Temporal Profiling of Gene Networks Associated with the Late Phase of Long-Term Potentiation *In Vivo*

Margaret M. Ryan1,2,3, Brigid Ryan1,2, Madeleine Kyrke-Smith1,2, Barbara Logan1,4, Warren P. Tate1,3, Wickliffe C. Abraham1,4, Joanna M. Williams1,2*

1 Brain Health Research Centre, University of Otago, Dunedin, New Zealand, 2 Department of Anatomy, Otago School of Medical Sciences, Dunedin, New Zealand, 3 Department of Biochemistry, Otago School of Medical Sciences, Dunedin, New Zealand, 4 Department of Psychology, University of Otago, Dunedin, New Zealand

Abstract

Long-term potentiation (LTP) is widely accepted as a cellular mechanism underlying memory processes. It is well established that LTP persistence is strongly dependent on activation of constitutive and inducible transcription factors, but there is limited information regarding the downstream gene networks and controlling elements that coalesce to stabilise LTP. To identify these gene networks, we used Affymetrix RAT230.2 microarrays to detect genes regulated 5 h and 24 h (n = 5) after LTP induction at perforant path synapses in the dentate gyrus of awake adult rats. The functional relationships of the differentially expressed genes were examined using DAVID and Ingenuity Pathway Analysis, and compared with our previous data derived 20 min post-LTP induction *in vivo*. This analysis showed that LTP-related genes are predominantly upregulated at 5 h but that there is pronounced downregulation of gene expression at 24 h after LTP induction. Analysis of the structure of the networks and canonical pathways predicted a regulation of calcium dynamics via G-protein coupled receptors, dendritogenesis and neurogenesis at the 5 h time-point. By 24 h neurotrophin-NFkB driven pathways of neuronal growth were identified. The temporal shift in gene expression appears to be mediated by regulation of protein synthesis, ubiquitination and time-dependent regulation of specific microRNA and histone deacetylase expression. Together this programme of genomic responses, marked by both homeostatic and growth pathways, is likely to be critical for the consolidation of LTP *in vivo*.

Introduction

Long-term potentiation (LTP) is widely regarded as a memory storage mechanism as it fulfills key properties of a mnemonic device. For example, LTP is specific to only closely grouped synapses activated following high-frequency stimulation [1,2]; LTP shows both cooperativity and associativity, such that for LTP induction a threshold intensity is required, as is coincident activity of the pre- and postsynaptic neurons. LTP is also remarkably persistent, when induced at perforant path synapses of awake freely moving animals, LTP has been shown to transition through distinct phases which culminate in a potentiation that can remain stable for months and in one documented case, even for a year [3].

The molecular mechanisms underlying the early events have been thoroughly characterised both *in vitro* and *in vivo*. Protein kinase activation and subsequent trafficking of AMPA-glutamate receptors to the cell surface, together with altered release of neurotransmitter [4], underpin the early phase of LTP. This is followed by trafficking of NMDA-glutamate receptors [5] and metabotropic glutamate receptors [6] that facilitate a change in the calcium dynamics of activated synapses and reflect metaplasticity events [7].

There is considerable evidence that late phases of LTP (LTP2 and LTP3) require new protein synthesis, both rapidly via regulated translation of synaptically located mRNA and over the long-term via new gene transcription [8]. The role that new gene expression plays in LTP has been debated, however, and there is evidence that LTP can persist under certain *in vitro* conditions without protein synthesis [9,10,11]. LTP-related gene expression might contribute indirectly to replenish proteins depleted in response to synaptic activity, while LTP itself is maintained by enzymatic storage mechanisms [12]. Nonetheless, evidence primarily from studies of LTP *in vivo* strongly supports the hypothesis that new gene expression is crucial for the persistence of LTP. General inhibitors of transcription and translation or knockdown of specific LTP-regulated genes inhibit the maintenance of LTP *in vivo* and clusters of genes have been identified as regulated by LTP-inducing stimulation [13,14,15].

Our proposal is that to fully understand the complex molecular mechanisms underlying LTP, it is essential to carry out genome-wide analyses to identify and interpret the gene networks regulated...
by the biology. Accordingly, we studied the early phase of LTP to identify interrelated networks of genes induced at perforant path synapses in the dentate gyrus in vivo [13]. This study and others consolidated the concept that a significant and co-ordinated alteration in gene expression is triggered during LTP [16,17]. However, few analyses have extended beyond this very early time frame (e.g. [16]). In the current study, we have broadened our analysis to identify gene sets differentially regulated during the late phases of LTP (5 h and 24 h post-LTP induction), elucidating their associated canonical pathways, biological functions and clustering into gene networks. The resulting holistic view of gene expression changes over 24 h has provided insight into the regulation and function of LTP-related gene expression in the hippocampus.

**Analysis**

1. Regulation of gene expression 20 min, 5 h and 24 h following LTP induction in vivo

To determine whether there are ongoing LTP-related alterations in gene expression and whether overlapping or distinct gene sets are regulated in the later stages of LTP, we established time-specific LTP-related gene (LRG) datasets. Our established LTP induction paradigm is well documented to produce a very persistent form of LTP at perforant path synapses in vivo (e.g. [3]). In this study, HFS resulted in a robust potentiation over 24 h of the fEPSP slope (21 ± 3%; mean ± SEM) and the population spike (293 ± 61%) (Figure 1). LRGs were identified by isolation and purification of total RNA from individual control and stimulated hemispheres (5 h and 24 h post-LTP induction; n = 5), followed by gene expression profiling using Affymetrix Rat 230.2 arrays. After normalization across all datasets and using a moderated paired t-test (Limma), an inclusive list of differentially expressed LRGs was produced for each time point using dual selection criteria ($\geq 1.15$ fold change; $p \leq 0.05$). This analysis identified 190 differentially expressed genes (73% increased and 27% decreased) at 5 h post-LTP induction, and 691 differentially expressed genes at 24 h post-LTP induction (31% increased and 69% decreased) (Figure 2; Table S1, S2). Overall our analysis, when compared with a re-analysis of our 20 min-LRG set [13], suggests that there is a rapid upregulation of gene expression which persists for at least 5 h, but by 24 h the proportion of upregulated genes is attenuated, and a generalised downregulation of gene expression (Figure 2). While little literature exists regarding the specific cohorts of genes regulated in the later phases of LTP [14,18,19], we explored our datasets for previously reported LTP-regulated genes. Several known LTP-regulated genes were identified within our new datasets, and a subset was confirmed using qPCR as having temporally specific gene expression (Figure 3A). We confirmed the upregulation of the immediate-early gene HOMER, observed that the metallopeptidase inhibitor TIMP1 is upregulated with a delayed time-course, and showed that the amyloid precursor protein (APP) is downregulated specifically 24 h post-LTP induction. Next, we confirmed the differential expression of several genes, which had not previously been reported to be regulated by LTP (Figure 3B). These included LINGO1, a member of the Nogo receptor signalling complex which regulates axon regeneration, the transcription factors SP1 and BHLHE22, HDAC1, a histone deacetylase, KCNE2, an ancillary protein of voltage-gated potassium channels as well as DNAJB5 (see below).

