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Alterations in the Brain Transcriptome in *Plasmodium Berghei* ANKA Infected Mice

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Abstract

We have used cDNA microarrays to compare gene expression profiles in brains from normal mice to those infected with the ANKA strain of *Plasmodium berghei*, a model of cerebral malaria. For each of three brains in each group, we computed ratios of all quantifiable genes with a composite reference sample and then computed ratios of gene expression in infected brains compared to untreated controls. Of the almost 12,000 unigenes adequately quantified in all arrays, approximately 3% were significantly downregulated ($P < 0.05$, ≥ 50% fold change) and about 7% were upregulated. Upon inspection of the lists of regulated genes, we identified a high number encoding proteins of importance to normal brain function or associated with neuropathology, including genes that encode for synaptic proteins or genes involved in cerebellar function as well as genes important in certain neurological diseases such as Alzheimer's disease or autism. These results emphasize the important impact of malarial infection on gene expression in the brain and provide potential biomarkers that may provide novel therapeutic targets to ameliorate the neurological sequelae of this infection.

Keywords

malaria; neurobiology; gene regulation; *Plasmodium berghei*; mouse models; vascular disease

1 Introduction

A large study in sub-Saharan Africa reported that almost 50% of malarial patients exhibited neurological deficits [15] encompassing a number of symptoms, including ataxia, seizures, hemiplegia, and eventually coma and death [13, 15,16]. In addition, greater than 20% of children who survive an episode of cerebral malaria sustain persistent cognitive deficits, which can include memory impairment, visuospatial deficits, and psychiatric disorders as well as motor coordination dysfunction [2,3,6,12,20]. While the precise etiology of cerebral malaria has not fully been elucidated, recently vasculopathy has been recognized as contributory to mortality during cerebral malaria [5]. We and others previously demonstrated that experimental cerebral malaria is associated with impairment of blood flow to the cerebral microvasculature, and that this directly correlated with neuronal and axonal damage [18,31]. Impairment of the cerebral blood flow and associated axonal damage has also been observed in children with cerebral malaria [1,35]. In addition, although cerebral

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malaria affects the vasculature, extensive immunological and inflammatory effects occur within the nervous system [4]. In fact, recent studies intimate that the development of deficits in human cerebral malaria is a complex issue which involves the progression of metabolic and physiologic processes in several regions of the brain [19].

Microarray analysis of differentially expressed genes offers the possibility to search for pathways responsible for disease in a broad, unbiased approach. Using such a strategy, a number of authors have previously identified interferon-regulated genes as prominently altered in brains of mice that are susceptible to cerebral malaria [33], with differential expression in mouse strains that are either susceptible or resistant to cerebral malaria [9,25]. Most recently, Lovegrove et al. [24] identified neuronal apoptosis as another pathway that was differentially regulated. Although many genes show strain-specific expression and there are numerous differences between mouse models and human disease, the utility of this novel approach for appreciating alterations in expression of neural genes was highlighted in the accompanying commentary by John [17].

We have recently obtained evidence for cognitive deficits in mice infected with the ANKA strain of *Plasmodium berghei*, a well-studied mouse model of cerebral malaria [8,10]. Because previous studies had observed such small numbers of altered genes in brain or had been focused on changes occurring in other tissues, we undertook the experiments described here, in which multiple biological replicas were used to provide statistically meaningful datasets of up- and downregulated genes. Our findings indicate profound changes in gene expression in the brains of infected animals, both with regard to total number of affected genes and the multiple signaling pathways that they encompass. Particularly surprising was the extraordinarily high number of affected genes that are associated with neurological disease. We conclude from this study that infection of mice with a Plasmodium strain which causes cerebral malaria leads to large-scale gene expression changes in the brain, emphasizing that the resulting disease is fundamentally neurological and identifying putative neural targets toward which therapy might be directed.

