Master's Thesis

Master of Science in Systems Biology

Investigating the β-adrenergic modulation of the acute stress induced molecular response in the mouse hippocampus

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Abstract

Acutely stressful experiences can trigger neuropsychiatric disorders and impair cognitive processes. The norepinephrine (NE) secreting locus coeruleus is highly involved in the stress reaction and has therefore been studied extensively. Previous studies have shown strong changes in transcription and phosphorylation of proteins under acute stress (AS) in the hippocampus (HC). We investigated the LC specific signalling in the ventral hippocampus through the adrenergic beta-receptors by blocking with propranolol and sotalol. We identified stress sensitive genes whose transcriptional changes could be prevented through injection of propranolol. In a meta-analysis of multiple experiments targeting the LC-NE-HC system we found *Dio2*, *Ppp1r3c*, *Ppp1r3g* and *Sik1* consistently reacting to NE signalling highlighting them as possible downstream targets of the beta-receptors. We successfully induced transcriptional changes in *Dio2*, *Ppp1r3c* and *Ppp1r3g* trough specific excitation of HC projecting LC neurons. Further we pursued the possibility of phosphorylation being the medium of signal transduction missing between receptor activation and transcriptional changes. In the many AS related changes in the phosphoproteome we found 16 phosphopeptides whose changes could be counteracted by blocking the beta-receptors of which the transcripts of *Cacna1a*, *Sipa1l1* and *Tprg1l* are likely targets involved in neuronal signalling and modulation and possible targets for future research.

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Introduction

Stress is often associated with disease, yet a healthy stress response to a stressor on physiological and behavioural level is essential for survival and health. Responding to a stressor in a healthy way is usually beneficial yet under certain circumstances acute stressful experiences can trigger numerous neuropsychiatric disorders and impair cognitive processes. (Romero et al., 2015).

When the brain detects a threat the stress machinery induces activation of a variety of physiological responses through a multitude of signalling pathways. One essential system in the stress response that has been studied extensively is the hypothalamus-pituitary-adrenal (HPA) axis. When triggered this system results in the production of glucocorticoids in the adrenal glands. These steroids act on the glucocorticoid receptors present in both neurons and glia cells of the brain through which they can act as transcription factors and possibly induce long lasting changes (de Kloet et al., 2005; Lupien et al., 2009).

There are numerous feedback loops acting on the HPA system to restore homeostasis, one of them originating from the hippocampus (HC). This brain region located in the medial temporal lobe and part of the limbic system is involved in memory formation among other things by binding stimuli to their spatiotemporal context (Manns et al., 2007; Teyler & DiScenna, 1985).

In Alzheimer's disease and other forms of dementia the HC is one of the first brain regions to show damage (Dubois et al., 2016). There are a multitude of possibilities for this damage, amongst other there is a link between Alzheimer's disease and chronic stress (Lyons & Bartolomucci, 2020). Overall, the hippocampus has been shown to be highly susceptible to stress and has been studies in this context extensively (Kim & Diamond, 2002).

The HPA axis targeting the HC through glucocorticoids is not the only stress related system with impact on the hippocampus. The locus coeruleus (LC) norepinephrine (NE) system acts throughout the brain and if activated the resulting increase of NE heightens arousal, alertness, readiness for action and stress response which helps memory retention (Benarroch, 2009). The response to NE follows an inverted U-shape meaning that alertness etc. only increases with NE abundance to a certain point and then regresses in a kind of "escape" mechanism (Baldi & Bucherelli, 2005).

The LC contains one of the several noradrenergic cell groups distributed through the brainstem. It sits in the hindbrain and only consists of a few hundred neurons in mice and estimated 15'000 in primates (Sharma et al., 2010). The neurons of the LC project far into the brain, reaching into the forebrain, brainstem, cerebellum as well as the spinal cord (Fig. 1) (Breton-Provencher et al., 2021). For the most part the LC is the only noradrenergic nucleus with axons projecting into the hippocampus, neocortex and cerebellum (Szabadi, 2013). Similar to the HC the LC also shows degeneration in Alzheimer's disease. Especially in cases with a high dementia score and relatively young age of death, loss of up to 80% of all neurons in the locus coeruleus has been observed (Bondareff et al., 1982).

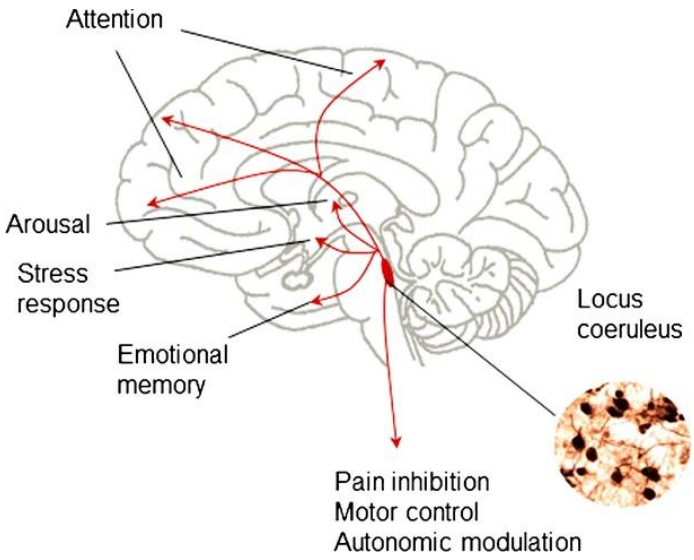
The neurotransmitter NE released from the LC is part of the catecholamine family also including epinephrine and dopamine. NE is synthesized in the synaptic vesicles of NE producing neurons starting from dopamine by the dopamine β-hydroxylase. Dopamine beforehand is synthesized in a series of enzymatic reactions from the amino acid tyrosine in the cytosol (Yaffe et al., 2018).

A catecholamine closely related to norepinephrine is epinephrine (E). Its structure is very similar only differing in methylation of its primary amine. Epinephrine is produced in the adrenal glands Like NE it is part of the fight or flight response and prepares the body increasing blood flow to the muscle (edited by Rodney A. Rhoades, n.d.), increasing heartrate (BROWN et al., 1979) and blood sugar level. NE and

E both can activate the same receptors but are released in different parts of the body. NE primarily acts as neurotransmitter in the brain while E is released into the bloodstream and acts as hormone throughout the body.

The NE released in the brain into the synaptic cleft transmits signals by binding to and subsequent activation of alpha- and beta-adrenergic receptors located on the cell surface. Both alpha- beta-adrenergic receptors consist of multiple subtypes but all of them function as G-protein-coupled receptors passing on the received signal through complex second messenger systems. Through alpha -2 receptors NE mostly exercises an inhibitory effect while binding to alpha-1 and all three beta-receptor subtypes (β_1, β_2 and β_3) induces excitatory effects. Alpha-1 are located presynaptically for the most part which results in a negative feedback-loop and less norepinephrine release (James Ritter, 2018).

Figure 1: Location and projections of the locus coeruleus (Benarroch, 2018)**.**



Binding of NE to these receptors can be inhibited using receptor antagonists. Two drugs competitively inhibiting beta-receptors are propranolol and sotalol, both in used for their antiarrhythmic effects to prevent abnormal heart rhythm sold under a variety of brand names. These drugs are unselective beta-blocker, meaning they inhibit all three subtypes of beta-receptors. Propranolol additionally weakly inhibits alpha-2 receptors while sotalol inhibits fast acting potassium channels, additionally reducing the heart rate(BERTRIX et al., 1986; Srinivasan, 2019).

Propranolol is more lipophilic and can pass the blood-brain barrier therefore also acting in the brain. Sotalol on the other hand is more lipophilic and cannot pass the blood brain barrier restricting its action only on the beta-receptors in the periphery.

Previous experiments have shown that acute stress (AS) induces vast molecular changes in the mouse hippocampus across different levels. AS triggers a fast but robust wave of phosphoproteomic changes that are followed by slower waves of transcriptional changes (von Ziegler et al., 2022). Although there is a good understanding of the different molecular changes induced by acute stress, the upstream processes responsible for them are not fully understood.

