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Temporal course of gene expression during motor memory formation in primary motor cortex of rats

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Running Head: Learning related gene expression in M1

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Abstract

Motor learning is associated with plastic reorganization of neural networks in primary motor cortex (M1) that depends on changes in gene expression. Here, we investigate the temporal profile of these changes during motor memory formation in response to a skilled reaching task in rats. mRNA-levels were measured 1h, 7h and 24h after the end of a training session using microarray technique. To assure learning specificity, trained animals were compared to a control group. In response to motor learning, genes are sequentially regulated with high time-point specificity and a shift from initial suppression to later activation. The majority of regulated genes can be linked to learning-related plasticity. In the gene-expression cascade following motor learning, three different steps can be defined: 1) an initial suppression of genes influencing gene transcription. 2) Expression of genes that support translation of mRNA in defined compartments. 3) Expression of genes that immediately mediates plastic changes. Gene expression peaks after 24 hours - this is a much slower time-course when compared to hippocampus-dependent learning, where peaks of gene-expression can be observed 6 to 12 hours after training ended.

Introduction

The primary motor cortex (M1) is thought to be one brain area where motor memories are formed and encoded (Monfils, Plautz, and Kleim, 2005). In response to motor training in rats, profound changes within the matrix of M1 have been described at multiple sites (Hosp, Pekanovic, Rioult-Pedotti, and Luft, 2011): at the cellular level, an increment in dendritic length and arborisation occurs in apical (Greenough, Larson, and Withers, 1985) and basal dendrites (Kolb, Cioe, and Comeau, 2008) of layer II/III and V motor neurons (Greenough et al., 1985; Withers and Greenough, 1989) contralateral to the trained limb. Furthermore, an initial increase in spine formation is followed by an enhanced turnover that reduces the number of spines to baseline levels but selectively preserves functionally relevant synapses (Xu, Yu, Perlik, Tobin, Zweig, Tennant, Jones, and Zuo, 2009). At the level of synaptic weights, motor skill learning induces a long-lasting increase of synaptic strength in M1 horizontal connections of layer II/III suggesting an association with long-term potentiation (LTP)-like plasticity (Rioult, 1998). In line with this assumption, capacity to induce LTP was reduced whereas long-term depression (LTD) was increased, suggesting that the learning-induced gain in synaptic strength reduced the capacity of LTP-formation (Rioult-Pedotti, Friedman, and Donoghue, 2000). Several weeks after skill acquisition, the ability to form LTP was restored while the horizontal connections of layer II/III remained strengthened (Rioult-Pedotti, Donoghue, and Dunaevsky, 2007). At the level of cortical physiology, motor learning induces an enlargement of the motor-cortical representation (motor maps) of the body-parts that became trained. This phenomenon can be observed in rodents, primates, and humans (Kleim, Barbay, and Nudo, 1998; Nudo, Milliken, Jenkins, and Merzenich, 1996; Pascual-Leone, Nguyet, Cohen, Brasil-Neto, Cammarota, and Hallett, 1995).

This enlargement is learning specific as it does not occur in response to mere motor activation and its magnitude is proportional to learning success (Kleim, Hogg, VandenBerg, Cooper, Bruneau, and Remple, 2004; Molina-Luna, Hertler, Buitrago, and Luft, 2008).

De novo synthesis of proteins is required for most of plastic changes that occur during motor learning (Alvarez, Giuditta, and Koenig, 2000; Bisby and Tetzlaff, 1992) and a learning-specific hippocampal protein expression has been demonstrated in response to spatial learning in rats (Monopoli, Raghnaill, Loscher, O'Sullivan, Pangalos, Ring, von Schack, Dunn, Regan, Pennington, and Murphy, 2011). In line with these findings, protein-synthesis inhibition in M1 interferes with the acquisition of a motor task in rats (Luft, Buitrago, Ringer, Dichgans, and Schulz, 2004).

Changes in gene expression are expected to precede the synthesis of novel proteins that further form the molecular basis of motor cortical neuroplasticity. Such changes have been demonstrated in the hippocampus of rats that were trained in the Morris water maze task (Cavallaro, D'Agata, Manickam, Dufour, and Alkon, 2002) and in a passive avoidance learning paradigm (D'Agata and Cavallaro, 2003). Regulated genes could be classified into the categories of "cell signalling", "synaptic proteins", "cytoskeletal proteins", "apoptosis" and "transcription and translation". Thus, these sets of regulated genes were ideally suited to mediate neuroplasticity processes including changes in morphology and synaptic weights (Monfils et al., 2005).

Besides the functional role of regulated genes, the temporal succession of gene regulating processes has to be taken into account, as gene-expression in memory formation is progressing through different stages (Alberini and Kandel, 2015; Paratore, Alessi, Coffa, Torrisi, Mastrobuono, and Cavallaro, 2006). For example, cascade-like alteration in gene expression has been observed within the

hippocampus following passive avoidance learning (O'Sullivan, McGettigan, Sheridan, Pickering, Conboy, O'Connor, Moynagh, Higgins, Regan, and Murphy, 2007). In the Morris water maze task, regulated genes within the hippocampus of animals belonging to the spatial learning group were largely overlapping with swimming controls but groups could be clearly distinguished due to the unique temporal profile of up- or down-regulation (Cavallaro et al., 2002). Thus, learning-specific gene expression is not only defined by the identity of regulated genes - but also by the temporal profile of their expression.

Recently, motor learning-related alterations in gene expression could also be demonstrated within M1 of rats that were trained in a reach and grasp task (Cheung, Deboer, Hanson, Tunesi, D'Onofrio, Arisi, Brandi, Cattaneo, and Goosens, 2013). As Cheung and colleagues focused on a single time-point during memory stabilization, the unique temporal profile and identity of regulated genes during early skill acquisition is still unknown.

As we hypothesized that gene regulation also occurs in non-discrete fashion early after motor skill acquisition, the objective of this study was to determine this temporal profile of changes in gene-expression within M1 in response to motor skill learning. We therefore assessed motor cortical mRNA levels of rats that were trained in a skilled reaching task using a microarray 1h, 7h and 24h after the end of the second training session – the time-point where the steepest phase of learning occurs (Buitrago, Ringer, Schulz, Dichgans, and Luft, 2004). To assure learning specificity of changes, mRNA levels of trained animals were related to a control group.

Materials and Methods

1. Animals and experiments

Twenty-one adult male Long–Evans rats (8-12 weeks old, raised within our own stock) were used in this study. The Animal Care and Use Committee of the State of Baden-Württemberg (Germany) approved all animal procedures. The rats were randomly assigned to groups trained either in a skilled reaching task (SRT) or a control task (CT) for 2 days. Trainings were performed at the beginning of the dark phase of a 12 h day/night cycle. For both tasks, exposure to a customized training cage, food, handling and pre-training were identical. Animals were euthanized 1 h (n = 4 for SRT and n = 3 for CT), 7 h (n = 4 per group) or 24 h (n = 3 per group) after training session two. The brains were removed for tissue processing.