To determine the overall similarity between our datasets we used Venn diagrams and investigated the intersections formed (Figure 4A; Table S3). This analysis showed that while the 20 min and 5 h datasets contain similar numbers of differentially expressed genes, few are in common and at 24 h there are 3 fold more downregulated genes than at the earlier time points. Analysis of the temporal expression profiles of the genes common

![Figure 1. Induction of robust LTP by 50×400 Hz, 10 impulse trains (HFS) at perforant path dentate granule cell synapses.](http://example.com/figure1.png)

Data are mean ± standard error (n = 5) for the fEPSP (A) and population spike (B). Inset waveforms are averages of 10 sweeps (5 min) for one animal, taken during baseline (1), 45–60 min (2) and 24 h (3) after HFS. Calibration bars: 5 ms, 5 mV. doi:10.1371/journal.pone.0040538.g001

![Figure 2. LTP-related gene expression 20 min, 5 h and 24 h post-LTP induction.](http://example.com/figure2.png)

Alterations in mRNA transcript number identified 20 min, 5 h and 24 h post-LTP, as detected using Affymetrix Rat 230 2.0 microarrays; note increase in proportion of downregulated genes at 24 h (dual selection criteria: fold change $\geq 1.15$ or $\leq 0.85$; $p \leq 0.05$). doi:10.1371/journal.pone.0040538.g002

![Figure 3A. Induction of robust LTP by 50×400 Hz, 10 impulse trains (HFS) at perforant path dentate granule cell synapses.](http://example.com/figure3a.png)

![Figure 3B. LTP-related gene expression 20 min, 5 h and 24 h post-LTP induction.](http://example.com/figure3b.png)
between the 3 data sets by heatmap (Figure 4B) demonstrates that there is a close relationship between the 20 min and 5 h data sets; however, the heatmap includes data that have not reached statistical significance and only comprises a small proportion of the differentially expressed genes within each timepoint. Thus we conclude that the three LRG sets represent distinct temporally defined datasets. Remarkably, there is only one gene in common between all 3 data sets, the class II histone deacetylases (HDACs)-interacting protein, DNAJB5 [20]. DNAJB5 is postulated to act as a chaperone involved in nuclear localisation of HDACs, which regulate gene transcription by removal of acetyl groups from ε-N-acetylated histones. This enhances histone-DNA association and prevents access of transcriptional machinery to DNA. Interestingly, also present within the intersection between the 5 h and 24 h gene sets is the class I histone deacetylase HDAC1, GMNN (Geminin), a molecule that has recently been shown to regulate the conformation of chromatin around neural genes [21] and MLL4, a histone methyltransferase which also regulates acetylation [22]. These findings highlight potential epigenetic control of LTP-related gene expression.

2. Temporal analysis of LRG sets

To explore the potential contribution that our temporally defined LRG datasets make to the late phases of LTP, the relationships between the contributing genes were analysed using Ingenuity Pathway Analysis (as described [13]) (IPA; Canonical Pathways, Biological Function and Network Analysis), and DAVID software. Our 20 min-LRG data set [13] was reanalysed alongside those from 5 h and 24 h to examine LTP-associated
A

B

Color Key

-0.5 0.5

Value

24h 5h 20min

DUSP5 NR4A3 VGF DNAJB6 HOMER NGFR RAASF1 FAM136A PLAUR KPN4A1 FOXO1 GMINN GAMK2D RFP1 MMP23 NET222 MLL4 ZNF775 ASPH MAN1A1 CHRM5 UTP5 UBE2G2 HDAC1 FOXP1 OSBPL1A FERMTZ DNM3 SAMD4A PEX5L IGBP5 ZP1 P7EB TB11X KDM8B DYNCLIL CEP350 NFE2L1 MYO5C FOXN3 ANKRD52
functions across our full temporal range using the most current versions of the software.

2.1 Ingenuity Pathway Analysis: Biological Functions. Biological Function analysis (Figure 5) highlighted the contribution that regulated gene expression plays in the maintenance of LTP. The function Gene expression was well above the threshold value within all three LRG datasets. Interestingly, Cell-Cell Signalling & Interaction, Molecular Transport and Protein Synthesis were all more significantly regulated at 24 h. Examination of the genes represented within the latter set (Table S4) indicate a potential downregulation of protein synthesis mediated via regulation of the pre-initiation complex and charging of tRNA with their cognate amino acids (EIF2, EPRS, EIF3) as well as a generalized disruption of the 40S initiation complex via reduction in levels of the scaffolding factor, eIF4G.

2.2 Ingenuity Pathway Analysis: Canonical Pathway Analysis and the Database for Annotation, Visualization and Integrated Discovery. The Canonical Pathways identified within the 20 min-LRG set analysis were associated with Cell growth, Cell death, Development and PDGF signalling (Figure 6). Interestingly, interleukin 6 (IL6) signalling was identified specifically within our 20 min-LRG set. This supports the observation that immune response-related genes are implicated in the LTP stabilization process [16]. Canonical analysis also highlighted an early involvement of Neurotrophin/TRK signalling in the LTP stabilization process, as previously described [23,24].

