

Transcriptional Changes in Insulin- and Lipid Metabolism-Related Genes in the Hippocampus of Olfactory Bulbectomized Mice

Peter Gass,^{1*} Fernando Leonardi-Essmann,² Maha Zueger,¹ Rainer Spanagel,² and Peter J. Gebicke-Haerter²

¹RG Behavioral Biology, University of Heidelberg, Mannheim, Germany

²Department of Psychopharmacology Central Institute of Mental Health, University of Heidelberg, Mannheim, Germany

Affymetrix chips were used to perform a hypothesis-free large-scale screening of transcripts in the hippocampus of olfactory bulbectomized mice, an established animal model of depression. Because only 11 transcripts were significantly changed, the statistically subsequent 25 transcripts below the significance level were additionally included in a first round of qRT-PCR evaluations. Furthermore, all 36 genes were then tested for mutual interactions or interactions with other molecules in a physiological context using PathwayArchitect software. Thirty of them were displayed in a network interacting with at least one partner molecule from the list or with other partner molecules known from the literature. All partner molecules from the most prominent 10 molecules of this network were then identified and put together into a new list. On those grounds, the hypothesis was made that metabolic network components of the insulin signaling pathway are perturbed in the disease. This pathway was subsequently tested by a second round of qRT-PCR, adding also a few additional candidate molecules belonging to this pathway. It turned out that the key target—FABP7—fell into the group of transcripts not significantly regulated within the chip data, and another key target—IRS1—did not show up in the chip experiments at all. In conclusion, our data reveal a problem with adhering to statistical significances in microarray experiments, insofar as molecules important for the disease may fall into the range of statistical noise. This approach may also be useful to find new targets for pharmacotherapy in affective disorders. © 2008 Wiley-Liss, Inc.

Key words: olfactory bulbectomy; affective disorders; insulin signalling; fatty acid binding protein; molecular networks

Animal models for psychiatric disorders represent valuable tools for studying molecular changes in tissues from specific brain regions, something that usually cannot be done in patients (Nestler et al., 2002; Licinio

and Wong, 2004). A robust depression model in mice and rats is the bilateral destruction of the olfactory bulbs, which creates a chronically altered brain state with complex changes of behavioral, neurochemical, neuroendocrinological, and neuroimmunological parameters, many of which are comparable to those seen in patients with major depression (Willner, 1990; Harkin et al., 2003; Cryan and Holmes, 2005; Song and Leonard, 2005). Thus, the olfactory bulbectomy in rodents has been proposed to represent a model for chronic psychomotor agitated depression, which also has a high predictive validity (Lumia et al., 1992; Kelly et al., 1997; Harkin et al., 2003). The major behavioral change in this model is a hyperactive response in a brightly illuminated open field arena, which is reversed almost exclusively by chronic, but not acute, antidepressant treatment (Leonard and Tuite, 1981; Jesberger and Richardson, 1986; Kelly et al., 1997; van Riezen and Leonard, 1999). Furthermore, olfactory bulbectomy leads to different signs of anhedonia, combined with deficits in spatial learning, avoidance learning, conditioned taste aversion, and food-motivated behaviors (Kelly et al., 1997; Harkin et al., 2003; Song and Leonard, 1994, 1995, 2005).

Structural consequences of this model apart from the artificial removal of the olfactory bulbs and reminiscent of the human disease are changes in the limbic system, in particular amygdala and hippocampus (Carlsen et al., 1982; Duman et al., 1997). Similar to neuroimag-

Contract grant sponsor: Deutsche Forschungsgemeinschaft (to P.G.); Contract grant number: SFB-636/B3 (to P.G.); Contract grant number: GA-427/9-1 (to P.G.).

*Correspondence to: Peter Gass, MD, Central Institute of Mental Health (CIMH), University of Heidelberg, J5, D-68159 Mannheim, Germany. E-mail: peter.gass@zi-mannheim.de

Received 11 January 2008; Revised 10 March 2008; Accepted 13 March 2008

Published online 9 June 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21753

ing findings in depressed patients, a decrease in hippocampal volume and enlargements of lateral and third ventricles have been reported after olfactory bulbectomy in rodents, most likely resulting from reduced numbers of synapses and dendritic spines in CA1 and CA3 regions of hippocampus (Wrynn et al., 2000; Song and Leonard, 2005). Recently, a decrease in hippocampal neurogenesis—another cellular process that has been associated with pathogenesis and therapy of depression—has also been described as a consequence of olfactory bulbectomy (Jaako-Movits and Zharkovsky, 2005; Jaako-Movits et al., 2006).

So far, only very little is known about the molecular mechanisms underlying the changes of hippocampal plasticity induced by olfactory bulbectomy (Hellweg et al., 2007). Therefore, we wanted to study in a hypothesis-free approach potential changes in hippocampal gene expression. We have used Affymetrix chips to reveal changes in transcriptional activity in the hippocampus of bulbectomized mice at 3 weeks postsurgery, a time when the typical depression-like syndrome was fully developed.

MATERIALS AND METHODS

Animal Experiments

Olfactory bulbectomy was essentially performed as described earlier for adult male 10–12-week-old C57BL/6 mice (purchased from Charles River, Sulzfeld, Germany; Zueger et al., 2005). Prior to the operation, animals were allowed to acclimatize for 2 weeks to a reversed 12 hr dark/light cycle and housed individually with access to food and water ad libitum.

Olfactory bulbectomy was carried out under anesthesia with xylazine (80 mg/kg bw, i.p.; Bayer, Leverkusen, Germany) and ketamine (90 mg/kg bw, i.p.; Aventis, Bad Soden, Germany). Olfactory bulbs were removed bilaterally by suction with a hypodermic needle. Sham operations were performed leaving the bulbs intact. At day 21 after surgery, locomotor activity was analyzed in the open field test as described elsewhere (Kelly and Leonard, 1994; Zueger et al., 2005). Only bulbectomized mice with significant hyperlocomotion, the key behavioral feature of olfactory bulbectomy, were used for transcriptional analyses. Moreover, only male mice were used to avoid compounding factors resulting from the more complex hormonal status of females. At day 23 after surgery, mice were decapitated and the hippocampi were dissected, frozen, and stored at -80°C until further use. Hippocampi from six bulbectomized mice and six sham-operated controls were processed for chip experiments. All animal experiments were in accordance with the regulations issued by the German animal welfare office, Regierungspräsidium Karlsruhe, Germany.

