Acetyl-L-carnitine (ALC) is a naturally occurring substance that, when administered at supra-physiological concentration, is neuroprotective. It is involved in membrane stabilization and in enhancement of mitochondrial functions. It is a molecule of considerable interest for its clinical application in various neural disorders, including Alzheimer’s disease and painful neuropathies. ALC is known to improve the cognitive capability of aged animals chronically treated with the drug and, recently, it has been reported that it impairs forms of non-associative learning in the leech. In the present study the effects of ALC on gene expression have been analyzed in the leech Hirudo medicinalis. The suppression subtractive hybridisation methodology was used for the generation of subtracted CDNA libraries and the subsequent identification of differentially expressed transcripts in the leech nervous system after ALC treatment. The method detects differentially but also little expressed transcripts of genes whose sequence or identity is still unknown. We report that a single administration of ALC is able to modulate positively the expression of genes coding for functions that reveal a lasting effect of ALC on the invertebrate, and confirm the neuroprotective and neuromodulative role of the substance. In addition an important finding is the modulation of genes of vegetal origin. This might be considered an instance of ectosymbiotic mutualism.

Abstract

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Introduction

Acetyl-L-carnitine (ALC) is the acetyl ester of the trimethylated amino acid L-carnitine that plays an essential role in energy production as a “shuttle” of long-chain fatty acids between the cytosol and the mitochondria for subsequent β-oxidation. ALC is involved in the control of ATP levels, the mitochondrial acyl-CoA/CoA ratio, peroxisomal oxidation of fatty acids, and mitochondrial enzyme activities. It is known that ALC has neuromodulatory, cytoprotective, antioxidant, neurotrophic, anti-apoptotic, and anti-aging effects, and, in addition, it improves the cognitive capability of aged animals chronically treated with ALC (1–4, for a review see [5]). In previous studies, the effects of ALC on simple forms of nonassociative learning have been analyzed in the model of the swimming induction in the leech Hirudo medicinalis [6]. In particular, ALC reduces the sensitization process induced by brush strokes in a dose- and time-dependent manner, and provokes a reduction of the dishabituation [6]. In H. medicinalis, it is known that sensory stimulation initiates swimming activity by recruiting the mechanosensory neurons (T, P and N cells) which drive the flow of information to the swim-related muscles through a complex neuronal network [7]. Either T or N cell activity was affected by ALC: Lombardo et al. [8] reported that ALC increases the activation threshold of N cells, reducing the probability of activating them, and, in so doing, exerts an antinociceptive action. This effect is in accordance with studies in invertebrates demonstrating that ALC has analgesic properties and it is significantly effective in reducing neuropathic pain [9–12], although its mechanisms of action are not fully known. ALC also affects leech T cells, producing an increase in the amplitude of the afterhyperpolarization (AHP) which accompanies T cells bursting, by potentiating the Na+K+-ATPase activity [13]. This modulation of the AHP amplitude leads to changes in the synaptic efficacy of T cells [13,14,15]. Since previous studies showed that a single treatment with ALC has persistent effects which increase in time [6], it is possible to hypothesize a modulation of gene expression. At present, there is evidence for a modulation of ALC-induced gene expression in the nervous system of rats chronically treated with the drug [16–22], but nothing is known about this in the leech. Therefore, in order to clarify whether a single ALC treatment is capable of regulating gene expression in H. medicinalis, suppression subtractive hybridisation (SSH) methodology has been used for generating subtracted cDNA libraries and subsequently identifying differentially expressed transcripts in the leech nervous system.
Materials and Methods

Animals

Adult leeches *H. medicinalis* (8–10 months old) purchased from Ricarimpex (Eysines, France) were utilized. The animals were kept in aquarium at 15–16°C, exposed to natural light/dark cycle. ALC or saline was supplied dorsally by two injections (one in the rostral and the other one in the caudal art of the body of the leech), ALCl or saline was supplied dorsally by two injections (one in the T/A cloning vector using the 

Total RNA Isolation

A group of leeches has been injected with physiological solutions (control group, C), whereas another group with 2 mM ALC (Sigma Tau Laboratories, Pomezia Italy) (treated group, T). Eleven days after the treatment, total RNA was isolated according to Macchi et al. [23] and stored at −80°C until the RNA isolation.