Canonical pathway analysis of the 5 h-LRG set identified two separate pathways associated with G-protein coupled signalling and calcium dynamics (cAMP, Ca²⁺ signalling), with the majority of the genes upregulated (Figure 6; Table S5). In support of these findings, when the 5 h-LRG set was submitted to functional analysis using DAVID software, a Calcium and Calmodulin annotation cluster was identified as significantly enriched (enrichment score: 1.42; see Figure S1). Furthermore, DAVID analysis identified clusters of genes with annotations related to Ion channels (enrichment score: 1.59; see Figure S2) and Synapse-related proteins (enrichment score: 1.37; see Figure S3). DAVID analysis of our 24 h-LRG set highlighted a significant regulation of protein kinase and phosphorylation-related genes (enrichment scores 1.98, 2.58 respectively; Figure S4, S5). Closer analysis of the 24 h-LRG set showed that it contained 39 genes encoding protein kinases, 25 of which were downregulated, as well as 11 phosphatases, 9 of which were downregulated. Thus the DAVID analysis predicts regulation of AMP and G-protein coupled receptor linked pathways 5 h post-LTP that is followed by downregulation of protein kinases/phosphatases at 24 h. Together these analyses predict that gene expression upregulates ongoing modulation of pivotal cell-signalling systems, potentially contributing to a metaplastic shift in calcium dynamics or signalling [25].

Canonical pathway analysis of the 24 h-LRG set identified NFκB (nuclear factor kappa B), Reelin, PTEN (phosphatase and tensin homolog), DHA (omega-3 fatty acid DHA; docosahexaenoic acid) and Clathrin-mediated endocytosis pathways as specifically regulated (Figure 6). Reelin and PTEN signalling are linked to neurogenesis, synaptic plasticity [26], neuronal migration and maturation [27] or local protein synthesis [28]. DHA signalling has previously been linked to LTP [29] and cell survival [30]. Thus these data predict an ongoing role of gene expression supporting synaptic growth and plasticity. Interestingly, PTEN associates with PSD95 and is recruited to the PSD in response to NMDA receptor activation [31] and has been linked to AMPAR trafficking [32]. These data interpreted alongside the observation that Clathrin-mediated endocytosis is a significantly regulated canonical pathway specifically within the 24 h-LRG set suggests that late phase LTP-related gene expression may contribute to homeostatic glutamate receptor regulation.

3. Ingenuity Pathway Analysis: Network analyses

To further explore our LRG datasets we used the network analysis tools of the IPA software to predict potential molecular networks functioning during the consolidation of LTP. These analyses revealed both effector pathways and highlight regulatory mechanisms engaged in response to LTP.

3.1 Calcium signalling and LTP persistence. The most significant network formed at 5 h (5 h-Network 1; Figure 7A; Score = 42; direct interactions) included the biological functions Calcium, Protein kinase A and phospholipase C signalling. This supports the primary conclusion from the IPA Canonical pathway and DAVID analysis that regulation of calcium signalling is a key marker of the 5 h-LRG set. Within this network calmodulin forms a central hub that is linked to a type 1 and type 2 calcium/calmodulin-dependent protein kinases cluster. Interestingly, this network also contains a hub centred on the transcription factor CREB, which interacts with the known LTP-related protein kinase PRKGA (aka PKCξ) and its substrate Growth associated protein-43 (GAP43). Co-regulation of PRKGA and GAP43 is particularly pertinent as phosphorylation of GAP43 and the resulting enhanced release of glutamate have been mooted as a potential mechanism contributing to LTP persistence [33]. As CAMKI phosphorylates and activates synapsin I, a molecule also known to be LTP-regulated [34] and involved in neurotransmitter release, these data suggest a role for gene expression in supporting enhanced release of glutamate 5 h post-LTP, perhaps at downstream mossy fiber terminals in area CA3 [35].

CAMK signalling may contribute to LTP persistence in multiple ways. Recent literature has shown CAMKI-gamma (CAMKIG) to be enriched within the dentate gyrus and linked to dendritogenesis [36,37] and CAMKI-alpha to axonal elongation [38]. As expression of CAMKIG was upregulated in our 5 h-LRG set as well as other molecules linked to postsynaptic reorganisation such as the protein kinase ROCK1 (Rho-associated coiled-coil forming protein kinase 1) and its binding partner RHOD, this network may reflect LTP-responsive cytoskeletal reorganisation [39]. Together these data suggest that a key function of the 5 h-LRG network is related to synaptic reorganisation.

3.2 Negative regulation of gene expression at 5 h post-LTP. Several molecules involved in negative regulation of gene expression are present in 5 h-Network 1. For example, HistoneH3 forms a central hub and is linked to the epigenetic repressor of transcription HDAC1 (a member of the histone deacetylase complex) and its binding partner RUNX1T1, both of which are upregulated in our 5 h-LRG set. This complex removes acetyl moieties from histones, resulting in condensation of chromatin and transcriptional repression [40]. This network also includes transcriptional repressors such as NUMBL (numb homolog), which acts as a negative regulator of NFκB signalling [41] and HES1 (hairy and enhancer of split 1), which inhibits expression of genes regulated by bHLH (basic helix-loop-helix) proteins [42]. Furthermore, several specific transcriptional activators are down-regulated in our 5 h-LRG set: for example, SMARCA4 (SWI/
SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4), a member of the CREST-BRG1 transcriptional coactivator complex [43], ROCK1, an activator of SRF [44] and CAMKI otherwise known as CREB kinase. Such molecular correlates of repression of gene expression are consistent with our observation that after the rapid LTP-induced increase in expression observed 20 min post-LTP (Figure 2; [13]) there is an attenuation of gene expression. The
emerging theme of negative regulation of gene expression is
further exemplified in a later highly significant network (5 h
Network 4: Score 21; Figure 7D), where ubiquitin forms a central
hub. This network predicts an LTP-associated increase in protein
degradation which is mediated by upregulation of TOLLIP, (Tol
interacting protein), a molecule which forms part of a complex
which promotes endosomal trafficking of clathrin and ubiquiti-
nated proteins to endosomes [45], and the ubiquitin-conjugating
enzyme, UBE2G2 [46].

