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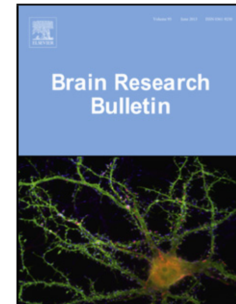

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A proteomic signature for CNS adaptations to the valence of environmental stimulation

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Graphical abstract

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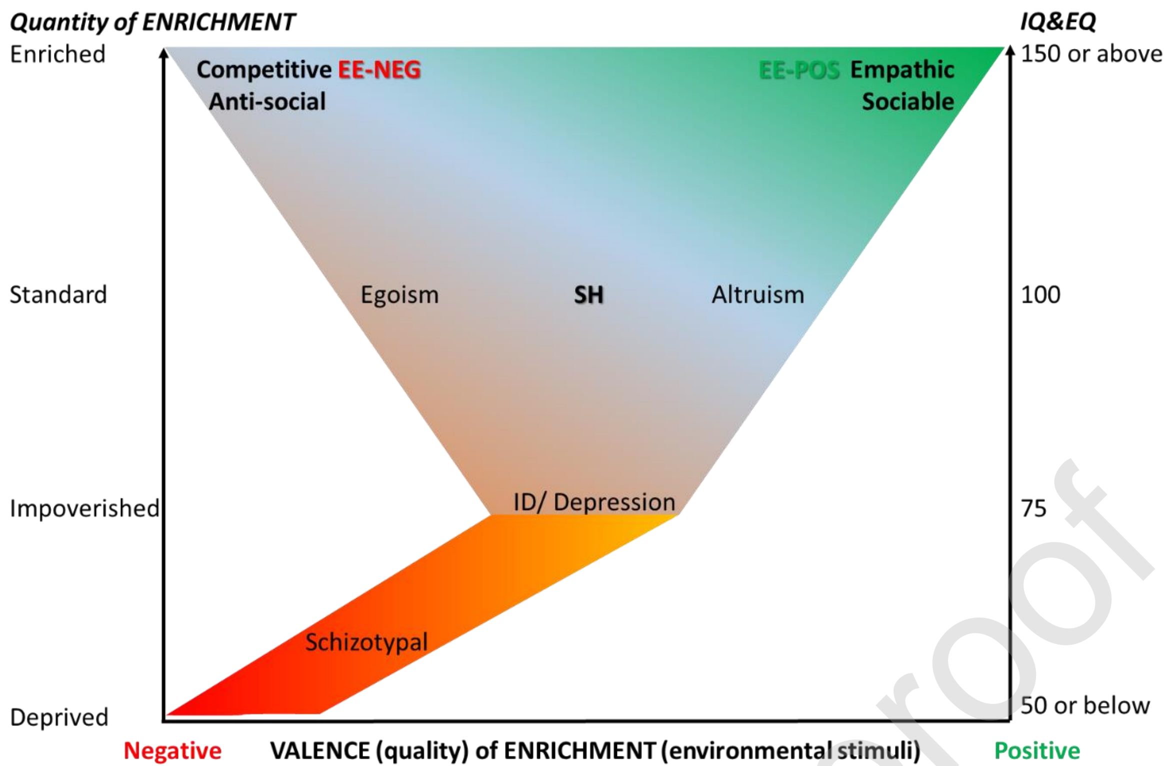

Highlights

- The valence of mice living environment can be heavily modulated in laboratory settings
- Environmental enrichment with stimuli carrying a negative valence enhances social competition
- The valence of environmental enrichment is associated with a specific proteomic signature in the CNS
- Negative enrichment is associated with the upregulation of proteins involved with mitochondrial metabolic processes related to TCA cycle and respiration

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Abstract

Environmental Enrichment leads to a significant improvement in long-term performance across a range of cognitive functions in mammals and it has been shown to produce an increased synaptic density and neurogenesis. Nevertheless it is still an open question as to whether some key aspects of spatial learning & memory and procedural learning might be embodied by different molecular pathways to those of social cognition. Associated with synaptic changes and potentially underlying conditions, the Ras-ERK pathway has been proposed to be the primary mediator of *in vivo* adaptations to environmental enrichment, acting via the downstream Ras-ERK signalling kinase MSK1 and the transcription factor CREB. Herein, we show that valence of environmental stimulation increased social competition and that this is associated with a specific proteomic signature in the frontal lobe but notably not in the hippocampus. Specifically, we show that altering the valence of environmental stimuli affected the level of social competition, with mice from negatively enriched environments winning significantly more encounters—even though mice from positive were bigger and should display dominance. This behavioural phenotype was accompanied by changes in the proteome of the fronto-ventral pole of the brain, with a differential increase in the relative abundance of proteins involved in the mitochondrial metabolic processes of the TCA cycle and respiratory processes. Investigation of this proteomic signature may pave the way for the elucidation of novel pathways underpinning the behavioural changes caused by negative enrichment and further out understanding of conditions whose core feature is increased social competition.