Total RNA Isolation and Quality Control

Tissue was lysed in Trizol reagent (Invitrogen, Karlsruhe, Germany) and homogenized by passing the suspension 30 times through a 22-gauge needle. Total RNA was extracted by adding chloroform. To achieve better separation

of organic and aqueous phases, Phase Lock Gel Heavy tubes (Eppendorf, Hamburg, Germany) were used. Upper phases were carefully removed by pipetting, and total RNA was purified by using a RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA quality was evaluated by optical density (OD) measurements (260 nm/280 nm) in a GeneQuant (Pharmacia, Freiburg, Germany) in 10 mM Tris-HCl, pH 7.6, and its integrity was determined by measuring ribosomal 28S/18S ratios using RNA 6000 Nano Assay RNA chips run in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Ratios of 1.9–2.2 (optical density 260/280) and >1.6 (28S/18S rRNA) as well as an absence of a peak of DNA contamination in electropherograms at 29S were chosen as inclusion criteria.

Target Labeling, Murine Genome MG-U74Av2 GeneChip Hybridization, Scanning, and Quality Control

Double-stranded (ds) cDNA synthesis and in vitro transcription (IVT) of ds cDNA into cRNA and GeneChip MG-U74Av2 (Affymetrix, Santa Clara, CA) hybridizations were carried out according to the manufacturer's protocol. Briefly, 5 μg total RNA was reverse transcribed using T7-oligo(dT)24 primer (GeneSet Oligos, Evry, France) and SuperScriptII Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Second-strand cDNA was synthesized by adding DNA polymerase I (*Escherichia coli*), ribonuclease H, *E. coli* DNA ligase, and T4 DNA polymerase. After ds cDNA precipitation and resuspension, IVT was performed using the BioArray HighYield RNA Transcript Labeling Kit (Enzo, Farmingdale, NY). Biotin-labeled cRNA was purified with RNeasy Mini Kit (Qiagen) and fragmented with RNA fragmentation buffer. IVT and cRNA fragmentation quality controls were carried out by running an mRNA Nano assay in the Agilent 2100 Bioanalyzer. cRNA electropherograms showed a single, broad peak beginning at 22 sec and ending at 74 sec. Fragmented cRNAs were resolved in a single peak starting at 19 sec and declining at 26 sec. GeneChip MG-U74Av2 arrays were filled with hybridization cocktails containing 10 μg fragmented cRNAs (after correction for total RNA carryover). After 16 hr of hybridization in a GeneChip Hybridization Oven (Affymetrix), chips were stained with streptavidin/R-phycoerythrin conjugate (Molecular Probes, Eugene, OR) in a GeneChip Fluidics Station (Affymetrix) and immediately scanned in a GeneArray Scanner (Agilent Technologies). Chips were checked for performance using five evaluation criteria: 1) comparable scaling factors among the chips; 2) single chip average background <100 and similar among the chips; 3) signal (3'/5') of rat housekeeping genes GAPDH, hexokinase, and β -actin <2 ; 4) increasing signals of hybridization controls within a chip and similar intensities for each hybridization control among the chips; and 5) visual assessment of scanned image (DAT files) and grid alignment.

Data Mining

Micro Array Suite 5.0 (Affymetrix)-derived cell intensity files (CEL) were processed in the R 2.1.1 language and envi-

TABLE I. Differential Gene Expression Profiling by Affymetrix Chips With Fold Changes and P Values and Reevaluation by qRT-PCR (Right Column, Arrows Indicate Up- or Down Regulation, Respectively)

Probe set (Affymetrix)	Gene name	# Fold change (array)	P value (array)	Up/down (qRT-PCR)
160714_at	Gab 1	1.19	0.000222	↑
160911_at	SOS 1	1.16	0.006021	±
94947_9_at	Map3K3(MEK3)	-1.22	0.006542	↓
94391_at	Gjp6	1.18	0.007205	↑
97017_f_at	SPARC	1.20	0.008493	↑
101753_s_at	Lzp-s	1.15	0.010434	↑
104034_at	Shyc	1.09	0.014072	↑
160941_at	cnPD8a	1.13	0.014456	↓
97381_s_at	Tcp11	1.08	0.024196	↑
161231_r_at	Reelin	-1.21	0.031748	±
162152_r_at	CRHR1	-1.23	0.047832	↑
161923_at	Psmb1	-1.22	0.080042	↑
160614_at	Plen	-1.35	0.096322	±
100526_f_at	Adam3	-1.48	0.098158	↓
102752_at	Shyc	-1.28	0.101713	↑
161832_r_at	Csf1R	-1.31	0.106826	↓
97759_at	Kcnma1	-1.39	0.109817	↑
94689_at	simPAFHyr	-1.26	0.116811	↓
98967_at	Fabp7	-1.21	0.117498	↓
161614_r_at	Tubb2	-1.22	0.119298	↓
101787_f_at	intracistApart	-1.25	0.124835	↓
98477_s_at	Ankyrin3	-1.24	0.126957	±
100947_at	Tcf20	-1.69	0.142622	↓
95677_at	Prp8bind	-1.2	0.152002	↑
98122_at	Lmo4	-1.22	0.157812	±
103546_at	Fosl2	-1.25	0.157845	↑
161408_r_at	dishev2	-1.29	0.179596	±
92945_at	GluRB (AMPA)	-1.35	0.192738	±
104205_at	Aggrecan1(AGC1)	-1.24	0.245638	±
98909_at	LipAcSynth	-1.25	0.258675	↓
92906_at	EphrinR	-1.22	0.260715	±
98096_f_at	VPSA4	-1.2	0.26604	±
97770_s_at	LAMB-hom.	-1.23	0.275752	↓
94781_at	Hba-a1	-1.3	0.37706	±
102431_at	Mapt	-1.22	0.386916	↓
104169_at	Zic1	-1.2	0.400869	↑

ronment (R Development Core Team, 2005) by using Bioconductor 1.6 (Gentleman et al., 2004) packages. Among them, package Affy 1.6.7 (Irizarry et al., 2005) for chip performance quality control and for robust-multichip-average (RMA) global normalization was used. Genes differentially expressed between olfactory bulbectomized and sham-operated groups were identified by the following series of filtering steps: 1) a probability of differential expression of $P < 0.05$ from a t -test, 2) a 100% of “present” call in at least one group, and 3) a -fold change (FC) in gene expression $-1.3 > FC > 1.3$. No differentially expressed genes were identified by Bonferroni correction.