SSH Library Construction

Poly (A)+ RNAs were isolated from the pools of total RNAs of control and treated leeches using the PolyATrac® mRNA Isolation Systems (Promega, Madison, Wi, USA) according to the protocol described by the manufacturer. The SSH (subtractive suppressive hybridisation) was performed according to Datchenko et al. [24] using the BD PCR-Select cDNA Subtraction Kit (BD Biosciences) after the use of BD SMART™ PCR cDNA Synthesis Kit (BD Biosciences Clontech). The SMART procedure requires 0.025–1 μg of poly (A)+ mRNA to allow the amplification of the complete mRNA population contained in each sample (treated and control). The cDNAs from treated samples were directly inserted as test and driver for the forward and reverse subtraction according to the BD PCR-Select cDNA Subtraction Kit.

Differentially expressed clones were sequenced by automated sequencing (MWG Biotech Ebersberg, Germany). Homology searches of all sequences were compared to the GenBank EMBL-EBI database using the BLAST algorithm. The obtained clones were cultured in LB medium with 10 mg/ml ampicillin in 96-well plates at 37°C. The cDNA fragments were amplified by PCR with nested PCR primers 1 and 2R, which were complementary to the adaptors, to check the presence and size of the individual fragments.

PCR reactions (25 μl) contained 18.5 μl sterile water, 0.6 μl of each primer (10 μM each), 2.5 μl 10× reaction buffer (Euroclone, Italy), 1 μl MgCl2 (Euroclone), 0.6 μl of dNTP mix (2.5 mM each), 0.25 μl Euro Taq polymerase (Euroclone) and 1 μl bacterial culture. Samples were denatured at 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 1 min 30 sec, with a final extension at 72°C for 10 min. All PCR products were analyzed by agarose-gel electrophoresis. Two identical blots were created by spotting heat denatured PCR products (1 μl) from each of the clones of the subtractive libraries onto nylon membranes positively charged (Roche, Germany) and cross-linked by UV. The membranes were pre-hybridized for 3 h in prehybridization buffer (DIG easy Hyb, Roche), then incubated overnight at 42°C in hybridization buffer (DIG easy Hyb containing the labeled cDNAs obtained by DIG-DNA Labelling Kit Nonradioactive, Roche). To eliminate the background due to the presence of the adapter sequences (BD PCR-Select cDNA Subtraction Kit; BD Biosciences) in the probes, a high concentration (3 μg/ml) of oligonucleotides corresponding to the nested primers and the complementary sequences (competitors) previously used for the libraries construction have been added into the prehybridisation and hybridisation solution. Moreover, the adaptors have been removed by digesting cDNAs with RsaI restriction enzyme prior to probe labeling. The fragments that hybridized only with the labeled forward cDNA or that showed at least higher signals than the signals obtained with the reverse labeled cDNA were subjected to sequencing analysis.

Clone Sequencing and Analysis

Differentially expressed clones were sequenced by automated sequencing (MWG Biotech Ebersberg, Germany). Homology searches of all sequences were compared to the GenBank EMBL-EBI database using the BLAST algorithm.

Semi Quantitative Relative RT-PCR

Reverse transcriptions were carried out with total RNA (4 μg), previously treated with DNase I (Roche), isolated from ALC treated and control leeches with SuperScript™ II RNase H- Reverse Transcriptase kit (Invitrogen) and Oligo(dT)12–18 Primer (Invitrogen) according to the manufacturer’s instructions.

1 μl of first-strand cDNAs were used for each PCR amplifications and PCRs were performed using gene-specific primers and 5.8S rDNA gene of *Xenopus laevis* (accession No. X02995.1) or alpha- tubulin of *Hirudo medicinalis* (accession No. U67677.1) were used as housekeeping genes. Primer sequences are reported in Table 1.

The relative amounts of each PCR product were readily quantified by direct scanning with a densitometer of ethidium bromide-stained 2% agarose gel electrophoresis with Quantity One® Software (Bio-Rad, USA). To standardize the total RNA and the efficiency of cDNA synthesis of the samples, the band intensities were standardized with the average intensity of the 5.8S or Tubulin product across the samples investigated. The ratio between the value of the analyzed gene product level and the 5.8S or Tubulin product level of each sample was calculated from three independent experiments performed for each gene. The statistical analysis was done with the Unpaired T test (GraphPad Prism 4.00 software). All data are expressed as mean values ± SE.