Keywords

Environmental enrichment, Social competition, Ras-ERK pathway, Proteomics, Hippocampus, Frontal pole

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Introduction

Environmental Enrichment (EE) leads to a significant improvement in long-term performance across a range of cognitive functions in humans and other animals [1-3]. EE has been shown to produce an increased synaptic density and neurogenesis [4]. In mammals, the Ras-ERK pathway has been proposed to be the primary mediator of *in vivo* adaptations to the environment [4-6]. Previous work in mice has shown that much of the cognitive enhancing effects of environmental enrichment act via the downstream Ras-ERK signalling kinase MSK1 and a relevant portion of spatial cognitive flexibility is mediated by the transcription factor CREB phosphorylation [4, 7, 8]. It remains still an open question whether some key aspects of spatial learning & memory and procedural learning might be governed by different molecular pathways than those controlling social cognition [9].

Most studies on anxiety and stress disorders affecting social cognition, apply one or more stressors which are often accompanied by social isolation and/or environmental impoverishment [10-14]. Stressful experiences, such as reductions in maternal care, changes in diet, and exposure to aversive stimuli, are known to lead to long lasting epigenetic modifications in the degree of DNA methylation/demethylation, or histone post-translational modifications such as acetylation [15-17]. While these models well recapitulate some endophenotypes of Post-Traumatic Stress Disorder (PTSD) and similar conditions where stress levels are high, they somehow fail to address the ecological validity of some other conditions that develop when levels of environmental stimulation are high and there is high competition within the social group. Highly competitive environments are a core feature of nowadays western societies and might also have a pathological manifestation in a spectrum of antisocial behaviours defined as Antisocial Personality Disorder (APD). APD subjects, including sociopaths and psychopaths, contribute significantly to violent offences among the general population [18]. The essential diagnostic feature of APD is a pervasive pattern of disregard for—and violation of—the rights of others that begins in childhood or early adolescence and continues into adulthood; DSM 5 Antisocial Personality Disorder 301.7 (F60.2).

In domestic violence crimes, APD prevalence is high in major offenders, which poses a serious and expensive threat for modern society. A European-wide investigation launched in 2014 (http://fra.europa.eu/sites/default/files/fra-2014-vaw-survey-main-results-apr14_en.pdf) has shown alarming figures of violence against women in EU countries; in the course of 12 months prior to survey interviews, 13 million women experienced physical violence and 3.7 million women had experienced sexual violence. APD or psychopathy are by no means a direct synonymous of physical aggression—yet they are regarded as a major risk factor.

It is well known that early life exposure to violence and abuse in humans leads to an increase in the violent crime rate—known as "the cycle of violence" [19]. Furthermore, in recent years, a growing body of work has shown how early life epigenetic modifications may underlie some of the behavioural

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changes observed in later life, including increased anxiety levels and a higher incidence of cognitive disorders and psychopathy [20].

Unfortunately, little is known regarding the mechanistic molecular basis of this complex disorder and consequently there are no effective pharmacological or therapeutic strategies available to help correct its aberrant behaviours. Hence, a key step forward towards a therapeutic strategy must be to uncover the biochemical and molecular basis of increased social competition and elucidate the pathways responsible for the development of associated behavioural traits.

In this experiment, mice were raised in two different enriched environmental conditions, one of which with high levels of competition so as to induce the conditions to trigger an APD-like adaptation. This was to mimic real-life conditions in human society that see sociopaths develop as highly intelligent individuals who show enhanced social competition and disregard for other people [21-25].

Three major environmental manipulations were utilised to increase social competition; (1) Female pheromones; (2) Predator pheromones; and (3) Overcrowding.

The presence of female scents is a powerful trigger for dominance fighting in adult male mice, with predator pheromones also known to induce a generalized level of anxiety and trigger defence behaviours [26-29]. Similarly, overcrowding was employed to increase social competition. Mice have preferred areas where they feel safe from predators and like to sleep and hide (e.g. shuttle box or inside some toys) [30]. In our overcrowding settings, there was not enough space in the safe areas for approximately half of the mice; thus, there would be continuously competing for these prime areas.