2. Experimental setup and behavioral experiments

Training sessions were performed at the beginning of the dark phase. Animals were food-restricted for 24 hours before the first pre-training session. During training animals were kept slightly over their initial weight (336.7 ± 31.2 g) by providing 50 mg/kg of standard lab diet after each training session. Water was given ad libitum. The reaching task was performed as previously described (Buitrago et al., 2004). The training cage was a 15 x 40 cm chamber (height 30 cm) with a vertical window (1 cm wide, 5 cm high, lower edge 2 cm above ground) in the front wall and a small light sensor in the rear wall (7 cm above ground). Animals were first pre-trained for five days learning to open the motorized sliding door that covered the front window by nose-poking the sensor in the rear. Opening the window gave access to one food pellet (45 mg, Bio-serve, Frenchtown, NJ, USA) located on a small horizontal board

in a distance of 0.5 cm relative to the outside edge of the window. During pre-training pellets were retrieved by tongue. Upon retrieval a pellet dispenser automatically replaced the pellet. In SRT rats, pre-training was followed by motor skill training that was initiated by removing the board and placing the pellet on a small vertical pedestal 1.5 cm away from the window. In this position pellets were only retrievable by using the forelimb. Because the diameter of the pedestal was approximately that of the pellet, the pellet was in an unstable position and easily kicked off. During the first 10 door openings (= trials) of the first training session forelimb preference was determined and the pedestal was shifted to one side of the window to allow for reaching with the preferred limb only. At each of the two consecutive training days rats were allowed to perform 60 trials. To retrieve the pellet rats had to extend the forelimb towards the target, pronate, open the paw, grasp, and pull the forelimb back while supinating to bring the pellet towards the mouth (Whishaw and Pellis, 1990). Each reaching trial was scored as "successful" (reach, grasp and retrieve) or "unsuccessful" (pellet pushed off pedestal or dropped during retraction).

Reaching performance between sessions was measured using the success rate defined as the ratio of the number of successful trials and the total number of trials per session, i.e. 60. The CT group (n = 10) received the same pre-training like SRT rats. Pre-training was then continued for two additional sessions (equally 60 door openings on consecutive days). Thus, animals in the CT group were not required to reach outside the cage using their forelimb and were not exposed to the new motor skill. This task bears the disadvantage that changes in response to mass movements of the forelimb can hardly be differentiated from changes due learning the skilled grasp with the paw. However, a task that included gross forelimb movements also required motor learning to certain degree and induced plastic changes within M1 as

shown in previous work from our group (Hosp, Mann, Wegenast-Braun, Calhoun, and Luft, 2013). To enable a sharp-cut differentiation of motor-learning related genes, we decided to choose a control paradigm that lacks an involvement of forelimb movements.

3. Tissue and RNA preparation

The animals were decapitated 1 h, 7 h and 24 h after the session on training day 2 (SRT group) or pre-training day 7 (CT group). At this time-point, a clear improvement in reaching performance is usually not present as the largest increase in reaching performance (i.e. "the steepest phase of the learning curve") is expected to occur between training day two and three. Thus, the processes that mediate this step are expected to occur within the 24 hours after the second training session ended. To display gene-expression in this particular time-window, rats were killed at 1h, 7h and 24h after day two of training. Thus, reaching performance at day three could not be measured and the formal proof of an improvement in performance is consequently lacking. The intact brains were rapidly removed from the skull and the forelimb area of M1 contralateral to the trained forelimb was dissected en-bloc (all cortical layers) at ice temperature according to published coordinates (+2.0 – 0.0 mm to bregma, 2.0 – 5.0 mm parasagittal; (Neafsey, Bold, Haas, Hurley-Gius, Quirk, Sievert, and Terreberry, 1986). All tissue samples were shock frozen in liquid nitrogen and stored at -80°C until use. Tissue was then treated with buffered solution containing mRNase treated, sonicated for homogenization and centrifuged. RNA was isolated using guanidine isothiocyanat (Qiagen, Hilden, Germany), DNase treated and cleaned up (RNeasy Lipid Mini Kit, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. RNA quality was assessed and quantified by UV spectrophotometry. Samples were used only if OD260/280 nm ratio was greater than 1.8. The integrity of

each sample was checked on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) prior to array processing.

4. Labelling and hybridization

Production of biotinylated cRNA and hybridization was performed at the Microarray Facility Tübingen, Germany. In brief, 1.5 µg of total RNA were transcribed to double-stranded cDNA (SuperScript Choice System, Invitrogen, Karlsruhe, Germany) and then via in vitro transcription to biotinylated cRNA, using Enzo BioArray High Efficiency RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA). The quality of the RNA amplification was supervised by capillary electrophoresis. 15 µg of fragmented cRNA of each sample was hybridized to a RAE 230A microarray chip (Affymetrix, High Wycombe, UK) overnight at 45°C in the presence of biotinylated control oligonucleotides. Each individual animal sample was hybridized with a separate chip. Then the chips were washed in order to remove cRNA that has not hybridized to its complementary oligonucleotide probe and were fluorescently labeled using phycoerythrin-conjugated streptavidin (SAPE, Bioscience, San Diego, CA). After chips had been washed and stained, they were scanned with the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

5. Signal Analysis

Raw expression data were collected by Affymetrix Microarray Suite MAS 5.0 software. CEL files were then imported to the software package R, version 2.0.1. The statistical analysis for the DNA microarray data was carried out using the libraries gcrma and limma of the Bioconductor Project, version 1.5. The data preprocessing steps, background-adjustment, normalization and computation of GCRMA gene expression measures (on $\log_2$ scale), were performed according to Wu and

colleagues (Wu and Irizarry, 2004). For the statistical analysis empirical Bayes inference for linear models with factors training group (CT, SRT) and time point (1 h, 7 h, 24 h) and their interaction was used (Smyth, 2004). From there moderated t-statistics based on shrinkage of the estimated sample variance towards a pooled estimate and corresponding p-values were calculated for the comparison SRT vs. CT group for all three time points separately. A gene was regarded as up-regulated if the expression value on the "SRT membrane" was greater than $2^{0.5} \sim 1.4$ (log2 expression ratio (training/control) ≥ 0.5) than that of the corresponding spot on the "CT membrane", corresponding to a fold change (FC) of 1.4. A gene was regarded as down-regulated if the expression value on the "SRT membrane" was less than $2^{-0.5} \sim 0.7$ than that of the corresponding spot on the "CT membrane" corresponding to a FC of 0.7. Only differences with a p-value ≤ 0.05 (t-test) were regarded as significant.

