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SYNAPTIC ACTIVITY-INDUCED GLOBAL GENE EXPRESSION PATTERNS IN THE DENTATE GYRUS OF ADULT BEHAVING RATS: INDUCTION OF IMMUNITY-LINKED GENES

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Abstract—Gene expression in adult neuronal circuits is dynamically modulated in response to synaptic activity. Persistent changes in synaptic strength, as seen during high-frequency stimulation (HFS)–induced long-term potentiation (LTP), require new gene expression. While modulation of many individual genes has been shown, an understanding of LTP as a complex dynamical response requires elucidation of the global gene expression signature and its impact on biologically meaningful gene sets. In this study, we demonstrate that LTP induction in the dentate gyrus of awake freely moving rats was associated with changes in the expression of genes linked to signal transduction, protein trafficking, cell structure and motility, and other processes consistent with the induction of mechanisms of synaptic reorganization and growth. Interestingly, the most significantly over-represented gene sets were related to immunity and defense, including T-cell-mediated immunity and major histocompatibility complex (MHC) class I–mediated immunity. Real-time PCR confirmed the upregulation of a panel of immune-linked genes including the *rt1-a/ce* family, and the MHC class II members *cd74*, *rt1-Ba* and *rt1-Da*. These genes were *N*-methyl-D-aspartate receptor-independent and not induced following HFS-LTP induction in anesthetized rats, indicating a gene response specific to behaving rats. Our data support recent assumptions that immunity-associated processes are functionally linked to adaptive neuronal responses in the brain, although the differential expression of immunity-linked genes could also be related to the HFS per se.

Key words: LTP, microarray, gene expression, hippocampus, immunity, MHC.

Long-term potentiation (LTP) is an experimentally evoked strengthening of synaptic connections by high-frequency stimulation (HFS) of afferent input (Bliss et al., 2007). Activity-dependent changes in synaptic strength, like LTP, are thought to subserve adaptive responses in the adult nervous system of importance for a broad range of functions including memory formation, pain control and synapse maturation ((Milner et al., 1998; Bramham, 2007), for review). LTP induction in most excitatory pathways requires a rapid increase in postsynaptic calcium levels which is mediated by an activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, activation of voltage-dependent calcium channels, and mobilization of calcium from intracellular stores. Both behavioral memory and experimental HFS NMDA receptor (NMDAR)-dependent LTP in the hippocampus of freely behaving rats were recently demonstrated to depend on similar inductive mechanisms (Pastalkova et al., 2006; Whitlock et al., 2006).

Maintenance of LTP is characterized by an early transcription-independent phase, lasting between 1 and 3 h, followed by a late transcription-dependent phase that is required for enduring LTP (Nguyen et al., 1994). Although the early LTP does not require new gene expression, transcriptional events during this period are crucial for the development of late phase LTP. Microarray-based global gene expression profiling has provided insight into behavioral-linked memory processes and LTP-inductions *in vitro* (Cavallaro et al., 2002; Leil et al., 2002, 2003; Levenson et al., 2004; Zhang et al., 2005; Park et al., 2006b). In the dentate gyrus (DG) *in vitro*, LTP modulated the expression of more than 1600 genes of which many were physically clustered at specific locations on the genome (Park et al., 2006a). Functional classification linked several gene sets to cell–cell interaction, neurite outgrowth, and synapse formation and remodeling. In addition, some gene groups were specifically regulated by the cAMP response element-binding protein (CREB). This factor has been linked to LTP (Davis et al., 2000; Barco et al., 2005), but conditional knockout mice apparently display normal LTP and learning capabilities (Balschun et al., 2003).

We hypothesized that the LTP induced in awake behaving rats may elicit transcriptional responses that are

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Abbreviations: AB, Applied Biosystems; CPP, (R,S)-3-22-carboxypiperazin-4-yl-propyl-1-phosphonic acid; *c3*, *complement component 3*; DG, dentate gyrus; DIG, digoxigenin; EC, electrode control; *erm*, *ets-related molecule*; FC, fold change in mRNA levels; fEPSP, field excitatory postsynaptic potential; GSEA, gene set enrichment analysis; HFS, high-frequency stimulation; IPA, Ingenuity pathways analyses; LTD, long-term depression; LTP, long-term potentiation; MHC, major histocompatibility complex; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; *nptxr*, *neuronal pentraxin receptor*; Panther, Protein analysis through evolutionary relationships; PSD-95, postsynaptic density protein-95; RT-PCR, reverse transcription polymerase chain reaction; SAM, significance analysis of microarrays; *sez6*, *seizure-related gene 6*; S/N, signal-to-noise ratio; TF, transcription factor.

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distinct from those observed in hippocampal slices *in vitro*, or in anesthetized rats *in vivo*. In behaving animals, sleep–wakefulness state strongly modulates LTP induction and the expression of synaptic activity-dependent genes (Cirelli and Tononi, 2000). For instance, expression of LTP-associated genes such as *activity-regulated cytoskeleton-associated protein* (*arc*) and *brain-derived neurotrophic factor* (*bdnf*) in rat is markedly higher during wakefulness than sleep. Also, the genome-wide transcriptome profile of specific brain regions has been demonstrated to change considerably between behavioral states and time of day (Cirelli et al., 2004). Functional classification of the genes whose expression was affected by the animal's arousal state revealed that processes of neuronal plasticity and membrane trafficking were markedly involved. In order to discover not only new genes, but also biological meaningful processes, HFS-LTP should be induced in awake animals and characterized by functional annotation of changes in the global gene expression pattern as an experimental model on learning and memory.

In the present study we aimed at providing a functional classification of HFS-LTP-related changes in global gene expression in the DG of awake and freely moving rats. HFS-LTP was associated with significant induction and over-representation of biological processes consistent with the induction of mechanisms underlying neuronal signaling, and synaptic reorganization and growth. Interestingly, however, the most significantly over-represented group of genes was linked to immunity and defense, especially major histocompatibility complex (MHC) class I–mediated immunity and T-cell-mediated immunity.

EXPERIMENTAL PROCEDURES

LTP induction in awake behaving rats

Electrophysiological, pharmacological (NMDAR inhibition) and surgical procedures that were employed on male Sprague–Dawley rats (Møllegaard Breeding Centre, Denmark) have previously been described (Havik et al., 2003). Briefly, animals were anesthetized (pentobarbital/chloral hydrate cocktail) and electrodes were chronically implanted for unilateral stimulation of the medial perforant pathway in the angular bundle and recording of evoked response in the hilar region of the DG. Rats remained in the animal facility for at least 20 days, after which they were handled daily and habituated to the recording chamber. All recordings were done while the rat was quiet and awake (still–alert behavioral state). HFS was composed of three stimuli sessions separated by 5 min. Each session included eight pulses at 400 Hz, repeated four times at 10 s intervals. The stimulus intensity for HFS was set to evoke 50% of that needed to evoke a maximal population. In experiments on NMDAR blockade, the competitive NMDA receptor antagonist CPP ((R,S)-3-22-carboxypiperazin-4-yl-propyl-1-phosphonic acid; Tocris Cookson, UK) was dissolved in saline and injected i.p. at a dose of 10 mg/kg, 2 h prior to HFS. All other animals received 0.9% saline i.p. 2 h prior to HFS.

