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Distinct functional brain regional integration of *Casp3*, *Ascl1* and *S100a6* gene expression in spatial memory

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Short title: Integration of gene expression in memory formation

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Abstract

Evaluating the brain structural expression of defined genes involved in basic biological processes of neurogenesis, apoptosis or neural plasticity may facilitate the understanding of genetic mechanisms underlying spatial memory. The aim of the present study was to compare *Ascl1*, *Casp3* and *S100a6* gene expression in the hippocampus, prefrontal cortex and cerebellum of adult rats in water maze spatial memory performance. After four days training, the mean platform time (<10 sec) was evidence of stable long-term spatial memory formation. Real time PCR analysis revealed a positive inter-structural correlation for *S100a6*/*Casp* gene expression between the prefrontal cortex and the cerebellum but a negative correlation for *S100a6/Ascl1* transcribed genes between the prefrontal cortex and hippocampus during swimming in the active controls. However, during spatial memory performance there was only one inter-structural correlation between the prefrontal cortex and cerebellum with respect to *Casp3* expression, though there were intra-structural correlations between *Casp3/Ascl1* transcriptions within the prefrontal cortex and hippocampus as well as between *Ascl1/S100a6* in the cerebellum. In active learners versus naive controls, the transcrption of all genes was augmented in the prefrontal cortex but *Casp3* and *Ascl1* were also elevated in hippocampus whilst only *S100a6* increased in the cerebellum. The findings endorsed the role of the hippocampus in memory acquisition in addition to an integrative relationship with the prefrontal cortex and cerebellum. This structural and molecular configuration is important for creation of novel neural circuitry for consolidation and reconsolidation of memory trace with an involvement of coupled processes of neurogenesis, apoptosis or neural plasticity.

Keywords:

Memory

Casp3

Ascl1

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hippocampus

cortex

cerebellum

1. Introduction

Spatial memory is regarded as a staged process which includes acquisition, consolidation storage, retrieval and extinction [1,2]. Recent studies on the molecular basis of these component processes have found that numerous signaling molecules are involved in the memory phenomenon but, in some cases, molecular pathways may be selectively recruited during certain memory stages [3]. The use of the genetic approach, in addition to pharmacological manipulation [4] and lesioning [5], has contributed towards the delineation of brain systems and molecular mechanisms involved in spatial memory performance. In this connection, functional studies on brain gene transcriptional activity are particularly informative in identifying regional and molecular specificities [6,7].

As a result of transcriptional and behavioral training studies with the water maze, a dynamic and intricate pattern of gene expression with respect to memory has emerged [8] in addition to changes in early gene expression [9,10,11]. Disclosure of the precise genomic contribution towards long-term memory formation [12,13], together with an understanding of the roles of orchestrated mRNA and protein synthesis in this process, will undoubtedly facilitate the perception or comprehension of memory storage, retrieval and extinction both in normal and pathological states [14,15,8]. The functional properties of specific brain regions are determined in large part by the genes that are expressed within individual cells and together with developmental times, these mechanisms are dynamically regulated [16]. The subcellular processes of gene induction and expression, particularly in the hippocampus, are likely to underlie spatial learning and long term memory formation [11]. However, transcriptional processes probably vary between other brain areas such as the cerebral cortex and cerebellum in addition to the hippocampus and these are likely to adjust during retrieval and transformation into motor activity [8,17]. It has been demonstrated that spatial memory is associated with the hippocampus [18,19], there being translocation to the cortex for long term memory consolidation [8,20]. The traditional view on the core functions of the cerebellum consists of the regulation of motor coordination, balance and motor speech [21]. In addition to coordinating motor activity, the cerebellum has been implicated in motor learning and higher cognitive functions, but the circuitry involved in these activities is not yet understood [22] though there is recent evidence of anatomical and functional connectivity supporting a cerebello-hippocampal interaction [23]. In this context, cerebellar long-term depression may subserve a general sensorimotor adaptation process shared by both motor and spatial learning functions [24].

Defined gene expression analysis in different brain structures will also be useful in addressing any potential involvement of two coordinated processes - neurogenesis and programmed cell death (apoptosis) underlying spatial memory generation [25,26]. Previously, we have demonstrated molecular integration between neurogenesis and apoptosis at the genetic level in experiments

concerning expression of various gene transcripts which participate in mechanisms of cell birth, development and death in discrete brain regions in naïve adult rats. Thus, in the cerebellum, the content of mRNA coding for: *Ascl1* (*Mash1*), *Bax, Bcl2, Casp3, Casp8, Casp9, Dffb, Myh10, Naip2, Napa, Notch2, Numb, Pura, S100a6* and *Tnf*, excluding *Apaf1*, were significantly augmented against their levels in the hippocampus and prefrontal cortex. In comparison with the prefrontal cortex, hippocampal *Apaf1* expression was significantly reduced in contrast with higher expression levels of *Ascl1, Pura, S100a6* and *Tnf* genes. These data were further validated by disclosed correlations between gene regional expression rates [27]. It was particularly striking that the expression of three genes (*Ascl1, Casp3* and *S100a6*) was most consistent in the brain structures studied, and this finding prompted their selection as archetypical in terms of neurogenesis and/or apoptosis in the hippocampus, prefrontal cortex and cerebellum.

In order to investigate functional brain regional integration of gene expression in spatial memory performance in the current work, we focused on those transcriptional response examplars which play an integral role in regulating the coupled processes of neurogenesis and apoptosis.

Using the Gene Ontology bioinformatics database (www.geneontology.org) and the Mammalian Adult Neurogenesis Gene Ontology database (MANGO; http://adult-neurogenesis.de/resources/mango) common biological processes were verified for the products of the selected genes for current study: *Casp3* and *Ascl1* i.e. positive (GO:0043525) and negative (GO:0043066) regulation of apoptosis and neural differentiation (GO:0030182). In the case of the protein product of the *S100a6* gene, which is designated as the S100 calcium binding protein A6 - S100A6 or calcyclin [28,29] the transmembrane transport of ions is categorized separately in the database, i.e. GO:0034220. Hence, S100A6 is important for neuronal function through its involvement in calcium homeostasis [30].

The aim of the present work therefore, was to perform a comparative study on the level of transcriptional activity for *Ascl1* (*Mash 1*), *Casp3* and *S100a6* in the hippocampus, prefrontal cortex and cerebellum and analysis of any possible intra-and interstructural links within the examined gene activities during hippocampal dependent spatial memory performance in adult Wistar rats.

2. Materials and Methods

All experimental procedures were carried out in accordance with: the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996); the UK Animals Scientific Procedures Act 1986 and associated guidelines; the European Communities Council Directive of 24 November 1986 (86/609/EEC) for care and use of laboratory animals. They were also approved by the Animal Care and Use Committee of the P. K. Anokhin Institute of Normal Physiology, Russian Academy of Medical Science.

2.1 Animals

The study was performed using adult male Wistar rats weighing 220±20 g (n=30). They were allowed food and water *ad libitum* and housed in groups of three in standard laboratory cages under 12h–12h light-dark conditions. They were divided into three experimental groups: Group 1 - naïve rats (n=10), Group 2 - active control (swimming) rats (n=10), Group 3 - training rats in water maze (n=10).

2.2 Water maze paradigm

The water maze consisted of a grey coloured circular pool (160 cm in diameter and 60 cm in height) filled to a depth of 40 cm with water rendered opaque by the addition of a small quantity of powdered milk according to Sewell et al., [31]. The temperature of the water was maintained at 22.0±1.0^{o}C and the pool was divided into four quadrants. A transparent circular escape platform (12 cm in diameter) was located in one quadrant of the pool 2.0 cms beneath the water surface and hidden from animal view. The platform had a rough surface which facilitated animal access onto the platform once its presence was detected. A videomonitoring system (TSE, Germany) was used for recording the main parameters (time to reach the platform and length of travel) of training in the paradigm.

The maze was positioned in a well-lit room with several posters and other distal visual stimuli on the walls to provide external spatial cues. Animals were trained to spatially locate the hidden platform on 4 consecutive days. On each day, they received four consecutive training trials during which the hidden platform was kept in a constant location. Each trial was commenced by carefully placing each animal into the water facing the wall of the pool at one of three random start positions avoiding the quadrant including the platform. In each trial, the time taken to escape onto the hidden platform (swimming latency, sec) was recorded and followed by a 30 sec occupation of the platform. The inter-trial period was 60 sec. Any animal that did not find the platform within 60 sec was gently guided to it by the experimenter. Animals from the active control group were compelled to swim in the absence of the platform daily for 4 days (4 trials daily). The experimental protocol was designed so that the

swimming time of the control animals in each trial was matched to the mean time required by the trained rats to reach the platform and the inter-trial period was 90 sec.

2.3 Real-time PCR analysis

Immediately after the final trial on the 4th day in the water maze, rats were killed and cerebellar, hippocampal and prefrontal cortical structures were dissected on ice then stored at -80^{o}C for real-time PCR analysis.