6. Quantitative Real-Time RT-PCR

In order to validate the expression levels detected by the microarrays, a subset of the regulated genes **(Table 1)** was verified by quantitative real-time RT-PCR (Gibson, Heid, and Williams, 1996). Genes were chosen with respect to their biological significance for neuroplasticity in a preliminary literature search using the NIHS Public Archive For The Refereed Literature (PUBMED; https://www.nvbi.nlm.nih.gov). TaqMan technology (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, Foster City, CA), TaqMan Universal PCR MasterMix and TaqMan Gene Expression Assay kits were used. RNA samples collected for the gene chip experiment were used as a template for cDNA synthesis (SuperScript II RT, Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. The PCR primers and TaqMan probes were obtained from Applied Biosystems, Foster City,

CA. The 5`reporter dye for all probes was FAM and the 3`quencher TAMRA. A passive reference dye (ROX) provided an internal standard for normalization of FAM fluorescence, correcting for fluctuations resulting from volume changes. A total volume of 20 µl PCR reaction mixture containing 9 µl cDNA (or dH$_2$O), 1 µl TaqMan probe (250 nmol/l) and primer mix (900 nmol/l, 20x), and 10 µl TaqMan Universal PCR Master Mix (2x) was amplified. Two-step PCR cycling was carried out: first cycle Uracil-N-glycosylase incubation at 50°C for 2 min then at 95°C for 10 min to activate AmpliTaq Gold DNA polymerase, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. The 18S rRNA was used as housekeeping gene for each target gene. All samples were run in duplicate for the target gene and the housekeeping gene. cDNA was quantified using the "delta-delta Ct" method (Livak and Schmittgen, 2001).

7. Functional categorization of regulated genes

The physiological role of regulated genes was assessed using the functional annotation chart tool of DAVID Bioinformatics Resources ver. 6.7 (Huang da, Sherman, and Lempicki, 2009). The annotation sources included the Clusters of Orthologous Groups (COG), Swiss-Prot (SP), Protein Information Resources (PIR), Uniprot Sequence Feature (UP), Gene Ontology (GO), Protein Analysis Through Evolutionary Relationships (PANTHER), Pubmed ID, InterPro, and KEGG Pathway. Analyses were performed at default settings with the rat genome set as the gene population background. Up- and down-regulated genes were assessed separately. Only analyses that integrated at least 40% of the regulated genes into a functional annotation chart (i.e. categorized genes, CG) were considered to be relevant. To reduce redundancy, functionally related or similar annotation terms were condensed into superordinate gene categories (Cheung, Deboer, Hanson, Tunesi, D'Onofrio,

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Arisi, Brandi, Cattaneo, and Goosens, 2013). The functional annotation analysis bears the risk of neglecting single relevant genes that are regulated independently from others. Thus, highly regulated uncategorized genes (HUG) that were not integrated into a functional annotation chart (i.e. uncategorized genes, UG) were selected using the algorithm Fold change$_{UG}$ > Mean fold change$_{CG}$. To cross-check the validity of the functional annotation analysis for processes involved in neuronal plasticity we performed a literature search using the NIHS Public Archive For The Refereed Literature (PUBMED; https://www.nvbi.nlm.nih.gov) with the search criteria "gene symbol, neuron", "gene symbol, brain" and "gene symbol, learning". For up-regulated CGs and HUGs, functional interactions were analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; http://string-db.org; Szklarczyk et al., 2015). Interactions with confidence score of 0.7 or higher were integrated to the interactome. Clusters were determined by MCL algorithm and presented with different node colors.

8. Statistical analysis

Statistical analyses and graph presentations were performed using Statistica (version 7; StatSoft, Tulsa, OK, USA), Prism (version 5; GraphPad Software, La Jolla, CA, USA) and Plotly (Plotly Inc, Montréal, Québec, Canada). Paired t-tests were used to analyze evolution of speed (trials/time) and reaching performance (successful trials/trials per session) for the SRT group. A p-value less than 0.05 was considered to be significant. Numerical results are expressed as mean and standard error of the mean (SEM).

Results

Animals that were trained in the SRT improved in speed (trials/minute) between training days 1 and 2 (day 1: 3.8 ± 0.48 day 2: 5.1 ± 0.52; p=0.04) indicating the acquisition of the operant component of the task. There was only a slight and not significant improvement in skill performance (successful trials/trials per session) between day 1 and 2 (day 1: 0.3 ± 0.04; day 2: 0.35 ± 0.03; p=0.19). This is expected, as a significant performance gain is expected to occur between day 2 and 3 (Buitrago et al., 2004).

Using detection thresholds of +40% or -30% difference of mRNA levels between groups we identified 296 genes out of the probe sets (15.805 genes; 1.9%) that were up- or down-regulated at any sampling time point. The number of regulated genes increases with time (**Figure 1A**; 44 genes at 1h, 75 genes at 7h, 177 genes at 24h) and an initial preponderance of down-regulation becomes detached by increased up-regulation. Only three genes were regulated at more than one time point indicating a high time point-specificity of gene expression (**Figure 1B**). No gene was regulated at all three time points.

To assess the functional relevance of up-/down-regulated genes (**Supplementary Table 1**) annotation terms were condensed into the functional categories "Regulation of transcription", "Cytoskeleton", "Development", "Posttranscriptional regulation", "Intracellular signaling", "Synapse" and "Oxygen transport" (**Table 2**). The expression pattern of individual genes and their affiliation to different functional categories were separately displayed for each time point (1h: **Figure 2**, 7h: **Figure 3** and 24h: **Figure 4**). Between functional categories, a broad overlap of affiliating genes indicates that particular genes are involved in different biological processes. For "Development" this

overlap is especially large as there are only two genes that are not included otherwise in functional categories. Thus, to avoid redundancy, we did not incorporate this category in the schematic view of sequential gene expression in M1 (**Figure 5**).

Over all, functionally categorized genes (CG) are significantly stronger regulated than the uncategorized ones (UG; 70.1 ± 0.06% vs. 51.0 ± 0.02%; Paired t-test: p=0.0002). Thus, the functional annotation analysis may have "filtered" biologically relevant genes (i.e. "signal") and separated them from irrelevant information (i.e. "noise"). However, to avoid neglecting single genes that are biologically relevant but do not fit in categories, we retained UGs that were regulated above average (i.e. HUGs: highly regulated UG; **Supplementary Table 2**). To cross-check the validity of the functional annotation analysis for neuronal plasticity, we performed a Pubmed literature research for every CG and HUG using the terms "brain", "neuron" and "learning". Thus, 53% of CGs - but only 28% of HUG could be directly linked to learning-induced or -related plasticity within the brain (**Supplementary Table 3**).

For the validation of the microarray data, ten selected genes (**Table 1**) were analyzed using real-time RT-PCR for the time point of 24h. Gene expression in microarray analysis correlated completely with real-time RT-PCR results, p-values for comparing SRT versus CT group by real-time RT-PCR were all significant (**Table 3**).

