Environmental temperature variation affects brain protein expression and cognitive abilities in adult zebrafish (Danio rerio): A proteomic and behavioural study.

Environmental temperature variation affects brain protein expression and cognitive abilities in adult zebrafish (*Danio rerio*): a proteomic and behavioural study

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Abstract

Water temperature is an important environmental parameter influencing the distribution and the health of fishes and it plays a central role in ectothermic animals. The aim of this study is to determine the effects of environmental temperature on the brain proteome and the behavioural responses in zebrafish, a widely used animal model for environmental "omics" studies. Adult specimens of wild-type zebrafish were kept at 18°C, 34°C and 26°C (control) for 21 days. Proteomic data revealed that several proteins involved in cytoskeletal organization, mitochondrial regulation and energy metabolism are differently regulated at the extreme temperatures. In particular, the expression of proteins associated to synapses and neurotransmitter release is down-regulated at 18°C and 34°C. In both thermal conditions, fish exhibited a reduced interest for the novel environment and an impairment of cognitive abilities during Y-Maze behavioural tests. The observed pathways of protein expression are

possibly associated to functional alterations of the synaptic transmission that may result in cognitive functions impairment at central nervous system level as those revealed by behavioural tests. This study indicates that temperature variations can elicit biochemical changes that may affect fish health and behaviour. This combined approach provides insights into mechanisms supporting thermal acclimation and plasticity in fishes.

Keywords: environmental temperature, behaviour, *Danio rerio*, shotgun proteomic, Y-Maze

Introduction

All organisms have to cope with changes in environmental parameters during their life. Among these, particularly relevant is the variation of the environmental temperature that can deeply alter the habitat of the species [1, 2] and impact at all the biological levels from population to molecule affecting the survival and longevity of the organisms. The poikiloterm animals such as fish, whose body temperature varies with the environmental temperature, are particularly sensitive to thermal variations and water temperature is considered the major ecophysiological variable for aquatic ectotherms, influencing physiology, behaviour and distribution of animals [3].

The ecological response of fish to temperature changes is behavioural and involves the movement of animals to more favourable areas of the environment [4]. If the animal fails to reach such areas, it has no choice but to acclimatize and adapt to the environmental changes to survive. Thermal acclimatization is based on complex and not fully understood physiological processes known as *temperature compensation* that allow acclimated (or adapted) animals to modify their thermal sensitivity [5]. The variation of enzyme concentration by transcriptional and translational regulation is thought to play a crucial role in temperature compensation.

Recent advances in the characterization of fish genomes together with the application of functional genomic and proteomic techniques [6] gave a strong impulse to studies on the effect of thermal variations on eurytherm and stenotherm fish and this has become a robust and growing research field [7]. Data obtained in different species clearly demonstrated that thermal stress impacts on all the biological levels, as the variation in environmental temperature can influence nutrition, growth, morphology, gonadal development, sex determination, swim performance, anti-predatory behaviours, protein use and metabolism [8]. Recent transcriptional studies have shown that thermal stress at the brain level induces the differential expression of many genes such as chaperones, transcription factors, gene products involved in signal transduction pathways, lipid metabolism, and translation [7]. Moreover, thermal stress affects the synthesis, release and kinetic properties of neurotransmitters such as acetylcholine [9-12].

The advances in proteomics techniques allow analysing the variation of the expression of proteins belonging to specific pathways and the progress in neuroscience have allowed identifying specific pathways involved in specific cognitive processes. This gives us the opportunity to carry out a more in-depth analysis of the effect of thermal variation on the animal's cognitive abilities compared to transcriptomic studies, since transcript variations do not necessarily reflect the actual variations in protein expression for the possible existence of post-transcriptional regulatory mechanisms.

Cognitive abilities are extremely complex and only partially understood processes that involve synaptogenesis, synaptic plasticity and rearrangements of the neuronal cytoarchitecture. Numerous proteins and signalling pathways are known to play a role in cognitive processes, as those triggered by growth factors, i.e. the platelet derived growth factor (PDGF) [13] and the epidermal growth factor (EFG) [14-16], or adhesion receptors, i.e. some integrins [17, 18].

Thermal stress and adaptation to temperature require changes in brain protein expression that potentially compromise sensory perception, integrative functions and/or motor control. Such alterations of nervous functions could in turn induce behavioural variations that can be investigated by reliable ethological tests.

In this study, we performed a proteomic and behavioural analysis on adult specimens of wild-type zebrafish (*Danio rerio*, ZF) kept at three different temperatures: 18°C, 34°C and 26°C (used as control) for 21 days to investigate the effect of temperature changes on the Central Nervous System (CNS).

The ZF is a poikiloterm and euriterm cyprinid characterized by wide thermal tolerance from 6.7 to 41.7°C [19, 20] that is widely used as a model organism in many research areas. Wild ZF lives in freshwater streams of the South East Asian regions that are characterized by daily temperature fluctuations of ∼ 5°C and wide seasonal temperature variations ranging from 6°C in winter to more of 38°C in the summer [21].

The expected results may provide useful information not only in the neurochemical and ethological field, but also in the ecological field, considering that the global warming occurring in our planet will induce considerable environmental temperature variations [22] together with extreme climatic events such as heat waves and droughts periods [23].

Materials and Methods

Subjects

A total of 99 adult (6–7 month old) wild type ZF, purchased from commercial dealers, was used in the present study.

Sex ratio was about 50:50 male:female and the mean weight was 0.4 grams. Fish were randomly placed in three tanks (40 x 30 x 30 cm, width x depth x height) of 33 litres

(hometanks) and maintained at 26°C (control temperature) for 10 days to acclimate to the tank (adaptation period). Fish, 33 for each tank, were maintained under an artificial photoperiod (12:12 light/dark cycle) and fed three times a day (10 am, 2 pm and 6 pm) with commercial dry granular food (TropiGranMIX, Dajanapet) by using automatic fish feeders (Eden 90, Eden Water paradise, Germany). 1.3 grams/day of food, corresponding to 10% of body mass, were administrated to each tank. The water used throughout the experimental phase was produced by reverse osmosis pumps (Reverse Osmosis AquiliOS2) and reported to the appropriate salinity adding aquarium salt (1g/L, Aqua Medic 301.01). In order to ensure good water quality, a constant flow of filtered water (600 l/hr) was maintained by external filter systems (Eden 511h) in each tank and water was also continuously aerated (7.20 mgO_2/L) by aerator for aquaria (SicceAIRlight, 3300 cc/min 200l/h). The chemical/physical characteristics of tank water were checked at least two times per week by measuring the values of water hardness, pH, ammonium (NH_4), ammonia (NH_3), nitrate (NO_3), nitrite (NO_2), phosphate (PO_4), copper (Cu) and calcium (Ca^{2+}) with the Sera aqua-test box kit (Sera Italia srl). The salinity was checked with a hand-held refractometer. Faeces and the remaining food waste were removed from the animal tanks at least three times per week. During the tank-cleaning operations, a water exchange of about 20–30% per week was performed to restore the correct volume of water and to maintain its chemical–physical parameters.

Thermal treatment

Subjects were exposed to thermal treatment. In two of the three hometanks the water temperature was gradually brought from 26°C to 18°C or 34°C in 72 hours. Fish were then maintained at the two experimental temperatures, 18±1°C and 34±1°C, for 21 days. Fish maintained at 26±1°C were used as control. The three temperature values were chosen according to Vergauwen et al. [24] within the ZF vital range and correspond to temperatures

the fish cope with in the natural environment. The water temperature was kept constant by external water chiller (TK 150 Teco) or digital thermostats (Eden 430) connected to a heating coil (Eden 415, 230V, 50/60Hz, 80W).

The water temperature was further checked daily using a hand thermometer. The interior enrichment of each tank (consisting of a heating coil, inlet and outlet pipes of the filters and aerator) was replicated identical in all the tanks. During the thermal treatment only three fishes died: two at 18°C and one at 34°C. All the experimental procedures were approved by the Animal Care Committee and authorized by the Italian Ministry of Health (protocol number 290/2017-PR).

At the end of the thermal treatment, of the total of 33 fishes present in each hometank, 20 individuals were subjected to the behavioural test, 9 were used for proteomic analysis and the remaining fish were stored for mRNA expression analysis. All the individuals enrolled in the study were euthanised individually by a prolonged immersion in a solution of the anaesthetic tricaine methanesulfonate MS-222 (300 mg/L). All the procedures were performed between March and May 2017.

Proteomic analysis

Total protein expression in fish brain homogenates was analysed and compared under the three thermal regimes. Although this analysis is not able to discriminate proteins expressed in the different cell populations of the brain (neurons, glial cells, endothelial cells), it provides a useful tool for assessing the impact of thermal treatment on the brain function and neurochemistry.

At the end of thermal treatments, the brain proteome from ZF adapted at the three temperatures (not subjected to behavioural tests) was analysed by a shotgun label free proteomic approach for the identification and quantification of expressed proteins. At each

temperature, 9 whole brains were homogenized using a Potter homogenizer in 500 µL of extraction buffer (8 M urea, 20 mM Hepes pH 8, with protease inhibitors Complete Mini) at full speed for 1-3 min. The homogenate was centrifuged at 10000 rpm for 10 minutes to sediment unhomogenized tissue and large cellular debris. The pellet was discarded and the protein content was determined by a bicinchoninic acid assay (Thermo Fisher Scientific). Proteins were subjected to reduction with 13 mM dithioerythritol (30 min at 55°C) and alkylation with 26 mM iodoacetamide (IAA; 30 min at RT). Peptide digestion was conducted using sequence-grade trypsin (Roche) for 16 hours at 37°C using a protein:trypsin ratio of 20:1 [25]. The proteolytic digest was desalted using Zip-Tip C18 (Millipore) before mass spectrometric (MS) analysis [26]. LC-ESI-MS/MS analysis was performed on a DionexUltiMate 3000 HPLC System with a PicoFritProteoPrep C18 column (200 mm, internal diameter of 75 µm) (New Objective, USA). Gradient: 2% ACN in 0.1 % formic acid for 10 min, 2-4 % ACN in 0.1% formic acid for 6 min, 4-30% ACN in 0.1% formic acid for 147 min and 30-50 % ACN in 0.1% formic for 3 min at a flow rate of 0.3 µL/min. The eluate was electrosprayed into an LTQ-OrbitrapVelos (Thermo Fisher Scientific) through a Proxeon nanoelectrospray ion source (Thermo Fisher Scientific) [27]. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350-2000) in the Orbitrap (at resolution 60000, AGC target 1000000) and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan (normalized collision energy of 35%, 10 ms activation). Isolation window: 3 Da, unassigned charge states: rejected, charge state 1: rejected, charge states 2+, 3+, 4+: not rejected; dynamic exclusion enabled (60 s, exclusion list size: 200) [28]. Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

Statistical and bioinformatic analysis of the proteomic data

MS spectra were searched against the ZF Uniprot sequence database (release 01.04.2015) by MaxQuant (version 1.3.0.5). The following parameters were used: initial maximum allowed mass deviation of 15 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks, trypsin enzyme specificity, a maximum of two missed cleavages, carbamidomethyl cysteine as fixed modification, N-terminal acetylation, methionine oxidation, asparagine/glutamine deamidation and serine/threonine/tyrosine phosphorylation as variable modifications. False protein identification rate (5%) was estimated by searching MS/MS spectra against the corresponding reversed-sequence (decoy) database. Minimum required peptide length was set to 6 amino acids and minimum number of unique peptide supporting protein identification was set to 1.

