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Expression of Pea3 protein subfamily members in hippocampus and potential regulation following neuronal stimulation

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Short Title : Pea3 protein regulation in the hippocampus

Abstract

Pea3 proteins belong to a subfamily of the E-twenty six (ETS) domain superfamily of transcription factors, which play various roles during developmental processes. Polyoma Enhancer-Activator 3 (Pea3) proteins Pea3, ERM and Er81 are particularly involved in tissues with branching morphogenesis, including kidney, lung, mammary gland and nervous system development. A recent transcriptomic study on novel targets of Pea3 transcription factor has revealed various axon guidance and nervous system development related targets, supporting role of Pea3 proteins in specific motor neuron connectivity, as well as novel targets in signaling pathways involved in synaptic plasticity. This study focuses on the expression of Pea3 family members in hippocampal neurons, and regulation of putative Pea3 targets in Pea3-overexpressing cell lines and following Long Term Potentiation (LTP) or seizure induction *in vivo*. We show that Pea3 proteins are expressed in hippocampus in both neuronal and non-neuronal cells, and that Pea3 represses Elk-1 but activates Prkca and Nrcam expression in hippocampal cell lines. We also show that mRNA and protein levels of Pea3 family members are differentially regulated in the dentate gyrus and CA1 region upon MECS stimulation, but not LTP.

Keywords: Pea3, hippocampus, LTP

Introduction

ETS (E26 Transformation Specific) domain transcription factors superfamily is involved in tissue patterning and lineage commitment during development [1]. PEA3 subfamily members include PEA3 (Polyoma enhancer activator 3) / E1AF (E1A enhancer binding protein) / ETV4 [2,3], ER81/ETV1 [4] and ERM/ETV5 in mammals [5-8]. Characteristically, PEA3 subfamily members are particularly important for tissues showing branching morphogenesis [9]. They are also expressed in the developing nervous system of mouse from E9.5 to birth [1,10]: strong *erm* expression was seen in the developing brain, while both *pea3* and *erm* were strongly expressed in posterior neural plate and *er81* in the embryo was found by E9.5 [1,8,11]. In adults, ER81 transcript is present in a variety of tissues, including heart, brain and lung [4], whereas Pea3 RNA is most abundant in brain and spinal cord [3,12]. Regulation of Pea3 through FGF18 signaling is important for the laminar positioning of developing neocortex [13].

In concert with their pattern of expression, PEA3 family members are important in the formation of functional neuronal circuitry [10,14,15]. They are selectively expressed in specific classes of motor neurons and corresponding muscle afferent sensory neurons at limb levels of the spinal cord in chick [16]. Er81/ETV1 are expressed in rodent and primate layer 5 neocortical neurons [17]. Interestingly, transcriptional targets of Pea3 members have only recently been revealed [18-21]; a transcriptomic analysis in SH-SY5Y cells over-expressing Pea3-VP16 constitutively active protein had focused on neuronal targets, particularly those in axon guidance or nervous system development [19].

In this study, we focused on hippocampal expression and function of Pea3 family members. We identified components of canonical Long Term Potentiation (LTP) pathway genes to be

targets of Pea3-VP16, along with glutamergic and dopaminergic synapse pathways [19]. We then showed that Pea3 family members Pea3, ERM and ER81 are expressed in the hippocampus, and analyzed and validated some of the identified targets in hippocampal cells and examined hippocampal regulation in two models of plasticity, LTP and electroconvulsive shocks *in vivo*.

Materials and Methods

Microarray analysis

Data previously submitted to EBI ArrayExpress on microarray results from SH-SY5Y human neuroblastoma cell line overexpressing constitutively active Pea3-VP16 (accession E-MTAB-5324 [19]), were analyzed as follows: Gene IDs were converted to official gene symbol, then *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway tools were used for functional enrichment of the list of genes and identification of affected pathways. KEGG pathway tools were analyzed as previously reported [19].