A separate group of rats served as controls for electrode implantation and surgery ($n=5$). Rats were implanted unilaterally with stimulation and recording electrodes as described above, followed by recovery period for at least 20 days. The rats did not receive HFS. mRNA was extracted from paired DG samples (electrode side vs. contralateral control side) and used as template for real-time reverse transcription polymerase chain reaction (RT-PCR) analyses.

LTP induction in anesthetized rats

Rats were anesthetized with urethane (1.4–1.8 g/kg, i.p.) and placed in a stereotaxic frame in the flat skull position. Rectal temperature was maintained at 36 °C with a thermostatically-controlled electric heating pad to prevent heat loss. Test-pulses were applied (one/30 s) throughout the experiment and LTP was induced used the same protocol employed in awake rats. This protocol has been shown to evoke stable late phase LTP in both awake and anesthetized rats (Bramham and Srebro, 1987; Havik et al., 2003).

Tissue collection

Immediately after electrophysiological recording, each rat was rapidly anesthetized (pentobarbital/chloral hydrate, awake rats only) and decapitated with subsequent removal of the brain. The brain was dissected rapidly on ice and kept cold by repeated rinses in oxygenated ice-cold artificial cerebrospinal fluid (aCSF, pH 7.4) (in mM: NaCl, 124.0; $NaHCO_3$, 25.0; D-glucose, 10.0; KCl, 3.4; KH_2PO_4, 1.2; $MgSO_4$, 1.0; $CaCl_2$, 2.5). The DG from the HFS and control samples was immediately frozen on a dry-ice/ethanol slurry and stored at −80 °C. DG samples were collected at 40 min and 2 h post-HFS in saline- injected animals, and 40 min post-HFS in the CPP group.

For the microarray analysis we employed paired DG samples (HFS-stimulated vs. contralateral control side) obtained at 40 min ($n=4$) and at 2 h ($n=4$) post-HFS. At each time point, three samples were from single rats and one sample was obtained by pooling DG from five rats. In CPP-treated rats, three paired DG samples were obtained, each based on five pooled DG. The pooling was performed during a previous study, in which a part of the pooled DG homogenate was fractionated for the isolation of the synapto-dendritic compartment (Havik et al., 2003).

RNA samples

mRNA was purified and characterized from microdissected DG samples as previously described (Havik et al., 2003). mRNA was purified from DG homogenates using Dynabeads mRNA Direct Kit (Dynal, Norway).

Microarray analysis

Microarray analysis was essentially performed as previously described (Stansberg et al., 2007). Digoxigenin (DIG)–UTP-labeled cRNA was generated and labeled from 50 ng mRNA of each sample using Applied Biosystems (AB, USA) chemiluminescent RT-IVT labeling kit v.2.0 according to the manufacturer's protocol (AB). The yield of synthesized cRNA was typically in the range of 30–40 μg. Twenty micrograms of fragmented DIG-cRNA samples was hybridized onto AB rat genome survey microarrays for 16 h at 55 °C. Chemiluminescence detection, acquisition and analysis were performed using the AB chemiluminescence detection kit and AB 1700 Chemiluminescent Microarray Analyzer following the manufacturer's instructions.

The AB Expression System software was used to extract signals and signal-to-noise ratios (S/N). Signal intensities were imported into J-Express Pro V2.7 software (MolMine, Norway) (Dysvik and Jonassen, 2001), where inter-array quantile normalization was performed. The majority of genes that were differentially expressed in HFS-stimulated DG, as compared with the un-stimulated contralateral controls, were identified on the basis of statistical paired *t*-tests ($P<0.05$, linear signal intensities). NMDAR-dependent gene expression was determined as significant fold changes (FC) in expression level in the HFS group relative to the CPP-treated group (unpaired *t*-test, $P<0.05$).

Panther classification system

Functional classification of differentially expressed gene 40 min and 2 h post-HFS was performed by the Panther Classification System 1.2 (http://www.pantherdb.org/) (Mi et al., 2006). Each of the lists of differentially expressed probes was compared with the entire list of probes that showed detectable expression in unstimulated control DG (11,639 probes; 11,311 genes, S/N=5) on the AB Rat Genome Survey Array. Statistically significant over-represented annotation categories were determined by binomial statistics, using the observed number of genes versus the numbers expected by chance within a certain annotation group. Over-represented categories, or categories with any sub-category with *P*-values less than 0.05 are shown.

Gene set enrichment analysis (GSEA)

GSEA searches for differential expression in a set of related genes (Subramanian et al., 2005), here defined as belonging to the same biological process. GSEA starts by ranking the genes of the entire data set according to a test statistic (e.g. SAM and t-score), also including genes below the single gene statistical test threshold. The null hypothesis of GSEA is that the genes belonging to a particular gene set are evenly distributed across the ranked gene list. If the genes of a gene set are overrepresented toward the top or the bottom of the ranked list, then this may be an important set of genes for the study.

Paired GSEA was performed using the J-Express Pro software v.2.8 (MolMine) (Dysvik and Jonassen, 2001). Gene sets were created using the Panther Biological Process terms (http://www.pantherdb.org/). The Panther annotation file dated 30.09.2006 was used to create a database of 250 gene sets.

Probes were collapsed to gene level before running GSEA. Gene sets smaller than 5 or larger than 900 were excluded from the analysis. Paired significance analysis of microarrays (SAM) scores, based on log2-transformed microarray signal intensities, was used to rank the genes. t-Score-based ranking was also performed, giving similar results for the top-ranked enriched gene sets (data not shown). Significance was assessed by permuting the gene labels (1000 iterations). Default values were used for all other parameters.

Ingenuity pathways analysis (IPA)

Interaction-based relationships between possible proteins encoded by LTP modulated genes were constructed on the basis of the IPA Knowledge Base (Ingenuity Systems, USA) (https://analysis.ingenuity.com). IPA provides functions of, and interactions between, cellular proteins mined from peer-reviewed literature. Gene lists containing Entrez identifiers and expression values (microarray-based mRNA FC values) were uploaded as Excel spreadsheets according to the IPA guidelines. The top-ranking generated protein–protein networks were analyzed for functional significance in relation to LTP.

