Chapter 2

Time-dependent changes in the hippocampal synaptic membrane proteome after contextual fear conditioning

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Chapter 2

Abstract

Changes in efficacy of hippocampal synapses are important for the consolidation of contextual fear memories. In the contextual fear conditioning paradigm, the temporal placement of the shock during the conditioning session is critical to whether an aversive memory is formed; a delayed shock resulting in the formation of an associative fear memory, an immediate shock resulting in a memory deficit. In this study we performed a quantitative proteomics analysis to identify temporal changes in the hippocampal synaptic membrane proteome induced by these two conditioning protocols. One and four hours after conditioning, synaptic membrane fractions were compared by mass spectrometry. Of the 724 proteins identified, no changes in protein expression were found 1 hour after conditioning and 423 proteins were significantly regulated between the delayed and immediate shock groups 4 hours later. However, the majority of this regulation was due to temporal regulation within each conditioning group over the time course of 4 hours. On further analysis, 164 proteins were found regulated over time in the delayed shock group. Surprisingly, 273 proteins were temporally regulated after an immediate shock, pointing towards a global change in the hippocampal proteome by the presentation of an aversive stimulus alone. From the proteins that were regulated between the delayed and immediate shock groups at 4 hours after shock exposure, 48 proteins were regulated in both protocols, but in opposite directions. These data give insight into 1) changes that take place at the synapse over the time-course of contextual fear-memory consolidation, 2) changes in the synaptic membrane proteome induced by a non-associated aversive stimulus which may underlie the immediate shock deficit and, 3) differential changes in protein expression that depend on the temporal placement of an aversive stimulus during conditioning, which can be critical to whether an aversive fear memory is consolidated.
Introduction

The hippocampus is involved in memory processing and the regulation of negative emotion, in particular the control of contextual fear\textsuperscript{220}. Contextual fear learning is a form of associative aversive learning, wherein a delayed foot shock (Unconditioned Stimulus, US) that is paired with a conditioning chamber (Conditioned Stimulus, CS) elicits detectable fear responses in rodents\textsuperscript{159}. However, a foot shock given immediately upon placement in the conditioning chamber results in an immediate shock deficit, wherein the contextual cues are not associated with the aversive stimulus\textsuperscript{221}. This deficit has been attributed to failures in both CS\textsuperscript{159} and US\textsuperscript{222} processing, the molecular mechanisms of which have remained elusive\textsuperscript{223}.

The formation of lasting and stable neural representations of both the context and the aversive stimulus is dependent on the synthesis of new proteins\textsuperscript{122,223,123,126}, resulting in long-lasting changes in synaptic plasticity and efficacy in key neural connections within the hippocampus. Over the years some individual genes, proteins and pathways involved in the temporal process of memory consolidation from a transient newly learned short-term memory (STM) to a stable and lasting long-term memory (LTM) fear memories have been identified\textsuperscript{179}. Depending on the type of conditioning protocol, consolidation of aversive memories has been shown to be dependent on either one or two phases of protein translation\textsuperscript{124,224}. The first phase occurs early, i.e., within the first hour, and has been interpreted as being one in which transcription factors and immediate early genes are being expressed\textsuperscript{124,224}. The later phase, several hours after conditioning, is presumed to underlie synaptic remodeling required for longer-term memory formation during which structural and plasticity genes are being translated and their protein products trafficked into synaptic structures\textsuperscript{124,224}. For contextual fear conditioning, wherein the conditioning context is paired with a single delayed 0.7 mA foot shock, two sensitive windows of protein synthesis have been identified, one at the time of conditioning, and the second 4 h later\textsuperscript{107}. The focus thus far has been on identifying the underpinnings of glutamate receptor plasticity and its role in memory formation\textsuperscript{148,179}. To this end, by the use of pharmacologic, genetic and molecular investigation, various targets have been identified\textsuperscript{126,225}. Overall, these studies suggested that a cascade of molecular and cellular events results in a durable form of synaptic plasticity and modification\textsuperscript{90,126,148}. Moreover, both somatic and local dendritic production of new proteins including specific transcription factors, synaptic structural components, receptors and adhesion molecules have been shown to be important for hippocampal memory-associated
synaptic plasticity\textsuperscript{90,226-228}. However, profiles of protein changes at the hippocampal fear conditioned synapse over the time-course of consolidation has remained elusive.

