Final Report

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Title of Project: ***Decoding the rogue cellular signals of Zika virus in infected neurons and neural progenitor cells by quantitative proteomics and FDA-approved drugs***

Summary: (approximately 1,000 words)

We started the Brain Foundation-funded project in October 2019 and finished the experiments to optimize proteomic and bioinformatic analyses of neurons undergoing excitotoxic cell death. As Dr. Paradkar and his colleagues in the CSIRO Australian Centre for Disease Preparedness were deployed to work on projects related to Covid19 since January 2021, we were unable to generate the Zika virus-infected neurons and neural progenitor cells for proteomic analysis as proposed in this application. Summaries of our findings for experiments we conducted for the two aims are presented below. These are presented in detail in a preprint (*1*), which is an earlier version of the manuscript we submitted and the appendix to this document.

Aim 1. To define the role of the newly discovered excitotoxicity-activated kinases in ZIKV-induced neuronal death using inhibitors of these kinases and the quantitative phosphoproteomic approach.

Findings (October, 2019 – April, 2021): Using the proposed proteomic approaches, we discovered a novel mechanism of activation of the protein tyrosine kinase Src in neurons undergoing excitotoxic cell death. In brief, Src was cleaved by a protease called calpain to generate an active stable truncated fragment. The proteomic approaches also allowed us to map the cleavage site. Based upon the findings, we developed a cell–permeable inhibitor called Tat-SRC capable of blocking aberrant activation of the Src in excitotoxic neurons. More importantly, this inhibitor could protect against excitotoxic neuronal death *in vitro* as well as *in vivo* in a rat model of neurotoxicity. Exploring if this cell-permeable inhibitor Tat-SRC can protect against excitotoxic neuronal loss caused by Zika virus infection may lead to the development of neuroprotectants as therapeutics to reduce the brain damage caused by Zika virus infection.

Findings (April, 2021-Dec, 2021): In collaboration with Ms. Nane Griem-Krey and Prof. Petrine Wellendorph of the University of Copenhagen, Demark and Dr. Andrew Clarkson of University of Otago in New Zealand, we discovered a new mechanism of dysregulation of calmodulin-dependent protein kinase II (CaMKII). Our proteomic data revealed that neuronal CaMKIIα and CaMKIIβ were cleaved by calpains at two sites during excitotoxicity to generate two active truncated fragments consisting of the intact kinase domain. We confirmed the findings by in vitro experiments and in vivo in a mouse model of excototoxic brain damge. Relevant to this, Griem-Krey, Clarkson, Wellendorph and their colleagues previously developed a group of CaMK2a-specific inhibitors capable of protecting against brain damage caused by excitotoxic neuronal loss induced by ischemic stroke (*2*). Our findings suggest that these inhibitors are potential neuroprotectants to protect against excitotoxic neuronal death induced by Zika virus infection.

Aim 2. To identify the substrates of NS2B-NS3 in ZIKV-infected NPCs and neurons

Findings (October, 2019 – April, 2021): Using a specific inhibitor of calpains called calpeptin and the N-terminomics approach detailed in our grant application, we successfully identified for the first time over three hundred neuronal proteins undergoing proteolytic processing to form stable fragments in neurons undergoing excitotoxic cell death. Furthermore, the N-terminomics method enabled us to define the exact sites of calpain cleavages in these proteins. Since synaptic loss and dysfunctions underpin excitotoxic neuronal death (*3*), we hypothesise that the proteolytically processed proteins identified by us that are critical to neuronal survival are located in synapses where they play significant role in synaptic functions. Using a newly available bioinformatic tool called SynGO (*4*), we were able to assign the proteolytically processed proteins to specific locations and biological processes of synapses.

Findings (April, 2021 – December, 2021):

Publication:

The first version of a manuscript describing our findings [deposited in Biorxiv (*1*) The manuscript link: https://www.biorxiv.org/content/10.1101/2020.06.15.151456v1] was assessed by editors and two external reviewers of Review Commons (https://www.reviewcommons.org/). All editors and external reviewers gave positive comments and our manuscript was recommended for submission to their member journals.