3.3 Dynamic epigenetic regulation of LTP-related gene
expression. In 5 h-Network 2 (Figure 7B; Score 33) the
microRNA MIR24-1 formed a hub, while MIR34A is present

![Figure 6. IPA Categorization of LRG sets according to their Canonical Pathways.](doi:10.1371/journal.pone.0040538.g006)
Within 5 h-Network 3 (Figure 7C; Score 18). While regulation of microRNA was not directly assessed in this study using Affymetrix RAT 230 2.0 arrays, the prediction generated by the IPA software that microRNA form central hubs within our networks is of particular note as microRNA are proposed to be central regulators of functionally related genes [47]. This is mediated by binding to the 3’ untranslated region of target mRNAs, which results in translation repression and/or mRNA degradation. Interestingly, this process has been shown to occur in dendrites, and microRNA accordingly have also been proposed as key regulators of protein synthesis-dependent synaptic plasticity [48,49,50].

As MIR24-1 is linked to 5 genes that are upregulated in response to LTP, the prediction from this network analysis is that MIR24-1 is downregulated in response to LTP, thus allowing the
expression of a subset of linked genes. We tested this hypothesis using TaqMan microRNA qPCR and found a significant downregulation of MIR34A \((0.53 \pm 0.16 \text{ average } \pm \text{ S.E.M})\), \(n = 5\), \(p < 0.05\) two-tailed t-test and MIR24-1 \((0.59 \pm 0.12, \ n = 9, \ p < 0.05\) two-tailed t-test) 5 h post-LTP (Figure 8). This is the first report of microRNA regulation following perforant path LTP in awake freely moving rats and is particularly relevant as the seed recognition sequence of the MIR34 family has recently been reported to rescue learning impairment [31]. Potentially, as antagonism of MIR24-1 has been shown to promote cell proliferation [52] and MIR34A regulates neural stem cell differentiation [53], these data suggest microRNA may also regulate LTP-related neurogenesis.

To investigate the potential mRNA targets of MIR34A and MIR24-1 we used the microRNA target prediction algorithms TargetScan, PicTar and miRanda. Prediction of MIR34A targets revealed 239 genes according to TargetScan, 438 according to miRanda, and 354 according to PicTar. Prediction of MIR24-1 targets revealed 329 genes according to TargetScan, 386 according to miRanda, and 369 according to PicTar.

Working on the principle that if a target is consistently predicted by multiple algorithms which use different selection criteria, it is more likely that the target prediction will be accurate, we investigated the intersection of the lists predicted by the three algorithms for each microRNA. This resulted in the identification of 30 genes that were predicted to be targeted by MIR34A and 19 genes predicted to be targeted by MIR24-1 (Table S7 and S8). Interestingly, a number of these genes are involved in synaptic transmission, specifically vesicle trafficking, endocytosis and exocytosis. However, when we screened our LTP datasets (5 h or 24 h) for the algorithm-predicted MIR34a and MIR24-1 target genes, we found no crossover between the groups. This does not discount the involvement of these microRNAs in LTP consolidation but predicts that MIR34a and MIR24-1 at least contribute to LTP consolidation at the level of translational repression.

Taking an alternative approach, we used the web application miRvestigator Framework to test whether individual microRNA seed sequences were enriched within the mRNA of our LRG data sets. We found evidence for enrichment of the MIR30-3p seed sequence within our 5 h data set \((p = 7.3e-04)\). Interestingly, the genes predicted to be targeted by MIR330-3p appear to primarily fall into the functional categories of transcription regulation and ubiquitination (Table S9).

Epigenetic regulation of gene expression emerges as a central theme of the 24 h-LRG network analyses. Within 24 h-Network 1 (Figure 9A; all interactions; Score = 41), the microRNA MIR-1 and MIR-124 form central hubs, with the majority of the genes in this network downregulated. MIR-124 has been implicated in the long-term plasticity of synapses in the mature nervous system and as a central controller of neurogenesis. MIR-1, a microRNA which is expressed in human neuroblastoma cells [54], targets CREB1 and regulates cell growth [55], and differentiation of embryonic stem cells [56]. Interestingly MIR-1 was also shown to inhibit apoptosis via modulating PTEN/Akt signalling, a canonical pathway we identified as LTP-regulated at 24 h post-LTP induction. Together these findings suggest a potential role for LTP-regulated microRNA in growth and/or neurogenesis.

HDACs also form central hubs within 24 h Network 1 (Figure 9B; direct interactions; Score 37). It is of note that HDAC2 is upregulated. This HDAC has previously been implicated in negative regulation of learning and memory [57]. In contrast HDAC1, which was upregulated within the 5 h-LRG set, is now downregulated, indicating a shift in the mode of action within the HDAC repressor complex similar to that shown to occur during development [58]. This network also contains evidence of direct transcriptional repression via downregulation of the transcription factor, SPI (two probes: −1.5 fold), and downregulation of RBM39, which acts as an activator of the AP1-transcription factor complex. Taken together, these data suggest that regulation of microRNA, HDACs and transcription factors represents a homeostatic response 24 h post-LTP induction.

### 3.4 Persistent involvement of NFKB in LTP-related gene expression.

Having identified an LTP-related homeostatic response underpinned by gene expression, we reasoned that a targeted analysis of the subset of upregulated genes within the 24 h-LRG set may identify positive or effector functions. Using IPA Network analysis we found evidence for ongoing activity of the transcription factor NFKB (Figure 9C; Score 39; all interactions). This factor, previously implicated in the regulation of LTP-associated gene expression [59], forms the major hub of our most significant network generated from this 20 min LRG-dataset [13] and was identified in our Canonical Pathway analysis (Figure 4). Interestingly, the positive regulators of NFKB, AZ12 (5 h) and RIPK1 (24 h), were identified as upregulated post-LTP induction, while the negative regulator, NFKBIZ, is upregulated within the 24 h LRG-set suggesting multiple, dynamic mechanisms of NFKB activity regulation in the hours post-LTP induction. Interpretation of the genes present within the 24 h “upregulated only” network predicts that this gene network underpins two functions. One is modification of transcription mediated by, for example, recruitment of HDACs (NR1D1) and histone modification (IL1A and PHC1). The second is a theme of neuronal growth via both proliferation (STAP2) and differentiation mediated upregulation of NGFR (aka TNFR1; p75NTR) and its interacting protein SALL2. SALL2 is a transcription factor, which constitutively binds NGFR, and upon activation of the receptor dissociates and migrates to the nucleus and promotes cell cycle arrest and neurite outgrowth [60]. This analysis reinforces the conclusion that LTP is associated with ongoing regulation of gene expression and that the gene expression may function to promote neurotrophic processes.

