Brief isoflurane anaesthesia affects differential gene expression, gene ontology and gene networks in rat brain

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Running title: Gene networks after isoflurane anaesthesia

Abbreviations: GOEAST: Gene Ontology Enrichment Analysis Software; MAC: minimum alveolar concentration; SNARE: N-ethylmaleimide-sensitive factor attachment protein receptor.

Abstract

Much is still unknown about the mechanisms of effects of even brief anaesthesia on the brain and previous studies have simply compared differential expression profiles with and without anaesthesia. We hypothesised that network analysis in addition to the traditional differential gene expression and ontology analysis, would enable identification of the effects of anaesthesia on interactions between genes. Rats (n=10 per group) were randomised to anaesthesia with isoflurane in oxygen or oxygen only for 15min, and 6h later brains were removed. Differential gene expression and gene ontology analysis of microarray data was performed. Standard clustering techniques and principal component analysis with Bayesian rules were used along with social network analysis methods, to quantitatively model and describe the gene networks. Anaesthesia had marked effects on genes in the brain with differential regulation of 416 probe sets by at least 2 fold. Gene ontology analysis showed 23 genes were functionally related to the anaesthesia and of these, 12 were involved with neurotransmitter release, transport and secretion. Gene network analysis revealed much greater connectivity in genes from brains from anaesthetised rats compared to controls. Other importance measures were also altered after anaesthesia; median [range] closeness centrality (shortest path) was lower in anaesthetized animals (0.07 [0-0.30]) than controls (0.39 [0.30-0.53], $p<0.0001$) and betweenness centrality was higher (53.85 [32.56-70.00]% compared to 5.93 [0-30.65]%, $p<0.0001$). Simply studying the actions of individual components does not fully describe dynamic and complex systems. Network analysis allows insight into the interactions between genes after anaesthesia and suggests future targets for investigation.

Key words

Brain; rat; isoflurane; network; gene; anaesthesia

1. Introduction

Despite the use of volatile anaesthesia for over 100 years, there is still much to be identified regarding effects on tissues and cells at the molecular level to explain both anaesthetic effects on the brain and also non-anaesthetic effects such as cognitive impairment, conditioning or immune modulation. Much remains unknown about the mechanisms of anaesthesia or the persistent effects of isoflurane on the brain.

The transcriptome (messenger RNA, mRNA) can vary with external environmental conditions and is more dynamic than the genome which is relatively fixed in an individual. Using microarray technology it is possible to investigate changes in the transcriptome across the entire known transcribed genome of several species including mouse, rat and human in organs, or regions of organs, or blood, including in response to anaesthesia.

A few studies have reported the effect of anaesthesia with volatile agents on the transcriptome in cells or various organs from rats [1-5] and have showed that changes can persist even several days after exposure to anaesthetics.[4] Clearly, changes in the transcriptome itself cannot cause molecular effects, but rather the consequent protein and functional metabolic changes. Most studies of effects of anaesthesia on gene transcripts have compared differential expression profiles in samples from animals exposed or not to anaesthesia and grouped the altered genes according to gene ontology groupings by function.[2] We hypothesised that using a systems biology approach with gene connections identified using Bayesian rules and with further analysis using social network tools[6-8] in addition to the traditional differential gene expression and ontology analysis, we would be able to identify novel gene interactions for further investigation. We therefore undertook network analysis on the brain transcriptome of rats several hours after a brief period of isoflurane anaesthesia and also studied differential gene expression using conventional techniques.

Methods

2.1 Animals

All animal studies were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986, following relevant aspects of the ARRIVE guidelines.[9] Groups of 20 week old male Sprague Dawley rats were studied (250-300g). Animals were conventionally housed, allowed food and water *ad libitum* and kept on a 12h light-dark cycle. All experiments began mid-morning to control for any diurnal effects. Rats were conditioned to handling and the anaesthetic equipment prior to experimentation. Animals were allocated by random number to either exposure to isoflurane in oxygen at a steady state concentration of 2% (1.4 x minimum alveolar concentration, MAC) (n=10) or oxygen only (n=10) in a transparent gas tight tank, about 30cm x 20cm x 20cm, into which gas flowed at about a litre/minute. After 15min were

placed on a heating mat and allowed to recover and were then returned to their cages. Six hours after the start of the anaesthetic/oxygen exposure, animals were briefly exposed to isoflurane and immediately killed by rapid cervical dislocation and decapitated. The brains were quickly and atraumatically removed and the entire cortex was dissected and placed in Ringer's solution (155mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 2mM NaH_2PO_4, 10mM HEPES, 10mM glucose) at 4°C to wash away blood before being placed immediately into RNAlater (Life Technologies, Paisley, UK) to stabilise the RNA. All samples from individual rats were analysed separately and independently without pooling of samples.