In accordance with a previously reported protocol [27], the total RNA fraction from samples of rat hippocampus, prefrontal cortex and cerebellum (median horizontal fragment including both hemispheres and the vermis) was isolated using TRIzol (Invitrogen, USA), the RNA preparations were then purified from genome DNA admixture by DNase 1 treatment (RQ1 RNase-Free DNase 1, Promega, USA). The concentration of RNA was measured on a Qubit fluorometer (Invitrogen, USA) using a Qubit RNA Assay Kit (Invitrogen, USA). Reverse transcription was carried out with 50 ng total RNA, 200 U revertase M-MLV and oligo-$(dT)_{15}$ with 0.5 U RNAse inhibitor (Promega, USA) for 1.75 h at 37^{o}C. The resultant complementary DNA was diluted 10-fold with deionized water and stored at -80^{o}C. Samples with deionized water instead of RNA served as the negative controls. Real-time PCR was carried out repeatedly, for every sample, the test volume being 25 µl, with 1 µl diluted cDNA, 0.5 µl ready primer mixture (SABiosciences, USA), 5 µl qPCRmix-HS SYBR (Eurogen, Russia), and 18.5 µl deionized water. The protocol was as follows: amplification at 94^{o}C, 1.5 min; 50 cycles: denaturation at 94^{o}C, 30 sec; primer annealing at 64^{o}C, 15 sec; elongation at 72^{o}C, 30 sec. The amount of mRNA was evaluated by automated registration of the threshold cycle (C_t) by the amplifier DTlite (DNA Technology, Russia). No amplification was found in the negative control samples. Specificity of PCR products was confirmed by electrophoresis in 1.5% agarose with ethidium bromide with marker of sizing and approximate quantification of a wide range of double-stranded DNA GeneRuler 50 bp DNA Ladder (Fermentas, Lithuania). ß-actin gene was used as the reference gene for counting the relevant level of gene expression by method $2^{-\Delta\Delta Ct}$ [29]. Mean C_t values of each sample's repeats were used to calculate the level of gene mRNA expression (R) by the $2^{-\Delta\Delta Ct}$ and $2^{-\Delta\Delta Ct'}$ methods suggested previously [32]. The R values determined by the $2^{-\Delta\Delta Ct}$ method could not be used to detect the correlations between the genes in the prefrontal cortex, as the R values for all test genes were fixed and equal to 1. The R values determined by the 2 -ΔΔCt' method allowed detection of correlations between the genes in all structures, including the prefrontal cortex, as the R values for all experimental genes varied.

2.4 Statistics

The results were analyzed using Statistica 7.0 software. The empirical data did not conform to a normal distribution (as shown by Lilliefors test, $p<0.01$). Hence, several independent samples were compared

using Kruskal-Wallis with one-way analysis of variance. Post-hoc analysis for two samples involved the Mann-Whitney U test. In the case of dependent samples obtained in the water maze, comparison of multiple dependent samples was performed using Friedman ANOVA and Wilcoxon's matched pairs test. The significance level was taken as 5%. For mRNA expression, the significance of differences between two samples was verified by the Mann-Whitney U test. Correlations between the median levels of mRNA expression were detected using Spearman's ranked coefficient (r_S).

3. Results

3.1 Memory performance in the water maze

In behavioural experiments on long-term spatial memory performance in the water maze, the mean times to reach the platform for rats in the second and subsequent trials were progressively lower than the first trial (except of the 2nd day): Friedman ANOVA Chi Square (n = 10, df = 3) = 9.434, P = 0.024; (n = 10, df = 3) = 6.034, P = 0.109 and (n = 10, df = 3) = 9.218, P = 0.026 respectively. However, up to the end of the last trial on the fourth training day, the mean platform time was less than 10 seconds which is evidence of stable long-term spatial memory formation (Fig. 1). Comparing the mean values for the 2nd, 3rd and 4th trials with the first trial on each particular day, the following significance of differences in mean platform times were found: 1st day - 2nd trial=P<0.05, 3rd and 4th trials=P<0.01; 2nd day-4th trial=P<0.05; 3rd day-2nd trial=P<0.05, 3rd and 4th trials=P<0.01; 4th day-2nd, 3rd and 4th trials=P<0.01 (see Fig. 1).

Analysis of group times to reach the platform showed the following significant differences: On days 2 and 4, the platform times for the first trials were significantly different from the first trials on days 1 and 3, respectively (P<0.05). The overall means for all trials on days 2, 3 and 4 were significantly different from the means for all trials on previous days respectively (Kruskal-Wallis test: H (3, n= 40) =11.395, P=0.0125). Such analysis is necessary for detailed validation of long term memory processing. Since the platform times at the end of the fourth trial on the 4th training day were less than 10 seconds this indicated that stable long-term spatial memory had been established (Fig. 1).

3.2 Gene expression analysis

In molecular genetic experiments, it was found that there was regional specificity in *Casp3*, *Ascl1* and *S100a6* gene expression. Examination of *Casp3* transcription in brain structures showed that for this particular gene, expression levels were significantly lower in training animals versus active controls in the hippocampus (Z(1,17)=2.79, P<0.05) and prefrontal cortex (Z(1,17)=3.46, P<0.05) but not the cerebellum (Fig. 2). However, in the hippocampus and prefrontal cortex, *Casp3* gene expression was

elevated in both experimental groups relative to naïve control. Thus, in the hippocampus of the active control animal group, expression of the *Casp3* gene was increased 21.7 fold ($Z(1,17)=3.82$, $P<0.01$) ($P<0.01$) and only 3.0 fold in the training group ($P<0.01$). In the prefrontal cortex, a similar pattern of *Casp3* gene expression was found compared to the hippocampus. In the active controls there was a 28.8 fold ($P<0.05$) increase in *Casp3* mRNA in the prefrontal cortex and only 3.0 fold ($Z(1,17)=3.75$, $P<0.01$) in the training group compared to naïve controls (see Fig. 2).

It is interesting to note that the pattern of *Ascl1* gene expression in brain structures during physical activity and spatial memory performance differed from the *Casp3* gene. Hence, there was significant augmentation of *Ascl1* gene transcription in both experimental groups in the hippocampus ($P<0.01$) and prefrontal cortex ($P<0.01$) and also the cerebellum of trained rats ($P<0.01$) compared to naïve controls (Fig. 3). Assessment of *Ascl1* expression in the hippocampus and cerebellum also disclosed statistical enhancements in the training animal group versus active controls ($P<0.01$). In the hippocampus, the increase in *Ascl1* gene expression was 5.2 fold and 9.9 fold higher respectively in active control and training groups, against naïve rats ($P<0.01$). In comparison with naïve control in the cerebellum there was a 5.0 fold increase in *Ascl1* gene expression only in the training group and there was no difference from active control. In the prefrontal cortex however, there was no significant reduction in expression of *Ascl1* gene during spatial memory performance (training group) versus active control. Active controls displayed a 7.2 fold increase in *Ascl1* gene expression in the prefrontal cortex but during long-term memory formation there was a 5.0 fold increase in comparison with naïve rats (Fig. 3). The pattern of *S100a6* gene expression was similar in the cerebellum and prefrontal cortex but not in the hippocampus. Thus, in the training group, the mRNA level of this calcium/zinc binding protein was higher in the cerebellum ($P<0.05$) and prefrontal cortex ($P<0.05$) than in the active controls (Fig. 4). In the training rats, expression of this gene (*S100a6*) in comparison with active controls was statistically higher in the cerebellum (5.0 fold) and prefrontal cortex (1.6 fold) but in the hippocampus it was not statistically changed ($P=0.29$). In comparison with naïve controls, differences in both experimental groups ($P<0.05$) were found only in the prefrontal cortex (Fig. 4).

3.3 Correlations between memory performance parameters and intra- and inter-structural brain gene expression

Correlative analyses were performed with the aim of probing any possible interlinks between memory performance parameters and the transcriptional gene activities investigated. Comparison of the levels of *Casp3* gene expression in the prefrontal cortex and the time to platform in the first trial on each day of training yielded the following Spearman's rank correlation coefficients and significances: 1st day - $r_s = 0.71$, $P<0.05$; 2nd day - $r_s=0.83$, $P<0.01$; 3rd day - $r_s=0.81$, $P<0.01$; 4th day - $r_s=0.86$, $P<0.01$.

It can be seen in Table 1 that in the active control group, positive correlations between total swimming time for each training day and the level of *Casp3* expression in the prefrontal cortex existed and they were most marked on the second experimental day (r_s=0.904; P=0.002). A positive correlation was also drawn between overall swimming time (4 days) and *Casp3* expression in prefrontal cortex (Table 1). Moreover, negative correlations between the level of *S100a6* expression in the hippocampus and swimming time which reached a statistically significant level (P<0.05) on the 3rd and 4th day of experiments were obtained in the active controls. This was in contrast to the first two days of experiments where these negative correlations were not significant (i.e. P>0.05) (Table 1).

