Environmentally enriched pigs have transcriptional profiles consistent with neuroprotective effects and microglial depletion

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Abstract

Environmental enrichment (EE) is widely used to study the effects of external factors on brain development, function and health in rodent models, but very little is known of the effects of EE on the brain in a large animal model such as the pig. Twenty-four young pigs (aged 5 weeks at start of study, 1:1 male: female ratio) were housed in EE pens and provided with additional enrichment stimulation (bag filled with straw) once daily. Litter, weight and sex matched controls n= (24) were housed in barren (B) conditions. Behaviour was recorded on alternate days from study day 10. After 21 days, RNA-sequencing of the frontal cortex of male piglets culled one hour after the enrichment stimulation, but not those at 4 hours after stimulation, showed upregulation of genes involved in neuronal activity and synaptic plasticity in the EE compared to the B condition. This result is mirrored in the behavioural response to the stimulation which showed a peak in activity around the 1 hour time-point. By contrast, EE piglets displayed a signature consistent with a relative decrease in microglial activation compared to those in the B condition. These results confirm those from rodents, suggesting that EE may also confer neuronal health benefits in large mammal models, through a potential relative reduction in neuroinflammation and increase in neuroprotection driven by an enrichment-induced increase in behavioural activity.

1. Introduction

Environmental enrichment (EE) paradigms are widely used in laboratory rodents as a method to study the effects of environmental influences on brain development and function (1). EE utilises multiple methods of environmental modification to facilitate cognitive and motor stimulation in a species appropriate manner. In rodents this is often achieved through the use of novel objects, running wheels, nesting material and group housing (2).

The beneficial effects of EE on physiology and brain function in rodents are well documented (reviewed in (3)). EE paradigms have been shown to have a neuroprotective effect through the

38 inhibition of spontaneous apoptosis (4) and through increased cell proliferation and neurogenesis in
39 the hippocampus (5-7) and prefrontal cortex (8). Indeed, EE has been shown to limit
40 neurodegeneration in models of aging (9, 10), and can rescue some behavioural and physiological
41 brain effects of early life stress (11, 12). Current evidence suggests this is most likely precipitated by
42 the mechanisms underlying neuronal plasticity, due to increased expression of trophic factors and
43 immediate early genes (IEG) in the brains of EE animals (13-15).
44 Expression of neurotrophins has also been proposed as a contributory factor in mood disorders, in
45 particular stress-induced depression and anxiety. While the main focus has been around expression
46 of brain derived neurotrophic factor (BDNF) (16-18), there are a number of other trophic factors and
47 IEGs with a role in stress resilience and mood. Neuronal growth factor (*Ngf*) mRNA has been shown
48 to be reduced in the dentate gyrus in response to restraint stress in rats (19). Environmental
49 deprivation during early life in rats reduces IEG expression in the prefrontal cortex both as a chronic
50 effect (20) in response to social interaction (21), and after chronic social defeat stress in mice(22).
51 Levels of IEG *Arc* mRNA in the frontal cortex and hippocampus of rats exposed to predator scent
52 stress were reduced in those animals which displayed an extreme anxiety-like behavioural response,
53 and in the dentate gyrus. They also correlated with levels of circulating corticosterone(23).
54 Interestingly, depressive-like behaviour in rodents induced by social isolation in early life not only
55 results in decreased expression of a number of IEG involved in synaptic plasticity, including *Bdnf, Egr-*
56 *1* and *Arc* ((24), mice), but also in increased microglial activation ((25), rats). Microglia are specialised
57 macrophages of the central nervous system that play a role in neuromodulation, phagocytosis and
58 inflammation (reviewed in(26)). There is increasing evidence to suggest that microglial mediated
59 brain inflammation is a contributing factor to a number of mental health and nervous system
60 disorders, such as multiple sclerosis(27), Alzheimer's disease(28), schizophrenia (29, 30), and major
61 depression(31). While the exact mechanism by which microglia contribute to these disorders is not
62 yet known, psychological stress has been shown to result in increased levels of microglia in the
63 prefrontal cortex of rats (32) while early life stress, in the form of maternal deprivation, has also been

shown to lead to increases in microglial motility (33) and phagocytic activity (34) that persist into adulthood. Anti-inflammatory drugs such as minocycline (which blocks microglial activation) produce antidepressant effects in rodent models (35). Several anti-psychotic medications have also been shown to block microglial activation (36, 37).

The physiological and behavioural effects of a number of nervous system disorders can be modulated by EE in rodent models (38), and it is now widely used as an experimental tool in studies of neurodegeneration and neural development (39). Due to its beneficial effects on behaviour, EE has long been used as a method to improve the welfare of production animals, by providing environmental manipulations that allow the animal to behave in a more species-typical way (reviewed in (40)). However there has been little work on the effects of EE on the brain in a large animal model, such as the pig. The aim of the present study was to investigate the effects of EE on gene expression within the frontal cortex of young pigs with the aim of identifying potential gene expression signatures of a healthy brain in a large animal model, and to potentially identify early signatures of neural ill-health in the absence of abnormal behaviours.

2. Materials and Methods

2.1 Ethical review

All work was carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 under EU Directive 2010/63/EU following ethical approval by SRUC (Scotland's Rural College) Animal Experiments Committee. All routine animal management procedures were adhered to by trained staff and health issues treated as required. All piglets remaining at the end of the study were returned to commercial stock.

2.2 Animals and general experimental procedures

Post-weaning behavioural observations were carried out on litters from six commercial cross-bred mothers (Large White x Landrace); the boar-line was American Hampshire. Litters were born within a

72 hour time window. At weaning piglets were weighed, vaccinated against Porcine Circoviral Disease and ear tagged for identification. No tooth resection was performed and males were not castrated. Post-wean diet was in the form of pelleted feed (Primary Diets Prime Link Extra) provided *ad libitum*. At 4 days post weaning, 8 piglets per litter were selected as being as close to the litter average weight as possible while balancing for sex (1:1 male to female). These 8 piglets per litter were split into sex balanced quartets to give final group sizes of 4 piglets per matched group. One group was then housed in an enriched (EE) pen and one in a barren (B) pen, all in the same room (Figure 1).

