# Glucocorticoid control of gene transcription in neural tissue

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# Chapter 2

# Acute activation of hippocampal glucocorticoid receptors results in different waves of gene expression throughout time

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## ABSTRACT

Several aspects of hippocampal cell function are influenced by adrenal-secreted glucocorticoids in a delayed, genomic fashion. Previously, we used Serial Analysis of Gene Expression to identify glucocorticoid receptor (GR)-induced transcriptional changes in the hippocampus at a fixed time point. However, since changes in mRNA levels are transient and most likely precede the effects on hippocampal cell function, the aim of the current study was to assess the transcriptional changes in a broader time window by generating a time curve of GR-mediated gene expression changes.

Therefore, we used rat hippocampal slices obtained from adrenalectomized rats, substituted in vivo with low corticosterone pellets, predominantly occupying the hippocampal mineralocorticoid receptors. To activate GR, slices were treated in vitro with a high (100 nM) dose of corticosterone and gene expression was profiled 1, 3 and 5 hours after GR-activation. Using Affymetrix GeneChips, a striking pattern with different waves of gene expression was observed, shifting from exclusively downregulated genes 1 hour after GR-activation to both up and down regulated genes 3 hours after GR-activation. After 5 hours, the response was almost back to baseline. Additionally, real-time qPCR was used for validation of a selection of responsive genes including genes involved in neurotransmission and synaptic plasticity such as the CRH receptor 1, monoamine oxidase A, LIM Kinase 1 and calmodulin 2. This allowed confirmation of GR-responsiveness of 15 out of 18 selected genes.

In conclusion, direct activation of GR in hippocampal slices results in transient changes in gene expression. The pattern in which gene expression was modulated suggests that the fast genomic effects of glucocorticoids may be realized via transrepression, preceding a later wave of transactivation. Furthermore, we identified a number of interesting candidate genes which may underlie the glucocorticoid-mediated effects on hippocampal cell function.

## INTRODUCTION

Glucocorticoids, which are secreted by the adrenals in response to stress, control hippocampal cellular function (1). Acute activation of the hippocampal glucocorticoid receptor (GR) affects neuronal excitability and energy metabolism within hours, whereas chronic activation results in atrophy of dendrites of pyramidal neurons (2-4). The delayed onset of these changes and the fact that the GR belongs to the superfamily of ligand regulated nuclear receptors (5,6) suggests a genomic mode of action for these glucocorticoid-dependent effects.

We previously used Serial Analysis of Gene Expression to assess the GR-induced transcriptional changes in the hippocampus against an occupied mineralocorticoid receptor background and identified over 100 GR-responsive genes 3 hours after a single corticosterone injection in rats (7). However, with respect to neuronal excitability, the precise mechanisms underlying the GR-induced changes still remain unknown, most likely due to the fact that changes in mRNA levels do not necessarily have to coincide with the effects on cell function (8,9). For instance, the GR-dependent increase in 5HT1A-receptor mediated hyperpolarization has so far not been directly linked to an increase in 5HT1A-receptor mRNAs. This could suggest that 1) transient changes in receptor mRNA levels precede the effects on neuroexcitability and/or 2) other, receptor function modifying proteins, which are transcriptionally regulated by GRs, are responsible for these effects. Therefore, the aim of the current study was to elucidate GR-regulated genes throughout a defined time interval by large scale gene expression profiling, thereby identifying candidate genes possibly involved in the GR-mediated effects on hippocampal cell function.

The hippocampal slice preparation is an ideal model system for monitoring the transcriptional responses since the hippocampus has a laminated structure and therefore the architecture of the hippocampal network is largely maintained after slicing. As a result, the histology strongly resembles that of in situ preparations (10). Furthermore, the influence of projections from other brain areas to the hippocampus is removed and the GRs can be directly activated without the interference of peripheral effects. Additionally, the slice preparation can be maintained for up to 12 hours (11) and neuronal activity can be measured throughout this time period. In these slices the effects of GR-activation on cellular electrophysiology have been well described, allowing changes in gene expression to be directly correlated with the changes observed in the electrophysiological recordings.

Activation of GR by a 20 minute exposure to a high concentration of corticosterone results in increased calcium currents and serotonin mediated hyperpolarization with a delay of 1 to 4 hours, through a mechanism requiring DNA-binding of GR-homodimers (12,13). Additionally, nuclear accumulation studies (14,15) in primary neurons and hippocampal slices indicate maximal nuclear uptake 30 to 60 minutes after ligand activation.

Moreover, studies assessing glucocorticoid-responsive genes in vivo (16-18) have shown that hippocampal transcriptome modulation takes place between 1 and 6 hours after the initial GR-activation. Therefore, a time window of 1 to 5 hours was chosen to profile GR-mediated changes in gene expression.

We used hippocampal slices obtained from adrenalectomized animals replaced with low corticosterone pellets, occupying the high affinity mineralocorticoid receptors. In order to activate the GRs, slices were treated with a brief *in vitro* corticosterone pulse and gene expression was profiled 1, 3 and 5 hours afterwards.

## MATERIALS & METHODS

### Animals

All experiments were carried out with the approval of the Animal Care Committee of the Faculty of Medicine, Leiden, The Netherlands (DEC nr. 02095).

Young male Wistar rats (<200 g) obtained from Charles Rivers Laboratories Inc. (Germany) were used for the experiments and housed under a 12 h light : 12 h dark cycle (lights on at 7:00h.) with food and drinking water (and after adrenalectomy also a 0.9% NaCl drinking solution) available *ad libitum*.

In order to obtain constant basal plasma levels of corticosterone, 15 rats (5 per time point) were adrenalectomized under isoflurane anesthesia and substituted with a subcutaneous pellet releasing a low concentration of corticosterone (20 mg corticosterone / 80 mg cholesterol). After 3 days, the rats were decapitated and from each rat both hippocampi were immediately isolated and subjected to slicing.

### Hippocampal slice preparation and treatment

Identical conditions were used as for previously performed electrophysiological recordings in order to facilitate comparison. Per time point, 5 rats (e.g.10 hippocampi) were used. Briefly, after isolation the hippocampi were sliced with the McIlwain Tissue Chopper™, generating approximately twenty 400 µm slices for each hippocampus. Per hippocampus, slices were collected in separate containers containing carbogenated (95% $O_2$, 5% $CO_2$) artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.5 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.25 mM $NaH_2PO_4$ and 10 mM glucose).

Throughout the experiment all slices originating from the same hippocampus were always kept separately from the others. After an equilibration period of 1 hour in ACSF at room temperature all the slices obtained from the left or right hippocampus of each animal (n=5 per time point) were used either for GR-activation or as controls, resulting in a paired setup allowing within-animal comparisons between treatment and control groups.

GRs were activated by *in vitro* treatment with a high concentration (100 nM) 20 minute pulse of corticosterone applied to ACSF which was pre-heated to 32°C. The control slices were treated with a 20 minute 0.009% ethanol (vehicle) pulse in pre-heated ACSF.

Twenty minutes after corticosterone or vehicle treatment, slices were placed back into normal ACSF at room temperature. One, three or five hours after the initial addition of corticosterone, all the slices originating from the same hippocampus were pooled again and total RNA was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.



#### RNA preparation and Affymetrix array hybridization

After isolation, total RNA was purified using the QIAGEN RNEasy® Mini Kit RNA Cleanup procedure (QIAGEN Inc., Valencia, CA, USA). RNA quality was assessed with the LabChip® RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Per RNA sample, 10 µg was used as input into the Affymetrix procedure as recommended by Affymetrix (www.affymetrix.com). Briefly, total RNA was converted to double-stranded cDNA after which the mRNA portion was amplified and biotin-labeled using the ENZO BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA, USA). The amplified RNA was purified with the QIAGEN RNEasy® Mini Kit RNA Cleanup procedure and the quality was checked with the LabChip® RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies). The RNA samples were hybridized to Rat Genome U34A GeneChips (Affymetrix) at the Leiden Genome Technology Center (LGTC®), Leiden University, The Netherlands.

Per time point, 10 Affymetrix arrays were used, 5 for the corticosterone-treated slices and 5 for the control slices. Each array included ~8000 probe sets, representing ~7000 full-length or annotated sequences and ~1000 EST clusters.

#### Affymetrix data analysis

Microarray Analysis Suite 5.0 (MAS5.0; Affymetrix) was used to estimate signal intensities plus signal reliabilities and to normalize the array signals by total intensity normalization (19).

Transcripts that generated present and/or marginal calls throughout all 10 arrays per time point were selected for further analysis, removing all transcripts that generated one or more absent calls.

Significance Analysis of Microarrays (SAM) (20) was used to identify responsive genes. Briefly, when using microarrays, a large number of hypotheses is tested in a single experiment (8000 genes), resulting in many false positives (multiple testing problem). SAM is a non-parametric test which allows the user to control the False Discovery Rate (FDR), i.e. the relative number of false positives generated. For all three time points SAM was applied to the paired data sets derived from left and right hippocampi and the lowest FDR was chosen to assess the most significant responsive genes.

Gene Ontology Biological Process classifications were obtained using the NetAffx Analysis Center (21) (www.affymetrix.com), allowing genes involved in similar biological processes to be grouped together.

Furthermore, the obtained data set was compared with our previously generated SAGE data set. To allow cross-platform comparisons, the Expression Analysis Systematic Explorer (22) was used to couple Affymetrix probes to UniGene clusters. The NCBI SAGE-map ftp-site was used to couple these UniGene clusters to SAGE tags (Figure 2). Both the Expression Analysis Systematic Explorer (EASE) and the NCBI SAGEmap used UniGene build 139.

