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ORIGINAL ARTICLE

Involvement of individual hippocampal signaling protein levels in spatial memory formation is strain-dependent

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Abstract Although a series of signaling cascades involved in spatial memory have been identified, their link to spatial memory and strain-dependent expression has not been reported so far. Hippocampal levels of the above-mentioned signaling proteins were determined in laboratory inbred strain C57BL/6J, the wild-derived inbred strain PWD/PhJ and the wild caught mouse *Apodemus sylvaticus* (AS) by immunoblotting. The resulting hippocampal protein levels were correlated with results from MWM. Hippocampal signaling protein (hSP) levels were tested also in yoked controls. Within-strain comparison between trained and yoked controls revealed significant differences between levels of Phospho-CaMKII (alpha), Phospho-CREB, Egr-1, c-Src, Phospho-ERK5, Phospho-MEK5 and NOS1 in all of the three strains tested. In addition, the three strains revealed different involvement of individual hSP levels clearly indicating that individual mouse strains were linked to individual hSPs in spatial memory. Phospho-ERK5 levels were not detectable in hippocampi of yoked controls of each strain. We learn from this study that a series of hSPs are associated with spatial memory and that different hSPs are linked to spatial memory in different strains that show different outcome in the MWM. Even correlational patterns in the individual hSPs differed between mouse strains. This is of importance for the interpretation of previous studies on the abovementioned signaling cascades as well as for the design of future studies on these hippocampal proteins. It is intriguing that individual mouse strains, laboratory or wild caught, may use different signaling pathways for spatial memory in the Morris water maze.

Keywords *Apodemus sylvaticus* · Morris water maze · PWD/PhJ · Western blotting · Signaling proteins

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Introduction

From the last decade, memory-related signaling proteins in the hippocampus are holding center stage and forming the basis for neurochemical and neuropharmacological studies of learning and memory (Arnsten et al. 2005; Grant 2003; Greer et al. 2009). A series of proteins are representing specific cascades and signaling pathways known to be involved in the processes of learning and memory:

cAMP response element-binding protein (CREB) a member of the basic region leucine zipper (bZIP) family of proteins is regulated through phosphorylation on Ser133. It belongs to a family of nuclear transcription factors with profound impact on the understanding of signaling-induced gene transcription. Wu et al. showed timed activation of CREB by the calmodulin kinase and MAPK pathway (Bito et al. 1996; Wu et al. 2001). The phosphorylation of CREB is required for hippocampus-dependent spatial memory formation (Colombo et al. 2003; Porte et al. 2008) while dephosphorylation generates inactivation (Hagiwara et al. 1992).

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), activated at high Ca^{2+}/calmodulin concentrations, is required for LTP (Griffith et al. 2003). CaMKII shows four isoforms, α, β, γ and δ, with predominant CNS expression of α and β isoforms (Miller and Kennedy 1985; Strack et al. 1997). CaMKII can undergo autophosphorylation, which is triggered after binding to the NMDA receptor (Lisman et al. 2002). Mutant mice deficient for α-CaMKII have defective spatial learning in the MWM and BM (Bach et al. 1995; Silva et al. 1992).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of secretory peptides activating Trk (tyrosine kinase) receptors (Kafitz et al. 1999) and phosphorylates the hippocampal NMDA receptor (Suen et al. 1997). BDNF is shown to enhance excitatory synaptic transmission (Canas et al. 2004) and spatial learning (Mizuno et al. 2003; Yamada et al. 2002). Protein synthesis is required for BDNF-dependent LTP (Takei et al. 2001) and CREB is an activator of BDNF at synapses to control the neuronal response (Tao et al. 1998).

Extracellular signal-regulated protein kinase 5 (ERK5) is a member of mitogen-activated protein kinases (MAPKs) regulated by a wide range of mitogens and cellular stresses. It is also known as big MAPK 1 (BMK1), because it is twice the size of other MAPKs (Lee et al. 1995). Ras, MEKK2, MEK5 and c-Src are activators of the ERK5 pathway (Chao et al. 1999; Zhou et al. 1995) while downstream effectors are MEF2 (Liu et al. 2003) and CREB (Watson et al. 2001). Kasler et al. (2000) have provided evidence that ERK5 in turn, acts as transcription activator.

Nitric oxide (NO) is a diffusible gas released after activation of nitric oxide synthase (NOS), and has been found to play many diverse physiological roles as a neurotransmitter. Pharmacological and genetic inhibition NOS was shown to block LTP and impair spatial learning (Böhme et al. 1993; Kirchner et al. 2004). The effects of NO were found to be dependent on species, strain and training paradigm (Susswein et al. 2004). The NOS–PSD-95 interaction was proposed to lead to multi-axon connections (Nikonenko et al. 2008) and neuroprotection (Cao et al. 2005).

Mitogen-activated protein kinases (MAPKs) are regulators of cell division, differentiation and apoptosis. p38 is one of MAPKs (Chen et al. 2001). Phosphorylation of Thr180 and Tyr182 by MKK3 and MKK6 activates p38 MAPK (Dérijard et al. 1995; Raingeaud et al. 1996). The role of MAPK signaling in neuronal plasticity and memory formation (Mazzucchelli and Brambilla 2000; Sweatt 2001), regulation of CREB (Adams et al. 2000) and spatial learning (Selcher et al. 1999) is well documented.

Mitogen-activated protein kinase kinase 5 (MEK5) a dual specificity protein belongs to the Ser/Thr kinase family. MEK5 inhibition prevents phosphorylation and activation of ERK5 in behavioral experiments, indicating the pivotal role of MEK5 in synaptic plasticity (English and Sweatt 1997) and learning (Atkins et al. 1998).

Fyn is one of the non-receptor type tyrosine protein kinases of the Src family, with diverse biological functions (Resh 1998). It is abundant in hippocampus associated with learning, memory (Grant et al. 1992) and synaptic plasticity (Kojima et al. 1997). Fyn regulates AMPA receptor expression (Narisawa-Saito et al. 1999) and activates NMDA by phosphorylation (Tezuka et al. 1999).

Early growth response (Egr-1) protein belongs to a family of DNA-binding zinc finger proteins. The precise role of Egr-1 in spatial learning is still elusive (Guzowski et al. 2001), and is NMDA receptor-dependent (Cole et al. 1989).

Hippocampal Egr-1 levels are upregulated in spatial memory (Pollak et al. 2005) and fear conditioning (Ko et al. 2005).