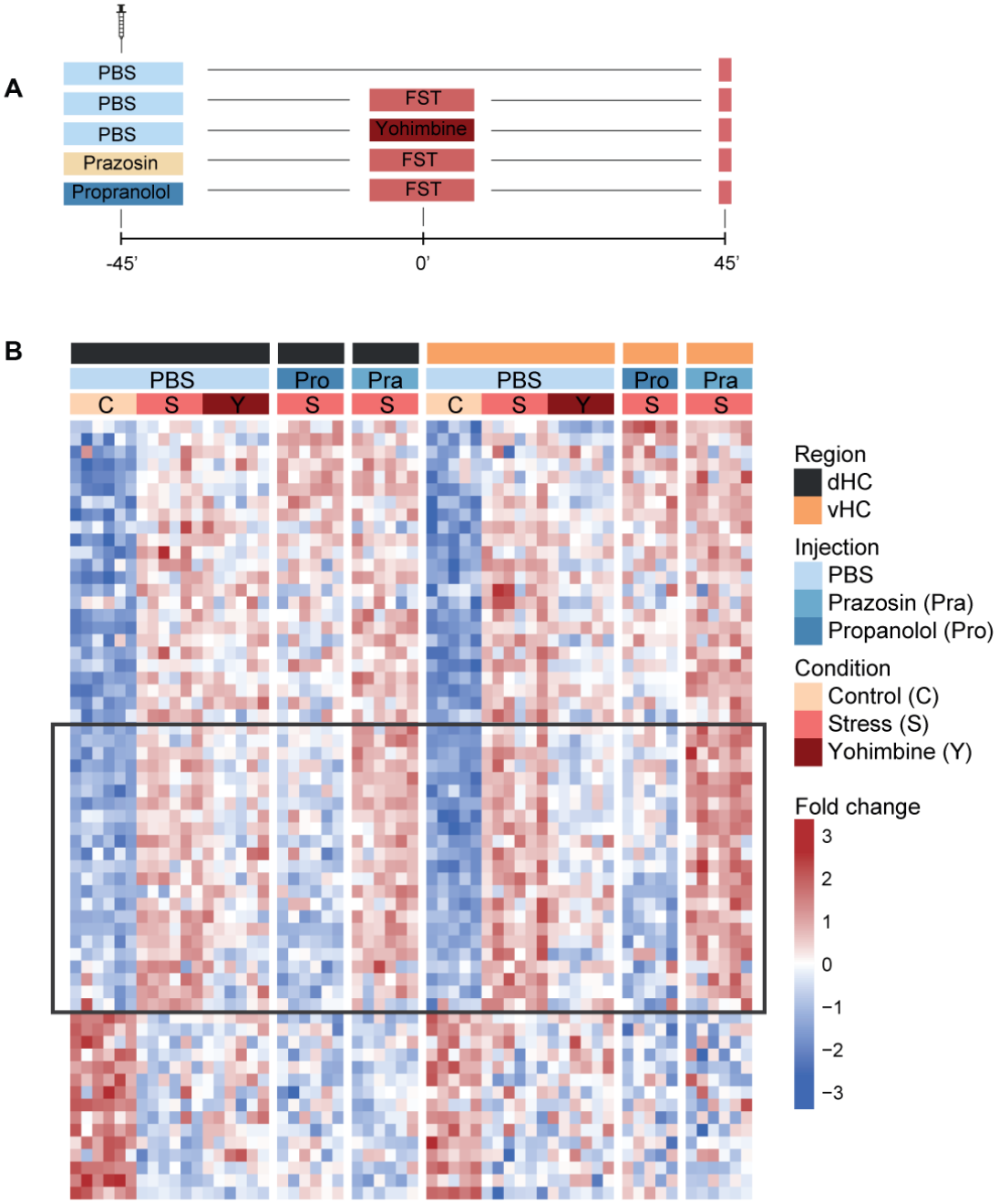
Going forward the goal is to try to elucidate how other brain regions contribute to the molecular events in the hippocampus through the release of signalling molecules.

One probable upstream node point are the adrenergic receptors which were the target of a preliminary experiment (not published). Looking at the transcription using mRNA-sequencing our group found in a preliminary experiment that propranolol, a systemic β-adrenergic antagonist, significantly altered stress induced transcriptional activity of multiple genes (Fig. 2B) in both dorsal (dHC) and ventral (vHC) hippocampus. It was found that while these genes were altered both by the injection of yohimbine (a compound that increases LC dependent NE release) and swim stress, injection of propranolol prior to swim stress prevents some of these effects. On the other hand, the tested alpha-receptor blocker prazosin did not prevent but even reinforce the same transcriptional changes. Reinforcement of the changes could possibly be explained by an increase in free NE levels as prazosin prevents NE alpha-receptors access. With more free NE there is an increase on the beta-receptor signalling.

While these preliminary results are promising and pinpoint towards the beta-receptors, the incompleteness of the experimental design leaves uncertainty and open questions:

1) Due to the absence of a propranolol injected control group, it remains unclear if the observed propranolol effects are truly stress related or mere baseline effects of propranolol injection.
2) Propranolol interacts with beta-adrenergic receptors in the brain and in the periphery. In the periphery beta-receptors are activated by epinephrine released by the adrenal glands and are not dependent on the LC. This leads to numerous physiological changes such as increased hearth rate, changes in vasodilation as well as activation of glycolysis and lipolysis. All these changes may have an indirect effect on hippocampal transcription. To further pin-point the LC specific effect on the hippocampal transcriptome through NE, methods to separate systemic from brain specific effects are needed.
3) Sex differences have been described in the stress literature before, however the experiment was performed exclusively with male mice. It remains unclear if male and female mice may react differently to propranolol administration.
4) It remains unclear through which upstream cascades adrenergic receptor blocking could induce the observed alterations in transcription.

Figure 2 Design and results of preliminary experiment blocking beta- and alpha-receptors during stress.



A) Experimental design. Animals were injected with either PBS, prazosin or propranolol and exposed to stress through forced swim test (FST) or yohimbine injections. **B)** Transcriptional changes after acute stress exposure with indication of transcripts effected by propranolol injection in dorsal (dHC) and ventral (vHC) hippocampus. Black box highlights genes that are inhibited by propranolol.

Results

Transcriptomics

To verify the preliminary findings of propranolol affecting the transcriptome in the hippocampus after acute stress, a more comprehensive experiment was needed with unstressed propranolol and sotalol groups to single out stress dependent drug effects. We used sotalol in combination with propranolol as it should not pass the blood brain barrier. Then by comparing the results with propranolol injections we can differentiate brain specific and peripheral effects.

Acute stress (AS) induces strong transcriptional changes in a variety of genes.

Male and female mice were injected intraperitoneally (i.p.) with either PBS, propranolol or sotalol (Fig. 3A, injection group). 45 minutes after injection animals were subjected to swim stress while control animals were kept in the homecage (treatment). 45 minutes after stress exposure the ventral hippocampus (vHC) was collected and analysed using illumina sequencing.

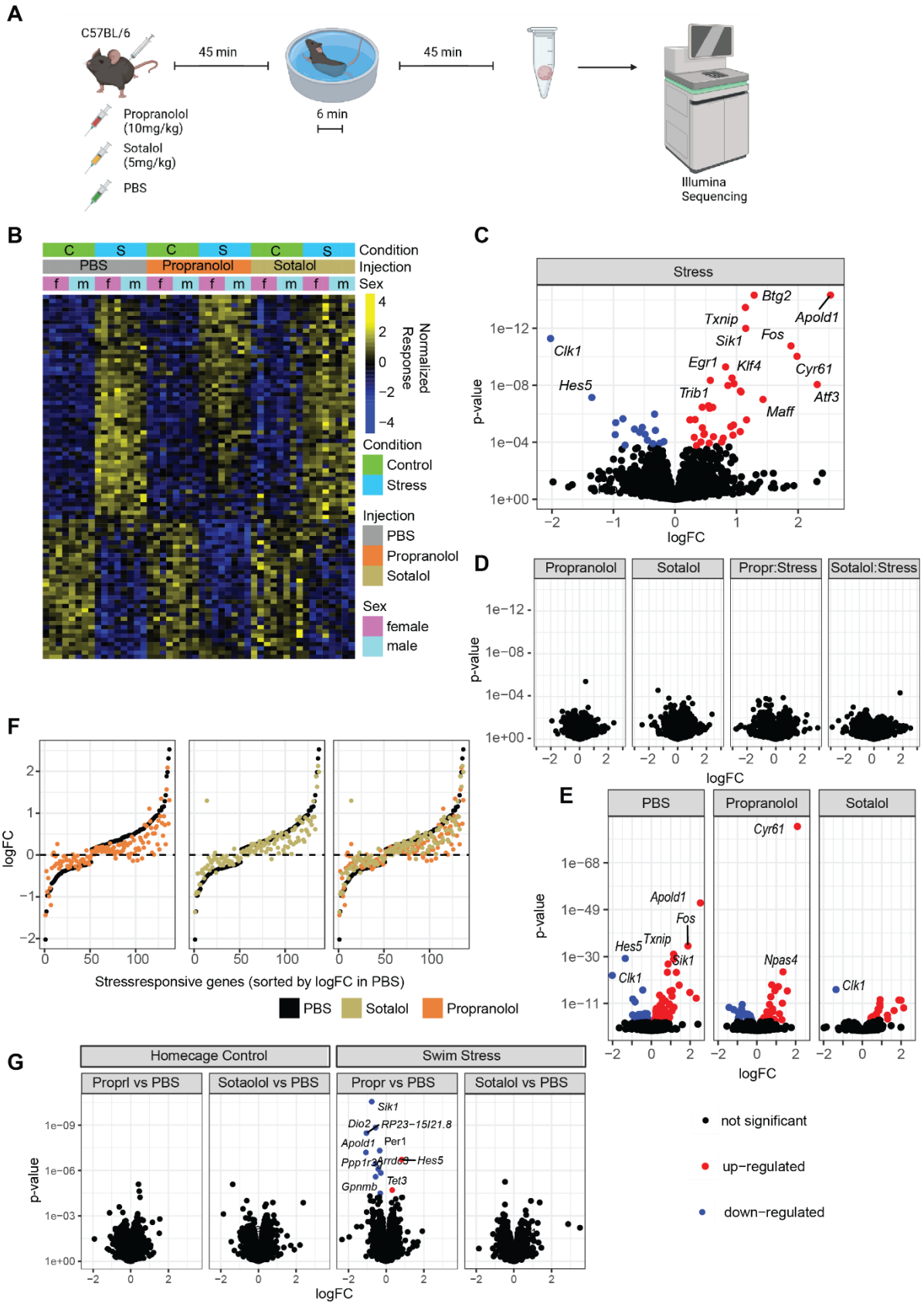
Gene wise fitting of negative binomial generalized models (glmQLF) to the sequencing data indicated the successful induction of many strong changes in transcription through AS (Fig. 3B/3C). We observed multiple significant changes in expression of genes already known for their involvement in the stress reaction of the HC such as *Apold1*, *Fos* and *Egr1* (Floriou-Servou et al., 2018; Roszkowski et al., 2016). This indicates that our methodology was sound and reproduced previously described gene expression changes. Two-group comparisons within injection group show that the stress model permeated all groups no matter the injected drug (Fig. 3E). However, no statistically significant drug effects or drug stress interactions could be detected with negative binomial generalized linear models (Fig 3D). This is most probably due to the high number of tests which was corrected for rigorously leading to a loss of sensitivity, especially in combination with complex statistical designs.