Real-time RT-PCR

cDNA samples (50 μl) were synthesized from 20 ng mRNA using the SuperScript II First-Strand Synthesis System for RT-PCR (Life Technologies, Sweden). Three technical replicates with 0.2 μl cDNA template each were used for real-time PCR analysis on an AB Prism 7900HT Sequence Detection System (10 μl total reaction volume; AB). The relative gene expression levels were determined with the comparative Ct-method, and similar results were obtained using Ct standard curves. Two endogenous controls, ribosomal protein P0 mRNA and eukaryotic 18S mRNA, and one no-template-control were also run in technical triplicates. The relative gene expression levels were presented as fold-change values normalized to P0. Commercially designed TaqMan®Gene expression assays (AB) were used to detect expression of the following genes (AB assay name in parentheses): *cd74* (Rn00565062_m1), *rt1-Da* (Rn02346209_g1), *rt1-Ba* (Rn01428452_m1), *complement component 3* (*c3*) exons 27–28 (Rn00566466_m1), *c3* exons 34–35 (Rn01527743_g1), *c3* exons 35–36 (Rn01527744_m1), *centaurin 1*α (Rn00554894_m1), *baiap-2* (Rn00589411_m1), *scnb1* (Rn00441210_m1), *casein kinase 1*ε (Rn00581130_m1), *prickle1* (Rn01407797_m1), *pscd1* (Rn00588204_m1), *zif268* (Rn00561138_m1), *neuronal pentraxin receptor* (*nptxr*) (Rn00578183_m1), eukaryotic *18S* (4352930E).

The following PCR primers (forward, reverse) were used for quantitative SYBR green real-time RT-PCR (Eurogentec; purchased from Medprobe, Norway):

ets-related molecule (*erm*): 5′-AACTGCACTGCATCCCTTAGGT, 5′-CTCAGGCTGCAGCGTCTTC;

bmi1: 5′-TTACACCTGGAGACGGAATGG, 5′-TTGCAAGTTGGCCGAACTC;

rt1-a/ce assay 1: 5′-GATACCTGGAGCTCGGGAA, 5′-CCTCAGGGTGACATCACCTT;

rt1-a/ce assay 2: 5′-GATACCTGGAGCTCGGGAA, 5′-CCTTCAGGTCTGGGGTGAAG;

np2/narp: 5′-GGCAAGATCAAGAAGACGTTG, 5′-TCCAGGTGATGCAGATATGGT;

neuritin: 5′-GGGACTTAAGTTGAACGGCA, 5′-ACCCAGCTTGAGCAAACAGT;

P0: 5′-CATTGAAATCCTGAGCGATGTG, 5′-AGATGTTCAACATGTTCAGCAGTGT.

RESULTS

LTP-associated gene expression profiles in awake rats

Fig. 1 presents an overview of the experimental design. Global gene expression profiles were determined at 40 min and 2 h after LTP induction in the DG of awake adult rats. As previously reported (Havik et al., 2003), LTP was induced by HFS to the left DG, while the contralateral DG served as a paired un-stimulated control. Another group of animals received systemic injection (i.p.) of the NMDAR blocker CPP 2 h prior to HFS. LTP of the field excitatory postsynaptic potentials (fEPSPs) and population spike (PS) was blocked in CPP-treated rats.

Data analysis of the un-stimulated DG control samples showed reliable hybridization signals from 11,639 probes (11,311 genes), using a conservative S/N≥5. The S/N threshold was set by evaluating the impact of increasing S/N from 0 to 15 on the number of excluded genes. A marked threshold was observed at S/N=5, after which further stepwise increases in S/N showed low and stable fractions of excluded genes. Thus, genes rated as expressed in this analysis have a reliable basal expression level.

The genes with robust hybridization signals were subsequently analyzed for differential expression in response to HFS-LTP (paired *t*-tests: $P<0.05$). The early transcriptional response at 40 min post-HFS was linked to differential expression of 1021 genes (Fig. 2A). At 2 h post-HFS, 375 differentially expressed genes were observed, indicating a marked reduction in the transcriptional response at the later time point (Fig. 2B).

HFS-LTP at medial perforant path synapses is strongly NMDAR-dependent. We therefore used CPP treatment to identify genes linked to NMDAR-dependent LTP. By compar-

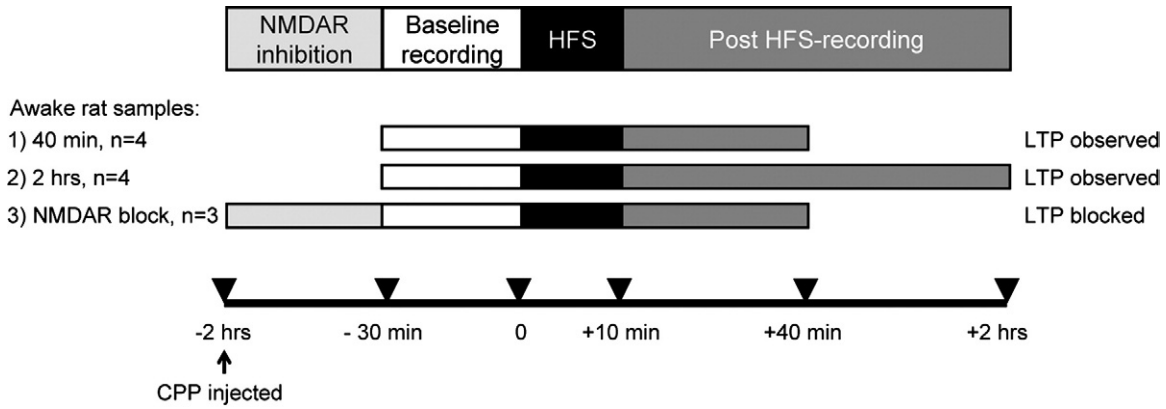

Fig. 1. Experimental setup for HFS-LTP in awake rats. Schematic presentation of the experimental design. Time points for LTP induction, CPP injection (NMDAR inhibition) and sample collections are marked. CPP treatment lasted throughout the experiment. Rats not exposed to CPP, were injected with saline solvent only. Three groups of paired DG samples were collected for microarray analyses: 40 min, NMDAR inhibited 40 min, and 2 h samples. DG samples from awake and anesthetized rats were used for real-time RT-PCR verification of selected LTP-response genes (see text).

ing gene-specific FC-values (FC_{40min} and $FC_{NMDAR\text{-}inactive}$) from rats receiving only HFS to those also receiving CPP treatment, we found that 413 (41%) of the 1021 differentially expressed genes at 40 min post-HFS had significantly different expression levels ($P<0.05$, *t*-test). The percentage of NMDAR-dependent genes was similar among up- and downregulated genes.

Functional categorization of HFS-regulated genes

The lists of differentially expressed genes were mapped to the Panther annotation categories to search for gene-sets of over-represented biological processes. Almost all differentially expressed genes defined above classified to one or more of the categories listed in Fig. 3A–C, and over-represented categories were ranked according to statistical significance.

Interestingly, the biological process immunity and defense (31 genes) with the subgroups T-cell-mediated immunity (10 genes) and MHCI-mediated immunity (6 genes), were the top ranked over-represented processes, as seen 2 h post-HFS (Fig. 3A). Notably, genes linked to immunity and defense were also expressed 40 min post-HFS, but, however, in comparison with the 2 h time point they are nearly non-redundant. Few other gene sets showed a significant over-representation, except various sub-categories of transport; nucleoside, nucleotide, and nucleic acid metabolism; and carbohydrate metabolism (at 40 min and at 2 h post-HFS: Fig. 3A).