A revised manuscript entitled "An Atlas of Phosphorylation and Proteolytic Processing Events During Excitotoxic Neuronal Death Reveals New Therapeutic Opportunities" was assessed by editors and two external reviewers of Science Advances. The external reviewers suggested us to present the results of the TAILS findings and phosphoproteomic findings as two separate manuscripts. We have prepared the draft of the manuscripts describing our TAILS findings for submission.

Request for the use of the reminder of the funds of the Brain foundation-funded project to cover the cost of manuscript publication:

The cost of publication of the two manuscripts describing our findings is around Aus$8000-9000 (e.g. the publication fee for a manuscript in Elife is US$3000). We would like to request permission of using the reminder of the fund in our account to contribute towards the publication cost of the manuscripts.

Hypothesis vs Findings

Aim 1. To define the role of the newly discovered excitotoxicity-activated kinases in ZIKV-induced neuronal death using inhibitors of these kinases and the quantitative phosphoproteomic approach.

Hypothesis: Zika virus infection induces excitotoxic neuronal death by aberrant activation of multiple protein kinases in neurons.

Hypothesis vs findings: Our findings include: (i) multiple protein kinases are activated in neurons undergoing excitotoxic cell death and (ii) blockade of activation of one of the kinases Src can protect against excitotoxic neuronal death in vivo in a rat model of neurotoxicity.

Aim 2. To identify the substrates of NS2B-NS3 in ZIKV-infected NPCs and neurons

Hypothesis: The proteomic method TAILS can identify the substrates and define the cleavage sites in the substrates of the Zika virus protease NS2B-NS3 in ZIKV-infected cells.

Hypothesis vs findings: The TAILS method allowed us to identify over 300 neuronal proteins cleaved by the excitotoxicity proteases in neurons undergoing excitotoxicity cell death. These findings indicate that TAILS can identify direct substrates of activated proteases in neurons

Unanswered Questions

Unanswered question 1 for Aim 1: Can blockade of aberrant activation of Src by Tat-Src protect ZIKV-infected neurons against excitotoxic cell death?

Follow-up studies to be conducted in future: As we proposed in the application, we will examine if Src is proteolytically processed by calpains to form the stable active truncated fragment in neurons infected with Zika virus. Furthermore, we will examine the efficacy of Tat-Src in blocking calpain cleavage of Src in the infected neurons. Results of the proposed experiment will reveal if this cell-permeable inhibitor Tat-SRC is a potential neuroprotectant to protect against excitotoxic neuronal loss caused by Zika virus infection.

Unanswered question 2 for Aim 1: Can inhibitors of CaMKII protect ZIKV-infected neurons against excitotoxic cell death?

Follow-up studies to be conducted in future: In light of the importance of CaMKIIα and CaMKIIβ in directing excitotoxic neurons, our discovery of proteolytic processing of the two CaMKII isoforms by calpains represents a new mechanism of dysregulation of CaMKII. Our collaborator Nane Griem-Krey, Petrine Wellendorph and Andrew Clarkson developed several small molecule inhibitors of CaMKIIα, which when applied three days after ischemic stroke in mice, could protect against exxcitotoxic neuronal loss *in vivo*. (REF). We will examine if these neuroprotective CaMKIIα inhibitors can protect ZIKV-infected neurons against excitotoxic cell death.

Unanswered question 1 for Aim 2: Can the N-terminomic procedure TAILS identify cellular proteins cleaved by the Zika virus protease NS2B-NS3 in ZIKV-infected cells?
Follow-up studies to be conducted in future: We will use the same N-terminomics procedure to identify cellular proteins undergoing proteolytic processing in Zika virus-infected neurons and neural progenitor cells as we proposed in grant application. We will also treat the infected cells with Novobiocin a specific inhibitor of NS2B-NS3 protease prior to N-terminomics analysis to define which of the proteolytically processed proteins are potential direct substrates of the viral protease.

What these research outcomes mean

Significance: From the results of the follow-up study and the findings described in Aims 1 and 2, we can (i) identify the cellular proteins that are proteolytically processed by NS2B-NS3 and/or calpains in Zika virus-infected cells and (ii) assign their synaptic locations and functions. The findings we made so far will contribute to our understanding of the pathophysiological mechanism governing cell death in Zika virus-infected neurons. More importantly, the cell-permeable Tat-Src peptide inhibitor and the neuroprotective CaMKIIα inhibitors developed by our collaborators are potential lead compounds for the development of neuroprotectants to reduce brain damage induced by Zika virus infection.