---

**Figure 8. MicroRNAs identified by network analysis (5 h LRG-set) and validated by qPCR.** Results are expressed as mean fold change \(\pm\) SEM. \(*p<0.05,\) Student’s t-test \((n=5–9)\). qPCR samples were normalised to the housekeeping gene Y1 using the \(2^{-\Delta\Delta CT}\) method.

doi:10.1371/journal.pone.0040538.g008
We have used Ingenuity Pathway Analysis and DAVID to interpret how the differentially expressed genes identified at 20 min, 5 h and 24 h post-LTP induction at perforant path synapses \textit{in vivo} are related. This analysis has confirmed that the persistence of LTP is associated with ongoing regulation of gene expression and has highlighted both an integrated network of regulatory mechanisms as well as effector mechanisms likely to contribute directly to the consolidation of LTP.

Our analysis has identified regulation of G-protein coupled signalling, calcium dynamics and ion channels as important biological functions that are regulated 5 h post-LTP induction.

Figure 9. IPA network analysis at 24 h. The three highest scoring networks from the LRG list are shown. See Figure 5 legend for further details.

doi:10.1371/journal.pone.0040538.g009
and clathrin-mediated endocytosis at 24 h. This suggests that the LTP-associated gene response may contribute to an alteration in the synaptic calcium response and potentially contribute to metaplasticity mechanisms proposed to facilitate or inhibit further plasticity within neuronal networks [7]. It is of note that the CaMKI gene family, previously linked to synaptic reorganisation was differentially regulated. The conclusion that LTP-related gene expression contributes to structural alterations at synapses is strengthened by the observation that multiple pathways identified both at 5 h and 24 h converge on this or related functions. Furthermore, as a number of the differentially expressed genes (e.g. FOXO1, NUMBL, MIR24-1) or molecules predicted by the IPA software (e.g. MIR124) have reported roles in neurogenesis these highlight a potential role of gene expression in LTP-stimulated neurogenesis.

LTP persistence involves an intricate interplay of molecules and this is reflected in our results, which indicate that control of LTP-regulated gene expression occurs in multiple layers. We show evidence for ongoing positive regulation of gene expression via CREB (5 h-Network 1) and NFκB (24 h: Canonical Pathway; 24 h-Network “up only”), the functions of which appear to be at least in part the regulation of GAP43 and neurotrophic signalling respectively. These findings suggest that these two transcription factors that are activated immediately after LTP induction continue to play significant roles throughout the period of LTP consolidation.

Our network analysis has also highlighted mechanisms that account for the generalised downregulation of gene expression observed at 24 h. This response may contribute to resetting the transcriptional programme in response to LTP towards underlying homeostatic mechanisms, or it may facilitate expression of the “positive” LTP-related features, such as neurotrophin signalling, structural modification or neurogenesis. We identified multiple pathways that mediate downregulation of gene expression. These function by directly modulating protein levels either by ubiquitination, endocytosis, inhibition of protein synthesis or microRNA-driven translation arrest. Control of gene expression was also demonstrated through temporally controlled upregulation of histone acetylases and downregulation of transcription factors such as SP1. Importantly, as modification of histone acetylation has previously been shown to occur in other models of memory [57,61], our data reinforce the importance of epigenetic mechanisms in synaptic plasticity. In summary, our data suggest that LTP-related gene expression contributes directly to both regulation of calcium dynamics as well as structural or neurogenic modifications of the network, which are likely to be involve ongoing activation of the CREB and NFκB transcription factors. Furthermore, we have identified a complex set of transcriptional regulatory mechanisms that appear to coalesce to form a transcriptional environment that facilitates the consolidation of LTP. These data should provide a solid platform for the development of biologically realistic neurogenetic computational models of memory storage processes.

Materials and Methods

Induction of LTP in freely moving animals

Ethics Statement. The surgical protocols, previously described in detail [13] were approved by the University of Otago Animal Ethics Committee (Permit number: 115–09) and were in accordance with New Zealand animal welfare legislation. All experiments were conducted on perforant path dentate gyrus synapses in adult male Sprague-Dawley rats (400–550 g, 4–6 months old at the time of surgery). Teflon-coated stainless steel wire stimulating and recording electrodes were implanted bilaterally to establish perforant path-evoked field potentials, which were recorded in the dentate hilus. The methods of LTP induction are the same as those described in [3] and [13], with the exception that the animals were euthanized either 5 h or 24 h following LTP induction (n=5 per group). In order to reduce variation all animals were tetanized unilaterally so that the unstimulated hemisphere could serve as a within-animal control. Our standard experimental protocols reliably produce an increase in the eEPSP that lasts for at least several weeks [3].

RNA purification and microarray hybridization

RNA isolation and array hybridization were carried out as described previously [13]. RNA was isolated from individual snap-frozen dentate gyrus using TRIzol reagent (Invitrogen) followed by purification through RNeasy Mini columns (Qiagen, USA). Samples meeting our quality control criteria (average RNA integrity number >8, [62]) were bioin-labeled and hybridized to Affymetrix RAT 230 2.0 arrays at the Centre for Proteomics and Genomics, University of Auckland. RNA isolation for microRNA analysis was performed similarly, but using Norgen Total RNA purification columns (Biotek Corporation) to retain these small RNA molecules.