2.2 RNA analysis

Total RNA was extracted from the cortex of each brain with TRIzol reagent (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions. Total RNA was further purified using Qiagen column based methodology (Qiagen Ltd., Manchester, UK). The integrity of total RNA from each sample was established using an Agilent 2100 Bioanalyser and RNA 6000 Pico II Labchips® (Agilent Technologies UK, Cheshire, UK). Complementary RNA (cRNA) targets for hybridization were prepared and hybridized to the Rat 230 2.0 Genechip® for 16h at 45°C according to the Affymetrix protocol and identified using biotin-streptavidin-phycoerythrin labelling.[10] [11] To control for hybridization, washing, and staining procedures, the array also contains probe sets for various non-eukaryotic cDNA transcripts which were added to the hybridization mix. Fluorescence patterns of the microarrays, which indicates gene expression, were scanned at 3μm resolution using an Affymetrix gene array scanner and the digital images were analyzed using Affymetrix Microarray Software Suite 5.0 and dChip™ version 2010 (http://www.dchip.org). Differential gene expression was determined by comparing data from isoflurane exposed rats to that seen in control rats exposed to oxygen only. Only genes which changed by at least 2 fold with a fluorescent intensity of >100 units were included ($P<0.05$). In accordance with the recommended analysis strategy for small data sets, the results were permutated 1000 times and the false discovery rate was <15 out of ~30,000.

2.3 Data analysis methodology

2.3.1 Network analysis.

Fluorescence data from each animal from both groups were mined using standard clustering techniques and principal component analysis. Essentially this means that genes having similar expression levels were grouped. Quantitative modelling of the direct interactions between genes was then performed using graphical Gaussian models that represent causal dependencies in biomolecular networks. We used the GeneNet software package which computes the direct interaction between pairs of genes by the partial correlation matrix.[12] The partial correlation of a given pair of genes is the correlation of the expression data for that gene pair conditioned on all the other genes; the partial correlation matrix is the matrix with all these pairwise partial correlations. The use of partial correlations allowed us to obtain, from

multiple samples of gene expression data, a measure of the causal (direct) interactions between genes, as opposed to a simple correlation, which may have nothing to do with causation. The output of the GeneNet analysis is a graphical network where the nodes are genes, and the links represent genes that have strong interactions. This is a tried and tested method based on Bayesian probability principles, used successfully in previous gene expression studies to unravel metabolic networks in, for example, breast cancer data sets.[13]

Further analysis was undertaken using techniques originally described for analysis of social networks.[14] [15] There are various measures which are used to describe networks. Networks consist of *nodes*, which in this case are the genes, and each connection to another gene is called an *edge*; the number of edges connected to a single node is called the *degree* of the node. The topology of the network can be presented graphically where the direction of the interactions is identified (i.e. where a gene acts to increase or decrease expression of another). The thickness of the edges indicates the strength of the interaction. The *degree distribution* is how many nodes (genes) have a certain degree number. The 'importance' of each node can be described in several ways: the *degree centrality* is simply the degree of a node (i.e. the number of edges or interactions it has) and highly connected nodes with a high degree (5 or more) are called *hubs. Closeness centrality* is the average of the pathlength of one node to every other node such that the most important nodes have a low value, describing shortest paths to other nodes. *Betweenness centrality* is a measure of a node's influence as a 'gatekeeper' and is essentially the % of shortest paths that include a given node. Finally the *cohesion* of the network is the number of nodes which would need to be removed to render the network disconnected. A schematic diagram depicting the key features of an example network is shown in Figure 1 and the various measures are described in Table 1.

2.3.2 Functional clustering.

Differential gene expression between anaesthesia and control rats was clustered into functional groups using the Gene Ontology Enrichment Analysis Software Toolkit (GOEAST).[16] For this, the gene names with known functions were grouped into key functional categories identified by accepted GO numbers to enable unambiguous groupings.

2. Results

3.1 Network analysis

The expression network diagrams shown in Figure 2A and 2B show the interactions between the genes. The networks consisted of 18 connected genes in the non-anaesthetized control rats (Figure 2A) and 15 connected genes in the anaesthetised rats (Figure 2B). The full names and functions of the proteins encoded by these genes are listed in Table 2. It can be seen that the network after anaesthesia (Figure 2A) is completely different from that seen in the non-anaesthetized rats (Figure 2B). Genes which are most highly connected have a high degree

centrality and dominate the network topology –i.e. hub genes. Two hub genes (nodes), both with 6 edges (connections), were identified in the control animals: *Mll5* and *VHL* (Figure 2A). Details of the proteins encoded by these genes and their functions are given in Table 3. In the rats which had been anaesthetised for 15 min there were 8 hub genes seen, with a clique of 5 strongly interacting genes *Bcat1*, *Nr1d1*, *Ntrk2, Rn415* and *Tpm3* (Figure 2B, Table 2). After anaesthesia the interactions were dominated by this clique. Three genes expressed in the control rat network were not seen in anaesthetized animals: *Cacng2, Hipk2* and *GRM3* (Figure 2).

