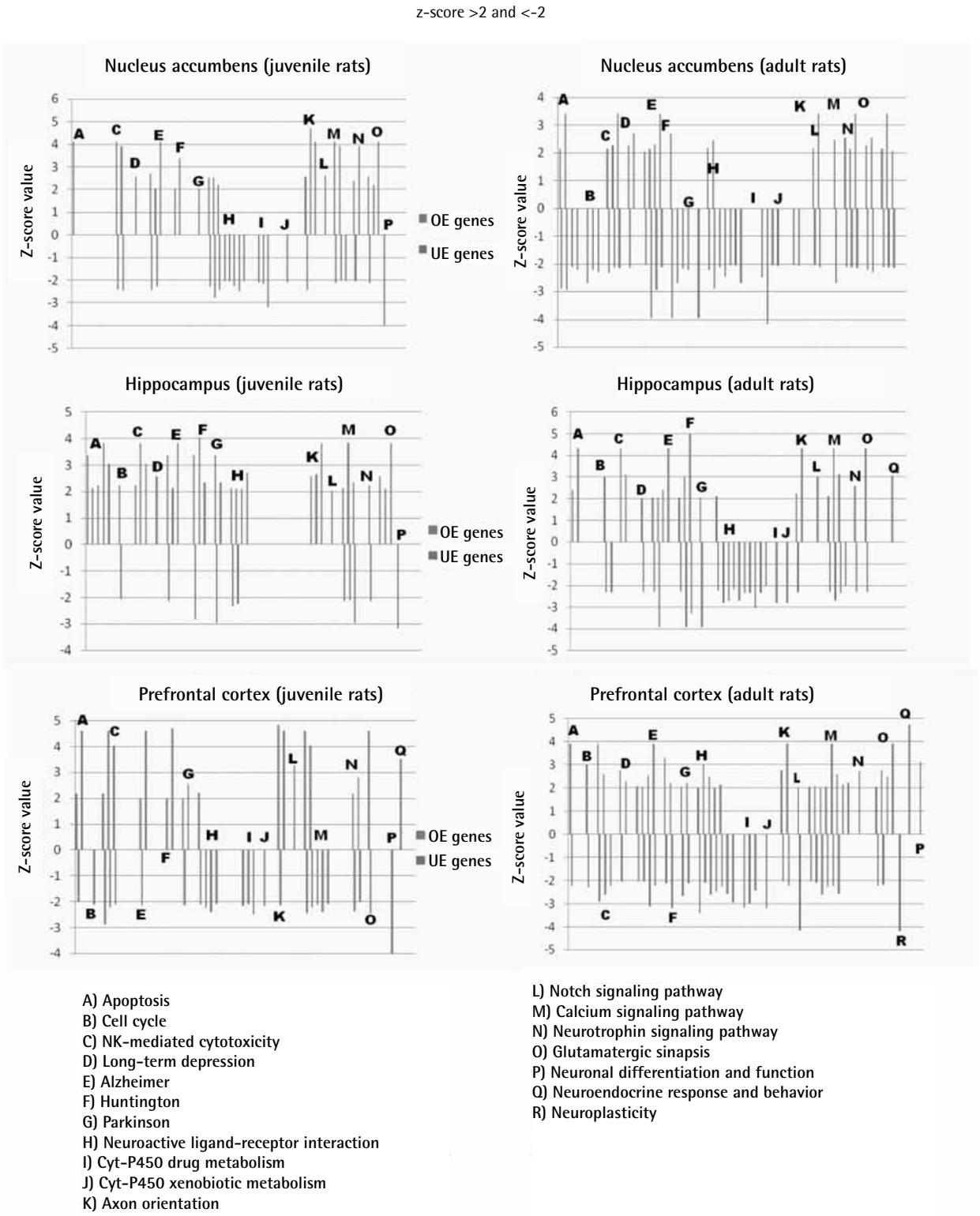

Figure 1 — *The graphs show the number of overexpressed and underexpressed genes involved in pathways of interest in the group of juvenile and adult rats. Each gene is plotted relative to its Z-score value. (Alma D. Genis-Mendoza)*