Next we examined whether upregulated genes and downregulated genes were linked to the same or different biological processes (Fig. 3B and 3C). At 40 min post-HFS, the upregulated genes constitute a group of over-represented biological processes that have previously been associated with LTP-induction (Fig. 3B), such as signal transduction (93 genes), protein modification and metabolism (including proteolysis) (106 genes), transport (56 genes), intracellular protein traffic (52 genes) and the cell cycle sub-group mitosis (17 genes). In contrast, none of the downregulated genes at 40 min post-HFS grouped into any overrepresented biological processes (Fig. 3C). The

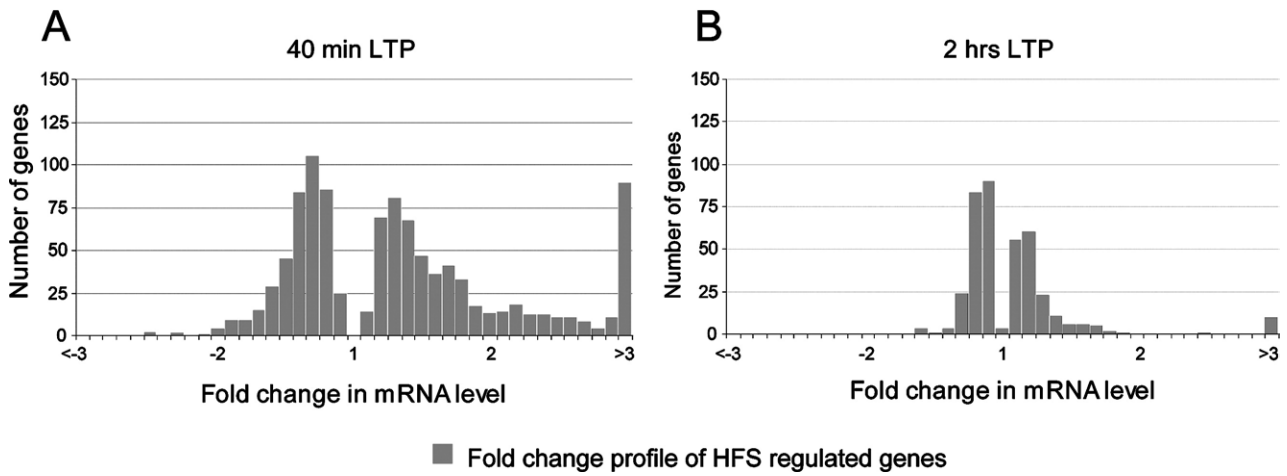

Fig. 2. FC distribution for HFS-regulated genes detected in the DG at 40 min and at 2 h post-HFS. Differentially expressed genes were selected on the basis of paired *t*-test ($P<0.05$). (A) FC distribution of 1021 differentially expressed genes at 40 min post-HFS. (B) FC distribution of 375 differentially expressed genes at 2 h post-HFS.

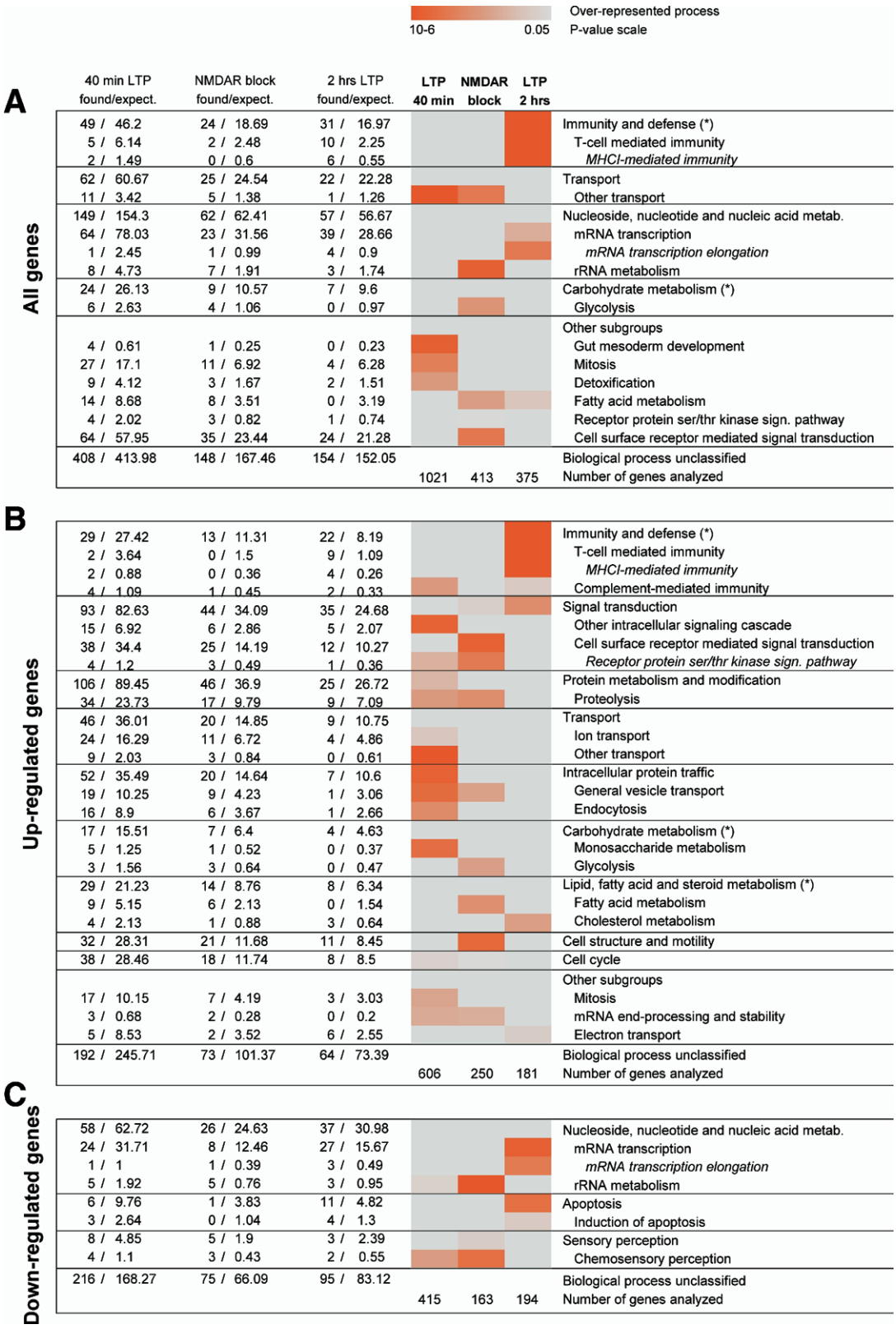

Fig. 3. Classification of HFS-regulated genes according to their biological processes. Gene sets are grouped according to gene-specific Celera-based functional annotations. Over-representation was measured in terms of statistical significance, calculated by the use of the Panther classification software. Biological processes are listed if at least one functional sub-group was over-represented ($P<0.05$). The number of identified HFS-responding genes (found) for each biological process, and the theoretically expected number of genes (expected) are listed on the left side. (A) Classification of all HFS-responding genes into biological processes. (B) Classification of up-regulated HFS-responding genes. (C) Classification of down-regulated HFS-responding genes. The number of HFS-responding genes used for each classification in A, B and C is listed under the respective rows denoting over/under-representation. (*) Asterisks marks novel biological processes, not previously described induced by HSF-LTP.

upregulated gene sets that were shown to depend most on NMDAR-activity (set as processes with 60% or more NMDAR-controlled genes) were functionally linked to cell structure and motility (32 genes), and cell surface receptor–mediated signal transduction (38 genes) (Fig. 3B). Interestingly, we also observed NMDAR-dependency for glycolysis (all three upregulated genes NMDAR dependent) and fatty acid metabolism (9 genes), for which enzymes encoded by the glycolytic genes previously have been shown associated to LTP (Tekkok and Krnjevic, 1995).