Results and Discussion

The main behavioural results are reported in Figure 1. Mice raised in the Negatively Enriched group (NEG) showed a more competitive/aggressive approach than the Positively Enriched group (POS)—this in spite of their lower body weight (Figure 1a; S1). Lower body mass would normally see a mouse as recessive in the wild, because a smaller mouse would almost inevitably lose an encounter with a heavier mouse [31, 32]. Moreover, these groups belonged to the EE housing condition, which made them quite fit with little extra body fat compared to standard housing. Further it should be noted that the environmental manipulations in the Negative group were introduced after weaning so to avoid overwhelmingly levels of stress, which would have induced PTSD [33], rather these mice successfully adapted to their more competitive environment as proven by their normal growth and gross behaviour.

The increased competition of the NEG group was also more directly tested with the T-tube paradigm (Figure 1b), whereby one POS and one NEG had to physically compete with one another to gain access to the exit leading to the oestrous female.

Several factors that theoretically could have affected the results of the two tests were investigated. One hypothesis being that the NEG were more active regardless of their environment; however, this was not

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the case, with POS group in fact being more active in the Open Field and showing similar responses to a novel object inserted after the initial stage (Figure S2).

A different explanation could also be argued to the T-tube test; this would see mice raised in different enriched environments as having different pattern of exploration in enclosed environment. This was tested in the Elevated Plus maze, which revealed no difference between POS and NEG in investigating the closed zones of the maze (Figure S3).

Furthermore, as expected, mice in these groups did not substantially differ in their spatial working memory ability, nor in their short-term memory (Figure S4). Interestingly, they did show a different pattern of interaction to objects and conspecifics (Figure S5, S6, S7), thus indicating a major effect of this oppositely enriched environment to have exerted its effects in the fronto-ventral portion of the brain, rather than on the hippocampus.

To investigate this, a bottom-up “shotgun” proteomics study was carried out using LC-MS/MS in these two separated brain areas important for social cognition, spatial memory, and action planning, known to be altered in APD patients: the frontal pole and the hippocampus (Figure 2) [7, 34-38]. LC-MS/MS analysis identified 977 proteins from the hippocampi and frontal lobes of mice from positively and negatively enriched groups and determined their normalised abundance (see supplementary data for raw data tables).

Of the total number of proteins identified, none were increased or decreased in the hippocampus (supplementary data). However, after correction for false discovery rate (FDR), the relative abundance of 47 proteins were seen to be significantly increased in the frontal lobes of mice from the negative enrichment group, compared to those from the positive enrichment group (Figure 2).

Gene ontology (GO) enrichment analysis of the altered proteins from the frontal lobes identified several Biological Processes and Cellular Components affected by the negatively enriched environmental stimuli (Figure 3). The functional characterisation of the 47 differentially produced proteins in the frontal lobe identified a clear signature of upregulated proteins related to the mitochondrial processes of TCA cycle activity and aerobic respiration, in response to negative enrichment (Figure 3C). The proteins included alpha-actinin-3, citrate synthase, dihydrolipoyl transacetylase (an enzyme component of the pyruvate dehydrogenase (PDH) complex), and succinate dehydrogenase complex flavoprotein subunit A.

Adding to this clear metabolic proteomic signature, mitochondrial respiratory processes related to electron transport and oxidative phosphorylation were identified in the upregulated proteins of negatively enriched frontal lobes (Figure 3B). These included the components of complex I (Ndufs6a, Ndufv1) and complex II (Sdha) of the electron transport chain (ETC), but also the mitochondrial calcium-binding carrier protein Slc25a12.

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Other processes identified centred on ATPase activity and transmembrane proton transport, which included components of the vacuolar-type H^+-ATPase complex (Figure 3A). Moreover, a number of upregulated calcium-related proteins were identified to be associated with the presynaptic cytosol (calbindin 1, protein kinase c alpha, and protein kinase c epsilon) in frontal lobes due to negative enrichment (Figure 3 D).

Whilst the Ras-ERK pathway is thought to control transcriptional events in Neuro Developmental Disorders and ageing [7, 39-41], little is known about the downstream mechanisms. However, metabolic regulation of mitochondrial ATP production lies central to neuronal and glial function and processes related to neurotransmission [42-44].

Mitochondria are thought to play a pivotal role in neuronal function and synaptic regulation in neuropsychiatric disorders [45, 46]. Furthermore, mitochondria and reactive oxygen species (ROS) have been implicated in the developmental control of neuronal processes such as synapse pruning [47]. That we have identified a striking proteomic signature related to upregulation of proteins involved in the mitochondrial processes of the TCA cycle and the electrons transport chain in frontal lobes of mice due to negative enrichment, may be important in the elucidation of novel mechanistic pathways underlying the development of endophenotypes proper of APD.