C-Src (cellular-Src) is a non-receptor selective tyrosine protein kinase, abundant in synaptic vesicles and growth cones and phosphorylates synaptic vesicle proteins such as synapsins (Onori et al. 2007), synaptophysin (Barnekow et al. 1990) and synaptogyrin (Janz and Südhof 1998).

The absence of information on the link of hippocampal levels of the abovementioned signaling pathway components to spatial memory formation along with corresponding and missing data on wild-caught mice formed the rationale for the current study.

Therefore, it was the aim of the study to show memory-dependent hippocampal signaling protein levels using hippocampi also of yoked controls, i.e. animals placed into the Morris water maze without the platform for the same time period as the trained mice (Sunyer et al. 2008a). Yoked controls were used to rule out the effect of stress on protein expression. Moreover, we intended to determine strain-dependent hippocampal levels of signaling proteins in C57BL/6J mice, wild-derived inbred PWD/PhJ mice and wild-caught *Apodemus sylvaticus* (AS) mice. Last but not the least, signaling protein levels determined, herein, were compared between "poor, good and excellent" learners based on the previously published MWM work in the identical animals.

Materials and methods

Animals used

C57BL/6J ($n = 20$), PWD/PhJ ($n = 20$) and AS ($n = 20$) mice, male, aged 12–14 weeks were used for protein chemical studies. MWM data from C57BL/6J and AS were taken from a recent study (Patil et al. 2008) to link

cognitive data with protein chemical work in hippocampus. MWM data on PWD/PhJ were not published before. These mice were chosen on the basis of background information, including an own previous study (Patil et al. 2008) C57BL/6J mice were obtained from Charles River Laboratory (Germany). PWD/PhJ mice were obtained from Jackson Laboratory (USA). AS were caught in a hangar at a military airport LOXT (Patil et al. 2008). AS were bred in laboratory and second generation mice were used for the study. Animals were maintained in cages made of Makrolon, filled with wood chips and bred and kept in the core unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna.

All animals were housed in their individual home cages prior to experiments. An autoclaved standard rodent diet (Altromin 1314ff) and water were available ad libitum. Room temperature was 22 ± 1°C and relative humidity was 50 ± 10%. The light/dark rhythm was 14:10. Ventilation with 100% fresh air resulted in an air change rate of 15 times per hour. The room was illuminated with artificial light at an intensity of about 200 lx in 2 m from 5 a.m. to 7 p.m. Behavioral tests were performed between 8 a.m. and 1 p.m. Experiments were done under licence of the federal ministry of education, science and culture, which includes an ethical evaluation of the project (Project: BMWF-66.009/0152-C/GT/2007). Housing and maintenance of animals were in compliance with European and national regulations.

Morris water maze

The MWM paradigm was carried out as described previously (Höger et al. 2008; Sunyer et al. 2009). The MWM consisted of a circular pool (122-cm diameter, walls 76-cm depth) in which mice were trained to escape from water by swimming to a hidden platform (1.5-cm beneath water surface) whose location could be only identified using distal extra-maze cues attached to the room walls. Visual cues had different colors and dimensions and were kept constant during the whole experiment. Water temperature was maintained at 21 ± 1°C.

The pool was divided into four quadrants (compass locations: NE, NW, SW and SE) by a computerized tracking/image analyzer system (video camcorder: 1/3″ SSAM HR EX VIEW HAD coupled to a computational tracking system: TiBeSplit). The platform was placed in the middle of the SW quadrant and remained at the same position during the whole experiment.

The spatial acquisition phase consisted of 16 training trials: 4 training trials per day and 4 training days with an inter-trial interval of 20 min. Mice were released randomly with their heads facing the pool wall from the four compass locations, and allowed to swim and search for the platform for 120 s. If mice did not locate the platform after 120 s, animals were manually placed on the platform and allowed to remain on it for 30 s.

On the first training day, mice were given an acclimatization training session in the water maze; mice were placed on the hidden platform, allowed to swim for 30 s, and subsequently were guided back to the platform. The latency and path length to reach the hidden platform and speed was recorded.

On the fifth day, the acquisition phase, subjects received a probe trial, in which the platform was removed. Mice were released from the NE start point and were allowed to swim freely for 60 s. The path the mouse swam was tracked and analyzed for the proportion of swim time and/or path length spent in each quadrant of the pool and swim speed was recorded.

On the twelfth day, subjects once again received the probe trial for 60 s to check retention memory. Mice were released from the NE start point and were allowed to swim freely for 60 s. The path, the mice swam was tracked and analyzed for the proportion of swim time and/or path length spent in each quadrant of the pool and swim speed was recorded. Mice were not trained during the time period between fifth and twelfth day.

Yoked controls, 20 animals per group, were not trained in the MWM because the platform was omitted to rule out the effect of swimming and water stress.

Immunoblotting

Hippocampal samples

For protein chemical analysis, 6 h after the last probe trial on day 12, animals were killed by decapitation and hippocampi were rapidly dissected and kept at −80°C until analysis (Sunyer et al. 2009). Hippocampal tissue was obtained from the three mouse strains, 20 per group, of trained and yoked control samples. All efforts made to minimize animals suffering and reduce number of animals used.

Sample preparation

The sample preparation was conducted as described by Sunyer et al. (2009). Hippocampi were homogenized and suspended in 1.2 mL sample buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4% w/v CHAPS, 10 mM 1, 4-dithioerythritol, 1 mM EDTA,

1 mM PMSF, 1 tablet Complete™ from Roche Diagnostics, and 0.2% v/v phosphatase inhibitor cocktail from Calbiochem). After sonication of suspension approximately 30 s on ice, the suspension was left at room

temperature for 1 h and centrifuged at 14,000×*g* for 60 min at 12°C. Desalting was carried out with an Ultrafree-4 centrifugal filter unit with a cutoff molecular weight of 10,000 Da (Millipore, Bedford, MA, USA) at 3,000×*g* at 12°C until the eluted volume was about 4 mL and the remaining volume reached 100–200 µL (John et al. 2009). The protein content of the supernatant was determined by the (Bradford 1976) assay.

