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Effects of Cold Shock on Responses of Phosphomonoesters and Free Amino Acids in Phospholipid-Rich Organs in the Amur Sleeper *Perccottus Glehni*

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Similarities and differences were found in the mechanisms of adaptation of the brain and liver to cold shock in the freshwater fish *Perccottus glehni*. The quantity of phosphoethanolamine in the brain on day 4 of cold shock at +4°C increased 84-fold from the level in controls (+20°C), accounting for 22.3% of the total pool of free amino acids and ninhydrin-positive compounds. Phosphoserine was absent from the brain at +20°C, but cold shock initiated its accumulation to 1.8% of the total pool. The taurine pool at +4°C decreased from 28.3 to 20% of the total pool, yielding first place to phosphoethanolamine. However, in the liver, phosphoethanolamine (PE) and phosphoserine (PS) were not seen in the free form either in controls or at 4°C. The dominant amino acid in the liver at both temperatures was taurine, the quantity at +4°C being significantly increased. Th glutamate pool decreased nine-fold at +4°C. It is suggested that the intense accumulation of PE and PS in the brain at low temperatures is evidence of the specific features of the adaptation of the neuron membrane, reflecting quantitative changes in phospholipids, possibly including sphingosine-1-phosphate.

Keywords: phosphomonoesters, cold shock, liver, brain, protectors, bony fish.

Studies of the responses of free amino acids and ninhydrin-positive compounds (NPC) when the environmental temperature decreases to 0°C have showm that on amino acid chromatograms of brain extracts from eurythermic freshwater – the Amur sleeper *Perccottus glehni* (Eleotridae, Perciformes, Dyb. 1877) – the taurine elusion zone contains two peaks not previously seen in the muscles or plasma of these fish [1, 2] or in any of the organs studied in other poikilothermic animals [4, 5, 11, 14, 16]. These peaks were shown to correspond to two phosphomonoesters: the major to phosphoethanolamine (PE) and the minor to phosphoserine (PS) [3, 17]. PE is an ester of phosphoric acid and ethanolamine and, like amino acids, forms a blue color with ninhydrin, such that it can be detected [25]. The quantity of phosphomonoesters detected was found to reach a maximum by the beginning of the winter period [3]. We then established that in the brain (central neural ganglia) of the freshwater mollusk *Lymnaea stagnalis*, PE and PS are present constantly (at both low and normal environmental temperatures), though only in very small quantities [6]. It can be suggested that the quantitative and temporal (season-dependent) parameters of the presence of free PE and PS in the brain reflect a hierarchy of biochemical features underlying evolution. As PE and PS have the structure of phospholipids and are directly linked with their metabolism [27–29], this suggestion may make sense if the effect is specific for the brain while other phospholipid-rich organs in bony fish show no accumulation of these phosphomonoesters at low temperatures.

The liver, like the brain, is rich in phospholipids (the content in fish liver is about 65% [24, 27]), so the liver therefore provides a suitable system for seeking an answer to the question of the specificity of this phenomenon for the brain. Thus, the aim of the present work was to identify the presence or absence of PE and PS in the liver of eurythermic

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TABLE 1. Pools of Amino Acids and NPC in the Liver of *P. glehni* after Summer Exposure to Cold Shock (nmol/g wet weight)