Microarray data analysis

All array data were assessed using the Bioconductor software packages [63] as described previously [13], with an initial evaluation performed using the affyQCReport package, which confirmed that all quality control measures were within the recommended ranges [64]. The Robust Multichip Average (RMA) package (version 1.16.0) was used to normalize the data derived from control and stimulated hemispheres 20 min [13], 5 h and 24 h datasets. As outlined before [13], inclusive LTP-related gene (LRG) sets suitable for network analysis (either direct or indirect interactions) were derived using dual selection criteria: a threshold fold change cutoff (1.15) and a moderated paired t-test between matched stimulated and control samples (significance criterion of p<0.05). The t-statistic was generated using the Limma package, which utilizes a standard error moderated across all genes using a simple Bayesian model and produces p-values with greater degrees of freedom and hence greater reliability [65]. More stringent selection criteria were not used in this study to avoid the risk of Type 2 error and unnecessarily limited datasets at 5 h and 24 h for network analysis. All data comply with MIAME guidelines and is available through ArrayExpress (Accession number to be confirmed).

Real-time quantitative PCR: mRNA

Selected genes identified as differentially expressed by DNA microarray analysis were also analysed by quantitative PCR (qPCR). RNA was extracted as described above and reverse-transcribed to first strand cDNA using Superscript III (Invitrogen). Primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) and obtained from Sigma or Integrated DNA Technologies. Primer sequences are described in Table S6. qPCR was performed using SYBR green Roche mastermix on either an ABI 7300 or a Roche Lightcycler 480. qPCR samples were normalised to the housekeeping gene HPRT using the 2\(^{-\Delta\Delta CT}\) method [66]. Standard curves were established and amplification and dissociation curves were analysed. Significance was assessed using Student’s t-tests with the criterion set at p<0.05.
Real-time quantitative PCR: microRNA

TaqMan® MicroRNA Assays were used to analyse MIR-34-a, MIR-24-1 and the endogenous control, Y1. RNA was extracted using TRIzol reagent (Invitrogen) followed by purification through Norgen Total RNA purification columns (Biotek Corporation) and reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) with an input of 10 ng total RNA per reaction. PCR reactions were carried out as per manufacturer’s instructions. Ct values were normalized using the 2^{-}\Delta\Delta Ct method [66]. Significance was assessed using Student’s t-tests with the significance criterion set at p<0.05.

Identification of biologically relevant networks and biological pathways

Ingenuity Pathway Analysis, version 7 (IPA; Ingenuity Systems, USA; https://www.analysis.ingenuity.com) The individual LRG sets, containing Affymetrix identifiers and corresponding expression values (p<0.05) were uploaded to the IPA application and analysed, as described previously [13], to identify biological functions, canonical pathways and gene networks that may contribute to LTP maintenance and stabilization. IPA builds gene networks from input data utilizing the IPA knowledge base, which contains manually curated information sourced from published literature regarding the functions and interactions of molecules. A graphical representation of the predicted interactions of so-called focus genes is produced, which may contain molecules important to the structure of the network, but not identified as differentially expressed in the input data set (e.g. transcription factors activated by phosphorylation, or microRNA not assessed by mRNA array analysis). Each focus gene is represented as a node, where shape indicates functional groups (e.g. protein kinases) and colour indicates direction of regulation of the gene in the input dataset (green indicates downregulated; red indicates upregulated). Nodes are linked by lines (edges), which indicate proposed interactions. Solid lines indicate direct interactions while dotted lines indicate indirect interactions. In our analysis each network contained up to 35 genes and has an associated score derived from a p-value that indicates the expected likelihood of the focus genes being present in a network compared to that expected by chance. Scores of 3 or above have at least a 99.9% likelihood of not being generated by chance.

The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.abcc.ncifcrf.gov/; [67]) was used for functional annotation clustering. This measures relationships among annotation terms on the basis of the degree of their co-association with genes within the inputted list. Enrichment scores of ≥1.3 are equivalent to non-log scale ≤0.05. A 2D-associated view tool was also used to examine the internal relationships among the clustered terms and genes.

microRNA target prediction

The following computational algorithms were used to predict mRNA that may be targeted by microRNA of interest: TargetScan (www.targetscan.org/), PicTar (pictar.mdc-berlin.de/), and miRanda (http://www.ebi.ac.uk/). Lists of putative mRNA targets generated by each of the three algorithms were compared. To investigate microRNA seed sequence enrichment in our data we used the web application miRvestigator Framework (http://mirvestigator.systemsbiology.net/).

Supporting Information

Figure S1 Functional Annotation Clustering (DAVID Analysis) of LRGs at 5 h. Genes with annotations related to calcium and calmodulin. Enrichment score 1.42. Associations between the 5 h LRG sets and their annotations are illustrated above. The region in grey illustrates the common annotation across the cluster with areas in black illustrating the differences in annotation.

Figure S2 Functional Annotation Clustering (DAVID Analysis) of LRGs at 5 h. Genes with annotations related to ion channels. Enrichment score 1.59. Associations between the 5 h LRG sets and their annotations are illustrated above. The region in grey illustrates the common annotation across the cluster with areas in black illustrating the differences in annotation.

Figure S3 Functional Annotation Clustering (DAVID Analysis) of LRGs at 5 h. Genes with annotations related to the synapse. Enrichment score 1.37. Associations between the 5 h LRG sets and their annotations are illustrated above. The region in grey illustrates the common annotation across the cluster with areas in black illustrating the differences in annotation.

Figure S4 Functional Annotation Clustering (DAVID Analysis) of LRGs at 24 h. Genes with annotations related to protein kinases, Enrichment score 1.98. Associations between the 24 h LRG sets and their annotations are illustrated above. The region in grey illustrates the common annotation across the cluster with areas in black illustrating the differences in annotation.

Figure S5 Functional Annotation Clustering (DAVID Analysis) of LRGs at 24 h. Genes with annotations related to phosphorylation. Enrichment score 2.58. Associations between the 24 h LRG sets and their annotations are illustrated above. The region in grey illustrates the common annotation across the cluster with areas in black illustrating the differences in annotation.

Table S1 Annotated genes differentially expressed at 5 h post-tetanization ranked by p-value.