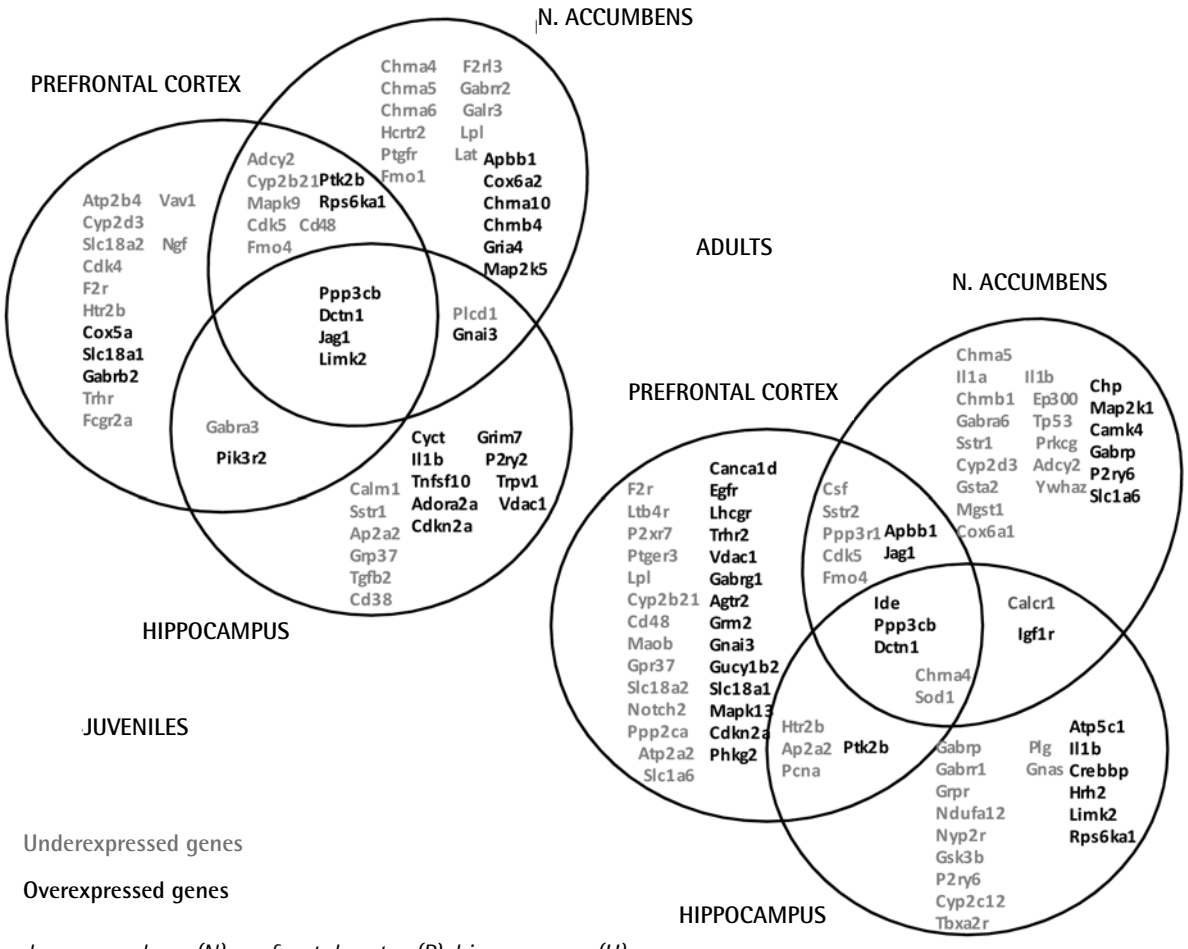

Figure 2 — *This figure shows the candidate genes obtained from the following three criteria: 1) Z-score values >2 and <-2); 2) expression in more than one brain area; and 3) participation in relevant metabolic pathways of the central nervous system*

its expression in the three areas of the brain in both groups. In this category, we found another gene with a high z-score, *cpz* 4.37.

Finally, after taking into account z-score values, expression in more than one brain area and participation in metabolic pathways of interest, eight candidate genes were proposed for the schizophrenia study. In Table 2 we summarize the results of the z-score values for the proposed genes; it can be observed that *ppp3cb* and *dctn1* had the highest z-score values in all three areas evaluated.