The stress response of multiple genes is altered by propranolol

The genes that change in expression following acute stress are of primary interest when examining beta-receptor signalling. The drug effect was not picked up in the transcriptome wide analysis however when looking at the stress responsive genes they seem to be consistently altered by drug injections (Fig. 3F). Propranolol and Sotalol tend to reduce the strength of fold change in transcription compared to PBS. Both up and down regulation is dampened for several stress responsive genes while propranolol shows a stronger overall effect than sotalol.

To assess if these trends can be statistically resolved we employed more sensitive analyses. First, we used direct two-group comparisons between PBS and drug injected animals within treatment groups (Fig 3G). These analyses were performed independently for propranolol and sotalol resulting in 4 independent analyses. Reducing the variables within each test to one gives the advantage of a simpler statistical model with higher power. Application of two-group comparisons indeed resolved significant differences between PBS and propranolol injected stressed animals, indicating a propranolol dependent alteration of the stress response. Using this method did not resolve a significant sotalol effect. Furthermore, when analysing if either propranolol or sotalol injections influence the transcriptome of control groups we did not observe any effects, indicating that in absence of stress the drugs have no effect on the transcriptome. However, it must be noted that independent two-group analyses can be subjected to bias and misinterpretation as distribution and noisiness of data may not be consistent across groups.

Figure 3: Acute Stress (AS) induced transcriptional changes partly prevented by propranolol.



A) Experimental overview to investigate transcriptional changes 45min after AS. For each injection a group control of the same size (not depicted in the illustration) were kept in their homecage and not subjected to stress. Groups were equally balanced in male and female mice numbers. N per group = 7-8 animals. **B)** Expression of stress responsive genes across all biological replicates (FDR in glmQLFTest ≤ 0.05). **C**-**D)** Fold changes with adj. sig. in full testing of gene wise generalized linear models in the stress variable (C) and injection variables (propranolol, sotalol) as well as drug/durg:stress interaction terms (D) (propr; propranolol). Black = not significant, red / blue = significant (up-regulated/ down-regulated, FDR ≤ 0.05) **E)** Two group comparison: Stress induced changes within injection group. **F)** LogFCs of stress responsive genes sorted by FC in PBS group (black). Overlayed logFC of the same genes for propranolol (yellow) or sotalol injections (orange). From left to right: PBS vs. propranolol; PBS vs. sotalol; all 3 overlayed in one plot. **G)** Two group comparison: Difference in expression of PBS vs. drug injection within treatment groups (Propr; propranolol).

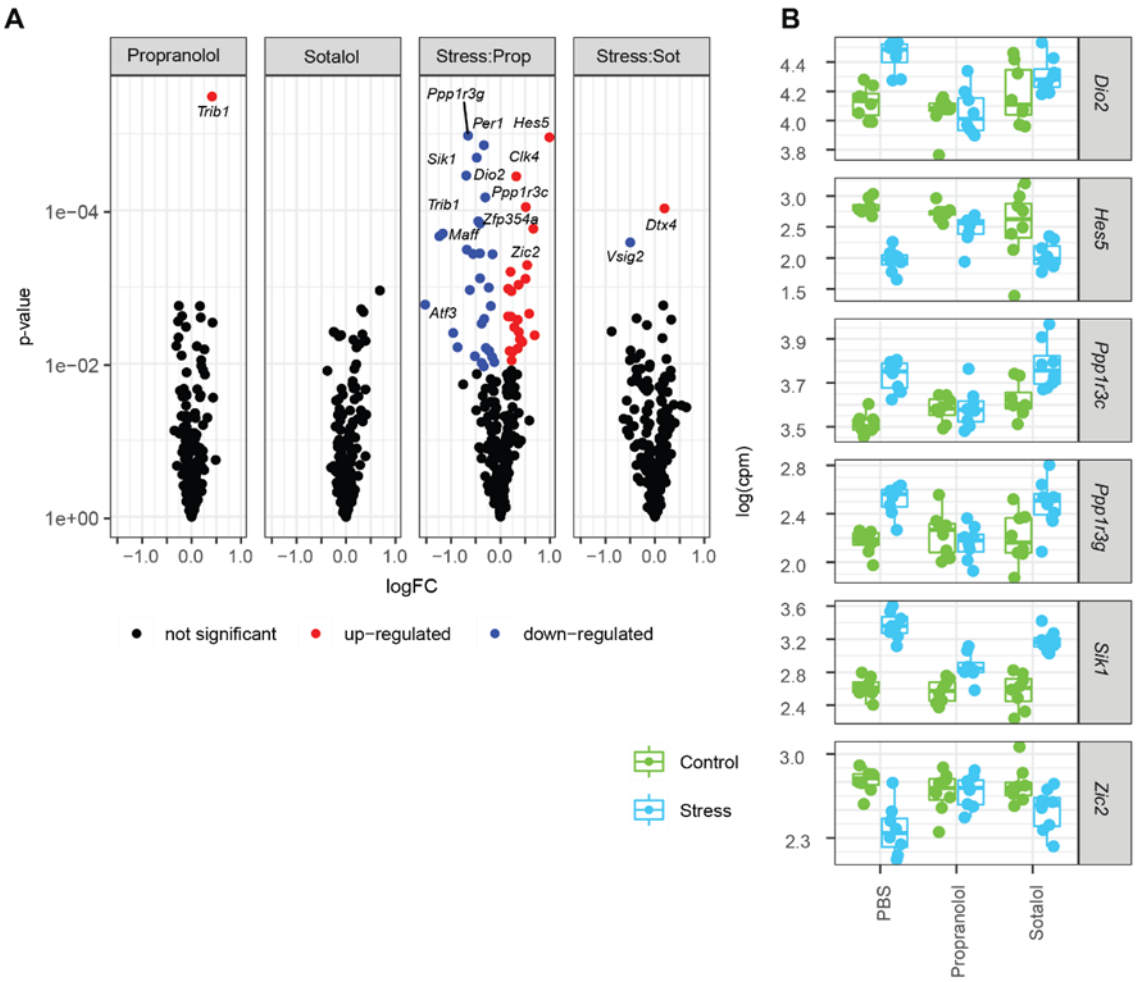
A different method to increase sensitivity while not sacrificing robustness is the reduction of tested hypotheses and therefore the false discoveries that must be corrected for.
We did this by only considering genes showing a response to stress (all displayed in Fig. 3C) as we want to investigate the possible prevention of those changes through beta-blocker. The test and the fitted models are the same as in the full analysis.

Using this approach, we were able to detect significant interaction between AS and injection of propranolol for multiple genes (Fig. 4A). The propranolol variable attributes significance to one gene. *Trib1* is associated with adapter function promoting protein degradation (Dedhia et al., 2010). This is the only stress independent propranolol effect detected. Sotalol shows interaction with AS in 2 genes (*Dtx4*, *Vsig2*) and no stress independent effects.

There is a variety of genes affected by propranolol in interaction with AS, while there are close to none purely propranolol induced differences. This indicates a strong interaction of genes responsive to stress and beta-receptor blocking under AS. Being affected only under combined conditions suggest the genes of changed transcripts being downstream targets of the NE signalling over beta-receptors. By interrupting this signalling pathway those specific transcriptional changes under AS are prevented. The statistically resolved effect is visible in the raw data (Fig. 4B).

The small number of transcripts significantly affected by sotalol can either mean this drug did not pass the blood-brain barrier as intended and hence what we observe are some indirect peripheral effects on the hippocampus. Alternatively, sotalol passed the brain barrier better than expected but the effective dose in the hippocampus was not high enough to induce strong changes as observed with propranolol.

Figure 4: Drug effect of propranolol and raw data of few affected genes.