The degree distribution frequencies showed that there were more genes with a higher degree number after anaesthesia (Table 3). There were two genes with a degree number of 5 or more in the control group and eight genes with a degree of 5 or more after anaesthesia; ie. the degree centrality was higher after anaesthesia (Table 3). In terms of the other importance measures, median [range] closeness centrality (shortest path) was lower for all genes in anaesthetized animals (0.07 [0-0.30]) than controls (0.39 [0.30-0.53], $p<0.0001$). In contrast, betweenness centrality was almost ten-fold higher for all genes after anaesthesia (53.85 [32.56-70.00]%) than controls (5.93 [0-30.65]%, $p<0.0001$) (Table 3). The two most 'important' genes in the network after anaesthesia were *Bcat1* and *Nr1d1* with a high degree number (both 8) and betweenness centrality of 70% and 63% respectively.

3.2 Differential gene expression

Analysis of microarrays identified the effect of isoflurane anaesthesia on differential gene expression. We identified 416 probe sets which were differentially regulated, as determined by our pre-defined criteria of at least a 2-fold change. Of these, 343 were assigned to named full length gene sequences and 73 were either associated with expressed sequence tag clusters with no known function or assigned gene name, or were redundant (gene duplication) probe sets. The entire dataset described here has been deposited in the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO)[32] and is accessible through the GEO series accession number 64617 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64617).

3.3 Gene ontology enrichment

GO enrichment analysis of the differentially expressed genes resulted in identification of 23 genes that could be used to determine shared gene functionality. Twelve common genes which were different between control and anaesthetized rats were functionally classed as regulators of neurotransmitter release (GO group number 0001505), transport (GO:0006036) and secretion (GO:0007269). Of these 12, only two genes were up-regulated. In addition, 2 genes were assigned as chromo shadow domain genes (GO:0070087) and were also up-regulated; 6 were classed as organ morphogenesis genes (GO:009887), three were classed as genes for

generation of signals involved in cell signalling (GO:0003001) and signal release (GO:0023061), with some genes also being functionally classed as vasculogenesis (GO:001570), glutamate secretion (GO:0005030), retinal rod development (GO:0046548), regulation of neurotransmitter secretion and transmission (GO:0046928) and ontogenesis of dentin-containing tooth genes (GO:0051588); these were all down-regulated (Table 4).

3. Discussion

Gene expression analysis of the transcriptome in rat cortex showed that a very brief period of isoflurane anaesthesia resulted 6h later in marked changes both in differential gene expression and the network interactions between expressed genes.

Recovery after anaesthesia with volatile agents is associated with elimination of the agent from the brain. Despite this, exposure to isoflurane impairs subsequent learning[33] whilst tolerance of an ischaemic insult is improved even 2 weeks after anaesthesia.[34] The persisting effects of anaesthetic exposure on gene expression and interaction may explain these post-anaesthesia mechanisms. We therefore wished to determine the effects several hours after a brief period of anaesthesia on the rat brain transcriptome. Traditional microarray analysis focuses on finding genes or classes of genes whose differential expression levels differ between two states (i.e. non-anaesthetised versus anaesthetised). However, by focusing on *changes* in expression of genes, most traditional differential analyses neglect the *interactions* between the genes. Biological systems are both dynamic and complex such that it is difficult to determine the behaviour of the whole system by studying only the actions of individual components.

Our conventional differential gene expression analysis revealed that 343 known genes were changed by at least 2 fold, with 235 up-regulated and 108 down-regulated (see GEO submission) after a brief period of anaesthesia with isoflurane. Previous studies also found altered expression of a roughly similar number of genes in the brain of rats at various lengths of time after varying durations of anaesthesia with inhalational agents.[2-5] Two days after rats were anaesthetised with isoflurane and nitrous oxide for 4h, Culley et al described upregulated expression of genes involved in a range of cellular functions, whilst many of the downregulated genes were those involve in in signal transduction and synaptic plasticity.[4] Our gene ontology analysis showed that the majority of the enriched genes were associated with the neurotransmitter release machinery, termed soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) associated genes which mediate docking of synaptic vesicles with the presynaptic membrane.[35] SNARE proteins are a superfamily of over 60 structurally diverse proteins that are involved in the process of neurotransmitter secretion. They all have a SNARE motif that allows them to interact with each other to form 'SNARE complexes' that facilitate secretion of neurotransmitters through interaction with the plasma

membrane. Isoflurane disrupts multiple pre-synaptic targets, and may also affect SNARE-mediated fusion.[36] Evidence suggests that syntaxin1A, a key membrane-bound component of the SNARE complex, is required for neurotransmission[37] and this gene was down-regulated 4-fold by isoflurane anaesthesia in our study. This protein both binds to, and is regulated by, some other proteins which are associated with SNARE, which may also be disrupted by anaesthesia.[38] In addition, GO enrichment analysis identified crossover of some of these genes with those that also are involved in cell signalling, repression of gene expression, organ morphogenesis and ontogenesis (Table 4).