All piglets were marked on the back with spray marker paint to allow individual identification. B pens measured 1.8m x 1.8m with concrete floors and rubber matting (1.8m x 1.2m) in one corner to provide some thermal insulation. Kennels were provided for the first 10 days. EE pens measured 3.6m x 1.8m also with concrete flooring and with large quantities of straw covering the floor (covering half the pen to a depth of 0.15m). The same rubber matting was also provided in the enriched pens. Piglets in the six EE pens were provided with additional enrichment once daily (enrichment stimulus) in the form of a plastic bag (approximately 400mm x 750mm x 150mm) filled with straw (approximately 2kgs). This bag was placed in the EE pens after morning husbandry was complete (at around 10 am). At the same time the gate of the matched B pen was approached to account for the stimulating effect of human presence. Pen cleaning and enrichment order was randomised daily. In accordance with the Defra Code of Recommendations for the Welfare of Livestock(41), temperature within the room was automatically controlled at 22°C and artificial lighting was maintained between the hours of 0800 to 1600, with low level night lighting at other times.

2.3 Behavioural observations

114 Piglets were digitally recorded in their home pen using Sony LL20 low light cameras with infra-red
115 and a Geovision GV-DVR. Two cameras were set up per EE pen, one at the rear and one at the front
116 to provide maximal coverage, and one camera per B pen. Piglets were observed in their home pens (
117 on five days between the ages of 41 and 53 days (+/-3 days due to staggered farrowing times). Piglets
118 were all observed on the same days during the same time periods to negate any time of day effects
119 on sampling. Observations began after bedding was replaced, 15 minutes prior to the time point
120 when the additional enrichment was provided to the EE pigs, and finished 4 hours after the provision
121 of additional enrichment. Behaviours were recorded by scan sampling with a frequency of one
122 minute(42). The observer used a 10 second window to aid in accurate identification of the behaviour
123 occurring at the start of every scan sample. A condensed ethogram based on previous work in
124 enrichment (43) and play (44) in pigs (Table 1) was used. All behaviours were recorded as
125 frequencies. One observer completed all video analysis to remove any reliability issues relating to
126 multiple observers.
127
128 **Table 1:** Ethogram used for behavioural scoring. Within the category of "Locomotion without object
129 or social", walking and running were combined to make up "locomotion-no interaction" in the
130 analysis. Individual postures were considered inactive behaviours and all other behaviours as active
131 because when active the posture is included in the active behaviour description and not scored
132 separately.

Category/Behaviour	***Definition/type***
Social	
Belly nosing penmate	A distinctive, rhythmic up-and-down movement of a piglet's snout against the belly of another piglet. Piglets may be standing, sitting or lying when preforming or receiving belly nosing
Tail biting	Manipulation, sucking or chewing tail of pen mate

Locomotion without object or social	
Walking	A steady 4 beat gait with 2 or 3 legs bearing weight at any one time (depending on the phase of the movement).
Running	Energetic running and hopping in forward motions often associated with excitability, using large areas of the pen, occasionally coming into marginal/accidental contact with other piglets
Hop/flop/pivot	High energy movements on the spot in an upwards (hop), downwards (flop) or circular (pivot) direction but with little or no forward propulsion
Object and pen Interaction	
Object interaction_bag	Any active interaction with the enrichment stimulus. Piglet may be locomotory or stationary. Includes biting/chewing of bag, walking or running with bag, climbing in bag, shaking bag vigorously and lying on bag.
Object interaction_no bag	Active interaction with other pen components, such as straw, rubber matting and shavings. As above may include walking or running with the object, and chewing/biting object
Nosing/exploring pen	Piglet walking/running or stationary with nose to floor or pen walls performing a gentle nosing motion
Feeding/drinking	Piglet oriented with snout in trough/drinker appearing to be chewing or swallowing
Postures	
Lateral lying	Piglet lying on its side
Ventral lying	Piglet lying on its front

Sitting	Piglet sitting on its haunches with front legs straight
Standing	Piglet standing still. Not performing any other visible behaviour
Other	
Not visible	Piglet out of view of camera

133

134 **2.4 Statistical analysis of behaviour**

135 The full dataset was condensed to give one data point for each behaviour per hour period per day for
136 each pen being the cumulative recordings for all piglets in that pen in that hour. Analysis was
137 performed on these mean values for each hour period per pen across the 5 observation days.
138 Analysis of the behavioural data was performed in Minitab version 17.3 (2016) using a generalized
139 linear model with treatment as a fixed effect and time point as a random effect.

140 **2.5 Tissue collection**

141 At 54 days of age (+/- 2 days) one male piglet per pen was sedated at one hour after the introduction
142 of the additional enrichment stimulus, and one male per pen at 4 hours post enrichment stimulus.
143 An intramuscular injection was used with a combination of Domitor (1mg/ml medetomidine
144 hydrochloride) (0.01ml/kg), Ketamine (0.1ml/kg), Hypnovel (5mg/ml medazolam) (0.1ml/kg) and
145 Stresnil (40mg/ml azaperone) (0.025ml/kg). Piglets were left for 15 min to allow sedation to take full
146 effect before being euthanized via intercardial injection with Euthatal (200mg/ml sodium
147 pentobarbital) (0.7ml/kg) for brain tissue collection. Piglet brains were removed whole and dissected
148 over dry ice. Frontal cortex was placed in RNAlater (Ambion) and agitated on a tube rotator for 15
149 minutes to assist in the perfusion of RNAlater through the tissue. Samples were frozen at -20˚C until
150 required.

151 **2.6 Preparation of tissue samples**

152 Frontal cortex samples in RNAlater were removed from -20˚C and allowed to reach room
153 temperature. RNA extraction was performed using the Qiagen RNeasy lipid tissue kit as per
154 manufacturer's instructions. 80mg of tissue was used from each sample and a TissueRupter (Qiagen)

155 used for homogenization. Extracted RNA was stored at -20˚C until required (<3months). Samples
156 were quantified and quality checked using a 2200 Tapestation (Agilent).

157 **2.7 RNA-seq and differential expression analysis**

158 Poly A+ library preparation and RNA sequencing (RNA-seq) of the prepared samples was carried out
159 by Edinburgh Genomics Next Generation Sequencing Service
160 (http://genomics.ed.ac.uk/services/sequencing) using the Illumina HiSeq 4000 platform, generating
161 >30 million stand-specific 75bp paired-end reads per sample. RNA-seq reads were processed using
162 the high-speed transcription quantification tool Kallisto(45), version 0.42.4, generating transcript-
163 level expression estimates both as transcripts per million (TPM) and estimated read count. Kallisto
164 quantifies expression by building an index of k-mers from a set of reference transcripts and mapping
165 the reads to these directly. For this purpose, we used the combined set of cDNAs and ncRNAs from
166 annotation Sscrofa11.1 (ftp://ftp.ensembl.org/pub/release-
167 90/fasta/sus_scrofa/cdna/Sus_scrofa.Sscrofa11.1.cdna.all.fa.gz and
168 ftp://ftp.ensembl.org/pub/release-90/fasta/sus_scrofa/ncrna/Sus_scrofa.Sscrofa11.1.ncrna.fa.gz,
169 respectively, accessed 1st November 2017). Transcript-level read counts were then summarised to
170 the gene level using the R/Bioconductor package tximport v1.0.3 (46), with associated HGNC gene
171 names obtained from Ensembl BioMart v90 (47). The tximport package collates Kallisto's output into
172 a count matrix, and calculates an offset to correct for differential transcript lengths between samples
173 (the consequence of differential isoform usage). The count matrix was imported into the
174 R/Bioconductor package edgeR v3.14.0 (48) for differential expression analysis, with the 'trimmed
175 mean of M values' method used to normalise gene counts. Genes with expression level estimates
176 lower than 2 TPM in both treatment groups were filtered out as noise. After filtering lowly expressed
177 genes, the final dataset comprised 21,971 genes. Genes were considered to be differentially
178 expressed (EE vs B) when they returned a between group fold-change greater than +/-1.2, with a *p*-
179 value <0.02. The sequencing data generated for this project is deposited in the European Nucleotide