A) Fold changes vs. p-values in generalized linear model of preselected, stress responsive genes (stress FDR in glmQLFTest over all genes ≤ 0.05). testing injection variables (propranolol, sotalol) as well as drug/durg:stress interaction terms. Black = not significant, red / blue = significant (up-regulated/ down-regulated, FDR ≤ 0.05) **B)** Raw data of interesting candidates who have shown statistically significant injection:stress interaction effects. (Prop = Propranolol; Sot = Sotalol; log(cpm) = log(counts per million))

Male and female mice show comparable molecular response to acute stress and to tested drugs

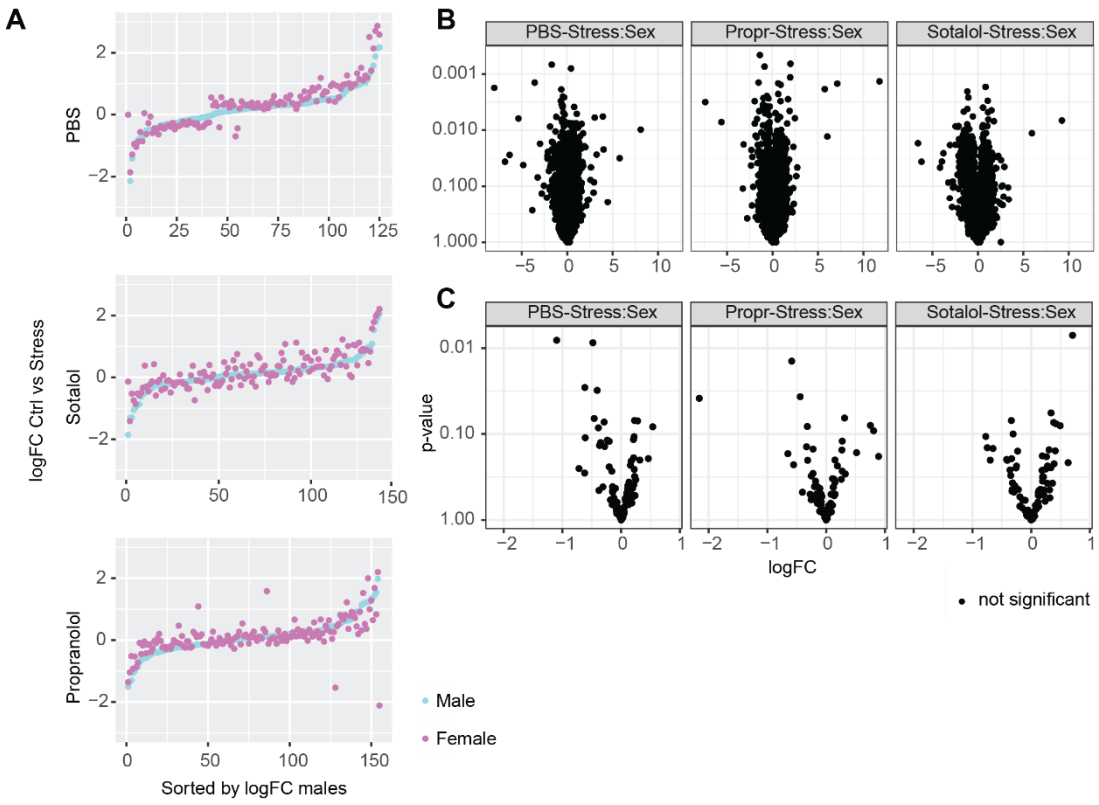
It is unclear if the observed changes in transcription are sex specific, differences in molecular stress response between sexes have been reported in the hippocampus (Marrocco et al., 2017) and the LC-NE arousal system (Bangasser et al., 2016).

As both male and female mice were incorporated in this experiment a direct comparison can be made. Stress responsive genes in male and female mice show similar trends (Fig. 5A) with increased noisiness in the drug injected groups. The increased variation in the sotalol group could be partly due to stressed mice in the homecage group.

Applying glmQLF and testing for injection-stress:sex interactions in either all (Fig. 5B) or only the stress affected genes (Fig. 5C) does not point out significant sex differences. As neither way of testing picks

up AS induced expression changes of stress, the effect on genes in male and female mice seem comparable. We therefore conclude that assessing sex differences while studying the NE dependent stress transcriptome of the hippocampus is not of high importance.

Figure 5: Sex differences in response to acute stress and drugs.



A) LogFCs (control vs stress) of stress responsive genes sorted by logFC in males (blue). Overlayed logFC of the same genes in females (pink). Injection groups from top to bottom: PBS, Sotalol, Propranolol. **B**-**C)** Fold changes between sexes with adj. p-values in all (B) and only stress responsive genes (C). (Propr; Propranolol)

Several genes are dependent on NE in the hippocampus

While the genes highlighted show responsiveness to stress and dependence of NE binding to beta-receptors it cannot be determined if NE is the sole actor or if it has a more modulatory mechanism that interacts with stimulation of other neurotransmitters. Previous experiments in our lab (not yet published) investigated the isolated effect of LC NE release. In two experiments the adrenergic neurons of the LC were either chemogenetically or optogenetically stimulated to release NE 45 min prior to mRNA sequencing. Multiple genes were found that reacted to LC stimulation without any other treatment.

To find the most promising NE responding genes we performed a meta-analysis that considers the responsiveness of genes across all experiments. Responsiveness of genes was assessed by the stress:propranolol interaction p-value in pharmacological experiments, while in the other experiments (chemogenetic and optogenetic LC stimulation) we considered the stimulation p-value. Summing the rank (awarded by p-value) in each experiment of each gene singles out a hand full of genes that show very consistent changes across pharmacological, chemogenetic and optogenetic experiments (Fig 6A).

These clearly separated genes include *Dio2*, *Ppp1r3c*, *Ppp1r3g* and *Sik1*. *Dio2* translates to a thyroxine 5'-deiodinase which plays an important role in supplying body and brain with thyroid hormone, especially during development(Friedrichsen et al., 2003). *Ppp1r3c* and *Ppp1r3g* translate to the regulatory subunits 3C and 3G of the phosphatase 1. Those subunits are associated with activation of the glycogen synthase and reduction of glycogen phosphorylase activity where subunit 3G plays a special role in the liver in postprandial glucose homeostasis (Israelian et al., 2021; Luo et al., 2011; Printen et al., 1997). *Sik1* transcribes to a serine/threonine kinase involved in numerous processes and generally a tumour repressor. A mutation in the *Sik1* gene causes epilepsy and shows abnormalities in the MEF2C-ARC pathway of neuronal development and synapse activity response (Pröschel et al., 2017).

Using this ranking approach, we conclude that a small number of genes are associated with the noradrenergic part of the transcriptional stress response mediated by the LC.

Transcription of NE dependent genes induced by direct LC-HC connections

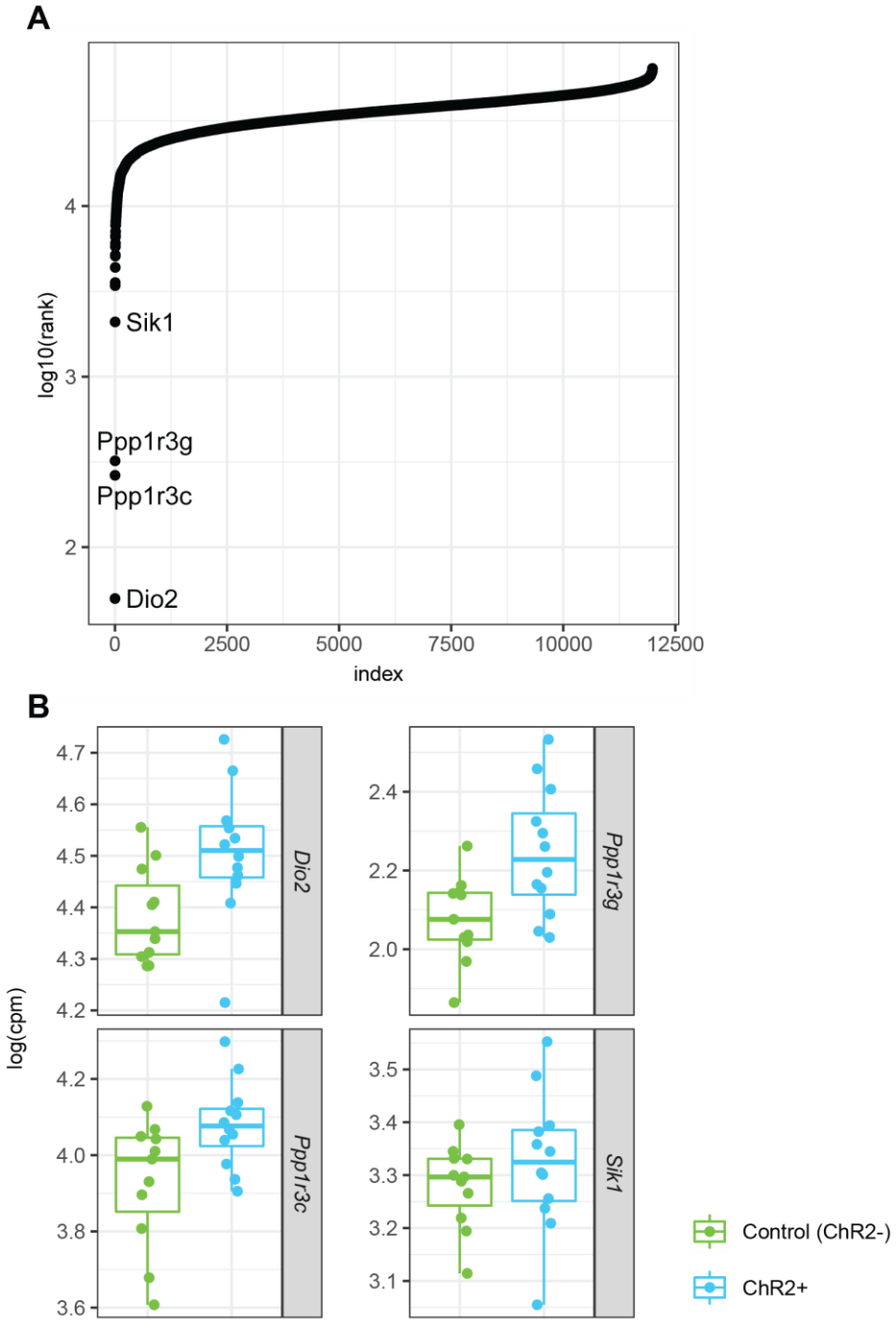
While we could show that several genes in the hippocampus depend strongly on NE and LC it remains unclear if direct noradrenergic projections from the LC are responsible for these genes or if these are indirect effects. To further investigate this proposition, we used samples from a different model (not yet published experiment of Mattia Privitera) where Channelrhodopsin-2 (ChR2) was retrogradely expressed in only the subset of LC neurons that project directly to the hippocampus. Mice were injected with a retrovirus that contained either a construct that included ChR2 (ChR2+), or a control construct devoid of ChR2 (ChR2-) in the hippocampus (see methods). then optogenetic activation in the LC of this selected subset of neurons was used 42 min prior to tissue collection (vHC). As part of this thesis, we performed a mRNA sequencing and analysis.