Amino acid	Control, nmol/g	Shock day 4, nmol/g
Phosphoserine	Not detected	Not detected
Taurine	5257 ± 298 (28.5)	6600 ± 280$^{\#}$ (35.4)
PE	Not detected	Not detected
Aspartate	440 ± 36 (2.4)	460 ± 35 (2.5)
Serine + threonine	1579 ± 150 (8.5)	1737 ± 165 (9.3)
Glutamate	460 ± 34 (2.5)	53 ± 6* (0.3)
Glycine	3800 ± 310 (20.1)	5100 ± 425$^{\#}$ (27.4)
Alanine	4800 ± 420 (26)	3100 ± 260$^{\#}$ (16.7)
Valine	474 ± 36 (2.5)	426 ± 40 (2.3)
Methionine	300 ± 23 (1.6)	450 ± 31$^{\#}$ (2.4)
Isoleucine	250 ± 19 (1.3)	44 ± 5* (0.2)
Leucine	Not detected	Not detected
GABA	Not detected	Not detected
Tyrosine	Traces	Traces
Histidine	509 ± 47 (2.7)	286 ± 24$^{\#}$ (1.5)
Lysine	593 ± 50 (3.2)	378 ± 32$^{\#}$ (2.0)
TP	18462 ± 1790 (100 %)	18634 ± 1805 (100 %)
NH_3	2333 ± 220	2167 ± 200

Notes (here and Table 2). Experiments were performed in mid-July. Shock day 4 at +4°C. Cysteine, proline, arginine not detected. TP – Total pool of free amino acids and NPC. GABA – γ-aminobutyric acid. Data are means of three parallel experiments (M ± s.e.m.), each with three individuals. *$p < 0.001$; $^{\#}p < 0.05$. Control – +20°C. Values in parentheses are percentages of the total free amino acids + NPC pool.

freshwater fish in normal conditions and at low temperatures as compared with the corresponding characteristics of the brain. In addition, the study addressed the task of identifying the specific characteristics of adaptive responses of liver and brain free amino acids to the actions of low temperatures, supplementing data on the low-temperature adaptation of these organs.

Methods. *Study system*. Amur sleepers, *P. glehni*, were caught in the second 10 days of July in a lake in the basin of the Oka river at 54°50′ N, 37°42′ E. Individuals of mean weight 25 ± 2 g were selected from the caught fish.

Cold shock was imposed by exposure to a temperature of +4°C for four days by placing groups of three animals in a cold chamber in 5-liter plastic containers with a daily two-fold change of water at the same temperature.

After decapitation on day 4 of exposure to cold, brains were homogenized by slow manual rotation of the pestle of a microhomogenizer. Liver was homogenized in a Dounce homogenizer. Biological material was mixed with double-distilled water at a ratio of 1:9 and centrifuged at 15000 *g* for 20 min at 0°C in a K-24 centrifuge (Germany). The post-centrifugation supernatant (S_1) was stored at –10°C. After thawing, samples were centrifuged at 12000 *g* for 15 min before use and the resulting supernatant (S_2) was diluted with 2 N hydrochloric acid (1:1 by volume) to precipitate dissolved protein. After centrifugation of S_2, at 10000 *g* for 20 min, supernatant S_3 was diluted 1:1 with 2 N sodium citrate buffer solution pH 2.2 [3].

Phosphoester and free amino acid concentrations were determined by ion exchange chromatography on a T339 amino acid analyzer (Mikrotekh, Czech Republic) with cation exchange resin Ostion LG AB with a three-step gradient sodium citrate regime as eluant [25]. Post-column derivatization of amino acids was performed with ninhydrin, which was followed by detection at 570 nm. A standard chromatogram consisting of a mixture of 21 amino acids and PE was prepared for each series of experiments. The standard mix contained: taurine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, GABA, cystathionine, phenylalanine, histidine, lysine, arginine, PS, and PE. Amino acid contents were expressed as nmol/g wet weight.

Statistical analysis was run in Microsoft Excel 2010. Data were expressed as the means of three parallel samples ($n = 3$), each of which used three individuals, ± error of the mean (M ± s.e.m.).

All chemical reagents were from Sigma Chemical Co (USA).