2 Materials and methods

2.1 Infection of mice

Experiments were performed with the approval of the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine. Four- to five-week old C57BL/6 female mice (Jackson laboratory, Bar harbor, ME) were either infected with *Plasmodium berghei* ANKA (PbA) or left uninfected for comparison. Blood containing either 5×10^5 red blood cells (RBCs) parasitized with PbA or uninfected blood was diluted in PBS, and 200 microliters were injected via the intraperitoneal route. The mice were then separated into two groups of infected or uninfected mice. Parasitemia, or the percentage of parasitized RBCs, was evaluated by examining Giemsa stained blood smears on day 6 postinfection (PI). On day 6 PI, mice were rapidly euthanized using carbon dioxide and the brains were harvested, frozen in liquid nitrogen, and then stored at −80°C for future analysis.

2.2 RNA extraction and hybridization

We used a previously published protocol [14] and a composite reference RNA sample (R) prepared in sufficient quantity for the entire experiment from ten adult mouse tissues (aorta, brain, heart, kidney, liver, lung, ovary/testicles, spleen, and stomach—equal amounts from males and females). This combination of source tissues provided a high diversity of genes expressed in the midrange of the detection system for the AECOM mouse cDNA microarrays. Briefly, 60 μg total RNA, extracted in Trizol® (Invitrogen, Carlsbad, CA) from brains of three infected (I) and three control (C) mice, purified with RNeasy® mini kit (Qiagen, Valencia, CA), were reverse transcribed into cDNA incorporating fluorescent Cy3-

dUTP. The composite reference was reverse transcribed to incorporate Cy5-dUTP. Each of the six Cy3-labeled brain extracts was cohybridized overnight at 50°C against the Cy5-labeled reference with 32 k 70-mer oligonucleotide mouse microarrays produced by the Microarray Facility of the Albert Einstein College of Medicine (platform described in http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL5371). After hybridization, the slides were washed at room temperature, using solutions containing 0.1% sodium dodecyl sulfate (SDS) and 1% SSC (3M NaCl + 0.3M sodium citrate) to remove the nonhybridized cDNAs.

2.3 Acquisition, filtering, and normalization

All microarrays were scanned with an Axon GenePix® 4000B scanner[1] and data acquired through GenePixTM Pro 6.0 software[2]. Spots with substantial local imperfections (customarily flagged by the acquisition program), those for which the medians of the foreground signals were not at least twice as high as the medians of the background signals in both channels, and those with saturated pixels were eliminated from the analysis to avoid inadequate quantification. Background-subtracted signals were normalized through an iterative algorithm, alternating within-array normalization with interarray normalization until the average corrected ratio differed by less than 5% in subsequent steps. Normalized relative expression levels were then organized into redundancy groups, each composed of all spots probing the same gene and each group then represented by the weighted average of the individual spot values.

2.4 Detection of differentially expressed genes

Detection of differentially expressed genes relied on both absolute $>$1.5x fold-change and $<$ 0.05 *P*-value of the heteroscedastic *t*-test applied to the means of the background subtracted normalized fluorescence values in the four biological replicas of the compared transcriptomes. The *P*-values (two samples, unequal variance) were computed with a Bonferroni-type correction applied to the redundancy groups [14].

3 Result and discussion

Data complying with the “Minimum Information About Microarray Experiments” (MIAMEs) were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24086). Of the 11,669 genes whose expression was adequately quantified on all arrays, 335 (2.87%) were significantly downregulated and 793 (6.90%) upregulated in infected mouse brain. These differentially expressed genes, along with their expression ratios and *P*-values are listed in Table 1.

When pathways of regulated genes were analyzed using GenMapp software, the most prominent pathways of upregulated genes (Table 1A) were those related to rhodopsin like receptors and related categories of olfactory, G-protein coupled, hormone and transmembrane receptors, and perception of smell, including the beta adrenergic receptor (*Adrb3*), muscarinic receptors *Chrm2* and *Chrm3*, the orexin receptor *Hetr1*, the H1 histamine receptors *Hrh1* and *Htr1a1*, the prostaglandin receptor *Ptgfr*, and the vomeronasal receptors V1rc20, V1rc8, V1rel18, and v1rd13. Other GO terms with disproportionately high number of regulated genes were cytokine production (*Tnfs15, Inhbb, Cdlb1, Il17f, Cdld1, Nod1, and Spin*), voltage gated chloride channels (*Clic2, Clic4, Clic5*), chromatin remodeling (*Mta2, Smarcc1, Suv39h2, Suv39h1, Nasp*), and genes related to defense and acute phase response (*Fn1m Pxp, Serpna3n*).