Table 1. Primer sequences used in semi-quantitative RT-PCR assay.

| Gene               | Primer sequence                  | Accession No. |
|--------------------|----------------------------------|---------------|
| 5.8S               | F: CTTACGCGGGTATCATCCGGCTC       | X02995.1      |
|                    | R: GCCACGCTGACAGCGGGTAC          |               |
| Tubulin            | F: CCAACTGTGAAGCCCTTTGAGGAGGAGG | U67677.1      |
|                    | R: GCTCAAATGACACAGCGGCCAGCAGC   |               |
| Actin              | F: ACTCTTCTACAACTTGGGCCTAGCAGC  | HE962534      |
|                    | R: GAATGGTCAACTCATTCTCGAGGGG    |               |
| Hsp90              | F: ATGTCACCCAGGATGAAAGGGAGG     | HE962533      |
|                    | R: GCATATTCTGGAATATGACATCCACC   |               |
| Thiazole           | F: TAATTGAGCAATCTTGAGTCTGAGC    | HE962538      |
|                    | R: GGAAGTAAAGAGCCGGCTGAGC       |               |

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Results

The aim of this research was to single out genes that are differentially expressed in the leech nervous system in response to a single ALC treatment. In order to detect the differentially expressed genes, two groups of leeches were used: the first group was subjected to a single administration of 2 mM ALC and the second one was subjected to a single administration of saline solution. Eleven days after the treatment, chains of ganglia from both groups of animals were extracted, and the total RNA was isolated. We performed the suppression subtractive hybridization (SSH) method [24] for the construction of two subtractive cDNA libraries: the forward and reverse libraries consisting of transcripts positively and negatively modulated, respectively, by the treatment with ALC. The efficiency of subtraction was evaluated by PCR amplification of the housekeeping gene for 5.8S rRNA. The unsubtracted sample. The fragments are detectable as faint bands in the subtracted sample.

About 400 cDNA clones for each cDNA library were collected and the clones showing a single band after PCR amplification were sequenced. About 70% of the analyzed cDNA clones from all libraries resulted differentially expressed. We sequenced 40 cDNA clones, belonging to different cDNA libraries. With the information gathered from different databases we identified and assigned a putative function to more than half of the clones showing a single band after PCR amplification, while they are clearly detectable in the unsubtracted sample.

Table 2: we report also some alien sequences in Table 3 that pose some interesting questions that will be analysed in the discussion.

As shown in Tables 2–3 IA56, IAAB and IA55 clones code for Actinin, HPS90 and Thiazole biosynthetic enzyme respectively. Surprisingly the last clone codes for a protein, the Thiazole biosynthetic enzyme, which is generally expressed only in plants. The proteins Actinin and HPS90 are involved at different levels in neuronal activity. Because we previously observed that a single treatment with ALC in the leech affects forms of non associative learning, (6), here we thought it would be interesting to point out whether the treatment with ALC modulates the expression of the genes coding for these proteins. The technique of relative RT-PCR was used to analyze the expression of the IA56, IAAB and IA55 clones. The results obtained showed that ALC positively modulates the expression of genes coding for: Actinin (ALC 0.757 ± 0.034, Control 0.326 ± 0.029; n = 3; t = 9.645, df= 4, p = 0.0006; Fig.1A), HPS90 (ALC 0.766 ± 0.043, Control 0.383 ± 0.021; n = 3; t = 8.004, df= 4, p = 0.0013; Fig. 1B) and Thiazole biosynthetic enzyme (ALC 1.310 ± 0.040, Control 0.991 ± 0.081; n = 3; t = 3.531, df= 4, p = 0.0242; Fig. 1C). The aim of this research was to single out genes that are differentially expressed in the leech nervous system in response to a single ALC treatment. In order to detect the differentially expressed genes, two groups of leeches were used: the first group was subjected to a single administration of 2 mM ALC and the second one was subjected to a single administration of saline solution. Eleven days after the treatment, chains of ganglia from both groups of animals were extracted, and the total RNA was isolated. We performed the suppression subtractive hybridization (SSH) method [24] for the construction of two subtractive cDNA libraries: the forward and reverse libraries consisting of transcripts positively and negatively modulated, respectively, by the treatment with ALC. The efficiency of subtraction was evaluated by PCR amplification of the housekeeping gene for 5.8S rRNA. The fragments are detectable as faint bands in the subtracted sample.