***Appendix*: Findings made from October 2019 to December 2021**

Finding 1: Blockade of aberrant activation of neuronal Src protein kinase can protect against excitotoxic neuronal death

Previous findings by us and others revealed that Zika virus infection induces neuronal death by aberrant stimulation of neuronal NMDA receptors (*5, 6*). Even though we were not able to use Zika virus to induce excitotoxic neuronal death, we treated cultured primary neurons with glutamate to induce excitotoxic cell death. Using the proteomic and biochemical procedures detailed in the application, we successfully defined the mechanism of dysregulation of Src, a protein tyrosine kinase critical to neuronal survival. In both the glutamate-treated and Zika virus-infected neurons, the over-stimulated NMDA receptors cause over-activation of calcium-dependent protease calpains, which in turn proteolytically process a number of neuronal proteins. Src is a one of these processed proteins. In our study, we found that Src was cleaved by calpains in neurons and defined the cleavage site ($F^{63}\downarrow G^{64}$) (Figure 1A). We also identified calpains as the upstream proteases cleave Src in neurons (Figure 1B).

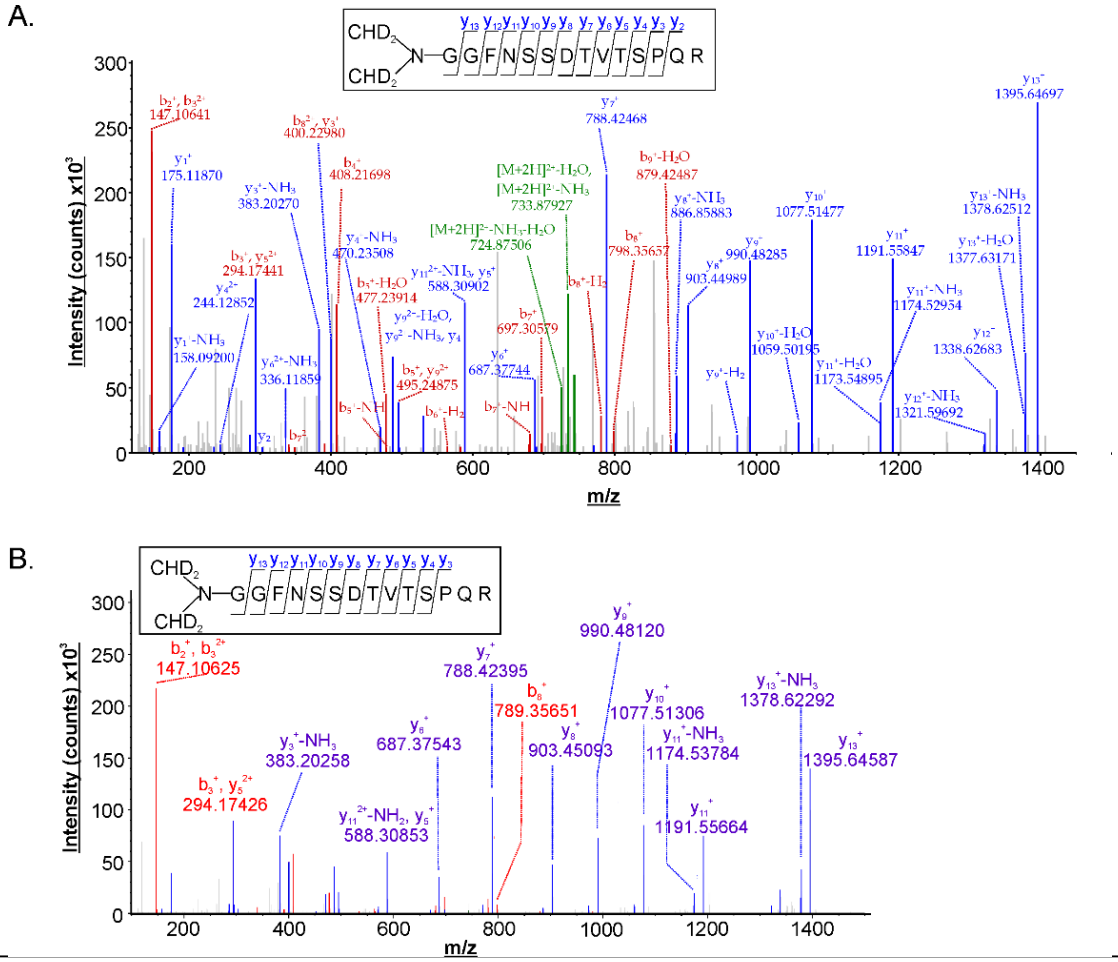

Figure 1 Identification of the neo-N-terminal peptide derived from the truncated Src fragments in excitotoxic neurons and in the reaction of *in vitro* cleavage of recombinant Src (R-Src) by calpain 1 (A) Fragment ion chromatogram identifying a neo-N-terminal peptide encompassing residues 54 to 77 