Two lists of genes were obtained with up- or down-regulated genes, respectively. At the threshold of statistical significance there remained 11 genes, the majority of which were up-regulated. At this point, it was decided to include more genes of those that did not reach the $P < 0.05$ significance level. The new cutoff was arbitrarily set to 36 genes, which corresponded to a confidence level close to 60% (see Table I and Results).

Quantification by Real-Time Polymerase Chain Reaction

Relative quantification by real-time polymerase chain reaction (qRT-PCR) was carried out for confirmation of the 36 differentially expressed genes found in hippocampal formation based on our gene expression profiling. qRT-PCR was run in a total reaction volume of 20 μ l using Power SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) in an ABI 7900 HT RT-PCR System (Applied Biosystems, Foster City, CA). β -Actin cDNA was used as an internal standard, because it showed stable expression across samples. Thermal profile was 95°C for 3 min, followed by 40 cycles of 15 sec at 95°C and 40 sec at 60°C. qRT-PCR data were imported into Statistica 6.1 (StatSoft, Inc., Tulsa, OK). Relative quantification was done according to the $\Delta\Delta C_t$ method. Differences in transcript abundance between bulbectomized (“ob”) and control (“sham”) groups were analyzed using a t -test for independent samples and were considered statistically significant at $P < 0.05$.

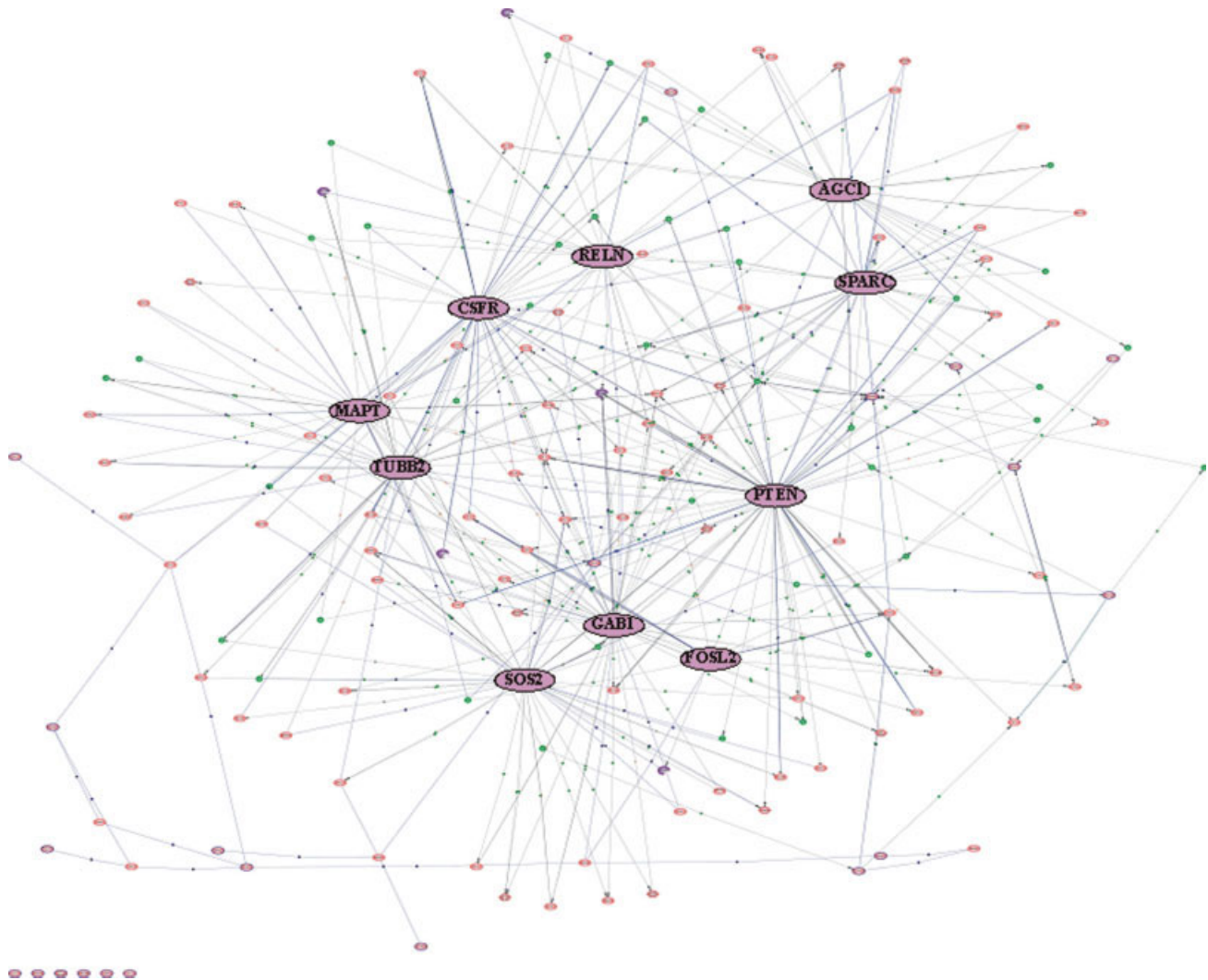


Fig. 1. Results from Stratagene's PathwayArchitect after evaluation of the transcripts from Table I. The 10 most prominent were checked for each of their interactions (with themselves and with any other molecule known from the literature), which gave rise to Table II.

sitol-3 kinases or -phosphatases (PIK3, INPP5D, PtdIns) are additional nodal components; 3) growth factors or their (kinase-coupled) receptors (PDGF, TGF- β , EGFR, IGF1, IGF1) are frequent nodal components; and 4) in terms of IGFs, insulin-related components (INS1, IRS1, IRS2, glucose, GAB1, SOS) often occur, too. Taken together, most of those genes can seamlessly be related to the insulin-signaling pathway consisting of: INS1, INS2, INSR, IRS1, IRS2, AKT1, GRB2, IGF1, IGF2, IGF1R, IGF2R, GSK3-beta, GYS1, PIK3cb, PIK3r1, SHC1, SOS1, and others. This led us to hypothesize that insulin-related molecular subnetworks may be affected in the hippocampus during the depressive-like syndrome evoked by olfactory bulbectomy.