Three of the four genes highlighted in previous experiments show significant changes in transcription (*Dio2*, *Ppp1r3c*, *Ppp1r3g* but not *Sik1*). As we have a specific excitation of the LC neurons projecting to the HC the observed response should be induced directly by NE release. The individual mice show high variation within group which might have been brought about by the complexity of the experimental procedure (Fig. 6B).

Table 1: Statistical data of highlighted genes.

	logFC	logCPM	F	p-value	FDR
Dio2	0.207037	6.413619	17.89225	0.000242	0.477785
Ppp1r3c	0.174617	5.774389	8.633074	0.006697	0.896466
Ppp1r3g	0.239514	2.986446	9.116692	0.005493	0.896466
Sik1	0.090923	4.726947	2.834406	0.10384	0.922387

Figure 6: Highlighted norepinephrine target genes in the hippocampus.



A) Sum of rankings of genes in multiple experiments. Ranking based on stimulation p-value in chemo- and optogenetic experiments and propranolol:stress p-value for pharmacological experiments. **B)** Raw data of the highlighted candidate genes in specific stimulation of HC protruding LC neurons through retrograde optogenetics (N = 11 per group).

The identification of presumably NE induced transcriptional changes opens the question of the mediator between beta-receptor and transcriptional machinery. As phosphorylation of proteins is changed in numerous ways after AS it is a possible medium of signal transduction between the beta-receptors and the observed transcriptional changes.

Phosphoproteomics

Acute Stress (AS) leads to great differences in phosphorylation patterns of proteins.

To investigate the effects of beta-adrenergic receptors on the phosphoproteome we conducted a new experiment (Fig 7A). Since we observed a much weaker effect of sotalol in the previous experiment we focused exclusively on propranolol injections. Furthermore, since changes in protein phosphorylation are induced much faster and are short lived, we used a different time-point. Samples were collected immediately after stress exposure which has previously been shown to be a suitable time-point (L. M. von Ziegler et al., 2022). We then use quantitative phosphoproteomics to investigate changes in protein phosphorylation.

We applied tests based on linear models and empirical Bayes statistics to compare swim stressed and homecaged mice within their respective injection group. We saw a multitude of phosphopeptides change in abundance induced by exposure to AS (Fig 7C). We observed similar changes as were described previously (L. M. von Ziegler et al., 2021).

Although strong, stress related changes in phosphorylation are detected no drug effects are picked up. The broad analysis over all measured peptides demands severe statistical correction for the high number of hypotheses which leaves us with less statistical power. This does not mean there are none, only that with the used approach we cannot statistically verify them. We noted a number of phosphopeptides in the bottom of the Heatmap (Figure 7B) which differing in abundance between the propranolol and PBS swim stress groups (bottom right corner).

Propranolol induced prevention of stress related phosphorylation events.

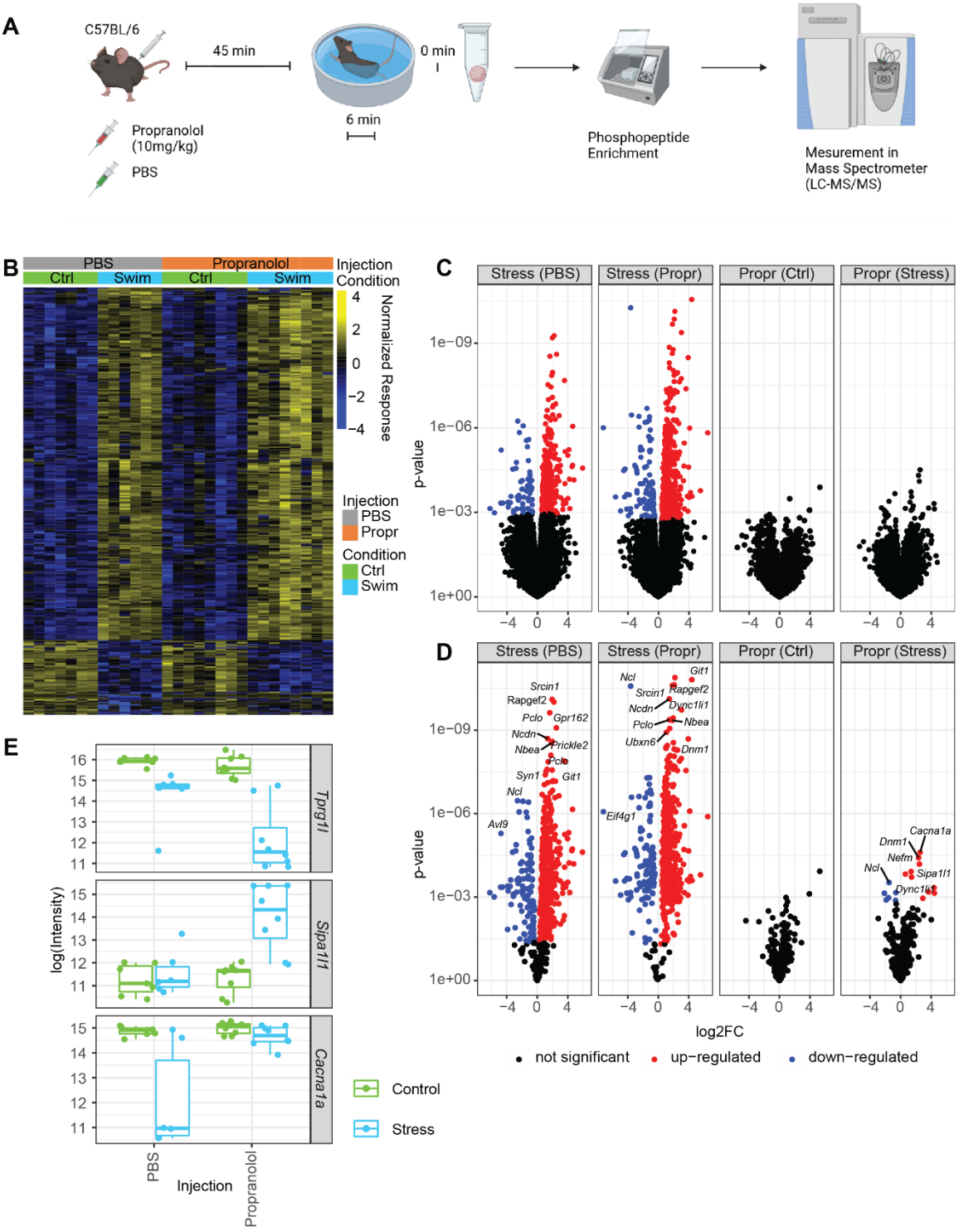
To assess if these trends can be statistically resolved we again employed more sensitive analyses, where we limited the analysis of propranolol effects to phosphopeptides that were effected by acute stress in the first place. Applying the same models but only testing for the preselected phosphopeptides yielded promising results (Fig 7D). When comparing the two stress groups the effect of propranolol injection is picked up and a small number (16) of phosphopeptides are highlighted. They all originate from different proteins. There is no detectable effect of propranolol in non-stressed animals which suggest a propranolol:stress interaction and not just a baseline shift by propranolol.

Three of the 16 genes encoding for the highlighted phosphopeptides are particularly interesting (Fig. 7E): *Cacna1* encodes for a calcium channel and its mutations are associated with attacks of neurological dysfunction. In tottering mice, which exhibit a mutation in this gene, alpha-blocker were shown to prevent stress induced attacks resembling paroxysmal dyskinesia. Propranolol was tested as well yet showed no prevention (Fletcher et al., 1996; Fureman & Hess, 2005). *Sipa1l1* knockout mice show hyperactivity, enhanced anxiety, learning impairments, social interaction deficits, and enhanced epileptic seizure susceptibility suggesting a possible association of *SIPA1L1* deficiency with neuropsychiatric disorders related to dysregulated GPCR signalling, such as epilepsy, attention deficit hyperactivity disorder (ADHD), autism, or fragile X syndrome (Matsuura et al., 2022). *Tprg1l* is suspected to play a role in synaptic transmission tuning and has been shown to decrease the Ca^{2+} sensitivity of release in rats (Körber et al., 2015).