Cell lines and cell culture

SH-SY5Y, mHypoA2/12 and mHippoE-14 cell lines were maintained in DMEM (4.5g/L glucose, Gibco) containing 10% FBS (10500–064, Life Technologies), 1X L-Glutamine, Pyruvate and 1X penicillin streptomycin solution (10378016, Gibco). Cells were grown in medium at 37^{o}C in 5% CO_2 incubator until they reach confluency.

Electrophysiology

Electrophysiology was conducted in adult male Sprague Dawley rats (250-300 g, Iffa Credo, France) in accordance with the European Communities Council Directive 86/609/EEC. Details of the LTP-inducing procedures used in this study have been published previously [22]. Briefly, animals were anesthetized with urethane carbamate (1.5 g/kg, i.p.), then placed in a stereotaxic frame to implant a recording electrode in the hilus of the dentate gyrus (DG) and a stimulating electrode in the angular bundle of the perforant path. Electrodes were lowered under electrophysiological guidance and adjusted to stimulate maximum DG response to perforant path stimulation. Following stabilization, a 30 min baseline was

recorded under low-frequency stimulation (0.033 Hz), then, a tetanus (6 series of 6 trains of pulses @ 400 Hz, 20 ms) to induce LTP (n=8), or a pseudotetanus (n=6) to mimic this stimulation pattern without inducing LTP, were delivered. The intensity of stimulus was kept constant throughout the experiment. Evoked responses were recorded before and after induction of LTP and pseudotetanus. fEPSPs (field excitatory postsynaptic potentials) were stored for offline analyses of the slope of the fEPSP and population spike amplitude. Values were normalized to baseline and data were analyzed using ANOVA [19]. At the end of the experiment brains were prepared for Western blotting analysis (n=5; LTP, n=3; pseudotetanus) and Real-time PCR (n=3; LTP induced, n=3; pseudotetanus).

Maximal Electroconvulsive Shock (MECS)

Rats were briefly anesthetized with fluothane and given MECS (200 V, 2s, 50 mA, x 2) via ear clip electrodes using a constant current generator [23]. Control rats (n=6) were treated identically with the exception that no current was delivered via the ear clip electrodes. They were returned to home cages for 10 (n=5), 30 (n=5) or 90 (n=5) minutes prior to decapitation to harvest tissue bilaterally from CA1 and DG. Brains were rapidly dissected on ice and frozen at -80^{0}C for Western blotting and Real-time PCR analyses.

RNA isolation and qPCR

Dissected tissues were homogenized with 1 ml of Trizol (15596026, Invitrogen) using a Precelly homogenizer (4776, Bertin Technologies), and lysates were centrifuged at 12,000xg for 5 min at 4^{0}C. Each supernatant was incubated in 0.2 ml of chloroform (3 min). Samples were centrifuged at 12,000xg for 15 min at 4^{0}C. Aqueous phases (RNA) were transferred to new tubes and 500µl of isopropanol was added. Lysates were incubated for 10 min and centrifuged at 12,000xg for 10 min at 4^{0}C. The pellets were resuspended with 1 ml of 75% ethanol by vortexing. Then, samples were centrifuged at 7,500xg for 5 min at 4^{0}C.

Supernatants were discarded, and pellets dried at room temperature for 10 min and resuspended in 30µl of Nuclease-free water by pipetting and stored at -80^{0}C.

For cell culture experiments, SH-SY5Y, mHypoA2/12 and mHippoE-14 cells were transfected with pCDNA3 and pCDNA3-mPea3, pCDNA3-Erm or pCDNA3-Er81 (courtesy of Prof. A.D. Sharrocks) using PEI reagent (Polysciences) in (1µg DNA: 3.5µl PEI), 3 replicas per sample. RNA samples were isolated 48 hrs post-transfection using Invitrogen PureLink RNA Mini Kit and Agilent Absolutely RNA Microprep Kit using manufacturer's instructions.

500ng of total RNA was used to synthesize a first cDNA strand as described (1708891, BioRad); 1ng of cDNA was used as a template in 10µl reactions with SsoAdvance Universal SYBR Green Supermix (172-5271, BioRad) for qPCR with CFX96 Touch Real-Time PCR detection system (primer list is given in Table 1). The qPCR reaction and relative gene expression level calculation using $2^{-\Delta\Delta Ct}$ method was carried out [19].