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As shown in Tables 2–3 IA56, IA56 and IA55 clones code for Actinin, HPS90 and Thiazole biosynthetic enzyme respectively. Surprisingly the last clone codes for a protein, the Thiazole biosynthetic enzyme, which is generally expressed only in plants. The proteins Actinin and HPS90 are involved at different levels in neuronal activity. Because we previously observed that a single treatment with ALC in the leech affects forms of non associative learning, (6), here we thought it would be interesting to point out whether the treatment with ALC modulates the expression of the genes coding for these proteins. The technique of relative RT-PCR was used to analyze the expression of the IA56, IA56 and IA55 clones. The results obtained showed that ALC positively modulates the expression of genes coding for: Actinin (ALC 0.757 ± 0.034, Control 0.326 ± 0.029; n = 3; t = 9.645, df= 4, p = 0.0006; Fig.1A), HPS90 (ALC 0.766 ± 0.043, Control 0.383 ± 0.021; n = 3; t = 8.004, df= 4, p = 0.0013; Fig. 1B) and Thiazole biosynthetic enzyme (ALC 1.310 ± 0.040, Control 0.991 ± 0.081; n = 3; t = 3.531, df= 4, p = 0.0242; Fig. 1C).

Discussion

Studies on the effects of ALC on nonassociative learning forms in Hirudo medicinalis showed that ALC is able to produce a long-lasting effects on sensitization, dishabituation, and habituation processes [6]. In particular, a single administration of ALC brings about long-term effects that occur even 11 days after the treatment. The fact that ALC has long lasting effects suggested that it could lead to
a modulation of gene expression and, consequently, a differential mRNA synthesis in the leech nervous system. This hypothesis was evaluated with a molecular approach using the technique of Suppression Subtractive Hybridisation (SSH). SSH permits the construction of cDNA libraries containing differentially expressed transcripts in a tissue in response to a treatment, or to a different physiological condition. The analysis of expression of the clones considered has shown that ALC modulates the expression of several genes: among them, the genes coding for Actinin, HSP90 protein and the biosynthetic enzyme for Thiazole were chosen for

| Clone  | Accession No. | Accession No. of matching Sequence | Putative identification | Biological process |
|--------|---------------|-----------------------------------|-------------------------|-------------------|
| 1AA10  | HE962535      | AEX60843                          | Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit | Photosynthesis/Photorespiration |
| 1AB6   | HE962536      | AAP35043.1                        | Chlorophyll a/b binding protein | Photosynthesis, light harvesting |
| 7AG3   | HE962537      | AAP35043.1                        | Chlorophyll a/b binding protein | Photosynthesis, light harvesting |
| 1AE5   | HE962538      | ADG27845                          | Thiazole biosynthetic enzyme | Thiamine biosynthetic process |

*Accession number of the clones;  
*Accession number of the best match sequence;  
*Identification based on sequence similarity;  
*Biological process according to http://www.uniprot.org/.  

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Figure 1. Semiquantitative analysis. Relative RT-PCR (left) of Actinin (A), HSP90 (B) and Thiazole biosynthetic enzyme (C) transcripts. The relative expression levels (right) were calculated as ratio of each analysed transcripts with respect to the Tubulin or 5.8S product levels. The values followed by different letters are significantly different at the 0.05 probability level according to Unpaired T test. ALC and C, treated and control leeches respectively. M, molecular weight marker.  