Results. The results presented in Table 1 showed that PE and PS in *P. glehni* liver, in contrast to brain (Table 2), were not detected either in conditions of the natural summer environmental temperature or on exposure to cold shock for four days at +4°C. A feature of the liver amino acid and NPC pattern on cold shock was a significant increase in the level of sulfur-containing compounds: taurine (a sulfoacid) and methionine (a sulfoamino acid). The dominant amino acids in the liver at normal temperature were taurine, alanine, and glycine, making up a total pool accounting for 75% of the total amino acids pool (Table 1). Cold shock produced an almost 10-fold drop in the glutamate pool and an almost

TABLE 2. Pools of Amino Acids and NPC in the Brain of *P. glehni* after Summer Exposure to Cold Shock (nmol/g wet weight)

Amino acid	Control, nmol/g	Shock day 4, nmol/g
Phosphoserine	Not detected	172 ± 23*(1.8)
Taurine	3340 ± 240 (28.3)	1998 ± 170# (20.0)
PE	27 ± 7(0.2)	2257 ± 205* (22.3)
Aspartate	64 ± 6 (0.5)	125 ± 20# (1.2)
Threonine	Not detected	410 ± 55* (4.0)
Serine	Not detected	1207 ± 133* (12.0)
Glutamate	1806 ± 161 (15.3)	1673 ± 153 (16.6)
Glycine	948 ± 88 (8.0)	623 ± 50# (6.2)
Alanine	806 ± 77 (6.9)	370 ± 50# (3.6)
Valine	183 ± 17 (1.5)	60 ± 10# (0.6)
Methionine	Not detected	26 ± 5* (0.3)
Isoleucine	23 ± 5 (0.2)	Not detected
Leucine	30 ± 5 (0.2)	Not detected
GABA	995 ± 80 (8.4)	762 ± 72 (7.5)
Tyrosine	63 ± 9 (0.5)	46 ± 13 (0.4)
Histidine	2960 ± 263 (25.1)	177 ± 25* (1.7)
Lysine	542 ± 45 (4.6)	195 ± 16# (1.9)
TP	11787 ± 998 (100 %)	10101 ± 925 (100 %)
NH_3	1350 ± 155	246 ± 20*

five-fold drop in the isoleucine pool. These were accompanied by less significant decreases in the quantities of alanine and histidine. Marked increases in the levels of taurine, glycine, and methionine significantly compensated for the decreases in the pools of the amino acids named above.

In the brain, in contrast to the liver, among the identified amino acids and NPC, those showing large increases in levels in response to the sharp decrease in temperature were not the sulfur-containing compounds but, rather, the phosphorus-containing compounds – PE and PS (Table 2). In summertime, PE was also detected in controls at +20°C, though its level was extremely low, at only 0.2% of the total pool of free amino acids and NPC. Cold shock increased the pool of this phosphomonoester by a factor of 84, and the overall proportion of PE in this pool increased 111-fold, to 22.3% (Table 2).

In contrast to PE, the other phosphomonoester, PS, was absent from the brain at +20°C, though cold shock initiated its appearance and accumulation, such that the quantity of PS on day 4 was quite significant, at 172 ± 23 nmol/g (1.8%). The percentage content of the PS and PE pools in the brain essentially corresponded to those seen in our previous studies of the effects of the seasonal drop in temperature: 3.7% PS and 33.5% PE [3]. Similarity of the responses in the brain in different temperature conditions (shock in the present study and the seasonal drop in the preceding work [3, 17]) was also apparent in the negative correlation of changes in the taurine and PE pools: the taurine level, in contrast to that in the liver, decreased at +4°C to a level comparable with the increase in the quantity of PE (Table 2). It was of note that the seasonal drop in the taurine level was much more marked – from 3897 to 905 nmol/g [3].

In parallel with the decrease in taurine in the brain, there was a large, brain-specific, decrease in the quantity of histidine (Table 1). The previous study also noted a reduction in the histidine pool in the brain (during the seasonal temperature drop), though it was not as large as that resulting from cold shock (from 3290 to 2167 nmol/g) [3]. In the blood, as noted previously [2], cold shock decreased the histidine pool only three-fold, while muscles even showed a significant increase. Decreases in the pools of several protein amino acids in the brain on exposure to cold shock (glycine, alanine, some essential amino acids) and the liver may suggest that they have a role in protein synthesis (desaturases and other enzymes, chaperonins, etc.), which occurs along with proteolysis. These two processes are in relative equilibrium with each other and differ in different organs [20].