Western blot

Tissue samples were homogenized, and proteins were detected using Western blotting [24]. Briefly, total proteins was separated by SDS-PAGE and transferred to the nitrocellulose membrane. Blots were blocked and then incubated with primer antibodies including anti-actin (1:10000, Abcam A2066), anti-PEA3 (1:500, Abcam, ab101455), anti-ERM (1:1000, Abcam, ab102010) and anti-ER81 (1:1000, Abcam, ab81086). After washing, HRP conjugated secondary antibodies were applied for 1 hr at room temperature before applying ECL solution (Amersham Biosciences), exposed to film for 10-20 min, and developed by hand. Protein bands were quantified using GeneSnap acquisition and GeneTools analysis software.

Immunohistochemistry

Animal experiments were performed under the approval of İstanbul Medipol University Animal Experimentation Local Ethical Committee (IMU-HADYEK-no.E.15421). Briefly, adult (6-8 week old) Balb-C mice were perfused transcardiacally with 1X PBS/heparin (10 U/ml) and 4% PFA under Ketamine/Xylazine anesthesia. Brain tissue were dissected and incubated in 4% PFA overnight at 4^{o}C for further fixation. Afterwards, brain tissue was washed in cold PBS three times for 1h to remove excess PFA and directly sectioned without dehydration steps. For sectioning, brains were wiped gently, embedded in 3 % agarose and cut into coronal sections (75μm) using a Vibratome (Leica VT1000S) as per routine procedures at Medipol University Animal Facility.

For enhancing immunostaining of transcription factors, DNAse pretreatment was performed [25]. Sections were rinsed in PBS and incubated in 3% H_2O_2 in PBS (30 min), re-rinsed (PBSx3) and incubated in 1:1000 Triton-X in PBS (10 min), rinsed again in PBS (x3). Sections were then equilibrated in 1XHBSS (5 min), incubated in 500 U/ml DNAse I in HBSS (15 min) and washed extensively with PBS.

Sections were incubated in blocking solution (3% BSA, 1% goat serum, 0.1% Triton-X and 0.1% sodium azide) for 1 h at room temperature and incubated with primary antibodies (mouse anti-NeuN (abcam), rabbit anti-ER81 (Novus), rabbit anti-ERM (abcam), rabbit anti-Pea3 (Santa Cruz)) diluted in 3% BSA, 1% goat serum, 0.1% Tween-20, 0.1% sodium azide overnight at 4°C. Sections were rinsed (x3) in PBS-T and incubated in secondary antibodies (1:1000) goat anti-mouse Alexa Fluor 488, goat anti-Rabbit DyLight 555 (ThermoFisher) for 2h at room temperature. Sections were rinsed in PBS-T (x3) and incubated in DAPI (1ug/ml) for 3min, rinsed (x2) in PBS-T, mounted using Fluoromount mounting medium (Sigma) and stored at 4^{o}C for imaging. Z-stack images were taken with confocal microscope (Zeiss LSM

800) using 20x Plan-Apochromat objective and maximum intensity images were constructed using ZEN Blue software.

Results

Regulation of nervous system related pathways by Pea3-VP16 in transcriptomic analysis

In previous studies, genes related to axon guidance and neurotrophin signaling pathways were identified as Pea3 targets (array EBI ArrayExpress, accession E-MTAB-5324 [19]). Using this microarray data and String Database, *Kyoto Encyclopedia of Genes and Genomes* (KEGG) annotation and enrichment analysis was used to visualize map cluster of 4138 genes in common pathways and processes. Microarray data was run 5 times and among the 142 pathways, those related to neuronal function were selected (Table 2).