Table 2: Phosphopeptides showing response to propranolol under acute stress.

Gene Symbol	Protein	#Phos	Phospho-sites	Distibution of phosphorylation	log2FC stress (Propr vs PBS)	log2FC ctrl (Propr vs PBS	p-value (adj.) (Propr vs PBS in stress)
Dnm1	P39053	1	S822; S834	S822 %1	2.36	-0.196	0.011743
Cacna1a	P97445	1	T1077; S1085; T1092; S1096; T1100; S1103; S1104; T1105; S1109	T1092 %0.09; S1096 %0.91	2.56	0.114	0.011743
Sipa1l1	Q8C0T5	1	S1078; S1083; S1087; T1090; S1091	S1083 %0.02; S1087 %0.3; T1090 %0.14; S1091 %0.54	2.47	0.0817	0.013182
Speg	Q62407	1	S2451	S2451 %1	0.633	0.183	0.018141
Nefm	P08553	2	S715; S720; S723; S727	S715 %0.35; S720 %0.6; S723 %0.53; S727 %0.53	1.38	-0.89	0.018141
Dync1li1	Q8R1Q8	2	S405; T408	S405 %1; T408 %1	1.44	-0.118	0.019399
Ncl	P09405	1	S577; T580; T581; T584	S577 %1	-1.5	-0.248	0.026067
Atp2b2	Q9R0K7	1	S1155; S1156; S1159; S1160	S1155 %0.66; S1156 %0.34	4.39	0.0636	0.034748
Tprg1l	Q9DBS2	2	T7; S10; T13; S14; T16; T34	T7 %0.02; S10 %0.98; T34 %1	-2.12	-0.258	0.036738
Dmxl2	Q8BPN8	1	S659; T666; S667; S668; T675; S678; S684; S686	T666 %0.15; S667 %0.15; S668 %0.15; T675 %0.56	4.44	-0.202	0.036738
Arhgap35	Q91YM2	1	S1150; S1153; Y1158	S1150 %1	-0.616	-0.419	0.036738
Dst	Q91ZU6	1	S236; S237	S236 %0.5; S237 %0.5	3.67	0.17	0.036738
Rbm17	Q8JZX4	1	S155; Y161	S155 %0.77; Y161 %0.23	2.92	-0.0232	0.048001
Itpka	Q8R071	1	Y184; S185; T193; S195	Y184 %0.03; S185 %0.97	-1.58	-0.872	0.048001
Clip2	Q9Z0H8	1	S353; T355; T356	S353 %1	-0.653	-0.155	0.049942
Cdc42bpb	Q7TT50	1	S984; S986; S988; T991; S992; T993	S984 %0.96; S986 %0.01; S988 %0.01; T991 %0.01; S992 %0.01; T993 %0.01	-1.89	0.0407	0.049942

Fig 7: Acute Stress (AS) induced phosphorylation modulated by propranolol.



A) Experimental overview to investigate phosphorylation of proteins immediately after AS. For each injection a group control of the same size (not depicted in the illustration) were kept in their homecage and not subjected to stress. (N = 8 per group) **B)** Expression of stress responsive phosphopeptides across all biological replicates. **C**-**D)** Differential phosphopeptide abundance with adj. sig.: stress effect within injection groups and drug effect within treatment group in all (C) or preselected stress responsive detected phosphopeptides (D) labelled with their gene of origin. Black = not significant, red / blue = significant (up-regulated/ down-regulated, FDR ≤ 0.05) **E)** Raw data of interesting phosphopeptides affected by propranolol in stressed animals.

Discussion

Regulation of transcription through beta-receptors

With a more complete experimental design we were able to validate the preliminary findings. We identify multiple candidate genes whose transcriptional changes under exposure of acute stress probably are induced through beta-receptor signalling. We showed this by preventing said changes through competitive blocking of beta-receptors with propranolol and inducing said effects by releasing NE in the LC neurons projecting to the HC trough optogenetic and chemogenetic activation. Two different approaches, one broadly impeding, one accurately inducing, support both the same hypotheses which gives high confidence for the results. Moreover, our observations are supported across multiple experiments investigating NE release in the HC shown by ranking candidates across experiment based on their p-value. *Dio2*, *Ppp1r3c*, *Ppp1r3g* and *Sik1* all are top candidates ranked in that order. The top candidate *Dio2* has been under investigation in our lab for a while due to indication of involvement in dementia together with the thyroid hormones (not yet published). The enzyme DIO2 is responsible for transforming T4 into the active T3. Patients of senile dementia and Alzheimer's disease have been found to have a smaller T3/T4 ratio while showing on average similar (Nakanishi, 1990) or increased (Quinlan et al., 2020) T4 serum levels. This indicates lower deiodination rates and therefore lower DIO1 and DIO2 activity. *Dio2* has further been implicated as people with a certain SNP (Thr92AlaD2) in this gene have higher susceptibility to develop Alzheimer's disease. Based on our observations the NE released from LC likely influences *Dio2* expression in the HC. All this makes deregulation of LC a possible driver to evoke T3 deficiency and strongly links the LC-NE system to Alzheimer's and comparable neurodegenerative diseases. Interestingly *Dio2* shows upregulation under stress, respectively relative downregulation when blocking with propranolol. This might indicate that short term stress is not responsible for deregulation of the T3/T4 ratio but rather insufficient LC activity. As in Alzheimer's disease damage and neuron loss LC often is reported, the LC might be the vanguard of neurodegeneration. After the LC can no longer sufficiently distribute NE, lack of DIO2 might drive neurodegeneration in projection targets. Assuming this connection was verifiable the widespread use of beta-blockers, many lipophilic, might be of concern (Cojocariu et al., 2021). If DIO2 homeostasis is not restored in another way beta-blockers could play a role in T3 deficiency.

*Ppp1r3c*and *Ppp1r3g* both are regulative subunits of the protein phosphatase 1 which in neurons is attributed to regulate AMPA-type glutamate channels (Yan et al., 1999) which are important for neuron plasticity. There is the possibility that these two genes help longer term adaptation to perceived stress.

Brain specific prevention of stress effects.

We cannot say for certain if the observed prevention effects of propranolol are truly brain specific or at least partly peripherally induced. Effects of sotalol were minute when compared to those of propranolol yet comparison of two drugs with different efficiencies and administered in different

doses is inadvisable. We used a smaller dose of sotalol as it is attributed with higher bioavailability and to not facilitate blood-brain barrier passing with too high of a concentration. Injections in rats of propranolol have shown to reduce fear while injection of sotalol did not affect fear expression (both 10mg/kg)(Rodriguez-Romaguera et al., 2009). That suggests no penetration of the blood brain barrier of our lower sotalol dosage. Still, we cannot say if the peripheral blockage of beta-receptors through sotalol was comparable to propranolol.

To be able to tell if a sotalol effect was induced in the periphery but not in the brain a dose response curve would be needed. To be able to tune the potency of the drugs while ensuring only propranolol passes the blood-brain barrier, dose dependent transcription changes in the brain and the periphery should be known. While this would be interesting, we do not think it is of high priority:

While the results from injecting sotalol have limited power they do not contradict the other findings. The NE targets propranolol points to show response to specific NE release in the HC. This cannot rule out peripheral effects totally but shows transcriptional changes in response to NE detached from system exposure to AS and therefore epinephrine involvement.

Sex differences in transcriptional response to stress.

Studies looking at the post-stress ribosome-bound mRNA from hippocampal CA3 neurons (Marrocco et al., 2017) and the LC-NE arousal system (Bangasser et al., 2016) highlight numerous weighty differences between the stress response of male and female rodents on molecular and structural level. However, recent meta-analyses have highlighted that some of these studies may have overestimated reported molecular sex effects (L. von Ziegler et al., 2020). In this thesis we did not observe a noticeable sex effect. It is probable that we lack the needed sensitivity to find underlying differences but on the applied models the two groups are statistically very similar. Therefore, the probable sex effects should not interfere in studying the stress related NE induced transcriptional changes in the ventral hippocampus. We therefore conclude that assessing for sex differences in our specific case, a severely delimited snapshot, it is not of high importance and datasets should be comparable between sexes.

That said, based on the studies finding differences and the morphological and psychological differences between male and female overall, it is very unlikely there are no differences on a neuro systemic level.

Acute stress induced phosphorylation changes.

We successfully induced AS and observed numerous strong changes in abundance of phosphopeptides in the hippocampus as previously reported (L. M. von Ziegler et al., 2022). Further we observed changes in the abundance of these stress sensitive phosphopeptides when blocking the beta-receptors with propranolol which is an indication of NE, resp. beta-receptor dependent phosphorylation changes.
Activity of enzymes and receptors is regulated through phosphorylation, and it therefore plays a major role in cell signalling (Ardito et al., 2017). A shown before (L. M. von Ziegler et al., 2022) phosphoproteomic preempt transcriptional changes. This supports the possibility of signal transduction through phosphorylation to the transcriptional level. Phosphorylation is a prevalent mechanism in regulation of transcription factors (Ardito et al., 2017). Ideally one would disable the kinase pathways one by one and observe the impact on the transcriptional stress response. In this manner the specific signalling pathway could be identified.