[1]For more information visit http://www.moleculardevices.com/pages/instruments/gngenepix4000.html
[2]For more information visit http://www.moleculardevices.com/pages/software/gngenepixpro.html

Pathways with substantially higher expression in the malarial brain were chromatin remodeling genes (*Htaitip, Nptxs, Mbd3, H2cfx, Hist 1h2b, Myst3, Nap1l3*), cell development, chiefly genes related to negative regulation of apoptosis (*Pten, Rblcc1, Stat5b, Eefk2, Polb, Nsh2*), lipid metabolism (*Adipor1, Fads2, Lysla1, Psap, Scd2*), hydrolase activity (specifically, notrophenylphospatase), magnesium ion binding (*Arsa, Atp1a1, Atp2a2, Brsk1, Cno16l*), adult walking behavior (*Cacnb4, Glrb*), axon (*Alcam, Dpysl2, Pacb1*), and regulation of muscle contraction (*Atp2a2, Atp1a1*). Interestingly, both neuronal and vascular endothelial cell apoptosis have been described in human and experimental cerebral malaria, in association with glial cell damage and dysfunction [22,29,36] demonstrating that protein regulation occurs both at the transcription and the translation levels. The finding that substantial numbers of genes involved in chromatin remodeling are regulated in the infected brain raises the possibility that, in addition to involvement of epigenetic factors in the parasite itself [30], a major consequence of infection may be the epigenetic reprogramming of host nervous tissue.

These analyses of pathways affected by infection revealed that genes expected to be important in cerebral function (receptors cytokines, apoptosis, lipid metabolism, walking, axon, and muscle contraction) were disproportionately affected in these brains. As an additional method to mine the data corresponding to gene alteration in the brains of infected mice, we determined whether genes whose expression was altered were known to be associated with human neurological disease.

As can be seen from Table 2, the major subcategories of genes linked to neurological diseases that are up- and downregulated in malaria brain are channels/receptors/transporters and components of the synapse including cytoskeletal linkages to vesicles. Channel genes with altered expression include *Glrb* (the beta subunit of the glycine receptor), *Scn1b* (the beta subunit of the voltage gated sodium channel), *Cacnb4* (a beta subunit of voltage-dependent calcium channel), *Cabp1* (a neuron-specific regulator of calcium channel activation), *Accn2* (the neuronal amiloride sensitive cation channel2), Slc33a1 (the acetyl co-A transporter involved in gangliosides), and *Vdac3* (the mitochondrial voltage-dependent anion channel). Genes whose encoded proteins are involved in synaptic or other contact between cells include *Adam23* (which mediates integrin cell adhesion in brain), *Ap2a1* (calcium Huntington interacting protein that links clathrin to receptors in vesicles [34], *Akap8l* and *Akap9* (the latter of which also known as *Yotaio,* anchor protein kinase A and binds the *Nr1* subunit to the cytoskeleton to the vesicle fusion protein *Snap29* [23]), *Scrib* (a presynaptic scaffolding molecule), *Pcdha6* and *Pcdh9* (proto cadherins that link neural cells), *Nlgn3* (neuroligin3, a neuron cell surface protein involved in formation of remodeling of CNS synapses), *Nrnx2* (neurexin2, a neuron cell surface protein required for normal transmitter release), *Rims3* (which enhances neurotransmitter release [21]), *Cntnab1* (a contactin associated protein important for neurite outgrowth and differentiation), *Gabarap* (which links the *Gabaa* receptor with cytoskeleton), *Gripap1* (a neuron-specific guanine release factor associated with the AMPA complex), *Itsn1* (intersection 1, which associates with *Ehb2* tyrosine kinase and the cytoskeletal protein *Wasp* to mediate dendritic spine morphogenesis). Several mitochondrial genes are regulated in the malaria brain whose dysfunction is associated with neurological disorders [7], including *Vdac3* as mentioned above, *Tmem70, Sncoa2*, and *Acad8.*