In this study we aimed to quantitatively identify proteins that show altered hippocampal synaptic membrane expression over time, induced by a CS-US association compared with those regulated by the US alone during the period of contextual fear memory consolidation. Specifically, we assessed biochemically enriched synaptic membrane fractions and performed proteomic analyses using an isobaric tag for relative and absolute quantification (iTRAQ) followed by tandem mass spectrometry and immunoblotting. This approach identified proteins whose expression levels were altered over time due to associative learning as well as changes resulting from only the aversive attributes of contextual fear conditioning.

**Results**

Mice were conditioned using a standard contextual fear conditioning paradigm and received a single foot shock (0.7 mA, 2 s) either immediately on placement in the conditioning chamber (immediate-shock group) or after a delay of 180 s (delayed-shock group) (Fig. 1a), only the latter of which results in the formation of a fear memory (Fig. 1b). The dorsal hippocampus synaptic membrane proteome of animals that received either a delayed or an immediate shock during training was then analyzed 1 h and 4 h after conditioning (Suppl. Fig. 1).

We included proteins with 2 or more quantifiable peptides in all 6 samples of the three 8-plex replicates. Using these strict selection criteria we identified a total of 724 proteins (Table S1, online). We next categorized these into 19 functions groups as described previously\textsuperscript{27} (Fig. 1c).

Using this experimental set-up, we first analyzed changes in protein expression caused by the association of a foot-shock to the context, by comparing the proteome of delayed shock animals to immediate shock controls, 1 h and 4 h after conditioning.

**Changes in the hippocampal synaptic membrane proteome composition 1 h and 4 h after fear conditioning**

We first looked at significant changes in protein expression at fixed time points (1 h and 4 h) (Fig. 2a). In order to discern changes in protein expression specific to associative learning, the immediate shock group was used as a control, as these animals receive a foot shock but do not form a fear memory to the context (Fig. 1b)\textsuperscript{221,229}. SAM analysis revealed no significant
Figure 1. Hippocampal synaptic membrane proteome after fear conditioning. 

a) Experimental setup of proteomic analysis of hippocampal synaptic membrane fractions after fear conditioning. Delayed and immediate shock groups were analyzed 1 h and 4 h after conditioning. b) Retrieval was carried out 24 hours after conditioning in the same context. During retrieval, only animals that received a delayed shock at the end of the training exhibited a fear response, while animals that received an immediate shock during conditioning exhibit an immediate shock deficit and no fear response. Data points show mean ±SEM. c) Using a strict selection criteria of 2 or more quantifiable peptides in all 6 samples of the three 8-plex replicates, we identified a total of 724 proteins that were assigned to 19 different functional groups. 

Changes in protein expression between the delayed and immediate shock groups 1 h after fear conditioning (DS1h–IS1h) (Table S1, online). However 4 h after conditioning 423 proteins were significantly regulated in the delayed shock group when compared to the immediate shock group, with 250 proteins up regulated and 173 down regulated (Fig. 2b, Table S1, online; FDR q-value<5%). Of the 19 functional groups, the 5 groups with most regulation were ‘Cell adhesion/Transsynaptic signaling’ (77%), ‘Endocytosis’ (92%), ‘Ion balance/Transport’ (80%), ‘LGIC signaling’ (80%), and ‘Tyrosine kinase signaling’ (89%) (Fig. 2c, Suppl. Fig. 2), of which example proteins are shown (Fig. 2d). Since no significant changes were observed 1 h after conditioning and protein expression remains unchanged at this time point (Table S1, Fig. 2b), we hypothesized that the changes observed at the 4 h time point...
point could be attributed to either 1) temporal changes in protein expression in the delayed shock group which would underlie associative learning or 2) temporal changes in protein expression in the immediate shock group which cannot be attributed to learning, but to the delivery of the foot-shock alone. Indeed, we found that a majority of proteins regulated at 4 h between the delayed and immediate shock groups (67%) were also temporally regulated within each group over the time-course of 4 hours (Table S1 online, Fig. 2b).

Figure 2. Changes in the hippocampal synaptic membrane proteome composition 1 h and 4 h after fear conditioning.

a) Experimental setup of proteomic analysis of hippocampal synaptic membrane fractions after fear conditioning (see Fig. 1). Four different comparisons could be made, indicated by different colors; delayed vs. immediate shock at 1 h (green) and at 4 h (purple), and regulation over time in the delayed shock group (red) or the immediate shock group (blue).

b) Venn diagram displaying overlap in regulation after FDR analysis for the different comparisons. c) Proteins that were significantly up-regulated (red) or down-regulated (green) for the delayed vs. immediate shock at 4 h (DS4h–IS4h) were differentially enriched over 19 functional groups, with most regulation observed in 5 groups (bold). d) Representative expression changes (DS4h–IS4h; log2) as measured by iTRAQ of individual proteins belonging to the 5 most significantly regulated functional groups. Bar graphs show mean ± SEM.
Thus, we next focused on significant differences in the hippocampal synaptic membrane protein composition over the time course of 4 hours induced by either the delayed shock (DS4h–DS1h) or the immediate shock (IS4h–IS1h), given that no differences were observed at the 1 h time-point.