In addition to differential gene expression and GO analysis we also used a complex systems approach to identify changes in networks of gene expression in non-anaesthetised and anaesthetised rats. By comparing the functional moduli i.e. the pattern of the gene in the network- of the anaesthetised compared to non-anaesthetised control rats, a better understanding of how the genetic circuitry adapts or is affected by inhalational anaesthesia is possible. We examined the regularities in these gene expression profiles with respect to the topology of the gene networks as a way to automatically extract active pathways and their associated patterns of activity. This approach allows the relationships between the genes to be determined and potential inferences about changes in functional interconnectedness of those genes can be made.[14 15] The causal links between the genes is determined via statistical analysis of their correlations, using the well-known method of partial correlations (also known as conditional correlations) to determine causation from the correlation pattern. Simply examining the correlation between expressions of two genes will not establish that there is a direct influence of one over the other: the expression of both genes might be influenced by a third gene, for example. Conditional correlations of the two genes, given the expression of all the other genes is close to zero if both genes are being "driven" by other genes. So in essence, the method allows the removal of links that do not involve direct causation – subject, of course, to statistical errors and finite sample size. The topological analysis of the connections between the genes can then be described to create the network. We then went on to undertake network analysis originally developed for the social sciences and which has recently also been applied to investigating gene networks of antibiotic resistance[39] and inflammatory gene networks in coronary artery disease.[40] Although there are no reports of the effect of anaesthesia on gene networks, changes in brain networks after traumatic brain injury, have been reported, using a similar approach.[41]

We discovered a marked effect on the network of genes in rat brain cortex from functionally independent pathways 6h after 15min of isoflurane anaesthesia. The analysis, instead of focussing on the actions of individual components, describes the relationships between them. Such methods have now spread beyond social sciences to include applications in fields ranging from computer science and crime research[15] to the life sciences.[39 40 41] This provides

information on the direction of interactions (i.e, which gene up-regulates or down-regulates another), the key players in the network (which genes are most 'important' to the network), and the cohesion of the network. The frequency of the degree distribution showed that there were more genes with a higher number of degrees (interactions or edges) after anaesthesia and the degree centrality, ie the number of connections to other genes, was higher i.e. there were more nodes (genes) with a higher degree number, (defined as 5 or more edges) after anaesthesia than in controls. Closeness centrality – in other words the measure of the time needed for a gene to interact with other genes, was decreased after anaesthesia, whilst the betweenness centrality, which indicates how important each gene is in terms of routes between two genes, was much higher after anaesthesia. These changes are very marked and show that 6h after brief anaesthesia there is a change in the gene network which enables more interactions with other genes and faster and better interactions with the other genes.

The genes in the networks were identified as having a variety of functions and these are described in detail in Table 2, some of which have clear relevance for the hypnotic and analgesic (*Atp1b2*) effects of anaesthesia, whilst others are involved in neurotransmitter release (*Rph3a*), neuroplasticity (GRM3), learning and memory (*Nr1d1, Ntrk2*), cell signalling (*Digap3, Cacng4*) or metabolism *(Bcat1*). Particularly interesting was the change in the interactions of genes involved in hypoxia inducible factor 1α (HIF1α) regulation, notably *VHL* and *HipK2*, both of which have roles in downregulation of HIF1α expression.[23 31] HIF1α has been implicated in isoflurane mediated cardiac pre-conditioning and effects on metastatic processes in cancer cells.[42 43] The most 'important' genes in terms of number of interactions were *Nr1d1* which relates to a transcription factor that negatively regulates expression of core clock proteins, and which has been shown to be related to memory and mood. The clock gene 'loop' also has a role in regulation of inflammatory pathways.[26] The other notable hub gene with degree 8 was *Bcat1* which encodes for a transaminase that catabolizes essential branched chain amino acids.[18]

Differential gene expression analysis has been used extensively to identify genes and deregulated molecular mechanisms by comparison of individual genes between two phenotypes, in this case anaesthetised and non-anaesthetised, in various areas of rat brain.[3 4] Most studies have addressed the effects during anaesthesia, rather than effects occurring later, apart from the study by Culley et al.[4] Other studies have also concentrated on fairly long periods of anaesthesia; we show here that even a very brief period of isoflurane exposure had marked effects on both differential gene expression and gene networks 6h later. Alterations to multiple genes with changes in the interactions with other genes are the controllers of normal biological functioning and determine the cell phenotype. Differential expression analysis does not identify which of the large number of differentially expressed genes may be responsible for the phenotype, since each gene is analysed independently without considering any of the gene

interactions or networks, such that the differentially regulated genes might not directly relate to the phenotype.