180 Archive (ENA) under study accession number PRJEB24165
181 (http://www.ebi.ac.uk/ena/data/view/PRJEB24165).

182 **2.8 Gene set and gene signature enrichment analysis**

183 Gene set enrichment analysis was performed on the differentially expressed gene lists using
184 Panther.db v13.0 (release 20170413) with default parameters. To assess the statistical significance of
185 enrichment-induced variation in gene expression for small groups of genes with a common function
186 ('signature genes'), we used a randomisation test as in (49). Two sets of gene signatures, derived
187 from previous human studies, were first obtained for IEG genes (e.g. (50-58)) and microglial genes
188 (59) (Table S3), and filtered to remove those genes not annotated in pigs (resulting in n = 123 and
189 569 genes, respectively). Subsets of x genes were drawn at random s = 10,000 times from the set of
190 all genes for which a fold change is quantified (i.e. for each comparison of treatment groups), where
191 x = number of signature genes. We calculated q, the number of times the set of signature genes had
192 a higher (for IEG genes) or lower (for microglial genes) median fold change than each randomly
193 chosen subset. Letting $r = s-q$, then the p-value of this test is $r+1/s+1$.

194 **2.9 Network analysis of transcriptomic changes**

195 These expression values were entered into the network analysis tool Graphia Professional (formerly
196 Miru, derived from BioLayout *Express*^{3D} (60, 61); http://kajeka.com/graphia-professional). A sample-
197 to-sample analysis was performed using a correlation coefficient threshold of 0.99 to assess the
198 impact of individual variation on all gene expression profiles. A gene-to-gene network was also
199 created from the whole dataset using a correlation coefficient threshold of 0.90. The nodes (genes)
200 were clustered using the Markov Clustering (MCL) algorithm (62) with an inflation value (which
201 determines cluster granularity) of 2.2. This algorithm identifies tightly coordinated sub-structures
202 within the overall network.

203

204 **3. Results**

205 **3.1 Additional enrichment stimulation increases active behaviour**

206 An analysis of the percentage of active behaviours in EE vs. B housed piglets found a treatment by
207 time-point effect ($F_{4,43}$=4.2, p=0.005). The coefficients show this difference to be attributable to an
208 increased proportion of active behaviours in the EE piglets at one hour post provision of the
209 additional enrichment stimulus (hereafter enrichment stimulus; t_4=3.99, p<0.001)(Figure 2).

210

211

212 A housing treatment x time effect was observed in locomotor behaviour ($F_{4,43}$=3.19, p=0.021),
213 interaction with the enrichment stimulus ($F_{4,43}$=59.42, p<0.001) and interaction with other pen
214 components, such as rubber matting ($F_{4,43}$=2.75, p=0.038). These effects peaked at one hour post
215 provision of the enrichment stimulus to the EE pens with greater performance of locomotor
216 behaviour (t_4=2.75, p=0.008) and interaction with the enrichment stimulus (t_4=15.08, p<0.001), and
217 at 2 hours post enrichment stimulus with greater interaction with other pen objects (t_4=2.07, p=0.04)
218 in the B pens. A greater proportion of lateral lying in B housed pigs was observed at one hour post
219 enrichment stimulus (t_4=2.69, p=0.01). An effect of housing treatment alone was observed in nosing
220 of pen-mates ($F_{1,47}$=25.9, p=0.007) with more nosing of pen-mates occurring in the B housed piglets.
221 An effect of time alone was found in feeding behaviour ($F_{4,43}$=37.13, p=0.002), nosing of the pen
222 ($F_{4,43}$=11.47, p=0.018) and a trend towards lateral lying ($F_{4,43}$=4.3, p=0.09). No effect of time or
223 treatment was observed in any of the other behaviours recorded.

224

225 There was no difference between piglet growth during the pre-weaning period, prior to the start of
226 the study ($F_{(1, 47)}$ = 0.027, p = 0.873). Piglet growth (as measured by Average Daily Gain in weight,
227 ADG) was observed to differ between environments ($F_{(1, 47)}$ = 5.68, p = 0.038) during the study period,
228 with EE piglets having on average a higher ADG than B piglets (EE mean = 688.9g SEM = 15.5 , B mean
229 = 654.0g SEM = 12.6).

230

231 **3.2 Transient up- and downregulation of genes in response to environmental enrichment**

232 Complete lists of differentially regulated genes are provided in Table S1. Initial analysis revealed that
233 for a large majority of genes, expression in the frontal cortex was unaffected by the environmental
234 enrichment (Figure 3).

235

236

237 When all 21,971 genes were included in a network analysis (see Materials and Methods), members
238 of the same litter showed similar expression patterns, regardless of treatment or time. This was
239 particularly noticeable for litters L3, L4 and L6 (Figure 4A). Using the full gene set there was no
240 association with treatment (Figure 4B) or time (not shown).

241

242

243 A set of 349 genes were up-regulated after 1 hour of environmental enrichment but appeared to
244 return to baseline after 4 hours (Table 2). A second set of genes was down-regulated at both one
245 hour and four hours (Table 2) in the EE cohort. The composition of these two gene sets is discussed
246 below.

247

248 **Table 2:** Numbers of genes differentially expressed in the frontal cortex of piglets housed in EE and B
249 environments at one and four hours post enrichment. The last two rows combine all piglets within
250 each treatment regardless of time-point.