Analysis of the Insulin Signaling Pathway

Some of the reaction partners in the insulin signaling pathway, such as AKT1, INSR, IRS1 and -2, PI3K

p110 and p85, and GSK3, had not been detected in chip experiments. They were evaluated along with a second round of qRT-PCR of the transcripts from Table I.

As expected, the majority were not significantly regulated. The trends below the significance level, however, could be largely confirmed by qRT-PCR (arrows in Table I). With respect to normalization to reference genes in qRT-PCR (β -actin, GAPDH, cyclophilin, or just a *t*-test without reference gene), it turned out that FABP7 was consistently and significantly down-regulated upon this evaluation (No. 22 in the rank order in chip experiments with a *P* value of 0.1175) in each test. Furthermore, IRS1, IRS2, PI3K p85 and p110 subunits, PAF-hydroxylase-related protein, and MEK3 reached significance level in at least two of those tests or were very close to significance (Table III). The M values for the three standards, as calculated in geNorm software (Vandesompele et al., 2002), showed only little variation,

TABLE III. QRT-PCR Results From Transcripts Selected Specifically with Respect to the Insulin Hypothesis*

Transcript	<i>P</i> value C_T	Up/down regulation	<i>P</i> value ΔC_T to β -actin	Up/down regulation	<i>P</i> value ΔC_T to cyclophilin	Up/down regulation	<i>P</i> value ΔC_T to Gapdh	Up/down regulation
FABP7	0.0301	↓	0.0031	↓	0.0036	↓	0.0037	↓
IRS1	0.0074	↑	0.0410	↓	0.1271	↓	0.2805	↓
IRS2	0.0054	↑	0.6931	-	0.5516	↑	0.2306	↑
PI3Kcat	0.0602	↑	0.1047	↓	0.2662	↓	0.3125	↓
PI3Kp85	0.0179	↑	0.0496	↓	0.0571	↓	0.0643	↓
GSK3B	0.5330	-	0.4188	-	0.4628	↑	0.8603	-
LPA _{synth}	0.4335	↓	0.8026	-	0.8756	-	0.5021	↓
Pten	0.7174	-	0.4725	-	0.5871	↑	0.9161	-
REEL	0.9701	-	0.2886	↑	0.4125	↑	0.6177	-
Tubb2	0.2248	↓	0.4165	↓	0.3581	↓	0.1694	↓
GluRB	0.2250	↓	0.6679	-	0.7424	-	0.1345	↓
MAPT	0.1841	↓	0.7415	-	0.5913	-	0.0848	↓
MEK3	0.1678	↓	0.4960	↓	0.5293	-	0.0320	↓
cnPDE8a	0.1345	↓	0.6674	-	0.0915	↑	0.9400	-
PAFH _{hydr}	0.0391	↓	0.5078	↓	0.8285	-	0.0318	↓
AKT1	0.4395	↓	0.1740	↑	0.1173	↑	0.2953	↑
INSR	0.7611	↓	0.7429	↑	0.6896	↑	0.8949	-
SOS1	0.6166	↓	0.4518	↑	0.4052	↑	0.7420	-

*Three “housekeeping” transcripts were chosen as references (Vandesompele et al., 2002), and also *t*-tests were carried out without reference (left column). Numbers in boldface, *P* < 0.05; numbers in italic, confidence intervals of approx. 88 %, ? questionable. M values for β -actin, 0.036; cyclophilin, 0.026; for Gapdh, 0.027.

with cyclophilin (0.026) and GAPDH (0.027) almost identical and β -actin (0.036) scoring worse. These results strongly suggest disturbances in the insulin-signaling pathway via lipid metabolism rather than directed to glucose turnover.

DISCUSSION

Methodological Approach

The present analytical approach adds a new facet to interpretations of biological data sets obtained from expression profiling studies. It suggests that many disease-related molecular changes in biological systems often do not fall in the range of statistical significance. Although there appears to be an overall agreement that changes in biological systems on the transcript or protein level do not necessarily adhere to statistical thresholds, it is a generally held view that only statistically significant results count in successful publications. This convention is challenged in this report, because the authors do not see good reasons why an 85% or 91% confidence interval should not be sufficiently justified to search for a biologically meaningful hypothesis of molecular perturbations in a given disease. Admittedly, in doing so, occurrence of false-positive results increases, but this drawback may be overcome by qRT-PCR testing.

The extent to which any given mRNA is translated into protein depends on a variety of factors. The rate of transcription and the rate of degradation are certainly important control mechanisms. The latter highly depends on the stability of the mRNA. When mRNA stability is low, a low production of gene product may be expected despite high rates of transcription. Other

possibilities are 1:1 translations or higher than 1:1 translation rates into protein with increasing longevity of mRNAs. This would mean that, especially with short-lived mRNAs, transcriptional changes (e.g., in disease) would be more easily detected at the statistical significance level of 95%. Very likely, those mRNAs also encode proteins with functions distinct from proteins encoded by more stable mRNAs. This would mean that, by adherence to the generally accepted confidence interval of at least 95%, a “technical” bias is introduced into the data evaluation process. Especially long-lived transcripts may not change in large quantities in disease, but their more subtle changes might be also important. Those changes, however, may easily fail to be revealed during data mining. Because of mRNA stability, those little changes may be grasped on the protein level rather than on the transcript level if based on a translational ratio of far more than 1:1 (Fig. 2). Taken together, this means that changes in the transcript level in disease likely translate into protein. However, the extent of this translation does not depend on the quantities of mRNAs determined in microarray or qRT-PCR examinations. The ratio of amount of transcript to amount of translated protein is unpredictable and, hence, can be assessed only by direct measurements of each single mRNA species and its translation product. Nevertheless, changes in the transcript level can be used as qualitative indications of changes in the protein level. In terms of confidence intervals, there appears to be no reason not to lower them below 95% when evaluating chip data.