Determination of the relationships among the expressed genes and identification of the changes in the associations between them may lead to a better understanding of the molecular mechanisms involved in the many disparate effects of anaesthesia. We found that after isoflurane exposure a strong central clique of gene interactions was apparent, with two major hub genes and involving genes encoding for metabolic processes, learning and memory, cytoskeleton stability and cell signalling. All the genes in this clique strongly positively regulated each of the other genes in the clique suggesting multiple co-ordinated cellular and molecular effects and demonstrating the extent of anaesthetic actions. Outide the clique there was strong positive upregulation of *Digap3,* a gene involved in signalling at excitatory glutamatergic synapses and which has been implicated in the effects of halothane anaesthesia,[21] and downregulation of *Atp1b2* which has been linked to pain responses.[17] However it is arguably the strong interactive networks between genes not previously known to be affected by anaesthesia which may suggest plausible functional and/or regulatory connections with exertion of multiple effects via a relatively small set of genes; such information can direct future work. It is important to stress that the gene network analysis reported here was undertaken without any prior functional knowledge.

Microarray data gives information on the transcriptome; some transcripts may not affect subsequent proteins. A previous study reported effects of 3h desflurane anaesthesia on the cytosolic proteome up to 72h later,[44] but since proteins can also be modified post-translationally, even protein expression may not determine function. The data presented here therefore give a snapshot of the interaction between transcripts and cannot predict ultimate functional effects. It was a previously held view that microarray data should be verified by analysing RNA expression by an additional method. However such further analysis only provides information on the small proportion of genes selected and cannot be extrapolated to others, limiting its value. When the field of expression analysis was in its infancy and used expensive technology, the high costs of replication prevented adequately rigorous experiments. It is generally accepted today that the high quality array platforms are sufficiently robust.[45-47]

The network analysis approach provides fascinating information on expression similarities in a tissue specific manner and enables identification of the key connections and changes in these gene interactions under given conditions. It has the potential to reveal interactions not seen using traditional differential gene expression analysis and has not been reported previously in relation to anaesthesia, to our knowledge. We suggest that undertaking such network analysis in addition to differential gene expression analysis may help to unravel the complex

mechanisms which are affected by anaesthetics including those unrelated to the hypnotic effects. Although the genes identified by the network analysis have not all been associated with the molecular mechanisms or targets involved in the actions of anaesthetics, they represent novel relevant targets that warrant further investigation.

Author contributions

We confirm that all authors made a substantial contribution to the conception and design, acquisition of data, or analysis and interpretation of data and contributed to drafting the article or revising it critically for important intellectual content..

DAL: conducted experiments, undertook microarray and gene ontology analysis, helped write the paper.
AM: design of complex systems network model, undertook network data analysis and helped write the paper.
HFG: helped design the study, helped conduct experiments, undertook data analysis and presentation and critically revised and edited the paper.
NRW: conceived of the study, helped conduct experiments, drafted the paper.

All authors have approved the submitted version and are all accountable for the accuracy and integrity of any part of the work

Declaration of interests

HFG is an Editor and NRA is Chairman of the Board of Management of the British Journal of Anaesthesia.

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Legends to figures

Fig 1.

Schematic diagram showing the main features of networks.

- Node 4 has 5 edges (degree 5) and is a hub.
- Node 4 has the highest degree centrality (highest degree).
- Node 4 has the lowest average number of shortest paths and so the highest closeness centrality.
- Node 5 has the highest betweenness centrality since it has the highest % of shortest paths which go through this node.
- Nodes 1, 2 and 3, and nodes 4, 7, 8 and 9 are 'cliques' since all nodes connect to the others in the group.
- Nodes can be connected by any path consisting of several edges. For example node 5 is connected to node 8 via node 4 and to node 1 via node 2.

Fig 2.

Gene network topology in brains from 10 non-anaesthetised control rats and 10 rats exposed to isoflurane for 15 min. Solid lines represent one of the genes enhancing the expression of the other, and dashed lines represent genes inhibiting the other. Arrows indicate an inferred causal direction of interaction, otherwise direction cannot be inferred. The darkest and thickest lines indicate the strength of the connections. The nodes (genes) outlined in red are hubs, each with 5 or more edges. The nodes filled in pink are cliques, since each of genes interact directly with each other. The nodes outlined in blue have fewer than 5 edges and are less connected than the other nodes; they are all present in both groups i.e. non-anaesthetised control rats and anaesthetised rats. Only 3 of the nodes seen in control rats were not seen in anaesthetised rats (outlined in purple). Gene names and their function are detailed in full in Table 2.

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Table 1 Key measures in network analysis

Measure	Describes	Further information
SIZE		
Number of nodes	Size of the network.	Number of nodes are genes in this network.
Number of degrees/edges	How 'busy' the network is.	Number of interactions in total in the network.
COHESION		
Components	Identification of sub-groups.	Number of distinct sub – groups in the network.
Cliques	Identification of interrelated groups.	Number of groupings where all nodes (genes) interact with each other.
Density	Extent of connections: lower density networks have fewer edges between nodes and fewer hubs.	Number of connections in total in the network and number of highly connected genes (hubs).
CENTRALITY		
Degree frequency	Number of nodes with a specific degree/edge number.	Connections of nodes (genes) in the network.
Closeness centrality	Importance of each node in the network.	Average number of shortest paths that pass through each node (gene).
Betweenness centrality	Importance of each node in the network.	Average number of paths that pass through each node (gene).