These pathways included neurotrophin signaling pathway [19], however dopaminergic and glutamatergic synapse pathways, as well as LTP-related signaling pathways were not yet addressed in detail (Table 2). As Pea3 family members Pea3/ETV4 and ERM/ETV5 had recently been shown to be important for hippocampal dendrite development, regulation of genes pathways involved in synaptic plasticity were particularly interesting [26]. Therefore, we first wanted to determine the expression of PEA3 family members in the adult mouse hippocampus and analyzed the putative LTP-related gene targets in different model systems.

Expression of Pea3, ERM and Er81 in the hippocampus

Although Pea3 family members are expressed at high levels in brain and spinal cord, no detailed analysis has been reported for the expression of Pea3 proteins in the vertebrate hippocampus. To address this, we used immunohistochemistry. When mouse hippocampal sections were stained for Pea3 together with the mature neuron marker, NeuN, strong co-localization was apparent in the DG, CA3 and CA1 regions, showing for the first time that this protein is expressed in hippocampal neurons (Fig.1A). Expression of Pea3 was not

restricted to mature neurons, as some $NeuN^-$ cells were also stained for Pea3, however identifying those cell types was beyond the scope of this study. When similar analyses were conducted with other family members, ERM (Fig. 1B) and Er81 (Fig. 1C), a similar co-localization with $NeuN^+$ cells was observed, albeit not exclusively. Whether Pea3 family members are expressed in all neurons of the hippocampus or whether they are restricted to specific subsets are yet to be determined.

Expression of Pea3 target genes in mouse hippocampal cell line

Among the many genes regulated by Pea3-VP16 in LTP-related pathways in microarray analysis, significant changes were observed in Egr1 (~5-fold repression), Shc2 (~3-fold repression), Elk-1 (~5-fold repression), Ntrk3 (~2-fold repression) and Prkca (1.5-fold up-regulation) (Fig. 2a). LTP is known to increase the expression of Egr1 in rat DG [27], and similar effect has been shown in mice in a strain-dependent manner [28]. Elk-1, an ETS protein of the TCF subfamily, was previously shown to be hyper-phosphorylated following LTP induction and to concur to regulation of LTP-dependent genes in DG [22], as well as long-term consolidation of NMDA signaling [29]. Neurotrophin tyrosine kinase receptor type 3 (Ntrk3) was shown to be one underlying molecule involved in panic attack and related hippocampal hyperexcitability and fear circuit activation [30]; Shc2 is downstream of neurotrophin receptors in neurons [31], and Protein kinase Ca (Prkca) was shown to be important for memory capacity in humans [32,33]. We have initially selected this subset of targets and investigated whether Pea3 protein subfamily regulates expression of these genes in mouse hippocampal cell line, mHippoE-14. Protein kinase A (PKA) catalytic subunit Cα (Prkaca), which was previously reported to be important in anxiety-like behaviors [34] and is affected by Pea3-VP16 overexpression, albeit not significantly, was also included in the study.

To this end, mHippoE-14 cell line was initially transfected with Pea3-VP16 expression vector, and expression levels of Prkca as well as Prkaca, Ntrk3 was investigated, together with a known Pea3 target as positive control, the neuronal cell adhesion molecule, Nrcam ([19]; Fig.4b). Relative to housekeeping control, Prkaca and Ntrk3 were found to be repressed nearly two-fold, while Prkca was up-regulated almost 1.5-fold and Nrcam by 3.5-fold, validating microarray results (Fig. 2b).

We next studied the regulation of Elk-1 by Pea3 proteins in SH-SY5Y cells, mHypoA-2/22 and mHippoE-14 cell lines following overexpression Pea3, Erm, Er81 and Pea3-VP16. Elk-1 was shown to be repressed by Pea3-VP16, Pea3 and Er81, but not Erm, in SH-SY5Y cells, validating previous reports (Fig. 2c, Table 2). However, Elk-1 was not significantly altered in either hypothalamic or hippocampal cell lines, indicating the cross-regulation between these ETS genes can be cell context-dependent (Fig. 2c).