of neuronal Src (inset) detectable exclusively in neurons treated with glutamate for 30 min. **(B)** The fragment ion chromatogram identifying the deuterated dimethyl-labelled Src (64-77) segment of R-Src (inset) as the neo-N-terminal peptide detected only in the reaction mixture containing R-Src and calpain 1 at 120 min of incubation. The insets show the confirmed amino acid sequence. Blue: y ions, Red: b ions.

Based upon the location of the calpain cleavage site in Src ($F^{63}\downarrow G^{64}$), we designed a cell-permeable peptide TAT-Src consisting of the cell-permeable TAT-sequence and the segment encompassing residues 49-79 in the unique domain of Src and a cell-permeable control peptide TAT-Scrambled (Figure 2A) to demonstrate blockade of cleavage of Src by TAT-Src in excitotoxic neurons (Figure 2B).

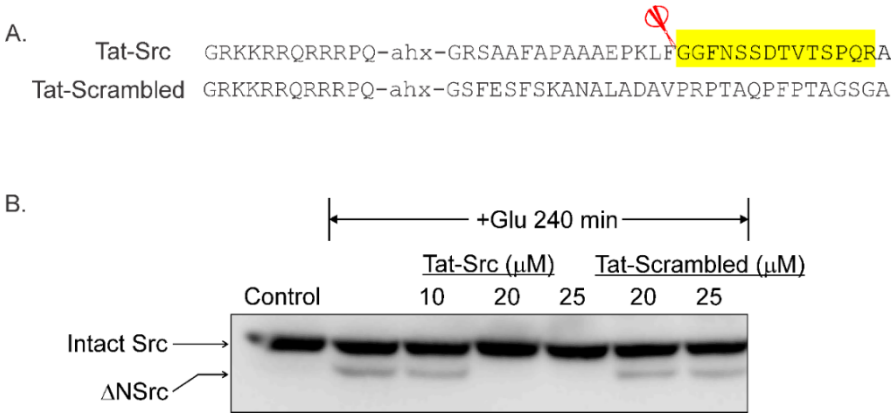

Figure 2 Tat-SRC but not Tat-Scrambled blocks cleavage of Src in neurons (A) Sequences of Tat-Src and Tat-scrambled-Src. Segment highlighted in yellow: the neo-N-terminal peptide corresponding to Src(64-77) detected exclusively in glutamate-treated neurons (Figure 13A). Red scissor: the cleavage site in neuronal Src targeted by the excitotoxicity-activated proteases in neurons and by calpain 1 *in vitro*. **(B)** Tat-Src or Tat-Scrambled of the designated concentrations were added to the culture medium 1 h prior to treatment of cultured neurons with 100 μM glutamate. Cleavage of Src during excitotoxicity was monitored by anti-Src Western blot. ΔNSrc: truncated Src fragment.