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selected clones from a 24-h regenerating ganglion subtracted stress [42]. Interestingly, Gass et al. [43] demonstrated that contribute to protein homeostasis in physiological conditions and receptors for steroid hormones and protein kinases, and it may be essential for cell survival because they help the correct folding and chaperone eukaryotes, even in absence of stress [41]. This protein is a major isoforms [29]. Man, and probably other vertebrates, express all four isoforms, while invertebrates and protists have only one isoform. The members of the α-Actinin family are very represented in the mammals CNS, in particular, in dendritic spines. There is evidence that α-Actinin is involved in the modulation of glutamatergic receptors [30,31]; α-Actinin is associated with both AMPA and NMDA receptors (NMDAR) [32–35] whose importance in the mechanisms of synaptic plasticity and neuronal development is well known, and it represents a key component of the macromolecular complex mediating the calcium-dependent inactivation of NMDAR. In the leech, there is evidence that sensory neurons such as tactile T neurons, trigger glutamatergic polysynaptic connections activating NMDAR-dependent mechanisms linked to activity-dependent synaptic plasticity and behavioural processes [36,37]. Our data show an ALC-induced up-regulation of the expression of the gene for α-Actinin suggesting a mechanism through which ALC might reduce the responsiveness to tactile stimuli in the behavioral performances that have previously been shown to be impaired by ALC treatment [6]. A modulation of the expression of the gene coding for α-Actinin during learning has also been observed in the rat. Experiments carried out to identify genes involved in synaptic plasticity during fear conditioning, have shown that after 30 minutes and up to 4 hours of conditioning, there is an increase in α-Actinin expression [38].

The heat shock proteins (HSPs) are a class of proteins produced in response to various stress conditions [39]. HSPs prevent the inappropriate aggregation of proteins and mediate the transport of immature proteins to the target organelles [40]. Therefore they are essential for cell survival because they help the correct folding and refolding of new proteins or the degradation of proteins damaged by stress. HSP90 is one of the most abundant cytosolic proteins in eukaryotes, even in absence of stress [41]. This protein is a chaperone, acting towards certain classes of proteins, such as receptors for steroid hormones and protein kinases, and it may contribute to protein homeostasis in physiological conditions and stress [42]. Interestingly, Gass et al. [43] demonstrated that HSP90 protein is constitutively expressed in neuronal cells of the adult rat brain and Blackshaw et al. [44] found an HSP90 from selected clones from a 24-h regenerating ganglion subtracted library just in H. medicinalis thus suggesting a functional role of HSP90 in the physiological molecular program of neurons. In 2004, Gerges et al. [45] identified the role of HSP90 in excitative synaptic transmission in the hippocampus, observing that this chaperone is necessary for efficient neurotransmitter release. HSP90 is an important component of the molecular machinery required for continuous cycling of AMPA type glutamate receptors. These results highlight the importance of HSP90 as a regulator of synaptic processes [43]. In the leech nervous system there is evidence of glutamatergic transmission at the level of sensory integration [35]. Our findings suggest the hypothesis that HSP90 modulation might influence synaptic control during plastic changes.

The biosynthetic enzyme for Thiazole is an enzyme involved in the biosynthesis of thiamin (vitamin B1) [46], necessary in the metabolism of proteins, carbohydrates and fats. Among its different functions, the coenzyme thiamine is also important in the synthesis of acetylcholine, glutamate and GABA [47] and its deficiency can play an important role in some neurodegenerative disorders [48,49,50] such as Alzheimer’s disease and Wernicke encephalopathy [51]. Finally, thiamine, and other B group vitamins seem to have important anti-nociceptive and anti-inflammatory effects [52–55], therefore they are useful for the treatment of certain pain conditions, such as low back pain, or trigeminal neuralgia [52,53]. ALC increases the expression of the gene coding for the biosynthetic enzyme for Thiazole, and this effect might be linked to the anti-nociceptive action of the drug.

The troubling problem that arises is that the biosynthesis of thiamin is normally codified in plant genomes but not in the animal genome.

As far as the finding of alien plant genes is concerned, it must be stressed the fact that several plant genes are found in different animal systems. In the comparative genome analysis of planarian ESTs, Mineta et al. [56] found that about 30% of planarian nervous system-related genes had homologous sequences in Arabidopsis and yeast. The authors speculate that during evolution many genes, that are functional in the nervous system or CNS, may have been recruited from genes used in unicellular systems. It is worth noting that in our system the alien sequences are present as clones, but the corresponding genes were not found in leech DNA (unpublished results).