Western blot

Aliquots of 10 mg of protein were denatured at 65°C for 15 min and applied on 7.5 and 12.5% (as per requirement to separate proteins with different apparent molecular weight; Cheon et al. 2008) ExcelGel SDS homogenous gels (GE Healthcare, Buckinghamshire, UK). Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins separated on the gel were transferred onto PVDF membranes. Membranes were probed with diluted primary rabbit polyclonal antibodies against phospho-MEK5 (1:5,000; Abcam, Cambridge, UK), NOS1 (1:5,000; Santa Cruz Biotechnology Inc, CA, USA), phospho-ERK5 (1:2,500; Cell signaling technology Inc, USA), phospho-CREB (1:5,000; Cell signaling technology Inc, USA), phospho-p38-MAPK (1:5,000; Cell signaling technology Inc, USA), Egr-1 (1:5,000; Santa Cruz Biotechnology Inc, CA, USA), BDNF (1:5,000; Santa Cruz Biotechnology Inc, CA, USA), c-Src (1:5,000; Santa Cruz Biotechnology Inc, CA, USA), anti-phospho-CaMKII (alpha) (1:5,000; BIOMOL International, L.P, USA), Fyn (1:5,000; Santa Cruz Biotechnology Inc, CA, USA), actin (1:5,000; Abcam, Cambridge, UK) and detected with horseradish peroxidase-coupled anti-goat (Abcam, Cambridge, UK), anti-rabbit IgG and anti-mouse IgG (Cell signaling Technologies Inc, USA) according to the supplier's protocol. Membranes were developed with the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL, USA). Densities of immunoreactive bands were measured by Image J software program (http://rsb.info.nih.gov/ij/). Selection of antibodies was based on the literature search**.** Actin immunoreactivity was used as loading control.

Statistical analysis

Behavioral test

The strain-dependent effect during the acquisition phase [expressed as the difference between latencies to reach the platform on days 1 (D1) and 4 (D4)] was analyzed by one-way analysis of variance followed by Tukey's LSD multiple comparisons.

For the short-term (D5) and long-term retention phase (D12), a paired *t* test was used for each strain separately to evaluate preference for the target quadrant (time spent in the target quadrant vs. average time spent in the three control quadrants).

Strain differences were analyzed with the repeated measurements analysis of variance followed by Tukey's LSD for post hoc comparisons. The secondary endpoints swimming speed and path length were analyzed as for comparison of latencies.

Western blot

The strain-dependent effects were analyzed by one-way ANOVA with factor strain, and post hoc analysis was performed by Tukey's LSD multiple comparisons. Between-group differences were performed by unpaired Student's *t* test. Values are expressed as mean ± SD.

The correlation between time (s) spent in the target quadrant in the MWM on day 12 and density of immunoreactive bands was analyzed by Pearson correlation. This test was also carried out to correlate individual hSPs in the individual mouse strains.

A probability level of $P < 0.05$ was considered statistically significant. Calculations were performed using SPSS for Windows 14.0 (SPSS Inc, Chicago, IL, USA).

Results

Cognitive test: MWM

Behavioral data were taken from a previous publication on the identical animals (data for MWM taken from a previous own publication with permission of the publisher, "Reprinted from Neurobiology of Learning and Memory, 89/4, Patil SS, Sunyer B, Höger H, Lubec G, AS (LOXT) is a suitable mouse strain for testing spatial memory retention in the Morris water maze, 552–559, Copyright (2008), with permission from Elsevier."

Acquisition phase: latency and path length to reach the hidden platform

Over the period of 4 days of water maze trials, there was a reduction in mean distance traveled and the latency to reach the hidden platform for the three strains, indicating mean improvement in the animal's performance (Fig. 1a–c). Speed remained constant over the training period for C57BL/6J while PWD/PhJ ($P = 0.0001$), and AS ($P = 0.008$) showed a statistically significant increase.