The relative abundance of various proton-transporting ATPase proteins was also seen to be increased in the frontal lobe due to negative enrichment. These ATPase proteins pump protons across the plasma membrane in order to acidify a wide-range of intracellular and extracellular environments, such as secretory synaptic vesicles [48]. Moreover, in cancer cells, increased V-ATPase expression is associated with various of metabolic and signalling adaptations [49]. Thus, changes in the abundance of proton-pumping ATPase enzymes in neurons of the frontal lobe may have far-reaching implications for both neurotransmitter release and neuronal/glial metabolism in the context of increased social competition.

In summary, we have proven that the valence of mice living environment can be heavily modulated in laboratory settings and that environmental enrichment with stimuli carrying a negative valence enhances social competition. Further we show evidence that the valence of environmental enrichment is associated with a specific proteomic signature in the CNS and Negative enrichment is associated with the upregulation of proteins involved with mitochondrial metabolic processes related to TCA cycle and respiration.

So a clear proteomic signature in the frontal lobes (but critically not the hippocampi) in the Negatively enriched mice paralleled increased social competition, due to a negatively enriched environment. Further work is required to investigate the molecular mechanisms underpinning the roles of mitochondrial metabolism and ATPase proteins in models of increased social competition.

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All efforts were made to minimize animal suffering and to reduce the number of mice used, in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Author contributions

AS performed and analysed the proteomics experiment. LM designed and analysed the behavioural paradigms. LP and LDA performed part of the experiments. AS, MKD and PDW oversaw the proteomics analysis. LM conceived and oversaw the study. LM and AS wrote the manuscript. All authors approved the final version of the manuscript.

Author Information

The authors declare no competing interests.
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The data that support the findings of this study are available from the corresponding author.

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Material and Methods

Mice and cages

Forty-two C56/BL6J mice were brought in from Charles River when they were P21 and housed as follows: 16 in Positive enrichment (POS) 8 per GR1800 cage (Techniplast) and 16 in Negative enrichment (NEG) in one GR1800 cage. Ten mice were housed in standard cages and kept as reference for normal growth. The mice were injected s.c. with a 0.8mm transponder (IMI-500) when they were P43.

Lights were on h07:00 – h19:00. Temperature was kept 21 (±1) ºC. Humidity was kept 50-70%.

The Enriched cages included one shuttle box, one seesaw tube, one big running wheel, multiple tubes, one ball, two wooden blocks for chewing and some bedding, along with food and water ad libitum. NEG also had one small wheel due to the number of mice within being double than POS to make sure they were getting the same opportunity to exercise.

Cages were cleaned each week. This included fresh sawdust and mouse bedding (fresh mouse bedding not added to NEG). Clean toys were also added to NEG at cleaning, as the intention was to induce strong environmental change, while toys were gradually changed in POS to minimize environmental disruption. Rat bedding was introduced to NEG two to three times a week for extra aversive stimulation. To add some extra stimulation with the intention to increase competition within NEG female mice's bedding was also introduced. Some females were also individually introduced into the cage protected inside a wire-cup for 10 minutes each to trigger intra-cage competition. This was done after the social recognition tests had been completed.

Behaviour software, statistics and experimental design

The order of the mice in behavioural experiments was semi-random so to balance for cage composition. Mice were video-tracked and analysed with Anymaze 6.1.

SPSS 25 was employed for statistical calculations. A mixed model ANOVA was employed with Housing condition and Enrichment as the between group factors. Within factors were used as appropriate (e.g. days, time).

Figures legend: *= $p<0.05$; **= $p<0.01$. If no otherwise stated graphs depict Mean and SEM.

Testing started at P50 and followed the order as below (one type of test per week);

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Physiology

Body weight

Mice were regularly weighed and checked to make sure they were healthy and growing with a scale having a 0.1g precision. Three of the initial 32 mice were culled for health reasons (hydrocephalus or teeth misalignment).

Social competition

Reciprocal Interaction with freely moving oestrous female

The bottom part of a GR1800 cage was placed under the camera. Clean sawdust was on the bottom. One NEG and one POS mouse were simultaneously released each into one of the sides of the cage, as far away as possible form the freely moving female. Further to starting point balancing, mice pairings were made accordingly to body weight so to avoid discrepancies over 10%. This test was carried out under Red-lights.

T-tube test

The bottom part of a GR1800 cage was placed under the camera. Clean sawdust was on the bottom. A set of mouse size tubes were set up to form an inverted T shaped figure (25 x 15cm). Fifteen cm apart from opening of the central tube, a female mouse in oestrous was placed inside a wire cup.