Long-term potentiation and regulation of Pea3 proteins

LTP induction in DG was previously shown to cause rapid phosphorylation of ERK MAPK, of the downstream transactivator of the ETS domain superfamily, Elk-1, and up-regulation of the transcriptional target *egr1*/*zif268* [22,35]. However, no study has yet analyzed the relation between Pea3 family members and LTP. To this end, we induced LTP in the DG of adult rats by repeated high-frequency stimulation of the perforant pathway.

Analyses of basal EPSP slope and spike amplitude, and of stimulation intensities used to evoke DG responses, were not significantly different between LTP and pseudotetanus groups (all p values >0.05) suggesting they did not contribute to any changes occurring after tetanic

stimulation for 30 (Fig. 3a) and 90 min (Fig. 3b). The tetanus induced a stable and rapid increase in both the population spike amplitude ($t=5.96$; $p<0.01$) and fEPSP slope ($t=3.71$; $p<0.05$), while there was no change in the pseudotetanus control group (Fig. 3a, b).

Since we have shown that Pea3 proteins are expressed throughout the hippocampal region (Fig. 1A-C), and that in transcriptomic studies Pea3 regulates genes in LTP signaling pathways, we next examined whether their expression was altered after LTP induction. Ninety minutes following induction of LTP we found no significant changes in Pea3, Erm and Er81 protein expression levels when compared between tetanus and pseudotetanus (Fig. 3c-e). However due to a lack of phosphor-specific antibodies for these proteins, it is yet to be determined whether any post-translational changes occur in response to LTP in this time frame.

Quantitative real-time PCR analysis showed no significant change in Pea3 (Fig. 4a), Erm (Fig. 4b) or Er81 (Fig. 4c) mRNA levels 30 minutes after induction of LTP compared with pseudotetanus or the non-stimulated DG. As a positive control, we analyzed *zif268* mRNA in the same brains and observed a strong increase in *zif268* mRNA levels (~6,5 fold) (Fig. 4d).

Effect of Maximal Electroconvulsive Shock (MECS) on Pea3 expression

As Pea3 proteins may not be fast-responding proteins, or may require stronger stimulation, we next tested whether their expression is affected by MECS, which induce seizures throughout the entire brain. MECS-induced seizures is a pathological condition that fundamentally affects neurons in a manner different to that induced by synaptic plasticity, or LTP. Following MECS, changes in expression of all three Pea3 proteins were observed, albeit to

different extents and with different temporal dynamics (Fig. 5). Whereas Pea3 protein levels in DG and CA1 gradually decreased compared to controls (Fig. 7a and 7b), Erm protein levels decreased more rapidly in DG, starting 10 min post MECS, and remained stable for at least 60 min (Fig. 7c), but not in CA1 (Fig. 5d). In contrast, the small decrease in Er81 expression in either DG or CA1 was not significant (Fig. 5e-f).

We then assessed mRNA levels of Pea3 family members at different time points following MECS. The relative abundance of Pea3 mRNA in DG decreased within 10 min of stimulation, thereafter gradually returning to control levels (Fig. 6a), as opposed to the gradual decline in protein levels (Fig. 5a). Similarly, ERM mRNA in DG showed a significant increase 1hr after MECS (Fig. 6a), while protein levels had decreased (Fig. 5c). Parallel to protein levels, no significant change in Er81 transcription was observed in DG (Fig. 6a).

A similar dissociation of Pea3 protein/mRNA levels was found in CA1: while Pea3 protein levels decreased in CA1 (Fig. 5b), mRNA levels were increased 30 min after MECS (Fig. 6b). Neither Er81 nor ERM transcript levels were significantly changed in CA1 (Fig. 6b).

Having observed MECS-dependent changes in the rat hippocampus, we examined a select subset of potential Pea3 target genes under the same paradigm: Egr1/Zif268, a transcription factor known to have a key role in synaptic plasticity [34], Elk-1, NTRK3, and Shc2. Egr1 expression increased rapidly 10 min after MECS, as in LTP, almost 3-fold in CA1 and 6-fold in DG, thereafter declining back to basal levels in CA1 and remaining elevated at around 3-fold over basal level in DG 60 min after MECS (Fig. 6c,d, compare to Fig. 4). This time point coincides with up-regulation of Pea3 family members (Fig. 6a,b). This parallels the

decrease observed in our transcriptomic studies where Pea3-VP16 overexpression resulted in Egr1 down-regulation [19].