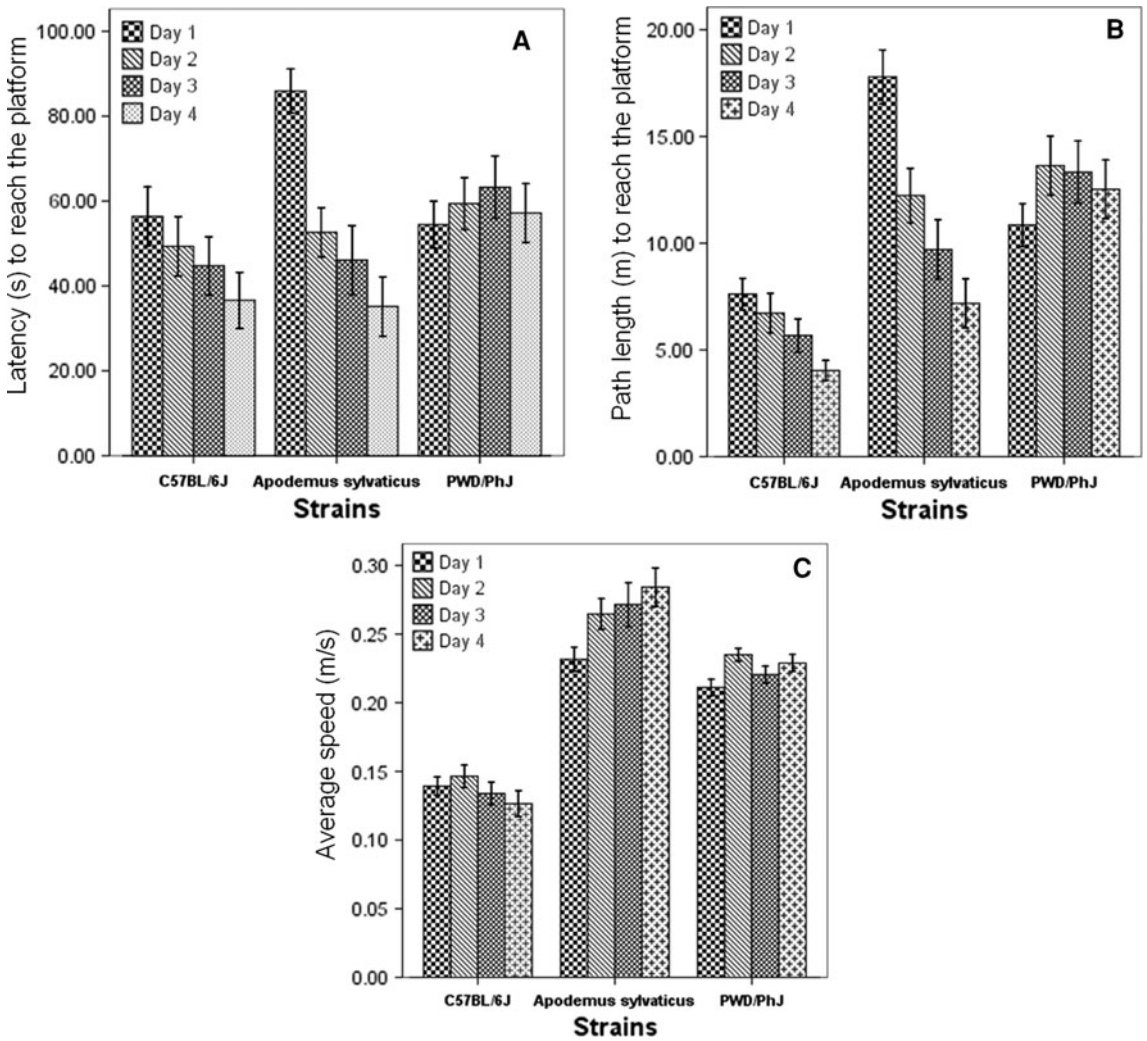

Fig. 1 Acquisition phase in the hidden platform water maze task. During the training days, there was a reduction in the mean latency (s) (**a**) and distance traveled to reach the hidden platform across the three strains (**b**). During the training days, there was no change in the mean speed (m/s) in the C57BL/6J mouse, but was increased in the AS and PWD/PhJ (**c**)

The difference between the latency to reach the platform on D1 and D4 (ΔL) was calculated for each strain to study their ability to learn the task.

Delta was significantly different between strains (ANOVA, $P = 0.0001$). Pairwise comparison of strains showed a statistically significant difference between C57BL/6J and AS (ANOVA, $P = 0.010$) and between PWD/PhJ and AS (ANOVA, $P = 0.0001$). No strain difference was observed between C57BL/6J and PWD/PhJ.

Path length during training days was analyzed in the same way as for latency. Statistically significant strain difference of path length between D1 and D4 was observed [(ΔP); ANOVA, $P = 0.0001$]. Pairwise comparison of strains showed a statistically significant differences of ΔP between C57BL/6J and AS (ANOVA, $P = 0.001$), PWD/PhJ and AS (ANOVA, $P = 0.0001$) and C57BL/6J and PWD/PhJ (ANOVA, $P = 0.012$).

Time (s) spent and path length (m) in the target quadrant (t–tq) at day 5 (probe trial at short-term retention memory)

Time spent in the target quadrant

During the probe trial on D5, t–tq = time spent in the target quadrant versus average of time spent in the adjacent quadrants, was measured. As given in Fig. 2a; all strains showed a clear preference for the target quadrant. This preference was confirmed applying the paired *t* test (for each strain separately) between the t–tq. The paired *t* test showed statistical significance in C57BL/6J ($P = 0.024$), AS ($P = 0.0001$) and no significant difference was observed in PWD/PhJ.

For strain comparison, univariate analysis of variance was performed using the time spent in the target quadrant

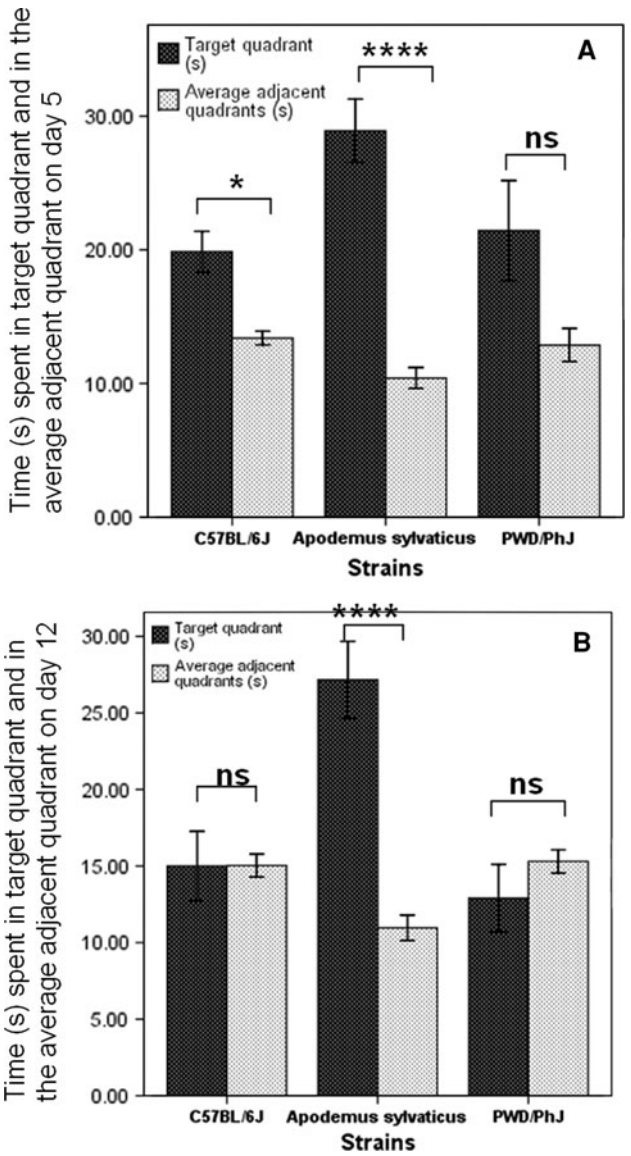

Fig. 2 **a**, **b** Probe trial, the figure shows time (s) spent in the target quadrant and the average time spent in the adjacent quadrants on day 5 (Fig. 2a) and time (s) spent in target quadrant and in the adjacent quadrants on day 12 (Fig. 2b). *Asterisks* indicate level of significance of difference in univariate analysis of variance, $*P<0.05$, $**P<0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, *n.s.* not significant)

as variable. No strain differences in the time spent in the target quadrant was observed. Pairwise comparison of strains showed a statistically significant difference between C57BL/6J and AS (ANOVA, $P = 0.024$), no significant difference was observed in PWD/PhJ and AS and C57BL/6J and PWD/PhJ.

Path length

Path length during the probe trial on day 5 (D5) was analyzed: the difference between the distance traveled in the target quadrant and the average of the adjacent quadrants was evaluated with paired *t* test for each strain. The paired *t* test showed significant differences in C57BL/6J, and AS ($P = 0.024$; $P = 0.0001$), no significant difference was observed in PWD/PhJ.

Strain differences were observed for the distance traveled in the target quadrant (ANOVA, $P = 0.0001$). Pairwise comparisons revealed statistically significant differences between C57BL/6J and AS (ANOVA, $P = 0.0001$) and PWD/PhJ and AS (ANOVA, $P = 0.0001$). No significant difference was observed between C57BL/6J and PWD/PhJ.