Discussion

In response to motor learning, genes are sequentially regulated within M1. The process of motor memory formation is characterized by an increasing number of regulated genes with time, a high time-point specificity and shift from initial suppression to later activation. The majority of regulated genes (i.e. CGs) can be related to functional categories that are known to play a role for motor learning-related plasticity in M1 (for review see Hosp and Luft, 2011). Altogether, three different steps can be defined within the gene-expression cascade following motor learning (**Figure 5**): first, an initial suppression of genes influencing gene transcription. This suppression might be a trimming in response to an earlier boost of immediate-early genes. Second, an expression of genes that support the spatio-temporally orchestrated translation of mRNA within defined compartments (e.g. postsynaptic site of an activated synapse) occurs. Third, genes coding for proteins that immediately mediate plastic changes e.g. by influencing neurite outgrowth, second messenger cascades, synapse formation or receptor distribution become expressed.

With respect to functional role of regulated genes, a broad similarity exists between hippocampus dependent learning and motor learning within M1 (functional categories in spatial learning and passive avoidance learning: "*Cell signalling*", "*Synaptic proteins*", "*Cytoskeletal proteins*", "*Apoptosis*" and "*Transcription and translation*" (Cavallaro et al., 2002; D'Agata and Cavallaro, 2003); "*Synapse*", "*Cytoskeleton*", "*Intracellular signaling*", "*Regulation of transcription*" and "*Posttranslational regulation*" in our study). With respect to the temporal profile of gene expression, an initial expression of genes influencing transcription and translation is followed by an

up-regulation of structural genes after three to six hours and a final down-regulation of plasticity mediating genes after twelve hours in response to passive avoidance learning (O'Sullivan et al., 2007). In contrast, consolidation of motor memory within M1 that is characterized by a peak of gene expression after twenty-four hours follows a much slower timescale. Thus, hippocampus dependent learning (i.e. explicit learning;(Squire, 2004) does not differ from motor learning (i.e. implicit learning) with respect to functional role of regulated genes, but with respect to the time schedule of their regulation. In addition, the shift from mere down-regulation towards prevailing up-regulation seems to be a unique feature of memory consolidation in M1.

Motor-learning specific gene expression within M1 has been investigated earlier in rats that were trained in a reaching task (Cheung et al., 2013). In this study, tissue was harvested twenty-four hours after training sessions at baseline, rising phase and at plateau of the learning curve. The authors could demonstrate the expression of particular genes that was selectively present during the learning phase, but not at baseline or plateau. Even though the identities of genes were only partially reported and only up-regulation was taken into account, the presence of the functional groups "Synapse", "Development" and "Intracellular signaling" fits well to our results. However, the functional groups "Fibroblast growth factor family" and "TGF-beta receptor" reported by Cheung and colleagues had no correlate in our analysis. This difference may be attributed to the different design of the two studies: as the work of Cheung and colleagues focuses on memory stabilization over the course of training (i.e. day 5), our study investigates the critical time window for skill acquisition and memory induction during the period of early steep learning i.e. after the second day of training (Buitrago et al., 2004). Furthermore, the study of Cheung and colleagues focused their assessment on a single time-point (24 hours after fifth day of training),

Within the first hours after training, the proportion of up- vs. down-regulation points to a suppression of gene-expression within M1. This is surprising, as learning usually induces an early up-regulation of the so-called immediate early genes (IEGs; Alberini and Kandel, 2015; Igaz, Vianna, Medina, and Izquierdo, 2002; O'Sullivan et al., 2007). In M1, the transcription factor Fos and the activity regulated cytoskeletal-associated protein (ARC) - two well-established IEGs - become induced during motor learning (Hosp et al., 2013; Kleim, Lussnig, Schwarz, Comery, and Greenough, 1996). Furthermore, the expression of ARC - that promotes the transcription of proteins influencing modification of the cytoskeleton and synaptic AMPA receptor trafficking (Bramham, Worley, Moore, and Guzowski, 2008) - is positively correlated with learning success (Hosp et al., 2013). However, in our present study, Arc is down-regulated at the time-point 1h. Given that mRNA of Fos and Arc have a peak at 30 minutes (Guzowski, 2002; Kovacs, 2008), their transcription occurs within the first minutes after the inducing event (Cortes-Mendoza, Diaz de Leon-Guerrero, Pedraza-Alva, and Perez-Martinez, 2013). Furthermore, an early decay in Arc expression is well established (Kelly and Deadwyler, 2003). Thus, we likely have missed the first wave of gene expression that is dominated by IEGs. The down-regulation of ARC one hour after training ended may be a compensatory trimming of an initial up-regulation related to consolidation processes (O'Sullivan et al. 2007).

It furthermore seems contradictory that "plasticity-supporting" genes (e.g. Top 1 or Actb) become down-regulated in response to motor training. However, regulation of genes may be different in different kind of cells (e.g. excitatory vs. inhibitory neurons, neurons vs. glial cells) and across cortical layers. Thus, analysing homogenized brain

tissue using a microarray chip can only reveal the net degree of gene regulation and does not provide any information regarding the balance of regulation between different classes of cells (Alberini and Kandel, 2015), which is a limitation of this study.

Genes **down-regulated** at the **time-points 1h and 7h** fall into the functional categories "*Regulation of transcription*" and "*Cytoskeleton*". As an example for the former category, the genes chromodomain helicase DNA-binding protein 8 (Chd8), topoisomerase 1 (Top1) and the FK506 binding protein 1a (Fkb1a) can be mentioned. Mutations of Chd8 - a regulator of a widespread transcriptional network comprising over 1700 genes - are frequently present in autism spectrum disorders, a deficiency that impacts pathways involved in brain and synapse development, neuron differentiation and axon guidance (Sugathan, Biagioli, Golzio, Erdin, Blumenthal, Manavalan, Ragavendran, Brand, Lucente, Miles, Sheridan, Stortchevoi, Kellis, Haggarty, Katsanis, Gusella, and Talkowski, 2014). Top1 is a transcription factor for genes with especially long transcripts. Its inhibition reduces excitatory and inhibitory neurotransmission by depleting synaptic proteins (King, Yandava, Mabb, Hsiao, Huang, Pearson, Calabrese, Starmer, Parker, Magnuson, Chamberlain, Philpot, and Zylka, 2013; Mabb, Kullmann, Twomey, Miriyala, Philpot, and Zylka, 2014). Fkb1a is an immunophilin that binds to immunosuppressant drugs such as FK506 and rapamycin thereby modulating the mTOR pathway. A deficiency enhances long-term potentiation (LTP) in hippocampal neurons in mice and leads to an increased contextual fear memory and a perseveration tendency in several behavioural tests (Hoeffer, Tang, Wong, Santillan, Patterson, Martinez, Tejada-Simon, Paylor, Hamilton, and Klann, 2008).