Korneev et al. [57] have constructed a subtractive cDNA library from regenerating Retzius cells of the leech: among the up-regulated sequences during nerve regeneration was found a hypothetical 39.4 yeast protein.

Blackshaw et al. [44] in their studies of the molecular basis of nervous system repair in H. medicinalis nerve cells found two clones coding for a heat shock protein HSP90 and a 16S ribosomal RNA as those reported in Table 2 of this paper. Moreover, they found a clone coding for an Arabidopsis thaliana ubiquitin-conjugating enzyme (UCE) and another coding for the cytochrome oxidase subunit II of the red alga Cyanidium caldarium; interestingly, these were up-regulated at 24 h post-axotomy.

One can hypothesize a symbiotic relationship with unicellular algae. This type of interactions is well known in different systems (for a review see Venn et al. [58]). More recently, an association was found between embryos of the salamander Ambystoma maculatum and the green alga Ophilia amblystomatis [59]. The authors considered this association as an instance of ecosymbiotic mutualism.

Aljamali et al. [60] in their transcriptome analysis of Amblyommia americanum salivary glands found a sequence of Pisum sativum root nodule estensin. Moreover, one contig of the library was highly similar to Lens culinaris nonspecific lipid transfer protein 1 precursor (LTP1). The authors hypothesized the possible involvement in a nonspecific uptake of arachidonic acid. It worth noting that in our library is present a LTP2 sequence.

A recent interesting and questioned paper by Zhang et al. [61] reported evidence of cross-kingdom regulation by exogenous plant microRNA in the sera and tissues of various animals. According to the authors, plant miRNAs are primarily acquired orally, through food intake.

In conclusion, the data collected in this paper reveal a lasting effect of ALC which modulates gene expression. Although our results do not fully explain the effects previously observed in behaviour, we show that a single administration of ALC is able to affect gene expression in the nervous system of the leech H. medicinalis. Previously, we have detected a modulation of gene expression by ALC in the rat brain [17–22]. By comparing the genome libraries obtained in both animal models we did not find common genes whose expression has been modulated by ALC.
except some HSPs. This difference in ALC-modulated gene expression might depend on different treatment modalities (single administration in the leech and chronic treatment in the rat). Also the time at which the analyses have been performed (11 days after the single treatment in the leech and immediately after the treatment in the rat) might account for the difference observed in gene expression as well as the phylogenetic distance between the animal models.

Nevertheless, the use of SSH has allowed to construct cDNA library in Hirudo medicinalis and to identify differentially expressed genes in response to a specific pharmacological treatment, thus representing a good tool for future investigations in the molecular biology of the leech nervous system.

**Author Contributions**

Conceived and designed the experiments: RB MB GT. Performed the experiments: MM Analyzed the data: GF RB MD GT. Wrote the paper: MD GT. Contributed to the editing and revising of the manuscript: GF RB RS MB MD GT. Supervisor of the project: MB MD GT.