Our study of transcriptomic regulation in the setting of cerebral malaria identified numerous genes whose altered expression or mutation has been associated with disorders of brain. Many of these regulated genes are associated with ataxia or altered cerebellum development or function (e.g.: *Camkk2, A2bp1, Ubqln4, Canx, Cacnb4, Psap, CCG1),* consistent with a common neurological consequence of the disease [32]. It is also noteworthy that several of the encoded proteins (*Fxr2h, Pip5k2c, Pcdh9*) have been associated with autism [27], which

in a small study of 20 children in Tanzania was diagnosed in a significant fraction of CM patients [26]. Another neurological overlap is with Alzheimer's disease. Delahaye et al. [9] reported overexpression of beta amyloid only in a mouse strain susceptible to cerebral malaria. In addition, axonal damage has been demonstrated as a feature of cerebral malaria with increased immunoreactivity of the amyloid precursor protein (APP) in the white matter adjacent to areas of vascular damage and of hemorrhage [28]. In Table 2, we demonstrate that *Apbb1* mRNA is significantly upregulated in infected brain.

4 Conclusion

The classification of cerebral malaria as a vascular disease [5,11] emphasizes the therapeutic potential of agents directed toward increasing brain perfusion. Long-term cognitive and motor deficits correspond with the geographical distribution of vascular damage in experimental cerebral malaria [8]. Likewise, decreased cerebral blood flow has been demonstrated to contribute to mortality in cerebral malaria, and vasodilitory agents increase survival in the experimental model [5]. However, focusing exclusively on microvascular damage presents an incomplete approach to a complex disease process, as cerebral malaria is also unquestionably a disease that affects neural components of the brain, resulting in impaired gait, cognition and neural processing, as well as cognitive and motor impairment [8,10]. The identification of altered genes encoding proteins within pathways prominently associated with neurologic disease in the present study provides alternative or adjunctive disease targets to improve treatment outcomes for the vast number of individuals who have recovered from acute parasitic infection but for whom there is neural damage that extends beyond impaired brain microcirculation.

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Table 1

Gene ontology terms of altered genes in CM brains.

GOID	GO name	Ttype	Number changed local	Number measured local	Number in GO local	Percent changed local	Z Score	Permuted P
A. GO terms upregulated in malarial brain								
1584	rhodopsin-like receptor activity	F	16	107	537	14.95327	3.388	0.001
3674	molecular_function	F	1	22	673	4.545455	2.383	0.024
4888	transmembrane receptor activity	F	1	15	88	6.666667	2.884	0.007
4930	G-protein coupled receptor activity	F	3	25	469	12	3.546	0
4984	olfactory receptor activity	F	28	193	1106	14.50777	2.61	0.013
5125	cytokine activity	F	5	21	162	23.80952	2.449	0.018
6338	chromatin remodeling	P	3	14	37	21.42857	2.326	0.042
7156	homophilic cell adhesion	P	7	37	122	18.91892	2.058	0.038
7166	cell surface receptor-linked signal transduction	P	3	13	140	23.07692	2.777	0.012
7186	G-protein coupled receptor protein signaling pathway	P	49	355	1776	13.80282	2.291	0.024
7608	sensory perception of smell	P	27	192	1095	14.0625	2.385	0.025
9968	negative regulation of signal transduction	P	1	15	34	6.666667	2.378	0.019
16503	pheromone receptor activity	F	6	25	150	24	2.572	0.022
42742	defense response to bacterium	P	5	12	69	41.66667	3.442	0.006
50896	response to stimulus	P	28	207	1194	13.52657	2.735	0.006
B. GO terms downregulated in malarial brain								
16791	phosphoric monoester hydrolase activity	F	1	18	12	5.555555	2.388	0.028
5925	focal adhesion	C	3	14	30	21.42857	3.437	0.009
30141	secretory granule	C	3	15	32	20	2.503	0.032
16323	basolateral plasma membrane	C	2	18	32	11.11111	3.157	0.01
3746	translation elongation factor activity	F	3	14	32	21.42857	3.437	0.016
16820	“hydrolase activity\, acting on acid anhydrides\, catalyzing transmembrane movement of substances”	F	2	13	33	15.38461	0.805	0.455
6333	chromatin assembly or disassembly	P	2	16	37	12.5	4.666	0
7179	transforming growth factor beta receptor signaling pathway	P	1	10	41	10	1.525	0.185