Elk-1 expression, however, showed a different response: it was substantially increased in the DG 60 min after MECS, or decreased (albeit not significantly) in CA1 (Fig. 6c,d). In previous microarray studies, Pea3-VP16 overexpression was found to repress Elk-1 transcription [19], suggesting context-dependent regulation of this target gene by Pea3. As for Ntrk3 expression levels, while there was no significant change in DG after MECS, mRNA level was strongly decreased after 1 hour in CA1 (Fig. 6c,d). mRNA levels of *Shc2*, on the other hand, were not significantly changed neither in DG nor CA1 (Fig. 6c,d) [19].

Discussion

Mice with Cop1 (constitutive photomorphogenesis 1; a critical regulator of Pea3 stability) deletion in neural stem cells, which resulted in higher levels of expression of all three Pea3 family members, showed densely packed frontal and parietal cortical layers 2 and 3, as well as reduced hippocampal thickness [36,37], yet no transcriptional targets have yet been studied in detail with respect to hippocampal function. In this study, we have confirmed expression of all three Pea3 proteins in various regions of the hippocampus, mostly associated with – but not restricted to – neuronal cells.

However, contrary to previous transcriptomic studies on Pea3-VP16 targets [19], LTP in DG *in vivo* has not caused any significant alteration in the expression of Pea3 family members at the transcript or protein levels at the time-points studied (Fig.4). Yet, Pea3 protein levesl in DG and CA1 were significantly decreased 1hr after MECS, while those of Erm and Er81 were not significantly altered (Fig.5), in spite of the fact that Er81 transcript is significantly decreased in CA1 and Erm transcription increased in DG 1hr after MECS (Fig.6a-b). The decrease in Pea3 transcript level in DG 10 min after MECS (Fig.6a) could correlate with the decrease in Pea3 protein levels 1hr after MECS (Fig.5a, Table 3). Pea3 proteins are regulated post-translationally through phosphorylation, SUMOylation and ubiquitylation, which are known to affect either activity or stability of Pea3 proteins [39-42], hence while a decrease in transcription level may correlate with a decrease in translation product, the reverse is not necessarily true.

In previous microarray studies, *egr-1/zif268*, *Elk-1, Shc2* and *Ntrk3,* among many other genes were down-regulated and *Prkca* was up-regulated by Pea3-VP16 overexpression in SH-SY5Y cells [19], which was confirmed in this study in mouse hippocampal cell line, mHippo-E14 (Fig.2b). Furthermore, in the context of MECS paradigm Pea3 decrease in DG at 10 min and Elk-1 up-regulation at 1hr after MECS was found to be in line with microarray data that indicated Elk-1 is negatively regulated by Pea3 (Fig.6c; Table 3). Together with results in mHippoE12/2 cell line, this implies an inverse correlation between Pea3 and Elk-1 expression levels (Fig.2c). It should be noted that while Pea3 deficient mice exhibited no significant symptoms except for male ejaculation dysfunction, most likely due to functional redundancy with other Pea3 family members, in Elk-1 deficient animals kainite-induced cortical and hippocampal CA1 expression of *c-fos* was reduced [12,43].

Electroconvulsive therapy was originally reported to induce LTP in rat hippocampal slices [44], while later studies reported LTP induction to be inhibited by MECS, although this was probably due to MECS-mediated induction of LTP to a degree that prevented further induction afterwards [45]. Electroconvulsive therapy is commonly used in humans for depression, yet the mode of action is still unclear: it is known, however, that hippocampus is among the regions affected in humans, and memory is disrupted [46]. Electroconvulsive shock was previously shown to regulate neurogenesis, along with induction of VEGF and BDNF [47]. When gene expression changes in the frontal cortex and hippocampus upon MECS was studied in rodents, 120 unique genes were identified in the neurogenesis, neuronal plasticity and neurite outgrowth pathways, including BDNF, FGFR1, VEGF, TrkB, as well as Neurofilament-L, Neurofilament-M and synapsin2, among many others [48], some of which are known Pea3 targets [19]. Although this study focused on LTP-related gene pathways

identified in Pea3-VP16 microarray study, a more in-depth analysis of neurotrophic and neurogenesis pathways should also be conducted in LTP and MECS paradigms in the future.