#### Corticosterone

In order to measure corticosterone levels, trunk blood was immediately collected in EDTA-coated tubes after decapitation. Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C after which the plasma was collected and stored at -20°C. Corticosterone levels were measured with the murine Corticosterone RIA Kit (ICN Biomedicals™, Costa Mesa, CA, USA).

#### Real-time quantitative PCR

A selection of responsive genes was validated in the same experimental RNA samples that were used for GeneChip analysis, using real-time qPCR on a DNA Engine Opticon® 2 Real-Time PCR Detection System (MJ Research, Inc., Waltham, Massachusetts, USA). Prior to cDNA-synthesis, all RNA samples were DNase-treated with DNase I (Invitrogen Life Technologies), according to the manufacturer's protocol. Synthesis of cDNA was performed in a total volume of 20 µl, using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). Per experimental sample, 50 ng of RNA was put into the cDNA-synthesis reaction (23). Standard curves were generated by performing cDNA-synthesis reactions on 5, 50, 100 and 500 ng of input RNA. As a control for genomic contamination, RT- samples were used. The PCR was performed in a total volume of 25 µl, consisting of 12.5 µl 2×PCR MasterMix with SYBR® Green I (qPCR™ Core Kit for SYBR® Green I, EUROGENTEC, Seraing, Belgium), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer (= 5 pmol), 6.5 µl water and per primer pair either 5 µl cDNA-sample, RT- sample or water (no template control).

The following heating protocol was used: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C + 1 minute at 60°C (for both annealing and extension). Afterwards, the temperature was gradually increased to 95°C in order to make dissociation curves.

Dissociation curves were examined for each primer pair and controlled for specificity of the reaction and genomic contamination by checking the RT- and no template control samples. Then, for each primer pair the standard curve was plotted and the PCR efficiency was estimated. All used primer pairs displayed reaction efficiencies between

80 and 100%. Target gene Ct-values ranged from 18 to 32 whereas RT- and no template control samples showed no products after 40 cycli.

Since the PCR efficiencies of the target and normalization genes were not equal, the standard curve method was used to quantify the expression differences (24). Expression levels of the target genes were normalized with the expression levels of beta-actin since 1) this gene is widely used as an internal control for real-time qPCR, 2) this gene was not found to be responsive to corticosterone in the current study and the previously performed SAGE study and 3) so far, no reports have been available in published literature indicating that beta-actin is a corticosterone-responsive gene.

In general, PCR-primers were designed in the regions where the probe sets used on the Affymetrix GeneChips were derived from.

The non-parametric Wilcoxon Signed Ranks Test was used to assess significant differential expression of GR-responsive genes.



**Primer sequences:**

The following primers were designed for validation of observed corticosterone-responsive genes:

| Gene Title | Probe set ID | Forward primer (5' - 3') | Reverse primer (5' - 3') | amplicon (bp) |
|---|---|---|---|---|
| Mineralocorticoid receptor | M36074_at | CCCGCGTGGGAAGTGTT | TGGAAGCGGGAAGAAGCA | 64 |
| Shaw-related potassium channel 2 | X62839mRNA_s_at | AGCTTCAAGAAATGCCCACAA | CTCTGTAATTTGCAGCAAAACCA | 73 |
| Atypical PKC-specific binding protein | AB005549_at | GGCCGGTCTATCCAGTCCTT | TTTCCTTGTCCCACTCTGTGC | 75 |
| Casein kinase II, alpha 1 polypeptide | L15618_at | GGACGTCCACCCTCTCCTTAA | GGTTCAGACACGGTGCTTCTG | 78 |
| Cytoplasmatic dynein intermediate chain | X66845_at | AGCCAGATATGATTGGGTGCA | AAACACCACATCTCAAGTCTTTGG | 75 |
| Metallothionein | M11794cds#2_f_at | ATGTGCCCAGGGCTGTGT | CGTCACTTCAGGCACAGCA | 64 |
| CRH receptor 1 | U53486mRNA_s_at | GCCCTGCAGCCTCAATTTC | GCTTGTGGCCCAGAAGGTC | 73 |
| EST196031 Rn.4183 | rc_AA892228_at | CAGGACATTAAGCAGCCTACTTACAG | GCATGCAGAGGCCACCTTAC | 77 |
| LIMK-1 | D31873_at | GAGAGAGGTCCAGTCCCATGTG | GGCTTTGATCAGGAAATGAGATG | 77 |
| Calcineurin subunit A alpha | D90035_s_at | TGACCACTTCCTGTTCACTTTTTTT | GCAAGAACATCCAACTGCTGAG | 80 |
| Calmodulin 2 | M17069_at | TTTAGGAACCGTCGGCATGT | GTACACGCTGTCGACTGTCCA | 74 |
| Apolipoprotein E | X04979_at | GCTGGGTGCAGACGCTTT | GTACCGTCAGTTCCTGTGTGACTT | 74 |
| Monoamine oxidase A | D00688_s_at | CGTCCAAGGTGTACAGAGGAAAAT | AAGGGTAGTGTGTATCACATGGAGC | 95 |

| Gene Title | Probe set ID | Forward primer (5' - 3') | Reverse primer (5' - 3') | ampli-con (bp) |
|---|---|---|---|---|
| EST190379 Rn.24136 | rc_AA800882_g_at | ACAAACAGCTTGACACTTGAC | AACCTCAGAGGGCCCAAGA | 69 |
| Beta-chain clathrin associated protein | M77246_at | GTGTGGGAGGCAGGTGGTAT | CCAGGAATCGGCACCG | 70 |
| Enolase 2, gamma | X07729exon#5_s_at | TTGCCTGAACACCGGAACA | CTATGGCGGGTCGGGAC | 77 |
| Sodium channel, voltage-gated, type 2 | M22254_at | TGGTGTCACTGGGTCCCTTAG | ATACAGCGGCATCAGCAAGA | 69 |
| BARS50 | AF067795_at | TTGGCATGAACCCCTTGTTC | GCACTGCAGACACACCTCAGA | 66 |
| Beta-actine | V01217_at | TGACCGAGCGTGGCTACA | CAGCTTCTCTTTAATGTCACGCA | 70 |

## RESULTS

### Hormone concentrations and RNA quality

Measurement of corticosterone concentrations demonstrated that all animals had basal levels of corticosterone (3-7 μg/dl), predominantly occupying the hippocampal MRs and partially occupying the hippocampal GRs, presumably leaving room for extensive GR-activation.

Since the effects of the slicing procedure and ACSF-storage on slice mRNA quality were unknown, we used the LabChip® RNA 6000 Nano Assay (Agilent Technologies) to assess integrity and amount of RNA. No evidence of RNA degradation was observed since the ratios of ribosomal 28S/18S intensities exceeded 1.5 for every sample.

### GR-responsive genes: dynamic pattern

To ensure the reliability of the data set, all transcripts that generated one or more absent calls were removed from the data set, resulting in reliable detection of 3335 genes 1 hour after GR-activation, 3289 genes 3 hours after GR-activation and 3184 genes 5 hours after GR-activation. Using these stringent selection criteria, the resulting detection efficiencies were 37% for both the 1 and 3 hours time point and 36% for the 5 hours time point, resulting in a detection overlap of 2867 genes in every time point.

Significance Analysis of Microarrays (SAM) revealed moderate responses 1 and 3 hours after GR-activation with FDRs of 43% and 37% respectively, and a weak response 5 hours after GR-activation (FDR=53%). The magnitude of gene expression responses varied between 10 and 60%.

Strikingly, a clear pattern in gene regulation was observed, with waves of gene expression occurring at different time points, shifting from exclusive downregulation of 81 genes 1 hour after GR-activation to 161 both up or downregulated genes 3 hours after GR-activation (Figure 1, Table 1 and Table S1). After 5 hours the response was almost

**Table 1.** GR-responsive genes for all three time points. FC = fold change.

| ***Probe Set ID*** | ***FC 1 hour*** | ***FC 3 hours*** | ***FC 5 hours*** | ***Gene Title*** | ***GO Biological Process Description*** |
|---|---|---|---|---|---|
| **Signal Transduction** | | | | | |
| AF030358_g_at | | 0.76887 | | chemokine (C-X3-C motif) ligand 1 | signal transduction /// chemotaxis |
| J05592_g_at | 0.79214 | | | protein phosphatase 1, regulatory (inhibitor) subunit 1A | signal transduction /// glycogen metabolism |
| L23219_at | | | 1.18458 | guanine nucleotide binding protein, gamma 7 | signal transduction |
| D26180_at | 0.71834 | | | protein kinase N1 | signal transduction |
| U90610_g_at | | 1.18337 | | chemokine (C-X-C motif) receptor 4 | G-protein coupled receptor protein signaling pathway |
| rc_AA891916_g_at | | 0.84722 | | membrane interacting protein of RGS16 | G-protein coupled receptor protein signaling pathway |
| U53486mRNA_s_at | | 1.42996 | | corticotropin releasing hormone receptor 1 | G-protein coupled receptor protein signaling pathway |
| M12672_at | | 0.75180 | | guanine nucleotide binding protein, alpha inhibiting 2 | G-protein coupled receptor protein signaling pathway |
| rc_AA859520_at | | 0.77783 | | Heterotrimeric guanine nucleotide-binding protein alpha q subunit | G-protein coupled receptor protein signaling pathway |
| M17069_at | | 0.88985 | | calmodulin 2 | G-protein coupled receptor protein signaling pathway |
| J05677mRNA_s_at | | 1.22537 | | natriuretic peptide receptor 1 | intracellular signaling cascade /// cGMP biosynthesis |
| AF026505_at | | 0.64875 | | Arg/Abl-interacting protein ArgBP2 | intracellular signaling cascade |
| S74351_s_at | | 1.60266 | | dual specificity phosphatase 1 | intracellular signaling cascade |
| U02553cds_s_at | | 1.56780 | | dual specificity phosphatase 1 | intracellular signaling cascade |
| D31873_at | | 1.26354 | | LIM motif-containing protein kinase 1 | intracellular signaling cascade |
| D78588_at | | 0.88000 | | diacylglycerol kinase zeta | intracellular signaling cascade |
| rc_AA875253_at | | 0.69295 | | ADP-ribosylation factor-like 1 | small GTPase mediated signal transduction |