As phosphorylation can regulate transcription factors, investigating them could also lead to further insight. We are trying to establish a protocol for immunoprecipitated chromatin proteomics. The goal

is to enrich for transcription factors and protein modifications in proximity of the DNA to gain a picture of the momentary transcriptional regulation.

Prevention of phosphorylation events in phosphopeptides worth pursuing.

The data highlights 16 phosphopeptides of which the encoding genes *Cacna1a*, *Sipa1l1* and *Tprg1l* are of special interest as based on literature they are very likely directly involved in neuronal signalling.

Sipa1l1 is believed to be part of the PSD-95/NMDA-R complex and localized to PSD. A new study contradicted this with convincing evidence (Matsuura et al., 2022). They found *SIPA1L1* localized throughout neurons and interacting with the neurabin family of proteins but not in PSD. Their results further suggest involvement of *SIPA1L1* in GPCR signalling. *SIPA1L1* might enhance α_2AR signalling by inhibiting spinophilin-α_2AR interaction. Our observed changes in phosphorylation might indicate a change of activity of *SIPA1L1*, resulting in changed interaction efficiency with spinophilin. This could mean a feedback loop for signalling over LC-HC synapses through modulation of α_2AR signalling. To investigate this the observe differences in modulation of beta-adrenergic signalling between *Sipa1l1*$^{-/-}$ and WT mice maybe similar experimental design to the one used in this study could be used, but in both genotypes.

Beta-andrenergic receptors are G-protein coupled receptors themselves therefore their modulation through *SIPA1L1* cannot be excluded. Previously *SIPA1L1* was supposed to be a PSD protein (Pak et al., 2001) but newer findings do not see notable *SIPA1LA* presence in PSD (Matsuura et al., 2022). This might suggest the postsynaptically located beta-receptors not being prime targets

Sipa1l1$^{-/-}$ mice show normal learning behaviour and motor coordination except for the trace paradigm (Matsuura et al., 2022) which among other forebrain sites greatly depends on the hippocampus (Bangasser, 2006). This highlights the importance of *SIPA1L1* in the hippocampus. As we suspect regulation of *SIPA1L1* phosphorylation in the hippocampus through beta-adrenergic signalling this might indicate involvement of LC-NE in regulation of trace conditioning. *Sipa1l1*$^{-/-}$ mice further show striking behavioural anomalies, such as hyperactivity, enhanced anxiety, learning impairments, social interaction deficits, and enhanced epileptic seizure susceptibility. This might indicate *SIPA1L1* deficiency in context of neuropsychiatric disorders related to dysregulated GPCR signalling, such as epilepsy, attention deficit hyperactivity disorder (ADHD), autism, or fragile X syndrome (FXS) (Matsuura et al., 2022). The locus coeruleus is implicated in ADHD (Aston-Jones et al., 2000). Linking this to probable change in activity of *SIPA1L1* and its probable importance in the hippocampus, this protein and its regulation might be worth pursuing.

Cacna1 is interesting in relation to AS as in a mouse model of neurological dysfunction attacks were stress dependent (Fureman & Hess, 2005). Blocking alpha-receptors has been shown to prevent those stress induced attacks but not beta-receptor blocking with propranolol. We found phosphorylation changes on *Cacna1* when blocking with propranolol. To compare ours and their results the observed connections in their model should be translatable to the WT and the preventive impact of the alpha-blocker should be visible on phosphoproteomic level. Given these lose assumptions were correct, it is probable that we observed a similar effect as supposed in the preliminary experiment with prazosin. By blocking the beta-receptors, we increase the free NE. This increases activation of alpha receptors and could subsequently lead to our observed *CACNA1* phosphorylation events.

TPRG1L knockdown has been shown to increase Ca^{2+} sensitivity (Körber et al., 2015). Therefore, it is implicated in modulating sensitivity by lowering Ca^{2+} release probability. Considering phosphorylation of proteins often impacts their activity the changes we observed could also impact Ca^{2+} sensitivity. This could be an adaptation to persistent NE signalling for the LC and adaptation to stress.

Deregulation of this system might lead to higher susceptibility or reduced excitability and might play a role in neural disorders associated with these extremes. If the Ca^{2+} release threshold in the hippocampus truly is modulated through beta-andrenergic signalling further experiments are needed. Maybe this can be done through Ca^{2+} caging in combination with blocking of beta-receptors.

Summary

Previous suppositions about transcriptional changes after AS induced through beta-receptors based on a preliminary experiment could be confirmed and additional insight regarding brain specificity and sex differences were gained. The identified genes supposedly influenced by NE trough the beta-receptors show consistent appearance across multiple different experiments and reaction to LC specific NE release.

Following the signal transduction to phosphorylation as a possible medium of transmittance lead to identification of multiple phosphopeptides changing abundance after AS under influence of propranolol. Three of them are attributed with processes very likely involved in neurological stress response and may warrant further investigation.

Material & Methods

General

Animals

All experiments were conducted in accordance with the Swiss federal guidelines for the use of animals in research and under license ZH161/17, approved by the Zurich Cantonal veterinary office. For experiments wild type animals, C57Bl/6J mice were either obtained from Janvier (France) or bred at the ETH Zurich animal facility (EPIC). Mice were housed in groups of 4-5 per cage in a temperature- and humidity-controlled facility on a 12-hour reversed light-dark cycle (lights off: 9:15 am; lights on: 9:15 pm), with food and water ad libitum, and used for experiments at the age of 2-5 months. All experiments were conducted during the animals' active (dark) phase and after 24-hour of single housing. Experiments were conducted with male, female mice, or both.

Swim stress paradigm

For all experiments, mice were single-housed 24 hours before exposure to stress. For cold swim stress, mice were placed for 6 min in a plastic beaker (20 cm diameter, 25 cm deep) filled with 18 ± 0.1°C water to 17 cm, in a room with dim red lighting. Immediately after stress exposure, mice returned to their assigned single-housing homecage.

Retro-optogenetic surgery and stimulation

For retro-optogenetic experiments C57BL/6-Tg(Dbh-icre)1Gsc mice at the age of 2-3 months were subjected to stereotactic surgery. The mice were anesthetized with 4% isoflurane and then placed in a stereotaxic frame with continuous anesthesia of 2% isoflurane. For analgesia, animals received a subcutaneous injection of 5 mg/kg Meloxicam and a local anesthetic (Emla cream; 5% lidocaine, 5% prilocaine) before and after surgery. After the skull was exposed, bregma was located and the skull placement corrected for tilt and scaling. Unilateral (right hemisphere) small holes were drilled above the LC at -5.4 mm AP and 0.9 mm ML from bregma. A pneumatic injector (Narishige, IM-11-2) and calibrated microcapillaries (Sigma-Aldrich, P0549) were then used to inject 1 µL of virus to the LC (coordinates from bregma: −5.4 mm AP, ± 1.0 mm ML, −3.8 mm DV). Viral vectors were obtained from the Viral Vector Facility (VVF) of the Neuroscience Center Zurich. For retrograde activation of LC-HC projecting neurons, animals received one injection of either ssAAV-retro/2-hEF1a-dlox-ChR2(H134R)_EYFP(rev)-dlox-WPRE-hGHp(A) (ChR2+) or ssAAV-retro/2-hEF1a-dlox-mCherry(rev)-dlox-WPRE-hGHp(A) (ChR2-) to the right dHC (coordinates from bregma: -2.10 mm AP, 1.5 mm ML; -1.8 mm DV) and vHC (coordinates from bregma: -3.30 mm AP, 2.75 mm ML; -4.0 mm DV). Additionally, animals were unilaterally implanted with an optic fiber (200 µm diameter, 0.22 NA) above the locus coeruleus (coordinates from bregma: -5.4 mm AP, 0.9 mm ML, -3.5 mm DV). The health of all animals was monitored over the course of 3 consecutive days post-surgery. Experiments on operated animals were conducted 7 weeks post-surgery to allow for recovery and sufficient virus expression.

Mice were under optogenetic stimulation (5Hz,10mW) for 21min alternating between off and on states every 3min starting with an off phase. The experimenter (Mattia Privitera) did not know which animals were ChR2-positive or controls. 45min after the start of the OFT mice were sacrificed and tissue was collected.

Tissue collection

At the appropriate time point after initiation of stress, respectively LC stimulation, mice were euthanized by cervical dislocation and decapitation. The brain was quickly dissected on ice and isolated hippocampi were cooled with PBS-ice for dissection of subregions as previously described (L.

M. von Ziegler et al., 2018) (prior to snap-freezing in liquid nitrogen and storing at −80°C for future processing.

Proteomics

Protein extraction

We used a block design for sample processing. Samples were split into multiple blocks, containing one replicate of each condition and region. Processing order within blocks was randomized. Proteins were extracted from pooled hemispheres of vHC using 150 µL TEAB buffer (100 mM triethylammonium bicarbonate, 0.1% SDS, 1:100 protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis MO, USA), 1:500 PMSF (50 mM in EtOH)) with 1:100 phosphatase inhibitor cocktail 2 (P5726, Sigma-Aldrich, St. Louis MO, USA), and phosphatase inhibitor cocktail 3 (P0044, Sigma-Aldrich, St. Louis MO, USA) were added.