Figure 3. Temporal changes in hippocampal synaptic membrane protein composition induced by delayed shock

a) Experimental setup (see Fig. 1) with the regulation over time in the delayed shock group (red) highlighted. b) FDR analysis revealed 164 proteins (87 up regulated, red, and 77 down regulated, green) as being significantly regulated over time after a delayed shock. Proteins that were significantly up- or down regulated were differentially enriched over 15 functional groups, with most regulation observed in 5 groups (bold). c) Representative expression changes (DS4h–DS1h, log₂) as measured by ITRAQ of individual proteins belonging to the 5 most significantly regulated functional groups. Bar graphs show mean ± SEM. d) Expression changes of non-mitochondrial proteins (DS4h–DS1h, red; log₂) of individual proteins significantly regulated by the delayed shock only, and showing a large expression difference when compared with temporal regulation by the immediate shock (IS4h–IS1h, blue). Bar graphs show mean ± SEM.
Temporal changes in hippocampal synaptic membrane protein composition induced by delayed shock

The hippocampal synaptic membrane proteome was analyzed 1 h and 4 h after conditioning with a delayed shock (Fig. 3a). FDR analysis revealed that treatment of mice with a delayed shock resulted in a total of 164 proteins being significantly regulated over time (4 h vs 1 h) (Table S1 online). There were 87 up regulated and 77 down regulated proteins (Table S1 online) distributed over 15 functional groups (Fig. 3b). The 5 groups with most regulation were ‘Endocytosis’ (36%), ‘G-protein relay’ (27%), ‘Mitochondrion’ (42%), ‘Neurotransmitter metabolism/transport’ (30%), and ‘Protein synthesis/folding/breakdown’ (27%) (Suppl. Fig. 2), of which examples are shown (Fig. 3c). When we omitted mitochondrial proteins from the analysis prior to normalization, similar regulations and P-values were observed (Regulations, $r^2=0.9983$; P-values, $r^2=0.9913$). Indeed, whereas many mitochondrial proteins were down regulated in the DS4h–DS1h comparison, a distinct set of mitochondrial proteins was down regulated in the immediate shock comparison (IS4h–IS1h; Suppl. Fig. 3).

Of the 164 proteins significantly regulated over time after conditioning with a delayed shock, 53 were regulated by this condition alone, and 51 were regulated by this condition and between DS4h–IS4h (Fig. 2b, Table S3). From these, a total of 61 proteins, i.e., 43 mitochondrial proteins (Suppl. Fig. 3) and 18 from other classes (Fig. 3d), showed a large difference in regulation when compared with the IS4h–IS1h dataset.

Temporal changes in hippocampal synaptic membrane protein composition induced by immediate shock

We next investigated temporal changes in protein expression using the immediate shock deficit paradigm, wherein mice do not form an aversive association between the context and the shock (Fig. 1b), since they are not allowed to explore the conditioning chamber prior to delivery of the foot shock. Mice were conditioned as described above, with the difference, that the foot shock was delivered immediately upon placement into the conditioning chamber, after which the mice were allowed to explore context for 3 min. Animals were sacrificed and the hippocampal synaptic membrane proteome was analyzed 1 h and 4 h after conditioning (Fig. 4a). FDR analysis revealed that treatment of mice with an immediate shock resulted in a total of 273 proteins being significantly regulated over time (4 h vs. 1 h) (Table S1). There were 92 up regulated and 181 down regulated proteins distributed over 19 functional groups
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Figure 4. Temporal changes in hippocampal synaptic membrane protein composition induced by immediate shock

a) Experimental setup where animals were sacrificed 1h and 4 h after immediate shock-conditioning and temporal changes in the hippocampal synaptic membrane proteome were measured at 4 h when compared to 1 h after conditioning with an immediate shock. b) FDR analysis revealed 273 proteins (92 up regulated, red, and 181 down regulated, green) as being significantly regulated over time after an immediate shock. Proteins that were significantly up- or down regulated were differentially enriched over 19 functional groups, with most regulation observed in 5 groups (bold). c) Representative expression changes (IS4h–IS1h, blue; log2) as measured by ITRAQ of individual proteins belonging to the 5 most significantly regulated functional groups. Bar graphs show mean ± SEM. d) Representative expression changes (log2) from 5 functional groups, as measured by the immediate shock only, and showing a large expression difference when compared with the temporal regulation by the delayed shock (DS4h–DS1h, red). Bar graphs show mean ± SEM.