Apart from Arc, the trafficking kinesin protein 2 (Trak2) and the kinesin light chain 2 (Klc2) can be mentioned as examples for the functional category "*Cytoskeleton*". Trak2 codes for a kinesin adaptor protein that is critically involved in the intracellular transport of mitochondria and GABAA-receptor subunits, thereby influencing inhibitory synaptic transmission (Stephenson, 2014). KLC2 is a key molecule of the kinesin cargo delivery system that amongst others mediates AMPA-receptor trafficking (Du, Wei, Liu, Wang, Khairova, Blumenthal, Tragon, Hunsberger, Machado-Vieira, Drevets, Wang, and Manji, 2010). Finally, Actin β (Actb) becomes down-regulated, a protein forming the backbone of the dendritic compartment and spines (Urbanska, Swiech, and Jaworski, 2012). Through dynamic changes, this protein is involved in dendritic and synaptic plasticity, synapse function (Dillon and Goda, 2005) and receptor trafficking (Hanley, 2008). Masked Actin β mRNA and ribosomes are present in neuronal dendrites, allowing a local translation in response to synaptic activity (Buxbaum, Wu, and Singer, 2014).

After its transcription, learning-induced mRNA has to be stabilized and shipped to specific compartments (e.g. postsynaptic membrane of an activated spine) where local translation takes place. Genes that contribute to this process become **up-regulated** at the **time-point of 7h and** can be subsumed into the functional category "*Posttranscriptional regulation*". As an example, the mago-nashi homolog (MAGOH) belongs to the exon-junction complex (EJC) that is required to allow spatial and temporal precise translation of mRNA into proteins (Tange, Shibuya, Jurica, and Moore, 2005) and stabilizes the interaction of the RNA helicase eIF4A3 with target mRNAs (Barker-Haliski, Pastuzyn, and Keefe, 2012). Furthermore, MAGOH is involved in regulating division of neuronal stem cells (Silver, Watkins-Chow, Schreck, Pierfelice, Larson, Burnetti, Liaw, Myung, Walsh, Gaiano, and Pavan, 2010). The fragile X mental retardation gene (FMR1) codes for FMRP that has four RNA-binding

domains that differentially affect transport, stability and translation of mRNA (Santos, Kanellopoulos, and Bagni, 2014). Dysfunction of FMRP leads to a dysregulation of translation, i.e. accumulation vs. reduction of certain proteins (Bagni, Tassone, Neri, and Hagerman, 2012). The absence of FRMP impairs axon growth and guidance (Doll and Broadie, 2014) and formation of dendritic spines (Penzes, Cahill, Jones, VanLeeuwen, and Woolfrey, 2011).

At the **time-point of 24h up-regulated genes** belong to the functional categories "*Synapse*", "*Cytoskeleton*" and "*Intracellular signalling*". To obtain a better insight into the functional impact of these genes, we performed a functional interaction analysis using the STRING software (**Supplementary Figure 1**) that highlighted two clusters of functionally interconnected proteins: the larger cluster contains key determinants of domapinergic signalling like the dopamine receptors 1 and 2, adenylate cyclase 5 and the adenosine A2a receptor. This is especially interesting, as dopaminergic signalling within M1 that activates D1 and D2-receptors is a prerequisite for successful acquisition of the skilled reaching task in rats (Molina-Luna, Pekanovic, Rohrich, Hertler, Schubring-Giese, Rioult-Pedotti, and Luft, 2009). Dopamine is provided by M1-projecting mesencephalic neurons (Hosp et al., 2011) and supports learning-related plasticity by inducing learning-relevant genes, enhancing cortical excitability, strengthening motor representations and supporting the formation of long-term potentiation (LTP, for review see (Hosp and Luft, 2013). With respect to the interaction analysis, DA also seems also to influence synapse formation by regulating actin fibre cross-linking via Actn2 (Hodges, Vilchez, Asmussen, Whitmore, and Horwitz, 2014) and the formation of the postsynaptic complex via Pde10a and Ppp2r2a (Russwurm, Koesling, and Russwurm, 2015). Within the smaller cluster, proteins are enriched that facilitate synapse-formation by regulation of fatty-acid uptake (Lpl; (Xian, Liu, Yu, Wang, Miao, Zhang, Yu, Ross, Karasinska, Hayden, Liu,

and Chui, 2009), promoting actin filament assembly (Dgkb; (Kobayashi, Hozumi, Ito, Hosoya, Kondo, and Goto, 2007) and spinogenesis (Sdc2; (Hu and Hsueh, 2014). Furthermore, it contains the mitogenic receptor tyrosine kinase Egfr that promotes neurogenesis and supports hippocampal LTP formation by influencing NMDA-receptor trafficking (Aguirre, Rubio, and Gallo, 2010; Tang, Ye, Du, Qiu, Lv, Yang, and Luo, 2015).

At the **time-point of 24h down-regulated genes** belong to the functional categories "*Synapse*", "*Oxygen transport*" and "*Intracellular signalling*". As an example for the functional category "*Synapse*", the adaptor molecule Grb2 can be mentioned. By mediating protein-protein interactions, it facilitates axon elongation in response to activation of the neurotrophin receptors (Shinoda, Taya, Tsuboi, Hikita, Matsuzawa, Kuroda, Iwamatsu, and Kaibuchi, 2007). Hemoglobin alpha and beta belong to the category "*Oxygen transport*". Both molecules are expressed in cortical neurons in response to stress and are required for the synthesis of peptides that act at opioid and cannabinoid receptors (Stankiewicz, Goscik, Swiergiel, Majewska, Wieczorek, Juszczak, and Lisowski, 2014). For the category "*Intracellular signalling*" the adenylate-cyclase activated peptide Adycap1 and Map-kinase10 can be highlighted. Adycap1 enhances NMDA and AMPA currents in hippocampal neurons and modulates contextual fear conditioning at the behavioural level (Schmidt, Myskiw, Furini, Schmidt, Cavalcante, and Izquierdo, 2015). Mapk10 enhances neurite outgrowth in dopaminergic midbrain neurons and decreases availability of metabotropic glutamate and AMPA receptors (Tonges, Planchamp, Koch, Herdegen, Bahr, and Lingor, 2011).

In summary, the process of encoding motor memory within M1 is characterized by a defined temporal course of gene regulation that is highly dynamic. Both, up- and down-regulation of specific genes evolve over time, likely with a synergistic purpose:

whereas functional categorization points toward an optimization of transcription/translation at early time-points (1 und 7h), genes mediating cellular modifications are expressed at a later stage (24h). While functional categories of regulated genes were roughly similar to comparative studies investigating hippocampus-dependent learning, the acquisition of skilled movements - as a form of implicit learning - is characterized by a unique and comparably longer-lasting times-schedule of gene regulation and a particular balance between up- vs. down-regulation over time.