In the trained (learning) rat group, positive correlations were found only in the prefrontal cortex between the level of expression of *Ascl1* gene and the mean time to platform on the first training day. Additional positive correlations were established between the expression of this gene and the mean platform time in the 1st trial on 3rd and 4th training days (Table 1). Furthermore, correlations between three gene expression rates in brain structures (Table 2) were investigated in active control and training animals. In the active controls, an intra-structural link was determined only in the hippocampus between expression of the *Casp3* and *S100a6* genes. Active control rats displayed inter-structural links between *Casp3* expression in the cerebellum and *S100a6* in the prefrontal cortex and also *S100a6* expression in the prefrontal cortex and *Acsl1* in the hippocampus (Table 2). During training, in comparison with active control, it was shown that there was a higher statistical correlation between the expression level of all three genes studied (*Casp3, Ascl1* and *S100a6*) (Table 2). In training rats, there were positive statistical correlations in all the structures studied: in hippocampus and prefrontal cortex between expression of *Casp3* and *Ascl1*; in cerebellum between *S100a6* and *Ascl1*. It is noteworthy that inter-structural correlations existed between all experimental animal groups. Thus, in training animals, there was a clear correlation between *Casp3* gene expression in the cerebellum and the prefrontal cortex. In contrast, active control animals exhibited correlations between *S100a6* gene expression in the prefrontal cortex with *Casp3* expression in the cerebellum and also with *Ascl1* expression in the hippocampus (Table 2).

4. Discussion

In the current study, levels of expression of the *Casp3, S100a6* and *Ascl1* genes were assessed in the hippocampus, prefrontal cortex and cerebellum at the late stage acquisition stage of spatial memory in comparison with naïve controls and active swimmers.

In order to investigate functional brain regional integration of gene expression in spatial memory performance in the current work, we focused on those transcriptional response exemplars which play

an integral role in regulating the coupled processes of neurogenesis and apoptosis.

For example, protein caspase-3 which is regarded as a key link in caspase dependent apoptosis [33] is known to be involved in molecular mechanisms of learning and memory [34,35] based on the principles of brain regional specificity and heterochrony [36]. Addressing this concern, a recent study reported that caspase-3 participates not only in processes of programmed cell death but also neuronal plasticity [37]. Moreover, an additional link between *Casp3* and *S100a6* genes together with their protein products arises from the fact that S100A6 may be involved in the processing of apoptosis by modulating the transcriptional regulation of *Casp3* [29]. In neuroblastoma cells, binding of S100A6 to RAGE [38] may induce neuronal apoptosis by reactive oxygen species (ROS)-dependent activation of JNK and caspases -3 and -7 [39]. JNK plays a critical role in death receptor-initiated extrinsic as well as mitochondrial intrinsic apoptotic pathways. Moreover, JNKs activate apoptotic signaling by the upregulation of pro-apoptotic genes [40]. It was also reported that activation of the JNK pathway ‘‘breaks the brake’’ on apoptosis, thereby regulating, but not initiating the apoptotic process [41] though protein S100a6 may be directly involved in this pathway and *S100a6* gene transcription may also be a player in this regulatory mechanism. Accordingly, the increase of cellular survival and apoptosis triggered by S100A6 may be mediated through the formation of ROS which themselves elicit a wide range of cellular functions from proliferation to cell death. Furthermore, ROS are recognized to be involved in several RAGE-mediated biological processes [38,42] and since S100A6 increases ROS formation it may support the hypothesis that S100A6 also modulates cell survival in a RAGE-dependent manner [38,43].

In the current study we investigated *Casp3, Ascl1 and S100a6* gene expression, the protein products of which mediate different functional processes in various pathways. We also examined the brain regional specificity of the expression of these genes relative to long-term spatial memory formation. There is no available experimental data in the literature concerning the time course of *Casp3, Ascl1 and S100a6* gene activation in connection with neurogenetic or apoptotic processes during naïve or training conditions in rats.

In order to compensate this issue and disclose adolescent gene potency, we have recently assessed the concurrent expression of a range of genes involved in neurogenesis and apoptosis (*Apaf1, Ascl1, Bax, Bcl2, Casp3, Casp8, Casp9, Dffb, Myh10, Naip2, Napa, Notch2, Numb, Pura, S100a6, Tnf*) in the hippocampus, prefrontal cortex and cerebellum of naïve adult rats [27]. The results concurred with the concept that because the hippocampus and cortex are both forebrain structures, they have many cellular and structural features in common, whereas the cerebellum has a distinct developmental origin and therefore a unique cellular composition. In relation to these findings, elevated neurogenetic and

apoptotic gene expression in adult brain, particularly in hippocampus, cortex, striatum and cerebellum has also been shown previously [44].

Thus, it can be concluded that in the brain there is a baseline supportive gene expression which can be changed in novel conditions, for example during memory formation in task performance. However, after other triggers such as tetanic or low frequency stimulation (LFS) analysis of S100A6 mRNA levels did not reveal any differences between treated and control samples at any of the time points studied [45]. Moreover, gene expression in the rodent brain is dependent on exercise [46,47], so the control (swimming rats) used in the current investigation were appropriate for the aim of the study.

The water maze is considered to be an aversive task and it is associated with a certain degree of stress [48]. The heterogeneous effects of acute stress on learning and memory depend on numerous parameters related to the stressor, the time the stressor is experienced, and the nature of the stimuli or task examined. Converging evidence from a number of behavioural tasks suggests that acute stress disrupts the retrieval of spatial and recognition memory regardless of whether the stress is experienced before or after learning. Few studies have attempted to discern whether these effects are due to specific failures in consolidation or late stage acquisition of task relevant information [49]. Moreover, acute stress impairs memory retrieval and facilitates the induction of long-term depression (LTD) in the hippocampal CA1 region of the adult rodent brain [50]. It is well known that at the molecular level, stress hormones modulate memory formation and the effects of stress on spatial configuration learning are moderated by the magnitude of endogenous cortisol secretion [51]. Chronic stress clearly impacts nearly every brain region and thus, how chronic stress alters hippocampal spatial ability is likely to depend upon the engagement of other brain structures during behavioral training and testing [52]. In our study the influence of stress on long term memory formation was taken into account by examination of the behavioural and genetic indicators in the active control group.

Analysis of the training process monitored in this study showed that the mean times for reaching the platform by animals in the second and all subsequent trials were statistically lower than in the first trial. This finding is evidence that after the first training trial, long-term spatial memory formation had occurred. Furthermore, since the platform times at the end of the fourth trial on the 4^{th} training day were less than 10 seconds this signified the instigation of explicit long-term spatial memory (Fig. 1). These results are in agreement with data obtained by other authors who showed that at the end of a fourth training day, animals reached a plateau level of spatial habit performance [53,54].

Correlations between the expression of the *Casp3* gene and swimming time in the active control animal group were obtained, possibly being a reflection of activation of apoptotic processes in the cortex under stressful conditions during physical activity [55]. The influence of physical activity (swimming) and

mild stress on the interlinked processes of neurogenesis/apoptosis are supported additionally by the currently observed correlations between the level of expression of the *S100a6* gene in the hippocampus and the swimming time of the active controls (Table 1).

In the training animal group, correlations with physiological indicators (time to reach the platform) were shown only with prefrontal cortical *Ascl1* gene expression (Table 1) the role of which in neurogenesis/apopotosis is still ambiguous [56]. The *Asc1l* gene is expressed in hippocampal dentate gyrus progenitor cells, but there is no evidence that *Asc1l* expression is essential for their generation, survival and development [57]. However, it has been reported that the level of *Ascl1* gene expression is one of the factors which determine the fate of neural cells during neurotrophin receptor activation [58].

Changes in *Ascl1* expression during various physiological conditions remain largely unstudied. It has been demonstrated that the appearance of *Ascl1* positive progenitor cells in the dentate gyrus of the hippocampus in adult rats may be induced by treadmill running over a seven-day period [59]. In our experiments, an increase in *Ascl1* transcription was also found in the hippocampus and in the other structures of active controls in comparison with naïve rats (Fig. 3) but there were no correlations with swimming times. Together with this, we found a decrease in transcription levels of *Ascl1* gene in the prefrontal cortex of trained animals at the late stage acquisition stage of spatial memory versus active controls. In this connection, correlations of mRNA levels of the *Ascl1* gene in the prefrontal cortex with platform time is of special interest. All analyzed gene/swimming time correlations in trainers/learners were positive, so a decrease in *Ascl1* gene expression in comparison with active controls may be associated with a faster task acquisition maintained by the hippocampus. In essence, the hippocampus mediates the acquisition of spatial memory but the memory trace is eventually transferred to the cortex [5,60] and our observation is evidence of this postulation because there is a reverse in *AScl1* expression profile between these two structures. It has been posited that memory formation during training procedures is based on neurogenetic and apoptotic processes in which Ascl1 production plays a role [61,62]. The elevated *Ascl1* mRNA concentration in the hippocampus after 4 days of training may signify the involvement of this protein in regulatory mechanisms of the above processes during all stages of spatial memory formation. In the prefrontal cortex, the migrated memory trace probably initiates similar cell processes at the stage of swimming activity and a low *Ascl1* expression heralds the initial phases of memory late stage acquisition in this structure substantiating its structural specificity in spatial memory. In this context, it has also been postulated that Ascl1 plays a significant role in cerebellum circuit formation [63].