(Table S1 online, Fig. 4b). The 5 groups with most regulation were ‘Cell adhesion/Transsynaptic signaling’ (64%), ‘Cell metabolism’ (55%), ‘Endocytosis’ (68%), ‘G-protein relay’ (53%), and ‘Ion balance/Transport’ (57%) (Supplemental Fig. 2), of which examples are shown (Fig. 4c). Of the 273 proteins significantly regulated over time after conditioning with a delayed shock, 28 were regulated by this condition alone, and 185 were regulated by this condition and between DS4h–IS4h (Fig 2b, Table S1 online). From these, a total of 125 proteins, i.e., 22 mitochondrial proteins (Suppl. Fig. 3) and 103 from other classes
(Table S1 online), showed a large difference in regulation when compared with the DS4h–DS1h dataset. The 103 proteins belonged to different classes, of which proteins from ‘LGIC signaling’, ‘G-protein relay’, ‘GPCR signaling’, ‘Exocytosis’, and ‘Excitability are shown (Fig. 4d).

**Differential changes in hippocampal synaptic membrane protein composition induced by immediate shock stress and by delayed shock-aversive learning**

Besides proteins that were found to be regulated over time by either delayed or immediate shock, we found a total of 60 proteins that were regulated in both conditions, of which 50 were shared in the DS4h–IS4h comparison (Fig. 2b, Table S1 online). From these 50, 48 had opposite regulation in the IS4h–IS1h comparison (Table S1 online). These proteins were represented by 12 functional groups. From these, 2 groups that stood out were ‘Cell metabolism’ (21%), and ‘Endocytosis’ (32%), each with 8 proteins up regulated in the DS4h–DS1h comparison and down regulated in the IS4h–IS1h comparison, from which examples are shown (Fig. 3c, Fig. 4c)

**Confirmation of iTRAQ analysis by immunoblotting**

It is interesting to note that of the 15 proteins identified involved in ligand-gated ion channel signaling, 12 are significantly down regulated in the DS4h–IS4h comparison (Fig 2d, Fig. 5a). Moreover, while not being regulated during aversive learning in our paradigm (Fig. 5a), these changes are merely the result of up regulation due to foot shock exposure. Furthermore, we observed a concomitant increase of several scaffolding proteins (protein clustering) after conditioning animals with an immediate shock (Fig. 5a), pointing to increased plasticity at hippocampal synapses after delivery of a foot shock.

To confirm the iTRAQ results for significant protein-regulation for the comparisons discussed above, immunoblotting was performed on an independent set of samples (Fig. 5b). To this end, following a conditioning session with either an immediate or a delayed shock, the level of expression for a representative set of proteins was measured. We observed a similar regulation of expression in this independent set of samples as seen with the iTRAQ data. In line with this, a significant up regulation over time was observed for the glutamate receptor subunits GluA2 (Gria2) and GluN2B (NR2B/Grin2b) in the immediate shock groups (Fig. 5b). In addition, a strong and specific temporal regulation of Disk large homolog 3 (DLG3) was observed over time with a delayed shock (Fig. 5c). Finally, significant changes in expression of Excitatory amino acid transporter 1 (EAAT1) and Protein kinase C and casein
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**Figure 5. Validation of glutamate receptor and related proteins by immunoblotting.**
a) Representative expression changes (mean±SEM, log₂) for subunits of NMDA (GluN) and AMPA (GluA) receptors and scaffolding proteins in the three different comparisons (DS4h–IS4h, purple; DS4h–DS1h, red; IS4h–IS1h, blue), with significant (*p<0.05) and trend (#p<0.1) indicated. b,c) Expression changes over time (log₂) as measured by iTRAQ (light grey) and immunoblot (dark grey). b) The up regulation of glutamate receptor subunits, Glu2 and GluN2b over time after the presentation of an immediate shock (IS4h–IS1h) was confirmed by immunoblotting in an independent set of samples (n=6, GluA2: p=0.020, GluN2b: p=0.036). c) The down regulation of Dlg3 over time after the presentation of a delayed shock (DS4h–DS1h) was confirmed by immunoblotting in an independent set of samples (n=6, p=0.024). The differential regulation in expression over time of Slc1a3 (EAAT1) and Pacsin1, depending on the conditioning protocol used (delayed shock or immediate shock) was confirmed by immunoblotting for the DS4h–DS1h comparison (n=6, Slc1a3: p=0.038; Pacsin1: p=0.042), and the IS4h–IS1h comparison (n=6, Slc1a3: p=0.013, Pacsin1: p=0.001). Bar graphs show mean ± SEM.