Time (s) spent and path length (m) in the target quadrant (t–tq) at day 12 (probe trial at long-term retention phase)

Time spent in target quadrant

During the probe trial on D12, the t–tq was measured. AS in contrast to C57BL/6J and PWD/PhJ showed a clear preference for the target quadrant. As given in Fig. 2b; this preference was confirmed applying paired *t* test for each strain separately to compare t–tq. The paired *t* test showed statistical significance in AS ($P = 0.0001$); C57BL/6J and PWD/PhJ showed no significant difference.

For strain comparison, univariate analysis of variance was performed using the time spent in the target quadrant as variable. Strain differences in the time spent in the target quadrant were observed (ANOVA, $P = 0.0001$). Pairwise comparison of strains showed a statistically significant difference between PWD/PhJ and AS (ANOVA, $P = 0.001$) and between C57BL/6J and AS (ANOVA, $P = 0.0001$). No significant difference was observed between PWD/PhJ and C57BL/6J on probe trial D12.

Path length

On D12 path length during probe trial was analyzed: differences between the distance traveled in the target quadrant and the average of the adjacent quadrants were evaluated with the paired *t* test (for each strain). The paired *t* test showed a significant difference in AS ($P = 0.0001$). In C57BL/6J and PWD/PhJ, no significant difference was shown.

The distance traveled in the target quadrant was significantly different between strains (ANOVA, $P = 0.0001$). Pairwise comparisons revealed statistically significant difference between C57BL/6J and AS (ANOVA, $P = 0.0001$) and PWD/PhJ and AS (ANOVA, $P = 0.0001$), no significant difference was observed in C57BL/6J and PWD/PhJ.

Comparison between probe trial on D5 and D12

Paired student *t* test was used to compare the time spent in the target quadrant on D5 with the time spent in the target quadrant on D12 for each strain separately (Fig. 3). C57BL/6J showed decreased performance on D12 (paired student *t* test, $P = 0.036$), while AS showed no statistically significant values thus keeping their memory until day 12 without any further training and PWD/PhJ showed no statistically significance although not having learned the task.

Swim strategies on training days

The strategy used during training was analyzed by categorizing each individual trial according to the predominant swim pattern. Seven categories were defined to capture the gradually improving spatial precision and efficiency during the learning process (Balschun et al. 2003).

To classify different swimming behavior into categories, the following criteria were used: a mouse naïve to the water maze initially tends to swim along the wall of the pool: wall hugging. As training progresses, a mouse begins to search the whole surface area of the pool, first randomly and later selectively scanning the inner area of the pool containing the escape platform. The development of spatial memory for the platform location is reflected by a focal search of a target quadrant or by direct swimming to the platform. Occasionally, a mouse may perform systematically searching the area of the pool at a constant distance of the platform location from the wall: chaining (Janus 2004).

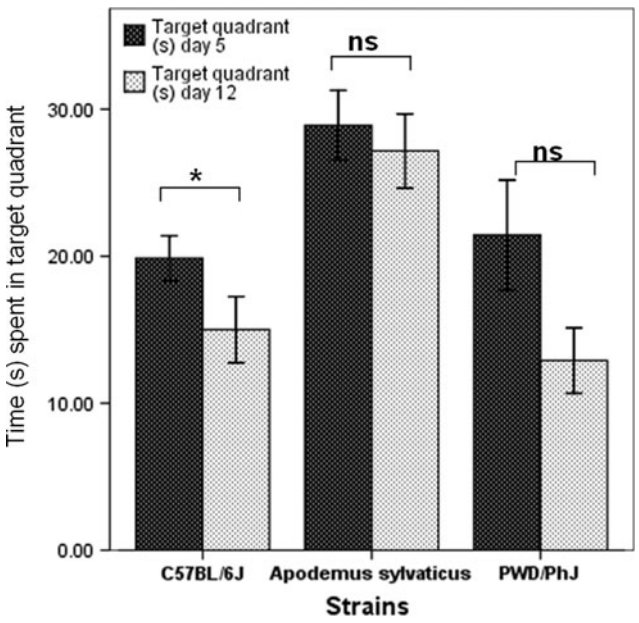

Fig. 3 The figure shows time (s) spent in the target quadrant and in the adjacent quadrants for each strain in days 5 and 12. *Asterisks* indicate level of significance of difference in univariate analysis of variance, $*P < 0.05$, $**P < 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, *n.s.* not significant)

The swim strategies described before can be categorized as spatial strategies (direct and focal) and non-spatial strategies (scanning, random, focal incorrect). Chaining and wall hugging would be described as strategies based on the repetitive looping (Patil et al. 2008).

For each day, all abovementioned strategies were quantified (%) individually.

As shown in Fig. 4, random strategies used by AS decreased during the training days [day 1 (D1) = 66.25%; day 4 (D2) = 5.0%]. On the last day of training AS used predominantly direct (28.7%) and focal searching (23.75%) strategies. Wall hugging strategy was observed only in AS. Random strategies in PWD/PhJ and C57BL/6J also decreased during training days (PWD/PhJ: D1 = 46.25% and D4 = 36.25%; C57BL/6J: D1 = 36.84 and D4 = 10.52). C57BL/6J showed more direct strategies than AS at the end of the training days (D4 = 39.47% for C57BL/6J and 28.7% for AS) while PWD/PhJ showed decreased direct strategies (D1 = 23.75% and D4 = 17.5%). On the other hand, focal searching was higher in AS (D4 = 23.75%) than in PWD/PhJ (D4 = 3.75%) and C57BL/6J (D4 = 17.10%).

C57BL/6J and AS showed a gradual increment of spatial strategies and a decrement of non-spatial strategies compared with PWD/PhJ to search for the platform during the training days. The swim pattern of C57BL/6J and AS strains shows clearly that the mice used spatial strategies to learn the task except PWD/PhJ.

Results of immunoblotting

Images of western blot results are presented in Fig. 5. As shown in Table 1, strain dependence of hippocampal signaling protein levels in trained mice (MWM) is shown. Table 2 revealed statistically different signaling protein levels in yoked controls indicating strain dependence of untrained mouse strains (Fig. 6a–c).

Table 3 shows the mean and standard deviation of protein levels in the three strains, both, in trained mice and yoked controls, indicating strain dependence and the result of statistical comparisons between trained and yoked controls.

Most proteins were represented by a single band. C-Src was represented by a double band standing for two known isoforms that herein were quantified together (http://www.uniprot.org/uniprot/P12931).

Arbitrary levels of optical density were normalized by actin.