On the first day of training, a correlation was found between *Ascl1* gene expression and the time to reach the platform (indicator of working spatial memory). However, in the following training days,

correlations with times of reaching the platform were found only in the first attempted trials (3rd and 4th training days – Table 2), which is the most important criterion for long-term spatial memory at that particular stage of training. Hence, it can be hypothesized that there is a specific involvement of mechanisms connected with changing *Ascl1* gene transcription within the prefrontal cortex to memory and learning processes (see Scheme 2). Neural and oligodendrocyte progenitor cells in the adult brain express AScl1 to promote differentiation/survival and oppose apoptosis in which Casp3 and S100a6 transcription factors are involved [56,64,65].

It is notable in the current study that in trained rats, the relative level of *Casp3* gene expression in the hippocampus and prefrontal cortex is lower than in the swimming active controls. In the cerebellum and prefrontal cortex, a unidirectional rise in *S100a6* gene expression occurs in memory performance relative to swimmers. This divergent effect in gene expression can be explained by an upsurge of apoptotic processes in which Casp3 is involved at the stage of physical training (i.e. swimming) and a simultaneous boost of cell survival processes occurs along with activation of *S100a6* gene expression (see Scheme 1). This result can be explained by the existence of intra- and inter-structural feedback links at the genetic level in the active controls, as corroborated by the correlations shown in Scheme 1. Furthermore, the level of *Ascl1* and *S100a6* expression in the cerebellum during training sessions increased five times compared to active control and this may be evidence of parallel activation of proliferation/differentiation and apoptotic mechanisms or coordinated processes of neuroplasticity. The cerebellum has been implicated in a variety of functions that lie outside its traditional domain of sensory-motor control [66]. However, our findings have shown that there is a positive inter-structural correlation between *Casp3* expression in the prefrontal cortex and cerebellum. This was the only inter-structural link that was observed during spatial memory performance (Scheme 2) which probably reflects neuroplasticity in the two structures and also the neuroanatomical network of cerebellar-cortical connections [17]. In this paradigm, the cerebellum is more likely to play a role in the articulation of stored cortical memory into a spatial performance and it is the preparation of this motor activity that is a probable contributory factor [66]. Nevertheless, the cerebellum has been shown to be a key structure in the navigation system and cerebellar long-term depression at parallel fiber Purkinje cell synapses is generally viewed as the neural correlate of cerebellar motor learning [60].

It is useful to bear in mind that there are also a number of other genes involved in synaptic plasticity or transmission that change their expression levels after learning. These include CamKIIα, ERK2, syntaxin 1a, akt/PKB, mGluR7, syndecan 3, cerebroside synthase, D1A receptor, erbB4 and TRKB. Another set of genes, although not as closely related to memory processing as the previous group, is additionally connected to brain functionality (PBPTP, CK2α, PLC-ß3, RL/IF1, neuromedin K, ATPases, potassium inward rectifier channel J8) [8,67].

Our data demonstrate a specific brain regional expression of *S100a6* gene in trainers/learners and swimmers which differs from that seen in naïve rats. Furthermore, during spatial training, no difference in *S100a6* expression level in the hippocampus was evident in comparison with the active controls but in both the prefrontal cortex and cerebellum, significant transcriptional augmentation was detected. What is more, in the cerebellum of active control animals, there was a 5 fold decrease in *S100a6* transcription in comparison with naïve rats though this returned to naïve levels during formation of the spatial habit (i.e. in the water maze training group). Direct correlations were ascertained between the mean platform times on various training days and the level of *Casp3* gene expression in the prefrontal cortex. Moreover, there was a rank correlation between training day progression and *Casp3* gene expression. Hence, it can be concluded that these data additionally confirm that caspase-3 participates in the process/mechanism of training and in the light of this, it has been reported that inhibition of caspase-3 activity blocks long-term potentiation [35].

The finding that *S100a6* gene expression is boosted in the hippocampus adds to data concerning the expression of this gene not only selectively in neurons but also to a limited extent in astrocytes in the amygdala and entorrhinal cortex [68]. Escalation of *S100a6* expression during the training procedure can be regarded as evidence of neuroapoptotic activation since it was recently shown *in vitro* that this member of the S100 protein family may initiate apoptosis through activation of caspase-3 [31]. It is well known that the training process is accompanied by increasing numbers of spontaneously active neurons [69] and fluctuating frequencies of neuronal impulse activity. It may be suggested therefore, that the currently observed expression profile of *Casp3*, *Ascl1* and *S100a6* genes in the three brain structures reflects functional idiosyncrasies of a genomic involvement in interlinked neurogenetic and neuroapoptotic maneuvers participating in learning and memory processes e.g. spatial memory.

Conclusions

A simultaneous study of gene expression resulting in generation of molecular factors involved in neurogenesis, apoptosis and neural plasticity which underlie the formation of spatial memory in distinct functional brain regions disclosed specific intra- and inter-structural links in swimming active controls (Scheme1) and spatial memory performance (Scheme 2). The outcomes endorse the role of the hippocampus as a major structural location of memory acquisition as well as an integrative relationship with the prefrontal cortex and cerebellum. This structural, genetic and molecular combination is important for creation of novel neural circuitry for consolidation and reconsolidation of memory trace with an involvement of coupled processes of neurogenesis and apoptosis or neural plasticity.

Conflict of interest

The authors declare no conflict of interest

References

[1] Anderson JR. Learning and memory; an integrated approach. 2nd Edition John Wiley 2000 pp 1-377.

[2] D'Hooge R, De Deyn P P. Applications of the Morris water maze in the study of learning and memory. Brain Research Reviews 2001;36:160-90.

[3] Abel T, Lattal KM. Molecular mechanisms of memory acquisition, consolidation and retrieval. Current Opinion in Neurobiology 2001;11:180-187.

[4] Diergaarde L, Schoffelmeer ANM, De Vries TJ. Pharmacological manipulation of memory reconsolidation: Towards a novel treatment of pathogenic memories European Journal of Pharmacology 2008;585:453-457.

[5] Broadbent NJ, Squire LR, Clark RE. Spatial memory, recognition memory, and the hippocampus Proceedings of the National Academy of Sciences 2004;101:14515-14520.

[6] Nalloor R, Bunting KM, Vazdarjanova A. Encoding of emotion-paired spatial stimuli in the rodent hippocampus. Frontiers in Bahavioural Neuroscience 2012;6:1-11.

[7] Wu K, Li S, Bodhinathan K, Meyers C, Chen W, Campbell-Thompson M, McIntyre L, Foster TC, Muzyczka N, Kumar A. Enhanced expression of Pctk1, Tcf12 and Ccnd1 in hippocampus of rats: impact on cognitive function, synaptic plasticity and pathology. Neurobiology of Learning and Memory 2012;97:69-80.

[8] Igaz LM, Bekinstein P, Vianna MR, Izquierdo I, Medina JH. Gene expression during memory formation. Neurotoxicity Research 2004;6:189-204.

[9] Kelly MP, Deadwyler SA. Experience-dependent regulation of the immediate-early gene arc differs across brain regions. Journal of Neuroscience 2003;23:6443-6451.

[10] Barry DN, Commins S. Imaging spatial learning in the brain using immediate early genes:

insights, opportunities and limitations. Reviews in Neuroscience 2011;22:131-142.

[11] Chawla MK, Penner MR, Olson KM, Sutherland VL, Mittelman-Smith MA, Barnes CA. Spatial behavior and seizure-induced changes in c-fos mRNA expression in young and old rats. Neurobiology of Aging. 2013;34:1184-98.

[12] Cavallaro S, D'Agata V, Manickam P, Dufour F, Alkon D L.Memory-specific temporal profiles of gene expression in the hippocampus. Proceedings of the National Academy of Sciences 2002;99:16279-16284.

[13] Clayton DF. The genomic action potential. Neurobiology of Learning and Memory 2000;74:185-216.

[14] Datson NA, van der Perk J, de Kloet ER, Vreugdenhil E. Expression profile of 30,000 genes in Rat hippocampus using SAGE. Hippocampus 2001;11:430-444.