Two important additional aspects have to be discussed at this point. Typically, data collection on the transcript or protein level is restricted to the instant

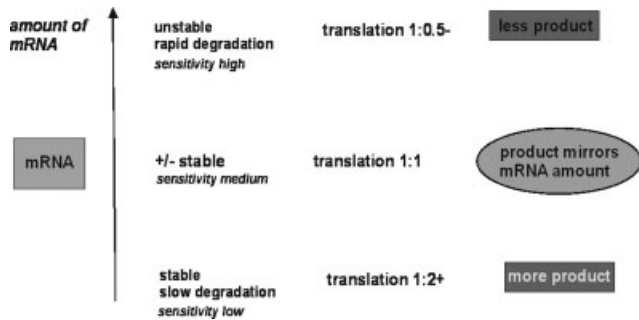


Fig. 2. Expression profilings on the mRNA level as performed here harbor a number of problems. A major one likely resides in differential stabilities of mRNAs and subsequent differential translations. On the transcript level (arrow) and with restriction to the statistical significance level of $P < 0.05$, one may detect transcripts with lower stability but that are more abundant. Up- or down-regulations on the level of five- or tenfold possibly do not translate into comparable amounts of protein. This technical bias requires reevaluation on the protein level for each identified transcript.

when the tissue is disrupted for molecular analyses. It is well known, however, that stability of any given RNA or protein modifications or degradation can change within short time intervals; i.e., there is always ongoing disease progression, pause, or remission. The molecular signatures obtained by expression profiling can only reflect the individual status of the tissue under investigation. This means that time course studies would greatly aid in elaborating better flow charts for expression changes of individual molecules at distinct disease states. The second aspect is the problem of regional specializations in brain. Expression patterns in other brain regions may be distinct from hippocampal patterns. Moreover, subregions of hippocampus, such as dentate gyrus, or pyramidal neurons as opposed to the pooled collection of all cell types may display distinct gene expression. In this study, both hippocampi have even been pooled to obtain sufficient material for all experiments. This measure may have introduced an additional compounding element into our statistical evaluations.

Moreover, it is obvious that only some of the genes from the list belong to the insulin-signaling pathway(s). This reveals a general problem with gene lists from expression profiling studies: often the gene products belong to distinct molecular networks, which are hard to identify by mere research experience or “educated guesses.” In this situation and with respect to the dynamic behavior of a disturbed system, only powerful computational software can help to develop clearer pictures of disease-related molecular events. It could step backward and reveal the true “culprits” hidden in statistical noise that give rise to the expression changes of others observed within statistical significance levels. This situation emphasizes the point that targets showing up at statistical significance often may not be the targets useful for drug therapy.

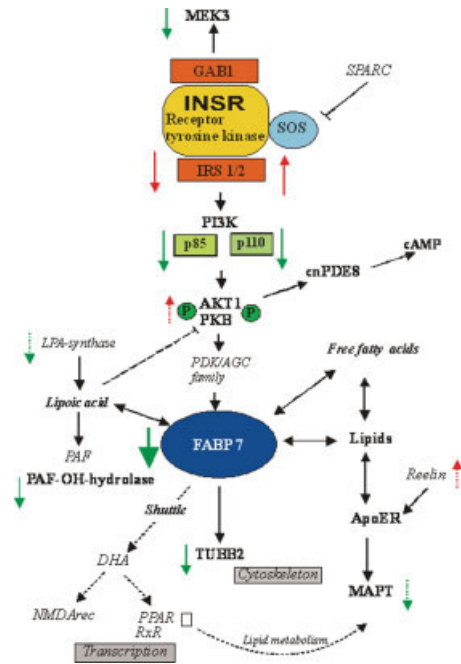


Fig. 3. Molecular network affected in our animal model of depression. Expression changes identified by hypothesis-free, genome-wide screenings gave rise to the hypothesis that insulin-related network components are associated with the disease. This hypothesis has been further supported by qRT-PCR measurements (red/green arrows: well supported; dotted red/green arrows: tendency).

Currently, programs are not available that are able to group a number of genes that show changed expression in a disease into a two- or three-dimensional image of molecular networks. Such graphic representations would greatly help the researcher to understand better disease-specific events and to develop better hypotheses. On the other hand, computational simulations heavily rely on the information available on each component of the network under investigation, which can be provided only by laboratory research.

Fatty Acid Binding Protein 7 (Brain Type)

Nine tissue-specific FABPs have been identified (Chmurzynska, 2006). FABPs serve as carriers for long-chain fatty acids such as docosahexaenoic acid (DHA) and arachidonic acid, but also cholesterol, across lipid membrane layers (Owada et al., 2006). Those long-chain fatty acids have been reported to be pivotal for brain development and important for spatial learning, memory, and emotional responses. Brain FABP (or FABP7), which was first described by Bennett et al. (1994), is strongly expressed in radial glia and immature astrocytes during development. In adult brain, substantial expression has been observed in olfactory ensheathing cells, in cerebellar Bergman glia, and in hippocampal radial glia (Kurtz et al., 1994). Additionally, FABP7 expression has been shown in astrocytes of the amygdala, septum, and