A. GO terms upregulated in malarial brain

GOID	GO name	Ttype	Number changed local	Number measured local	Number in GO local	Percent changed local	Z Score	Permuted P
151	ubiquitin ligase complex	C	1	15	45	6.666667	1.441	0.155
6874	calcium ion homeostasis	P	3	18	47	16.66667	2.892	0.03
43066	negative regulation of apoptosis	P	3	23	57	13.04348	2.753	0.014
785	chromatin	C	2	23	95	8.695652	2.797	0.015
3682	chromatin binding	F	5	48	110	10.41667	2.286	0.041
786	nucleosome	C	4	29	115	13.7931	2.805	0.019
42981	regulation of apoptosis	P	1	33	116	3.030303	1.601	0.139
4721	phosphoprotein phosphatase activity	F	3	12	120	25	2.601	0.025
7001	chromosome organization and biogenesis (sensu Eukaryota)	P	4	29	124	13.7931	3.221	0.001
17111	nucleoside-triphosphatase activity	F	1	31	125	3.225806	2.344	0.019
6334	nucleosome assembly	P	6	36	125	16.66667	4.029	0.001
5694	chromosome	C	4	58	166	6.896552	2.148	0.034
6629	lipid metabolic process	P	5	66	217	7.575758	3.019	0.005
74	regulation of progression through cell cycle	P	2	43	248	4.651163	2.346	0.028
6412	translation	P	2	67	459	2.985075	2.199	0.036
5488	binding	F	4	107	541	3.738318	3.217	0.003
3723	RNA binding	F	10	138	544	7.246377	2.481	0.017
5739	mitochondrion	C	18	276	813	6.521739	2.455	0.029
16787	hydrolase activity	F	24	315	1273	7.619048	2.344	0.019
3676	nucleic acid binding	F	11	245	1299	4.489796	2.664	0.008
5737	cytoplasm	C	21	501	1495	4.191617	2.591	0.01
3677	DNA binding	F	18	348	1725	5.172414	1.697	0.074
5622	intracellular	C	22	567	1962	3.88007	2.935	0.008
46872	metal ion binding	F	29	638	2173	4.545455	2.567	0.012
5634	nucleus	C	67	1268	3680	5.283912	2.993	0.003
5515	protein binding	F	84	1678	4425	5.00596	2.809	0.006

Number changed local = the number of genes altered in pathway; number measured local = number of genes in pathway with measurable signal; number in GO local = total number of genes in GO pathway.

Table 2

Neurological diseases corresponding to genes altered in malarial brain.

GB_Acc	NAME	SYMBOL	I/C	P-I/C	Neurological consequence of ablation/mutation
A. Channels receptors transporters and synapse components					
NM_010298	glycine receptor, beta subunit	Glrb	1.9	0.023	Hyperekplexia, autosomal recessive
NM_011322	sodium channel, voltage-gated, type I, beta	Scn1b	2.0	0.018	Generalized epilepsy with febrile seizures; regulates Na channel density and localization
NM_146123	calcium channel, voltage-dependent, beta 4 subunit	Cacnb4	2.2	0.033	Mouse “lethargic”: ataxic, first example of neurological disease in accessory subunit
NM_013879	calcium binding protein 1	Cabp1	−2.5	0.048	Neuronal signal transduction and memory
NM_009597	amiloride-sensitive cation channel 2, neuronal	Accn2	1.8	0.032	Enhanced fear conditioning; deletion protective in EAE
NM_015728	solute carrier family 33 (acetyl-coa transporter), member 1	Slc33a1	1.8	0.004	Spastic paraplegia42, autsomal dominant
NM_011696	voltage-dependent anion channel 3	Vdac3	1.5	0.017	Mitochondrial disease
XM_344168	vesicle-associated membrane protein 4 (predicted)	Vamp4	2.3	0.009	Vesicle component
B. Genes involved in synapse or other intercellular contact					
NM_017476	A kinase (PRKA) anchor protein 8-like	Akap8l	−3.4	0.042	Binds huntingtin in Huntington disease
NM_007458	Adaptor protein complex AP-2, alpha 1 subunit	Ap2a1	2.1	0.016	Huntingtin protein binding; vesicle trafficking
NM_194462	A kinase (PRKA) anchor protein (yotiao) 9	Akap9	2.1	0.011	Long QT syndrome 11 [kcnq1, ser1570leu]
NM_011780	A disintegrin and metalloprotease domain 23	Adam23	2.0	0.012	Brain specific
NM_023348	Synaptosomal-associated protein	Snap29	1.8	0.015	Cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma syndrome
NM_134089	Scribbled homolog (Drosophila)	Scrib	1.7	0.045	Craniorachischisis, a severe neural tube defect
XM_139187	Protocadherin 9	Pcdh9	2.2	0.015	Possibly autism
NM_172932	Neuroligin 3	Nlgn3	2.3	0.041	Ligand for neurexin: linked to autism, Asperger syndrome
NM_007767	Protocadherin alpha 6	Pcdha6	2.1	0.024	Synaptic junction protein
NM_020253	Neurexin II	Nrxn2	−2.4	0.001	Organize presynaptic terminals by functionally coupling Ca channels to presynaptic machinery
NM_153508	Calsyntenin 3	Clstn3	−2.8	0.006	Postsynaptic protein
NM_016782	Contactin associated protein 1	Cntnap1	−2.9	0.010	Essential for formation of axonal septate junctions
NM_182929	Regulating synaptic membrane exocytosis 3	Rims3	2.3	0.005	Enhances neurotransmitter secretion
NM_019749	Gamma-aminobutyric acid receptor-associated protein	Gabarap	1.9	0.030	Autophagy; binding GABA-A receptors
NM_207670	GRIP1-associated protein 1	Gripap1	1.8	0.038	Regulates neuronal Ras signaling and contributes to the regulation of AMPA receptor distribution