At 2 h post-HFS, the upregulated genes clearly displayed significant over-representation of immunity and defense–linked biological processes (22 genes) (Fig. 3B). In addition to the two sub-processes T-cell-mediated immunity (9 genes) and MHCI-mediated immunity (4 genes), induction of complement components was also indicated. Complement involvement during HFS-LTP was further supported by IPA, which was used to define possible protein–protein interaction networks. We identified a network of 35 HFS-regulated genes that were linked to cell signaling, immune response and small molecule biochemistry. The network uncovered a cluster of interactions between at least seven complement component members and their receptors (*c2, c3, c4a/b, seizure-related gene 6 (sez6), cfb, crry/cr1 and itgb2/cd18*). The group of complement factors was directly connected to the postsynaptic density protein-95 (PSD-95) via the complement-component receptor 1 (CR1) (network not shown, see Discussion). Among the downregulated genes at 2 h post-HFS, we observed an over-representation of genes linked to mRNA transcription (26 genes). This gene set was composed of both gene-specific transcription factors (TFs) (zinc finger TFs, homeobox TFs and KRAB-box TFs and others) as well as general TFs. Active transcriptional suppression may provide an explanation about why we observed fewer HFS-regulated genes at 2 h post-HFS, as compared with 40 min post-HFS (Fig. 2).

GSEA was performed as an alternative method to identify over-represented biological processes at 40 min and at 2 h post-HFS. Possible over-representation of gene sets was determined on the basis of gene distribution patterns within whole-transcriptome gene lists ranked according to SAM statistics (11,311 genes in each list) (see Experimental Procedures). At 2 h post-HFS, the top over-represented processes were complement-mediated immunity (total size of gene set: 19 genes), T-cell-mediated immunity (61 genes), MHCI-mediated immunity (14 genes) and MHCII-mediated immunity (9 genes) (Table 1) (Fig. 4). Thus, significant over-representation of immunity-linked processes was detected both by the GSEA (Table 1) and the Panther classification system (Fig. 3).

Among the other top-ranked gene sets, GSEA identified genes linked to the biological processes of protein metabolism and modification (translational regulation and protein phosphorylation), intracellular protein trafficking (exocytosis and general vesicle transport) and synaptic transmission (neurotransmitter release), both at 40 min and 2 h post-HFS (Table 1: GSEA). Similar categories of biological processes were also identified as over-represented by the Panther classification system at 40 min post-HFS (Fig. 3: Panther classification system), but GSEA more clearly identified the process of neuronal transmission.

GSEA also provides a tool to study the functional implication of NMDAR-activity during HFS (40 min post-HFS with CPP treatment/NMDAR blockade). In the CPP-treated samples, the SAM-ranked gene list did not show enrichment of any gene sets, except T-cell-mediated immunity and MHCII-mediated immunity (Table 1). Thus, consistent with the CPP-mediated blockade of NMDAR-activity and LTP response, none of the LTP-relevant gene sets described above were found to be over-represented (Fig. 1). The finding also demonstrate that the expression of immunity-linked gene sets are independent of NMDAR-activity, and, therefore, likely to depend on other receptor activities.

Specific synaptic activity-mediated expression of immunity-linked genes

As mentioned above, the immunity-linked gene sets that were differentially expressed at 40 min and at 2 h post-HFS were nearly non-redundant. Indeed, the two gene sets shared only *c3*, as determined by the microarray analyses. However, various antigen presenting molecules of both MHC class I and MHC class II, as well as genes linked to T-cell-mediated immunity were identified at both time points. Therefore, we investigated if such an immunity-linked gene expression could be related to tissue damage caused by chronic implantation of electrodes in the perforant pathways and DG, using an electrode control (EC) group which was chronically implanted with electrodes but did not receive HFS ($n=5$). Real-time PCR was performed for a panel of immunity-linked genes in microdissected DG. These genes included the MHC class I family *rt1-a/ce* (about 19 related genes amplified by two alternative pan-specific PCR primer-sets), and the MHC class II genes *cd74*, *rt1-Ba* and *rt1-Da*, and *c3*. There were no statistically significant changes in any of the mRNAs in the EC group, relative to contralateral control DG or to HFS-exposed DG. Thus immune genes as a whole seem not to be induced in response to electrode implantation. However, it should be noted that *c3* alone displayed borderline statistical significance when directly comparing FC-values of EC controls to that of HFS rats (*t*-test, *P*-values ranging from 0.012–0.107 as investigated by three different TaqMan® real-time RT-PCR assays) (Table 2).

Verification of HFS-response genes in awake rats

A selection of 17 HFS-regulated genes identified by the microarray analyses was independently reanalyzed by RT-PCR on paired DG samples from the awake behaving rats (Table 2). Real-time RT-PCR analyses were performed on three group of genes: (1) immunity-linked genes ($n=5$; including the *rt1-a/ce* family, *cd74, rt1-Ba, rt1-Da* and *c3*), (2) TFs ($n=3$), including the well-known LTP-induced gene *zif268*, and two other TFs with *zif268*-similar expression profiles (the *erm* and the polycomb complex protein Bmi1 (*bmi1*)), and (3) HFS-regulated genes with known or pre-

Table 1. GSEA

Gene Set	Size	FDR	
I. 2 h post-HFS			
Immunity and defense	458	>0.1	n.s.
T-cell-mediated immunity	61	0.001	
MHCII-mediated immunity	9	0.003	
MHCI-mediated immunity	14	0.006	
Complement-mediated immunity	19	0.001	
Intracellular protein traffic	596	0.007	
Exocytosis	91	0.005	
Regulated exocytosis	26	0.014	
Protein metabolism and modification	>900	>0.1	n.s.
Protein modification	636	0.006	
Protein phosphorylation	377	0.007	
Neuronal activities	310	>0.1	n.s.
Synaptic transmission	159	>0.1	n.s.
Neurotransmitter release	66	0.012	
II. 40 min post-HFS			
Neuronal activities	310	>0.1	n.s.
Synaptic transmission	159	0.106	
Neurotransmitter release	66	0.027	
Intracellular protein traffic	596	0.090	
General vesicle transport	179	0.061	
Carbohydrate metabolism	588	>0.1	n.s.
Glycogen metabolism	24	0.081	
Protein metabolism and modification	>900	>0.1	n.s.
Translational regulation	44	0.084	
Protein modification	636	0.105	
Protein phosphorylation	377	0.096	
Protein-lipid modification	12	0.104	
Nucleoside, nucleotide and nucleic acid metabolism	>900	>0.1	n.s.
Nucleoside, nucleotide and nucleic acid transport	26	0.106	
III. 40 min post-HFS/NMDAR blockade[a]			
Immunity and defense	458	>0.1	n.s.
T-cell-mediated immunity	61	0.015	
MHCII-mediated immunity	9	0.041	

Summary of GSEA results at 40 min and 2 h post-HFS (FDR<0.1). Calculated FDR-values for families and subfamilies of the 10 top-ranking gene sets are shown. Gene sets were categorized into biological processes according to the ontology terms of the Panther classification system (similar to Fig. 3). Main families are shown for all subcategories.