medial habenula. Its expression seems to be primarily regulated at the transcriptional level (Pelsers and Glatz, 2005). Apart from its function in fatty acid shuttling, it appears to bind to and activate transcription factors, to modify mRNA stability, and to influence transcription factor expression. The PPAR family of transcription factors, in particular, which control transcription of various genes involved in lipid metabolism, has been reported to cooperate with FABPs (Chmurzynska, 2006). FABP has also been shown to interact closely with the endogenous inhibitor [diazepam-binding inhibitor (DBI) or endozepine] of the peripheral benzodiazepine receptor. This receptor controls the entry of lipids into mitochondria and the cholesterol, pregnenolone, steroid synthetic pathway (Gavish et al., 1999). FABP's own expression has been found to be regulated by the transcription factor Pax6 (Arai et al., 2005) and by the Notch effector CBF1 (Anthony et al., 2005). FABP7 knockout mice develop normally but display enhanced anxiety and increased fear memory. Furthermore, isolated neurons from those animals showed reduced responses of DHA-stimulated N-methyl-D-aspartate (NMDA) receptors (Owada et al., 2006). Interestingly, FABP7 is also inserted in the reelin/apolipoprotein E receptor signaling pathway. Reeler mice display reduced levels of FABP7 in cerebral cortex, and reelin addition restores FABP7 (Hartfuss et al., 2003). FABP7 has also been shown in an Affymetrix gene chip study to be rhythmically expressed, being elevated during sleep phases (at ZT 6) in the SCN, in the ventrolateral preoptic area (VLPO), and in the lateral hypothalamus (LH; Gerstner et al., 2006). All in all, this is evidence for a role of FABP7 in features typically observed in affective disorders. However, so far, FABP7 had not been related to the pathogenesis of depressive disorders in humans nor to animal models of depression. Future studies will have to show in which cell types in the hippocampus this protein is down-regulated following bulbectomy. A direct functional role of FABP7 for the behavioral alterations observed in the bulbectomy model, such as hyperactivity or cognitive changes, could be demonstrated by investigating FABP7 mutant mice in behavioral tests. A functional role of FABP7 in depressive disorders would be further supported if one could identify a dysregulation of this gene/protein in other depression models. Finally, it is an interesting question whether FABP7 expression can be modulated by antidepressive treatments.

IRS and Insulin Receptor Scaffolding Proteins

The fine tuning of receptor activation largely depends on scaffolding proteins. For the NMDA receptor, there are presently 186 scaffolding proteins known to be assembled in the NRC/MASC complex (Pocklington et al., 2006). Much less is known about the insulin receptor in this respect. Insulin signaling has been investigated preferentially in view of glucose metabolism. Its influence on lipid metabolism has somewhat been neglected.

The adaptor proteins GAB1 and SOS1 have been indicative from the list of gene chip transcripts toward growth factor-insulin receptor scaffolding proteins. GAB1 is one of the initial signaling components of the MAPK pathway (Meng et al., 2005). Another insulin receptor- β -associated adaptor protein is PSM-1. It binds directly to and is activated by the phosphorylated kinase domain of the receptor (Nelms et al., 1999). Those molecules are listed in Table I. Presumably, these kinds of proteins can undergo larger changes of expression and, hence, reach statistical significance, because those changes may have less dramatic effects than comparable changes with other types of molecules. Those cues toward adaptor proteins of the insulin receptor have led to the inclusion of IRS1 and IRS2 as additional targets, which could be substantiated in qRT-PCR. Indeed, more recent reports revealed pivotal functions of those adaptor proteins in insulin receptor signaling. Apparently, both cooperate to maintain total PI3-kinase activity by mutual compensation (Fig. 3). Reduction of IRS1 resulted in increased expression of gluconeogenic enzymes, whereas reduced IRS2 increased MAPK-induced SREB(F1) expression. This has led to the notion that IRS1 is more closely linked to glucose and IRS2 more to lipid metabolism (Taniguchi et al., 2005). The present findings show increased expression of IRS2 and reduced expression of IRS1. This would be an indication of increased activity of the glucose metabolic pathway and reduction of the lipid metabolic pathway. For a clear key molecule in the latter, and as a confirmation of this notion, we observed a down-regulation of FABP7. Along those lines, there should also be changes in glucose-related signaling. AKT1 might be not a very suitable target, insofar as it is located exactly at another branching point between lipid- and glucose-related signaling. By contrast, GSK3 β may be a better target. Unfortunately, we did not observe any substantial changes in the transcript level (see above under Methodological Approach). It may, however, be rewarding to check this target molecule on the protein level. In summary, IRS1 and IRS2 constitute important branching points of insulin signaling at the insulin receptor. In keeping with this, IRS2-induced signaling has also been shown to be neuroprotective (Schubert et al., 2003). By contrast, ethanol impaired insulin-mediated actions in the developing brain (de la Monte et al., 2005); impaired insulin-stimulated neuronal survival (Xu et al., 2003); and reduced the levels of PI3-kinase, AKT1, and GAPDH but increased GSK-3 β and PTEN. In humans, major depression is associated with various metabolic complications similar to the metabolic syndrome: insulin sensitivity is impaired, abdominal obesity is prevalent, and the risk of incident diabetes mellitus is increased in subjects suffering from major depression (Everson-Rose et al., 2004; Carnethon et al., 2007). Insulin sensitivity and glucose tolerance improve in patients responding to pharmacological antidepressant treatment (Weber-Hamann et al., 2006). Although total cholesterol levels appear to be lower in depressed patients (Aijanseppa

et al., 2002), the composition of individual lipoproteins exhibits adverse changes that are improved by pharmacological antidepressant treatment (Kopf et al., 2004). These clinical findings support the relevance of the results of the present paper, which suggest an important role of key enzymes in glucose and lipid metabolism for the pathogenesis of depression. Various pathways of interaction between brain function and peripheral energy metabolism have been summarized in the “selfish brain hypothesis” by Peters et al. (2004).

PAF-ApoE

The phospholipid PAF (1-O-alkyl-2-O-acetyl-sn-glycerol-3-phosphocholine) is the substrate of PAF-acylhydrolase (PAF-AH). PAF is degraded by PAF-AH, a circulating enzyme having both pro- and antiinflammatory activities. PAF activates GSK-3 β (see above), one of the key enzymes in glucose metabolism and an important target of lithium and valproate, which inhibit its activity. PAF's major action, however, is linked to lipid metabolism. Elevated PAF levels (resulting from diminished activity of PAF-hydrolase) induce synaptic excitotoxicity tentatively mediated by NMDA receptors (Bellizzi et al., 2005). It is produced in neurons in response to NMDA receptor activation. Subsequently, it may increase glutamate release from presynaptic terminals. PAF-AH is an LDL- and HDL-bound enzyme that hydrolyzes and inactivates PAF and prevents LDL-cholesterol oxidation, thus delaying the onset of atherosclerotic disease (Campo et al., 2004). Accordingly, decreased enzyme activities, as observed in this report, should increase this kind of risk. However, there are also reports claiming that increased PAF-AH activity is a risk factor for coronary artery disease (CAD); however, whether PAF-AH has a causal role or is simply a marker of risk is unclear (Blankenberg et al., 2003).