DISCUSSION

Using microarray technology, we evaluated the expression of the genes in different brain regions using the VHLN animal model for schizophrenia. We worked with 4 experimental groups of Wistar male rats: VHLN juvenile rats, sham-injured juvenile rats, VHLN adult rats and sham-injured adult rats. The analysis was divided into 3 groups according to the z-score value (>1 and <-1, >2 and <-2, and >3 and <-3). Not all the routes observed were directly associated with mental disorders, so we identified 18 pathways relevant to the CNS (genes with z-score >2 and <-2). Finally, we examined the genes with z-score values >3 and <-3 to identify the candidate genes of interest with the greatest variations in expression.

Group of genes with z-scores >1 and <-1

This group included a large number of metabolic and signaling pathways that were found to be differentially altered in the three brain areas. The pathways with the largest number of modulated genes were cancer, neuroactive

Table 2 **Proposed candidate genes selected using the following 3 criteria: I) Z-score values >2 and <-2; II) expression in more than one area of the brain; III) participation in pathways of interest. The symbol of the gene, name, biological description, and z-score values in adults of each tissue are shown. Nucleus accumbens (N), prefrontal cortex (P), hippocampus (H)**

Symbol	Name	Description	A Score	P Score	H Score
Limk2	Kinase 2 with LIM domain	Proteins with LIM domains are involved in diverse cell-signaling processes, such as cytoskeletal organization, organogenesis and the cell cycle	4.710	4.834	2.659
Ppp3cb	Phosphatase 3 protein, catalytic subunit, beta isoform	Phosphatase-dependent calmodulin protein; it dephosphorylates nuclear factors of activated T cells and may play a role during skeletal muscle atrophy	4.130	4.600	3.846
Dctn1	Dynactin 1	Dynein microtubule component activated by ATPase, which acts as a microtubule engine	3.378	4.717	4.042
Jag1	Jagged 1	Ligand responsible for activation of the Notch1 receptor. Signaling through *Notch* is involved in the development of most tissues	2.625	3.254	2.036
Ide	Insulin-degrading enzyme	Enzyme involved in the degradation of bioactive peptides, including insulin, beta-endorphin, atrial natriuretic peptide and beta-amyloid	2.292	2.531	2.052
Sod1	Superoxide dismutase 1, soluble	Catalyzes the conversion from superoxide to hydrogen peroxide and molecular oxygen involved in the oxidative stress response	-2.193	-3.187	-3.301
Chrna4	Cholinergic receptor, nicotinic alpha-4	Belongs to the superfamily of ligand-activated gated ion channels that play a role in the rapid transmission of signals in synapses	-2.909	-3.420	-2.814

ligand-receptor interaction and calcium signaling; the last two pathways were of interest.

Group of genes with z-score >2 and <-2

In this segment, we detected 7 genes of interest whose expression was modified in the three brain areas and had ontologies related to the pathways of interest. The calcium signaling and neuroactive ligand-receptor interaction pathways continued to contribute a large number of differentially expressed genes, whereas neuronal differentiation and function, neuroendocrine response and behavioral pathways had less participation. It should be noted that there were overexpressed and underexpressed genes in most of the pathways, except for the route of drug and xenobiotic metabolism by Cyt-P450, where only underexpressed genes were found (Figure 1).

Group of genes with z-scores >3 and <-3

Finally, this analysis allowed us to propose 8 genes as potential candidates for the genetic approach to schizophrenia. Six of these genes *(ppp3cb, dctn1, ide, limk2, jag1* and *cpz)* were overexpressed, while the remaining two *(sod1* and *chrna4)* were underexpressed (Table 2).

Among the genes with the highest z-score values, *ppp3cb* (Table 2) was noteworthy for being overexpressed in the group of juvenile and adult rats. This gene encodes the catalytic subunit of the β isoform of phosphatase 3 protein. This enzyme interacts selectively and noncovalently with calmodulin in response to an increase in intracellular calcium levels[22]. Recently, a genome study in Taiwanese families with schizophrenia suggested the linkage of locus 10q22.3, where the *anxa7, dnajc9, zmynd17* and *ppp3cb* genes are located, the same genes associated with schizophrenia and with cognitive and attention deficit problems[23]. In addition, a meta-analysis was conducted to evaluate the entire genome of individuals with schizophrenia in various populations. Among the genes proposed as candidate genes was *ppp3cc*, which encodes the gamma isoform of the calcineurin enzyme. Other genes associated were *nos1ap, rgs4, uhmk1, nrg1* and *znf804a*, among others[24].