The samples were mechanically lysed by 15 strokes with a 26 G needle and sonicated for 5 mins. Samples were spun down at 16,000 g 1 hour at 4 °C and supernatants were collected. Proteins were quantified using a Qubit protein assay kit (ThermoFisher Scientific, Waltham MA, USA) following the manufacturer's protocol. Protein extracts were further processed with a filter assisted sample preparation protocol. 300 µg of protein were added to equal amount (v/v) of concentrated denaturation buffer (4% SDS (w/v), 100 mM Tris/HCL pH 8.2, 0.2 M DTT). For denaturation, samples were incubated at 95 °C for 5 mins. Samples were diluted with 200 µL UA buffer (8M urea, 100 mM Tris/HCl pH 8.2) and then loaded to regenerated cellulose centrifugal filter units (Microcon 30, Merck Millipore, Billercia MA, USA). Samples were spun at 14000 g at 35 °C for 20 mins. Filter units were washed once with 200 µL of UA buffer followed by centrifugation at 14,000 g at 35 °C for 15 mins. Cysteines were alkylated with 100 µL freshly prepared IAA solution (0.05 M iodoacetamide in UA buffer) for 1 min at room temperature (RT) in a thermomixer at 600 rpm followed by centrifugation at 14000 g at 35 °C for 10 mins. Filter units were washed three times with 100 µL of UA buffer then twice with a 0.5 M NaCl solution in water (each washing was followed by centrifugation at 35 °C and 14000 g for 10 mins). Proteins were digested overnight at RT with a 1:50 ratio of sequencing grade modified trypsin (6 µg per sample, V511A, Promega, Fitchburg WI) in 130 µL TEAB (0.05M Triethylammoniumbicarbonate in water). After protein digestion overnight at RT, peptide solutions were spun down at 14000 g at 35 °C for 15 mins and acidified with 10 µL of 5% TFA (trifluoroacetic acid). 100% ACN was added for a final concentration of 3% in the samples.

Enrichment of phosphopeptides

For phospho-enrichment digested samples were first lyophilized and resuspended in 200 µL loading buffer (1 M glycolic acid in 80% ACN, 5% TFA). Phosphopeptides were enriched using MagReSyn® Ti-IMAC HP magnetic beads (Resyn Biosciences). 20 µL of beads were used per sample. Microspheres were reactivated using 200 µL of resuspension solution (1% NH4OH) for 10 min. Microspheres were then equilibrated twice with 200 µL loading buffer for 1 min and then resuspended in 500ul 100% ACN per 20ul of beads. A Thermo Scientific™ KingFisher™ was used for enrichment of phosphopeptides. First, microbeads were loaded onto magnetic rods and washed with 500 µL loading buffer. Samples were loaded and washed in 500 µL loading buffer, in 500 µL wash 2 buffer (80% ACN, 1% TFA) and in 500 µL wash 3 buffer (10% ACN, 0.2% TFA). Samples were then eluted in 200 µL resuspension solution (1% NH4OH), snap frozen in liquid nitrogen and lyophilized in a speedvac then re-solubilized in 19 µL 3% ACN/0.1% FA (formic acid) prior to LC-MS/MS measurements. 1 µL of synthetic peptides (Biognosys AG, Switzerland) were added to each sample for retention time calibration.

LC-MS/MS measurements

Samples were measured on a Q Exactive HF (Thermo Fisher Scientific, Waltham MA, USA). Peptides were separated with an ACQUITY UPLC M-Class System (Waters, Milford MA, USA). We used a single-pump trapping 75-µm scale configuration (Waters, Milford MA, USA). 4 µL of each sample were injected. Trapping was performed on a nanoEase™ symmetry C18 column (pore size 100Å, particle size 5 µm, inner diameter 180 µm, length 20 mm). For separation a nanoEase™ HSS C18 T3 column was used (pore size 100Å, particle size 1.8 µm, inner diameter 75 µm, length 250 mm, heated to 50°C). Peptides were separated using a 60 minute long linear gradient of 5-35% ACN, 0.1% FA (using a flowrate of 300 nL / minute). Electronspray ionization with 2.6 kV was used and a DIA method with a MS1 in each cycle followed by 35 fixed 20 Da precursor isolation windows within a precursor range of 400-1100 m/z was applied. For MS1 we used a maximum injection time of 55 ms and an AGC target of 3e6 with a resolution of 30 K in the range of 350-1500 m/z. MS2 spectra were acquired using a maximum injection time of 55 ms an AGC target of 1e6 with a 30 K resolution in the range of 140 – (2 x the upper range of the precursor window) m/z. A HCD collision energy of 28 was used for fragmentation.

Peak picking DIA

We used Spectronaut™ (Biognosys, version 11) with directDIA for peak picking and sequence assignment. We used a Mus musculus reference proteome for C57BL/6J from uniprot (UP000000589) from the Ensembl GCA_000001635.8 assembly only including reviewed entries. We included a maximum of 2 missed cleavages, using a Tryptic specificity (KR/P). Sequences in a range of 7-52 AA were considered. We included carbamidomethyl as fixed modification for cysteine, oxidation as variable modification for methionine. As variable modification we included protein N-terminal acetylation and phosphorylation of serine, threonine or tyrosine as variable modification, either as full group or following a neutral loss of 98 PTM localization was enabled with a probability cutoff of 0.75 and quantification performed on the precursor level. Single hit was determined on the stripped sequence level. Major grouping was done by protein group ID and minor grouping by stripped sequence. For the minor and major group quantification the top 3 entries were used using the mean precursor/peptide quantity. Normalization strategy was set to automatic. We used a global imputation strategy. Machine learning was performed across Experiment and iRT profiling was enabled.

Statistical analysis of LC-MS/MS data

The measurements quantity values were exported from Spectronaut on the modified peptide level. We then used the R package “DEP” (version 1.12.0) for differential analysis of defined contrasts. We did perform multiple testing correction on our own using the Benjamini-Hochberg false discovery rate (FDR) method. This analysis was performed on the complete data set and separately on the preselected phosphopeptides showing response to stress in the full analysis.

Transcriptomics

Whole tissue RNA extraction

We used a block design for sample processing. Samples were split into multiple blocks, containing one replicate of each condition and processing order within blocks randomized. Samples were homogenized in 500 µL Trizol (Invitrogen 15596026) in a tissue lyser bead mill (Qiagen, Germany) at 4°C for 2 minutes, and RNA was extracted according to manufacturer’s recommendations. RNA purity and quantity were determined with a UV/V spectrophotometer (Nanodrop 1000), while RNA integrity was assessed with high sensitivity RNA screen tape on an Agilent Tape Station/Bioanalyzer, according to the manufacturer’s protocol.

Library preparation and sequencing was performed by Novogene Co (Cambridge, UK). For library preparation, the TruSeq stranded RNA kit (Illumina Inc.) was used according to the manufacturer's protocol. The mRNA was purified by polyA selection, chemically fragmented and transcribed into cDNA before adapter ligation. Paired-ended sequencing (150nt) was performed with Illumina Novaseq. Samples within experiments were each run on one or multiple lanes and demultiplexed. A sequencing depth of ~40M reads per sample was used. Kallisto (Bray et al., 2016) was used for pseudoalignment of reads on the transcriptome level using the genecode.vM17 assembly with 30 bootstrap samples. For differential gene expression (DGE) analysis we aggregated reads of protein coding transcripts and used R (v. 4.0.3) with the package "edgeR" (v 3.32.1) for analysis. A filter was used to remove genes with low expression prior to DGE analysis. EdgeR was then used to calculate the normalization factors (TMM method) and estimate the dispersion (by weighted likelihood empirical Bayes). For two group comparisons the genewise exact test (exactTest()) was used, for more complex designs we used a generalized linear model (GLM) with empirical Bayes quasi-likelihood F-tests (glmQLFTest()). For multiple testing correction the Benjamini-Hochberg false discovery rate (FDR) method was used.

For analyses of data-sets originating from multiple experiments and reducing batch effect we further employed SVA correction to correct for processing specific effects. Surrogate variables independent of experimental groups were identified using the sva package 3.38.0 (Leek et al., 2012) on data after DESeq2 variance-stabilization (Love et al., 2014), and were then included as additive terms in the GLMs.

Plot generation

Heatmaps were produced with the SEtools package. To avoid rare extreme values from driving the scale, the color scale is linear for values within a 98% interval, and ordinal for values outside it. Volcanoplots were produced using ggplot2 (v3.3.5).

Statistics & reproducibility

We used a block design for experiments. Animals and samples were split into multiple blocks, containing one replicate of each condition. Experimental and processing order within these blocks was randomized. Investigators were blinded during experiment and sample processing, but not during the analysis process. However, the same algorithmic analysis methods were used for all samples within each experiment. No statistical method was used to predetermine sample size. No data were excluded from the analyses.

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Declaration of originality

Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

Eigenständigkeitserklärung

Die unterzeichnete Eigenständigkeitserklärung ist Bestandteil jeder während des Studiums verfassten Semester-, Bachelor- und Master-Arbeit oder anderen Abschlussarbeit (auch der jeweils elektronischen Version).

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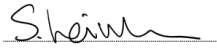
19.04.2022	

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