CONCLUSIONS

Lowering the conventional statistical significance limits in a classical microarray experiment on hippocampal tissue of bulbectomized mice and analyzing the results with the PathwayArchitect program led to the identification of a metabolic network that is disturbed in this model, i.e., the insulin signaling pathway affecting lipid metabolism. This approach indicated that molecules important for a disease state, here depression, may fall into the range of statistical noise. Moreover, molecules significantly changed may behave so as a consequence of former, more subtle changes. Those subtle expression changes can be identified by mathematical tools, such as clustering, or by computer simulations using the network components outlined. FABP7 and related molecules of the insulin signaling pathway are altered in the hippocampus, an important structure of the limbic system that has been associated with depression-like behavioral alterations in mice. This kind of approach not only is suitable for computer simulations to predict molecular responses within the network and/or with connected

networks but can also serve to find new targets for pharmacotherapy in affective disorders.

ACKNOWLEDGMENTS

The expert help of A. Tredup with qRT-PCR experiments and of E. Roebel with array studies is gratefully acknowledged. We are also very thankful to Dr. D. Kopf for critically reading the manuscript.

REFERENCES

- Aijanseppa S, Kivinen P, Helkala EL, Kivela SL, Tuomilehto J, Nissinen A. 2002. Serum cholesterol and depressive symptoms in elderly Finnish men. *Int J Geriatr Psychiatry* 17:629–634.
- Anthony TE, Mason HA, Gridley T, Fishell G, Heintz N. 2005. Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. *Genes Dev* 19:1028–1033.
- Arai Y, Funatsu N, Numayama-Tsuruta K, Nomura T, Nakamura S, Osumi N. 2005. Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. *J Neurosci* 25:9752–9761.
- Bellizzi MJ, Lu SM, Masliah E, Gelbard HA. 2005. Synaptic activity becomes excitotoxic in neurons exposed to elevated levels of platelet-activating factor. *J Clin Invest* 115:3185–3192.
- Bennett E, Stenvers KL, Lund PK, Popko B. 1994. Cloning and characterization of a cDNA encoding a novel fatty acid binding protein from rat brain. *J Neurochem* 63:1616–1624.
- Blankenberg S, Stengel D, Rupprecht HJ, Bickel C, Meyer J, Cambien F, Tiret L, Ninio E. 2003. Plasma PAF-acylhydrolase in patients with coronary artery disease: results of a cross-sectional analysis. *J Lipid Res* 44:1381–1386.
- Campo S, Sardo MA, Bitto A, Bonaiuto A, Trimarchi G, Bonaiuto M, Castaldo M, Saitta C, Cristadoro S, Saitta A. 2004. Platelet-activating factor acetylhydrolase is not associated with carotid intima-media thickness in hypercholesterolemic Sicilian individuals. *Clin Chem* 50:2077–2082.
- Carlsen J, De Olmos J, Heimer L. 1982. Tracing of two-neuron pathways in the olfactory system by the aid of transneuronal degeneration: projections to the amygdaloid body and hippocampal formation. *J Comp Neurol* 208:196–208.
- Carnethon MR, Biggs ML, Barzilay JI, Smith NL, Vaccarino V, Bertoni AG, Arnold A, Siscovick D. 2007. Longitudinal association between depressive symptoms and incident type 2 diabetes mellitus in older adults: the cardiovascular health study. *Arch Intern Med* 167:802–807.
- Chmurzynska A. 2006. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. *J Appl Genet* 47:39–48.
- Cryan JF, Holmes A. 2005. The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov* 4:775–790.
- de la Monte SM, Xu XJ, Wands JR. 2005. Ethanol inhibits insulin expression and actions in the developing brain. *Cell Mol Life Sci* 62:1131–1145.
- Duman RS, Heninger GR, Nestler EJ. 1997. A molecular and cellular theory of depression. *Arch Gen Psychiatry* 54:597–606.
- Everson-Rose SA, Meyer PM, Powell LH, Pandey D, Torrens JI, Kravitz HM, Bromberger JT, Matthews KA. 2004. Depressive symptoms, insulin resistance, and risk of diabetes in women at midlife. *Diabet Care* 27:2856–2862.
- Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G, Weizman A. 1999. Enigma of the peripheral benzodiazepine receptor. *Pharmacol Rev* 51:629–650.
- Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J. 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5:R80.