[a] GSEA for the CPP-treated group identified over-representation of only two biological processes. All other gene sets in the CPP-treated group displayed FDR>0.6, indicating limited HFS-mediated changes in the expression profile of other gene-sets. Abbreviations: FDR, false discovery rate; Size, number of genes in gene set; n.s., not significant (FDR>0.1).

dicted functions for neuronal signal transduction, synaptic activity or neurodevelopment ($n=9$; including *neuritin1, baiap1, prickle1, voltage-gated sodium channel (scn1b), pscd1*/*sec7, centaurin* $\alpha 1$ and *casein kinase* 1ε, *nptxr* and *pentraxin 2* (*np2*/*narp*). It should be noted that pentraxin is not present on the microarray, but we previously showed activity-mediated regulation of this gene (Wibrand et al., 2006), whose gene product can form a cell-surface protein complex with *ntpxr.*

In DG samples collected at 40 min and at 2 h post-HFS in awake rats, RT-PCR confirmed significant upregulation of 14 of the 17 selected genes (Table 2), whereas 3 genes were not verified (*pscd1*/*sec7*, *centaurin* $\alpha 1$ and *casein kinase* 1ε) (Table). Among the replicated genes, all except *rt1-a*/*ce, cd74, rt1-Ba, rt1-Da* and *c3* showed NMDAR- and LTP-dependent expression. Non-replicated genes were not re-analyzed by alternative RT-PCR assays.

Immunity-linked gene sets are not induced following LTP in anesthetized rats

In order to investigate the importance of the awake state, we compared the HFS-mediated gene expression in awake animals with that of anesthetized rats (Table 3). LTP was induced by the same stimulus parameters as those used in awake rats (Fig. 5). Notably, in anesthetized rats, HFS did not result in the expression of any immunity-linked gene products. In the acute experiments, activity-mediated expression was only observed for *zif268, prikle1, baiap2, neuritin, pentraxin 2* and *erm.*

DISCUSSION

In this study, global gene expression analysis was combined with systematic functional classification to identify genes sets that are modulated during HFS-LTP in the DG

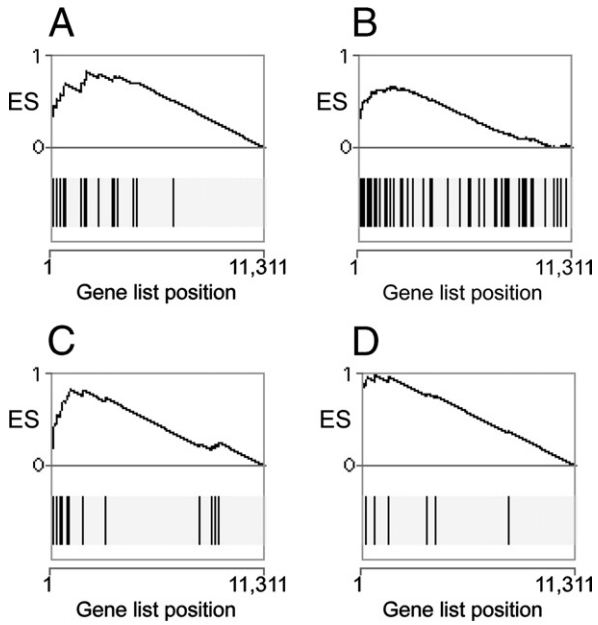

Fig. 4. Enrichment score (ES) behavior of four immunity and defense–linked gene sets at 2 h post-HFS. (A) The gene set of complement components, 19 gene members. (B) The gene set of T-cell-mediated immunity, 61 gene members. (C) The gene set of MHCI-mediated immunity, 14 gene members. (D) The gene set MHCII-mediated immunity, nine gene members. The position of individual genes within a gene set is marked by vertical lines.

of awake, freely moving rats. We show that HFS-LTP in awake animals is characterized by upregulation of genes encoding antigen-presenting molecules and T-cell linked factors.

LTP is a dynamic multi-cellular and compartmentalized process, gated by molecular detectors at multiple levels from the cell membrane, to signal transduction pathways and gene regulation. Stable forms of LTP require new RNA synthesis shortly after afferent HFS in hippocampal slices, and transcriptional inhibitors can block the LTP-response if applied within the first 2 h, but not later (Nguyen et al., 1994). This observation led to the assumption that the early phase of LTP is independent of new gene expressions, while consolidation of the late-phase required the presence of the early preceding transcriptional events.

In total we identified about 1300 genes with differential expression during HFS-LTP. Previous studies have reported modulation of a similar number of genes during LTP in hippocampal slices and simply as a function of sleep–wake cycle (Cirelli et al., 2004; Park et al., 2006a). The fact that a long-lasting form of LTP depends on early expression of many genes, should implicate linkage between such early genes and biological processes capable of differentiating or determining the functional fate of cells and subcellular compartments. In accordance with this rationale, several of our HFS-induced gene sets classified to protein metabolism and modification and cell structure and motility. While such processes may modulate long-lasting effects of HFS, we also identified processes likely to mediate rapid signaling and transport (cell surface receptor–mediated signaling, intracellular signal transduction pathways, and transport mechanisms for vesicles, proteins and other molecules).

However, the major finding in our study was the detection of increased expression of immunity-linked gene sets, in particular of antigen-presenting molecules. The brain was long believed to be immunologically privileged as its ability to respond by the classical humoral- and cellular-immune responses was absent. During the past years it has been shown that the immune system is active also in the brain, although with clear mechanistic and kinetic differences from other tissues (Dalziel et al., 1986; Fazakerley et al., 1992; Binder and Griffin, 2001; Ransohoff et al., 2003). In parallel with these findings, several reports have implicated CNS-expressed immunity genes, especially neuronal MHC class I family members, in brain development, neuronal differentiation and synaptic plasticity (Neumann et al., 1995; Corriveau et al., 1998; Huh et al., 2000; Syken and Shatz, 2003; Boulanger and Shatz, 2004; Oliveira et al., 2004). Although the functional implication of MHC class I is yet to be elucidated, MHC class I-deficient mice have impaired activity-dependent refinement of synaptic connection during development, and hippocampal slices from the adult mice display enhanced NMDAR-dependent LTP and lack long-term depression (LTD) (Huh et al., 2000). Recently, MHC class I molecules were found to colocalize with PSD-95 in the postsynaptic compartment, and their expression was important for the basal synaptic signaling-activity in cultures of hippocampal and cortical slices (Goddard et al., 2007).