[15] Izquierdo I, Vianna MR, Izquierdo LA, Barros DM, Szapiro G, Coitinho AS, Muller L, Cammarota M, Bevilaqua LR, Medina JH. Memory retrieval and its lasting consequences. Neurotoxicity Research 2002;4:573-593.

[16] Wang VY, Zoghbi HY. Genetic regulation of cerebellar development. Nature Reviews in Neuroscience 2001;2:484-491.

[17] De Smet HJ, Paquier P, Verhoeven J, Mariën P. The cerebellum: Its role in language and related cognitive and affective functions. Brain and Language 2013;
DOI: 10.1016/j.bandl.2012.11.001

[18] Ross RS, Slotnick SD. The Hippocampus is preferentially associated with memory for spatial context. Journal of Cognitive of Science 2008;20:432-446.

[19] Slotnick SD, Thakral PP. The hippocampus operates in a threshold manner during spatial source memory. NeuroReport 2013;24:265-269

[20] Ranganath C, Ritchey M. Two cortical systems for memory-guided behaviour. Nature

Reviews in Neuroscience 2012;13:713-726.

[21] Baillieux H, De Smet HJ, Paquier PF, De Deyn PP, Mariën P. Cerebellar neurocognition: insights into the bottom of the brain. Clinical Neurology and Neurosurgery 2008;110:763-773.

[22] Voogd J, Glickstein M. The anatomy of the cerebellum. Trends in Neuroscience 1998;21:370-375.

[23] Rochefort C, Lefort JM, Rondi-Reig L. The cerebellum: a new key structure in the navigation system. Frontiers in Neural Circuits 2013;7:1-12.

[24] Burguière E, Arleo A, Hojjati MR, Elgersma Y, De Zeeuw CI, Berthoz A, Rondi-Reig L. Spatial navigation impairment in mice lacking cerebellar LTD: a motor adaptation deficit? Nature Neuroscience 2005;8:1292-1294.

[25] Prickaerts J, Koopmans G, Blokland A, Scheepens A. Learning and adult neurogenesis: Survival with or without proliferation? Neurobiology of Learning and Memory 2004;81:1-11.

[26] Bruel-Jungerman E, Rampon C, Laroche S. Adult hippocampal neurogenesis, synaptic plasticity and memory: facts and hypotheses. Reviews in the Neurosciences 2007;18:93-114.

[27] Kolobov VV, Storozheva Z I, Gruden MA, Sherstnev VV. Regional Features of the Expression of Genes Involved in Neurogenesis and Apoptosis in the Brain of Adult Rats. Bulletin of Experimental Biology and Medicine 2012;153:707-711.

[28] Leclerc E, Fritz G, Weibel M, Heizmann CW, Galichet A. S100B and S100A6 differentially modulate cell survival by interacting with distinct RAGE (receptor for advanced glycation end products) immunoglobulin domains. The Journal of Biological Chemistry 2007;282:31317-31331.

[29] Joo JH, Yoon SY, Kim JH, Paik SG, Min SR, Lim JS, Choe IS, Choi I, Kim JW. S100A6 (calcyclin) enhances the sensitivity to apoptosis via the upregulation of caspase-3 activity in Hep3B cells. Journal of Cell Biochemistry 2008;103:1183-1197.

[30] Jastrzębska B, Filipek A, Nowicka D, Kaczmarek L, Kuźnicki J. Calcyclin (S100A6) binding protein (CacyBP) is highly expressed in brain neurons. Journal of Histochemistry

and Cytochemistry 2000;48:1195-1202.

[31] Sewell RDE, Gruden MA, Pache DM, Storozeva ZI, Kostanyan IA, Proshin AT, Yurasov VV, Sherstnev VV. Does the human leukaemia differentiation factor fragment HLDF6 improve memory via brain DNA and protein synthesis? Journal of Psychopharmacology 2005;602-608.

[32] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402-408.

[33] Creagh EM, Martin S J. Caspases: cellular demolition experts. Biochemical Society Transactions 2001;29:696-702.

[34] Dash PK, Blum S, Moore AN. Caspase activity plays an essential role in long-term memory. Neuroreport 2000;11:2811-2816.

[35] Gulyaeva NV, Kudryashov IE, Kudryashova IV. Caspase activity is essential for long-term potentiation. Journal of Neuroscience Research 2003;73: 853-864.

[36] Sherstnev VV, Yurasov VV, Storozheva ZI, Gruden MA, Yakovleva NE. Biochemical markers of apoptosis in different parts of the brain during learning. Neuroscience and Behavioral Physiology 2006;36:915-9.

[37] D'Amelio M, Cavallucci V, Cecconi F. Neuronal caspase-3 signaling: not only cell death. Cell Death and Differentiation 2010;17:1104-1114.

[38] Donato R. RAGE: a single receptor for several ligands and different cellular responses: the case of certain S100 proteins. Current Molecular Medicine 2007;7:711-24.

[39] Leśniak W, Słomnicki ŁP, Filipek A. S100A6 - new facts and features. Biochemical and Biophysical Research Communications 2009;390:1087-1092.

[40] Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. Oncogene 2008;27:6245-6251.

[41] Lin A. Activation of the JNK signaling pathway: breaking the brake on apoptosis. Bioessays 2003;25:17-24.

[42] Kang R, Tang D, Lotze MT, Zeh HJ. RAGE regulates autophagy and apoptosis following oxidative injury. Autophagy 2011;7:442-444.

[43] Leclerc E, Fritz G, Vetter SW, Heizmann CW. Binding of S100 proteins to RAGE: An update. Biochimica et Biophysica Acta 2009;1793:993-1007.

[44] Stansberg C, Vik-Mo AO, Holdhus R, Breilid H, Srebro B, Petersen K, Jørgensen HA, Jonassen I, Steen VM.. Gene expression profiles in rat brain disclose CNS signature genes and regional patterns of functional specialization. BMC Genomics. 2007;8:94. doi: 10.1186/1471-2164-8-94.

[45] Lisachev PD, Shtark MB, Sokolova OO, Pustylnyak VO, Salakhutdinova MY, and Epstein OI. A Comparison of the Dynamics of S100B, S100A1, and S100A6 mRNA Expression in Hippocampal CA1 Area of Rats during Long-Term Potentiation and after Low-Frequency Stimulation. Cardiovascular Psychiatry and Neurology. 2010;720958:1-6. doi: 10.1155/2010/720958].

[46] Mojtahedi S, Kordi MR, Hosseini SE, Omran SF, Soleimani M. Effect of treadmill running on the expression of genes that are involved in neuronal differentiation in the hippocampus of adult male rats. Cell Biology International. 2013;37:276/a-283/a doi: 10.1002/cbin.10022.

[47] Lee M-Y, Yu JH, Kim JY, Seo JH, Park ES, Kim CH, Kim H, Cho S-R. Alteration of synaptic activity-regulating genes underlying functional improvement by long-term exposure to an enriched environment in the adult brain. Neurorehabilitation and Neural Repair. 2013;27:561-574 doi: 10.1177/1545968313481277.

[48] Harrison F.E., Hosseini A.H, McDonald M.P. Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks. Behavioural Brain Research, 2009;198:247-251.

[49] Cazakoff BN, Johnson KJ, Howland JG. Converging effects of acute stress on spatial and recognition memory in rodents: a review of recent behavioural and pharmacological findings. Progress in Neuropsychopharmacology and Biological Psychiatry. 2010;34:733-741.

[50] Wong TP, Howland JG, Robillard JM, Ge Y, Yu W, Titterness AK, Brebner K, Liu L, Weinberg J, Christie BR, Phillips AG, Wang YT. Hippocampal long-term depression mediates acute stress-induced spatial memory retrieval impairment. Proceedings of the National Academy of Science U S A. 2007;104:11471-11476.

[51] Meyer T, Smeets T, Giesbrecht T, Quaedflieg CWEM, Marckelbach H. Acute stress differentially affects spatial configuration learning in high and low cortisol-responding healthy adults. European Journal of Psychotraumatology 2013;4:1-9 doi: 10.3402/ejpt.v4i0.19854.

[52] Conrad CD. A critical review of chronic stress effects on spatial learning and memory. Progress in Neuropsychopharmacology and Biological Psychiatry. 2010;34:742-755.

[53] Terry AVJr. Spatial Navigation (Water Maze) Tasks. In: Methods of Behavior Analysis in Neuroscience. Frontiers in Neuroscience. 2nd ed. Boca Raton (FL): CRC Press; 2009;Chapter 13. Ed: Buccafusco, J. J.

[54] Dupret D, Fabre A, Döbrössy MD, Panatier A, Rodríguez JJ, Lamarque S, Lemaire V, Oliet SHR, Piazza PV, Abrous DL. Learning depends on both the addition and removal of new hippocampal neurons PLoS Biology 2007;5:e214.

[55] Tong L, Shen H, Perreau VM, Balazs R, Cotman CW. Effects of exercise on gene-expression profile in the rat hippocampus. Neurobiology of Disease 2001;8:1046-56.