- Gerstner JR, Vander Heyden WM, Lavaute TM, Landry CF. 2006. Profiles of novel diurnally regulated genes in mouse hypothalamus: expression analysis of the cysteine and histidine-rich domain-containing, zinc-binding protein 1, the fatty acid-binding protein 7 and the GTPase, ras-like family member 11b. *Neuroscience* 139:1435–1448.
- Harkin A, Kelly JP, Leonard BE. 2003. A review of the relevance and validity of olfactory bulbectomy as a model of depression. *Clin Neurosci Res* 3:253–262.
- Hartfuss E, Forster E, Bock HH, Hack MA, Leprince P, Luque JM, Herz J, Frotscher M, Gotz M. 2003. Reelin signaling directly affects radial glia morphology and biochemical maturation. *Development* 130:4597–4609.
- Hellweg R, Zueger M, Fink K, Hortnagl H, Gass P. 2007. Olfactory bulbectomy in mice leads to increased BDNF levels and decreased serotonin turnover in depression-related brain areas. *Neurobiol Dis* 25:1–7.
- Irizarry RA, Gautier L, Bolstad BM, Crispin M, et al. 2005. Affy: methods for Affymetrix oligonucleotide arrays. R package version 1.6.7.
- Jaako-Movits K, Zharkovsky A. 2005. Impaired fear memory and decreased hippocampal neurogenesis following olfactory bulbectomy in rats. *Eur J Neurosci* 22:2871–2878.
- Jaako-Movits K, Zharkovsky T, Pedersen M, Zharkovsky A. 2006. Decreased hippocampal neurogenesis following olfactory bulbectomy is reversed by repeated citalopram administration. *Cell Mol Neurobiol* 26:1559–1570.
- Jesberger JA, Richardson JS. 1986. Effects of antidepressant drugs on the behavior of olfactory bulbectomized and sham-operated rats. *Behav Neurosci* 100:256–274.
- Kelly JP, Leonard BE. 1994. The effect of tianeptine and sertraline in three animal models of depression. *Neuropharmacology* 33:1011–1016.
- Kelly JP, Wrynn AS, Leonard BE. 1997. The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol Ther* 74:299–316.
- Kopf D, Westphal S, Luley CW, Ritter S, Gilles M, Weber-Hamann B, Lederbogen F, Lehnert H, Henn FA, Heuser I, Deuschle M. 2004. Lipid metabolism and insulin resistance in depressed patients: significance of weight, hypercortisolism, and antidepressant treatment. *J Clin Psychopharmacol* 24:527–531.
- Kurtz A, Zimmer A, Schnutgen F, Bruning G, Spener F, Muller T. 1994. The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. *Development* 120:2637–2649.
- Leonard BE, Tuite M. 1981. Anatomical, physiological, and behavioral aspects of olfactory bulbectomy in the rat. *Int Rev Neurobiol* 22:251–286.
- Licinio J, Wong ML. 2004. Translational research in psychiatry: pitfalls and opportunities for career development. *Mol Psychiatry* 9:117.
- Lumia AR, Teicher MH, Salchli F, Ayers E, Possidente B. 1992. Olfactory bulbectomy as a model for agitated hyposerotonergic depression. *Brain Res* 587:181–185.
- Meng S, Chen Z, Munoz-Antonia T, Wu J. 2005. Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. *Biochem J* 391:143–151.
- Nelms K, O'Neill TJ, Li S, Hubbard SR, Gustafson TA, Paul WE. 1999. Alternative splicing, gene localization, and binding of SH2-B to the insulin receptor kinase domain. *Mamm Genome* 10:1160–1167.
- Nestler EJ, Gould E, Manji H, Buncan M, Duman RS, Greshenfeld HK, Hen R, Koester S, Lederhendler I, Meaney M, Robbins T, Winsky L, Zalcman S. 2002. Preclinical models: status of basic research in depression. *Biol Psychiatry* 52:503–528.
- Owada Y, Abdelwahab SA, Kitanaka N, Sakagami H, Takano H, Sugitani Y, Sugawara M, Kawashima H, Kiso Y, Mobarakeh JI, Yanai K, Kaneko K, Sasaki H, Kato H, Saino-Saito S, Matsumoto N, Akaike N, Noda T, Kondo H. 2006. Altered emotional behavioral responses in mice lacking brain-type fatty acid-binding protein gene. *Eur J Neurosci* 24:175–187.
- Pelers MM, Glatz JF. 2005. Detection of brain injury by fatty acid-binding proteins. *Clin Chem Lab Med* 43:802–809.
- Pelers MM, Hanhoff T, Van der Voort D, Arts B, Peters M, Ponds R, Honig A, Rudzinski W, Spener F, de Kruijk JR, Twijnstra A, Hermens WT, Menheere PP, Glatz JF. 2004. Brain- and heart-type fatty acid-binding proteins in the brain: tissue distribution and clinical utility. *Clin Chem* 50:1568–1575.
- Pocklington AJ, Cumiskey M, Armstrong JD, Grant SG. 2006. The proteomes of neurotransmitter receptor complexes form modular networks with distributed functionality underlying plasticity and behaviour. *Mol Syst Biol* 2:23 [E-pub Jan 17].
- Possidente B, Lumia AR, McGinnis MY, Rapp M, McEldowney S. 1996. Effects of fluoxetine and olfactory bulbectomy on mouse circadian activity rhythms. *Brain Res* 713:108–113.
- R Development Core Team. 2005. R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. ISBN 3-900051-07-0; <http://www.R-project.org>.
- Schubert M, Brazil DP, Burks DJ, Kushner JA, Ye J, Flint CL, Farhang-Fallah J, Dikkes P, Warot XM, Rio C, Corfas G, White MF. 2003. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. *J Neurosci* 23:7084–7092.
- Song C, Leonard BE. 1994. The effects of chronic lithium chloride administration on some behavioral and immunological changes in the bilaterally olfactory bulbectomized rat. *J Psychopharmacol* 8:440–447.
- Song C, Leonard BE. 1995. Interleukin-2-induced changes in behavioral, neurotransmitter, and immunological parameters in the olfactory bulbectomized rat. *Neuroimmunomodulation* 2:263–273.
- Song C, Leonard BE. 2005. The olfactory bulbectomized rat as a model of depression. *Neurosci Biobehav Rev* 29:627–647.
- Taniguchi CM, Ueki K, Kahn R. 2005. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J Clin Invest* 115:718–727.
- van Riesen H, Leonard BE. 1999. Acts of psychotropic drugs on the behavior and neurochemistry of olfactory bulbectomized rats. *Pharmacol Ther* 47:21–34.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034.
- Weber-Hamann B, Gilles M, Lederbogen F, Heuser I, Deuschle M. 2006. Improved insulin sensitivity in 80 nondiabetic patients with MDD after clinical remission in a double-blind, randomized trial of amitriptyline and paroxetine. *J Clin Psychiatry* 67:1856–1861.
- Willner P. 1990. Animal models of depression: an overview. *Pharmacol Ther* 45:425–455.
- Wrynn AS, Mac Sweeney CP, Franconi F, Lemaire L, Pouliquen D, Herlidou S, Leonard BE, Gandon J, de Certaines JD. 2000. An in-vivo magnetic resonance imaging study of the olfactory bulbectomized rat model of depression. *Brain Res* 879:193–199.
- Xu J, Yeon JE, Chang H, Tison G, Chen GJ, Wands J, de la Monte S. 2003. Ethanol impairs insulin-stimulated neuronal survival in the developing brain: role of PTEN phosphatase. *J Biol Chem* 278:26929–26937.
- Zueger M, Urani A, Chourbaji S, Zacher C, Roche M, Harkin A, Gass P. 2005. Olfactory bulbectomy in mice induces alterations in exploratory behavior. *Neurosci Lett* 374:142–146.