Comparison	Direction of Change	# Differentially Expressed Genes
1hr EE vs. 1Hr B	Up	357
	Down	652
4hr EE vs. 4hr B	Up	46

	Down	83
All EE vs. All B	Up	125
	Down	184

Gene set enrichment analysis at one hour post enrichment highlights an over-representation of genes involved in synaptic transmission being up-regulated in the EE animals (Table 3), with an over-representation of genes involved in apoptosis and cellular defence being down-regulated in the EE animals. Further analysis of the gene lists revealed that approximately 8% of those genes upregulated at 1 hour in the EE animals are known immediate early genes while 15% of those genes down-regulated in the EE (up-regulated in B) animals at 1 hour are related to microglial processes. At four hours post enrichment no gene sets were found to be overrepresented using the Panther GSEA overrepresentation test.

Significant *p*-values from randomisation tests (up-regulated IEG signature *p*=9.99E-05, down-regulated microglial signature *p*=9.99E-05) would suggest these signatures do not occur by chance in EE animals.

Table 3: Panther GO-Slim Biological Process GO terms statistically overrepresented in the 1 hour EE vs 1 hour B comparisons. The 'direction of change' column signifies the direction of differential gene expression in the EE animals relative to the B animals. The observed column is the number of genes assigned this GO term in the submitted gene list; the expected column is the number of genes expected given the full *Sus scrofa* reference list.

Comparison	Direction of Change	GSEA Overrepresentation Test (PANTHER release 20170413)				
		GO Terms	Observed	Expected	Fold Enrichment	*p*-value

1hr EE vs. 1Hr B	Up	Synaptic transmission	15	4.41	3.4	1.12E-02
		System development	14	4.45	3.14	4.53E-02
		Regulation of molecular function	16	5.30	3.02	2.54E-02
		Regulation of phosphate metabolic process	18	6.34	2.84	2.05E-02
	Down	JAK-STAT cascade	8	0.61	13.16	6.07E-05
		Negative regulation apoptotic process	16	2.83	5.65	1.15E-05
		Cellular defense response	14	2.64	5.3	1.68E-04
		Cell Adhesion	24	7.98	3.01	6.52E-04
		Immune system process	21	7.02	2.99	2.93E-03

In a network analysis using only the differentially expressed genes, the EE samples were close together in a relatively tight group, while the B samples were more dispersed, but located together with little overlap with the EE samples (Figure 4). In addition there was no relationship with litter, indicating that for the differentially expressed gene set the effect of environment was stronger than any intrinsic difference based on genetics or early life (e.g. maternal) effects (Figure 5).

278

279 The expression profiles of the differentially expressed genes were analysed to create a gene-to-gene
280 network. As might be expected (as these were differentially expressed in both directions) there were
281 two clear elements in the network, comprising one element where the overall gene expression level
282 was higher in the EE animals and a second element where the level was lower in EE animals. Similar
283 results were seen when using the 1 hour, 4 hour or complete set of differentially expressed genes
284 (Figure S1: Panel A-C segregated gene cluster histograms by animal, Panel D Clusters for all animals
285 at 1 hour).

286

287 When the DEGs at 1 hour were used to construct a network graph, cluster 1 (which contains the
288 majority of genes with average expression reduced in the EE condition) contained microglia-
289 associated genes such as *CSF1R* (63)*, IRF8* (64) and *P2RY12* (65) alongside various connective tissue
290 genes including several collagen genes and *FBN1.* Cluster 4, where the average expression was
291 increased in EE samples, contained immediate early genes such as *ARC,* several EGR genes, *FOS* and
292 *IER2.* Cluster 2 contained largely neuronal genes which were not differently expressed between
293 environments. Cluster 3 contained additional connective tissue genes with high levels in four of the B
294 individuals. Clusters 5 and 6 represent groups of genes that were increased in a single individual. The
295 gene lists for each cluster and average expression profiles for clusters where treatment differences
296 were shown are presented in Table S2 and Figure S1.

297

298 The profiles for the 4 hour samples were largely determined by high or low expression in a single or
299 small number of individuals. Many of the genes in this list were non-coding RNAs or unannotated
300 genes. When both time points were included in the analysis, cluster 3 primarily contained immediate
301 early genes and showed a pattern in which the 1 hour EE samples had higher expression than the 1
302 hour B samples, but at 4 hours the two groups had similar expression. One individual in the B group

303 had a spike of IEG expression at 4 hours although most of the 4 hour B samples showed lower IEG
304 expression than the EE samples.
305

306 **4. Discussion**

307 The EE piglets in this study displayed more active behaviour (combining locomotion, social and object
308 interaction) than B piglets, but only in the first hour after the additional enrichment stimulus (bag
309 filled with straw) was provided. This suggests that the piglets' behavioural response to enrichment in
310 this experiment was largely defined by the period that the additional enrichment stimulus remained
311 novel. Despite the larger space allowance and fresh bedding (straw) provided daily to the EE piglets,
312 their overall activity profile was not different to the B housed piglets outside of the first hour post
313 additional enrichment (using a scan sampling methodology). Overall total activity levels were lower
314 in this study than in previous studies (see (66) for a review), but the proportion of active time spent
315 interacting with the enrichment stimulus or the pen environment was higher than previously
316 reported. Within the time spent active there were some effects of housing on specific behaviours. B
317 housed piglets displayed more nosing of pen-mates (primarily nosing of the belly of other pigs) over
318 the course of the observation, not dependent on time. This maladaptive behaviour is known to occur
319 more frequently in piglets when the opportunities to use the snout to forage and explore are not
320 provided(67).
321

322 The gene expression changes observed in this study mirror those of the behavioural differences, with
323 most genes differentially expressed at one hour post the provision of the additional enrichment
324 stimulation and few genes differentially expressed at four hours post his point. The observed
325 increase in genes involved in synaptic transmission at the one hour time point in EE animals supports
326 previous work describing a transient increase in cell activity in the hippocampus of rats following
327 environmental enrichment (reviewed in (68)). This increase in synaptic transmission is likely to be the