Quantification in MaxQuant was performed using the built-in label-free quantification algorithms (LFQ) based on extracted ion intensity of precursor ions [29, 30].

Four replicates were carried out for each group: 18°C, 34°C and 26°C used as the control. Only proteins present and quantified in at least 3 out of 4 technical repeats were considered as positively identified in a sample and used for statistical analyses. Statistical analyses of Max Quant results were performed using the Perseus software module (version 1.4.0.6, www.biochem.mpg.de/mann/tools/). A one-way analysis of variance (ANOVA) test was carried out to identify proteins differentially expressed among the different conditions. Proteins were considered to be differentially expressed if they were present only in 18°C, 34°C, or 26°C samples or showed significant t-test difference p-value (cut-off at 0.05 FDR). Focusing on specific comparisons, namely 26°C vs 18°C and 26°C vs 34°C, proteins were considered differentially expressed if they were present only in one condition or showed significant t-test difference (Welch's test p value = 0.05). Bioinformatic analyses were carried out by Panther software (Version 10.0) [31] and Revigo to reduce redundant GO terms [32], DAVID software (release 6.7) [33], BINGO and Enriched Map [34] to cluster enriched

annotation groups of Molecular Function, Biological Processes, Pathways and Networks within the set of identified proteins. Functional grouping was based on p value ≤ 0.05 and at least two counts. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [35] partner repository with the dataset identifier PXD009934.

Y-maze apparatus

The Y-maze was built by replicating exactly the same features of the maze successfully used on ZF by Cognato and collaborators [36]. Briefly, the Y-Maze was built with three arms of glass (25 x 8 x 15 cm width x depth x height) in which the single arm was identified by the presence of geometric shapes (square, circle and triangle) made of white paper and placed on the external walls and visible from the inside. The work by Cognato et al. [36] demonstrated the ability to recognize, distinguish and not to be feared by the geometric shapes chosen. In addition, in our setting, the external walls of the maze were coated by a 2 cm thick polystyrene panel to ensure the heat insulation and to keep constant the water temperature for the entire duration of behavioural tests.

Preliminary tests were carried out to ensure that the water temperature remained constant during behavioural testing. The difference in water temperature measured at the beginning and at the end of each experimental test was 1°C at maximum. A diffuse light was present in the room to avoid directional lighting that could interfere with the fish behaviour. All tests were video recorded by a webcam (Logitech C170) placed one meter above the maze.

Behavioural testing

The maze was filled with 4 litres of water at the same temperature at which the animal had been acclimated and the water depth was 6.5 cm, enough to submerge the geometric shapes

present on the sides of the arms. The total number of experimental subjects was 60 (20 for each temperature). Each single fish was captured by using a beaker and transferred from the hometank to waiting tank (size 15 x 10 x 10 cm, width x depth x height) for 30 minutes until the beginning of the behavioural test.

One single task consisted of four trials (T1, T2, T3 and T4) separated by a one-hour interval. Each trial consisted of a training phase (Tr) in which the fish could freely swim in the start (S) arm and in the other (O) arm for 5 minutes but it could not have access to the novel (N) arm for the presence of a dividing wall, and of a testing phase (Te) in which the wall was removed and the fish was free of swimming for 5 minutes all over the maze also exploring the novel environment constituted by the N arm. The assignment of circle, square, and triangle to the S, O and N arm was randomized for each experimental subject.

The behaviour was analysed using the ANY-Maze® software (Stoelting Co., Wood Dale, IL, USA). Locomotor activity was evaluated by measuring the mean speed, the total distance travelled, and the rotations of the body (clockwise, CW and counterclockwise, CCW). The exploratory activity and the interest in the novel environment were evaluated by quantifying the time spent in each arm, the number of passages among arms and the degree of exploration of the novel arm. In order to determine whether the temperature influences fish tendency to explore the novel environment in its entirety, the N arm was virtually divided into three equal parts named as sector 1, 2 and 3 starting from the centre of the maze. At the end of each trial the experimental subject was transferred to the waiting tank for one hour until the next trial. The temperature of the water contained in the waiting tank was the same to which the fish had been acclimated. After each trial the water contained in the maze was removed and the apparatus was rinsed and filled with clean water.

Statistical analysis of functional behavioural data

Results were expressed as mean±SEM. Data were subjected to ANOVA analysis with a post hoc test utilizing Bonferroni correction by ORIGIN® 2018 software. Depending on the data considered, one-, two- or three- way ANOVA analyses were performed (refer to caption of Figures 4 and 5 for details). Differences were considered to be statistically significant at $p \leq 0.05$ and $p \leq 0.01$.

Results and Discussion

Proteomic analysis

To evaluate the possible effects of temperature on protein expression, we have adopted a quantitative label free shotgun proteomic approach. This method allows to examine the impact of different conditions by achieving the simultaneous identification of thousands of proteins and their quantification in each sample. Therefore, it is well suited for studying differences in global protein expression between samples and provides substantial information to delineate cell signalling pathways involved in thermal responses. The whole brains of ZF acclimated for 21 days to three temperatures (18°C, 26°C and 34°C) were homogenized and submitted to tandem mass spectrometry analysis. The identification of the proteins from the MS/MS data was then achieved using a database search by MaxQuant which compares acquired mass spectra to a database of known sequences to identify the proteins. This strategy allowed the identification of 1735, 2077 and 1817 proteins at 18°C, 26°C and 34°C, respectively. The comparison of the three data sets highlights the presence of proteins exclusively expressed at a single temperature, as well as 1499 proteins present at all temperatures, among which 125 are differentially expressed at the thermal regimes tested. Bioinformatic analysis carried out on these proteins by DAVID software suggested that

different temperatures affect mainly cytoskeleton and ribosome and, to a lesser extent, carbon metabolism and mitochondrion related categories (Table 1).

Specific analyses were carried out by comparing 18°C and 34°C with 26°C, kept as the control condition. The lists of proteins differently expressed are documented in the supplementary material (Tables S1-S4) reporting the common proteins in the two comparisons whose differential expression is statistically significant according to Welch's test and also the proteins exclusively expressed at each of the temperatures tested. Figure 1A and B reports the corresponding Volcano plots. According to our results, 290 proteins are exclusively expressed at 18°C or down regulated at 26°C (Table S2) whereas 225 proteins are exclusively expressed at 34°C or down-regulated at 26°C (Table S4).

To disclose the effect of temperature treatment on the ZF brain proteome, the proteins differentially expressed in the comparison 26°C$_{vs}$18°C and 26°C$_{vs}$34°C were analysed for functional grouping. Interestingly, most proteins whose expression decreased in response to thermal changes (18 and/or 34°C) are associated to different steps of protein synthesis, from RNA translation to protein folding, localization and degradation (Table 2). All these proteins are more expressed or only expressed at 26°C suggesting that heat or cold temperatures may hamper the normal protein synthesis cascade.

GO enrichment analysis according to Panther software (Tables S5-S8), BINGO and Enriched Map (Figure 2) suggests that this effect is possibly mediated by a significant impact on the cytoskeleton. GO Slim Biological Processes (GOBP), GO Slim Molecular Function (GOMF) and GO Slim Cellular Component (GOCC) highlight changes in structural cytoskeleton constituents, cellular morphogenesis and organization at extreme temperature conditions and a more pronounced effect on motor activity, transmembrane movement and vesicle mediated transport at 18°C and 34°C (terms are highlighted in bold in Tables S5-S8). This finding is in agreement with the functional grouping analysis by DAVID that shows a decrease at 26°C of

proteins related to cytoskeleton and transport or motor activity (Table 3). At low temperature the GO enrichment analysis reveals a profound impact on morphogenesis, cytoskeleton organization and synaptic transmission, as well cell communication all decreased. Cold also induces a marked alteration of the actin cytoskeleton and actin binding proteins (Tables S5, S6, Figure 2).

The fold enrichment analysis gave more information on cold and heat effects. There are pathways exclusively enriched at 34°C or 18°C (Figure 3) and most pathways altered at the two temperatures decreased in comparison to controls at 26°C (Figure 3). The effects on WNT signalling pathways are quite interesting. Beside the canonical WNT pathway that leads to the regulation of gene transcription, two non canonical WNT pathways were described: the planar cell polarity pathway that regulates the cytoskeleton, and the WNT/calcium pathway that regulates calcium levels inside the cell [37]. By targeting the cytoskeleton directly or through Rho GTPase [38], WNT signalling alteration may be related to a significant impact of temperature on the cytoskeleton in ZF brain (Figure 3).