One NEG and one POS mouse were simultaneously released each into one of the two lateral openings of the T shaped tubing so that they will have to meet each other in the middle and competed to get out towards the female. The first mouse to exit the tube towards the female was scored.

Further to starting point balancing, mice pairings were made accordingly to body weight so to avoid discrepancies over 10%. This test was carried out under Red-lights.

Exploration and Anxiety

Open Field Novel Object

Four Open field boxes (Ugo Basile 44 x 44 x 44cm) were fit onto a table and tracked simultaneously. This had two 30-minute stages. In the first stage each mouse was released into the arena and video-tracked. For the second stage of the test, the novel object, a 50-ml Flacon tube was placed upside down in the centre of the arena. Horizontal locomotor activity was scored.

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Elevated Plus Maze

Four of the eight arms or a radial maze were left open to form a +; two of the open arms, facing each other, had walls (the closed area) and two were wall-less (the open area). Mice were released individually and tracked for 10 minutes.

Spatial working memory

Spontaneous Alteration

A mouse size eight-arm radial maze (Ugo Basile) was used keeping only 4 arms open so to form a cross. Mice were individually released in the central hub and left free to explore for 10 minutes. Number and sequence of arm entries was recorded. A minimum threshold of 20 entries was applied to all groups.

Short term memory

Novel Object Recognition

Novel object recognition used the same arenas as the open field and novel object. There were two stages. Acquisition with 2 identical objects positioned 30 cm apart lasted 5 minutes, the ITI was 60 minutes and the test stage lasted 3 minutes with an old and a new object (330 ml can and 350 ml juice bottle). Time spent directly investigating the objects by tracking the head position.

Social Recognition

Sample investigation

This test used the same arenas as the open field and novel object. There was a single 10-minute stage and two 50-ml beakers were presented 30 cm apart; one was filled with fresh clean sawdust and a the other with sawdust from a cage of 5 unfamiliar adult male mice. The set up for this was to have the more salient stimulus (the biological sample) in the opposite position to the set-up of the novel object. Latencies and time spent directly investigating the samples by tracking the head position.

Social interaction

3-Chamber

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Two mouse size 3-chamber arenas (60 x 40 x 30cm) were tracked simultaneously. SH mice were used as baits within the wire cup. They were habituated to the wire-cup 2 times for 10 minutes the day before the test. Test mice were habituated to the 3-chamber for 20 minutes the day before testing occurred. In stage 1, only the central compartment of the 3-chamber was free to the mouse to explore for 5 minutes, in the next 10 minutes (stage 2) all 3 chambers were free to explore, one had an empty and clean wire-cup while the other had a SH male mouse inside. Baits were balanced to test mice.

Brains withdrawal

Two weeks after behavioural testing was terminated mice were culled by cervical dislocation and the brains quickly removed;

Left hemispheres (sagittal cut) were included in OCT and snap frozen for later analysis.

Right hemispheres were quickly dissected and the whole hippocampus (H), frontal pole (Striatum, Nucleus accumbens, Amygdaloid nuclei and Frontal cortices) (F) and the rest of the brain (R) were put in 3 cryovials and snap frozen. These were the samples used for proteomics analysis.

After snap freezing (liquid nitrogen) all samples were promptly placed in dry ice and then stored at -80°C shortly after.

Sample preparation for proteomic analysis

Frozen brains sections (frontal lobe and hippocampus) were defrosted on ice and re-suspended in 300 µL ultrapure water with added cOmplete™ EDTA-free protease inhibitor cocktail (Roche). Samples were sonicated twice for 30 seconds each. A BCA protein assay was then performed on homogenised samples, which were then diluted to 1mg/mL protein in ultrapure water. Thereafter, a PreOmics iST 96x Sample Preparation Kit was used to prepare the samples for mass spectrometry proteomic analysis. Briefly, in order to digest the samples, 50 µL of kit lysis buffer was added to 100 µg protein/100 µL of each sample, which were incubated on a heating block at 95°C for 10 minutes. Samples were then sonicated again to remove DNA.

Using the supplied adapter, cartridges were placed above the waste plate and samples added. 210 µL of re-suspend reagent was added to the digest vial and vortexed. 50 µL of the re-suspended digest reagent was then added to each sample cartridge, before place the samples on a heating block at 37°C for 2 hours. 100 µL of reaction stop reagent was then added to each sample. In order to purify the samples, the cartridge plate was centrifuged at 3800 rcf for three minutes, before 200 µL of wash reagent was added and the plate spun again (wash repeated twice). The samples in the cartridge adapter were then placed on top of the MTP plate and 100 µL of the elution reagent was added, before the plate was

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centrifuged and the supernatant collected. Samples were placed in a SpeedVac and dried at 45° C until completely dry. 100 µL of LC-loading buffer was then added to each sample before sonication (4 x 30 seconds). Samples were then diluted 1:10 in loading buffer for injection into the LC-MS/MS.