| ***Probe Set ID*** | ***FC 1 hour*** | ***FC 3 hours*** | ***FC 5 hours*** | ***Gene Title*** | ***GO Biological Process Description*** |
|---|---|---|---|---|---|
| L12380_at | | 0.82114 | | ADP-ribosylation factor 1 | small GTPase mediated signal transduction |
| S49491_s_at | | 1.34028 | | preproenkephalin, related sequence | neuropeptide signaling pathway /// immune response |
| M63901_g_at | | 0.82117 | | secretory granule neuroendocrine protein 1 | neuropeptide signaling pathway /// protein folding |
| L07925_g_at | | 1.19847 | | ral guanine nucleotide dissociation stimulator | neuropeptide signaling pathway /// transport |
| M11794cds#2_f_at | | 1.26349 | | Metallothionein | nitric oxide mediated signal transduction |
| rc_AI102562_at | | 1.21683 | | Metallothionein | nitric oxide mediated signal transduction |
| U70050_at | | 1.30780 | | jagged 2 | Notch signaling pathway /// cell fate determination |
| M12112mRNA#3_s_at | 0.82293 | | | angiotensinogen | cell surface receptor linked signal transduction |
| AB017140_g_at | 0.83592 | | | homer homolog 1 (Drosophila) | metabotropic glutamate receptor signaling pathway |
| M59814_at | | 0.85764 | | Eph receptor B1 | transmembrane receptor protein tyrosine kinase signaling pathway |
| rc_AA893743_g_at | | 0.75135 | | Protein kinase inhibitor, alpha | negative regulation of protein kinase activity |
| L15618_at | 0.78114 | | | casein kinase II, alpha 1 polypeptide | Wnt receptor signaling pathway /// regulation of cell growth |
| rc_AA957896_s_at | 0.90426 | | | mitogen activated protein kinase kinase 2 | protein amino acid phosphorylation |
| **Regulation of transcription** | | | | | |
| M36074_at | 0.88568 | | | mineralocorticoid receptor (MR) | regulation of transcription, DNA-dependent |
| rc_AI176710_at | | | 1.28570 | nuclear receptor subfamily 4, group A, member 3 | regulation of transcription, DNA-dependent |
| D13309_s_at | | 0.79705 | | nuclease sensitive element binding protein 1 | regulation of transcription, DNA-dependent |
| rc_AI227715_at | | 1.28016 | | retinoblastoma-like 2 | regulation of transcription, DNA-dependent |
| U09228_at | | 0.67615 | | transcription factor 4 | regulation of transcription, DNA-dependent |
| M34253_g_at | | 0.71646 | | interferon regulatory factor 1 | regulation of transcription, DNA-dependent |

| *Probe Set ID* | *FC 1 hour* | *FC 3 hours* | *FC 5 hours* | *Gene Title* | *GO Biological Process Description* |
|---|---|---|---|---|---|
| AF023087_s_at | 0.85455 | 1.18488 | | nerve growth factor induced factor A | regulation of transcription, DNA-dependent |
| rc_AI014091_at | | 0.71600 | | Cbp/p300-interacting transactivator | regulation of transcription, DNA-dependent |
| rc_AI008639_at | 0.77806 | | | MAD homolog 4 (Drosophila) | regulation of transcription, DNA-dependent |
| rc_AI104524_s_at | | | 1.32296 | heterogeneous nuclear ribonucleoprotein A/B | regulation of transcription, DNA-dependent |
| D37951UTR#1_at | 0.89545 | | | human immunodeficiency virus type I enhancer binding protein 2 | regulation of transcription, DNA-dependent |
| X84210complete_seq_s_at | | 1.40662 | | nuclear factor I/A | regulation of transcription, DNA-dependent |
| U91679_at | | 1.29938 | | FEV (ETS oncogene family) | positive regulation of transcription |
| rc_AI171268_at | 0.74219 | | | Inhibitor of DNA binding 3 | negative regulation of transcription from Pol II promoter |
| U30381_at | | 0.74329 | | zinc finger protein 148 | negative regulation of transcription from Pol II promoter |
| AB000491_at | | 0.76938 | | proteasomal ATPase (SUG1) | negative regulation of transcription |
| AF030091UTR#1_g_at | 0.80233 | | | cyclin L1 (ania-6a) | RNA processing |
| **Regulation of translation** | | | | | |
| X62671cds#1_s_at | 0.88530 | 1.16128 | | hybrid protein (ubiquitin-like protein/rps30) | protein biosynthesis |
| X66370_at | 0.85376 | | | ribosomal protein S9 | protein biosynthesis |
| rc_AA892123_at | | | 1.08962 | ribosomal protein L36 | protein biosynthesis |
| rc_AI008641_at | 0.84630 | | | ribosomal protein L22 | protein biosynthesis /// ribosome biogenesis |
| X51707cds_s_at | 0.90951 | | | ribosomal protein S19 | protein biosynthesis /// ribosome biogenesis |
| rc_AA892895_r_at | | 1.25344 | | EST196698 Rn.3391 | protein biosynthesis /// ribosome biogenesis |
| rc_AI178750_at | | 1.06600 | | eukaryotic translation elongation factor 2 | protein biosynthesis /// translational elongation |
| X58465mRNA_g_at | | 1.08129 | | ribosomal protein S5 | protein biosynthesis /// ribosome biogenesis |

| ***Probe Set ID*** | ***FC 1 hour*** | ***FC 3 hours*** | ***FC 5 hours*** | ***Gene Title*** | ***GO Biological Process Description*** |
|---|---|---|---|---|---|
| X15096cds_s_at | | 1.15029 | | acidic ribosomal phosphoprotein P0 | protein biosynthesis /// translational elongation |
| rc_AI012604_at | | 0.86462 | | eukaryotic translation initiation factor 5 | protein biosynthesis /// translational initiation |
| **Energy metabolism** | | | | | |
| X56133_at | | 0.87735 | | F1-ATPase alpha subunit | ATP biosynthesis |
| X54510_at | 0.87307 | | | ATP synthase coupling factor 6 | ATP synthesis coupled proton transport |
| U07181_g_at | 0.82188 | | | lactate dehydrogenase B | glycolysis |
| X07729exon#5_s_at | 0.66408 | | | enolase 2, gamma | glycolysis |
| D49653_s_at | | 1.20581 | | leptin | energy reserve metabolism /// signal transduction |
| U96130_at | 0.84485 | | | glycogenin 1 | glycogen biosynthesis /// carbohydrate biosynthesis |
| | | | | | |
| **Oxidative stress metabolism** | | | | | |
| U86635_at | 0.86520 | | | glutathione S-transferase, mu 5 | metabolism |
| U86635_g_at | 0.91496 | | | glutathione S-transferase, mu 5 | metabolism |
| X02904cds_s_at | | 1.19923 | | glutathione-S-transferase, pi 1 /// glutathione S-transferase, pi 2 | xenobiotic metabolism |
| X07365_s_at | | 0.78354 | | glutathione peroxidase 1 | response to oxidative stress |
| L24896_s_at | 0.88171 | | | glutathione peroxidase 4 | response to oxidative stress |
| **Cholesterol metabolism** | | | | | |
| U36992_at | 0.83433 | | | cytochrome P450, family 7, subfamily b, polypeptide 1 | cholesterol metabolism /// electron transport |
| U53706_at | | 1.15474 | | mevalonate (diphospho) decarboxylase | cholesterol biosynthesis /// isoprenoid biosynthesis |
| **Miscellaneous metabolism** | | | | | |
| rc_AA997886_s_at | | 1.20486 | | cytochrome P450, family 2, subfamily d, polypeptide 22 | electron transport /// drug metabolism |

| Probe Set ID | FC 1 hour | FC 3 hours | FC 5 hours | Gene Title | GO Biological Process Description |
|---|---|---|---|---|---|
| E01524cds_s_at | | 0.84675 | | P450 (cytochrome) oxidoreductase | electron transport |
| X02918_at | | | 1.08883 | prolyl 4-hydroxylase, beta polypeptide | electron transport |
| D37920_at | 0.85716 | | | squalene epoxidase | electron transport /// aromatic compound metabolism |
| D88364_at | | 1.26212 | | phosphatidylinositol glycan, class L | GPI anchor biosynthesis |
| U72497_at | | | 1.24330 | fatty acid amide hydrolase | fatty acid metabolism |
| rc_AA894296_at | | 0.85359 | | phosphoglucomutase 1 | carbohydrate metabolism /// calcium ion homeostasis |
| AF034218_at | | 1.37420 | | hyaluronidase 2 | carbohydrate metabolism /// hyaluronan catabolism |
| rc_AI228110_s_at | | 0.82375 | | UDP-glucuronosyltransferase 8 | glycosphingolipid biosynthesis /// metabolism |
| rc_AI102868_g_at | 0.88055 | | | phosphoserine aminotransferase 1 | L-serine biosynthesis |
| AF067795_at | | 1.27671 | | BFA-dependent ADP-ribosylation substrate BARS50 | L-serine biosynthesis |
| rc_AA800745_at | 0.86907 | | | aminolevulinate, delta-, dehydratase | porphyrin biosynthesis /// heme biosynthesis |
| X55298_at | 0.86873 | | | ribophorin II | N-linked glycosylation /// N-linked glycosylation via asparagine |
| M57664_at | | 1.17637 | | creatine kinase, brain | phosphocreatine metabolism /// brain development |
| U95001UTR#1_s_at | 0.73694 | 0.79797 | | developmentally-regulated cardiac factor (DRCF-5) | phosphoinositide metabolism |
| J03572_i_at | | 1.22560 | | alkaline phosphatase, tissue-nonspecific | metabolism |
| U81186_g_at | 0.79409 | | | smooth muscle-specific 17 beta-hydroxysteroid dehydrogenase | steroid biosynthesis /// metabolism |
| AF093773_s_at | | 1.08950 | | cytosolic malate dehydrogenase (Mdh) | tricarboxylic acid cycle |
| AB000717exons#1-8_s_at | 0.82931 | | | methionine adenosyltransferase II | S-adenosylmethionine biosynthesis |
| **Cytoskeleton** | | | | | |
| rc_AA892373_at | | 0.87678 | | syndecan binding protein | actin cytoskeleton organization and biogenesis |