kinase substrate in neurons protein 1 (Pacsin1) were observed for both the delayed and immediate shock groups, in opposite directions (Fig. 5c). These results corroborate the protein expression changes measured by quantitative iTRAQ proteomics. Regulation measured by immunoblot was higher than that measured by iTRAQ (Fig. 5), which has been observed in several other studies.230,231
Discussion
Here we characterized the dorsal hippocampal synaptic membrane proteome after contextual fear conditioning using a proteomics approach that allowed us to identify changes in protein levels that take place up to 4 h after conditioning induced by both delayed and immediate shock protocols. We identified a large number of proteins that were altered over the time course of memory consolidation of aversive learning. However, presentation of a shock alone results in an even bigger change in the proteomic profile with time.

We focused this study on identifying protein expression changes after either a delayed or an immediate shock, only the former of which results in the formation of an aversive associative memory. Both these conditioning groups were taken along for comparison in order to monitor changes that were caused by learning (delayed foot shock), stress (immediate foot shock) and the interaction of the two in the consolidation of an aversive associative memory. The two time points chosen were based on the observation that the type of conditioning protocol we used results in two waves of protein synthesis required for consolidation, one around the time of conditioning (at 1 h) and one about 4 h later. It is important to note that only a small percentage of synapses and cells within the hippocampus actively encode the memory. Since this proteomic study was done on the entire dorsal hippocampus the significant changes in protein expression we observe could be due to 1) changes at synapses that are activated specifically by learning and 2) global changes at the cellular and network level in non-learning synapses of activated cells and cells that are not learning-specific. These comprehensive changes are also important as they might contribute to global protein-synthesis mechanisms in the hippocampus that establishes and regulates a state that can allow synaptic changes and plasticity in a smaller subset synapses that consolidate a long-term memory.

Using this experimental setup, it was possible to make four comparisons, namely 1) DS1h–IS1h, 2) DS4h–IS4h, 3) DS4h–DS1h, 4) IS4h–IS1h. The first two of these comparisons yield information on protein changes at a static time-point caused due to the association of a foot-shock to the conditioning context (delayed shock) versus the shock only (immediate shock), while the latter two comparisons result in a temporal profile of protein expression changes due to either learning (DS4h–DS1h), stress (IS4h–IS1h) or a combination of the two. With regard to the comparisons at a static time point, we found no differences between the delayed and immediate shock groups 1 h after conditioning. This could be attributed to the fact that we study synaptic membrane fractions and any changes in protein expression at this time-point may not yet be observed at the synapses. Alternatively, the
detection threshold using in an open screening might be too low to detect subtle changes, or changes in a few proteins. Using the current method, 1 h post conditioning synaptic protein expression appears to be unchanged irrespective of whether an animal learns to associate an aversive stimulus to a context or not. On the other hand, we found very large differences between the hippocampal synaptic membrane proteome of these groups 4 h after conditioning. Although interesting, 67% of these changes in protein expression could be attributed to temporal changes in protein expression caused by either the immediate shock or the delayed shock (by stress or by learning). In order to tease out these differences, we next focused on significant differences in protein expression over time caused by either the delayed or the immediate shock, namely DS4h–DS1h and IS4h–IS1h, given that no differences were observed at the 1 h time-point.