Temperature treatment: impact on metabolism and transport

Data reported in Table S8 show an increase in the transketolase and transaminase activity suggesting that the pentose metabolism (PPP) prevails at 34°C in keeping with the decrease of the oxidative phosphorylation at 34°C compared to 26°C [Tables 2, S7 (underlined terms)]. The need of the cell to counteract the oxidative stress induced by high temperatures could explain the increase associated with the PPP which produces NADPH necessary both for DNA production and for the regeneration of reduced glutathione, which in turn plays an important role in the regulation of the intracellular redox state by providing reduced equivalents for antioxidative pathways (Tables 3, S8, Figure 2B) [39]. However, the cell

redox balance is hampered by heat due to a decrease of the antioxidant and peroxidase activity (Tables 2, S7).

The alteration of mitochondrion-associated proteins found at 34°C suggests a perturbed cell energy metabolism that may compromise cell physiology and brain functioning. Most of the energy produced in the brain (75% -80%) is consumed by neurons [40] that are highly dependent on ATP amounts necessary to support synaptic vesicle mobilization, to generate the membrane action potential [41] and to ensure calcium homeostasis at the synaptic level [42, 43]. The different cellular districts need different amounts of energy and the synapse has a high energy requirement for restoration of neuronal membrane potentials following depolarization [44]. For these reasons, a reduced energy availability in the neuronal cells may induce synaptic impairments [45]. Moreover, the reduction of proteins involved in the mitochondrial transport (Table S7) at 34°C may suggest a defective mitochondria positioning in synaptic terminals [46] where they are involved in regulating neurotransmission [47-50] and synaptic plasticity [51, 52]. The defective transport of mitochondria is believed to contribute to the onset of neurodegenerative diseases [53-56]. Our proteome results are consistent with literature data showing that high temperature induces mitochondrial uncoupling and dysfunction by the reduction of cristae, oxidative phosphorylation and ATP synthesis [57-59]. Experimental evidence shows that the impairment of mitochondrial function can lead to synaptic degeneration [46, 51, 60, 61]; consistently, our results show a reduction in proteins associated to synapse, neurotransmitter secretion, receptor-mediated endocytosis and endosome suggesting an impairment of intercellular communication (Table S7).

Heat also induces the increase of ion transporter activity and transmembrane movement of substances, muscle contraction, motor activity, nuclear and protein transport and the increase in lipid and fatty acids metabolic processes (Table 3, Figure 2B).

At 18°C, the data reported in Figure 2 clearly show an increase of catalytic activity, catabolic processes and TCA cycle (Table S6, Figure 2B), in accordance with findings suggesting that cold stress increases the cellular content in ATP and ADP as possible strategy for offsetting kinetic effects of low temperatures on the reaction rates [62].

The increase in proteins associated with catalytic activity, catabolic processes, carbohydrate metabolism and TCA cycle (Table S6, Figure 2B) found at 18°C might be related to cellular production of a greater amount of ATP to counteract the reduced enzyme kinetics and vesicular mobility occurring at low temperatures [63-65]. The increased expression of proteins involved in the transport of vesicles and polypeptides (Table S6) could be also related to the restoring of the axon flow slowed by low temperatures. However, the reduction of proteins associated to synapse and neurosecretion was detected at both 18°C and 34°C (Table S5).

Consequently, the exposure to thermal extremes both determines synaptic function impairments that, associated with the modulation of signal transduction pathways, might be responsible for functional alterations of the CNS.

Temperature treatment: impact on cytoskeleton

As shown in Table 3 and Figure 2, heat treatment has a profound impact on cytoskeleton, mitochondrial organization and chromatin remodelling. We then asked if the temperature itself may lead to a change in cytoskeleton that can reverberate on cell signalling. It is known that temperature modulates stiffness and elasticity of cells and recent findings in rat demonstrated that neurons display a significant drop in the average elastic modulus with increasing temperature [66]. The decrease in neuron stiffness is linked to an increase in myosin II activity at high temperature since active myosin II fluidizes the cells while inactive myosin II acts as a cross linker for F-actin. Moreover, metabolic activity of the myosin II

motor is dependent on the temperature and availability of ATP [66-69], as well as on the length and orientation of actin filaments. In accordance, our GO analysis at 34°C shows a significant enrichment of proteins involved in muscle contraction, structural activity, intermediate and actin cytoskeleton while at 18°C the effect on microtubules are prevalent (Tables 2, 3).

Interestingly, at 18°C and 34°C there is also an increase of proteins involved in calcium binding (Table 3, Figure 2B). This finding is in line with the data reported in Amato & Christner [62] which suggest that the link between thermoregulation and neurotransmission is largely dependent on intracellular calcium homeostasis [70].

Therefore, the temperature treatment effects on proteins involved in actin rearrangements and calcium binding observed in ZF brains at 18°C and at 34°C can be framed in the mechano-biological interplay between cytoskeleton and calcium concentration.

Signal transduction pathway modulation

Several proteins involved in fundamental signalling pathways are modulated at both thermal extremes. Interestingly, many of the down-regulated pathways are involved in cognitive processes (Figure 3).

The expression of proteins involved in the integrins pathway was reduced at 34°C in comparison to 26°C. Integrins are a large family of heterodimeric transmembrane cell adhesion receptors involved in processes that can modify the brain cytoarchitecture by affecting axon growth and guidance, dendritic spine morphology, synaptogenesis [71], synaptic plasticity [17, 72, 73], cell migration and regeneration [74] and by supporting the differentiation and maintenance of neural stem cells [75]. Integrins are also engaged in learning mechanisms and their reduced expression in mice determined the impairment of long term potentiation (LTP) stabilization, memory acquisition and long-term storage of several

kinds of memories, including spatial and working memory [17], long-term object-location memory and novel-object recognition [17, 18].

The down-regulation of the arrestin pathway at 18°C and 34°C could provoke alterations of ZF cognitive abilities in analogy to β arrestin 1 KO-mice that showed deficits in learning tasks suggesting spatial learning deficiencies and general alteration in reward processing [76]. The down-regulation of the Ras pathway at 18°C and 34°C suggests that the exposition to the extreme temperatures could affect the cognitive abilities of the animals, as the Ras signalling is implicated in synaptic events leading to formation of long-term memories [77].

Focusing on pathways exclusively altered at 18°C or altered in the same way at 18°C and 34°C (Figure 3), cold acclimation leads to a lower expression of EGF and PDGF signalling in comparison to 26°C. Although these growth factors belong to different families and drive different biological roles, they share some overlapping targets. Both EGF and PDGF engage several well-characterised pathways as the Ras-MAPK, PI3K, and PLCG that are deeply involved in diverse cellular responses, and their dysregulation is common during oncogenesis and pathophysiological tissue remodelling [78]. The reduced expression of the PDGF pathway observed at 18°C suggests that ZF brain acclimated to low temperatures may be more susceptible to environmental stressors considering the neuroprotective role of the PDGF pathway demonstrated in different animal models during ischemy [79, 80], oxidative stress [81] and glutamate- or NMDA-induced excitotoxicity [82]. Moreover, PDGF increases neuronal cell survival, neurogenesis, angiogenesis and gliosis and its neuroprotective effect on dopaminergic neurons is well documented [83]. The reduced expression of the PDGF pathway could have a negative impact on animal cognitive abilities being PDGF involved in the regulation of synaptic plasticity and function, in the hippocampal LTP and hippocampus-dependent memory [84]. The EGF pathway down-regulation detected in fish at 18°C and 34°C could contribute to the altered cognitive performances on considering that EGF

administration ameliorates the cognitive decline [14] and memory deficit [15], and prevents brain injury upon hypoxia in mice [16].

The down-regulation of the ubiquitin pathway observed at 18°C could further contribute to the CNS impairment as the failure of the ubiquitin-proteasome system has been found in Parkinson's disease [85].

Behavioural data analysis

With the aim of verifying if the adaptation to thermal extremes determines impairments of cognitive abilities of the animals, experimental and control subjects maintained for 21 days at the three temperatures were subjected to behavioural tests by using a Y-Maze apparatus. The Y-Maze task is widely used on mouse model and has been also successfully used in ZF [36]. It allows evaluating both physical performances related to the locomotor activity and cognitive abilities related to the response to novelty and spatial orientation.

Behavioural parameters were analysed in both Tr and Te in order to evaluate the swimming performances and the dynamism of the fish together with its tendency to explore the novel environment.

During Tr, fish did not show preference for one or the other of the two arms, spending approximately the 50% of the total time in each arm. Neither temperature nor trial replication influenced these results (Figure 4A). As indicated by mean speed and distance travelled, animals maintained at 18°C and 34°C respectively showed a decrease and an increase in locomotor activity compared to controls (Figure 4 B,C). In the intra-temperature analysis, no differences were detected in mean speed and distance travelled between Tr and Te at 18°C and 26°C. At 34°C fish in Te showed higher values of mean speed and distance. The number of body rotations, which is the number of 360° rotations, was respectively lower and higher in fish at 18°C and 34°C compared to controls (Figure 4D). The three-way ANOVA analysis

among temperatures (18°C, 26°C and 34°C), experimental phases (Tr and Te) and directions of rotation (clockwise, CW and counter-clockwise, CCW) did not show significant differences in intra-test phase analysis between CW and CCW rotations, except at 34°C in which a higher number of CW rotations was detected in Te (Figure 4E).

The swimming activity and the dynamism of fish were also estimated by measuring the number of passages between the S and O arms. At both 26°C and 34°C, fish increased the number of passages from T1 to T4, whereas no differences were detected in fish at 18°C (Figure 4F).

The lower swimming performances showed by fish at 18°C in comparison to those kept at 26°C or acclimated to 34°C are consistent with literature data showing the increase in swimming activity at higher temperatures [86-88].

In Te, fish could freely swim in the three arms of the maze exploring the novel environment of the N arm for 5 minutes. Fish acclimated at 34°C showed the tendency to carry out a greater number of passages among the arms than those at 26°C and 18°C in all the trials, although only the comparison between 18°C and 34°C in T2 was statistically significant. No statistically significant differences were measured in the intra-temperature analysis (Figure 5A).