Proteomic analysis by LC-MS/MS

Peptide analysis was performed in positive ion mode using a Thermo LTQ-Orbitrap XL LC-MS^n mass spectrometer equipped with a nanospray source and coupled to a Waters nanoAcquity UPLC system. The samples were initially desalted and concentrated on a BEH C18 trapping column (Waters, Manchester, UK). The peptides were then separated on a BEH C18 nanocolumn (1.7 µm, 75 µm x 250 mm, Waters) at a flow rate of 300 nL/minute using an ACN/water gradient over 3 hours. MS spectra were collected using data-dependent acquisition in the range m/z 200-2000 using a precursor ion resolution of 30,000 following which individual precursor ions (top 5) were automatically fragmented using collision induced dissociation (CID) with a relative collision energy of 35%. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 s and exclusion duration of 180 seconds. Data were analysed using Progenesis QIP (Non-Linear Dynamics, Newcastle, UK) with the MASCOT (Matrix Science, London, UK) search engine against the UniProt database. The initial search parameters allowed for oxidation of methionine, a precursor mass tolerance of 10 ppm, a fragment mass tolerance of ± 0.5 Da, and a FDR of 0.01.

Proteomic data analysis

Analysis of variance (ANOVA) was performed for three-way comparison for normalised protein abundance. P-values adjusted for an optimised false discovery rate (FDR) were used to calculate q-values, whereby q-values ≤ 0.05 was considered statistically significant between normalised protein abundance means. Z-score were calculated from the fold change values of significantly altered protein abundances, which were then plotted on a heat map. Significantly alerted proteins were input into the ClueGO plug-in of Cytoscape in order to perform enrichment analysis and the output was plotted as clusters of genes in functionally grouped networks.

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Figure 1: Social Competition can be increased by modulating the Valence of Environmental Enrichment

Figure 1a. **Social interaction with oestrous female.** The number of initial interactions with the female (in oestrous) was superior in the negative group; independent samples t-test (26)= -2.895, p: 0.008 (two-tailed).

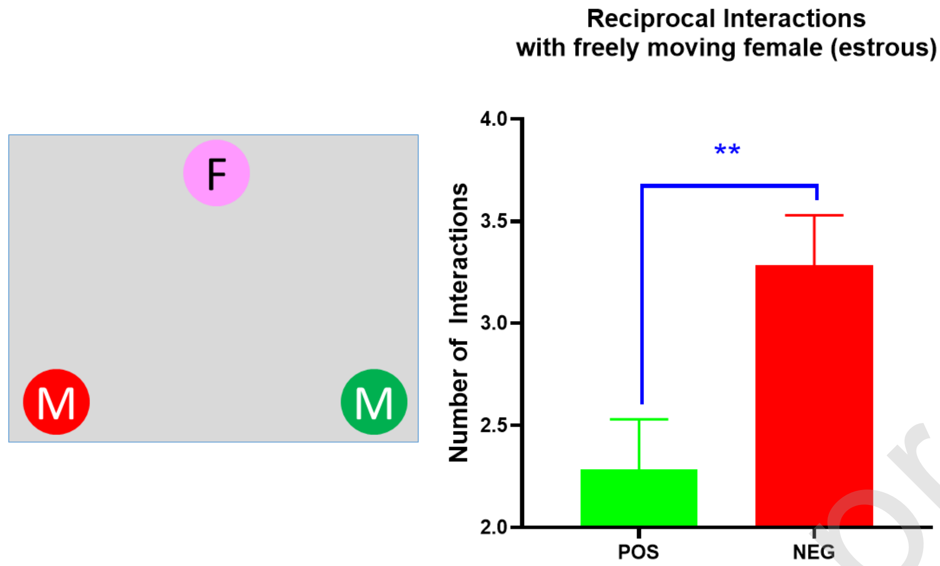

Figure 1b. **T-tube**. The Fisher's exact test gave a p: 0.007 (two-tailed) indicating the Negative group winning 11 out of the 14 encounters.