On the other hand, the human *dctn1* gene encodes the major p150 subunit of dynactin, a macromolecular complex consisting of 10 polypeptides, which is required for the retrograde axonal transport of cellular vesicles and organelles through the microtubule system. Numerous studies have shown the role of p150 in psychotic disorders associated with delusions and hallucinations[25], and neurodegenerative disorders such as Huntington disease[26] and amyotrophic lateral sclerosis (ALS)[27].

The differential regulation at the level of *ppp3cb* and *dctn1* transcripts was notable in both the juvenile and adult rats (z-score values >3), which suggests that both may contribute from the onset of the first psychotic symptoms.

Another of the genes that was overexpressed with a high z-score in all three brain areas studied was *ide,* which encodes insulin-degrading enzyme (IDE) (Table 2). This gene is situated in locus 10q23-q25, previously associated with schizophrenia[28], and encodes a zinc-dependent metallopeptidase that degrades insulin, glucagon, β-amyloid, β-endorphin, IGF-I and IGF-II. Deficit expression of this enzyme has been associated with Alzheimer disease and type 2 diabetes mellitus[29,30]. It has also been suggested that haloperidol could have an effect on IDE activity through changes in the level of expression of its substrates[28]. However, since our data revealed a significant increase in *ide,* it is possible that the regulation of both enzyme levels and activity occurs post-transcriptionally, as described in other members of the metallopeptidase family[31].

Another transcript expressed abundantly in this study was *jag1.* The resulting protein is the ligand of Notch 1, a transmembrane receptor activated before birth that induces radial glial differentiation[32], and that promotes the differentiation of progenitor cells into astroglial scaffolding cells after birth[33]. In patients with schizophrenia, it was recently shown that *jag1* expression is significantly increased, exhibiting positive correlation with canonical mediators of the Notch pathway, which influences cell proliferation and the cell cycle, and also participates in oligodendrocyte synthesis[34,35]. We observed *jag1* overexpression only in the group of juvenile rats. In this respect, we might speculate that *jag1* overexpression could reflect activation of this pathway to promote neurogenesis in response to injury caused by neonatal injury, thus reactivating the neuronal development of the animals.

The *chrna4* gene that encodes a nicotinic acetylcholine receptor belonging to the superfamily of ion channels was found to be underexpressed in the adult animals. As is well-known, this class of receptors plays a major role in the transmission of nerve signals in neuronal synapses. It has been suggested that cholinergic-nicotinic dysfunction may contribute to cognitive impairment in schizophrenia by mediating dopamine release. Furthermore, it has been seen that neurocognitive deficits in schizophrenia can be relieved temporarily by nicotine administration[36,37]; since then, several nicotinic receptor subtypes have been examined as candidates associated with this disease[37,38].

Another potential candidate gene for study in schizophrenia is *sod1,* which we found to be significantly underexpressed in adult rats. The superoxide dismutase 1 protein encoded by this gene is one of two human isoenzymes responsible for the destruction of superoxide free radicals in cells. Mutations in this gene have been associated with familial ALS[27]. A decrease in superoxide dismutase 1 enzyme expression may potentiate oxidative stress by increasing the vulnerability of neuronal cells to free radicals, which is consistent with reported brain damage in patients with schizophrenia[39].

It was noteworthy that the two underexpressed genes *sod1 and chrna4* only had decreased levels in the group of adult rats, suggesting that these changes may be associated with positive and negative symptoms of schizophrenia.

Moreover, our findings showed that *limk2* was overexpressed in the juvenile rat group. LIM kinase genes encode a group of protein kinases involved in diverse biological functions. Despite the fact that *limk2* has not been associated with schizophrenia, it has been associated with apoptosis as a protein that protects against ischemia in a rat model of diabetes[40,29], and in an animal model of Alzheimer disease, increased *limk2* expression was observed with degeneration of synaptic structures[41]. It is known that LIMK2 is phosphorylated and activated by ROCK kinases, thus phosphorylating cofilin, a protein that prevents actin polymerization and favors depolymerization, which are necessary for cell motility[42]. An increase in the expression of *limk2* may be disturbing cytoskeletal dynamics due to failure to regulate actin, which would have negative implications for early neuronal migration in injured juvenile rats and is consistent with our model of schizophrenia.