The number of times that fish entered into the N arm reaching the sector 3 was measured to evaluate whether temperature affects the exploration of the novel environment. Fish acclimated to the 34°C entered more often in the sector 3 of the N arm than fish at 18°C and 26°C. The number of entries was significantly higher at 34°C than at 26°C in T1 and T2 and at 34°C than 18°C in T1, T2, T3 (Figure 5B). Intra-temperature analysis revealed differences in number of entries in the sector 3 of the N arm only at 18°C between T4 and the other trials (Figure 5C). To better evaluate the temperature effect on the exploration of the novel environment, the ratio between the number of times the animal reached the sector 3 of the N

arm and the number of total entries in the N arm was calculated. No statistically significant differences were found both in the intra-trial and intra-temperature analysis (Figure 5D): in all conditions tested, fish tended to fully explore the N arm reaching the sector 3 the 60-80% of time.

The total time spent by the experimental subjects in each arm was also measured in Te to estimate the animal's interest in the novel environment. Fish maintained to the control temperature (26°C) during T1 spent most of the time in the N arm (54% of total time) (Figure 5E). This result demonstrates fish interest for the N arm, where they spent most of the time and is coherent with findings by Cognato and co-workers [36] thus confirming that control fish are able in spatial orientation and distinguish the unexplored from the explored arms. As expected from the results of proteomic analysis, fish acclimated to 18°C and 34°C showed a different behaviour compared to controls as they showed no preference for the N arm where they spent only 25-30% of the time. Different phenomena could explain the reduced interest in novelties such as the compromised ability in recognizing the geometric figures that distinguish the arms, difficulties in spatial orientation and the inability in recognizing the N arm like a novel environment.

In the subsequent trials at 26°C, there was a progressive significant reduction in the time spent in the N arm that gradually declines from T1 to T4 (45% in T2, 36% in T3 and 29% in T4) (Figure 5E, F), demonstrating a progressively lowered interest in the N arm that is no longer a novelty for the fish, as it has already been explored previously. These data indicate that past experiences affect behaviour of control fish. Therefore, they suggest that fish memorise experience made in previous trials. Differently, fish acclimated at 18°C and 34°C continued to exhibit a scarce interest in the N arm also in the following trials (Figure 5E, F). Their behaviour appears not to be modulated by past experiences and this may be related to an impairment of learning abilities due to thermal treatment.

Both the extreme temperatures seem to induce a similar effect on the exploratory behaviour. However, it is interesting that the number of entries in sector 3 of the N arm (Figure 5C) and the time spent in the N arm (Figure 5F) showed higher values in T4 compared to T1 at 18°C, while no differences were found at 34°C. This result may suggest differences in behavioural effects of the exposure to 18°C and 34°C, as if specimens acclimated to 18°C in T4 could recognize the N arm as a novel environment with a sort of delay in comparison to fish at 26°C. Further studies will be devoted to deeply investigate physiological and behavioural differences between low and high temperature acclimation.

Conclusions

The GOBP, GOMF, GOCC and Pathway enrichment analysis showed a strong impact of environmental temperature on the brain proteome. Overall, present results suggest that, in adult ZF, the exposure at 18°C and 34°C for 21 days provokes detectable alterations in the brain proteome possibly causing functional alterations of the CNS which can impair the exploratory behaviour of the animal (Figure 6).

Both thermal extremes induce quantitative alterations of proteins associated with metabolism, cytoskeleton organization and cellular transport suggesting a strong impact of environmental temperature on the cytoarchitecture and the energy state of the brain. In particular, the reduced expression of synaptic proteins and the down-regulation of pathways positively correlated to cognitive functions in fish acclimated to 18°C and 34°C, suggest that thermal treatment may alter the animal's cognitive abilities. This alteration is suggested by the Y-Maze tests in which the interest in novelties and spatial orientation abilities appear significantly reduced in specimens maintained at 18°C and at 34°C compared to controls.

The reduced interest in novelty at the extreme temperatures may be due to the compromised ability in recognizing the geometric figures that distinguish the Y-Maze arms. Thermal

treatment may cause a stress condition that prevent or impair fish from distinguishing new objects and recognizing familiar from unfamiliar parts of the environment.

In conclusion, although temperatures tested fall within the ZF vital range, present results demonstrate that long term acclimation to extreme temperatures strongly influences brain protein expression and the exploratory behaviour of animals suggesting the impairment of some cognitive abilities. Under a global warming scenario such temperature-dependent cognitive alterations could seriously compromise the survival of wild fish in the long-term perspective.

The following are the supplementary data related to this article.

Table S1 Proteins up regulated or exclusively expressed at 26°C in the comparison 26°C vs 18°C. Proteins were considered differentially expressed if they were present only in one condition or showed statistically significant difference (Welch's test $p \leq 0.05$).

Table S2 Proteins down regulated at 26°C or exclusively expressed at 18°C in the comparison 26°C vs 18°C. Proteins were considered differentially expressed if they were present only in one condition or showed statistically significant difference (Welch's test $p \leq 0.05$).

Table S3 Proteins up regulated or exclusively expressed at 26°C in the comparison 26°C vs 34°C. Proteins were considered differentially expressed if they were present only in one condition or showed statistically significant difference (Welch's test $p \leq 0.05$).

Table S4 Proteins down regulated at 26°C or exclusively expressed at 34°C in the comparison 26°C vs 34°C. Proteins were considered differentially expressed if they were present only in one condition statistically significant difference (Welch's test $p<0.05$).

Table S5 Gene Ontology classification of proteins up regulated or present only at 26°C in the comparison 26°C vs 18°C. The differentially expressed proteins were classified into different GO Slim Biological process (GOBPSlim), GO Slim Molecular Function (GOMFSlim) and GO Slim Cellular Component (GOCCSlim) using the Panther and Revigo software. Functional grouping was based on $p\leq0.05$ and minimum two counts.
In bold terms related to cytoskeleton and transport, whereas terms related to metabolism are underlined.

Table S6 Gene Ontology classification of proteins down regulated at 26°C or present only at 18°C in the comparison 26°C vs 18°C. The differentially expressed proteins were classified into different GO Slim Biological process (GOBPSlim), GO Slim Molecular Function (GOMFSlim) and GO Slim Cellular Component (GOCCSlim) using the Panther and Revigo software. Functional grouping was based on $p\leq0.05$ and minimum two counts. In bold terms related to cytoskeleton and transport, whereas terms related to metabolism are underlined.

Table S7 Gene Ontology classification of proteins up regulated or present only at 26°C in the comparison 26°C vs 34°C. The differentially expressed proteins were classified into different GO Slim Biological Process (GOBPSlim), GO Slim Molecular Function (GOMFSlim) and GO Slim Cellular Component (GOCCSlim) using the Panther and Revigo software. Functional grouping was based on $p\leq0.05$ and minimum two counts.

In bold terms related to cytoskeleton and transport, whereas terms related to metabolism are underlined.

Table S8 Gene Ontology classification of proteins down regulated at 26°C or present only at 34°C in the comparison 26°C vs 34°C. The differentially expressed proteins were classified into different GO Slim Biological process (GOBPSlim), GO Slim Molecular Function (GOMFSlim) and GO Slim Cellular Componenet (GOCCSlim) using the Panther and Revigo software. Functional grouping was based on $p \leq 0.05$ and minimum two counts. In bold terms related to cytoskeleton and transport, whereas terms related to metabolism are underlined.

Significance

Environmental temperature variation may impact on all levels of biological life. Understanding the impact of thermal variation on the nervous system and animal behaviour is of primary importance since the results obtained can be applied from the ecological to the biomedical fields.

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Disclosure and conflicts of interest

The authors declare no conflict of interest.

Figure 1 Volcano plot of the proteins differentially expressed in the comparison 26°C vs 18°C (A) and 26°C vs 34°C (B). Proteins were considered differentially expressed if they

were present only in one condition or showed significant t-test difference (Welch's test p=0.05). The proteins up- or down-regulated are indicated in green and red, respectively.

Figure 2 (A) Enriched Map analysis of gene sets up regulated or only expressed at 26°C in the comparison 26°C vs 18°C and 26°C vs 34°C (increased at 26°C). The differentially expressed proteins were classified into different biological processes according to the Gene Ontology Slim classification system using BINGO and Enriched Map software. Functional grouping was based on $p \leq 0.05$. Nodes represent gene-sets and edges represent GO defined relations. Gene-sets that did not pass the enrichment significance threshold are not shown. Nodes are coloured according to samples: black represents enrichment in the comparison 26°C vs 18°C whereas light grey represents the gene sets in the comparison 26°C vs 34°C. (B) Enriched map analysis of gene sets down-regulated at 26°C or only expressed at 18°C or 34°C in the comparison 26°C vs 18°C and 26°C vs 34°C (decreased at 26°C).

Figure 3. Gene Ontology Pathway classification of proteins differentially expressed in the comparison 26°C vs 18°C and 26°C vs 34°C. The differentially expressed proteins were classified into different GO Pathways using the Panther software. Functional grouping was based on $p \leq 0.05$ and minimum two counts. Negative value refer to fold enrichment of proteins less expressed at 26°C or only expressed at 18°C or 34°C (decreased at 26°C) whereas positive value refer to proteins more or only expressed at 26°C in the comparison 26°C vs 18°C and 26°C vs 34°C (increased at 26°C) .

Figure 4 Behavioural response to thermal treatment on Y-Maze test observed in Training phase (Tr) and Testing phase (Te). Swimming activity and behaviour measured as time spent in the Start arm (A), mean speed (B), total distance travelled (C), number of total rotations of the body (D), number of right (CW) and left (CCW) rotations (E), number of passages

between Start and Other arms (F). Data were analysed by two-way ANOVA (A-D, F) and three-way ANOVA (E) with a post hoc test that utilizes a Bonferroni correction.
#, $p \leq 0.05$ between Tr at 18°C and 26°C; @, $p \leq 0.05$ between Te at 18°C and 26°C; §, $p \leq 0.05$ between Tr at 34°C and 26°C; $, $p \leq 0.05$ between Te at 34°C and 26°C; °, $p \leq 0.05$ in intra-temperature Tr vs Te comparison; *, $p \leq 0.05$; **, $p \leq 0.01$.