| ***Probe Set ID*** | ***FC 1 hour*** | ***FC 3 hours*** | ***FC 5 hours*** | ***Gene Title*** | ***GO Biological Process Description*** |
|---|---|---|---|---|---|
| rc_AA817887_at | | 0.87757 | | profilin 1 | regulation of actin polymerization/depolymerization |
| M60666_s_at | 0.84978 | | | tropomyosin 1, alpha | muscle contraction /// muscle development |
| **Ubiquitin pathway** | | | | | |
| AB011369_s_at | 0.90069 | | | protein kinase C-binding protein Beta15 | ubiquitin cycle /// protein ubiquitination |
| U11760_at | 0.86057 | | | valosin-containing protein | ubiquitin cycle /// transport |
| D10756_at | 0.89500 | | | proteasome (prosome, macropain) subunit, alpha type 5 | ubiquitin-dependent protein catabolism |
| D17296_at | 0.80517 | | | ubiquitin C | ubiquitin-dependent protein catabolism |
| E03358cds_at | | 0.82688 | | proteasome (prosome, macropain) subunit, alpha type 2 | ubiquitin-dependent protein catabolism |
| U50842_at | | 0.79584 | | developmentally down-regulated gene 4A | ubiquitin cycle /// protein modification |
| **Transport miscellaneous** | | | | | |
| AB003515_at | | 0.79733 | | GABA(A) receptor-associated protein like 2 | transport /// intracellular protein transport |
| D12770_s_at | | 1.14520 | | solute carrier family 25 member 4 | transport /// mitochondrial transport |
| X66494_at | 0.91233 | | | choline transporter | transport /// neurotransmitter transport |
| M94918mRNA_f_at | | 1.09914 | | hemoglobin beta chain complex | transport /// oxygen transport |
| X56325mRNA_s_at | | 1.18004 | | hemoglobin alpha, adult chain 1 | transport /// oxygen transport |
| rc_AA892776_at | 0.85158 | | | solute carrier family 25 (mitochondrial carrier;), member 3 | transport /// phosphate transport |
| AF031642_at | | 1.43529 | | solute carrier family 14 (urea transporter), member 2 | transport /// response to water deprivation /// urea transport |
| X55572_at | | 0.90357 | | apolipoprotein D | transport /// tissue regeneration |
| M77246_at | 0.88904 | 0.81598 | 1.18781 | beta-chain clathrin associated protein complex AP-2 | intracellular protein transport /// protein complex assembly |
| rc_AA799784_at | | 0.66435 | | RAB6, member RAS oncogene family | Golgi vesicle transport |

| Probe Set ID | FC 1 hour | FC 3 hours | FC 5 hours | Gene Title | GO Biological Process Description |
|---|---|---|---|---|---|
| **Ion transport** | | | | | |
| X62839mRNA_s_at | 0.79486 | | | potassium voltage gated channel, Shaw-related, member 2 | potassium ion transport /// transport /// ion transport |
| X62840mRNA_s_at | 0.81548 | | | potassium voltage gated channel, Shaw-related, member 1 | potassium ion transport /// transport /// ion transport |
| X12589cds_s_at | | | 1.23072 | potassium voltage-gated channel, shaker-related, member 1 | --- |
| M22254_at | 0.84478 | | | sodium channel, voltage-gated, type 2, alpha 1 polypeptide | sodium ion transport /// generation of action potential |
| rc_AI169005_at | | 0.81965 | | chloride channel, nucleotide-sensitive, 1A | chloride transport /// regulation of cell volume |
| rc_AA955388_s_at | | 0.74288 | | ATPase, Ca++ transporting, plasma membrane 2 | calcium ion transport /// cation transport |
| U78977_at | | | 1.09489 | ATPase, class II, type 9A | cation transport |
| M58758_g_at | | | 1.18012 | ATPase, H+ transporting, lysosomal V0 subunit a isoform 1 | proton transport |
| **Cell adhesion** | | | | | |
| AB005549_at | 0.75895 | | | atypical PKC-specific binding protein | cell-cell adhesion /// cytokinesis /// cell cycle |
| S61868_at | | 1.18188 | | syndecan 4 | cell adhesion /// cell-cell signaling |
| AB008538_at | | 0.82303 | | activated leukocyte cell adhesion molecule | cell adhesion /// signal transduction |
| L14851_at | | | 1.33732 | neurexin 3 | cell adhesion |
| **Synaptic transmission** | | | | | |
| D00688_s_at | 0.91274 | 0.85613 | | monoamine oxidase A | neurotransmitter catabolism /// electron transport |
| X97374exon_g_at | | 1.41825 | | prepronociceptin | synaptic transmission /// neuropeptide signaling pathway |
| rc_AI102031_g_at | | 1.23401 | | bridging integrator 1 | synaptic transmission /// endocytosis /// cell differentiation |

| Probe Set ID | FC 1 hour | FC 3 hours | FC 5 hours | Gene Title | GO Biological Process Description |
|---|---|---|---|---|---|
| X04979_at | | 1.09126 | | apolipoprotein E | synaptic transmission, cholinergic |
| U14398_g_at | | 0.69717 | | synaptotagmin 4 | neurotransmitter secretion /// transport |
| **Cell cycle/proliferation** | | | | | |
| X63594cds_at | | 1.34022 | | nuclear factor of kappa light chain gene enhancer | regulation of cell proliferation |
| L26268_g_at | 0.87512 | | | B-cell translocation gene 1, anti-proliferative | negative regulation of cell proliferation /// cell proliferation |
| rc_AI177366_at | 0.84442 | | | Integrin beta 1 | regulation of cell cycle /// G1/S transition of mitotic cell cycle |
| D90035_s_at | | 0.82695 | | calcineurin subunit A alpha | G1/S transition of mitotic cell cycle /// calcium ion transport |
| **Immune response** | | | | | |
| AF029240_g_at | | 1.19300 | | RT1 class Ib, locus S3 | immune response |
| X16481_r_at | | 1.21903 | | parathymosin | immune response |
| rc_AI169802_at | | 0.89727 | | ferritin, heavy polypeptide 1 | immune response /// iron ion transport /// iron ion homeostasis |
| rc_AA859645_at | | 0.80486 | | attractin | inflammatory response /// heterophilic cell adhesion |
| **Regulation of apoptosis** | | | | | |
| L00981mRNA#2_at | 0.75489 | | | tumor necrosis factor superfamily, member 2 | apoptosis /// induction of apoptosis |
| U90261UTR#1_g_at | 0.84423 | | | SH3-domain kinase binding protein 1 | regulation of apoptosis |
| **Proteolyse** | | | | | |
| U62897_at | | | 1.23519 | carboxypeptidase D | proteolysis and peptidolysis |
| D13907_g_at | | 0.67629 | | peptidase (mitochondrial processing) beta | proteolysis and peptidolysis |
| D29683_at | | 1.24531 | | endothelin converting enzyme 1 | proteolysis and peptidolysis |

| Probe Set ID | FC 1 hour | FC 3 hours | FC 5 hours | Gene Title | GO Biological Process Description |
|---|---|---|---|---|---|
| **Protein folding** | | | | | |
| rc_AI170613_g_at | 0.87572 | | | heat shock 10 kDa protein 1 | protein folding /// response to unfolded protein |
| rc_AI170613_at | | | 1.25684 | heat shock 10 kDa protein 1 | protein folding /// response to unfolded protein |
| rc_AA859806_at | | 0.82780 | | Cysteine string protein | protein folding /// synaptic transmission |
| **Miscellaneous** | | | | | |
| rc_AI232374_at | 0.80048 | | | H1 histone family, member 0 | nucleosome assembly |
| D42137exon_s_at | 0.89193 | | | annexin A5 | blood coagulation |
| S49400_at | 0.91053 | | | protein tyrosine phosphatase, non-receptor type 5 | protein amino acid dephosphorylation |
| rc_AI180424_at | 0.79537 | | | 14-3-3 protein beta-subtype | protein targeting /// protein-mitochondrial targeting |
| U08290_at | | 1.22286 | | neuronatin alpha | development |
| rc_AI175539_at | | 1.58511 | | parvalbumin | muscle development |
| rc_AA866276_at | | 0.80044 | | myeloid-associated differentiation marker | myeloid blood cell differentiation |
| rc_AI113289_s_at | | 0.86044 | | protein tyrosine phosphatase, non-receptor type 1 | protein amino acid dephosphorylation |
| J03886_at | | 1.24701 | | myosin light chain kinase 2, skeletal muscle | protein amino acid phosphorylation |
| U32681_at | | 1.30676 | | deleted in malignant brain tumors 1 | response to wounding /// cell differentiation /// tissue regeneration |
| M91597_s_at | 0.92536 | | | expressed in non-metastatic cells 2 | GTP biosynthesis /// UTP biosynthesis /// CTP biosynthesis |
| rc_AA892832_at | | 0.79504 | | ELOVL family member 5 | fatty acid elongation |

From left to right are listed the GeneChip Probe Set IDs, the fold changes in the 1, 3 and 5 hours time point, the gene titles and the Gene Ontology Biological Process descriptions. Fold changes below 1 indicate downregulated genes; fold changes above 1 indicate upregulated genes. Please note that genes were placed into categories based on their first GO-component. Since this is an arbitrary division, many genes can be placed into multiple functional categories.