After a delayed shock 164 proteins were significantly regulated over time (4h–1h). There appeared to be an up regulation of a majority of proteins involved in endocytosis, G-protein relay and protein synthesis/folding/breakdown and a down regulation of mitochondrial proteins and those involved in neurotransmitter metabolism/transport. The direction of regulation of these proteins suggests that a majority of dorsal hippocampal synapses undergo a temporal decrease in synaptic efficacy and neurotransmission. It might be speculated that the changes we observe are more global, and are responsible for regulating and maintaining an optimal balance for plasticity events in a smaller subset of synapses that encode the memory. This is in line with the idea that within the hippocampus only a sparse population of neurons actively participates in the formation of a memory\textsuperscript{148,188}. A large number of the remaining non-activated synapses and cells may partake in post-learning cellular- and network-level information sculpting through LTD-like mechanisms that reduce basal firing rates and increase signal-noise ratio, thereby enabling the activated neurons to select and shape learning-acquired synaptic information for long-term encoding\textsuperscript{233}. Furthermore, these data also indicate that the activation of second messenger systems may result in protein synthesis and structural modifications underlying the remodeling of synapses that is required for memory consolidation\textsuperscript{90,126,149}. It is interesting to note that we see no temporal increase in glutamate receptor levels 4 h after conditioning, which is in contradiction to a previous study that showed that overexpressed GFP-GluA1 receptors were observed in the hippocampus starting from 2 h after conditioning and lasting for more than 24 h\textsuperscript{148}. There may be several reasons for this discrepancy, the main one being that this increase at early time points was observed for somatic receptors that were in large abundance due to overexpression, whereas our study focuses on expression changes at the synapse.
Somewhat surprisingly, we found that delivery of an immediate shock results in huge changes in the synaptic membrane proteome. A total of 273 proteins were significantly regulated, with most regulation being observed in proteins involved in cell adhesion/ transsynaptic signaling, cell metabolism, endocytosis, G-protein relay and ion balance/transport. This leads us to believe that shock alone causes large-scale changes in the hippocampal synapses, despite the fact that this does not lead to a fear memory of the event. Besides being an important brain locus for the processing of contextual memories, the hippocampus is vulnerable to stressful experiences and is also involved in the processing of the shock. In the immediate shock group, animals do not explore the environment prior to receiving the shock, and so perceive it different than with a delayed shock. In case of the latter, the context has been explored before delivery of the aversive stimulus resulting in the formation of an aversive association. This might result in the differential effect we observe on synaptic protein levels within the hippocampus, which in turn might affect the consolidation of memory and subsequent behavior. In line with this, pre-exposure to the conditioning context has been shown to alleviate the immediate-shock deficit in associative fear learning. Stress has been shown to engage the same underlying mechanisms as LTP in the hippocampus, impairing and occluding subsequent synaptic potentiation that underlies learning. Stress may also interfere with memory formation by affecting metaplasticity and thereby the optimal balance of synaptic plasticity within memory circuits. At the molecular level we see an exclusive up regulation of synaptic glutamate receptors in animals that receive an immediate shock. It would be interesting to test whether reversing this regulation rescues the immediate shock deficit in these animals.

In total, 60 proteins were significantly regulated over time in both the delayed shock and immediate shock groups (Fig. 2b). These are of interest because the direction of regulation of the majority, i.e., 48, of these proteins is different between the two groups. It is interesting to note that most of these proteins are involved in either cell metabolism or endocytosis, which points towards synaptic modification depending on the conditioning protocol used. For instance, Protein kinase C and casein kinase substrate in neurons protein 1 (Pacsin1, also named Syndapin1) shows a strong up regulation after a delayed shock, while being strongly down regulated over time by the presentation of an immediate shock stimulus. This protein is involved in the clustering of the endocytotic machinery and the activity-dependent endocytosis of NMDARs, potentially conferring a differential spatiotemporal role for these receptors that is dependent on the conditioning protocol used. Specifically, Pacsin1 is required for the endocytosis of the GluN3A (NR3A) subunit of the NMDAR.
thereby altering calcium permeability and magnesium sensitivity, in which GluN3A acts as a dominant negative regulator of NMDA current\textsuperscript{239,240}. A down-regulation of GluN3A, possibly through action of Pacsin1\textsuperscript{237}, is observed prior to critical periods of plasticity and is very important for synapse maturation and memory consolidation\textsuperscript{239}. In line with this, the temporal up regulation of Pacsin1 due to learning might result in increased NMDAR function and synaptic plasticity. Moreover, Pacsin1 has recently been found to interact with PICK, in which down regulation of Pacsin1 leads to less internalization of AMPAR\textsuperscript{241}. Thus, the increase in AMPAR after stress could be related to the concomitant down regulation of Pacsin1. Alternatively, a role for Pacsin1 on the presynaptic site is its involvement in vesicle recycling\textsuperscript{242,243}. In particular under high neuronal activity, synaptic vesicle retrieval occurs via a Dynamin-dependent way, in which a dephosphorylated form of Dynamin binds to Pacsin1. During learning, increased cellular activity might recruit this process, requiring an up regulation of Dynamin, which has a similar pattern of regulation in our paradigms as Pacsin1.

Thus, although the context and delivery of a shock (but not its temporal placement) are common features in our learning and stress paradigm, the association of a delayed foot shock with the conditioned stimulus appears to alter protein expression of this selected group that might underlie the difference in behavioral outcome observed (Fig. 1b). An active memory trace is formed only in the presence of a delayed shock and not with the delivery of an immediate shock\textsuperscript{12,229}. It can be speculated that exploration of the context prior to the delivery of the foot shock, results in a reversal of expression of a critical set of proteins regulated due to foot-shock stress and this might allow for an association between the context and shock to be consolidated.