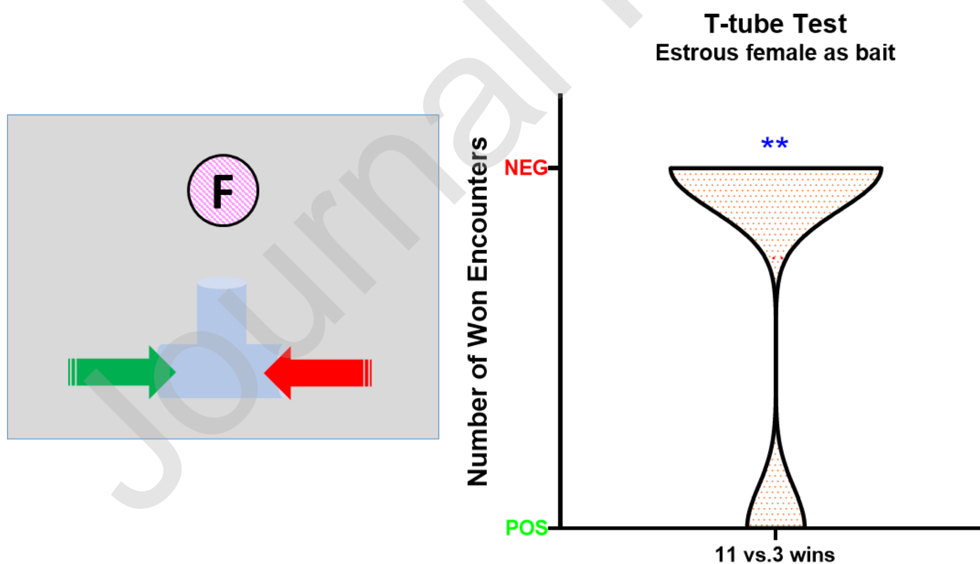

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Figure 2: Proteins differentially regulated in brain's fronto-ventral pole of mice raised in a highly competitive form of environmental enrichment. Heat map showing fold-change difference (plotted as Z-scores) of the 47 differentially produced proteins in the frontal lobes of mice from positively enriched environments (A1-5) and negatively enriched environments (B1-5) (ANOVA, n = 5; $q < 0.05$). No significant differences were found in hippocampi.

Data were ranked by fold difference between positive enrichment and negative enrichment, with higher abundance shown in red and lower abundance shown in blue.