Figure 5 Behavioural response to thermal treatment on Y-Maze test observed in Testing phase (Te). Total number of passages among the three arms (A), total number entries in the sector 3 of the N arm (B, C), ratio between the number of entries in sector 3 and the number of total entries in N arm expressed as percentage (D), time spent in N arm expressed as percentage of the total time (E, F) are represented. Data are shown as mean±SEM and analysed by two-way ANOVA with a post hoc test that utilizes a Bonferroni correction. Test with $p \leq 0.05$ and $p \leq 0.01$ represented by * and ** respectively. Ç refers to the comparison T4 vs T1 at 18°C with a $p \leq 0.05$ in a one-way ANOVA among values at 18°C.

Figure 6 Schematic summary of Gene Ontology classification of brain proteins expressed after thermal treatment fully reported in Tables 2, 3, S5-S8. Down regulation ↓, Up regulation ↑ and general variation ↑↓ of proteins are reported at 18°C on the left and at 34°C on the right.

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Table 1 DAVID functional grouping of the proteins differentially expressed at 18°C, 34°C and 26°C. The Table reports the proteins statistically differentially expressed among the three different conditions (ANOVA test FDR 0.05). The column "Counts" indicates the number of genes present in each category. Functional grouping was based on $p \leq 0.05$ and at least two counts.

Term	Count	PValue	Genes
Enrichment Score: 2.53			

GO:0005509~calcium ion binding	9	1.57 E-02	SRI, PVALB6, HPCAL4, MYLZ3, PVALB5, CALB2A, ANXA3B, LCP1, CELSR1A
Enrichment Score: 2.49			
GO:0005882~intermediate filament	4	2.06 E-03	PRPH, KRT18, KRT5, KRT8
GO:0005198~structural molecule activity	4	3.49 E-02	PRPH, KRT18, KRT5, KRT8
Enrichment Score: 2.12			
GO:0005874~microtubule	5	1.45 E-03	PAFAH1B1B, LOC100149074, MAP2, TUBA8L2, TUBA8L3
GO:0005856~cytoskeleton	5	3.72 E-02	PAFAH1B1B, MAP2, TUBA8L2, LCP1, TUBA8L3
Enrichment Score: 1.66			
GO:0006412~translation	7	1.47 E-03	TUFM, RPS8B, MRPL12, RPS19, EIF4A2, RPL4, EIF5A2
GO:0030529~intracellular ribonucleoprotein complex	5	8.94 E-03	HNRPDL, RPS8B, MRPL12, RPS19, RPL4
GO:0005840~ribosome	4	2.84 E-02	RPS8B, MRPL12, RPS19, RPL4
dre03010:Ribosome	4	4.32 E-02	RPS8B, MRPL12, RPS19, RPL4
Enrichment Score: 0.96			
dre01200:Carbon metabolism	4	3.63 E-02	SDHB, TPI1B, IDH3G, PGAM1B
Enrichment Score: 0.84			
GO:0000166~nucleotide binding	14	3.85 E-02	TUFM, MYHC4, RBM4.2, HSPA4A, ADRBK2, SYNCRIP, NCL, TUBA8L2, TUBA8L3, HNRPDL, SRSF5B, UBE2D3, EIF4A2, ACTA1A
Enrichment Score: 0.75			
GO:0005739~mitochondrion	8	4.77 E-03	TUFM, SDHB, IDH3G, COX6B1, CHCHD2, ACADL, VDAC2, TIMM8A

Table 2 DAVID functional grouping of the proteins differentially expressed at 26°Cvs18°C and 26°Cvs34°C. The table reports the proteins up regulated or exclusively expressed at 26°C. The column "Counts" indicates the number of genes present in each category. The first column reports a manual clustering of the Terms reported in column 2. Functional grouping was based on $p \leq 0.05$ and at least two counts.

INCREASED AT 26°C					
		26°C vs 18°C_26°Cup_only26°C		**26°C vs 34°C_26°Cup_only26°C**	
Clustering	**Term**	**Counts**	**Genes**	**Counts**	**Genes**

RNA translation	nucleotide binding	51	CKBA, PSMC1A, SYNCRIP, ATP2B1B, LONP1, LARSA, DHX37, U2AF1, ADCK3, AGAP1, GLULA, RABL3, ARL16, ABCC12, SARS2, EIF4A3, NME3, EIF4BA, CAMK4, EIF4BB, GNAS, GNAI3, MYO10L3, SNRPB2, MYO10L1, SEPT7A, CHEK2, GMPPAB, SF3B4, IARS, UBE2D3, EIF3G, GSK3AB, SEPT8A, PRKCDA, SNRNP70, YES1, RHOAA, DHX9, MATR3L1.1, MYO6A, RBM4.2, ADCY1A, MAPK10, TIA1L, GNA15.3, PSMC2, HUG, PABPC1A, MAP2K2A, ATP8A2	43	HNRNPH1L, SEPT3, PSMC1A, ATL1, MYO10L3, SYNJ1, SYNCRIP, SEPT7A, CHEK2, SF3B4, GMPPAB, ATP2B1B, SRSF5B, LARSA, EIF3G, DHX37, TUBB5, U2AF1, SNRNP70, YES1, MARS, RPS24, MATR3L1.1, DHX9, MYO6A, SI:ZFOS-588F8.1, HNRNPA0L, OLA1, ARL16, ELAVL3, ABCC12, MAPK10, NCL, TUBA8L2, SARS2, HNRPDL, NME3, ZGC:63587, MAP2K2A, PFKMB, EIF4BB, ATP8A2, KATNAL2
	intracellular ribonucleoprotein complex	9	RPS25, DHX9, LSM6, HUG, RPL24, RPS10, SNRNP70, MRPL46, RPL28	11	HNRPDL, DHX9, RPS28, LSM6, RPS12, SNRPC, ELAVL3, SNRNP70, MRPL46, RPS24, RPS7
	nucleosome assembly	8	ZGC:153405, HISTH1L, NAP1L1, SETB, H1FX, ZGC:163061, ZGC:110216, SI:CH211-103N10.5		
	chromosome	7	ZGC:153405, HISTH1L, H1FX, ZGC:163061, ZGC:110216, SI:CH211-103N10.5, SMC3		
	ribonucleoprotein			10	HNRPDL, RPS28, LSM6, RPS12, SNRPC, ELAVL3, SNRNP70, MRPL46, RPS24, RPS7
	cytosolic small ribosomal subunit			5	RPS28, RPS12, ZGC:114188, RPS24, RPS7
Protein folding	protein folding	7	ERP44, PFDN1, PPIFB, PFDN6, PPID, VBP1, AIP	7	PFDN1, GRPEL1, TXNDC5, PPID, PPIAA, VBP1, PDIA4
	prefoldin complex	3	PFDN1, PFDN6, VBP1		
Protein localization	protein transport			10	SEC23A, BC2, SLC7A6OS, AP1G1, TOM1L2, TIMM10, AP3S1, VPS26BL, AP4S1
	protein transport			10	SCAMP1, SEC23A, BC2, SLC7A6OS, AP1G1, TIMM10, EXOC4, AP3S1, VPS26BL, AP4S1
	protein transporter activity			5	AP1G1, TIMM10, AP3S1, VPS26BL, AP4S1
PT modification	phosphoprotein phosphatase activity	7	PPM1BB, PGAM5, PPM1BA, PTPRNB, PPP3CB, UBLCP1, PPP1CAB	8	PPP3CCB, PPM1BB, PGAM5, PPM1BA, PTPRNB, PPP3CB, UBLCP1, PPP1CAB
	protein serine/threonine phosphatase activity			4	PPM1BB, PPM1J, PPM1BA, UBLCP1
	protein phosphatase			5	PPM1BB, PPM1BA, PTPRNB, UBLCP1, PPP1CAB
Degradation	proteasome complex	7	PSMB7, PSMB6, PSMC1A, PSMC2, PSMB2, PSMD7, PSMA6B	5	PSMB7, PSMC1A, PSMB3, PSMA3, PSMD7
	proteasome core complex	4	PSMB7, PSMB6, PSMB2, PSMA6B		
	proteolysis involved in cellular protein catabolic process	5	PSMB7, PSMB6, PSMB2, CTSH, PSMA6B		
	protein catabolic process	4	LONP1, PSMC1A, PSMC2, CTSD		
Redox balance	cell redox homeostasis			5	TXNDC5, PRDX2, PDIA4, SH3BGRL3, GLRX
	mitochondrial respiratory chain complex I			3	NDUFS5, NDUFS4, NDUFB9

	oxidoreductase activity			16	HADHAA, GLUD1B, GMPR2, AIFM2, AKR1A1B, CBR1L, FDXR, PRDX2, GPD1L, SDHB, IDH3G, MICAL2A, CAT, CYP8B1, GPX1A, GLYR1
Other	endopeptidase activity	5	PSMB7, PSMB6, PSMB2, SI:DKEY-21C19.3, PSMA6B		
	threonine-type endopeptidase activity	4	PSMB7, PSMB6, PSMB2, PSMA6B		
	ATP-dependent RNA helicase activity	5	DHX9, EIF4A3, DDX39AB, DHX37, DDX39B		
	calcium ion binding			19	SRI, CALUA, LRP1AA, HPCAL4, PVALB6, PVALB5, SI:DKEY-110K5.6, NID1B, ANXA4, EFHD2, SCGN, NCALDA, NCALDB, CAPNS1A, SYT1B, F2, CELSR1B, S100A10B, LCP1
	isomerase activity			5	TXNDC5, PPID, PPIAA, PGAM1B, PDIA4

Table 3 DAVID functional grouping of the proteins differentially expressed at 26°Cvs18°C and 26°Cvs34°C. The Table reports the proteins down regulated at 26° C or exclusively expressed in stress conditions. The column "Counts" indicates the number of genes present in each category. The first column reports a manual clustering of the Terms reported in column 2. Functional grouping was based on $p \leq 0.05$ and at least two counts.