To the best of our knowledge, we show for the first time that HFS-LTP can induce MHC class I mRNA expression in awake animals. However, regeneration of axons and synaptic pruning after axotomy depend on MHC class I molecules on motor neurons (Oliveira et al., 2004). Therefore, the activation of antigen-presenting molecules in hippocampal slices could reflect tissue damage. This possibility was ruled out as MHC class I expression was not enhanced in awake non-HFS control rats with chronic electrode implantations or during HFS-LTP in anesthetized rats. This indicates that MHC class I molecules are modulated during activity-dependent neuronal functions that are specific for the behaving animal.

Co-expressed with MHC class I, we observed an over-representation of MHC class II mRNAs associated with T-cell-mediated immunity. The upregulated genes include cd*74, rt1-Ba* and *rt1-Da*. Increased expression of *cd74* was previously reported in a non-invasive emotional learning task using a genetically selected rat line (Zhang et al., 2005). In theory, systemic infections and tissue damage could direct T-cell entry through the blood–brain barrier and activation of microglia (Moalem et al., 1999; Yoles et al., 2001; Kipnis et al., 2002; Ransohoff et al., 2003). However, the electrode implantation controls were negative for changes in MHC class II expression, and rats used in our study were healthy, behaved normally and showed no signs of infection (see below). More likely, neuronal-activity-mediated MHC class II expression could reflect a recently proposed autoantigen T-cell response in the DG (Kipnis et al., 2004; Ziv et al., 2006). It was shown that

Table 2. Verification of HFS-regulated genes by real-time RT-PCR

Group	Gene	40 min LTP	Significance vs.		2 hrs LTP	Significance vs.		40 min CPP	Significance vs.	
			CON	EC		CON	EC		CON	EC
Immunity-linked genes	*cd74*	2.90±0.45	*	*	3.72±0.52	*	*	3.27±0.57	*	*
	rt1-Da	3.56±0.56	*	*	4.55±1.38	ns	*	3.30±0.41	*	*
	rt1-Ba	3.63±1.28	ns	ns	3.39±0.47	*	*	4.13±0.45	*	*
	c3 (exon 27–28)	2.78±0.41	*	ns	2.76±0.47	*	*	2.65±0.29	*	*
	c3 (exon 34–35)	3.69±1.08	*	ns	3.94±1.04	ns	ns	3.20±0.46	*	*
	c3 (exon 35–36)	3.47±0.94	*	ns	2.44±0.40	*	ns	2.85±0.41	*	*
	rt1-a/ce assay 1	1.55±0.16	*	*	1.49±0.11	*	*	1.60±0.16	*	*
	rt1-a/ce assay 2	1.45±0.16	ns	ns	1.48±0.06	*	*	1.51±0.05	*	*
	np2	2.26±0.43	ns	–	1.98±0.20	*	–	0.91±0.07	ns	–
	nptxr	1.58±0.06	*	–	1.55±0.06	*	–	0.96±0.09	ns	–
TFs	*zif268*	3.75±0.94	*	–	1.26±0.05	*	–	1.15±0.05	ns	–
	erm	2.60±0.51	*	–	2.14±0.18	*	–	1.09±0.09	ns	–
	bmi1	1.70±0.05	*	–	1.69±0.07	*	–	0.95±0.05	ns	–
Signaling/development	*scnb1*	1.92±0.39	*	–	1.46±0.16	*	–	0.84±0.06	ns	–
	prickle 1	2.06±0.24	*	–	1.31±0.27	*	–	1.01±0.17	ns	–
	baiap2	1.53±0.08	*	–	1.23±0.15	*	–	0.92±0.10	ns	–
	neuritin	1.75±0.28	*	–	0.84±0.15	ns	–	1.06±0.18	ns	–
	sec7	1.28±0.16	ns	–	1.13±0.11	ns	–	0.88±0.12	ns	–
	centaurin α1	1.26±0.10	ns	–	1.24±0.04	ns	–	0.89±0.04	ns	–
	ck1e	1.25±0.15	ns	–	1.20±0.27	ns	–	0.93±0.01	ns	–

Real-time RT-PCR validation of selected HFS-induced genes at 40 min and at 2 h post-HFS, and in CPP-treated awake rats at 40 min post-HFS. Based on microarray data, three groups of HFS-induced genes were investigated: (1) immunity-linked genes, (2) TFs, and (3) genes with known or predicted functions in intracellular and synaptic signaling, and during development. The numbers represent FC (mean±S.E.M.) in mRNA expression levels between HFS-exposed and contralateral (CON) DG. Statistically significant changes in expression levels (HFS vs. CON DG) are marked by asterisks (CON-labeled columns). For the immunity-linked genes, HFS-induced FC-values were compared to the respective FC-values in non-HFS EC rats, and statistically significant changes in FC-values are marked by asterisks (EC-labeled columns). Apart from the immunity-linked genes, none of the other HFS-induced genes showed significant expression in CPP/HFS-treated rats. ns, Not significant; –, not determined.
* Statistically significant different expression (paired *t*-test, $P<0.05$).

mice deficient for mature T-cell populations perform suboptimally during Morris water mice behavioral tests, as compared with T-cell-replenished or normal mice. T-cell dependent progenitor-cell proliferation, cell survival and neurogenesis took place in the DG of rodents during spatial learning, or when exposed for several days to enriched environments (Ziv et al., 2006). The neurodevelopmental process possibly involved MHC class II expression on microglia cells (Butovsky et al., 2006; Ziv et al., 2006). These findings are of importance as they demonstrate learning-associated T cell activity in the brain of non-traumatized animals. Indeed, HFS-LTP and learning have clearly been associated with enhanced granule cell neurogenesis (Gould et al., 1999; Derrick et al., 2000; Bruel-Jungerman et al., 2006; Chun et al., 2006).

Table 3. Real-time PCR analyses of potential

Gene	40 min LTP	*P*-value	2 h LTP	*P*-value
np2	29.9±4.41	*	31.8±6.18	*
erm	3.83±0.11	*	3.16±0.34	*
baiap2	4.21±0.28	*	3.83±1.05	ns
prickle 1	4.14±0.28	*	4.14±1.24	ns
zif268	3.55±0.76	*	1.32±0.19	ns
bmi	1.05±0.06	ns	1.01±0.31	ns
nptxr	0.93±0.31	ns	1.67±0.51	ns
scnb1	1.13±0.12	ns	1.10±0.07	ns
cd74	1.09±0.05	ns	1.46±0.63	ns
rt1-Da	1.19±0.13	ns	0.83±0.17	ns
rt1-Ba	1.13±0.20	ns	0.88±0.19	ns
c3 (exon 27–28)	0.92±0.17	ns	1.06±0.35	ns
rt1-a/ce assay 1	1.15±0.04	ns	0.89±0.11	ns

HFS-regulated genes in anesthetized rats. Real-time RT-PCR of HFS-regulated genes during NMDAR-dependent LTP in urethane-anesthetized rats ($n=4$). FC values (mean±S.E.M.) in mRNA expression levels of HFS-stimulated DG versus contralateral non-stimulated DG were determined at 40 min and at 2 h post-HFS. FC values for *neuritin* are not shown. Expression of *neuritin* was previously shown by *in situ* hybridization (Wibrand et al., 2006).
* Statistically significant different expression (*t*-test, $P<0.05$).