Tables

Table 1: List of genes assessed by quantitative real-time PCR assay for validation of microarray data

Gene symbol	Gene name	Accession No.	TaqMan Assay ID	Functional categories
Tac1	tachykinin 1	NM_012666	Rn00562002_m1	SYN, CYT, IS
Rarb	retinoic acid receptor, beta	NM_031529	Rn01537833_m1	IS
Gpr6	G Protein-coupled receptor 6	NM_031806	Rn00582568_m1	SYN
Slc5a7	solute carrier family 5, member 7	NM_053521	Rn00585367_m1	SYN
Nexn	nexilin	NM_139230	Rn01538866_m1	CYT, DEV
Egfr	epidermal growth factor receptor	NM_031507	Rn00580398_m1	SYN, CYT, IS, DEV
Rxrg	retinoid X receptor, gamma	NM_031765	Rn01483462_m1	IS, DEV
Drd2	dopamine receptor D2	NM_012547	Rn00561126_m1	SYN, CYT, IS, DEV
Rgs2	regulator of G protein signaling 2	NM_053453	R00584932_m1	UG
Rgs9	regulator of G protein signaling 9	NM_019224	Rn00570117_m1	IS

SYN: synapse; CYT: cytoskeleton; IS: intracellular signaling; DEV: development; UG: uncategorized genes.

Table 2: Summary of functional annotation analysis of regulated genes at different time points using the DAVID database

Time point	up- vs. down-regulated genes	% of genes included in analysis	Functional categories
1h	9 up-regulated		• no analysis possible due to low n
	35 down-regulated	51%	• Regulation of transcription • Development • Cytoskeleton
7h	45 up-regulated	47%	• Posttranscriptional regulation
	30 down-regulated	65%	• Regulation of transcription • Cytoskeleton
24h	122 up-regulated	59%	• Synapse • Cytoskeleton • Intracellular signaling • Development
	55 down-regulated	42%	• Oxygen transport • Intracellular signaling • Synapse

Table 3: Validation of microarray data of selected genes at time point 24h using quantitative real-time PCR

Gene symbol	Microarray FC	p-value	RT-PCR FC	p-value
Tac1	5.46	0.002	4.64	0.003
Rarb	2.52	0.003	2.56	0.005
Gpr6	2.15	0.001	7.73	0.001
Slc5a7	2.09	0.001	4.18	0.007
Nexn	1.65	0.001	3.78	0.019
Egfr	1.43	0.007	7.47	0.001
Rxrg	2.91	0.001	5.32	0.007
Drd2	3.49	0.007	4.31	0.038
Rgs2	1.91	0.006	1.79	0.038
Rgs99	3.93	0.001	4.47	0.049

Figures legends

Figure 1. Learning-specific modulation of gene expression in M1. A The number of regulated genes in response to motor learning increases with time. An initial preponderance of down-regulation turns into up-regulation at later time-points. **B** Venn diagram demonstrating the high time-point specificity of regulated genes.

Figure 2. Regulated genes at time-point 1h. Heat-maps indicate genes that were significantly up- or down regulated at the time-point 1h. Color intensities reflect the fold change, i.e. the degree of deviation with respect to controls (see scale at the bottom for reference). For down-regulated genes, the result of a functional annotation analysis using the DAVID database is indicated to the right of the heat-map. Circles indicate the affiliation of an individual gene to functional categories: "Regulation of transcription" (RT), "Development" (DEV), "Cytoskeleton" (CYT). For genes that were not assignable to a functional category, i.e. "Uncategorized genes" (UG), red circles indicate an especially high degree of regulation (i.e. fold change$_{UG}$ > Mean fold change$_{categorized genes}$). For up-regulated genes, a functional annotation analysis could not be performed due to the low number of regulated genes.

Figure 3. Regulated genes at time-point 7h. Heat-maps indicate genes that were significantly up- or down regulated at the time-point 7h. Color intensities reflect the fold change, i.e. the degree of deviation with respect to controls (see scale at the bottom for reference). The result of a functional annotation analysis using the DAVID database is indicated to the right of the heat-map. Circles indicate the affiliation of an individual gene to functional categories: "Posttranscriptional regulation" (PTR), "Regulation of transcription" (RT), "Cytoskeleton" (CYT). For genes that were not assignable to a functional category, i.e. "Uncategorized genes" (UG), red circles

indicate an especially high degree of regulation (i.e. fold change$_{UG}$ > Mean fold change$_{categorized\ genes}$).

Figure 4. Regulated genes at time-point 24h. Heat-maps indicate genes that were significantly up- or down regulated at the time-point 24h. Color intensities reflect the fold change, i.e. the degree of deviation with respect to controls (see scale at the bottom for reference). The result of a functional annotation analysis using the DAVID database is indicated to the right of the heat-map. Circles indicate the affiliation of an individual gene to functional categories: "Synapse" (SYN), "Cytoskeleton" (CYT), "Intracellular signaling" (IS), "Development" (DEV) and "Oxygen transport" (OT). For genes that were not assignable to a functional category, i.e. "Uncategorized genes" (UG), red circles indicate an especially high degree of regulation (i.e. fold change$_{UG}$ > Mean fold change$_{categorized\ genes}$).

Figure 5. Schematic view of sequential gene expression in M1 after motor learning. Functional categories of genes that were up- or down-regulated in response to training are displayed in a time-schedule. "Development" was discarded from the scheme due to its large overlap to the other functional categories.

References

Aguirre, A., Rubio, M. E., & Gallo, V. (2010). Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. *Nature, 467*, 323-327.

Alberini, C. M., & Kandel, E. R. (2015). The regulation of transcription in memory consolidation. *Cold Spring Harb Perspect Biol, 7*, a021741.

Alvarez, J., Giuditta, A., & Koenig, E. (2000). Protein synthesis in axons and terminals: significance for maintenance, plasticity and regulation of phenotype. With a critique of slow transport theory. *Prog Neurobiol, 62*, 1-62.

Bagni, C., Tassone, F., Neri, G., & Hagerman, R. (2012). Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics. *J Clin Invest, 122*, 4314-4322.

Barker-Haliski, M. L., Pastuzyn, E. D., & Keefe, K. A. (2012). Expression of the core exon-junction complex factor eukaryotic initiation factor 4A3 is increased during spatial exploration and striatally-mediated learning. *Neuroscience, 226*, 51-61.

Bisby, M. A., & Tetzlaff, W. (1992). Changes in cytoskeletal protein synthesis following axon injury and during axon regeneration. *Mol Neurobiol, 6*, 107-123.

Bramham, C. R., Worley, P. F., Moore, M. J., & Guzowski, J. F. (2008). The immediate early gene arc/arg3.1: regulation, mechanisms, and function. *J Neurosci, 28*, 11760-11767.

Buitrago, M. M., Ringer, T., Schulz, J. B., Dichgans, J., & Luft, A. R. (2004). Characterization of motor skill and instrumental learning time scales in a skilled reaching task in rat. *Behav Brain Res, 155*, 249-256.

Buxbaum, A. R., Wu, B., & Singer, R. H. (2014). Single beta-actin mRNA detection in neurons reveals a mechanism for regulating its translatability. *Science, 343*, 419-422.

Cavallaro, S., D'Agata, V., Manickam, P., Dufour, F., & Alkon, D. L. (2002). Memory-specific temporal profiles of gene expression in the hippocampus. *Proc Natl Acad Sci U S A, 99*, 16279-16284.