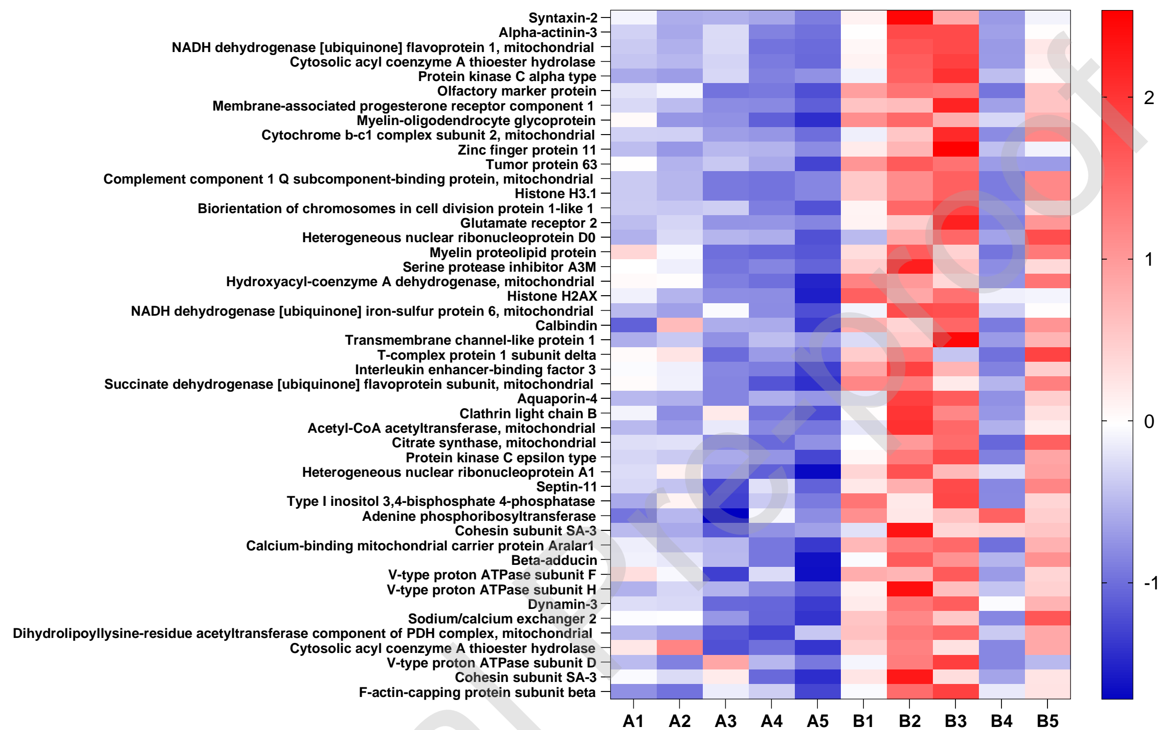

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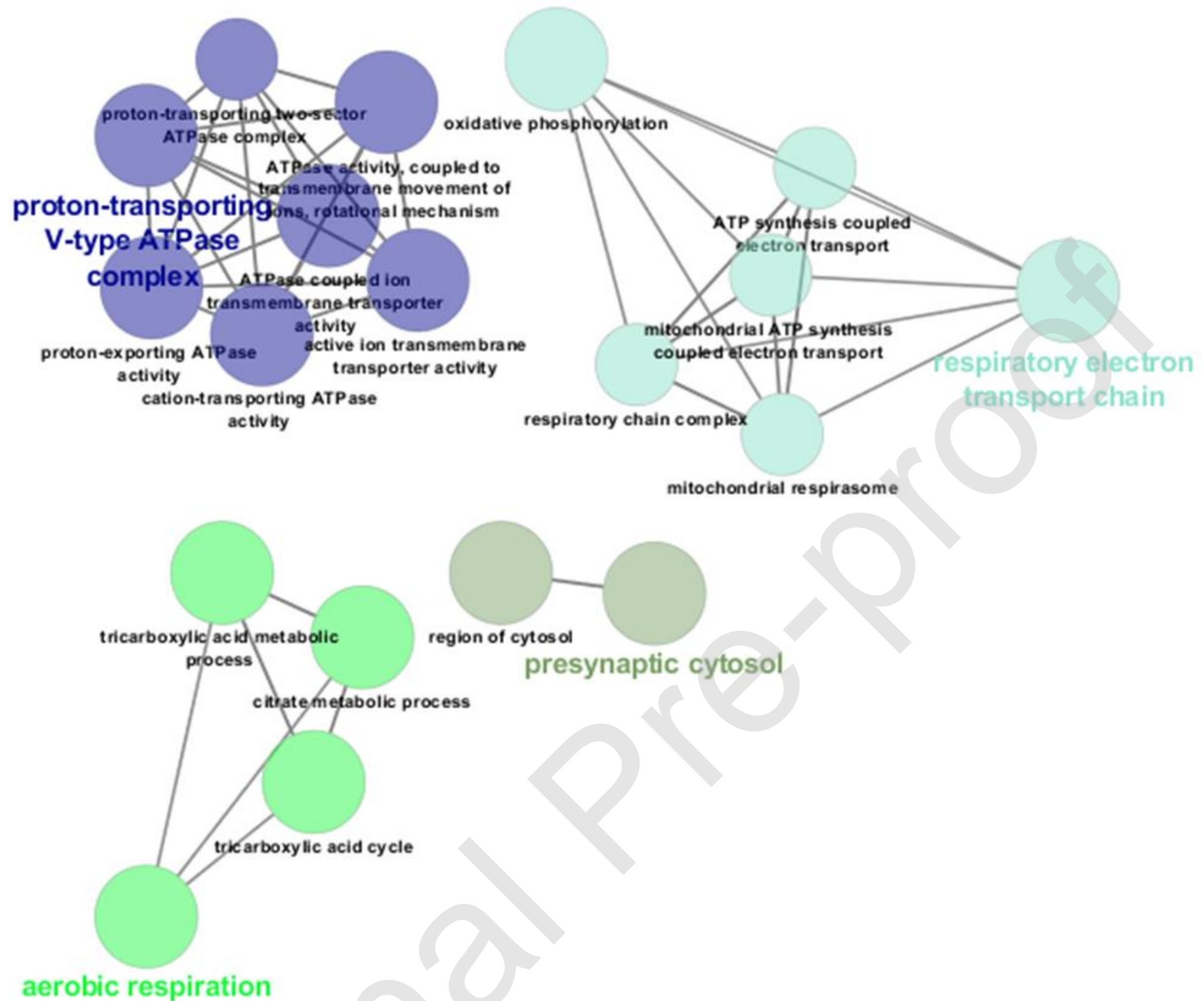
Figure 3. Network analysis of functional interactions was performed using the Cytoscape plugin ClueGo. The 47 differentially produced proteins measured between positively enriched and negatively enriched groups were mapped to GO categories (Biological Processes, Molecular Function, and Cellular Component). The most significant term in each cluster is highlighted in bold.



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