We then examined if blockade of cleavage of neuronal Src could protect against excitotoxic neuronal loss *in vivo* in a rat model of neurotoxicity (Figure 3). We first performed stereotaxic infusion of FITC-TAT-Src with a fluorescent tag covalently attached at the N-terminus of TAT-Src, into the cortical and striatal regions of rat brains (Figure 3A). FITC-TAT-Src was detected in neurons but not astrocytes and microglia in the infused regions of the rat brain (Figure 7A) at one-hour post infusion, suggesting that it could enter neurons to exert its neuroprotective action. To examine the ability of TAT-Src to protect against excitotoxic neuronal death *in vivo*, TAT-Src, vehicle (water) or TAT-scrambled was stereotaxically injected at four sites in the cortical and striatal regions of rat brains (Figures 3B and 3C). One hour after the injection, neurotoxic dose of NMDA was infused to the same sites to induce excitotoxic brain damage. At 24 h after the infusion of NMDA, the rats were sacrificed, and brain sections were prepared to measure the infarct volume and the number of surviving neurons (Figure 3D). The absence, or reduction in NeuN immunoreactivity, revealed NMDA-induced lesions within the motor and parietal cortex (Figures 3D and 3E). The plot in left panel of Figure 7F show that total lesion volume was consistent across all treatment groups with no significant difference in the volume of damage detected between groups. Stereological point counting of NeuN positive cells within the lesion revealed treatment-specific effects where the number of neurons in rats treated with TAT-Src was significantly higher than in rats receiving vehicle or scrambled Tat-Src control (Figure 3F, right panel). Thus, injection of TAT-Src prior to NMDA infusion could protect against excitotoxic neuronal loss caused by the injected NMDA. Since FITC-TAT-Src entered neurons but not astrocytes and microglia (Figure 3A), the ability of TAT-Src to protect against neuronal loss in NMDA-induced brain damage is presumably attributed to its blockade of calpain cleavage of neuronal Src to form the neurotoxic truncated Src fragment (ΔNSrc). In conclusion, our results illustrate the therapeutic potential of blockade of pathologically activated cellular events in excitotoxic neurons uncovered by our proteomic analysis.