[56] Yi S-H, Jo A-Y, Park Ch-H, Koh H-Ch, Park R-H, Suh-Kim H, Shin I, Lee Y-S, Kim J, Lee S-H. Mash1 and neurogenin 2 enhance survival and differentiation of neural precursor cells after transplantation to rat brains via distinct modes of action. Molecular Therapy 2008;16:1873-1882.

[57] Galichet C, Guillemot F, Parras CM. Neurogenin 2 has an essential role in development of the dentate gyrus. Development 2008;135:2031-2041.

[58] Nikoletopoulou V, Plachta N, Allen ND, Pinto L, Götz M, Barde YA. Neurotrophin receptor-mediated death of misspecified neurons generated from embryonic stem cells lacking Pax6. Cell Stem Cell 2007;1:529-540.

[59] Uda M, Ishido M, Kami K. Features and a possible role of Mash1-immunoreactive cells in the

dentate gyrus of the hippocampus in the adult rat. Brain Research 2007;1171:9-17.

[60] Rochefort C, Lefort JM, Rondi-Reig L. The cerebellum: a new key structure in the navigation system. Frontiers in Neural Circuits 2013;7:1-12.

[61] Castro DS, Guillemot F. Old and new functions of proneural factors revealed by the genome-wide characterization of their transcriptional targets. Cell Cycle 2011;10:4026-31.

[62] Dong C, Zhao H, Chen W, Wang L, Zhang L, Zhang X, Shi J, Li H, Jin G. The dynamic expression of Mash1 in the hippocampal subgranular zone after fimbria-fornix transection. Neuroscience Letters 2012;520:26-31.

[63] Sudarov A, Turnbull RK, Kim EJ, Lebel-Potter M, Guillemot F, Joyner AL. Ascl1 genetics reveals insights into cerebellum local circuit assembly. Journal of Neuroscience 2011;31:11055-11069

[64] Lo L, Dormand E, Greenwood A, Anderson DJ. Comparison of generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development 2002;29:1553-1567.

[65] Kim EJ, Leung CT, Reed RR, Johnson JE. In vivo analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis. Journal of Neuroscience 2007;27:12764-74.

[66] Nixon PD, Passingham RE. The cerebellum and cognition: cerebellar lesions do not impair spatial working memory or visual associative learning in monkeys. European Journal of Neuroscience 1999;11:4070-4080.

[67] Kaczmarek L. Gene expression in learning processes. Acta Neurobiologiae Experimentalis (Warsaw) 2000;60:419-424.

[68] Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 2002;110:429–441.

[69] Aleksandrov AA, Sabanov VS, Samoĭlova LA. Inhibitory responses of sensomotor cortex neurons in the rabbit to the initial stages of elaboration of conditioned defensive reflexes. Zhurnal Vysshei Nervnoi Deiatelnosti Imeni I.P. Pavlova. 1983;33:755-757.

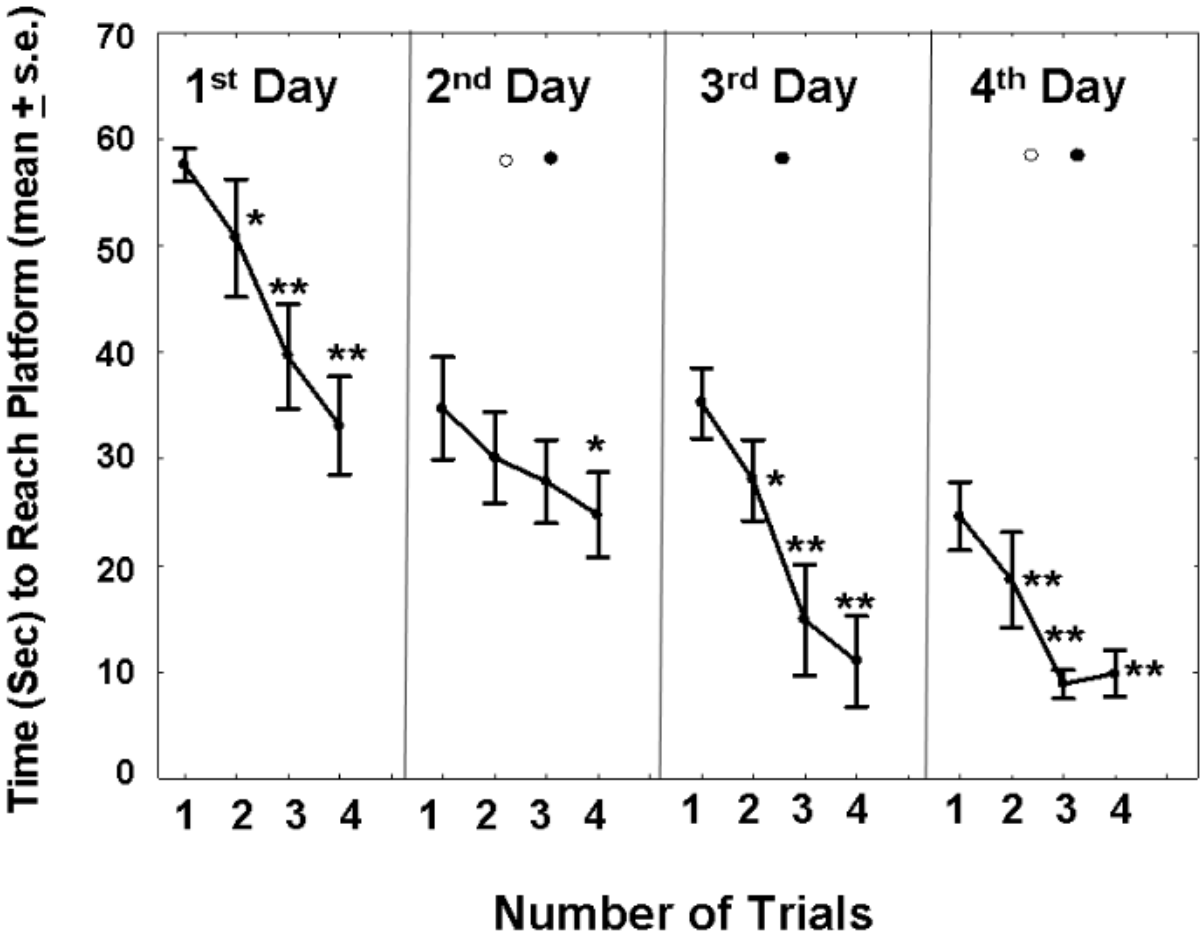


Fig. 1. Long-term spatial memory performance of rats in the water maze over four consecutive days

○ - *P*< 0.05 comparison of the mean time to reach the platform in the first trial with the mean platform time for the first trial on the previous training day

● - *P*< 0.05 comparison of the overall mean time to reach the platform for all trials with the mean platform time for all trials on the previous training day

*=*P*<0.05, **=P<0.01- comparison of the mean time to reach the platform with the first trial for each individual training day.

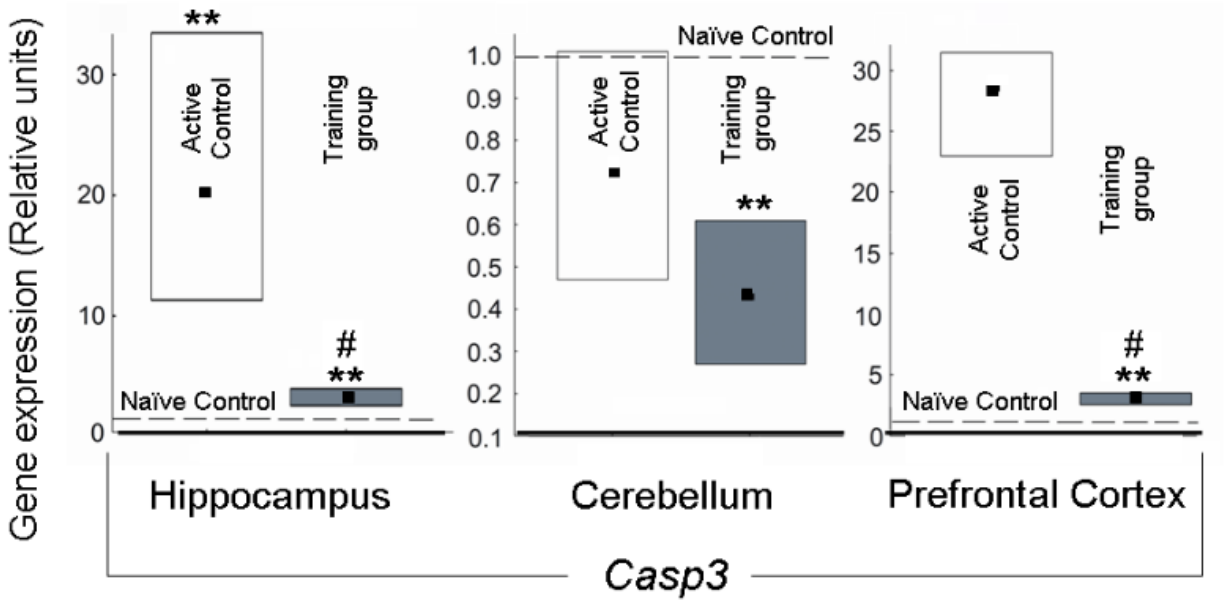


Fig. 2. *Casp3* gene expression in rat hippocampus, cerebellum and prefrontal cortex during spatial memory formation.