328 mechanism by which EE leads to increased brain weight (69), greater cortical depth (70), increased
329 neurogenesis (6, 71) and increased synapse formation (72). The set of transiently-induced genes at
330 one hour shows significant overlap with recent curated sets of immediate-early response genes
331 defined in stimulated neurons based upon single cell RNA-seq (73, 74). Increases in expression of
332 neurotrophic factor proteins (neuronal growth factors) have previously been observed in multiple
333 brain regions of rats housed in enriched cages (14) and were observed as gene expression changes in
334 the frontal cortex of the piglets in the current study, although not all growth factors fall within the
335 gene set enrichment analysis. Neuronal growth factors (such as *NGF, BDNF* and *VGF,* all of which
336 were upregulated in this sample set) are considered to be regulators of cell growth and survival in
337 both the developing and adult nervous system (reviewed in(75)) and have been shown to acutely
338 potentiate synaptic plasticity (76, 77).
339 Experience dependant plasticity, the way by which synaptic connections are strengthened or
340 weakened, or indeed formed or eliminated, in response to experience is a well established
341 mechanism (reviewed in(78)). Intracellular scaffold protein and regulator of membrane trafficking
342 GRASP (mRNA upregulated in the EE pig frontal cortex) has multiple protein interacting domains
343 allowing a wide range of protein assemblies to be trafficked at the synapse (79). GRASP is known to
344 be involved in dendritic outgrowth (80) and in synaptic plasticity through its interaction with group I
345 and II metabotrophic glutamate receptors (81). Reductions in GRASP protein expression have been
346 observed in post mortem prefrontal cortex of schizophrenia patients (82) with no correlation to
347 illness duration or history of medication. Similarly, decreases in mGluR2 have been observed in
348 multiple psychiatric conditions including schizophrenia (83) and addiction (84), while treatment with
349 the antidepressant medication imipramine has been shown to increase mGluR2 protein expression in
350 the hippocampus of wild-type rats (85) but not to increase mRNA expression. Another G-protein
351 coupled receptor upregulated in the EE pigs, GPR3, has been shown to be involved in anxiety related
352 behaviours, with *Gpr3-/-* mice displaying increased anxiety-like behaviours that could be rescued
353 with the use of the anxiolytic diazepam (86). Upregulation of these G-protein coupled receptors and

354 their targets may suggest that EE piglets in the current study were experiencing reduced levels of
355 anxiety and a more positive affective state compared to their B counterparts.
356
357 A large proportion of the set of genes downregulated in the brains of the pigs exposed to
358 environmental enrichment (relative to those in the B environment) are clearly associated with
359 microglia. Numerous recent studies have identified microglia-associated genes in mice and humans
360 (e.g. (59, 87-90)) however there has been little work focused on the pig. The set of genes down-
361 regulated after 1 hour post the additional enrichment stimulation included generic myeloid markers
362 such as *PTPRC* (*CD45*) and *CD68*, as well as most well-documented microglial markers (*C1QA, MYD88,*
363 *P2RY12, TGFBR2, TREM2*). We suggest that these changes most likely reflect a selective 1.5 to 2-fold
364 relative reduction in the number of microglia in EE pigs when compared to B pigs. Current literature
365 suggests that in mice and rodents the number of microglia in the brain is relatively stable over time
366 (91) however in our dataset the differential expression of generic markers of microglia and not just
367 those found in active cells would suggest otherwise. With our study design it is impossible to
368 determine whether the EE or B animals are the baseline so any changes in expression are relative,
369 however whether this is microglial depletion in EE animals or an increase in microglia in the B animals
370 this result would suggest that the reduction of microglia is a longer term adaptation with benefits for
371 brain health (92, 93). Interestingly, at the 4 hour time-point there is a high proportion of 5s ribosomal
372 and spliceosomal RNAs identified as differentially expressed (29% of the down regulated gene list,
373 compared with 0.3% at one hour), which may be adding noise to the dataset in the later time-point.
374 As observed in the network analysis, inter-individual variability in gene expression was greater at 4
375 hours, perhaps due to the varying rates of decay of the effect of the additional enrichment stimulus,
376 or due to the animals no longer having a behavioural 'focus' (which may also explain the greater
377 variability in gene expression between B individuals than between EE individuals). This is consistent
378 with the behaviour analysis, which indicated that the benefit from the additional enrichment
379 experience (bag of straw) was only felt while the object remained novel. This may contribute to the

380 noise in the data at 4 hours, as animals may 'cope' with the lack of behavioural stimulation in
381 different ways, or may be being stimulated by other factors within the home pen (such as other
382 interactions with pen mates).
383 Experimental depletion of microglia in rat neonates results in lifelong decreases in anxiety-like
384 behaviours and increased locomotory behaviour (94), while increases in microglial numbers and
385 activation states have been observed in stress-induced depression models (reviewed in (31)). In
386 human patients, increased microglial activation has been observed using PET scanning in major
387 depressive disorder (95), and from histology samples from brains of depressed suicides (96). This
388 suggests that B housed animals may be experiencing greater anxiety and/or lower mood than their
389 EE housed counterparts. This may provide a functional link as to why pigs housed in enriched
390 environments display more optimistic judgement bias than those in barren conditions (97).
391 The current study would suggest the benefits of enrichment may occur in a pulse-like fashion, with
392 bursts of activity being followed by a return to behavioural baseline, these bursts mirroring the active
393 engagement with the behavioural opportunities offered by the additional enrichment stimulus. It
394 would be of great interest to determine if these bursts of activity (and of related differential gene
395 expression) alter the structure and function of the brain in a way that facilitates long term
396 developmental change. Confirmation of the longer term effects of EE on microglial numbers and
397 activation are required to determine if the observed gene expression changes are an indicator of
398 lower neuronal health in B housed animals or if they are transient expression changes with little long
399 term consequence.
400

401 **5. Conclusions**

402 In this study, behaviour was mainly organised around the time point when a daily enrichment
403 stimulus was provided. This resulted in a 'pulse' of active behaviour that was mirrored by temporal
404 changes in gene expression profiles in the frontal cortex. These gene expression changes primarily

affected genes involved in synaptic plasticity, neuroprotection and the immune response, with a substantial fraction (15%) of known microglial signature genes showing relative down-regulation with enrichment. Our analyses suggest that relative to piglets in barren environments, those in enriched environments are likely to experience reduced anxiety, increased neuroprotection and synaptic plasticity, and an immune response consistent with reduced inflammatory challenge.

6. Acknowledgements

We would like to acknowledge funding support for this work from the BBSRC Strategic funding to The Roslin Institute, and from the Scottish Government's Rural and Environment Science and Analytical Services Division (RESAS). Technical support was provided by the SRUC technicians Marianne Farish Agnieszka Futro, Jo Donbavand and Mhairi Jack. Farm manager Peter Finnie provided general animal husbandry assistance throughout the experimental period. Some behavioural data collection was performed by summer student Manon Poux and initial expression analysis by Edinburgh Genomics bioinformatician (Dr Frances Turner).

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656

657 **8. Figure Legends**

658 **Figure 1:** Diagram of the layout of the experimental room. Double lines signify the outer room doors
659 used for access. Grey circles indicate the location of cameras and filled black boxes show the location
660 of feeders. Litter of origin is identified as L1-L6. Empty pens are indicated by hatched lines. EE pens
661 were twice the size of B pens as indicated in the diagram. All pens were solid floored. EE pens had
662 the addition of straw over two thirds of the flooring.