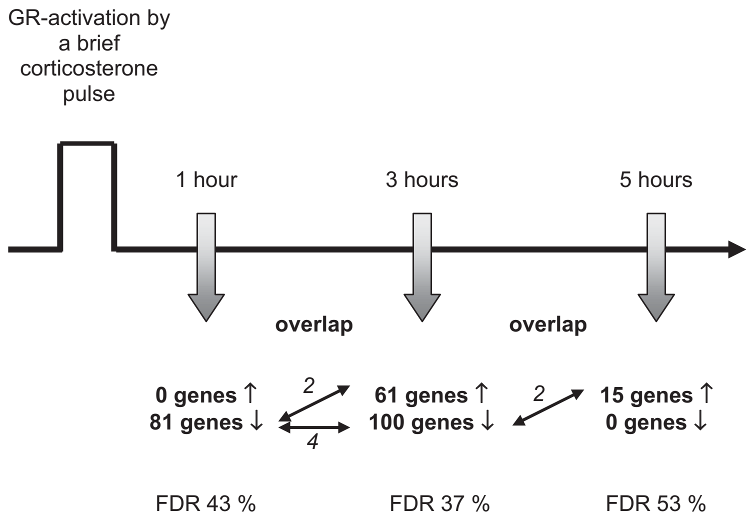


**Figure 1.** Different waves of gene expression after glucocorticoid receptor (GR)-activation according to a pattern of 81 exclusively downregulated genes 1 hour after GR-activation, 161 both up or downregulated genes 3 hours after GR-activation and 15 up regulated genes 5 hours after GR-activation. The arrows indicate overlap between the time points. The user-defined False Discovery Rate (FDR) from Significance Analysis of Microarrays (SAM) analysis is indicated.

back to baseline, with only 15 genes being upregulated. Furthermore, there was very little overlap between the time points (Table 2). Except for four genes at the 3 hours time point, all the other responsive genes at each time point generated reliable signals on the Affymetrix GeneChips at the other time points, thereby eliminating the possibility that these genes were not picked up as responsive genes in the other time points due to lack of signal on the Affymetrix GeneChips.

In order to determine whether changing the user-defined FDR would influence the observed pattern, the FDRs were increased to the highest possible values. This did not alter the observed pattern of 1) exclusive downregulation of genes 1 hour after GR-activation, 2) both up- and downregulated genes 3 hours after GR-activation and 3) the small overlap between the time points. However, increasing the FDR for the 5 hours time point resulted in the addition of downregulated genes to the list of responsive genes.

### GR-responsive genes: functional classification

In order to assign functions to the responsive transcripts, Gene Ontology classifications were obtained using the NetAffx Analysis Center. Genes were grouped together into a large variety of functional classes. Activation of GR clearly affected the expression of genes involved in different types of cellular metabolism, transcription, translation, different aspects of signal transduction, protein/vesicle trafficking, ion transport, cell adhesion, the cytoskeleton and synaptic transmission. Hence, in general, the different waves of gene expression at different time points contained similar functional gene groups.

**Table 2.** Overlapping genes between the three time points. FC = fold change

| Probe set ID | Gene title | FC 1 hour | FC 3 hours | FC 5 hours |
|---|---|---|---|---|
| AF023087_s_at | nerve growth factor induced factor A | 0.85455 | 1.18488 | |
| X62671cds#1_s_at | hybrid protein (ubiquitin-like protein/rps30) | 0.88530 | 1.16128 | |
| U95001UTR#1_s_at | developmentally-regulated cardiac factor (DRCF-5) | 0.73694 | 0.79797 | |
| rc_AA892851_g_at | EST196654 Rn.3616 | 0.74547 | 0.74772 | |
| D00688_s_at | monoamine oxidase A | 0.91274 | 0.85613 | |
| M77246_at | beta-chain clathrin associated protein complex AP-2 | 0.88904 | 0.81598 | 1.18781 |
| rc_AA800882_at | EST190379 Rn.24136 | | 0.62222 | 1.36565 |

Genes that overlap between the three time points. From left to right are listed the GeneChip Probe Set IDs, the gene titles and the fold changes in the 1, 3 and 5 hours time point.



Some of the interesting responsive genes were classified as signal transduction components and included corticotropin releasing hormone (CRH) receptor 1, mitogen activated protein kinase kinase 2, LIMK1 and calmodulin 2. Additionally, genes from other functional groups such as ania-6 (RNA processing), monoamine oxidase A (neurotransmitter catabolism), two potassium ion channels and prepronociceptin (synaptic transmission) were also identified as hippocampal GR-responsive genes.

#### Overlap with the SAGE data set

We previously used adrenalectomy in combination with low corticosterone pellets (MR-occupation) and high corticosterone injections in vivo (GR occupation) to assess corticosteroid-regulated gene expression in rat hippocampus 3 hours after a high corticosterone injection, using Serial Analysis of Gene Expression (7). Briefly, in SAGE, gene expression profiles are established by sequencing and counting 10 base pairs long SAGE tags which are derived from a defined position within the 3' untranslated regions of the transcripts (25). In order to compare the current data set with the SAGE data set, we used both the Expression Analysis Systematic Explorer (EASE) and the NCBI SAGEmap ftp-site as described in the materials and methods section (Figure 2). Since 1) not every SAGE tag and Affymetrix probe set could be annotated with a UniGene cluster and 2) not every annotated SAGE tag was present on the Affymetrix GeneChip, only seventy of the 203 responsive SAGE tags could be annotated as Affymetrix probe sets. However, still 4 (6%) of the responsive genes found with SAGE were also regulated 1 hour after GR-activation in this study, whereas 7 genes (10%) were regulated 3 hours after GR-activation (Table 3). The majority of these genes showed the same direction of regulation (8 genes out of 11) providing good positive controls for the current data set. Two of these genes (malate dehydrogenase and F1-ATPase alpha subunit) play a role in cellular metabolism whereas another, apolipoprotein E, functions as a lipid transporter and is involved in synaptic

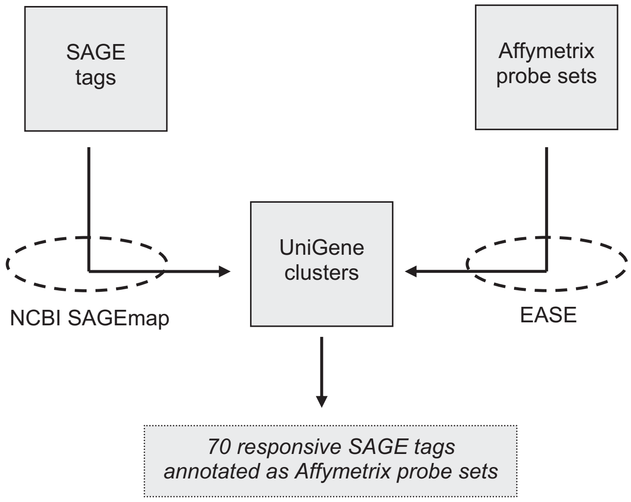


**Figure 2.** Cross-platform comparison between Serial Analysis of Gene Expression (SAGE) tags and Affymetrix probe sets. Affymetrix probe sets were annotated with UniGene clusters using Expression Analysis Systematic Explorer (EASE) whereas SAGE tags were annotated using the NCBI SAGEmap. Both annotation tools provide information on the UniGene builds used in the annotation process and therefore allow comparison of data sets via the same builds (build 139).

**Table 3.** Overlap with SAGE. FC = fold change

| SAGE tag | Unigenes | Probe set ID | Gene title | FC 1 hour | SAGE change |
|---|---|---|---|---|---|
| GAACATATTT | Rn.11273 | X66845_at | dynein, cytoplasmic, intermediate chain 1 | 0.79120 | GR down |
| TGGTGGAATG | Rn.4231 | L15618_at | casein kinase II, alpha 1 polypeptide | 0.78114 | MR down |
| TATAATCTGT | Rn.29774 | M63485_at | matrin 3 | 0.80711 | GR up |
| ACTTAGTTGT | Rn.5790 | X54510_at | ATP synthase coupling factor 6 | 0.87307 | MR up |
| **SAGE tag** | **Unigenes** | **Probe set ID** | **Gene title** | **FC 3 hours** | **SAGE change** |
| TTTGTGACTG | Rn.3946 | AF067795_at | BFA-dependent ADP-ribosylation substrate BARS50 | 1.27671 | MR up |
| ACGTAAAAAA | Rn.13492 | AF093773_s_at | cytosolic malate dehydrogenase (Mdh) | 1.08950 | MR up |
| GAGAGCTAAC | Rn.5785 | U08290_at | neuronatin alpha | 1.22286 | MR up |
| ACCAGCCAGG | Rn.32351 | X04979_at | apolipoprotein E | 1.09126 | GR up |
| GTGGGTGTGT | Rn.3391 | rc_AA892895_r_at | EST196698 Rn.3391 | 1.25344 | GR down |
| AATAAAAGTT | Rn.40255 | X56133_at | F1-ATPase alpha subunit | 0.87735 | GR down |
| TTGCTGTTGA | Rn.5968 | M17069_at | calmodulin 2 | 0.88985 | MR down |

Genes that overlap between the current and the Serial Analysis of Gene Expression (SAGE) study. The upper panel displays the genes that overlap between the 1 hour time point and the SAGE study; the lower panel displays the genes that overlap between the 3 hours time point and the SAGE study. In the SAGE change column genes are responsive to the mineralocorticoid receptor or glucocorticoid receptor and the direction of regulation is designated as up or down.

transmission. Furthermore, calmodulin 2, which was downregulated in both data sets, plays a role in calcium channel functioning. Interestingly, 6 of the GR-regulated genes in the current data set were MR-regulated in the SAGE data set.

