Universiteit Leiden
The Netherlands

Glucocorticoid control of gene transcription in neural tissue

Morsink, M.C.

Citation

Morsink, M. C. (2007, June 26). *Glucocorticoid control of gene transcription in neural tissue*. Retrieved from https://hdl.handle.net/1887/12094

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/12094

Note: To cite this publication please use the final published version (if applicable).

Chapter 2

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

Morsink M.C.[1], Steenbergen P.J.[1], Vos J.B.[2], Karst H.[3], Joëls M.[3], De Kloet E.R.[1], Datson N.A.[1]

[1]Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden, The Netherlands, [2]Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands, [3]Center for NeuroScience, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

Journal of Neuroendocrinology, 2006, Vol. 18, 239-252

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 SAGEmap 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′)	ampli-con (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

Chapter 2

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

Chapter 2

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 G0-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.

ACKNOWLEDGEMENTS

We thank E.P. Buddingh' and L. Meijer for technical assistance with the corticosterone measurements, R. De Jong for assistance with the real-time qPCR and O.C. Meijer for critical reading of the manuscript. This work was supported by the Netherlands Organization for Scientific Research (NWO) grants 903-42-197 and 903-42-085. E.R. De Kloet is supported by the Royal Netherlands Academy of Arts and Sciences (KNAW).

REFERENCES

1. de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 2005; **6:** 463-475.
2. McEwen BS. Stress and hippocampal plasticity. *Annu Rev Neurosci* 1999; **22:** 105-122.
3. de Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 1998; **19:** 269-301.
4. Horner HC, Packan DR, Sapolsky RM. Glucocorticoids inhibit glucose transport in cultured hippocampal neurons and glia. *Neuroendocrinology* 1990; **52:** 57-64.
5. Beato M, Sanchez-Pacheco A. Interaction of steroid hormone receptors with the transcription initiation complex. *Endocr Rev* 1996; **17:** 587-609.
6. McEwan IJ, Wright AP, Gustafsson JA. Mechanism of gene expression by the glucocorticoid receptor: role of protein-protein interactions 4 *Bioessays* 1997; **19:** 153-160.
7. Datson NA, et al. Identification of corticosteroid-responsive genes in rat hippocampus using serial analysis of gene expression. *European Journal of Neuroscience* 2001; **14:** 675-689.
8. Joels M. Steroid hormones and excitability in the mammalian brain. *Front Neuroendocrinol* 1997; **18:** 2-48.
9. Meijer OC, de Kloet ER. Corticosterone suppresses the expression of 5-HT1A receptor mRNA in rat dentate gyrus. *Eur J Pharmacol* 1994; **266:** 255-261.
10. Lipton P, Aitken PG, Dudek FE, Eskessen K, Espanol MT, Ferchmin PA, Kelly JB, Kreisman NR, Landfield PW, Larkman PM, Making the best of brain slices: comparing preparative methods. *J Neurosci Methods* 1995; **59:** 151-156.
11. Blitzer R, Lombroso PJ. Genetics of childhood disorders: LIII. Learning and memory, part 6: induction of long-term potentiation. *J Am Acad Child Adolesc Psychiatry* 2003; **42:** 998-1001.
12. Karst H, Wadman WJ, Joels M. Corticosteroid receptor-dependent modulation of calcium currents in rat hippocampal CA1 neurons. *Brain Res* 1994; **649:** 234-242.
13. Karst H, Karten YJ, Reichardt HM, de Kloet ER, Schutz G, Joels M. Corticosteroid actions in hippocampus require DNA binding of glucocorticoid receptor homodimers. *Nat Neurosci* 2000; **3:** 977-978.
14. Nishi M, Ogawa H, Ito T, Matsuda KI, Kawata M. Dynamic changes in subcellular localization of mineralocorticoid receptor in living cells: in comparison with glucocorticoid receptor using dual-color labeling with green fluorescent protein spectral variants. *Mol Endocrinol* 2001; **15:** 1077-1092.
15. De Kloet R, Wallach G, McEwen BS. Differences in corticosterone and dexamethasone binding to rat brain and pituitary. *Endocrinology* 1975; **96:** 598-609.
16. Hansson AC, Cintra A, Belluardo N, Sommer W, Bhatnagar M, Bader M, Ganten D, Fuxe K. Gluco- and mineralocorticoid receptor-mediated regulation of neurotrophic factor gene expression in the dorsal hippocampus and the neocortex of the rat. *Eur J Neurosci* 2000; **12:** 2918-2934.
17. Bhargava A, Meijer OC, Dallman MF, Pearce D. Plasma membrane calcium pump isoform 1 gene expression is repressed by corticosterone and stress in rat hippocampus. *J Neurosci* 2000; **20:** 3129-3138.
18. Schaaf MJ, de Jong J, de Kloet ER, Vreugdenhil E. Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. *Brain Res* 1998; **813:** 112-120.