Taken together, this proteomic study gives a new insight in the change of protein levels in the synaptic membrane proteome of the dorsal hippocampus after contextual fear conditioning. These changes contribute to the remodeling of synapses and synaptic plasticity that is required for the processing of an aversive and stressful stimulus, and the association between this aversive stimulus and a context. This study shows that temporal placement of a foot shock during conditioning greatly influences temporal protein expression that can be critical to whether an aversive fear memory is consolidated or not.
Methods

Animals and fear conditioning

Animals - All experiments were carried out in accordance to the Animal User Care Committee of the VU University. Adult male C57BL/6J mice (20–25 g, Charles River) were individually housed at a 12-h light/dark cycle with ad libitum access to food and water. Experiments were performed during the light phase. All mice were 9–10 weeks of age during testing.

Contextual fear conditioning - All experiments were carried out as described previously in a fear conditioning system (TSE-Systems, Bad Homburg, Germany). Training was performed in a Plexiglas chamber with a stainless steel grid floor with constant illumination (100–500 lx) and background sound (white noise, 68 dB sound pressure level). The chamber was cleaned with 70% ethanol prior to each session. For the delayed shock groups, training consisted of placing mice in the chamber for a period of 180 s after which a 2 s foot shock (0.7 mA) was delivered through the grid floor. Mice were returned to their home cage 30 s after shock termination. For the immediate shock group a 0.7 mA, 2 s foot shock was delivered immediately on placement in the conditioning chamber, after which the mice were allowed to explore the context for 210 s (180 s + 30 s). Using this protocol, we have previously shown that a fear memory is consolidated on presentation of a delayed shock, whereas a memory deficit is observed in the immediate shock group.

Preparation of synaptic membrane fractions

We dissected the dorsal half of the hippocampus at the desired time points (1 h and 4 h after conditioning) from fresh brains and stored them at –80 °C (Suppl. Fig. 1). Synaptic membrane fractions were isolated (pooled from three mice, n=6 biologically independent samples/group) on a discontinuous sucrose gradient, as described previously. Protein concentration was measured by a Bradford assay (Biorad). For each sample, 75 µg of protein was transferred to a fresh tube and dried in a SpeedVac overnight.

Protein digestion and iTRAQ labeling

Detailed protocols have been published previously. Briefly, 75 µg of synaptic membrane protein samples were dissolved in 0.85% RapiGest (Waters Associates, Milford, MA, USA), alkylated with methyl methanethiosulfonate, and digested with trypsin (sequencing grade; Promega, Madison, WI, USA). Two samples for each condition were put on a single 8-plex (Suppl. Fig. 1), and a total of 3, 8-plex iTRAQ experiments were performed resulting in a
total of 6 biological replicates. The labeling with iTRAQ reagents for each experiment was as follows: Immediate shock + 1 hour (IS1h): 113, 117; Immediate shock + 4 hours (IS4h): 114, 118; Delayed shock + 1 hour (DS1h): 115, 119; Delayed shock + 4 hours (DS4h): 116, 121.

Two-dimensional liquid chromatography (2DLC) and MALDI-MS/MS

As described previously, lyophilized iTRAQ labeled samples were separated in two dimensions. First by a strong cation exchange column (2.1 x 150 mm polysulfoethyl A column, PolyLC), and subsequently on an analytical capillary reverse phase C18 column (150 mm x 100 μm i.d. column) at 400 nL/min using the LC-Packing Ultimate system. Following this, peptides were separated using a linear increase in concentration of acetonitrile from 4 to 72% in temporal gradients. The eluent was mixed with matrix (7 mg of recrystallized α-cyano-hydroxycinnaminic acid in 1 mL 50% acetonitrile, 0.1% trifluoroacetic acid, 10 mM ammonium dicitrate), delivered at a flow rate of 1.5 μL/min and robotically deposited onto an Applied Biosystems matrix-assisted laser desorption ionization for a total of 384 spots. The samples were then analyzed on an ABI 4800 proteomics analyzer (Applied Biosystems, Forster City, CA). Peptide collision-induced dissociation was performed by air at 2 kV. MS/MS spectra were collected from 2500 laser shots. Peptides with signal-to-noise ratios over 50 at the MS mode were selected for MS/MS analysis, at a maximum of 30 MS/MS per spot. The precursor mass window was set to a relative resolution of 200. Peaklists were extracted from the instrument database using TS2Mascot software (MatrixScience).