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Additional Information

The authors declare no conflict of interest.

Figure Legends

Figure 1. A. Pea3 expression in hippocampus. Representative images of Pea3 (yellow), NeuN (green) and DAPI (blue) staining (Scale bar: 200 μm) (i). Magnified images illustrating CA1 (ii), CA3 (iii) and DG (iv) regions. Merged images show that Pea3 is expressed in $NeuN^+$ cells of DG and CA1/3 cells. **B.** ERM expression in hippocampus. Representative images of ERM (yellow), NeuN (green) and DAPI (blue) staining (Scale bar: 200 μm) (i). Magnified images illustrating CA1 (ii), CA3 (iii) and DG (iv) regions. Merged images show that ERM is expressed in $NeuN^+$ cells of DG and CA1/3 cells. **C.** ER81 expression in hippocampus. Representative images of ER81 (yellow), NeuN (green) and DAPI (blue) staining (Scale bar: 200 μm) (i). Magnified images illustrating CA1 (ii), CA3 (iii) and DG (iv) regions. Merged images show that ER81 is expressed in $NeuN^+$ of DG and CA1/3 cells.

Figure 2. Expression of LTP-related Pea3 targets in cell lines. (A) Fold changes in selected putative Pea3 targets identified in microarray experiment [19]; (B) qPCR analysis of putative Pea3 targets in Pea3-VP16 overexpressing mHippoE-14 cell line; (C) Validation of *elk-1* mRNA levels in SH-SY5Y (light grey), mHypoA-2/12 (grey) and mHippoE-14 (black) cells transfected with Pea3, Erm, Er81 and Pea3-VP16 expression plasmid. qPCR reaction was normalized using GAPDH and/or β-actin as housekeeping genes and reported as mRNA expression relative to control, i.e. empty pCDNA3 plasmid-transfected cells. Values are expressed as mean ± SEM for 3 independent experiments. *p* values: * < 0.05; ** < 0.01; *** < 0.001; Student's t-test.

Figure 3. LTP in the dentate gyrus (DG) *in vivo* followed for 30 and 90 min post-tetanus and expression of Pea3, Erm and Er81. (A) Top: sample evoked responses before and after LTP induction. Black responses are taken during the baseline, and red responses 15 min following LTP induction or a pseudotetanus. Responses on the left are from an animal receiving a pseudotetanus and on the right an animal in which LTP was induced. Point plots representing the fEPSP slope and population spike amplitude before and 30 min after tetanic stimulation to induce LTP (open circles) or pseudotetanus as control stimulation (black circles). Arrows indicate tetanus or pseudotetanus delivery. fEPSP and spike values are normalized to the mean of the baseline for responses evoked at 30-s intervals before and after LTP induction. (B) Point plots representing the fEPSP slope and population spike amplitude followed for 90 min following LTP induction (open circles) or a pseudotetanus as control stimulation (black circles). Each point represents fEPSP and spike values normalized as above. Arrows indicate tetanus or pseudotetanus; (C-E) Expression analysis of Pea3, Erm and Er81 90 min following LTP induction. Western blots of Pea3 (C), Erm (D) and Er81 (E) show that their protein levels did not change at this time point following induction of LTP in DG compared to pseudotetanus (pTET) controls. Percent change in expression was semi-quantified by normalizing band intensities of Pea3, ERM or Er81 to actin control; error bar ± SEM.