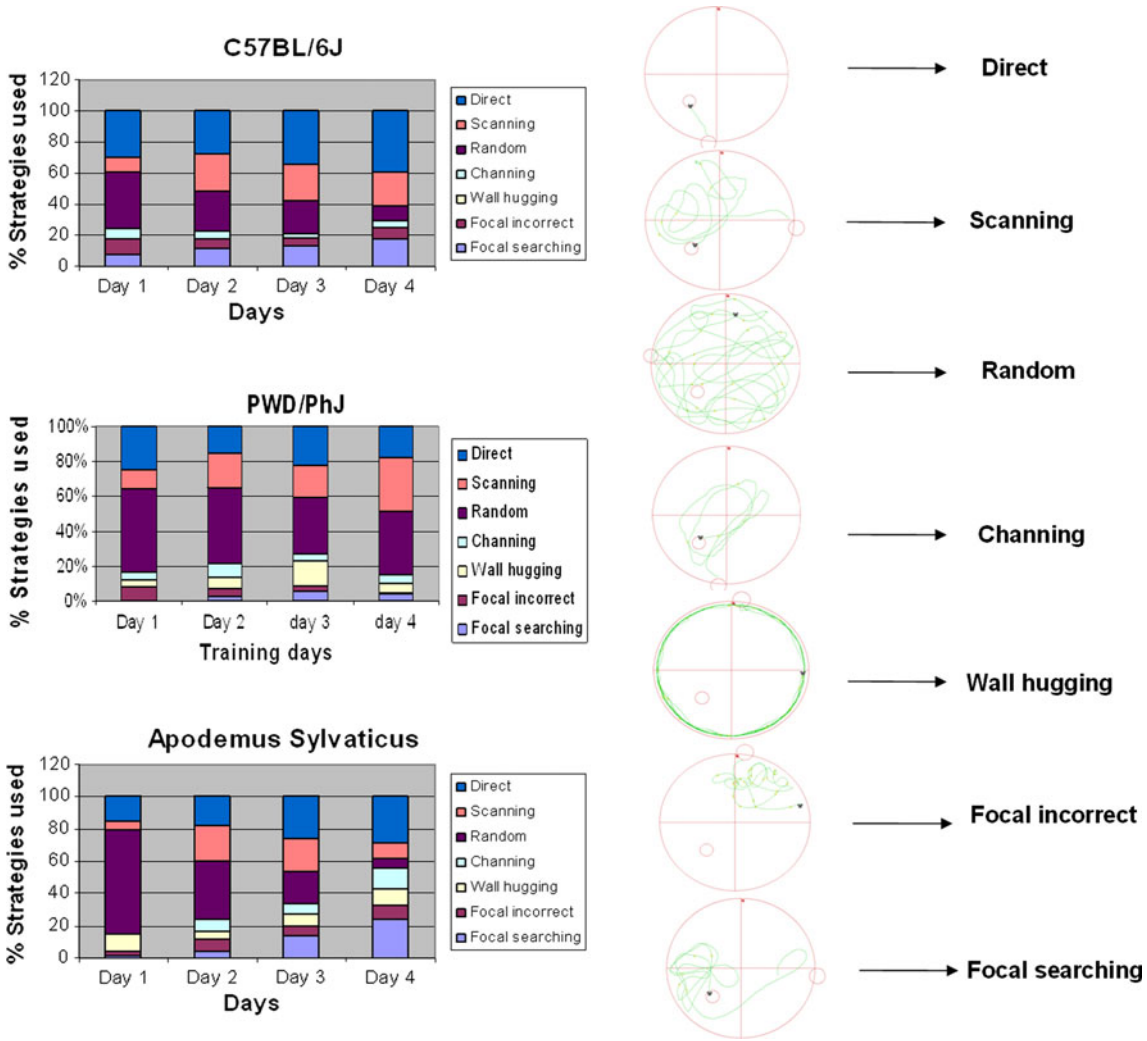

Fig. 4 Characterization of search strategy of three mouse strains in Morris water maze

Correlation between individual hSPs

As shown in supplemental tables 1a–c, significant correlations were observed between the individual hSPs. The correlation pattern, however, between individual hSPs varied between groups.

Discussion

The major outcome of the study is strain dependence of hippocampal signaling protein (hSPs) levels in a laboratory inbred strain, a wild-derived inbred strain and a wild-caught strain. The results were confirming a role for these hSPs in spatial memory formation. When the three mouse strains were categorized into "very good, good and poor memory" different protein levels for individual pathway components were observed (Tables 1, 2, 3).

Although it is known that memory is strain dependent in the MWM (Crawley et al. 1997; Klapdor and van der Staay 1996; Nguyen et al. 2000; Sunyer et al. 2008b; Upchurch and Wehner 1988) and hippocampal protein levels for some signaling proteins are strain dependent as well (Pollak et al. 2006), no corresponding information exists about wild-derived or wild-caught mice.

AS performed very well, both, in learning and memory: When spatial memory was tested in the MWM in the probe trials at days 5 (D5) and 12(D12), they learned the task and were performing well on D5 and were keeping the memory level from D5 at the probe trial at D12 (Patil et al. 2008), when they were killed 6 h following the probe trial and hippocampus was taken for the present study. In this panel, hippocampal levels of p-CaMKII, Egr-1. c-Src, p-ERK5, p-MEK5 and Fyn were highest in this group probably representing good memory performance. This finding was particularly prominent in the p-ERK5 (Fig. 5), where