DECREASED AT 26°C					
		26°C vs 18°C_26°Cdown_only18°C		**26°C vs 34°C_26°Cdown_only34°C**	
Clustering	**Term**	**Counts**	**Genes**	**Counts**	**Genes**
Cytoskeleton	structural molecule activity	9	SI:DKEY-178K16.1, PRPH, COPG2, KRT18, KRT5, COPB1, CLTCA, CLDNK, INAB	5	GFAP, KRT5, CLTCB, KRT8, KRT4
	intermediate filament	4	PRPH, KRT18, KRT5, INAB	4	GFAP, KRT5, KRT8, KRT4
	microtubule	8	FSD1, PAFAH1B1B, LOC100149074, MAP2, TUBA8L, SI:DKEY-77A20.5, TUBA8L2, TUBA8L3		
	cytoskeleton	12	FSD1, SI:DKEY-178K16.1, PAFAH1B1B, MYO15AA, ACTB1, MAP2, TUBA8L, SI:DKEY-77A20.5, LCP1, TUBA8L2, TUBA8L3, PLECA		
	microtubule-based process	4	DYNLL1, TUBA8L, TUBA8L2, TUBA8L3		
	structural constituent of cytoskeleton	4	SI:DKEY-178K16.1, TUBA8L, TUBA8L2, TUBA8L3		
	keratin filament			3	KRT5, KRT8, KRT4
	actin-binding			7	MYHC4, SYNE2B, SPTBN5, MYO18AB, CAPZA1B, FLNA, MYH10
	myosin complex			4	MYHC4, MYO15AA, MYO18AB, MYH10

Transport/motor activity	vesicle-mediated transport	7	COPG2, ZGC:92912, COPB1, CLTCA, AP3B2, STXBP1A, STXBP3		
	intracellular protein transport	8	COPG2, NAPG, CSE1L, COPB1, CLTCA, AP3B2, VPS35, ADPRH		
	lipid transporter activity			3	VTG4, VTG7, VTG5
	motor activity			4	MYHC4, MYO15AA, MYO18AB, MYH10
Ion binding	magnesium ion binding	5	IDH3G, PGM1, ENO2, ENO3, ADPRH		
	calcium ion binding			12	PVALB4, PCDH1A3, MYLPFA, HMCN1, LRP2A, MYLZ3, MYL1, PCDH1B, SPNA2, NOTCH1B, CELSR1A, CAPN2B
Other	nucleotide binding	30	TUFM, GNA11A, ATL1, ACTB1, CKMB, ADRBK2, IARS2, SI:DKEY-172J4.3, SRSF5B, SRSF2A, HNRNPM, MARK4A, PRKAA1, CSNK1A1, CSTF2, TUBA8L, ATP1A3B, RNPS1, NCL, TUBA8L2, TUBA8L3, SRSF6A, PSMC3, RARS, UBA2, EIF4A2, GSK3B, PFKMB, ACTA1A, CARS2	28	MYHC4, TUFM, HSP90AB1, ADRBK2, IARS2, HNRNPM, ACTR1, MYO18AB, GSK3AB, PRKAA1, HSPA5, HSPA9, HSPA4A, SSB, ABCA4A, EIF4A3, ATP2A3, RARS, UBA2, RRAS2, EIF4A2, REM2, ACTA1A, CCT8, FGFR1B, RHEB, KIF19, MYH10
	ribosome	10	RPS8B, MRPL12, RPS19, RPS16, RPS15, RPL10, RPL4, RPL10A, RPLP2L, RPS3		
	SM01391	4	PRPH, KRT18, KRT5, INAB		
	glycolytic process	4	ALDOAB, PFKMB, ENO2, ENO3		
	proteasome complex	5	PSMB4, PSMD13, PSMD12, PSMC3, PSMD6		
	oxidation-reduction process	15	SQRDL, KCNH6B, CYP2V1, FDXR, DLAT, ACADL, GLDC, SDHB, G6PD, IDH3G, YWHABL, UQCRH, ALDH4A1, GLYR1, RTN4IP1		
	mitochondrion	12	TUFM, SDHB, IDH3G, UQCRH, OTC, FDXR, SIRT5, ALDH4A1, IARS2, ACADL, RTN4IP1, FH		
	tricarboxylic acid cycle	3	SDHB, IDH3G, FH		
	GTPase activity	6	TUFM, GNA11A, ATL1, TUBA8L, TUBA8L2, TUBA8L3		
	ATP binding			26	MYHC4, HSP90AB1, ADRBK2, TTNB, IARS2, ACTR1, MYO18AB, GSK3AB, PRKAA1, HSPA5, DYNC1H1, HSPA9, HSPA4A, DDX1, ABCA4A, EIF4A3, MYO15AA, ATP2A3, RARS, UBA2, EIF4A2, ACTA1A, CCT8, FGFR1B, KIF19, MYH10
	stress response			4	HSP90AB1, HSPA4A, HSPA5, HSPA9
	RNA secondary structure unwinding			3	EIF4A3, EIF4A2, DDX1
	cytosolic small ribosomal subunit			3	RPSA, FAUA, RPS11

Graphical abstract

Highlights

- The acclimatization to low (18°C) and high (34°C) temperatures significantly alters the expression of proteins involved in important signal transduction pathways related to memory and cognition.
- Behavioural tests showed significant alterations of the exploratory behaviour confirming an impairment of animal cognitive abilities at thermal extremes.

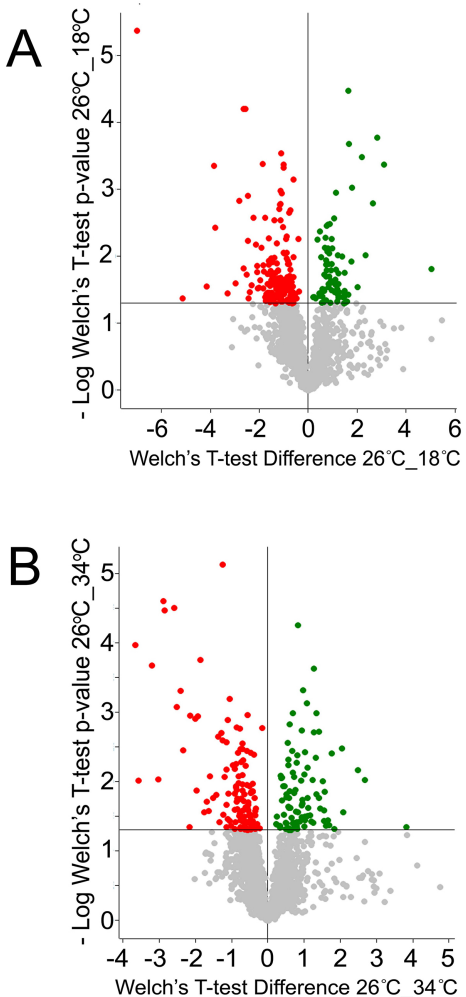
A
- Log Welch's T-test p-value 26°C_18°C
Welch's T-test Difference 26°C_18°C
B
- Log Welch's T-test p-value 26°C_34°C
Welch's T-test Difference 26°C_34°C

Figure 1

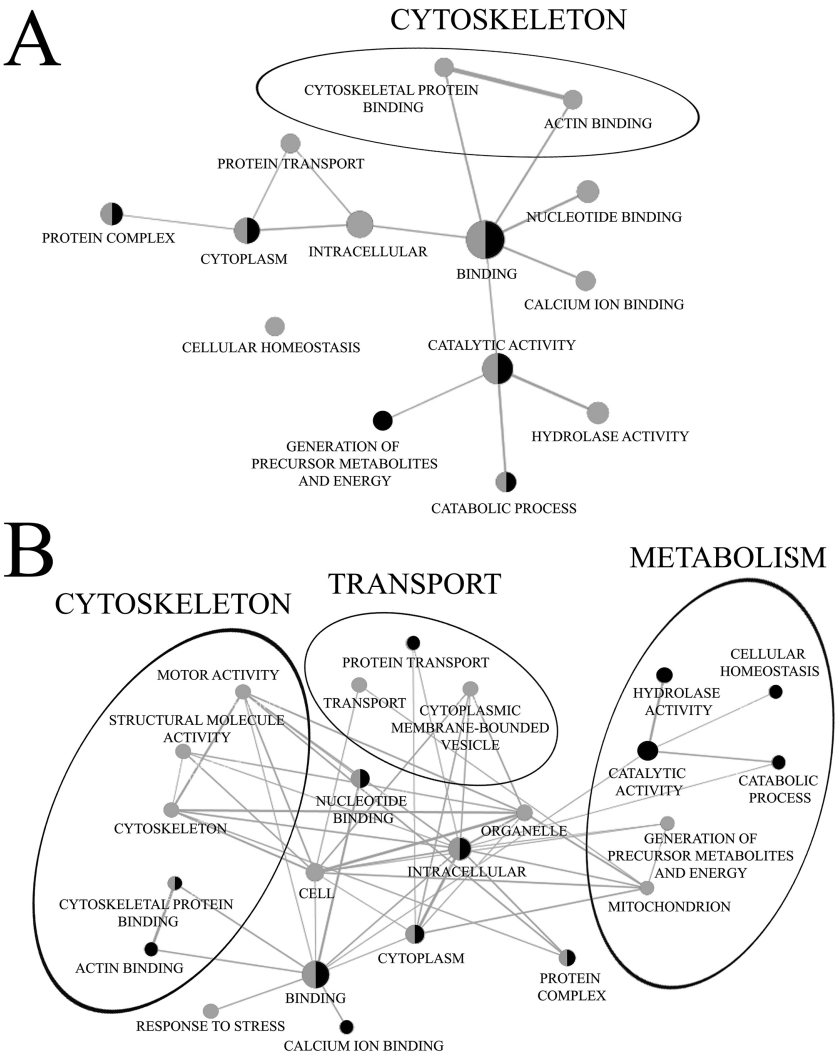
A
CYTOSKELETON
CYTOSKELETAL PROTEIN BINDING
ACTIN BINDING
PROTEIN TRANSPORT
NUCLEOTIDE BINDING
PROTEIN COMPLEX
CYTOPLASM
INTRACELLULAR
BINDING
CALCIUM ION BINDING
CELLULAR HOMEOSTASIS
CATALYTIC ACTIVITY
HYDROLASE ACTIVITY
GENERATION OF PRECURSOR METABOLITES AND ENERGY
CATABOLIC PROCESS
B
METABOLISM
TRANSPORT
CYTOSKELETON
PROTEIN TRANSPORT
CELLULAR HOMEOSTASIS
MOTOR ACTIVITY
HYDROLASE ACTIVITY
TRANSPORT
CYTOPLASMIC MEMBRANE-BOUNDED VESICLE
STRUCTURAL MOLECULE ACTIVITY
CATALYTIC ACTIVITY
CATABOLIC PROCESS
NUCLEOTIDE BINDING
CYTOSKELETON
ORGANELLE
GENERATION OF PRECURSOR METABOLITES AND ENERGY
INTRACELLULAR
CELL
CYTOSKELETAL PROTEIN BINDING
MITOCHONDRION
ACTIN BINDING
CYTOPLASM
PROTEIN COMPLEX
BINDING
RESPONSE TO STRESS
CALCIUM ION BINDING
BLACK 26°C vs 18°C_26°C DOWN + 18°C ONLY
LIGHT GREY 26°C vs 34°C_26°C DOWN + 34°C ONLY