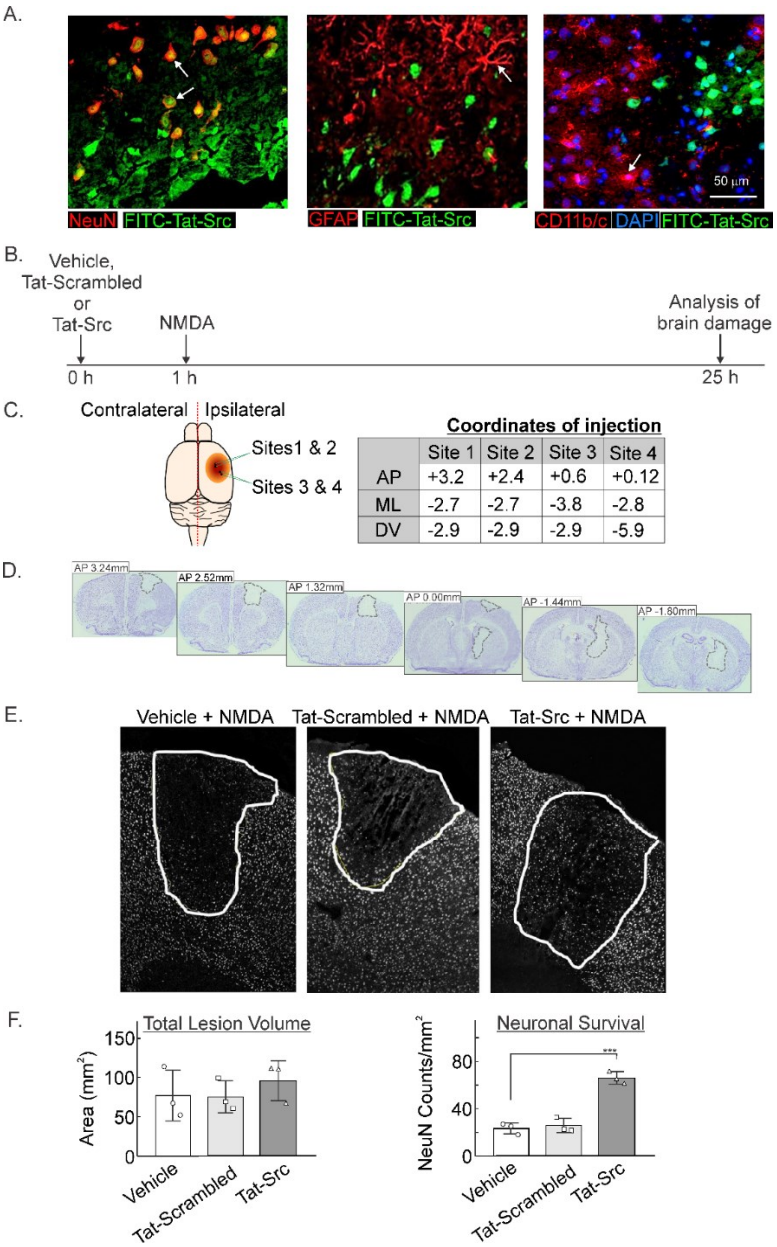

Figure 3 TAT-Src protects against neuronal loss *in vivo* in NMDA-mediated neurotoxicity
(**A**) Representative photomicrographs of FITC labelled TAT-Src infusion co-labelled against markers of neuronal cells (NeuN+ red); astrocytes (GFAP+; red) and microglia (CD11b/c OX-42+, red) and observed as orange. (**B**) Time line depicting treatment with Vehicle (Milli-Q H_2O, 3 µl), Tat-Src (5 mM in Milli-Q H_2O, 3 µl) or Tat-Scrambled (5 mM in Milli-Q H_2O, 3 µl) at 1 hour prior to NMDA-induced excitotoxicity. (**C**) Stereotaxic coordinates of the four injection sites (sites 1 - 4) used to cerebrally inject NMDA to induce excitotoxicity (70 mM in PBS, 1 µl per site). (**D**) Representative thionin-stained coronal images of rat brains infused with NMDA to demonstrate damage to the motor cortex and dorsal striatum. **(E**) NeuN+ cells (transposed white using Image J software) in all three treatment groups. (**F**) Left panel: the total lesion volumes in the treatment groups. Right panel: the total number NeuN+ cells in each treatment group was point-counted using image J software with the number of surviving neurons within the lesion significantly increased in rats treated with TAT-Src ($p < 0.0001$, n = 3/group, one-way ANOVA) followed by the Bonferroni post-hoc test. Data were analyzed using GraphPad Prism, version 8 and presented as mean ± SD. Statistical significance was defined as $p < 0.05$ for infarct volume and $p < 0.0001$ for NeuN cell count.