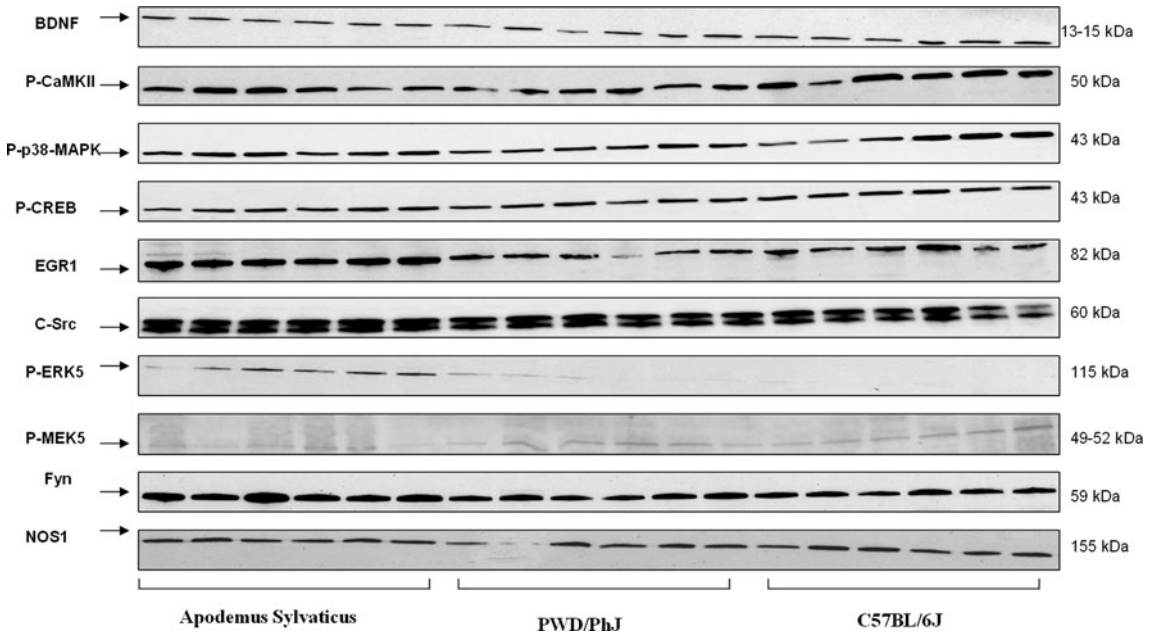

Fig. 5 Images of western blot results indicate strain dependence of hippocampal signaling protein levels in trained mice

Table 1 Strain dependence of hippocampal signaling protein levels in trained mice (MWM)

Protein	AS versus PWD/PhJ	AS versus C57BL/6J	C57BL/6J versus PWD/PhJ
BDNF			
p-CaMKII (α)			
p-p38-MAPK	0.004		0.0001
p-CREB			0.001
Egr-1	0.026	0.006	
c-Src	0.041	0.039	
p-ERK5	0.001	0.001	
p-MEK5	0.004	0.001	
Fyn	0.0001	0.002	0.014
NOS1	0.004		0.0001

Table 2 Statistically different signaling protein levels in yoked controls indicating strain dependence of un-trained mouse strains

Protein	AS versus PWD/PhJ	AS versus C57BL/6J	C57BL/6J versus PWD/PhJ
BDNF			
p-CaMKII (α)	0.001		
p-p38-MAPK			0.024
p-CREB	0.001		0.005
Egr-1	0.038	0.005	
c-Src	0.0001	0.0001	
p-ERK5			
p-MEK5	0.0001	0.0001	
Fyn	0.0001		0.002
NOS1	0.007	0.0001	0.0001

p-ERK5 was observed in the trained mice only but not in yoked controls. Moreover, p-ERK5 levels were manifold higher in AS than in the two other groups.

A series of correlations between individual hSPs were observed in this strain probably indicating links between signaling cascades (Supplementary Table 1a).

C57BL/6J mice performed very well, both, in learning and memory in the MWM like AS, but did not keep the memory level at D12 (Patil et al. 2008). In this group, BDNF, p-CREB and Egr-1 hippocampal levels were highest among the three strains.

Again, a series of individual hSPs levels were significantly correlated with individual signaling pathway components, but correlations different from AS and PWD/PhJ were observed (Supplementary Table 1b).

PWD/PhJ mice failed to learn the task in the MWM and failed at the probe trials at D5 and D12 as shown herein. Only BDNF, Egr-1 and p-MEK5, c-Src hippocampal levels were higher than in the other two groups.

Likewise; p-p38 MAPK, p-CREB, Egr-1, p-ERK5, p-MEK5, Fyn and NOS1 protein levels were lowest in the "poor memory performing" PWD/PhJ mouse strain.

And indeed, genetic or pharmacological inactivation of these proteins is known to lead to deficient memory formation (Bach et al. 1995; Silva et al. 1992; Böhme et al. 1993; Kirchner et al. 2004; Atkins et al. 1998; Grant et al. 1992; Bourtchuladze et al. 1994; Poirier et al. 2008). Correlations between individual hSPs levels were present, but were different from associations observed in AS and C57BL/6J. The most prominent difference in the