Figure 2

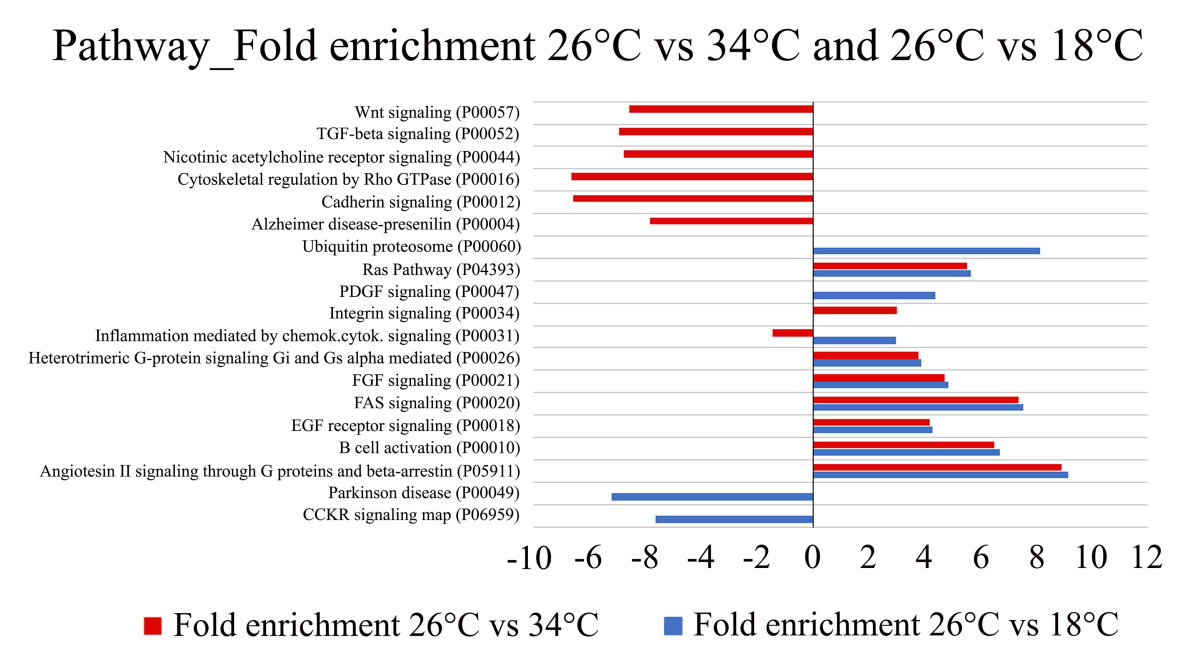
Pathway_Fold enrichment 26°C vs 34°C and 26°C vs 18°C
Wnt signaling (P00057)
TGF-beta signaling (P00052)
Nicotinic acetylcholine receptor signaling (P00044)
Cytoskeletal regulation by Rho GTPase (P00016)
Cadherin signaling (P00012)
Alzheimer disease-presenilin (P00004)
Ubiquitin proteosome (P00060)
Ras Pathway (P04393)
PDGF signaling (P00047)
Integrin signaling (P00034)
Inflammation mediated by chemok.cytok. signaling (P00031)
Heterotrimeric G-protein signaling Gi and Gs alpha mediated (P00026)
FGF signaling (P00021)
FAS signaling (P00020)
EGF receptor signaling (P00018)
B cell activation (P00010)
Angiotesin II signaling through G proteins and beta-arrestin (P05911)
Parkinson disease (P00049)
CCKR signaling map (P06959)
-10 -6 -8 -4 -2 0 2 4 6 8 10 12
Fold enrichment 26°C vs 34°C
Fold enrichment 26°C vs 18°C

Figure 3

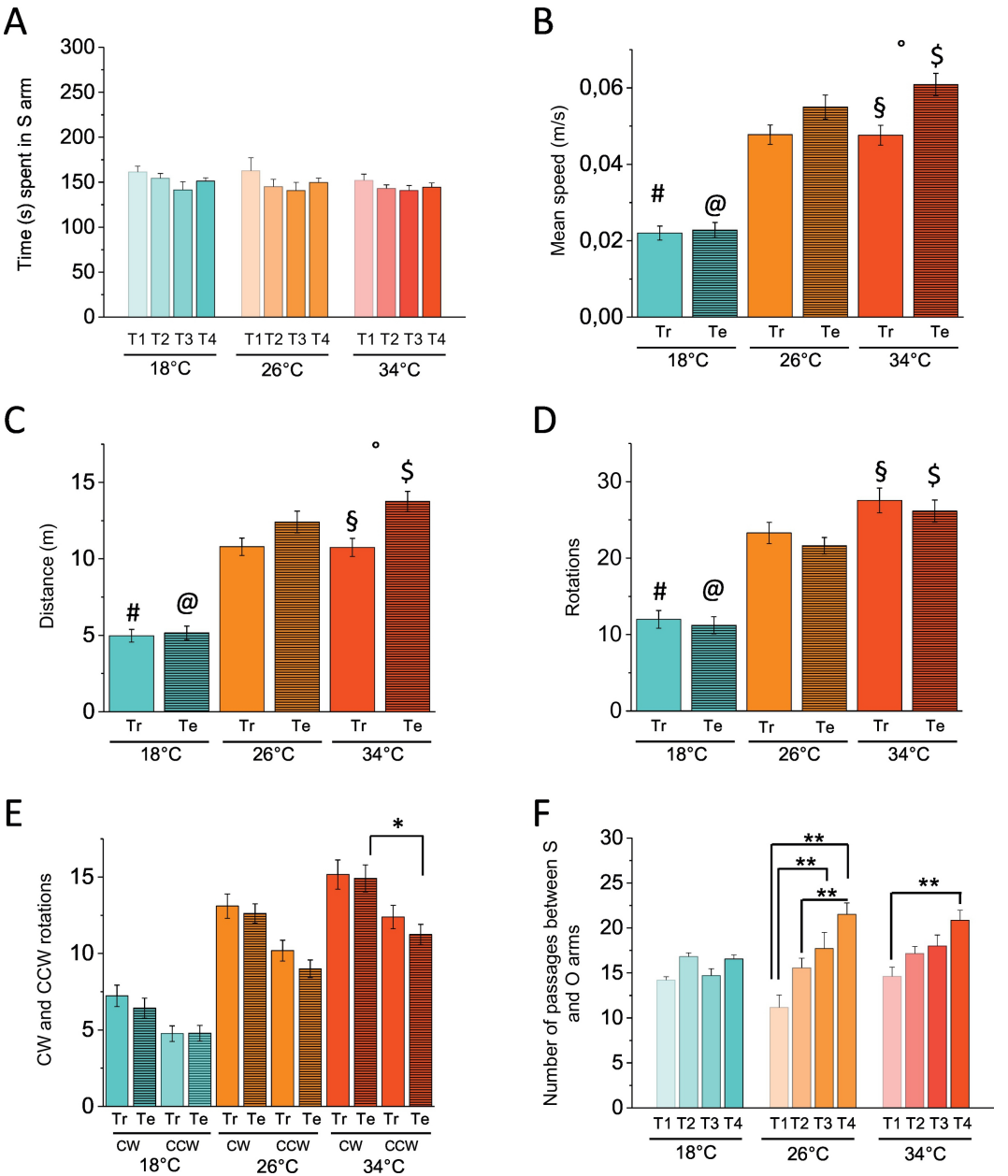
A
Time (s) spent in S arm
300
250
200
150
100
50
0
T1 T2 T3 T4
T1 T2 T3 T4
T1 T2 T3 T4
18°C
26°C
34°C
B
Mean speed (m/s)
0,06
0,04
0,02
0,00
#
@
§
°
$
Tr
Te
18°C
26°C
34°C
C
Distance (m)
15
10
5
0
D
Rotations
30
20
10
0
E
CW and CCW rotations
*
CW
CCW
F
Number of passages between S and O arms
**