### Validation of a subset of responsive genes

Since SAM revealed moderate gene expression responses with relatively high FDRs throughout the three time points, the GR-dependent change in expression of a subset of functionally interesting genes was validated with real-time qPCR.

Beta-actin, which is commonly used as normalization control and which was not responsive in the current and the SAGE data set, was used to normalize target gene expression values. In Table 4, the genes selected for validation and their validation results are listed. Furthermore, for some of the validated genes the expression pattern is illustrated in Figure 3.



**Table 4.** Real time qPCR validation results. FC = fold change.

| Gene title | Experiment | FC affymetrix | FC qPCR | Wilcoxon | Validation |
|---|---|---|---|---|---|
| Mineralocorticoid receptor | slice 1 hour | 0.9 | 0.5 | P<0.05 | true positive |
| Shaw-related potassium channel 2 | slice 1 hour | 0.8 | 0.8 | P<0.05 | true positive |
| Atypical PKC-specific binding protein | slice 1 hour | 0.8 | 0.8 | P<0.05 | true positive |
| Casein kinase II, alpha 1 polypeptide | slice 1 hour/SAGE | 0.8 | 0.7 | P<0.05 | true positive |
| Cytoplasmatic dynein intermediate chain | slice 1 hour/SAGE | 0.8 | 0.9 | P<0.05 | true positive |
| Metallothionein | slice 3 hours | 1.3 | 1.4 | P<0.05 | true positive |
| CRH receptor 1 | slice 3 hours | 1.4 | 1.5 | P<0.05 | true positive |
| EST196031 Rn.4183 | slice 3 hours | 0.7 | 0.7 | P<0.05 | true positive |
| LIMK-1 | slice 3 hours | 1.3 | 1.4 | P<0.05 | true positive |
| Calcineurin subunit A alpha | slice 3 hours | 0.8 | 0.7 | P<0.05 | true positive |
| Calmodulin-2 | slice 3 hours/SAGE | 0.9 | 0.7 | P<0.05 | true positive |
| Apolipoprotein E | slice 3 hours/SAGE | 1.1 | 1.1 | P<0.05 | true positive |
| Monoamine oxidase A | slice 1/3 hours | 0.9/0.9 | 0.9/0.9 | P<0.05 | true positive |
| EST190379 Rn.24136 | slice 3/5 hours | 0.6/1.4 | 0.9/1.4 | P<0.05 | true positive |
| Beta-chain associated protein complex AP-2 | slice 1/3/5 hours | 0.9/0.8/1.2 | 0.7/0.7/1.5 | P<0.05 | true positive |
| Enolase 2, gamma | slice 1 hour | 0.7 | X | X | false positive |
| Sodium channel, voltage-gated, type 2 | slice 1 hour | 0.8 | X | X | false positive |
| BARS50 | slice 3 hours/SAGE | 1.3 | X | X | false positive |

Validation of 18 genes. The fold changes obtained with Affymetrix GeneChip analysis and real-time qPCR are displayed as well as the result of statistical testing (Wilcoxon Signed Ranks Test). Genes were classified as true positives if the expression changes observed with Affymetrix GeneChips could be replicated with real-time qPCR.

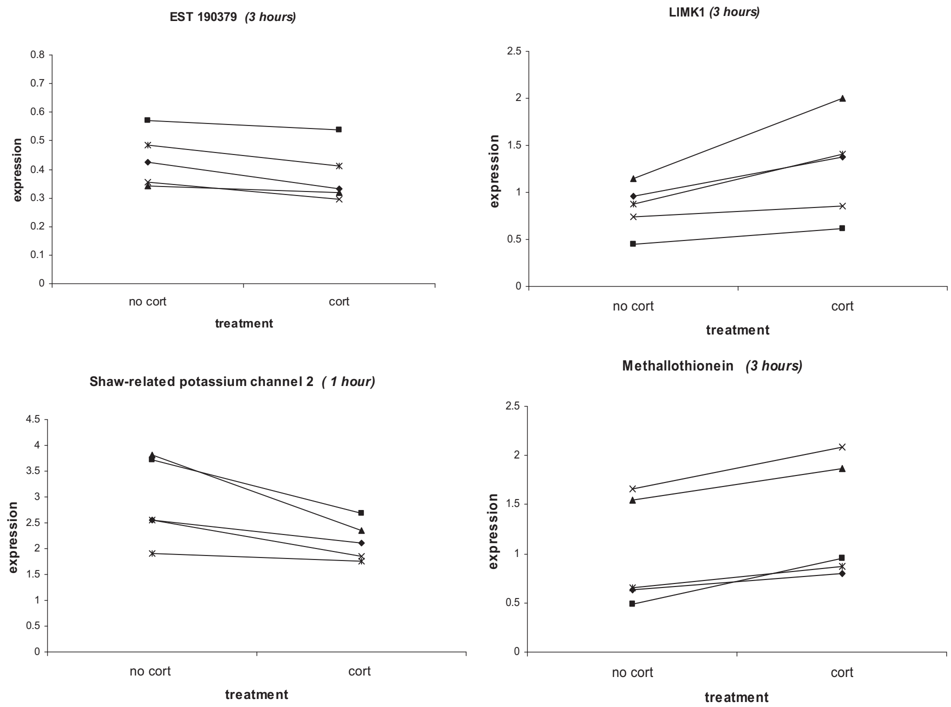


**Figure 3.** Real-time quantitative PCR results illustrated for four genes. Normalized gene expression levels obtained from corticosterone treated and vehicle treated hippocampal slices are plotted and for each separate animal connected with a line (paired setup).

Genes that were selected for validation belonged to interesting functional classes regulated throughout the time points. Additionally, a few genes that showed expression responses in multiple time points and some genes which overlapped with the SAGE data set were included for validation. In total 18 genes were selected and the GR-induced expression response of 15 genes could be confirmed ($P<0.05$, Wilcoxon Signed Ranks Test)

The fold-changes obtained by real-time qPCR were in good concordance with the fold-changes obtained by GeneChip analysis. Interestingly, despite the relatively high FDRs that were obtained by analyzing the GeneChip data with SAM, we could confirm GR-responsiveness of 15 out of 18 genes by real-time qPCR.

## DISCUSSION

The aim of this study was to elucidate potential molecular mechanisms underlying the action of corticosterone on the hippocampus by generating a time curve of predominantly GR-responsive genes. We identified over 200 likely GR-responsive genes and found a clear pattern of moderate changes in gene expression in all three time points.

Validation of a subset of genes by real-time qPCR resulted in a remarkable consistency of fold-changes since the changes observed in qPCR validation nicely followed the changes observed in GeneChip analysis. Furthermore, 83% of the selected genes could be confirmed as GR-responsive, indicating an overall FDR of 17%. Since the overall GeneChip FDR estimated by SAM was considerably higher, less false positives were found in the selected subset than expected. Although the subset was not selected randomly, these results could indicate that SAM to a certain extent overestimated the FDR, suggesting a higher number of false negatives.

In order to assess how specific the profiled genes are for neurons, we checked all the genes that were validated by real-time qPCR and found that all 12 genes with a known localisation in brain are expressed in principal neurons of the hippocampus, indicating that the majority of the profiled genes are specific for neurons (The Allan Brain Atlas; www.brainatlas.com).

The moderate changes in gene expression that were found in the current study are in agreement with previous reports on corticosteroid-responsive genes in the hippocampus, showing fold changes of less than 2 for the majority of genes in different hippocampal subregions by *in situ* hybridization (7,18,26,27). Although the observed differences in expression are remarkably consistent with what is reported in literature, it can be expected that by using whole hippocampus the expression differences are most likely diluted by hippocampal subregions and non-neuronal cell types which are not or less responsive to activated GRs (28,29). However, by using a paired study design we were able to enhance the detection of these subtle expression differences, increasing statistical power by observing very consistent changes of gene expression within the individual animals.

The current data set contained a number of genes that were already known to be responsive to corticosteroids in the hippocampus, such as metallothionein 1A (30), glutathione peroxidase (31,32) and the mineralocorticoid receptor (33). Therefore, these genes constitute good positive controls. Furthermore, we performed cross-platform comparisons with the previously generated SAGE data set and found 8 overlapping genes which showed similar changes in direction of expression. These 8 genes constituted approximately 10% of the responsive SAGE tags that could be detected on the GeneChips used in the current study. The current experiment differed from the SAGE experiment in the fact that in the current experiment prior to corticosterone treatment the hippocampus was excised from its surroundings. This resulted in loss of input from extra-hippocampal regions. Since these inputs may also be steroid responsive, the current data set does not reflect the total GR influence on hippocampal gene expression. Furthermore, since the corticosterone treatment was restricted to a 20 minute 100nM pulse whereas in the SAGE experiment GRs were activated with an (1 mg/kg bodyweight) in vivo injection, this most likely will lead to differences in hippocampal corticosterone exposure as well as additional peripheral effects of the injections. Finally, by comparing different gene expression platforms, a lot of genes are omitted from the actual comparison due to annotation differences. We believe that these experimental differences and the restrictions of cross-platform comparison can explain the relatively small overlap of 8 genes as well as the change in direction of expression regulation of 3 genes.