19. Quackenbush J. Microarray data normalization and transformation. *Nat Genet* 2002; **32 Suppl:** 496-501.

20. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 2001; **98:** 5116-5121.

21. Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, Sun S, Kulp D, Siani-Rose MA. NetAffx: Affymetrix probesets and annotations. *Nucleic Acids Res* 2003; **31:** 82-86.

22. Hosack DA, et al. Identifying biological themes within lists of genes with EASE. *Genome Biol* 2003; **4**.

23. Hoekstra M, Kruijt JK, Van Eck M, Van Berkel TJ. Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *J Biol Chem* 2003; **278:** 25448-25453.

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25:** 402-408.

25. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995; **270:** 484-487.

26. Vellucci SV, Parrott RF, Mimmack ML. Chronic dexamethasone-treatment alters mineralocorticoid receptor, truncated trkB and selected glutamate receptor subunit mRNA expression in the porcine hippocampus. *Neuropeptides* 2002; **36:** 291-298.

27. Neumaier JF, Sexton TJ, Hamblin MW, Beck SG. Corticosteroids regulate 5-HT(1A) but not 5-HT(1B) receptor mRNA in rat hippocampus. *Brain Res Mol Brain Res* 2000; **82:** 65-73.

28. Lein ES, Zhao X, Gage FH. Defining a molecular atlas of the hippocampus using DNA microarrays and high-throughput in situ hybridization. *J Neurosci* 2004; **24:** 3879-3889.

29. Datson NA, Meijer L, Steenbergen PJ, Morsink MC, van der LS, Meijer OC, de Kloet ER. Expression profiling in laser-microdissected hippocampal subregions in rat brain reveals large subregion-specific differences in expression. *Eur J Neurosci* 2004; **20:** 2541-2554.

30. Gasull T, Giralt M, Hernandez J, Martinez P, Bremner I, Hidalgo J. Regulation of metallothionein concentrations in rat brain: effect of glucocorticoids, zinc, copper, and endotoxin. *Am J Physiol* 1994; **266:** E760-E767.

31. McIntosh LJ, Cortopassi KM, Sapolsky RM. Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. *Brain Res* 1998; **791:** 215-222.

32. McIntosh LJ, Hong KE, Sapolsky RM. Glucocorticoids may alter antioxidant enzyme capacity in the brain: baseline studies. *Brain Res* 1998; **791:** 209-214.

33. Hugin-Flores ME, Steimer T, Aubert ML, Schulz P. Mineralo- and glucocorticoid receptor mrnas are differently regulated by corticosterone in the rat hippocampus and anterior pituitary. *Neuroendocrinology* 2004; **79:** 174-184.

34. Almon RR, DuBois DC, Pearson KE, Stephan DA, Jusko WJ. Gene arrays and temporal patterns of drug response: corticosteroid effects on rat liver. *Funct Integr Genomics* 2003; **3:** 171-179.

35. Fujikawa T, Soya H, Fukuoka H, Alam KS, Yoshizato H, McEwen BS, Nakashima K. A biphasic regulation of receptor mRNA expressions for growth hormone, glucocorticoid and mineralocorticoid in the rat dentate gyrus during acute stress. *Brain Res* 2000; **874:** 186-193.

36. Kasutani K, Itoh N, Kanekiyo M, Muto N, Tanaka K. Requirement for cooperative interaction of interleukin-6 responsive element type 2 and glucocorticoid responsive element in the synergistic activation of mouse metallothionein-I gene by interleukin-6 and glucocorticoid. *Toxicol Appl Pharmacol* 1998; **151:** 143-151.

37. Schoneveld OJ, Gaemers IC, Lamers WH. Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta* 2004; **1680:** 114-128.

38. Evans SJ, Datson NA, et al. Evaluation of Affymetrix GeneChip sensitivity in rat hippocampal tissue using SAGE analysis. *European Journal of Neuroscience* 2002; **16:** 409-413.