Edges and degrees both refer to connections between genes.

Nodes in this case are genes.

Hubs have 5 or more edges (connections).

Table 2. List of genes (nodes) and their functions identified in the network analysis

Gene ID	Protein	Function	Further information and key references
Atp1b2	ATPase, Na+/K+ transporting, beta 2 polypeptide	Responsible for establishing and maintaining electrochemical gradients of Na^+ and K^+ across plasma membrane.	Involved in nociception. [17]
Bcat1	Branched chain amino-acid transaminase 1, cytosolic	Catalyzes first reaction in catabolism of essential branched chain amino acids- leucine, isoleucine, valine.[18]	
Cacng2, *Cacng4*	Calcium channel, voltage-dependent, gamma subunit 2 or 4	Voltage-dependent calcium channel, functions as a transmembrane AMPA receptor regulatory protein.	Cacng1 protein shown to be increased after isoflurane anaesthesia in rats.[3]
Camk2	Calcium/calmodulin-dependent protein kinase II inhibitor 1	Modulates activity of a calcium-dependent protein kinase.	May have a role in synaptic plasticity and learning.[19]
Diras2	DIRAS family, GTP-binding RAS-Like 2	Ras GTPases controlling MAP kinase activity.[20]	
Dlgap3	Discs, large (Drosophila) homolog-associated protein 3	Role in organization of synapses and neuronal cell signalling at excitatory glutamatergic synapses.	Implicated in molecular effects of halothane anaesthesia.[21]
dUTPase	Deoxyuridine-triphosphatase	Involved in nucleotide metabolism and production of thymidine nucleotides.[22]	
HipK2	Homeodomain interacting protein kinase 2	Serine/threonine-protein kinase involved in transcription regulation of p53 and transcriptional co-suppressor of HIF1A.[23]	
GRM3	Metabotropic glutamate receptor 3	Regulator of pre-frontal cortex neuroplasticity.	Synthetic antagonists have antidepressant and anxiolytic activity.[24]
Mll5	Myeloid/lymphoid mixed-lineage leukemia 5	Key regulator of haematopoiesis and maintains expression of determination genes in quiescent cells.[25]	

Nr1d1	Nuclear receptor subfamily 1, group D, member 1	Transcription factor - negatively regulates expression of core clock proteins.	Also known as REV-ERBα : loss of expression related to memory and mood related changes. Clock gene 'loop' also in part regulates inflammatory pathways.[26]
Ntrk2	Neurotrophic tyrosine kinase receptor, type 2	Involved in learning and memory by regulating short-term synaptic function and long-term potentiation by mediating communication between neurones and glia.	Phosphorylates itself and mitogen associated phosphokinase (MAPK) family members. May be involved in molecular effects of alcohol sedation.[27]
Rph3a	Rabphilin 3A homolog protein	Involved with synaptic vesicle trafficking and synaptic vesicle fusion for neurotransmitter release.	Small GTP-binding protein involved in pre-synaptic neurotransmitter release.[28]
Scaf1	Serine arginine-rich pre-mRNA splicing factor	Role in pre-mRNA splicing.[28]	
Tpm3	Tropomyosin 3	Role in stabilizing non-muscle cell cytoskeleton actin filaments.	Can translocate with neurotrophic tyrosine kinase receptors and may form fusion proteins.[30]
VHL	Von Hippel Lindau tumour suppressor protein	Regulates angiogenesis, extracellular matrix formation and the cell cycle. Germ line mutation causes VHL disease, a hereditary cancer syndrome	Promotes degradation of the hypoxia responsive gene hypoxia-inducible factor α (HIF α) which has >70 direct targets.[31]

Table 3. Network analysis

Gene (node)	Control Degrees per node	Anaesthetized Degrees per node	Control Closeness centrality	Anaesthetized Closeness centrality	Control Betweenness centrality %	Anaesthetized Betweenness centrality %
Atp1b2	4	1	0.41	0.00	10.76	32.56
Bcat1	3	8	0.41	0.30	10.38	70.00
Cacng2	2	not expressed	0.30	not expressed	2.25	not expressed
Camk2	3	1	0.40	0.00	3.92	35.90
Cancg4	2	5	0.38	0.02	5.12	56.00
DIRAS2	3	4	0.47	0.17	12.90	53.85
Dlgap3	2	1	0.32	0.00	0.00	40.00
dUTPase	4	2	0.44	0.01	13.24	46.67
GRM3	2	not expressed	0.37	not expressed	8.87	not expressed
Hipk2	3	not expressed	0.39	not expressed	4.04	not expressed
Mll5	6	5	0.53	0.07	28.00	56.00
Nr1d1	3	8	0.35	0.25	1.04	63.63
Ntrk2	4	6	0.50	0.05	27.17	58.33
Rph3a	4	3	0.38	0.14	4.78	46.67
Scaf1	2	5	0.38	0.15	5.11	53.85
Tpm3	3	5	0.37	0.09	6.74	56.00
VHL	6	3	0.52	0.47	30.65	23.80
Median	**3**	**5***	**0.38**	**0.06***	**6.74**	**53.85***

* $P<0.0001$ compared to non-anaesthetized control rats (Mann Whitney U test). See Table 2 for full gene names.