663

664 **Figure 2:** Percentage of behaviours classified as 'active' at each time point for EE and B piglets. Active
665 behaviours include locomotion (running, walking, hopping, pivoting, jumping and climbing), object
666 interaction (with enrichment, i.e. bedding or enrichment stimulus, or components of pen) and nosing
667 (of pen-mates or pen structure). X-axis shows the time relative to the provision of the additional
668 enrichment stimulus.

669

670 **Figure 3:** Differentially expressed genes. (Panel A) Largely similar expression profiles of frontal cortex
671 in barren (x-axis) and enriched (y-axis) environments, with the exception of a subset of differentially
672 expressed genes. Each point is a gene, with expression level measured as the median transcripts per
673 million (TPM) across all replicates. The line y = x is shown in red. Differentially expressed genes are

674 more distant from this red line. (Panel B) Volcano plot of the fold change in expression for all genes.
675 X axis shows log2 fold change; Y axis shows –log10 *p*-value. Few genes demonstrate large fold
676 changes, with most clustering at the root of the graph.
677
678 **Figure 4:** Network analysis of all genes expressed in the frontal cortex. Each node (sphere) in the
679 network graph represents a sample (individual), and each edge (line) a Pearson's correlation
680 between samples ≥ 0.99. The local structure of the graph shows that gene expression profiles cluster
681 according to litter of origin (Panel A, where nodes are coloured by litter) and that there is little effect
682 of treatment (i.e. no clear clustering) upon overall gene expression (Panel B, where nodes are
683 coloured by environment).
684
685 **Figure 5:** Network analysis of differentially expressed genes in the frontal cortex. Each node (sphere)
686 in the network graph represents a sample, and each edge (line) a Pearson's correlation between
687 samples ≥ 0.93. There is no clustering of gene expression profiles based on litter of origin (Panel A)
688 and an unambiguous effect of treatment on gene expression (Panel B). Inter-individual variability in
689 expression profiles appears lower in the EE pigs than in the B pigs.
690
691
692 **9. Supplemental Information**
693 **Figure S1**: Cluster graphs from 1 hour Cluster lists found in Table S2 that show clear segregation
694 between EE and B animals. Panel (A) Microglia and connective tissue genes; Panel (B) Immune and
695 connective tissue genes; Panel (C) IEG; Panel (D) All clusters. Each column represents a single animal
696 with B house pigs as columns 1-6 and EE pigs columns 7-12. There is clear segregation in panel D of
697 the EE animals (top clusters) and B animals (bottom clusters)
698

699 **Table S1**: Differentially expressed genes at 1 and 4 hours. With log2 fold change, p value, average

700 counts and average transcripts per million for each treatment group.

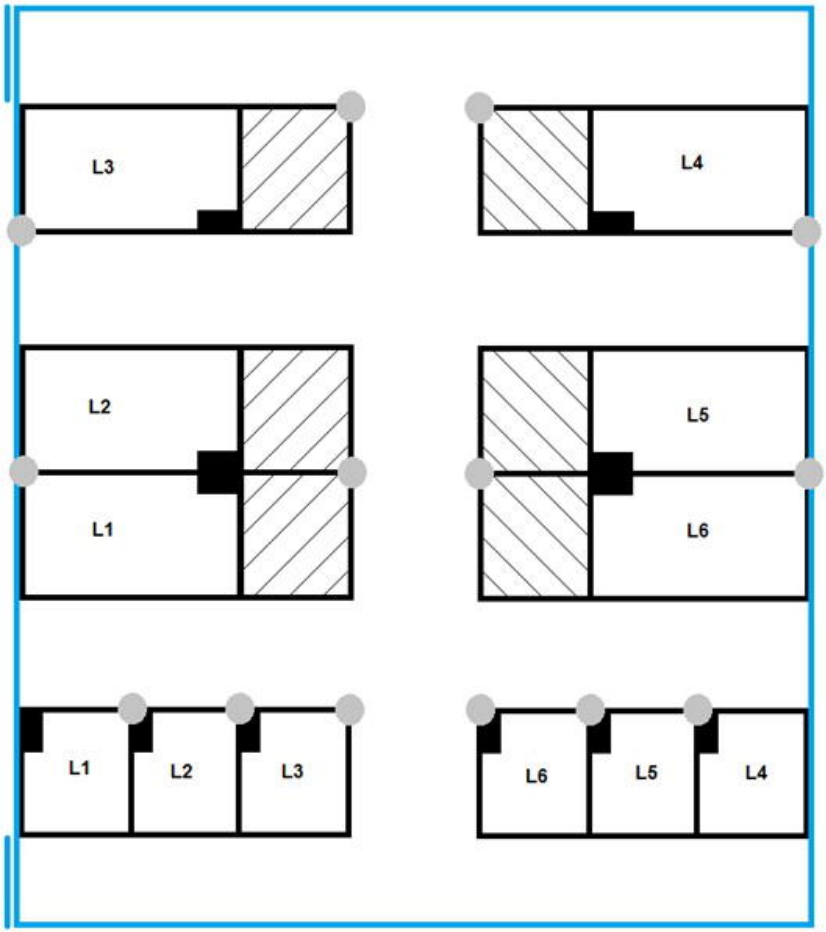
701

702 **Table S2**: Genes present in gene-gene cluster analysis.

703

704 **Table S3**: IEG and microglial reference gene list. Reference list used for randomisation tests.