Thus, three types of free amino acid responses to cold shock can be identified in the liver and brain: increasing, decreasing, and not altering the pool on exposure to subnormal temperatures (Table 3).

Discussion. Cold shock is a rapid reduction in the water temperature inducing a cascade of physiological and behavioral changes, the initial temperature and duration of the shock inducing different scales of consequences [12]. Rapid and slow decreases in temperature have similarities and differences in biochemical processes, though the differences are more clearly apparent after some period of time. Our previous data [2] indicate that changes in the pattern of free amino acids and phosphomonoesters in *P. glehni* brain and muscle tissue start to be apparent two days after cold shock at 0°C, though modification of pools occurred much more intensely over the next two days. The absence of PE and PS from the liver in eurythermic freshwater fish at normal sum-

TABLE 3. Type of the Response of Free Amino Acids and NPC on Exposure to Cold Shock

Type of response of amino acids and NPC	Liver	Brain
Increase in pool	Taurine, glycine, methionine	Phosphoserine, PE, aspartate, serine, methionine
Decrease in pool	Glutamate, alanine, isoleucine, histidine, leucine	Taurine, threonine, glycine, alanine, valine, isoleucine, leucine, histidine, lysine
Weak or no reaction	Aspartate, serine, threonine, valine, leucine, tyrosine	Glutamate, tyrosine

mer temperatures and on exposure to cold shock on the one hand was consistent with data reported for virtually all somatic organs in fish and reptiles in normal conditions and in hypometabolic states (though for some reason brains were not included in this study) [11, 14]. On the other hand, there is also a need to consider the fact that the use of the chromatography methods employed by these authors to analyze amino acids and NPC was unable to detect PE. Furthermore, the authors did not even address this task. Thus, this study finally established the absence of free PE and PS in the livers of fish at normal temperatures and the absence of their accumulation in the hypometabolic state. These substances in the free state are seen in small quantities in the somatic organs, including the liver, only in mammals [16].

In the brain, cold shock induced the accumulation of three closely related compounds: PS, PE, and serine, which are used in the synthesis of glycerophospholipids and sphingosides but are not detected at normal summer temperatures. PE and PS are of particular interest, as these phosphomonoesters were detected in the free form and in large quantities in the brains of bony fish as a result of low temperatures. The pools of these substances show abundant increases depending on the duration and extent of the low-temperature action: cold shock for several days (Table 1) and during seasonal reductions in temperature during the autumn period [3]. Especially interesting is the fact that the accumulation of PS, PE, and serine in the brain occur in both types of low-temperature exposure – both cold shock and seasonal reductions – which is evidence for the special importance of this phenomenon for adaptation. The same cannot be said of taurine – a "universal protector" substance characteristic of most somatic organs and blood, though accumulating in these only in the process of seasonal and not in the process of shock-related temperature reductions [1, 2, 16, 17], with increases in the pool on cold shock only in the liver (Table 1). There is another interesting point: a correlation between the ratio of these closely related compounds (phosphomonoesters and serine) and the quantity of taurine in the brain. Thus, the result of the seasonal decrease in temperature is that these substances in the brains of fish trapped from under the ice displace taurine at the beginning of winter, decreasing its pool four-fold [3, 17]. Exposure to cold shock also produce an analogous displacement of taurine, the extent depending on the temperature: cold shock with a temperature of 0°C decreased the size of the brain taurine pool four-fold [2], as in winter [3], while shock with a temperature of +4°C (this study) produced a less than four-fold decrease in the same time period (Table 2).