Figure 4

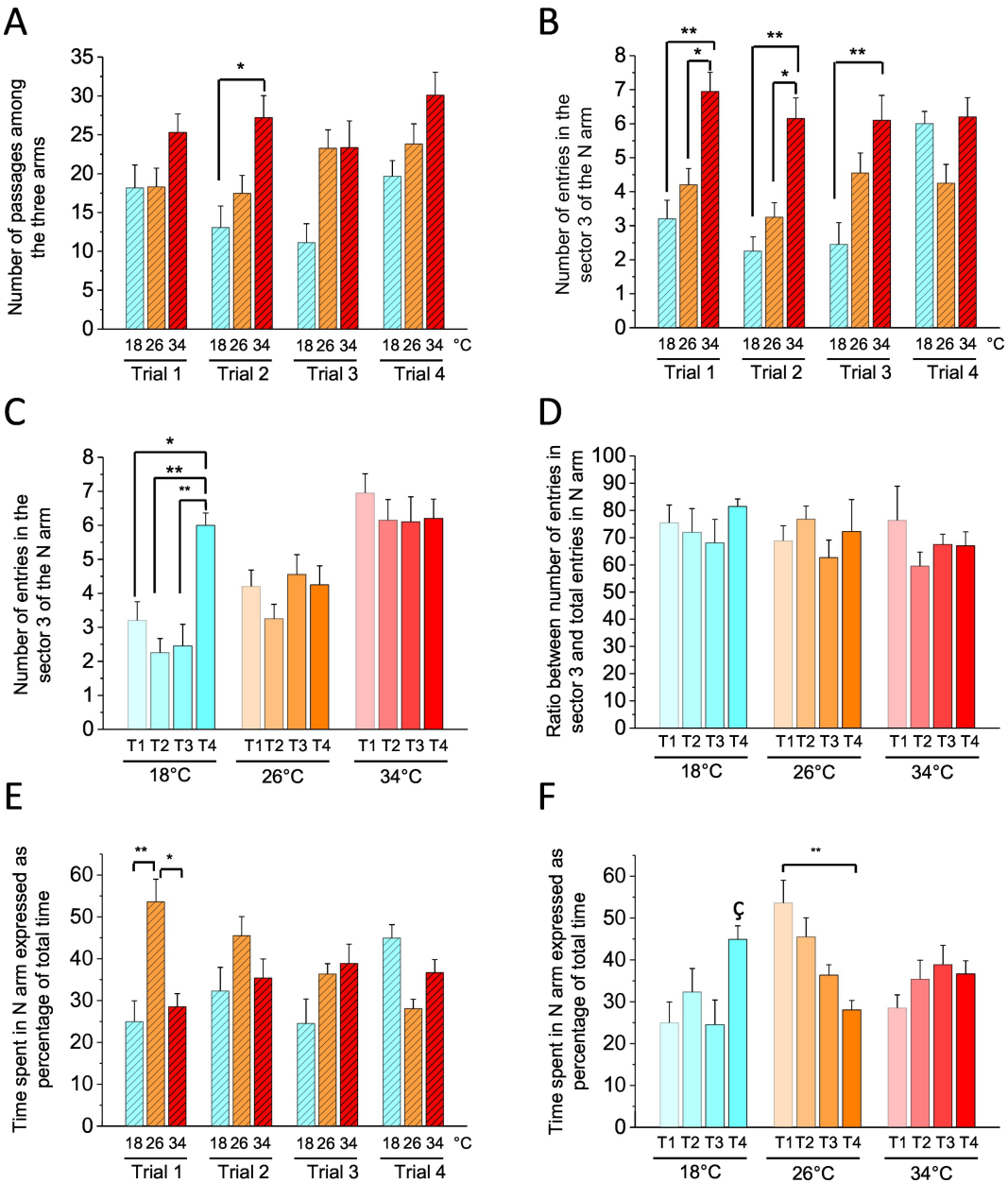
A
Number of passages among the three arms
35
30
25
20
15
10
5
0
*
18 26 34 °C
Trial 1
Trial 2
Trial 3
Trial 4
B
Number of entries in the sector 3 of the N arm
8
7
6
5
4
3
2
1
0
**
*
18 26 34 °C
Trial 1
Trial 2
Trial 3
Trial 4
C
Number of entries in the sector 3 of the N arm
8
7
6
5
4
3
2
1
0
*
**
T1 T2 T3 T4
18°C
26°C
34°C
D
Ratio between number of entries in sector 3 and total entries in N arm
100
90
80
70
60
50
40
30
20
10
0
T1 T2 T3 T4
18°C
26°C
34°C
E
Time spent in N arm expressed as percentage of total time
60
50
40
30
20
10
0
**
*
18 26 34 °C
Trial 1
Trial 2
Trial 3
Trial 4
F
Time spent in N arm expressed as percentage of total time
60
50
40
30
20
10
0
ç
**
T1 T2 T3 T4
18°C
26°C
34°C

Figure 5

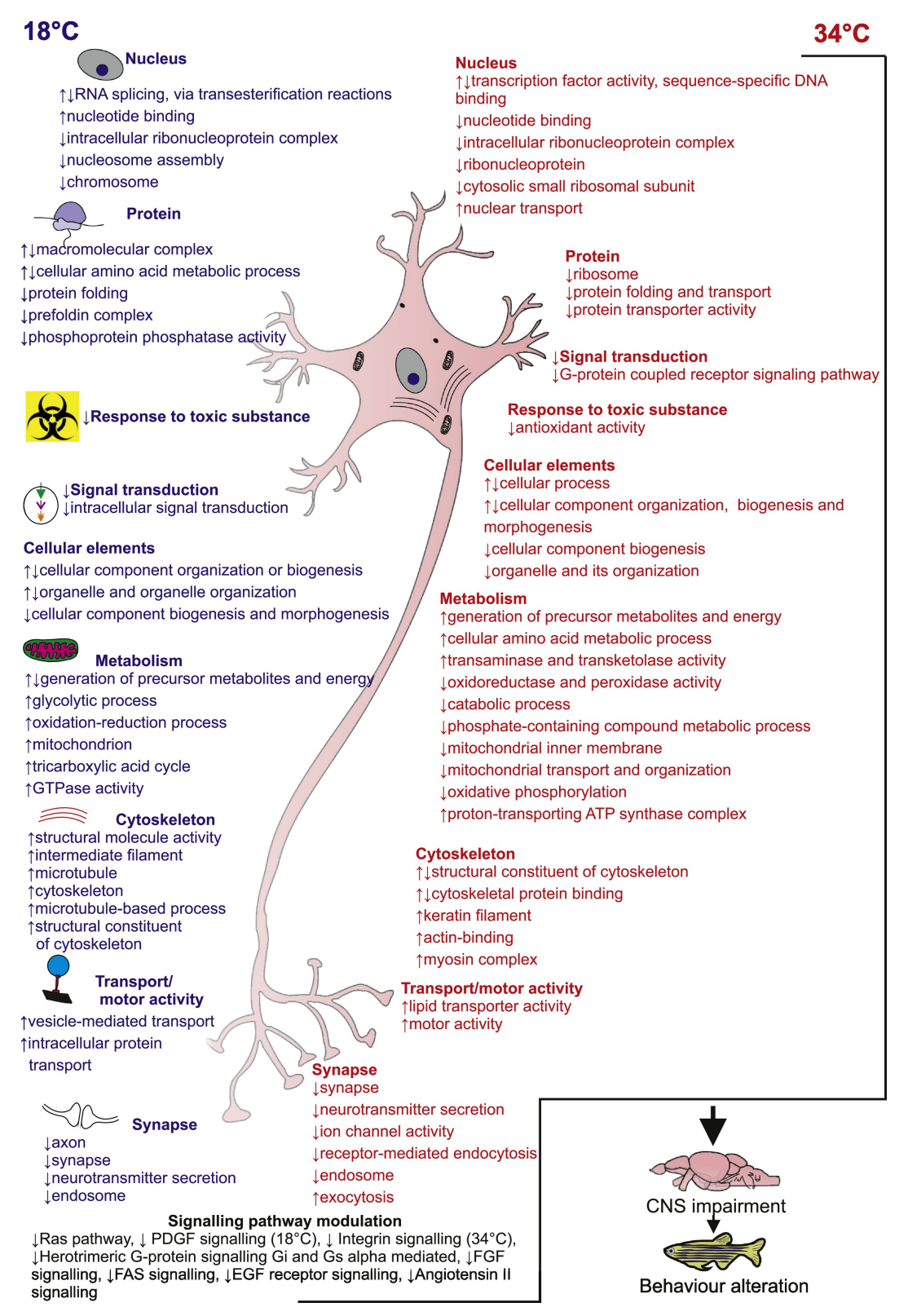
18°C
34°C
Nucleus
↑↓RNA splicing, via transesterification reactions
↑nucleotide binding
↓intracellular ribonucleoprotein complex
↓nucleosome assembly
↓chromosome
Protein
↑↓macromolecular complex
↑↓cellular amino acid metabolic process
↓protein folding
↓prefoldin complex
↓phosphoprotein phosphatase activity
↓Response to toxic substance
↓Signal transduction
↓intracellular signal transduction
Cellular elements
↑↓cellular component organization or biogenesis
↑↓organelle and organelle organization
↓cellular component biogenesis and morphogenesis
Metabolism
↑↓generation of precursor metabolites and energy
↑glycolytic process
↑oxidation-reduction process
↑mitochondrion
↑tricarboxylic acid cycle
↑GTPase activity
Cytoskeleton
↑structural molecule activity
↑intermediate filament
↑microtubule
↑cytoskeleton
↑microtubule-based process
↑structural constituent of cytoskeleton
Transport/ motor activity
↑vesicle-mediated transport
↑intracellular protein transport
Synapse
↓axon
↓synapse
↓neurotransmitter secretion
↓endosome
Nucleus
↑↓transcription factor activity, sequence-specific DNA binding
↓nucleotide binding
↓intracellular ribonucleoprotein complex
↓ribonucleoprotein
↓cytosolic small ribosomal subunit
↑nuclear transport
Protein
↓ribosome
↓protein folding and transport
↓protein transporter activity
↓Signal transduction
↓G-protein coupled receptor signaling pathway
Response to toxic substance
↓antioxidant activity
Cellular elements
↑↓cellular process
↑↓cellular component organization, biogenesis and morphogenesis
↓cellular component biogenesis
↓organelle and its organization
Metabolism
↑generation of precursor metabolites and energy
↑cellular amino acid metabolic process
↑transaminase and transketolase activity
↓oxidoreductase and peroxidase activity
↓catabolic process
↓phosphate-containing compound metabolic process
↓mitochondrial inner membrane
↓mitochondrial transport and organization
↓oxidative phosphorylation
↑proton-transporting ATP synthase complex
Cytoskeleton
↑↓structural constituent of cytoskeleton
↑↓cytoskeletal protein binding
↑keratin filament
↑actin-binding
↑myosin complex
Transport/motor activity
↑lipid transporter activity
↑motor activity
Synapse
↓synapse
↓neurotransmitter secretion
↓ion channel activity
↓receptor-mediated endocytosis
↓endosome
↑exocytosis
Signalling pathway modulation
↓Ras pathway, ↓ PDGF signalling (18°C), ↓ Integrin signalling (34°C), ↓Herotrimeric G-protein signalling Gi and Gs alpha mediated, ↓FGF signalling, ↓FAS signalling, ↓EGF receptor signalling, ↓Angiotensin II signalling
CNS impairment
Behaviour alteration

Figure 6