In conclusion, the reliability of the current data set was demonstrated by the validation results, the observed fold-changes, the presence of positive controls and the overlap with the SAGE study.

By generating a time curve of GR-responsive genes a striking dynamical pattern was revealed. We observed different waves of gene expression throughout the time frame, with only downregulated genes 1 hour after GR-activation. Since no protein synthesis inhibitors were used in the current study, the question whether these identified genes are primary GR-responsive genes or responsive to downstream GR-induced changes cannot be answered. Activated GRs can modulate gene expression either via transrepression, by interacting as a monomer with other transcription factors thereby inhibiting transcription, or via transactivation, by binding as a homodimer to glucocorticoid responsive elements (GREs) on the DNA, thereby inhibiting or stimulating transcription. The finding that all the genes which were responsive 1 hour after GR-activation were downregulated may indicate that at this time point these genes are primary GR-responsive genes and regulated via transrepression. Hence, this would mean that in general transrepression precedes and/or is faster than transactivation. In agreement with this, Almon and co-workers (34) found a similar dynamical pattern of transcription regulation by corticosteroids in liver, in which the majority of rapidly modulated genes (45 out of 50) were downregulated after injection of corticosteroids, whereas a robust, delayed wave of upregulated genes followed 2 hours later. Interestingly, some of the responsive genes which were regulated in more than one time point changed their direction of expression throughout the time points. A similar observation was found by Fujikawa and co-workers, showing a biphasic regulation of hippocampal mRNAs coding for growth hormone receptor, GR and MR throughout time during acute stress (35). This biphasic regulation could be the effect of downstream regulatory mechanisms, indicating that these genes change from primary responsive genes to downstream responsive genes throughout time. Therefore, the 3 and 5 hours time points would each contain at least 2 downstream responsive genes since both time points contain two genes which have changed their direction of expression in comparison with the previous time point (Figure 1). Additionally, one gene, metallothionein, for which the presence of a GRE has been reported (36) was also found to be regulated after 3 hours, indicating that at this time point most likely both primary and downstream responsive genes are present.

The different waves of gene expression at the different time points contained similar functional groups. Responsive genes were grouped into functional classes such as signal transduction, regulation of transcription, regulation of translation, metabolism, cell adhesion and synaptic transmission. Classification of GR-responsive genes into these functional groups is in agreement with previous studies showing similar functional categories in hippocampus and other tissues (7,34,37).

In terms of hippocampal neuronal excitability, both calcium channels and 5HT1A receptors comprise interesting genes. Glucocorticoid hormones have a clear effect on excitability of neurons in the CA1 area of the hippocampus, especially on voltage-gated calcium currents and 5HT1A receptor-mediated hyperpolarization (8). However, in the current study, the 5HT1A receptor was undetectable on the GeneChips used, which is consistent with several of our previous GeneChip studies in hippocampus (38-40). This is most likely due to expression levels below the detection limit of GeneChips. Therefore expression of the 5HT1A receptor could not be assessed using GeneChips. Furthermore, no GR-mediated transcriptional effects on calcium channels were found. Since stress-induced increases in calcium currents in vivo have been shown to be preceded by transient increases in calcium channel subunit mRNAs in the hippocampal CA1 region (41), the absence of calcium channels in the current study may indicate that 1) putative GR-mediated effects on calcium channel transcription in the CA1 subregions are undetectable due to the dilution of the response by other hippocampal subregions and non-neuronal cell types, or 2) calcium channel subunit expression is regulated at a different time point than currently profiled. Furthermore, it should be noted that by using GeneChips or microarrays in general, the possibility exists that false negative data is generated due to the large amount of hypotheses tested in a single experiment. This example underpins that a link between the corticosterone-induced changes in hippocampal neuronal excitability in the CA1 subregion and changes in gene expression using expression profiling is complex, even when the experimental protocols are matched as closely as possible within the current technical constraints. The same line of reasoning may hold true for other genes that are known to be responsive to corticosteroids such as brain-derived neurotrophic factor (BDNF) which was detectable on the GeneChips but not found to be regulated in the SAM analysis. BDNF is known to be downregulated in vivo 3 hours after a single corticosterone injection in both dentate gyrus and CA1 regions and in this case the differences between in vivo treatment and in vitro hippocampal slice treatment as previously described with respect to the comparison with the SAGE study should also be taken into account.

Our current expression profile corresponds with the well-documented effects of glucocorticoids on brain metabolism. For instance, it has been reported that corticosterone inhibits glucose utilization by neurons and astrocytes (42). In the current data set, lactate dehydrogenase B, which is involved in glucose utilization, is downregulated, possibly underlying (part of) this effect. Other downregulated genes playing a role in energy metabolism included the F1-ATPase alpha subunit and ATP synthase coupling factor 6 which are both involved in ATP synthesis. Since it is known that corticosterone accelerates ATP-loss after a metabolic insult (43) these two genes constitute interesting putative candidate genes for the glucocorticoid effects on ATP-synthesis. Beside the effects on energy metabolism, glucocorticoids also play a role in oxidative stress metabolism (44) and in the present study we observe a number of regulated genes which may be involved.

Strikingly, a number of genes playing a role in hippocampal neurotransmission and synaptic plasticity were found to be responsive to corticosterone. The functional implication of regulation of four of these genes is described in more detail below.

Firstly, monoamine oxidase A, which is involved in the catabolism of serotonin, dopamine and noradrenaline (45), was found to be downregulated at two time points, i.e. 1 and 3 hours after GR-activation. All three neurotransmitters have been associated with certain aspects of hippocampal functioning. The hippocampus is known to play a role in learning and memory formation and dopamine and noradrenaline have been associated with mnemonic processing and memory retrieval respectively (46,47). The hippocampus also plays an important role in anxiety and the dorsal hippocampal serotonergic system has been associated with anxiogenic responses (48). Hence, downregulation of monoamine oxidase A could modify these aspects of hippocampal function by increasing the availability of these neurotransmitters. To our knowledge, such a direct effect of corticosteroids on hippocampal neurotransmitter availability has not been observed before.

Secondly, CRH receptor 1 was found to be upregulated by corticosterone 3 hours after GR-activation. CRH is a neuropeptide that is released after stress by the hypothalamus and modulates many neuroendocrine and behavioral responses (49-51). In the hippocampus, the majority of the pyrimidal cells in CA1 and CA3 express the CRH receptor 1 (52). Since the GR is predominantly expressed in CA1, CA2 and DG, it may be that the effect of activated GRs on CRH receptor1 takes place in the CA1 area. Interestingly, administration of exogenous CRH into the hippocampus affects long-term potentiation (LTP) (53). Additionally, CRH-producing neurons have been found in interneurons in the pyramidal layers of the hippocampus (54) and there is evidence that stress-induced CRH of hippocampal origin activates hippocampal pyramidal cells via the CRH receptor 1 (55). Therefore, transient upregulation of the hippocampal CRH receptor 1 by stress-released corticosterone constitutes a very interesting phenomenon which could lead to an enhancement of the CRH-induced effects on hippocampal functioning.

Thirdly, calmodulin 2 was found to be downregulated both in the current study and the SAGE study. The calmodulins constitute a unique gene family in which all three different members (calmodulin 1, 2 and 3) code for exactly the same protein (56). Calmodulin has been hypothesized to function as a central regulator of synaptic plasticity (57) and, similar to CRH, to play a role in LTP, implying that corticosterone may influence this phenomenon via the regulation of expression of multiple genes.

Finally, LIMK1 was found to be upregulated 3 hours after GR-activation. In vitro it has been shown that this gene, via phosphorylation of ADF/cofilin, can enhance polymerization of actin filaments (58,59). Furthermore, studies with LIMK1 knock-out mice have shown that this gene is involved in actin cytoskeleton-dependent regulation of dendritic spine morphology and synaptic function (60). Hence, the transient upregulation of this gene by corticosterone could be a means to temporarily modify synaptic function. In this respect it is interesting to note that calcineurin A was downregulated 3 hours after GR-activation. This gene is known to be enriched in the dendritic spines of hippocampal neurons and to be involved in destabilizing both actin filaments and dendritic spine structure (61,62). Furthermore, Tojima and co-workers (59) demonstrated that calcineurin can inhibit protein expression of LIMK1 in neuronal NG108-15 cells, which would suggest that the corticosterone-induced downregulation of this gene could increase LIMK1-protein expression.

In conclusion, by profiling gene expression after GR-activation in hippocampal slices throughout a time window of 1 to 5 hours, we have found a very interesting dynamic pattern of gene regulation, with several subsequent waves of gene expression and only downregulated genes 1 hour after GR-activation. Furthermore, we have identified interesting candidate genes that may underlie the glucocorticoid-mediated effects on hippocampal cell function. However, due to 1) the resolution of the time frame profiled, 2) the technical difficulties with the detection of low abundant genes such as the 5HT1A receptor, 3) the use of whole hippocampi and 4) the transient changes observed in gene expression, establishing a correlation between changes in mRNA levels and changes in hippocampal function still remains complex. On the other hand, in the current study new unexpected genes such as LIMK1, calmodulin 2, monoamine oxidase A and CRH receptor 1 were found to be GR-responsive, thereby raising the question what the functional consequences are of GR-regulation of these genes in the hippocampus. Hence, the current study can be used to formulate new hypotheses about the effects of corticosterone on hippocampal cell function. Therefore, future studies need to be focused on the functional consequences of GR-regulation of presently identified interesting hippocampal genes.