In urethane-anesthetized rats, we evoked an enduring form of HFS-LTP that showed early elevated expression of known LTP-linked and NMDAR-dependent genes (*zif268, neuritin* and others). The anesthetized rats differed mainly from awake rats by the lack of induction of MHC class I and class II genes. Although exposure to large doses of urethane can induce cardiac, respiratory and endocrine effects, its dosage-dependent mechanism of action is similar to other anesthetics, enhancing the currents of inhibitory receptors ($GABA_A$, glycine) and inhibiting current responses of excitatory receptors (NMDA, AMPA) (Hara and Harris, 2002). Thus, in theory, HFS-induced MHC class I and II expression may be sensitive to the pattern of excitatory and inhibitory currents. Accordingly, MHC class I

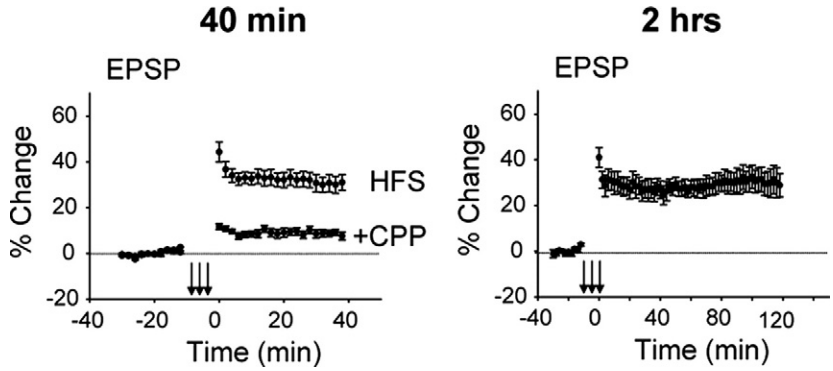

Fig. 5. Changes in fEPSP of HFS-treated anesthetized rats. Induction of NMDAR-dependent LTP in anesthetized rats. Changes in the fEPSP slopes in urethane-anesthetized rats, recorded for 40 min and for 2 h post-HFS (indicated by arrows). NMDAR-inhibited rats were recorded for 40 min only. They received CPP 2 h prior to HFS, resulting in reduced fEPSP. Values are groups means±S.E.M.

molecules are shown to be required for hippocampal LTD, and for synaptic pruning and maintenance of inhibitory synapses in motoneurons (Huh et al., 2000; Oliveira et al., 2004).

While we can link behavioral learning and activity-regulated neurodevelopment to the expression of MHC class I and class II molecules, less is known for the third group of HFS-induced immunity genes, the complement components. Seven complement-related or interacting factors were induced upon HFS (*c2, c3, c4a*/*b, sez6, cfb, crry*/*cr1* and *itgb2*/*cd18*). All complement components (C1–9) are expressed in the brain, and members like C3 are clearly implicated in neurogenesis and development (Benard et al., 2004; Lee et al., 2004). In adult brains, C3, C5 and C6 are detected in dentate granule cells, and C3 and C5 receptors are predominantly expressed in hippocampal and cortical neurons (Davoust et al., 1999; O'Barr et al., 2001; Yu et al., 2002). By a protein–protein network analysis, several of the HFS responding complement factors could be linked to PSD-95 in the post-synaptic density. Consistent with this prediction, it was recently reported that synaptic density, punctate staining of PSD-95 and LTP are reduced in the frontal cortex of *sez6* knockout mice (Kim et al., 2002; Gunnersen et al., 2006). *sez6* Is a membrane protein with five copies of short consensus repeat (SCR; complement C3b/C4b binding site) and was originally observed elevated after bursts of neuronal activities (Shimizu-Nishikawa et al., 1995).

The signaling mechanisms and biological significance behind the HFS-induced immune-responses discussed above are not yet fully understood. Our data suggest that most HFS-induced immunity-linked genes are independent of NMDAR-activity. It is therefore possible that HFS per se can cause immune-like signaling processes. Interestingly, HFS-LTP as evoked in the hippocampus of awake rats has been shown to activate several endogenous cytokines near the local site of stimulation, e.g. as demonstrated for *intelukin-6* expression in GFAP-positive astrocytes (Jankowsky et al., 2000). Also, repetitive patterns of afferent signals such as during seizures are known to enhance the expression of immunity-related genes in neuronal tissues (for review: (Jankowsky and Patterson, 2001)). Thus, it is clear that various forms of neuronal activities can regulate immunity-like signaling processes.

In our study we classified most of the HFS-induced immunity-linked genes as NMDAR-independent, both on the basis of a GSEA that was performed specifically on the HFS/CPP sample set (HFS given during NMDAR blockade) (Table 1, at 40 min post-HFS), and by real-time RT-PCR analyses with direct comparison of gene-specific FC-values from different sample sets (HFS FC vs. HFS/CPP FC) (Table 2). However, it should be noted that different tissue pooling procedures for the HFS/CPP- and HFS-sample sets, respectively (see Experimental Procedures, Tissue collection), could affect gene expression variances and thereby the statistical considerations regarding NMDAR-dependency (as deduced by the real-time PCR analyses: Table 2). However, GSEA detected over-representation of T cell– and MHC class II–linked genes in the HFS/CPP sample set, suggesting that such genes are independent of LTP and can be induced by afferent neuronal signaling per se.

CONCLUSION

In summary, this study has revealed early synaptic activity–mediated transcriptional responses in the DG of awake freely moving rats. Biological processes that require new gene expression for long-lasting activity-dependent changes are involved in the control of signal transduction, protein modification, transport/trafficking, and cell structure and motility. Interestingly, our results also linked HFS-LTP in the awake brain to the induction of immunity-like processes. Our findings suggest that the cross-talk between immunity-linked genes and neuronal activity in the normal brain might be more elaborate than previously understood.

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sykdomsmekanismer" and Mental Health program), Helse Vest RHF, Dr. Einar Martens Fund, and the EU RTN grant 504231. Author contributions: B.H. developed the study concept and design (together with V.M.S., C.R.B. and H.R.), performed all molecular biology experiments (mRNA extraction, RT-PCR and microarray), analyzed and interpreted data (RT-PCR, microarray, Panther classification system, and GSEA), and wrote the manuscript. H.R. performed and analyzed all electrophysiology experiments on the awake animals. A.-K.S. implemented the GSEA tools and assisted in the microarray-data analyzes. G.D. performed the electrophysiology on anesthetized rats. C.R.B. supervised the work on electrophysiology and assisted in the interpretation of data and writing of the manuscript. V.M.S. was responsible for microarray study design and supervision of data analyses, including interpretation of the results and writing of the manuscript. All co-authors have read and approved the final version of the manuscript.

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