**References**

1. Ghirardi O, Milano S, Ramacci MT, Angelucci L (1988) Effect of acetyl-l-carnitine chronic treatment on discrimination models in aged rats. Physiol. Behav. 43: 171–173.
2. Barnes CA, Markowska AL, Ingram DK, Kametani H, Spangler EL, et al. (1990) Acetyl- L-carnitine. 2: Effects on learning and memory performance of aged rats in simple and complex mazes. Neurobiol. Aging. 11: 499–506.
3. Ando S, Tadenuma T, Tanaka Y, Fuku J, Kobayashi S, et al. (2001) Enhancement of learning capacity and cholinergic synaptic function by carnitine in aging rats. J. Neurosci. Res. 66: 256–271.
4. Yasui F, Masugu S, Ishibashi M, Kajita T, Ezashi Y, et al. (2002) Effects of chronic acetyl-L-carnitine treatment on brain lipid hydroperoxide level and passive avoidance learning in senescence-accelerated mice. Neurosci. Lett. 334: 177–180.
5. Traina G (2011) Update on critical evidence for use of carnitine analogs in cerebrovascular disease. Neurotherapeutics. 8: 1–18.
6. Chiechio S, CaricaSeo A, Barletta E, Storto M, Catania MV, et al. (2002) L- Acetylcarnitine induces anaesthesia by selectively up-regulating mGlu2 metabotropic glutamate receptors. Mol. Pharmacol. 61: 989–996.
7. De Girardin D, Minardi G (2002) Acetyl-L-carnitine (levcarnitine) in the treatment of diabetic neuropathy. A long-term, randomised, double-blind, placebo-controlled study. Drugs R. D. 3: 223–231.
8. Lombardi P, Scutri R, Brunelli M (2003) Effects of acetyl-L-carnitine on sensory neurons of the leech Hirudo medicinalis. Pflugers Arch. Eur. J. Physiol. 445: R164.
9. Gadaleta MN, Cormio A, Pesce V, Lezza AM, Cantatore P (1998) Aging and heat shock proteins. J. Biol. Chem. 273: 9211–9218.
10. Fyrberg E, Kelly M, Ball E, Fyrberg C, Reddy MC (1990) Molecular Genetics of Drosophila alpha-actin-2: mutant alleles disrupt Z disc integrity and muscle insertions. J. Cell. Biol. 110: 1999–2011.
11. Cataldo E, Klemans L, Waterton RH (1991) Cloning, sequencing, and mapping of an alpha-actin gene from the nematode Caenorhabditis elegans. Cell Mot. Cytoskeleton. 20: 69–78.
12. Milli M, Yang N, Weinberger R, Vander Woude DJ, Berga AH et al. (2001) Differential expression of the actin-binding proteins, alpha-actin-2 and -3, in different species: implications for the evolution of functional redundancy. Hum Mol Genet 10: 1335–1346.
13. Dixson JD, Forster MJ, Garcia DM (2003) The alpha-Actinin gene family: a revised classification. J. Mol. Evol. 56: 1–10.
14. Rosenmund C, Feliz A, Westbrook GL (1995) Synaptic NMDA receptor channels have a low open probability. J. Neurosci. 15: 2788–2793.
15. Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit mediate Ca2+ dependent inactivation of NMDA receptors. J. Neurosci. 19: 1165–1178.
16. Schulz D, Sergeeva OA, Luhmann HJ, Haas HL, et al. (2004) Behavioural parameters in aged rats are related to LTP and gene expression of ChAT and NMDA-NR2 subunits in the striatum. Eur. J. Neurosci. 19: 1373–1383.
17. Nuriya M, Oh S, Huganir RL (2005) Phosphorylation-dependent interactions of alpha-Actinin-1/1QOGAPI with the AMPA receptor subunit GluR4. J. Neurochem. 95: 544–552.
18. Bouhaddan M, Yan HD, Yan BH, Bannon MJ, Audrade R (2006) Brain-specific regulator of G-protein signaling 2-9 selectively interacts with alpha-actinin-2 to regulate calcium-dependent inactivation of NMDA receptors. J. Neurosci. 26: 2522–2530.
19. Michailidis JE, Helton TD, Petrovi V, Mishali T, Ehlers MD, et al. (2007) Phosphatidylinositol-4,5-bisphosphate regulates NMDA receptor activity through alpha-actinin. J. Neurosci. 27: 5523–5532.
20. Burrell BD, Li Q (2008) Co-induction of long-term potentiation and long-term depression at a central synapse in the leech. Neurobiol. Learn. Mem. 90: 275–288.
21. Li Q, Burrell BD (2008) CNQX and AMPA inhibit electrical synaptic transmission: a potential interaction between electrical and glutamatergic synapses. Brain Res. 1229: 45–57.