Table S2 Annotated genes differentially expressed at 24 h post-tetanization ranked by p-value.

Table S3 Expression of significant LRG genes across time.

Table S4 Annotated genes associated with 24 h IPA Biological Function Protein Synthesis.

Table S5 Annotated genes associated with 5 h IPA Canonical Pathways.

Table S6 List of primers used for real-time qPCR.

Table S7 Targets of miR-34a predicted by TargetScan, miRanda and PicTar.

Table S8 Targets of miR-24-1 predicted by TargetScan, miRanda and PicTar.
Acknowledgments

The authors thank Diane Guévr몬트, Stephan Bisky and Sara Mason-Parker for technical support and Diane Guévr몬트 for proofreading of the manuscript.

References

1. Engert F, Bonhoeffer T (1997) Synapse specificity of long-term potentiation breaks down at short distances. Nature 388: 279–284.
2. Bonhoeffer T, Staiger V, Aertsen A (1998) Synaptic plasticity in rat hippocampal slice cultures: local “Hebbian” conjunction of pre- and postsynaptic stimulation leads to distributed synaptic enhancement. Proceedings of the National Academy of Sciences of the United States of America 85: 8113–8117.
3. Abraham WC, Logan B, Greenwood JM, Dragunow M (2002) Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. J Neurosci 22: 9626–9634.
4. Lisman J, Raghavachari S (2006) A unified model of the presynaptic and postsynaptic changes during LTP at CA1 synapses. Science’s STKE: signal transduction knowledge environment 2006: re11.
5. Williams JM, Guévrモント D, Mason-Parker SE, Luxmanan C, Tate WP, et al. (2007) Differential trafficking of AMPA and NMDA receptors during long-term potentiation in awake adult animals. The Journal of neuroscience: the official journal of the Society for Neuroscience 27: 14171–14178.
6. Cheyne JE, Montgomery JM (2008) Plasticity-dependent changes in metabotropic glutamate receptor expression at excitatory hippocampal synapses. Molecular and cellular neurosciences 37: 432–439.
7. Abraham WC (2008) Metaplasticity: tuning synapses and networks for plasticity. Nature reviews Neuroscience 9: 387.
8. Abraham WC, Williams JM (2008) LTP maintenance and its protein synthesis-dependence. Neuronol Learn Mem 8: 269–268.
9. Pang PT, Treat JK, Zaitsev E, Wos NT, Sakuta K, et al. (2004) Cleavage of proBDNF by iPA/plasmin is essential for long-term hippocampal plasticity. Science 306: 487–491.
10. Pang PT, La B (2004) Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins iPA and BDNF. Agering research reviews 3: 4–30.
11. Fonseca R, Valdua RM, Harlt FU, Bonhoeffer T, Nagerl UV (2006) A balance of protein synthesis and proteasome-dependent degradation determines the maintenance of LTP. Neuron 52: 239–245.
12. Pi HJ, Otmakhov N, Lemelin D, De Koninck P, Lisman J (2010) Autonomous CaMKII can promote either long-term potentiation or long-term depression, depending on the state of T305/T306 phosphorylation. The Journal of neuroscience: the official journal of the Society for Neuroscience 30: 6704–6709.
13. Ryan MM, Mason-Parker SE, Tate WP, Abraham WC, Williams JM (2011) Rapidly induced gene networks following induction of long-term potentiation at perforant path synapses in vivo. Hippocampus 21: 541–553.
14. Abraham WC, Williams JM (2003) Properties and mechanisms of LTP maintenance. Neuroscience 93: 463–474.
15. Abraham WC, Mason SE, Demmer J, Williams JM, Richardson CL, et al. (1993) Correlations between immediate early gene induction and the persistence for long-term potentiation. Neuroscience 56: 712–727.
16. Havik B, Rokke H, Dayge G, Stavrum AK, Brakman CR, et al. (2007) Synaptic activity-induced global gene expression patterns in the dentate gyrus of adult behaving rats: induction of immunity-linked genes. Neuroscience 148: 923–936.
17. Park CS, Gong R, Stuart J, Tang SJ (2006) Molecular network and chromosomal clustering of genes involved in synaptic plasticity in the hippocampus. J Biol Chem 281: 30095–30211.
18. Williams JM, Mason-Parker SE, Abraham WC, Tate WP (1998) Biphasic changes in the levels of N-methyl-D-aspartate receptor-2 subunits correlate with the induction and persistence of long-term potentiation. Brain research Molecular brain research 39: 621–627.
19. Matsuo R, Murayama A, Saitoh Y, Sakaki Y, Inokuchi K (2000) Identification of genes with sequence motifs from 5 h LRG list corresponding to rno-MIR330-3p. J Biol Chem 275: 13201–13204.
20. Dwek RA, Takemoto-Kimura S, Nonaka M, Adachi-Morishima A, Mano T, et al. (2007) Regulation of dendritogenesis via a lipid-raft-associated partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proceedings of the National Academy of Sciences of the United States of America 104: 12924–12929.
21. Nishimura H, Sakagami H, Ueza A, Fukumaga K, Watanabe M, et al. (2003) Cloning, characterization and expression of two alternatively splicing isoforms of Ca2+/calmodulin-dependent protein kinase I gamma in the rat brain. J Neurochem 85: 1216–1227.
22. Takenoto-Kimura S, Ageta-Ishihara N, Nonaka M, Adachi-Morishima A, Mano T, et al. (2007) Regulation of dendritogenesis via a lipid-raft-associated partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proceedings of the National Academy of Sciences of the United States of America 104: 12924–12929.
23. Ageta-Ishihara N, Takenoto-Kimura S, Nonaka M, Adachi-Morishima A, Suzuki K, et al. (2009) Control of cortical axon elongation by a GABA-driven Ca2+/calmodulin-dependent protein kinase cascade. J Neurosci 29: 13720–13729.
24. Linseman DA, Loucks FA (2008) Diverse roles of Rho family GTPases in neural fate acquisition of embryonic stem cells by maintaining chromatin in an open state. Molecular and cellular neurosciences 35: 675–687.
25. Lynch MA, Voss KL, Rodriguez J, Bliss TV (1994) Increase in synaptic vesicle protein expression at excitatory hippocampal synapses. Journal of the Society for Neuroscience 27: 14171–14178.