Figure 4. Pea3, Erm and Er81 mRNA expression 30 min following induction of LTP. The left side of the dentate gyrus (LDG) was stimulated and the right side (RDG) was used as a non-stimulated control. Total RNAs were subjected to real-time PCR for the measurement of mRNAs; β-actin was used as internal control for normalization and analyzed based on relative quantification method. Histograms represent relative expression levels observed in the non-stimulated control DG (right side, RDG, light grey) and in stimulated DG (left side, LDG, dark grey) in LTP (LTP) and pseudotetanus (Ptet) groups. qPCR results indicate that mRNA level of (A) Pea3, (B) Erm and (C) Er81 were equivalent in both groups and both sides. Error

bar ± SEM; (D) qPCR data indicates *zif268* expression is strongly induced (~6,5 fold, compared with pTET) after LTP. Error bar ± SEM.

Figure 5. Changes in expression levels of Pea3 (A,B), Erm (C,D) and Er81 (E,F) proteins at different time-points after MECS. DG (A,C,E) and CA1 (B,D,F) protein levels were analyzed 10 min, 30 min and 1 hour after MECS. In DG, Pea3 and Erm expression decreased rapidly after MECS whereas Er81 level decreased after 1 hour. Error bar ± SEM.

Figure 6. The effect of MECS on the abundance of Er81/ETV1, Pea3/ETV4 and Erm/ETV5, as well as selected putative targets in rat dentate gyrus and CA1. qPCR analyses of relative Er81/ETV1, Pea3/ETV4 and Erm/ETV5 expression at 10, 30 and 60 min after MECS in (A) DG and (B) CA1; error bar ± SEM; qPCR analysis of relative Elk-1, Ntrk3, Shc2 and Egr1 expression at 10, 30 and 60 min after MECS in (C) DG and (D) CA1 region; ; error bar ± SEM.

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Table 1. List of primers used in qPCR analysis

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
rEr81	GCAGGAGTACCACGACCCAGTCT ATG	AAATGGAGTGGCAGCTAGGCAC TTC
rPea3	ACTCAGATGTCCCTGGATGTG	TCACTCCATCACCTGAAGAGG
rErm	GAAGTGCCTAACTGCCAGTCACCC	GGCACCACGCAAGTGTCATCGA
rActb	ATTGCTGACAGGATGCAGAA	TAGAGCCACCAATCCACACAG
rEgr1	CACCTGACCACAGAGTCCTTTTC	AGAAGCGGCCAGTATAGGTGA
rElk1	GTACATTGGTGCACACCTACC	GCCATCCCAGGGGTACTATGT
rNtrk3	TTGGACTTGCTGCTTTTGCC	CAGCCACAGGACCCTTCATT
rShc2	AAGGGCGGCTTCATTCACAA	AATGCAGCCCATGTACCGAA

Table 2. Genes from microarray were classified to particular pathways with KEGG analysis using STRING (those in neuron-related pathways are presented; p values were calculated upon applying Bonferroni correction)

KEGG ID	KEGG Term	Term p values Corr Bonf	Times Found
KEGG:04720	Long-term potentiation	1,41E-08	35
KEGG:04730	Long-term depression	1,88E-06	6
KEGG:04722	Neurotrophin signaling pathway	3,01E-14	164
KEGG:04010	MAPK signaling pathway	4,83E-07	1
KEGG:04724	Glutamatergic synapse	1,13E-05	2
KEGG:04728	Dopaminergic synapse	2,92E-04	1

Table 3. A summary of findings presented in this study. Only the significant changes have been included in the table. qPCR, mRNA levels determined by quantitative PCR; prot, protein levels determined by Western blot; + denotes upregulation, - denotes downregulation

Expression	Microarray	qPCR	LTP	MECS

of:							CA1		DG	
	SH-SY5Y	mHippoE14	SH-SY5Y			qPCR	qPCR	prot	qPCR	prot
	Pea3VP16 overexp	Pea3VP16 overexp	Pea3 overexp	Pea3VP16 overexp	Er81 overexp					
Egr1	-					+	+		+	
Shc2	-									
Elk-1	-		-	-	-				+	
Ntrk3	-						-			
Prkca	+	+								
Nrcam		+								
Pea3							+	-	-	-
Erm									+	
Er81							-			