Protein identification

Protein identification and quantification have been described previously in detail. Briefly, Mascot (MatrixScience) searches were performed against the Swissprot database (release January 2009; 16,028 mouse entries) and the larger but more redundant NCBI database (release January 2009; 140,210 mouse entries) using the GPS Explorer software (version 3.6, Applied Biosystems) to annotate spectra. MS/MS spectra were searched with trypsin specificity and fixed iTRAQ modifications on lysine residues and N-termini of the peptides and methylthio modifications on cysteine residues. Oxidation on methionine residues was allowed as a variable modification. Mass tolerance was 150 ppm for precursor ions and 0.5 Da for fragment ions, while allowing a single site of miscleavage. The false discovery rate (FDR) for peptides identification was calculated using a randomized database. Protein redundancy in the result files was removed by clustering the precursor protein sequences at a threshold of 90% sequence similarity over 85% of the sequence length. Subsequently all
peptides were matched against the protein clusters and only those peptides were included that mapped unique to one protein. Proteins were considered for quantification if at least three unique peptides had a confidence interval of ≥95%, and at least three peptides were identified in all three experiments.

**Protein quantification and identification of differentially expressed proteins**

As described previously\(^{231}\), iTRAQ areas (m/z 113–121) were extracted from raw spectra and corrected for isotopic overlap using GPS explorer. Peptides with iTRAQ signature peaks of less than 1500 were not considered for quantification. To compensate for the possible variations in the starting amounts of the samples, the individual peak areas of each iTRAQ signature peak were log transformed to yield a normal distribution, and normalized to the mean peak area of every sample. Protein abundances in every experiment were determined by taking the average normalized standardized iTRAQ peak area of all unique peptides annotated to a protein. Finally, the standardized protein means were used to calculate the average difference between the 4 h and 1 h time points after a particular shock treatment. To assess whether differences had occurred by chance or could be deemed significant, we calculated permutation-derived false discovery rates (FDR), using Significance Analysis of Microarrays (SAM)\(^ {247}\) as implemented in the Multi Experiment Viewer software (MeV, version 4.6.1)\(^ {247,248}\). SAM uses a data resampling-based method and creates randomized data distributions in order to estimate false positive rates\(^ {247}\), and can be successfully applied to proteomics data\(^ {230,249-252}\). The SAM q-values reflect for each protein the number of empirically determined false-positives at the significance level of the respective protein. The false discovery rate (FDR) levels in our results thus hold information about individual proteins, and should not be interpreted as a global FDR. Changes in protein expression are considered to be significant when the FDR is <5%.

**Functional protein group analysis**

All quantified proteins assigned to one of 19 functional synaptic protein groups as defined previously\(^ {253,254}\).

**Immunoblot**

For all groups 5 μg/ sample was dissolved in SDS loading buffer and used for immunoblotting (Biorad) using antibodies against GRIA2 (Neuromab, 1:1,000), GluN2B (Neuromab, 1:1,000), EAAT1 (Santacruz, 1:1200), DLG3 (Neuromab, 1:1000), PACSIN
(Santacruz, 1:500). These proteins were chosen based on the ready availability of antibodies. To correct for input differences, we compared the total protein amount from each sample\textsuperscript{229,230,244}, as this is a reliable method that is not dependent on a single protein for normalization. Samples were loaded on Criterion Stain free gels (Biorad), activated and scanned using a Gel Doc Easy Scanner (Biorad) for 5 minutes. Gel volumes obtained from each lane were background subtracted and used for normalization with the corresponding background subtracted band intensity values from the western blot.
Supplemental material

Figures and legends

Supplemental table S1 can be obtained online by contacting the author.

Supplementary Figure S1. Experimental setup of proteomic analysis.
After the fear conditioning paradigm the dorsal hippocampus is dissected, and synaptic membranes are isolated using sucrose gradients from a pool of 3 mice, with n=6 biological replicates. Subsequent labeling by the eight different iTRAQ labels allows these eight samples to run in a single set, with 3 independent sets being run.
Supplementary Figure S2. Functional group regulation.
Proteins were divided over 19 different functional classes that were previously defined\(^\text{253}\). For the three different comparisons (DS4h–IS4h, purple; DS4h–DS1h, red; IS4h–IS1h, blue), the % of each class that showed significant regulation (FDR) is shown. The five most regulated classes (six for DS4h–IS4h) are colored and given in bold. Mind that each class contained at least 9 proteins, except ‘peptide/Neurotrophin signaling’ that contained only 2 proteins (bold, gray).

Supplementary Figure S3. Specific regulation over time for mitochondrial proteins.
Expression changes of mitochondrial proteins (log\(_2\)) of individual proteins significantly and specifically regulated by the delayed shock only (DS4h–DS1h; a) or the immediate shock only (IS4h–IS1h; b) over time. Bar graphs show mean ± SEM.