Finally, in the group of juvenile and adult rats we found overexpression of the carboxypeptidase Z gene *(cpz).* The protein has an N-terminal domain with 30% amino acid identity with the "frizzled" (Fzd) domain present in Wnt-interacting proteins[43,44]. Carboxypeptidase Z is abundant in the extracellular matrix of the placenta and is found in smaller quantities in brain, lung, thymus and kidney[38]. This protein had never been associated before with schizophrenia; in contrast, another enzyme of this family, glutamate carboxypeptidase II (GCPII) has been associated with mental disorders, including schizophrenia[45].

Although the exact function of CPZ is unknown, it is likely that it plays a role in the regulation of embryonic development by interaction with Wnt proteins. Furthermore, it has been reported that CPZ expression is much lower in adult tissues than in embryonic organs[46]. In turn, it has been shown in hippocampal neuron cultures that Wnt-mediated signaling is important for axonal development and guidance[46], regulating cytoskeletal dynamics in the axonal growth cone and branching[47], and leads to an increase in the formation and organization of new presynaptic terminals[48,49].

Our study showed that the *cpz* gene did not turn off after embryonic development and continued to be overexpressed in injured adult rats. If this protein remains highly expressed in the adult brain, it is very likely that dysregulation of Wnt-mediated signaling exists.

To date, *cpz* and *limk2* genes have never before been associated with schizophrenia and, because of their significant overexpression in our study model, these genes can be expected to play an important role in the development and course of the disorder. Undoubtedly, future research and new experimental strategies should be designed with the aim of clarifying the contribution of LIMK2 and CPZ proteins in this disease and establishing their role as potential biomarkers.

With respect to the three brain areas assessed (nucleus accumbens, hippocampus and prefrontal cortex) in the group of injured animals, a reduction was observed in the number of genes regulated compared with the group of sham injured animals. This is consistent with the cognitive impairment that has been observed in the brains of patients with schizophrenia, where neuronal deterioration and/or damage has been demonstrated in the hippocampus and prefrontal cortex[50]. Given the cognitive impairment manifested by patients with schizophrenia, it is possible that damage to the prefrontal cortex is observed in the long term, which in turn is consistent with the VHLN animal model of schizophrenia, which is based on the neurodevelopmental hypothesis and goes hand-in-hand with an early prenatal brain lesion[51,52] and the involvement of morphogenesis in the dorsolateral cortex region[53]. Furthermore, our findings suggest the possibility that the maturation of local inhibitory circuits in the prefrontal cortex may be altered in rats with hippocampal injury, as has been reported in a previous study[54].

One limitation of this study was that we evaluated gene expression profiles using the DNA 5k chip designed by the Cell Physiology Institute, UNAM. We know that late-generation DNA chips currently available on the market can be used to study more genes and other features; analyses with such chips might identify other genes that we did not observe with this chip. However, we believe that our findings are relevant because the modulation of some genes already associated with schizophrenia was observed, as well as two genes that so far have not been linked to schizophrenia and could play a role in the disease.

CONCLUSIONS

This work showed that the transcriptome of juvenile and adult rats with VHLN was differentially modified compared to the control group. Significant overexpression and underexpression of several genes was reported, some previously associated with schizophrenia and others not. Our contribution in this regard is to propose eight potential candidate genes for analysis in this disorder, two of which have not been previously associated with schizophrenia *(limk2* and *cpz).* These unpublished findings open new questions and broaden the horizons for future research at the functional level in both animal models and humans, which may allow reliable diagnostic molecular biomarkers to be established in schizophrenia.

ACKNOWLEDGMENTS

Isabel Beltran, Dr. Jorge Ramirez and his team: Lorena Chávez González, Simón Guzmán León and José Luis Santillán Torres for their technical assistance in the microarray determinations. Gerardo Coello, Gustavo Corral and Ana Patricia Gómez for their assistance with the genArise software.

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