■ = median values with upper and lower interquartile ranges for the following animal groups: white bars = active control; grey bars = training animals; dotted line = naïve control where 1 unit represents the level of *Casp3* gene expression against which relative gene expression levels were calculated. Statistical differences: * = $P< 0.05$, ** = $P< 0.01$ compared to naïve control; # = $P< 0.05$, ## = $P< 0.01$ compared to active control.

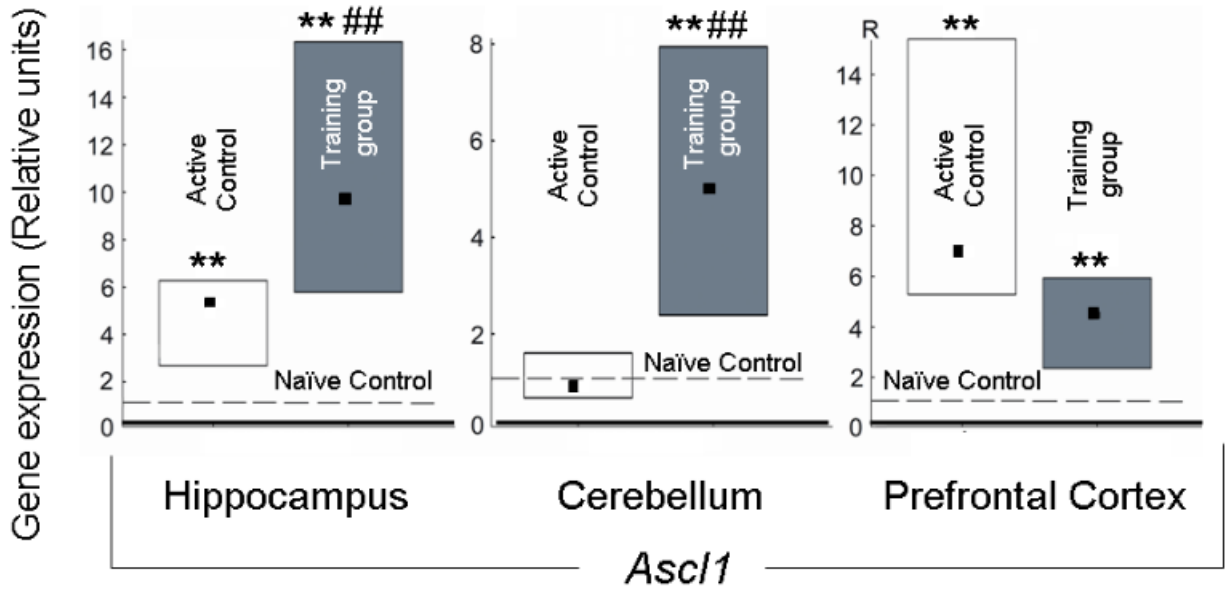


Fig. 3. *Ascl1* gene expression in rat hippocampus, cerebellum and prefrontal cortex during spatial memory formation.

■ = median values with upper and lower interquartile ranges for the following animal groups: white bar = active control; grey bar = training animals; dotted line = naïve control where 1 unit represents the level of *Ascl1* gene expression against which relative gene expression levels were calculated. Statistical differences: * = $P< 0.05$, ** = $P< 0.01$ compared to naïve control; # = $P< 0.05$, ## = $P< 0.01$ compared to active control.

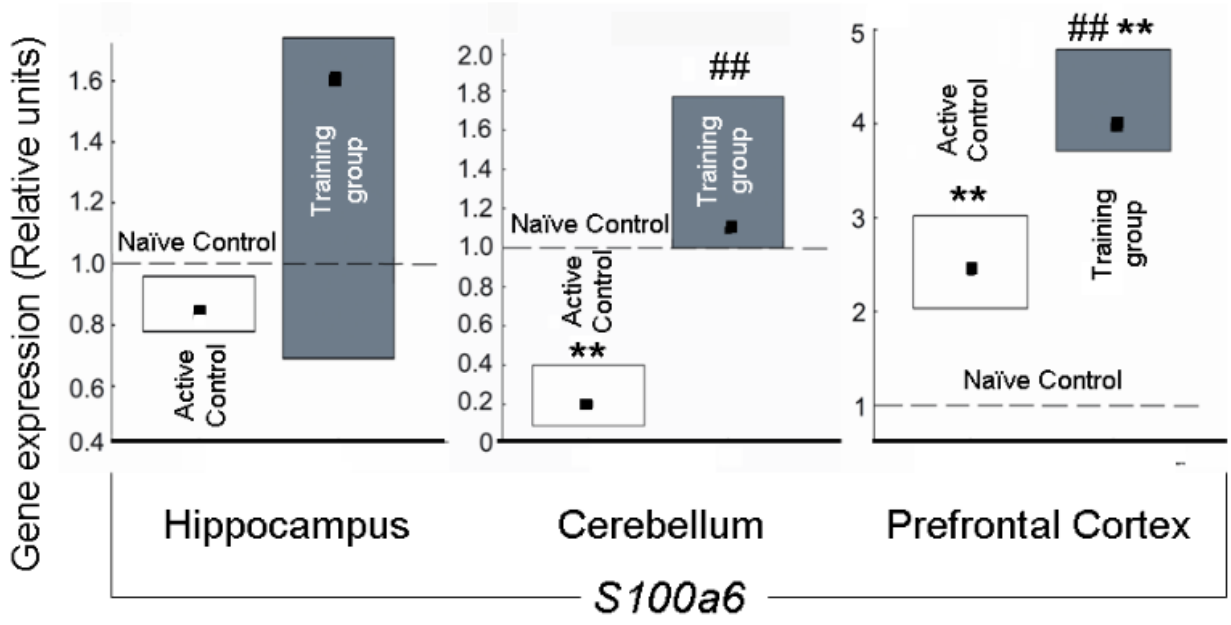
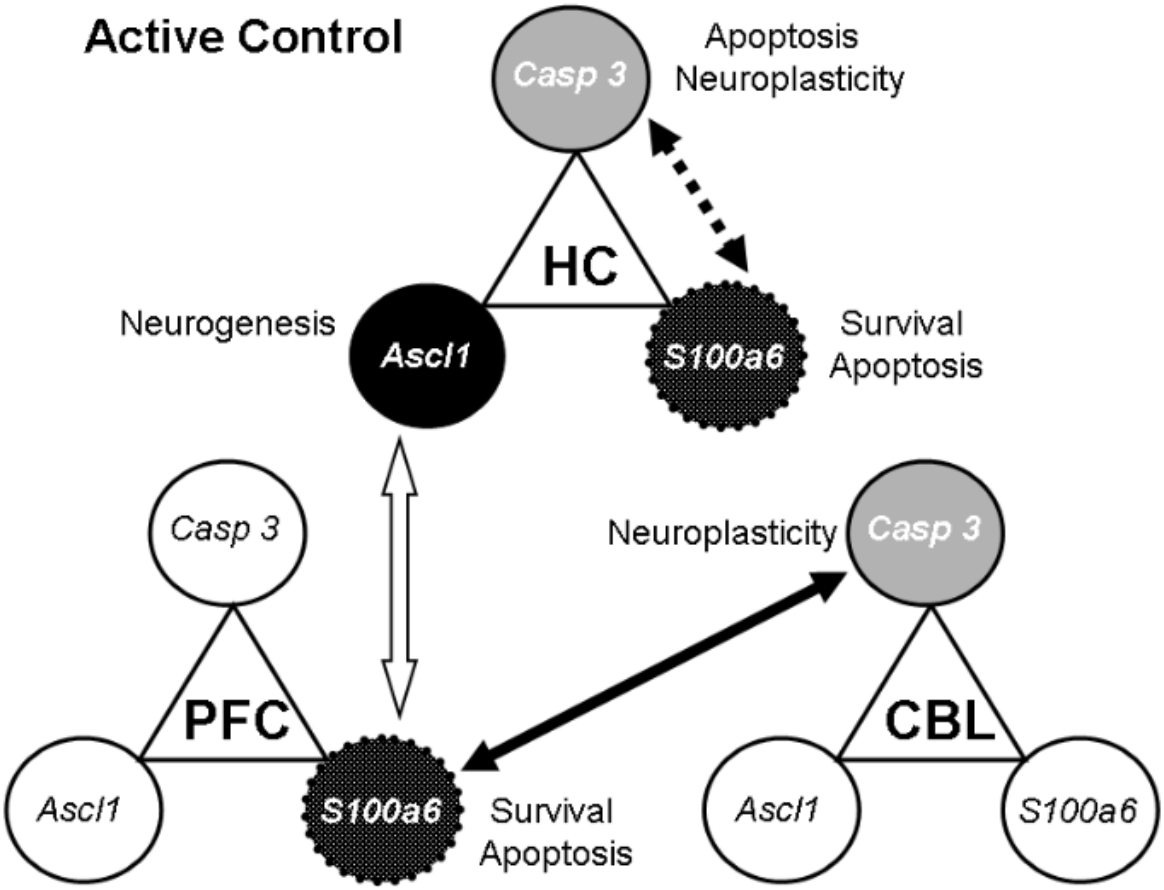


Fig. 4. *S100a6* gene expression in rat hippocampus, cerebellum and prefrontal cortex during spatial memory formation.