Table 4 Gene ontology enrichment analysis

Regulation of neurotransmitter release (GO 0001505), neurotransmitter transport (GO 0006036) and neurotransmitter secretion (GO 0007269)

Gene ID	Protein name	Fold change	Function
Nrxn3	Neurexin 3	2.40	Neuronal cell surface protein that may be involved in cell recognition and cell adhesion.
Cplx2	Complexin 2	2.85	Regulates the formation of synaptic vesicle and exocytosis.
Slc17a7	Solute carrier family member 17	-3.42	Mediates the uptake of glutamate into synaptic vesicles.
Stxbp1	Syntaxin binding protein 1	-3.04	Regulates synaptic vesicle docking and fusion.
Cacna1a	Class A calcium channel-1A	-3.49	Mediate the entry of calcium ions into excitable cells to induce neurotransmitter release.
Stx1a	Syntaxin 1a	-4.00	Potentially involved in docking of synaptic vesicles at presynaptic active zones. May play a critical role in neurotransmitter exocytosis.
Syt4	Synaptotagmin IV	-4.03	May be involved in Ca^{2+} dependent exocytosis of secretory vesicles.
ppfia3	Protein tyrosine phosphatase	-2.63	Involved in the molecular organization of presynaptic active zones.
Syn2	Synapsin II	-3.43	Regulation of neurotransmitter release
Slc6a17	Solute carrier family member 17	-2.40	Sodium-dependent vesicular transporter selective for proline, glycine, leucine and alanine.

Chromo shadow domain binding (GO 0070087)

Gene ID	Protein name	Fold change	Function
Nipbl	Nipped-B homolog (Drosophila)	2.72	Probably plays a structural role in chromatin
Atrx	Alpha thalassemia / mental retardation syndrome X-linked	11.38	Global transcriptional regulator

Organ morphogenesis (GO 009887)

Gene ID	Protein name	Fold change	Function
Igf2	Insulin-like growth factor II	-2.34	Growth factor
Col1a2	Collagen alpha-2(I) chain precursor	-2.30	Collagen synthesis
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	-2.18	Role in learning and memory via regulation of short-term synaptic function and long-term potentiation.
Nfic	Nuclear factor I/C	-3.54	Transcription factor
Sostdc1	Sclerostin domain-containing protein 1	-3.86	Enhances Wnt signalling and inhibits TGF-beta signalling.
Zmiz1	Zinc finger MIZ domain-containing protein 1	-2.51	Increases ligand-dependent transcriptional activity of AR and promotes AR sumoylation.

Generation of signals involved in cell-cell signalling (GO 0003001) and signal release (GO 0023061)

Gene ID	Protein name	Fold change	Function
Lphn1	Latrophilin-1 precursor	-2.18	Regulation of exocytosis.
Syn1	Synapsin-1	-2.27	Regulation of neurotransmitter release.
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	-2.18	Involved in neurotransmitter synthesis.

Vasculogenesis (GO 001570)

Gene ID	Protein name	Fold change	Function
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	-2.18	Involved in neurotransmitter synthesis.
Zmiz1	Zinc finger MIZ domain-containing protein 1	-2.51	Increases ligand-dependent transcriptional activity of AR and promotes AR sumoylation.

Glutamate secretion (GO 0005030)

Gene ID	Protein name	Fold change	Function
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	-2.18	Involved in neurotransmitter synthesis.

Retinal rod development (GO 0046548)

Gene ID	Protein name	Fold change	Function
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	-2.18	Involved in neurotransmitter synthesis.

Regulation of neurotransmitter secretion and transmission (GO 0046928)