GB_Acc	NAME	SYMBOL	I/C	P-I/C	Neurological consequence of ablation/mutation
NM_010587	Intersectin 1 (SH3 domain protein 1A)	Itsn1	2.0	0.029	Dendritic spine morphogenesis
C. Mitochondrial genes associated with neurological disorders					
AK181541	Transmembrane protein 70	Tmem70	2.1	0.038	Encephalomyopathy and mitochondrial DNA depletion syndrome
NM_025862	Acyl-Coenzyme A dehydrogenase family, member 8	Acad8	1.8	0.031	Isobutyryl-coa dehydrogenase deficiency
NM_011506	Succinate-Coenzyme A ligase, ADP-forming, beta subunit	Sucla2	2.7	0.032	Encephalomyopathy and mitochondrial DNA depletion syndrome
D. Ataxia and cerebellar function and development					
NM_145358	Calcium/calmodulin-dependent protein kinase kinase 2, beta	Camkk2	−3.1	0.003	Mouse null impaired spatial memory formation; altered cerebellar granule cell development
NM_021477	Ataxin 2 binding protein 1, transcript variant 2	A2bp1	2.7	0.045	Spinocerebellar ataxia 2
NM_033526	Ubiquilin 4	Ubqln4	2.7	0.009	Spinocerebellar ataxia 1
NM_007597	Calnexin	Canx	2.6	0.019	Unstable gait, truncal ataxia, abnormal relexes; loss of large nerve fibers
NM_011179	Prosaposin	Psap	1.9	0.047	Loss of cerebellar purkinje cells due to ceremide buildup
D26114	CCG1, complete cds	CCG1, complete cds	1.6	0.029	Neuropathy syndrome; spinocerebellar ataxia-17
E. Genes associated with autism					
NM_011814	Fragile X mental retardation gene 2, autosomal homolog	Fxr2h	2.0	0.028	Mental retardation
NM_054097	Phosphatidylinositol-4-phosphate 5-kinase, type II, gamma	Pip5k2c	1.9	0.025	Mental retardation
F. Genes associated with Alzheimer's disease					
NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1	Apbb1	1.6	0.031	Dementia of the Alzheimer's type
G. Other					
NM_009053	Radical fringe gene homolog (Drosophila)	Rfng	2.0	0.022	Notch signaling in cell fate determination in neurogenesis
NM_010487	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C)	Elavl3	1.8	0.018	Paraneoplastic neurologic disorder due to autoimmune neuronal destruction

GB_Acc = gene bank accession number; I/C = Fold change in gene expression of infected/control; P-I/C = p value.