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## SUPPLEMENTARY MATERIAL

**Table S1.** From left to right are listed the GeneChip Probe Set IDs, the fold changes in the 1, 3 and 5 hours time point, the gene titles and the Gene Ontology Biological Process descriptions. Fold changes below 1 indicate downregulated genes; fold changes above 1 indicate upregulated genes.

| *Probe Set ID* | *FC 1 hour* | *FC 3 hours* | *FC 5 hours* | *Gene Title* | *GO Biological Process Description* |
|---|---|---|---|---|---|
| AB016489_s_at | 0.88106 | | | jumping translocation breakpoint | --- |
| AF034582_g_at | 0.87415 | | | SEC31-like 1 (S. cerevisiae) | --- |
| C06598_at | 0.92833 | | | similar to binding protein | --- |
| L26292_g_at | 0.70512 | | | Kruppel-like factor 4 (gut) | --- |
| M31322_g_at | 0.86328 | | | sperm membrane protein (YWK-II) | --- |
| M63485_at | 0.80711 | | | matrin 3 | --- |
| M81639_at | 0.78973 | | | stannin | --- |
| rc_AA799726_at | 0.82732 | | | Similar to mKIAA1737 protein | --- |
| rc_AA800549_at | 0.80255 | | | Transcribed locus | --- |
| rc_AA800693_at | 0.81134 | | | Transcribed locus, moderately similar to XP_488563.1 | --- |
| rc_AA800693_g_at | 0.85774 | | | Transcribed locus, moderately similar to XP_488563.1 | --- |
| rc_AA874928_g_at | 0.86164 | | | sorting nexin 4 (predicted) | --- |
| rc_AA891314_at | 0.89191 | | | poly(rC) binding protein 4 (predicted) | --- |
| rc_AA946313_s_at | 0.80394 | | | secreted acidic cysteine rich glycoprotein | --- |
| rc_AA964320_at | 0.95849 | | | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 | --- |
| rc_AI009132_at | 0.82736 | | | similar to chromosome 13 open reading frame 12 (predicted) | --- |
| rc_AI010371_at | 0.84454 | | | Transcribed locus | --- |
| rc_AI112237_at | 0.77218 | | | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 | --- |
| rc_AI232012_at | 0.87777 | | | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8 | --- |
| rc_H33149_at | 0.84493 | | | similar to RIKEN cDNA 1810047C23 | --- |
| X52733cds_s_at | 0.88393 | | | ribosomal protein L27a (predicted) | --- |
| X66845_at | 0.79120 | | | dynein, cytoplasmic, intermediate chain 1 | --- |
| X76985_at | 0.85592 | | | latexin | --- |
| rc_AA892851_g_at | 0.74547 | 0.74772 | | EST196654 Rn.3616 | --- |
| AF069525_at | | 0.84834 | | ankyrin 3, epithelial isoform g | --- |
| AF095741_g_at | | 1.12291 | | Mg87 protein | --- |
| D13966_at | | 1.31674 | | insulin receptor-related receptor | --- |

| *Probe Set ID* | *FC 1 hour* | *FC 3 hours* | *FC 5 hours* | *Gene Title* | *GO Biological Process Description* |
|---|---|---|---|---|---|
| L40364_f_at | | 1.44847 | | MHC class I RT1.O type 149 processed pseudogene | --- |
| M13100cds#6_f_at | | 0.75022 | | similar to ORF2 consensus sequence encoding endonuclease | --- |
| rc_AA799369_at | | 0.83915 | | similar to RIKEN cDNA 1190002L16 (predicted) | --- |
| rc_AA799473_at | | 0.71189 | | Similar to R31449_3 | --- |
| rc_AA799550_at | | 0.81196 | | T-cell activation protein | --- |
| rc_AA799570_at | | 0.80322 | | Similar to mmDj4 | --- |
| rc_AA799607_at | | 0.69066 | | Transcribed locus | --- |
| rc_AA799824_at | | 0.87664 | | Similar to Vacuolar ATP synthase subunit C (V-ATPase C subunit) | --- |
| rc_AA800029_at | | 0.79104 | | pam, highwire, rpm 1 (predicted) | --- |
| rc_AA800296_at | | 0.79944 | | Similar to poly(A) polymerase V | --- |
| rc_AA800456_at | | 0.71941 | | Transcribed locus | --- |
| rc_AA800513_at | | 0.79192 | | Similar to transformation/transcription domain-associated protein | --- |
| rc_AA800753_at | | 0.71393 | | Similar to importin 7 | --- |
| rc_AA800882_g_at | | 0.66441 | | EST190379 Rn.24136 | --- |
| rc_AA858600_at | | 0.88112 | | leucine-zipper-like transcriptional regulator, 1 (predicted) | --- |
| rc_AA866291_at | | 0.78168 | | similar to cornichon-like protein (predicted) | --- |
| rc_AA866432_at | | 0.84821 | | LOC363015 | --- |
| rc_AA866459_at | | 0.84950 | | Cyclin D binding myb-like transcription factor 1 | --- |
| rc_AA874832_at | | 0.80373 | | anaphase-promoting complex subunit 5 (predicted) | --- |
| rc_AA874848_s_at | | 0.86137 | | Thymus cell antigen 1, theta | --- |
| rc_AA875084_at | | 0.70353 | | transducin-like enhancer of split 1, homolog of Drosophila E(spl) | --- |
| rc_AA875171_at | | 0.75744 | | N-acetylglucosamine-1-phosphotransferase, gamma subunit | --- |
| rc_AA891069_at | | 0.74698 | | serine/arginine-rich protein specific kinase 2 (predicted) | --- |
| rc_AA891161_at | | 0.66544 | | Transcribed locus | --- |
| rc_AA891476_at | | 0.87216 | | Transcribed locus | --- |
| rc_AA891824_at | | 0.88196 | | serine/arginine-rich protein specific kinase 2 (predicted) | --- |
| rc_AA891864_at | | 0.68641 | | ATP/GTP binding protein 1 (predicted) | --- |
| rc_AA892014_s_at | | 0.79215 | | HLA-B-associated transcript 1A | --- |
| rc_AA892228_at | | 0.74292 | | EST196031 Rn.4183 | --- |

| *Probe Set ID* | *FC 1 hour* | *FC 3 hours* | *FC 5 hours* | *Gene Title* | *GO Biological Process Description* |
|---|---|---|---|---|---|
| rc_AA892270_g_at | | 1.10753 | | similar to DNA polymerase epsilon p17 subunit | --- |
| rc_AA892376_at | | 0.82120 | | protein associated with PRK1 | --- |
| rc_AA892394_at | | 0.67246 | | Similar to CUG triplet repeat RNA-binding protein 1 (CUG-BP1) | --- |
| rc_AA892394_g_at | | 0.76976 | | Similar to CUG triplet repeat RNA-binding protein 1 (CUG-BP1) | --- |
| rc_AA892422_at | | 0.77592 | | mitochondrial ribosomal protein L11 | --- |
| rc_AA892465_at | | 1.18084 | | helicase with zinc finger domain (predicted) | --- |
| rc_AA892500_at | | 0.66947 | | Similar to mKIAA0623 protein | --- |
| rc_AA892506_at | | 0.76250 | | coronin, actin binding protein 1A | --- |
| rc_AA892548_at | | 0.69869 | | Transcribed locus | --- |
| rc_AA892666_at | | 1.22762 | | galactose mutarotase (aldose 1-epimerase) | --- |
| rc_AA892831_s_at | | 0.82520 | | proteasome (prosome, macropain) 26S subunit, non-ATPase, 11 | --- |
| rc_AA892842_at | | 0.72104 | | similar to capping protein alpha 2 subunit | --- |
| rc_AA893173_at | | 0.81139 | | vacuolar protein sorting 29 (S. pombe) (predicted) | --- |
| rc_AA893515_at | | 0.72324 | | translocation protein 1 (predicted) | --- |
| rc_H31887_at | | 1.28709 | | similar to RIKEN cDNA 1700037H04 (predicted) | --- |
| rc_H33656_at | | 0.92096 | | Transcribed locus | --- |
| U25264_at | | 0.93048 | | selenoprotein W, muscle 1 | --- |
| U48288_at | | 0.69457 | | A kinase (PRKA) anchor protein 11 | --- |
| X53581cds#5_f_at | | 0.75750 | | similar to RIKEN cDNA 9330196J05 (predicted) | --- |
| X83231_at | | 1.22802 | | inter-alpha trypsin inhibitor, heavy chain 3 | --- |
| rc_AA800882_at | | 0.62222 | 1.36565 | EST190379 Rn.24136 | --- |
| rc_AA891842_at | | | 1.34095 | Similar to death receptor 6 | --- |
| rc_AI639381_at | 0.81049 | | | --- | --- |
| X02412_at | 0.88790 | | | --- | --- |
| X51536cds_at | 0.90839 | | | --- | --- |
| U75404UTR#1_s_at | | 1.18993 | | --- | --- |
| X14210cds_at | | 1.07495 | | --- | --- |
| S46798cds#1_s_at | | 1.14169 | | --- | --- |
| U11071_f_at | | 1.14464 | | --- | --- |
| rc_AA965147_at | | 0.76936 | | --- | --- |
| rc_AI639002_i_at | | 1.36076 | | --- | --- |
| rc_AI639102_g_at | | 0.77318 | | --- | --- |

| *Probe Set ID* | *FC 1 hour* | *FC 3 hours* | *FC 5 hours* | *Gene Title* | *GO Biological Process Description* |
|---|---|---|---|---|---|
| rc_AI639427_at | | 0.80273 | | --- | --- |
| rc_H31610_at | | 0.82677 | | --- | --- |
| rc_AA892860_at | | 0.90096 | | --- | --- |
| rc_AA892864_at | | 0.75562 | | --- | --- |
| rc_AA875630_at | | 0.71008 | | --- | --- |
| rc_AA891054_at | | 0.66438 | | --- | --- |
| M61177_s_at | | 1.10081 | | --- | --- |