Cheung, V. C., Deboer, C., Hanson, E., Tunesi, M., D'Onofrio, M., Arisi, I., Brandi, R., Cattaneo, A., & Goosens, K. A. (2013). Gene expression changes in the motor cortex mediating motor skill learning. *PLoS One, 8*, e61496.

Cortes-Mendoza, J., Diaz de Leon-Guerrero, S., Pedraza-Alva, G., & Perez-Martinez, L. (2013). Shaping synaptic plasticity: the role of activity-mediated epigenetic regulation on gene transcription. *Int J Dev Neurosci, 31*, 359-369.

D'Agata, V., & Cavallaro, S. (2003). Hippocampal gene expression profiles in passive avoidance conditioning. *Eur J Neurosci, 18*, 2835-2841.

Dillon, C., & Goda, Y. (2005). The actin cytoskeleton: integrating form and function at the synapse. *Annu Rev Neurosci, 28*, 25-55.

Doll, C. A., & Broadie, K. (2014). Impaired activity-dependent neural circuit assembly and refinement in autism spectrum disorder genetic models. *Front Cell Neurosci, 8*, 30.

Du, J., Wei, Y., Liu, L., Wang, Y., Khairova, R., Blumenthal, R., Tragon, T., Hunsberger, J. G., Machado-Vieira, R., Drevets, W., Wang, Y. T., & Manji, H. K. (2010). A kinesin signaling complex mediates the ability of GSK-3beta to affect mood-associated behaviors. *Proc Natl Acad Sci U S A, 107*, 11573-11578.

Gibson, U. E., Heid, C. A., & Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Res, 6*, 995-1001.

Greenough, W. T., Larson, J. R., & Withers, G. S. (1985). Effects of unilateral and bilateral training in a reaching task on dendritic branching of neurons in the rat motor-sensory forelimb cortex. *Behav Neural Biol, 44*, 301-314.

Guzowski, J. F. (2002). Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. *Hippocampus, 12*, 86-104.

Hanley, J. G. (2008). PICK1: a multi-talented modulator of AMPA receptor trafficking. *Pharmacol Ther, 118*, 152-160.

Hodges, J. L., Vilchez, S. M., Asmussen, H., Whitmore, L. A., & Horwitz, A. R. (2014). alpha-Actinin-2 mediates spine morphology and assembly of the post-synaptic density in hippocampal neurons. *PLoS One, 9*, e101770.

Hoeffer, C. A., Tang, W., Wong, H., Santillan, A., Patterson, R. J., Martinez, L. A., Tejada-Simon, M. V., Paylor, R., Hamilton, S. L., & Klann, E. (2008). Removal of FKBP12 enhances mTOR-Raptor interactions, LTP, memory, and perseverative/repetitive behavior. *Neuron, 60*, 832-845.

Hosp, J. A., & Luft, A. R. (2011). Cortical plasticity during motor learning and recovery after ischemic stroke. *Neural Plast, 2011*, 871296.

Hosp, J. A., & Luft, A. R. (2013). Dopaminergic meso-cortical projections to m1: role in motor learning and motor cortex plasticity. *Front Neurol, 4*, 145.

Hosp, J. A., Mann, S., Wegenast-Braun, B. M., Calhoun, M. E., & Luft, A. R. (2013). Region and task-specific activation of Arc in primary motor cortex of rats following motor skill learning. *Neuroscience, 250*, 557-564.

Hosp, J. A., Pekanovic, A., Rioult-Pedotti, M. S., & Luft, A. R. (2011). Dopaminergic projections from midbrain to primary motor cortex mediate motor skill learning. *J Neurosci, 31*, 2481-2487.

Hu, H. T., & Hsueh, Y. P. (2014). Calcium influx and postsynaptic proteins coordinate the dendritic filopodium-spine transition. *Dev Neurobiol, 74*, 1011-1029.

Huang da, W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc, 4*, 44-57.

Igaz, L. M., Vianna, M. R., Medina, J. H., & Izquierdo, I. (2002). Two time periods of hippocampal mRNA synthesis are required for memory consolidation of fear-motivated learning. *J Neurosci, 22*, 6781-6789.

Kelly, M. P., & Deadwyler, S. A. (2003). Experience-dependent regulation of the immediate-early gene arc differs across brain regions. *J Neurosci, 23*, 6443-6451.

King, I. F., Yandava, C. N., Mabb, A. M., Hsiao, J. S., Huang, H. S., Pearson, B. L., Calabrese, J. M., Starmer, J., Parker, J. S., Magnuson, T., Chamberlain, S. J., Philpot, B. D., & Zylka, M. J. (2013). Topoisomerases facilitate transcription of long genes linked to autism. *Nature, 501*, 58-62.

Kleim, J. A., Barbay, S., & Nudo, R. J. (1998). Functional reorganization of the rat motor cortex following motor skill learning. *J Neurophysiol, 80*, 3321-3325.

Kleim, J. A., Hogg, T. M., VandenBerg, P. M., Cooper, N. R., Bruneau, R., & Remple, M. (2004). Cortical synaptogenesis and motor map reorganization occur during late, but not early, phase of motor skill learning. *J Neurosci, 24*, 628-633.

Kleim, J. A., Lussnig, E., Schwarz, E. R., Comery, T. A., & Greenough, W. T. (1996). Synaptogenesis and Fos expression in the motor cortex of the adult rat after motor skill learning. *J Neurosci, 16*, 4529-4535.

Kobayashi, N., Hozumi, Y., Ito, T., Hosoya, T., Kondo, H., & Goto, K. (2007). Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. *Eur J Cell Biol, 86*, 433-444.

Kolb, B., Cioe, J., & Comeau, W. (2008). Contrasting effects of motor and visual spatial learning tasks on dendritic arborization and spine density in rats. *Neurobiol Learn Mem, 90*, 295-300.

Kovacs, K. J. (2008). Measurement of immediate-early gene activation- c-fos and beyond. *J Neuroendocrinol, 20*, 665-672.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*, 402-408.

Luft, A. R., Buitrago, M. M., Ringer, T., Dichgans, J., & Schulz, J. B. (2004). Motor skill learning depends on protein synthesis in motor cortex after training. *J Neurosci, 24*, 6515-6520.

Mabb, A. M., Kullmann, P. H., Twomey, M. A., Miriyala, J., Philpot, B. D., & Zylka, M. J. (2014). Topoisomerase 1 inhibition reversibly impairs synaptic function. *Proc Natl Acad Sci U S A, 111*, 17290-17295.

Molina-Luna, K., Hertler, B., Buitrago, M. M., & Luft, A. R. (2008). Motor learning transiently changes cortical somatotopy. *Neuroimage, 40*, 1748-1754.

Molina-Luna, K., Pekanovic, A., Rohrich, S., Hertler, B., Schubring-Giese, M., Rioult-Pedotti, M. S., & Luft, A. R. (2009). Dopamine in motor cortex is necessary for skill learning and synaptic plasticity. *PLoS One, 4*, e7082.