39. Datson NA, van der PJ, de Kloet ER, Vreugdenhil E. Expression profile of 30,000 genes in rat hippocampus using SAGE. *Hippocampus* 2001; **11:** 430-444.

40. Feldker DE, Datson NA, Veenema AH, Proutski V, Lathouwers D, de Kloet ER, Vreugdenhil E. GeneChip analysis of hippocampal gene expression profiles of short- and long-attack-latency mice: technical and biological implications. *J Neurosci Res* 2003; **74:** 701-716.

41. Joels M, Velzing E, Nair S, Verkuyl JM, Karst H. Acute stress increases calcium current amplitude in rat hippocampus: temporal changes in physiology and gene expression. *Eur J Neurosci* 2003; **18:** 1315-1324.

42. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 2000; **21:** 55-89.

43. Lawrence MS, Sapolsky RM. Glucocorticoids accelerate ATP loss following metabolic insults in cultured hippocampal neurons. *Brain Res* 1994; **646:** 303-306.

44. Patel R, McIntosh L, McLaughlin J, Brooke S, Nimon V, Sapolsky R. Disruptive effects of glucocorticoids on glutathione peroxidase biochemistry in hippocampal cultures. *J Neurochem* 2002; **82:** 118-125.

45. Jahng JW, Houpt TA, Wessel TC, Chen K, Shih JC, Joh TH. Localization of monoamine oxidase A and B mRNA in the rat brain by in situ hybridization. *Synapse* 1997; **25:** 30-36.

46. Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA. A distinct role for norepinephrine in memory retrieval. *Cell* 2004; **117:** 131-143.

47. Fujishiro H, Umegaki H, Suzuki Y, Oohara-Kurotani S, Yamaguchi Y, Iguchi A. Dopamine D(2) receptor plays a role in memory function: implications of dopamine-acetylcholine interaction in the ventral hippocampus. *Psychopharmacology (Berl)* 2005; 1-9.

48. File SE, Kenny PJ, Cheeta S. The role of the dorsal hippocampal serotonergic and cholinergic systems in the modulation of anxiety. *Pharmacol Biochem Behav* 2000; **66:** 65-72.

49. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 1981; **213:** 1394-1397.

50. Rivier J, Spiess J, Vale W. Characterization of rat hypothalamic corticotropin-releasing factor. *Proc Natl Acad Sci U S A* 1983; **80:** 4851-4855.

51. Koob GF, Heinrichs SC. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res* 1999; **848:** 141-152.

52. Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* 2000; **428:** 191-212.

53. Blank T, Nijholt I, Eckart K, Spiess J. Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and acute stress: implications for hippocampus-dependent learning. *J Neurosci* 2002; **22:** 3788-3794.

54. Chen Y, Bender RA, Frotscher M, Baram TZ. Novel and transient populations of corticotropin-releasing hormone-expressing neurons in developing hippocampus suggest unique functional roles: a quantitative spatiotemporal analysis. *J Neurosci* 2001; **21:** 7171-7181.

55. Chen Y, Brunson KL, Adelmann G, Bender RA, Frotscher M, Baram TZ. Hippocampal corticotropin releasing hormone: pre- and postsynaptic location and release by stress. *Neuroscience* 2004; **126:** 533-540.

56. Toutenhoofd SL, Foletti D, Wicki R, Rhyner JA, Garcia F, Tolon R, Strehler EE. Characterization of the human CALM2 calmodulin gene and comparison of the transcriptional activity of CALM1, CALM2 and CALM3. *Cell Calcium* 1998; **23:** 323-338.

57. Xia Z, Storm DR. The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci* 2005; **6:** 267-276.

58. Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 1998; **393:** 809-812.

59. Tojima T, Takahashi M, Ito E. Dual regulation of LIM kinase 1 expression by cyclic AMP and calcium determines cofilin phosphorylation states during neuritogenesis in NG108-15 cells. *Brain Res* 2003; **985:** 43-55.

60. Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z. Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* 2002; **35:** 121-133.

61. Halpain S, Hipolito A, Saffer L. Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *J Neurosci* 1998; **18:** 9835-9844.

62. Lautermilch NJ, Spitzer NC. Regulation of calcineurin by growth cone calcium waves controls neurite extension. *J Neurosci* 2000; **20:** 315-325.

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		---	---