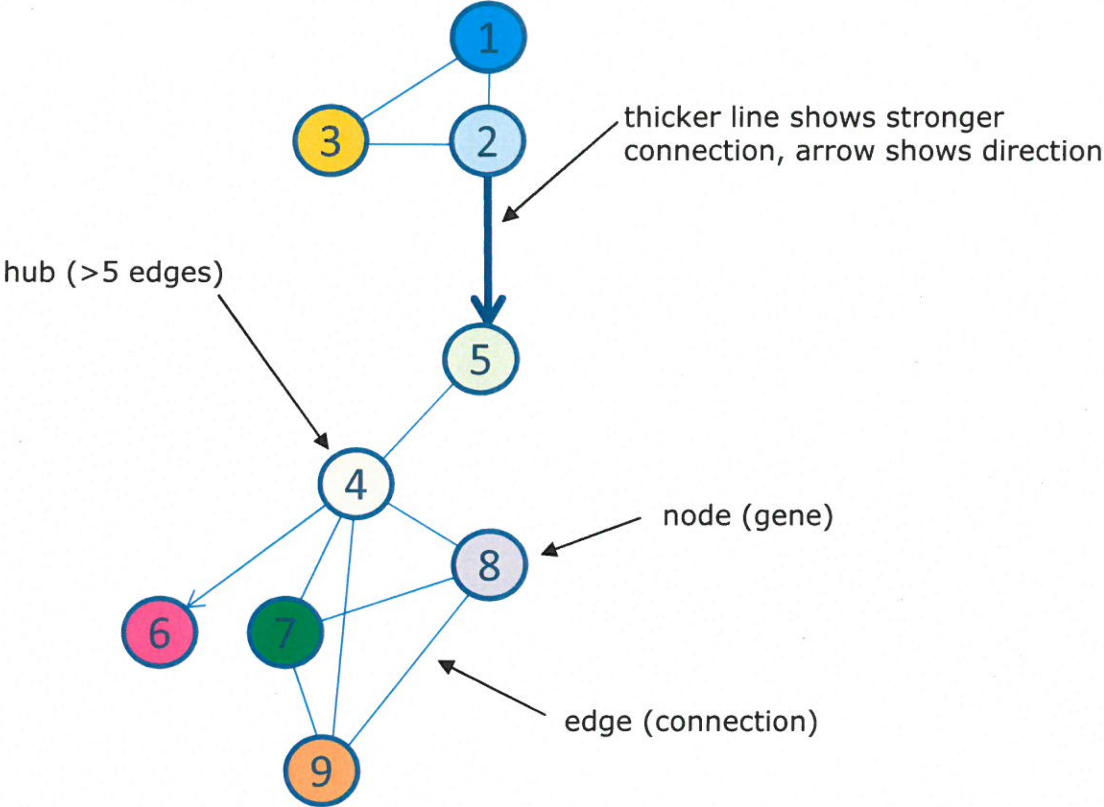
Gene ID	Protein name	Fold change	Function
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	-2.18	Involved in neurotransmitter synthesis.
Camk2a	Calcium/calmodulin-dependent protein kinase II alpha	-3.63	Functions in long-term potentiation and neurotransmitter release

Odontogenesis of dentin containing tooth (GO 0051588)

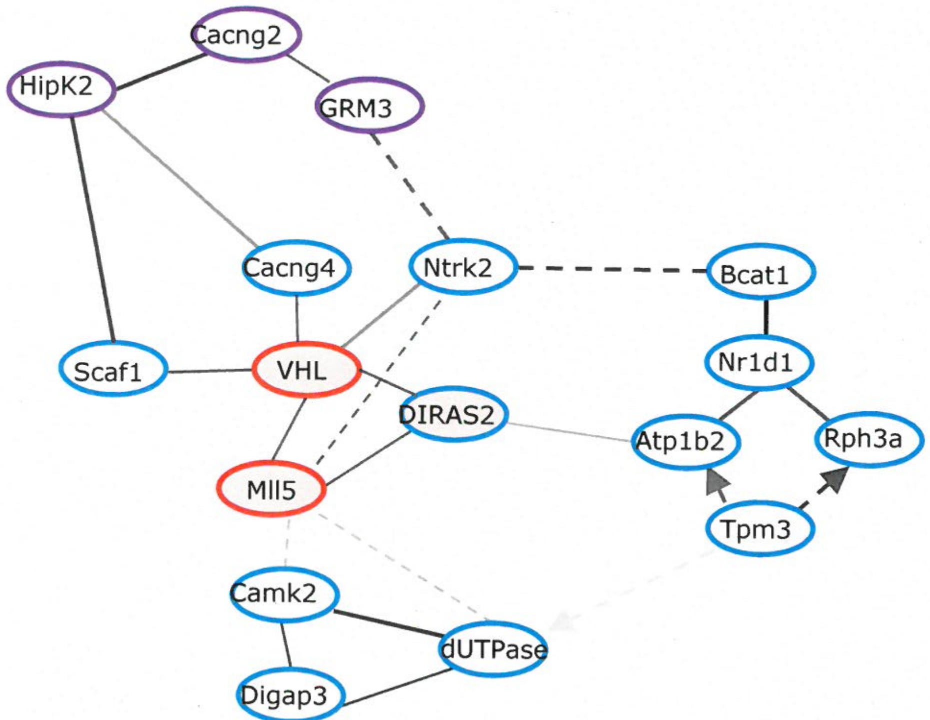
Gene ID	Protein name	Fold change	Function
Nfic	Nuclear factor I/C	-3.54	Transcription factor.
Sostdc1	Sclerostin domain-containing protein 1	-3.86	Enhances Wnt signalling and inhibits TGF-beta signalling.

Note: some genes appear in more than one GO group

Figure 1



Non-anaesthetised



Anaesthetised