22. Resler KJ, Paschall G, Zhou XI, Davis M (2002) Regulation of synaptic plasticity genes during consolidation of fear conditioning. J. Neurosci. 22: 7902–7904.
23. Sosnicka L, Cornellsen RN, Van Nieurenhoorn FA, Renan RS, Van Der Vusse GJ (2001) Heat shock proteins and cardiovascular pathophysiology. Physiol. Rev. 81: 1461–1497.
24. Calabrese V, Scapagnini G, Colombrita C, Ravagna A, Pennisi G, et al. (2003) Restoration of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach. Amino. Acids. 25: 437–444.
25. Welch WJ, Feramisco Jr. (1982) Purification of the major mammalian heat shock proteins. J. Biol. Chem. 257: 14944–14950.
26. Buchner J (1999) Hsp90 & Co. - a holding for folding. Trends Biochem. Sci. 24: 136–141.
27. Fyrberg E, Kelly M, Ball E, Fyrberg C, Reddy MC (1990) Molecular Genetics of Drosophila alpha-actin-2: mutant alleles disrupt Z disc integrity and muscle insertions. J. Cell. Biol. 110: 1999–2011.
28. Blackshaw SE, Babington JJ, Emes RD, Malek J, Wang WZ (2004) Identifying genes for neuron survival and axon outgrowth in Hirudo medicinalis. J. Anat. 204: 13–24.
29. Gerges NZ, Tran IC, Backos DS, Harrell JM, Chinkers M, et al. (2004) Independent functions of hsp90 in neurotransmitter release and in the continuous synaptic cycling of AMPA receptors. J. Neurosci. 24: 4738–4768.
46. Settembre E, Begley TP, Ealick SE (2003) Structural biology of enzymes of the thiamin biosynthesis pathway. Curr. Opin. Struct. Biol. 13: 739–47.
47. Thomson AD, Pratt OE (1992) Interaction of nutrients and alcohol: absorption, transport, utilization and metabolism. Watson RR and Watzl B, eds. Nutrition and Alcohol. 75–99.
48. Meador KJ, Nichols ME, Franke P, Durkin MW, Oberzan RL et al. (1993) Evidence for a central cholinergic effect of high-dose thiamine. Ann. Neurol. 34: 724–726.
49. Meador K, Loring D, Nichols M, Zamrini E, Rivner M, et al. (1993) Preliminary findings of high-dose thiamine in dementia of Alzheimer’s type. J. Geriatr. Psychiatry Neurol. 6: 222–229.
50. Benton D, Fordy J, Haller J (1995) The impact of long-term vitamin supplementation on cognitive functioning. Psychopharmacol. (Berl). 117: 298–305.
51. Heye N, Terstegge K, Sirtl C, McMonagle U, Schreiber K, et al. (1994) Wernicke’s encephalopathy-causes to consider. Intensive Care Med. 20: 282–286.
52. Mark J (1975) «A guide to the vitamins» MTP Press 73.
53. Mader R, Deutsch H, Siebert GK, Gerbershagen HU, Gruhn E, et al. (1988) Vitamin status of in patients with chronic cephalgia and dysfuction pain syndrome and effects of a vitamin supplementation. Int. J. Vitam. Nutr. Res. 58: 436–441.
54. Bromm K, Herrmann WM, Schulte H (1995) Do the B-vitamins exhibit antinociceptive efficacy in men? Results of a placebo-controlled repeated-measures double-blind study. Neuropsychobiol. 31: 156–165.
55. Wang ZB, Song XJ (2003) Antinoceceptive effect of the thiamine and cyanocobalamin in rats with primary sensory neurons injury. J. FASEB. 439–440.
56. Mineta K, Nakazawa M, Cebria F, Ito K, Agata K, et al. (2003) Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. Proc. Natl. Acad. Sci. U S A. 100: 7666–7671.
57. Korneev S, Fedorov A, Collin R, Blackshaw SE, Davies JA (1997) A subtractive cDNA library from an identified regenerating neuron is enriched in sequences up-regulated during nerve regeneration. Invert. Neurosci. 3: 163–192.
58. Venn AA, Loram JE, Douglas AE (2008) Photosynthetic symbioses in animals. J. Exp. Bot. 59: 1069–1080.
59. Kerney R, Kim E, Hangarter RP, Heiss AA, Bishop CD (2011) Intracellular invasion of green algae in a salamander host. Proc. Natl. Acad. Sci. U S A. 108: 6497–6502.
60. Aljamali MN, Hern L, Kupfer D, Downard S, So S, et al. (2009) Transcriptome analysis of the salivary glands of the female tick Amblyomma americanum (Acari: Ixodidae). Insect. Mol. Biol. 18: 129–154.
61. Zhang L, Hou D, Chen X, Li D, Zhu L, et al. (2012) Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell. Res. 22, 107–126.