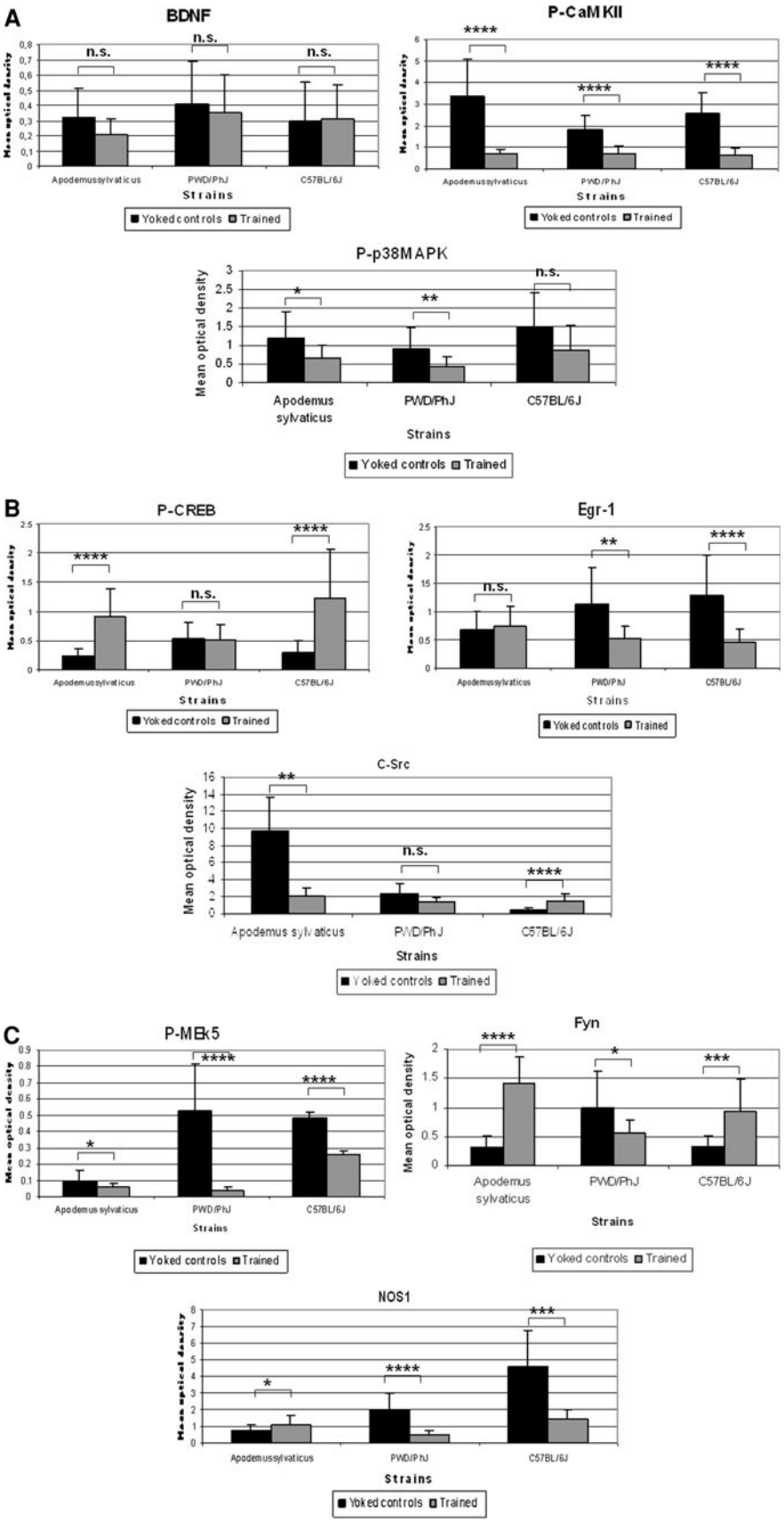

Fig. 6 Mean and standard deviation of the protein levels detected in western blotting. *Asterisks* indicate level of significance of difference with unpaired student *t* test, $*P < 0.05$, $**P < 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, *n.s.* not significant)

Table 3 Mean and standard deviation of protein levels in the three strains, both, in trained mice and yoked controls, indicating strain dependence and the result of statistical comparisons between trained and yoked controls

Protein	Treatment	Mean ± SD Apodemus Sylvativus	Mean ± SD PWD/PhJ	Mean ± SD C57BL/6J	*Apodemus sylvaticus*	PWD/PhJ	C57BL/6J
BDNF	Yoked	0.32 ± 0.19	0.41 ± 0.28	0.30 ± 0.25	n.s.	n.s.	n.s.
	Trained	0.21 ± 0.10	0.35 ± 0.25	0.31 ± 0.23	n.s.	n.s.	n.s.
p-CaMKII (α)	Yoked	3.40 ± 1.70	1.82 ± 0.67	2.56 ± 0.97	0.0001	0.0001	0.0001
	Trained	0.70 ± 0.22	0.71 ± 0.37	0.63 ± 0.32	n.s.	n.s.	n.s.
p-p38-MAPK	Yoked	1.20 ± 0.71	0.91 ± 0.55	1.51 ± 0.91	0.031	0.003	n.s.
	Trained	0.67 ± 0.32	0.43 ± 0.26	0.88 ± 0.64	n.s.	n.s.	n.s.
p-CREB	Yoked	0.23 ± 0.12	0.54 ± 0.27	0.30 ± 0.19	0.0001	n.s.	0.0001
	Trained	0.91 ± 0.48	0.52 ± 0.25	1.23 ± 0.83	n.s.	n.s.	n.s.
Egr-1	Yoked	0.67 ± 0.33	1.13 ± 0.65	1.28 ± 0.71	n.s.	0.004	0.0001
	Trained	0.74 ± 0.34	0.52 ± 0.22	0.46 ± 0.23	n.s.	n.s.	n.s.
c-Src	Yoked	9.75 ± 3.93	2.34 ± 1.17	0.37 ± 0.28	0.005	n.s.	0.0001
	Trained	2.10 ± 0.87	1.34 ± 0.62	1.45 ± 0.89	n.s.	n.s.	n.s.
p-ERK5	Yoked	±	±	±	n.a.	n.a.	n.a.
	Trained	0.20 ± 0.19	0.03 ± 0.02	0.03 ± 0.02	n.s.	n.s.	n.s.
p-MEK5	Yoked	0.10 ± 0.06	0.53 ± 0.29	0.48 ± 0.26	0.042	0.0001	0.0001
	Trained	0.06 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	n.s.	n.s.	n.s.
Fyn	Yoked	0.30 ± 0.21	0.99 ± 0.62	0.33 ± 0.18	0.0001	0.028	0.001
	Trained	1.41 ± 0.45	0.55 ± 0.23	0.94 ± 0.54	n.s.	n.s.	n.s.
NOS1	Yoked	0.71 ± 0.36	2.04 ± 0.98	4.57 ± 2.17	0.042	0.0001	0.001
	Trained	1.10 ± 0.56	0.52 ± 0.23	1.40 ± 0.61	n.s.	n.s.	n.s.

Protein levels were detected by Western blotting

Arbitrary units resulting from densitometry analysis are given

Data are mean ± SD

Statistical evaluation was performed by unpaired Student's *t* test

$P < 0.05$ was accepted as statistically significant (*n.a.* not analyzed, *n.s.* not significant)

correlations was the absence of any correlation between NOS1 and any other hSP (Supplementary Table 1c).

These data may indicate that memory performance may be probably associated with hSP levels (Table 4). The use of yoked controls (Sunyer et al. 2008a) allowed the assignment of changed protein levels to spatial memory because yoked controls were not allowed to perform the MWM task, as no platform was present in the circular pool.

Although the other hSPs studied are known to be involved in spatial memory formation, no significant correlation was obtained in any mouse strain investigated.

Taken together, we learn from the study that spatial memory is strain dependent and so are protein levels of individual hSPs. It is revealed that the individual hSPs are linked to spatial memory and the existence of different correlation patterns between the individual hSPs within the strains is shown. In a first approach, we attempted to assign hSPs to tentative categories of "very good, good and poor"

Table 4 Memory-related protein "relative abundance level scores" in hippocampus of trained mice on day 12

Proteins	Good performance at days 5 and 12	Poor performance at days 5 and 12	Good performance at day 5 and but not at day 12
BDNF	+	++	++
p-CaMKII (α)	+++	+	+++
p-p38-MAPK	++	+	+++
p-CREB	++	+	+++
Egr-1	++	++	+
c-Src	++	+	+
p-ERK5	+++	+	+
p-MEK5	+	++	+
Fyn	+	++	+++
NOS1	++	+	+++

Mean levels of hippocampal proteins were scored with +, ++ or +++

performance in the MWM at the time point 6 h following the probe trial at D12.

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