705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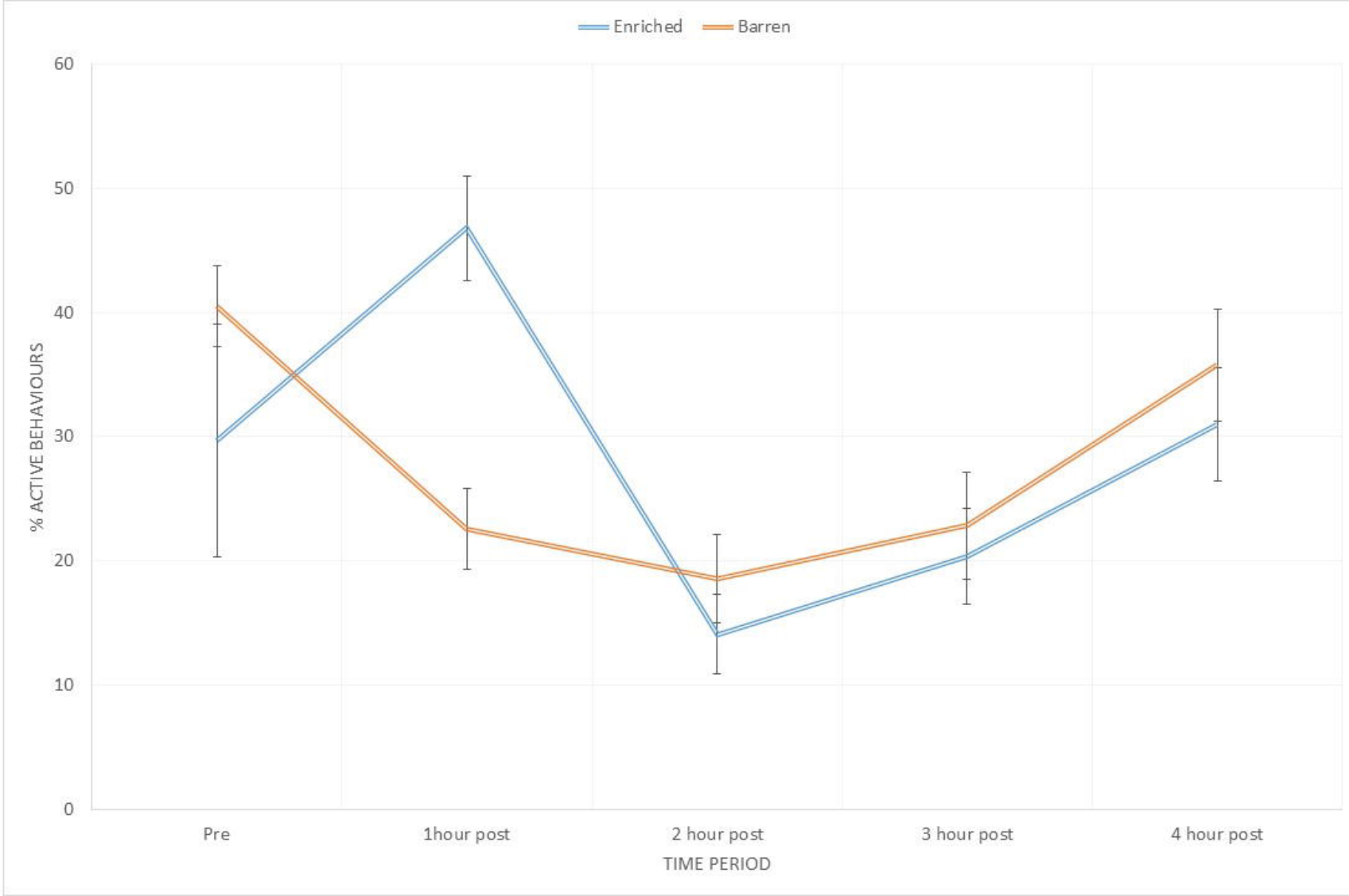
Enriched
Barren
60
50
40
30
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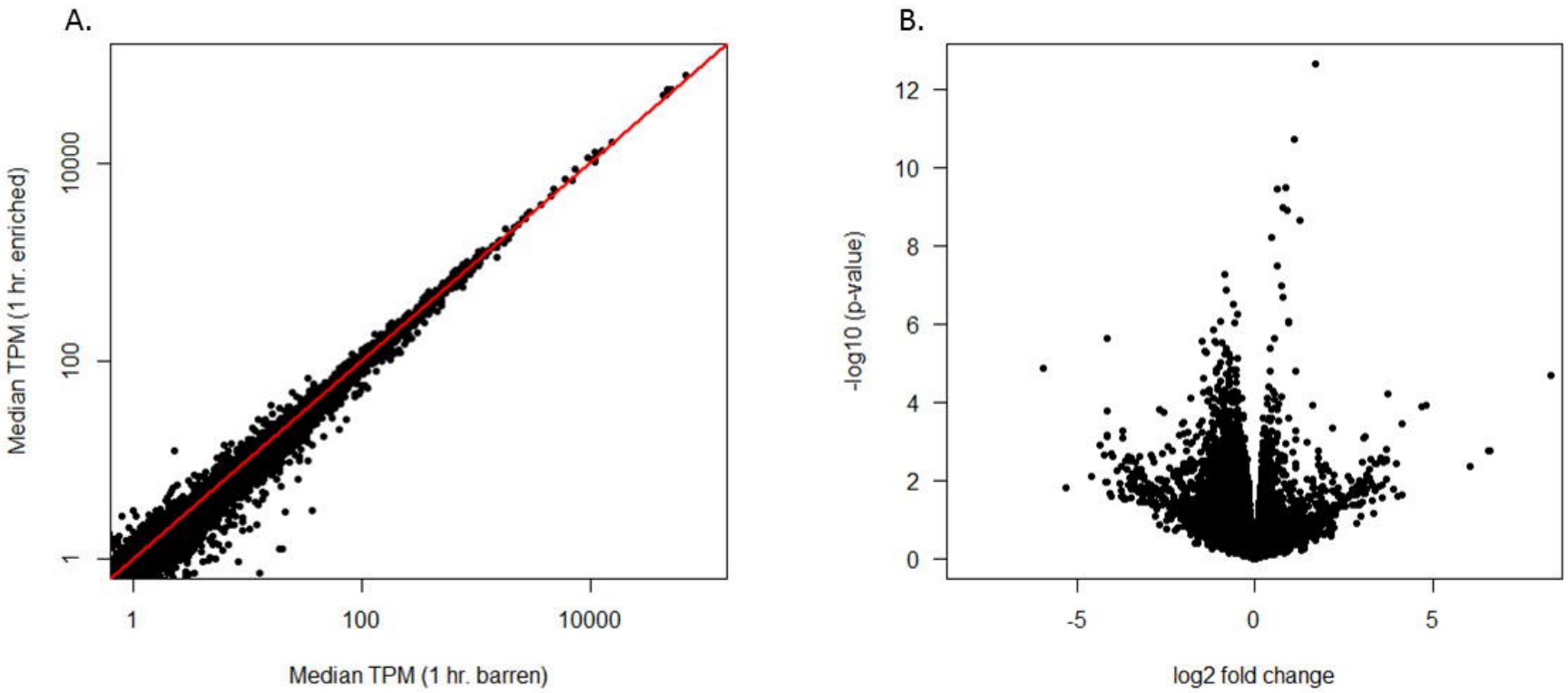
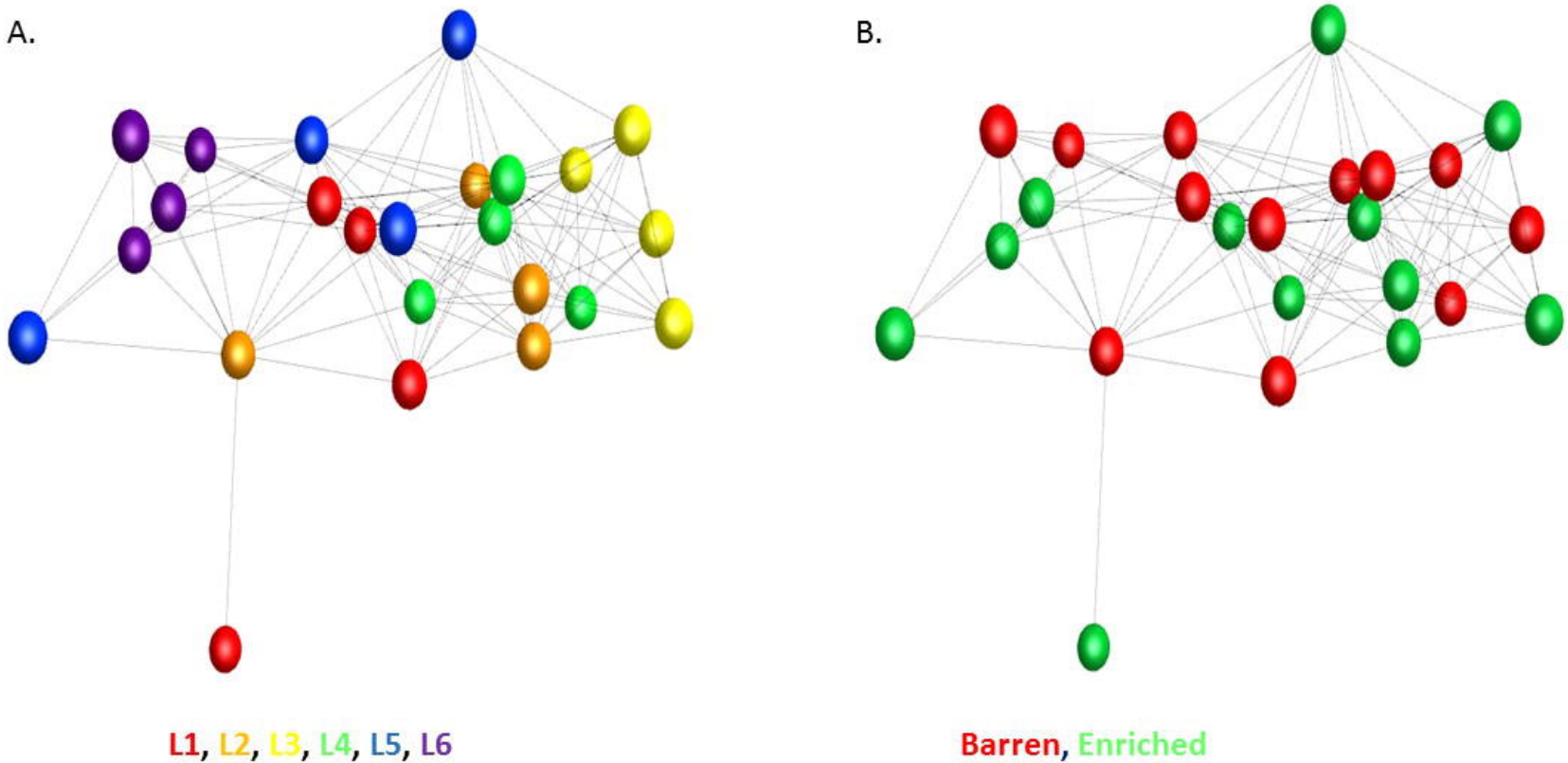
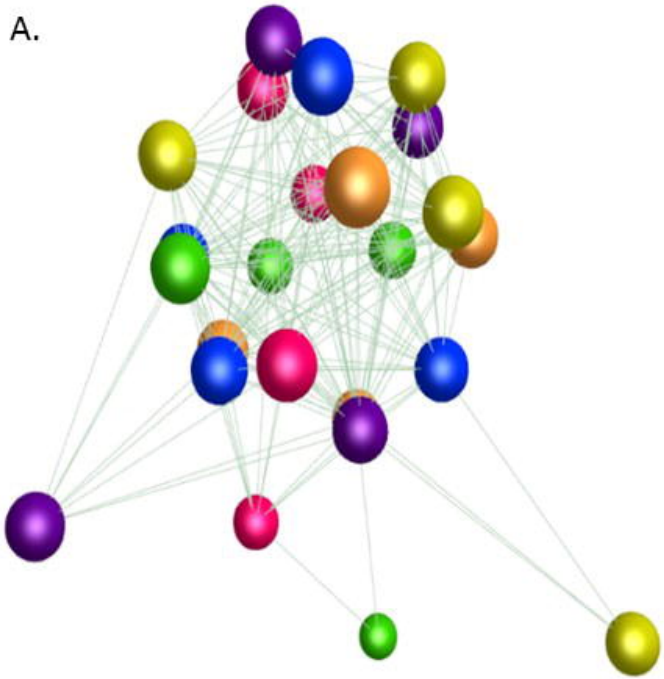
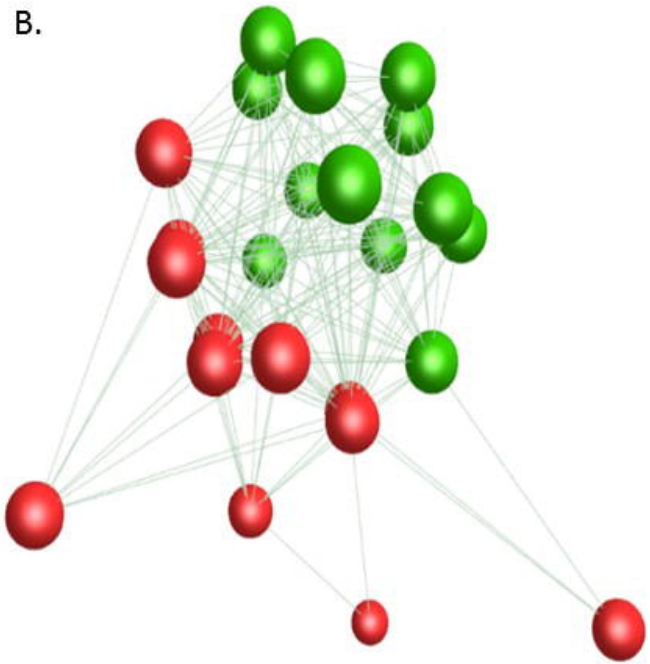
10
0
% ACTIVE BEHAVIOURS
Pre
1hour post
2 hour post
3 hour post
4 hour post
TIME PERIOD


A.
Median TPM (1 hr. enriched)
10000
100
1
1
100
10000
Median TPM (1 hr. barren)
B.
-log10 (p-value)
12
10
8
6
4
2
0
-5
0
5
log2 fold change


A.
B.
L1, L2, L3, L4, L5, L6
Barren, Enriched



L1, L2, L3, L4, L5, L6



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