■ = median values with upper and lower interquartile ranges for the following animal groups: white bar = active control; grey bar = training animals; dotted line = naïve control where 1 unit represents the level of *S100a6* gene expression against which relative gene expression levels were calculated. Statistical differences: * = $P< 0.05$, ** = $P< 0.01$ compared to naïve control; # = $P< 0.05$, ## = $P< 0.01$ compared to active control.



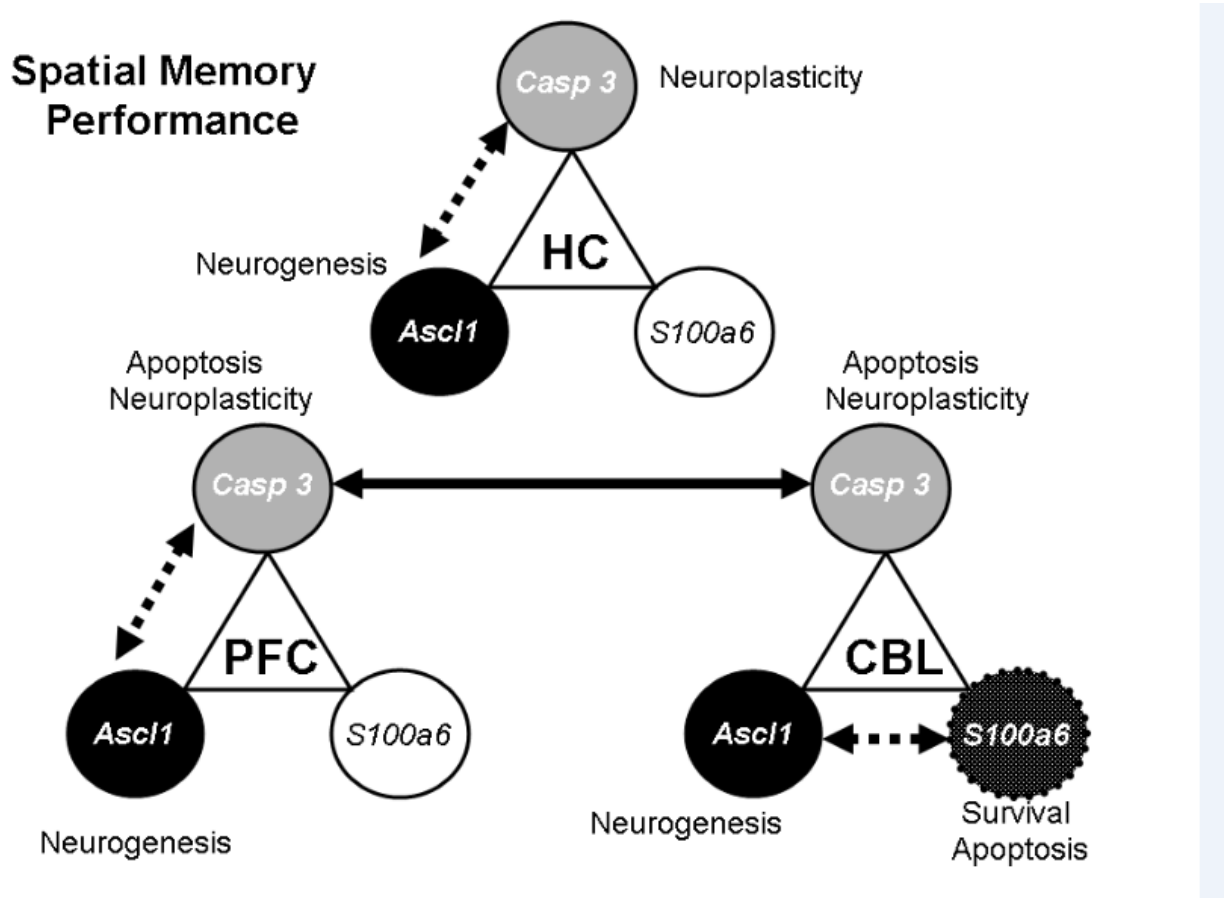
Scheme 1. Correlations between *Casp3, Ascl1* and *S100a6* gene expression showing brain inter- and intra-structural links with biological processes in active control animals.

Correlated gene expression: *Casp3* = grey circles, *Ascl1* = black circle, *S100a6* = stippled circles. Non-correlated gene expression = white circles.

Brain structures: HC = Hippocampus; PFC = Prefrontal cortex; CBL = Cerebellum.

Dotted black arrow = positive intra-structural correlation; solid black arrow = positive inter-structural correlation; white arrow = negative inter-structural correlation.

Gene-associated biological processes are shown as text.



Scheme 2. Correlations between *Casp3, Ascl1* and *S100a6* gene expression showing brain inter- and intra-structural links with biological processes during spatial memory performance.

Correlated gene expression: *Casp3* = grey circles, *Ascl1* = black circles, *S100a6* = stippled circle. Non-correlated gene expression = white circles.

Brain structures: HC = Hippocampus; PFC = Prefrontal cortex; CBL = Cerebellum.

Dotted black arrows = intra-structural correlations; solid black arrow = inter-structural correlation.

Gene-associated biological processes are shown as text.

Table 1. Correlations specifically between gene transcriptional activity of *Casp3* and *S100a6* in active controls plus *Ascl1* in training rats with indicators of spatial performance in the water maze over 4 days.

Animal Group	Correlation parameters	r_s	T(n-2)	*P*
Active Control	*Casp3*, prefrontal cortex /mean swimming time on 1st day	0.714	2.28	0.047
	Casp3, prefrontal cortex/mean swimming time on 2nd day	0.904	3.69	0.002
	Casp3, prefrontal cortex/mean swimming time on 3rd day	0.81	3.37	0.015
	Casp3, prefrontal cortex/mean swimming time on 4th day	0.57	1.39	0.1
	Casp3, prefrontal cortex/overall swimming time (4 days)	0.81	3.38	0.015
	S100a6, hippocampus/mean swimming time on 1st day	-0.69	-2.15	0.057
	S100a6, hippocampus/mean swimming time on 2nd day	-0.67	-2,05	0.071
	S100a6, hippocampus/mean swimming time on 3rd day	-0.79	-2.95	0.02
	S100a6, hippocampus/mean swimming time on 4th day	-0.69	-2.27	0.046
	S100a6, hippocampus /overall swimming time (4 days)	-0.79	-2.94	0.021
Training	*Ascl1, prefrontal cortex/mean time of reaching platform on the 1st training day*	0.67	2.25	0.049
	Ascl1, prefrontal cortex/mean time of reaching platform on the 1st trial on 3rd training days	0.71	2.68	0.031
	Ascl1, prefrontal cortex/mean time of reaching platform on the 1st trial on the 4th training day	0.75	2.97	0.019

Table 2. Correlations between the relative levels of *Casp3, Ascl1* and *S100a6* gene expression in the hippocampus, cerebellum and prefrontal cortex in active control and spatial memory performance rat groups in the Morris water maze.

Animal Group	Correlation parameters	r_s	T(n-2)	*P*
Active Control	***Casp3* / *S100a6*; hippocampus**	**0.738**	**2.74**	**0.0365**
	Casp3, cerebellum / *S100a6*, prefrontal cortex	0.714	2.28	0.0465
	S100a6, prefrontal cortex/ *Acsl1*, hippocampus	–0.881	-3.41	0.0039
Training	***Casp3* / *Ascl1*; hippocampus**	**0.87**	**3.65**	**0.0023**
	Casp3, cerebellum/ *Casp3*, prefrontal cortex	0.783	3.02	0.0126
	***Casp3* / *Ascl1*; prefrontal cortex**	**0.7**	**2.78**	**0.0358**
	***S100a6* / *Ascl1*; cerebellum**	**0.817**	**3.49**	**0.0072**

Intrastructural correlations are indicated in bold