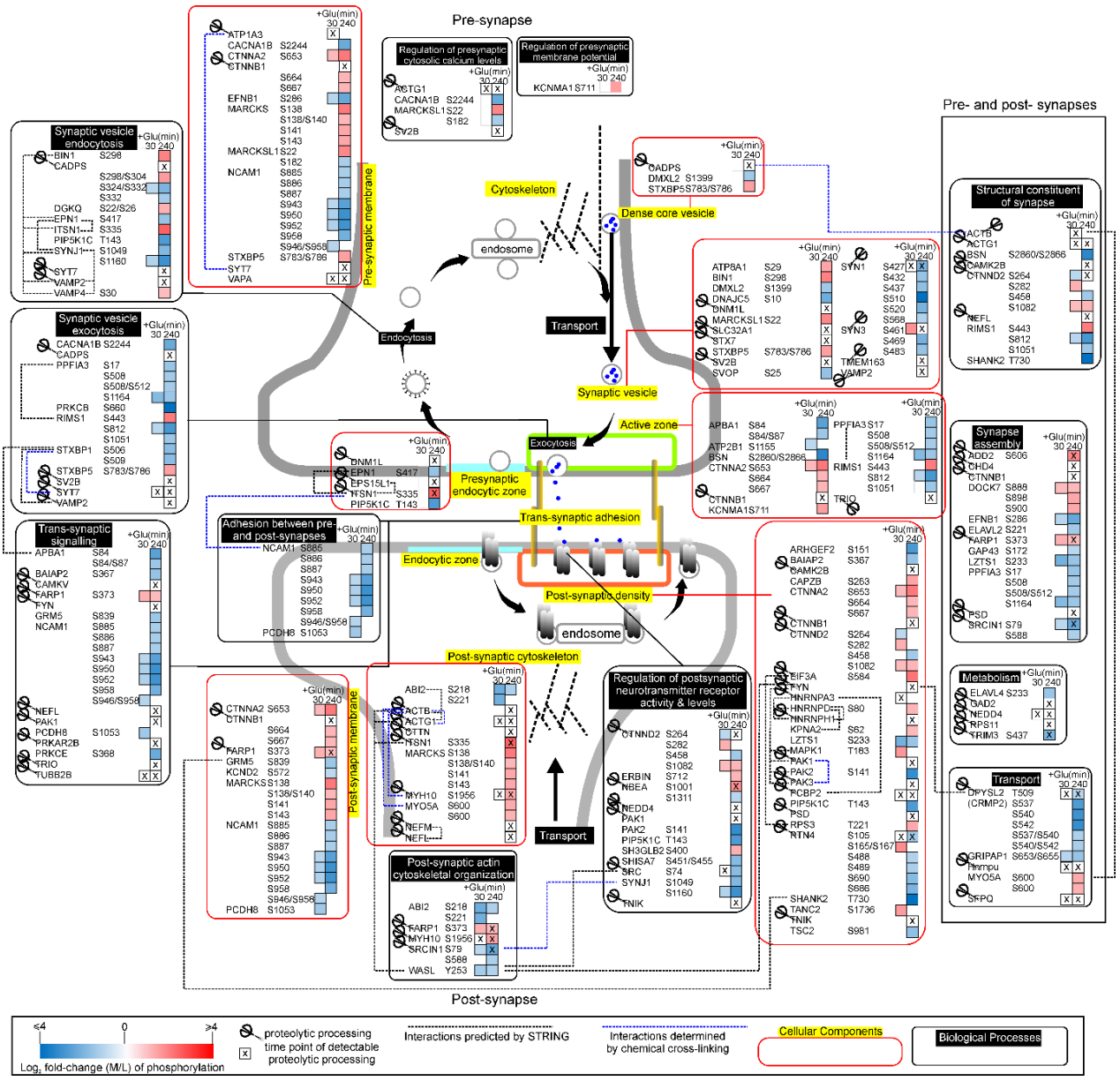

Figure 4 Synaptic proteins undergo significantly changed phosphorylation and enhanced proteolytic processing Neuronal proteins undergoing significantly changed phosphorylation and enhanced proteolytic processing were analyzed by SynGO for their synaptic locations (highlighted in yellow and grouped in red boxes and biological processes (white fonts in black background and grouped in black boxes). The heatmaps show the changes in phosphorylation (presented as Log_{10} normalized M/L ratio) of the identified phosphosites. Proteins undergoing significantly enhanced proteolytic processing to form stable truncated fragments are marked with black scissors and the time points at which the neo-N-terminal peptides were detected are marked with "×". Proteins forming complexes identified by cross-linking mass spectrometry by Gonzalez-Lozano are linked by blue dotted lines. Proteins forming complexes documented in STRING and with the complex formation confirmed experimentally are linked by black dotted lines. STRING parameters for analysis of the interaction networks are (i) network type: physical subnetwork, (ii) meaning of network edges: evidence, (iii) active interaction sources: experiment and database and (iv) minimum required interaction score: highest confidence (0.900).

Finding 2 Many neuronal proteins undergoing significant changes in proteolytic processing and phosphorylation are key participants of synaptic organisation and functions

In agreement with previous findings (*3*), bioinformatic analysis of our proteomic data with the IPA and PhosphoPATH software predicted significant perturbation of biological processes at synapses during excitotoxicity. We then performed further analysis to define how the significantly modified neuronal proteins participate in organization and functions of synapses using SynGO, X-link-MS and STRING databases (*4, 7, 8*). The analysis revealed that these proteins are mapped to all key synaptic components and many of them form stable protein complexes (Figure 4). More importantly, they are participants in most synaptic biological processes (Figure 4). Assignment of neuronal proteins undergoing significant modifications during excitotoxicity to specific locations and biological processes of synapses as depicted in Figure 4 provide valuable insights into their roles in the neurotoxic signalling pathways. Since Zika virus-infected neurons undergo excitotoxic cell death, we hypothesise that the proteins we identified in our studies to be significantly proteolytically processed are also cleaved by calpains and/or the Zika virus encoded protease NS2B-NS3 in Zika virus-infected neurons.

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