There are no data on the actual biochemical reactions lading to accumulation of PE and PS, though it can be suggested that one source of the increase in the PE level is linked with the catabolism of the signal lipid sphingosine-1-phosphate. The reaction whose degradation product is PE is catalyzed by sphingosine-1-phosphate lyase [9, 15, 21, 23]. An alternative source may be related to phosphatidylethanolamine (PtdEA) and the plasmalogen phosphatidylethanolamine, i.e., the appearance of a more active isoform of phosphoethanolamine kinase could increase the quantity of PE with a corresponding change in the quantity of PtdEA. It should be noted that changes in the quantities of phospholipids in the brains of poikilothermic, homoiothermic, and heterothermic animals in response to temperature are often contradictory [7, 10]. In addition, changes in phospholipids also occur differently in nerve and glial cells; they are also nonidentical in the membranes of different subcellular structures [8].

PS is an ester of serine and phosphoric acid and is a component of many proteins, where it is present as a result of posttranslational modification – such that it is a nonprotein-forming amino acid. In contrast to fish, PS in the free form is detected in humans not only in the brain, but also in many biological fluids, where it is a normal metabolite [18]. The quantity of PE decreases and the quantity of PS increases significantly in Alzheimer's disease and its accompanying degradation of neuron membranes [13, 19].

It should be noted that PS can also be made from glucose via phosphoglycerate and phosphohydroxypyruvate [26]. Hydrolysis involving phosphatase can convert PS into the protein-forming hydroxyamino acid serine [26], accumulation of which can be explained not only by a "phospholipid origin) but also by synthesis from glycine or, more likely, proteolytic processes occurring in cells as a result of cold shock [20]. As the brain accumulates not only serine, but also threonine at low temperatures, the possibility that signal proteins in which these amino acids have been phosphorylated are degraded should not be excluded.

We can add to this the observation that PE and PS are also detected in the brains of the eurythermic freshwater

mollusk *Lymnaea stagnalis* [6], though in contrast to fish it is present in both summer and autumn, albeit in small quantities (6% of the total pool for PE and 0.7% for PS). During the seasonal drop in temperature, the brain PE pool in mollusks increases only slightly, while the PS pool shows no change at all [6], which is evidence that mollusks, unlike fish, lack any particular need for the brain to contain large quantities of these substances at low temperatures.

In contrast to PE and PS, the dynamics of modifications to the pools of various brain amino acids on exposure to cold shock (Table 1), as compared with previous results in relation to seasonal adaptation [3], show radical differences. Thus, glutamic acid – one of the amino acids dominating the brain at moderate temperatures and the main excitatory neurotransmitter in mammals [22] – in summer at normal temperatures has the largest brain pool after histidine and taurine, accounting for more than 15% of the total pool of free amino acids and NPC. During the seasonal drop in temperature at the beginning of winter, the pool decreases six-fold and becomes the least abundant among other amino acids [3, 17]. However, exposure to cold shock produces virtually no changes in glutamic acid in the brain, in contrast to the profound drop in the liver (Tables 1 and 2).

These observations lead to the following conclusions. The liver of eurythermic freshwater fish was found to respond to cold shock by increasing its content of sulfur-containing amino acids, while the brain shows increases in phosphorus-containing compounds, which are absent from the liver at both normal temperature and on exposure to cold shock. This phenomenon of adaptation of the brains of bony fish to low temperatures is important as a biochemical sign of evolution predetermining the further phylogenetic transformation of the brain. This sign is due to the characteristics of a given stage of phylogenesis, when evolutionary adaptation based on new and more advanced genetic mechanisms are apparent only on exposure to low temperatures. Accumulation of PE and PS in the brain induced by low temperatures may be due to specific modifications to neuron membranes, simultaneously supporting the brain's needs for low-temperature protectors. The clear specific features of low-temperature adaptation of the brain are also apparent in the accumulation of large quantities of serine and a massive drop in the histidine level.

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