Monfils, M. H., Plautz, E. J., & Kleim, J. A. (2005). In search of the motor engram: motor map plasticity as a mechanism for encoding motor experience. *Neuroscientist, 11*, 471-483.

Neafsey, E. J., Bold, E. L., Haas, G., Hurley-Gius, K. M., Quirk, G., Sievert, C. F., & Terreberry, R. R. (1986). The organization of the rat motor cortex: a microstimulation mapping study. *Brain Res, 396*, 77-96.

Nudo, R. J., Milliken, G. W., Jenkins, W. M., & Merzenich, M. M. (1996). Use-dependent alterations of movement representations in primary motor cortex of adult squirrel monkeys. *J Neurosci, 16*, 785-807.

O'Sullivan, N. C., McGettigan, P. A., Sheridan, G. K., Pickering, M., Conboy, L., O'Connor, J. J., Moynagh, P. N., Higgins, D. G., Regan, C. M., & Murphy, K. J. (2007). Temporal change in gene expression in the rat dentate gyrus following passive avoidance learning. *J Neurochem, 101*, 1085-1098.

Pascual-Leone, A., Nguyet, D., Cohen, L. G., Brasil-Neto, J. P., Cammarota, A., & Hallett, M. (1995). Modulation of muscle responses evoked by transcranial magnetic stimulation during the acquisition of new fine motor skills. *J Neurophysiol, 74*, 1037-1045.

Penzes, P., Cahill, M. E., Jones, K. A., VanLeeuwen, J. E., & Woolfrey, K. M. (2011). Dendritic spine pathology in neuropsychiatric disorders. *Nat Neurosci, 14*, 285-293.

Rioult, C. (1998). [The therapeutic touch to enrich nursing practice]. *Soins*, 26-29.

Rioult-Pedotti, M. S., Donoghue, J. P., & Dunaevsky, A. (2007). Plasticity of the synaptic modification range. *J Neurophysiol, 98*, 3688-3695.

Rioult-Pedotti, M. S., Friedman, D., & Donoghue, J. P. (2000). Learning-induced LTP in neocortex. *Science, 290*, 533-536.

Russwurm, C., Koesling, D., & Russwurm, M. (2015). Phosphodiesterase 10A Is Tethered to a Synaptic Signaling Complex in Striatum. *J Biol Chem, 290*, 11936-11947.

Santos, A. R., Kanellopoulos, A. K., & Bagni, C. (2014). Learning and behavioral deficits associated with the absence of the fragile X mental retardation protein: what a fly and mouse model can teach us. *Learn Mem, 21*, 543-555.

Schmidt, S. D., Myskiw, J. C., Furini, C. R., Schmidt, B. E., Cavalcante, L. E., & Izquierdo, I. (2015). PACAP modulates the consolidation and extinction of the contextual fear conditioning through NMDA receptors. *Neurobiol Learn Mem, 118*, 120-124.

Shinoda, T., Taya, S., Tsuboi, D., Hikita, T., Matsuzawa, R., Kuroda, S., Iwamatsu, A., & Kaibuchi, K. (2007). DISC1 regulates neurotrophin-induced axon elongation via interaction with Grb2. *J Neurosci, 27*, 4-14.

Silver, D. L., Watkins-Chow, D. E., Schreck, K. C., Pierfelice, T. J., Larson, D. M., Burnetti, A. J., Liaw, H. J., Myung, K., Walsh, C. A., Gaiano, N., & Pavan, W. J. (2010). The

exon junction complex component Magoh controls brain size by regulating neural stem cell division. *Nat Neurosci, 13*, 551-558.

Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol, 3*, Article3.

Squire, L. R. (2004). Memory systems of the brain: a brief history and current perspective. *Neurobiol Learn Mem, 82*, 171-177.

Stankiewicz, A. M., Goscik, J., Swiergiel, A. H., Majewska, A., Wieczorek, M., Juszczak, G. R., & Lisowski, P. (2014). Social stress increases expression of hemoglobin genes in mouse prefrontal cortex. *BMC Neurosci, 15*, 130.

Stephenson, F. A. (2014). Revisiting the TRAK family of proteins as mediators of GABAA receptor trafficking. *Neurochem Res, 39*, 992-996.

Sugathan, A., Biagioli, M., Golzio, C., Erdin, S., Blumenthal, I., Manavalan, P., Ragavendran, A., Brand, H., Lucente, D., Miles, J., Sheridan, S. D., Stortchevoi, A., Kellis, M., Haggarty, S. J., Katsanis, N., Gusella, J. F., & Talkowski, M. E. (2014). CHD8 regulates neurodevelopmental pathways associated with autism spectrum disorder in neural progenitors. *Proc Natl Acad Sci U S A, 111*, E4468-4477.

Tang, Y., Ye, M., Du, Y., Qiu, X., Lv, X., Yang, W., & Luo, J. (2015). EGFR signaling upregulates surface expression of the GluN2B-containing NMDA receptor and contributes to long-term potentiation in the hippocampus. *Neuroscience, 304*, 109-121.

Tange, T. O., Shibuya, T., Jurica, M. S., & Moore, M. J. (2005). Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. *RNA, 11*, 1869-1883.

Tonges, L., Planchamp, V., Koch, J. C., Herdegen, T., Bahr, M., & Lingor, P. (2011). JNK isoforms differentially regulate neurite growth and regeneration in dopaminergic neurons in vitro. *J Mol Neurosci, 45*, 284-293.

Urbanska, M., Swiech, L., & Jaworski, J. (2012). Developmental plasticity of the dendritic compartment: focus on the cytoskeleton. *Adv Exp Med Biol, 970*, 265-284.

Whishaw, I. Q., & Pellis, S. M. (1990). The structure of skilled forelimb reaching in the rat: a proximally driven movement with a single distal rotatory component. *Behav Brain Res, 41*, 49-59.

Withers, G. S., & Greenough, W. T. (1989). Reach training selectively alters dendritic branching in subpopulations of layer II-III pyramids in rat motor-somatosensory forelimb cortex. *Neuropsychologia, 27*, 61-69.

Wu, Z., & Irizarry, R. A. (2004). Preprocessing of oligonucleotide array data. *Nat Biotechnol, 22*, 656-658; author reply 658.

Xian, X., Liu, T., Yu, J., Wang, Y., Miao, Y., Zhang, J., Yu, Y., Ross, C., Karasinska, J. M., Hayden, M. R., Liu, G., & Chui, D. (2009). Presynaptic defects underlying impaired learning and memory function in lipoprotein lipase-deficient mice. *J Neurosci, 29*, 4681-4685.

Xu, T., Yu, X., Perlik, A. J., Tobin, W. F., Zweig, J. A., Tennant, K., Jones, T., & Zuo, Y. (2009). Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature, 462*, 915-919.