26. Dong E, Caruncho H, Liu WS, Smalheiser NR, Grayson DR, et al. (2003) A reelin-integrin receptor interaction regulates Arc mRNA translation in synaptoneurosomes. Proceedings of the National Academy of Sciences of the United States of America 100: 10924–10929.
27. Li, Dong E, Caruncho H, Liu WS, Smalheiser NR, Grayson DR, et al. (2003) A reelin-integrin receptor interaction regulates Arc mRNA translation in synaptoneurosomes. Proceedings of the National Academy of Sciences of the United States of America 100: 10924–10929.
28. Dong E, Caruncho H, Liu WS, Smalheiser NR, Grayson DR, et al. (2003) A reelin-integrin receptor interaction regulates Arc mRNA translation in synaptoneurosomes. Proceedings of the National Academy of Sciences of the United States of America 100: 10924–10929.
29. Fujita S, Ikeya Y, Nishikawa M, Nishiyama N, Matsuki N (2001) Docosahexaenoic acid improves long-term potentiation attenuated by phospholipase A2 inhibitor in rat hippocampal slices. British journal of pharmacology 132: 1412–1417.
30. Zhao Y, Calon F, Julien C, Winkler JW, Petasia NA, et al. (2011) Docosahexaenoic acid-derived neuroprotectin D1 induces neuronal survival via secretase- and PPARgamma-mediated mechanisms in Alzheimer’s disease models. PLoS one 6: e13016.
31. Jurado S, Benoist M, Lario A, Kaño S, Petrov CN, et al. (2010) PTEN is recruited to the postsynaptic terminal for NMDA receptor-dependent long-term depression. The EMBO journal 29: 2927–2940.
32. Moult PR, Cress A, Santos SD, Carvalho AL, Lindsay V, et al. (2010) Leptin regulates AMPA receptor trafficking via PTEN inhibition. The Journal of neuroscience: the official journal of the Society for Neuroscience 30: 4088–4101.
33. Errington ML, Galley PT, Bliss TV (2003) Long-term potentiation in the dentate gyrus of the anaesthetized rat is accompanied by an increase in extracellular glutamate: real-time measurements using a novel dialysis electrode. Philosophical transactions of the Royal Society of London Series B, Biological sciences 358: 675–687.
34. Lynch MA, Voss KL, Rodriguez J, Bliss TV (1994) Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. Neuroscience 60: 1–5.
35. Smirnova T, Larocco S, Errington ML, Hicks AA, Bliss TV, et al. (1993) Transsynaptic expression of a presynaptic glutamate receptor during hippocampal long-term potentiation. Science 262: 433–436.
36. shirts}
43. Mohrmann L, Verrijzer CP (2005) Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. Biochimica et biophysica acta 1681: 59-73.
44. Brown JH, Del Re DP, Susman MA (2006) The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. Circulation research 98: 730-742.
45. Katoh Y, Imakagura H, Futatsumori M, Nakayama K (2006) Recruitment of clathrin onto endosomes by the Tom1-Tollip complex. Biochem Biophys Res Commun 341: 143-149.
46. Vembar SS, Brodsky JL (2008) One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 9: 944-957.
47. Ambros V (2001) microRNAs: tiny regulators with great potential. Cell 107: 823-826.
48. Miki M, Vendra G, Doyle M, Kiebler MA (2010) miRNA localization in neurite morphogenesis and synaptic regulation: current evidence and novel approaches. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 196: 321-334.
49. Saha R, Schratt GM (2010) mRNAs in neuronal development, function and dysfunction. Brain Res.
50. Schratt G (2009) microRNAs at the synapse. Nat Rev Neurosci 10: 842-849.
51. Zovoilis A, Agbeneyah HY, Agis-Balboa RC, Stilling RM, Ethauer D, et al. (2011) microRNA-34c is a novel target to treat dementias. The EMBO journal 30: 4299-4308.
52. Lal A, Navarro F, Maher CA, Maliszewski LE, Yan N, et al. (2009) miR-24 inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to “seedless” 3’UTR microRNA recognition elements. Molecular cell 35: 610-625.
53. Aranha MM, Santos DM, Sola S, Steer CJ, Rodrigues CM (2011) miR-34a regulates mouse neural stem cell differentiation. PloS one 6: e21396.
54. Chen H, Shalom-Feuerstein R, Riley J, Zhang SD, Tucci P, et al. (2010) miR-7 and miR-214 are specifically expressed during neuroblastoma differentiation, cortical development and embryonic stem cells differentiation, and control neurite outgrowth in vitro. Biochem Biophys Res Commun 394: 921-927.
55. Leone V, D’Angelo D, Ferraro A, Pallante P, Rubio I, et al. (2011) A TSH-CREB1-microRNA loop is required for thyroid cell growth. Molecular endocrinology 25: 1819-1830.
56. Glass C, Sunga DK (2011) MicroRNA-1 transfects embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart. American journal of physiology Heart and circulatory physiology 301: H2038-2049.
57. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, et al. (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459: 55-60.
58. Haberland M, Montgomery RL, Olson EN (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nature reviews Genetics 10: 32-42.
59. Meberg PJ, Kaney WR, Valcourt EG, Rosatoenberg A (1996) Gene expression of the transcription factor NF-kappa B in hippocampus: regulation by synaptic activity. Brain research Molecular brain research 38: 179-190.
60. Pinheiro R, Barerwal M, Dunbar JD, Donner DB (2009) Safl2 is a novel p75NTR-interacting protein that links NGF signalling to cell cycle progression and neurite outgrowth. The EMBO journal 28: 261-273.
61. Bredy TW, Wu H, Creo C, Zellhoefer J, Sun YE, et al. (2007) Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. Learning & memory 14: 268-276.
62. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, et al. (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7: 3.
63. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dietling M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80.
64. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy—analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307-315.
65. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3.
66. Winer J, Jung CK, Shackel I, Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Analytical biochemistry 270: 41-49.
67. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols 4: 44-57.