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A Bidirectional Switch in the Shank3 Phosphorylation State Biases Synapses toward Up or Down Scaling

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24 **ABSTRACT**

25   Homeostatic synaptic plasticity requires widespread remodeling of synaptic signaling

26 and scaffolding networks, but the role of posttranslational modifications in this process has not

27 been systematically studied. Using deepscale, quantitative analysis of the phosphoproteome

28 in mouse neocortical neurons, we found wide-spread and temporally complex changes during

29 synaptic scaling up and down. We observed 424 bidirectionally modulated phosphosites that

30 were strongly enriched for synapse-associated proteins, including S1539 in the ASD-

31 associated synaptic scaffold protein Shank3. Using a parallel proteomic analysis performed on

32 Shank3 isolated from rat neocortical neurons by immunoaffinity, we identified two sites that

33 were hypo-phosphorylated during scaling up and hyper-phosphorylated during scaling down:

34 one (rat S1615) that corresponded to S1539 in mouse, and a second highly conserved site, rat

35 S1586. The phosphorylation status of these sites modified the synaptic localization of Shank3

36 during scaling protocols, and dephosphorylation of these sites via PP2A activity was essential

37 for the maintenance of synaptic scaling up. Finally, phosphomimetic mutations at these sites

38 prevented scaling up but not down, while phosphodeficient mutations prevented scaling down

39 but not up. Thus, an activity-dependent switch between hypo- and hyperphosphorylation at

40 S1586/ S1615 of Shank3 enables scaling up or down, respectively. Collectively our data show

41 that activity-dependent phosphoproteome dynamics are important for the functional

42 reconfiguration of synaptic scaffolds, and can bias synapses toward upward or downward

43 homeostatic plasticity.

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INTRODUCTION

Synaptic scaling is an important form of homeostatic plasticity that bidirectionally adjusts synaptic weights in response to prolonged perturbations in firing, in the correct direction to stabilize neuron and circuit activity (Turrigiano, 2008; Turrigiano et al., 1998; Turrigiano & Nelson, 2004). Synaptic scaling is expressed through changes in the postsynaptic accumulation of glutamate receptors that then lead to increases or decreases in postsynaptic strength. This form of plasticity is triggered by changes in calcium influx, transcription, and translation (Dörrbaum et al., 2020; Ibata et al., 2008; Mao et al., 2018; Schanzenbächer et al., 2016, 2018; Schaukowitch et al., 2017; Steinmetz et al., 2016) and involves a complex remodeling of the postsynaptic density that relies on a number of scaffolding and trafficking pathways within individual neurons (Gainey et al., 2015; Hu et al., 2010; Louros et al., 2018; Steinmetz et al., 2016; Sun & Turrigiano, 2011; Venkatesan et al., 2020). While synaptic scaling protocols are known to induce complex changes in the phosphoproteome (Desch et al., 2021), the causal roles these might play in homeostatic plasticity are largely unexplored. Here we show that synaptic scaling is accompanied by widespread and dynamic changes in the phosphoproteome that are especially enriched in synapse-associated proteins, and find that activity-dependent bidirectional changes in the phosphorylation state of the synaptic scaffold protein Shank3 are essential for the induction of synaptic scaling up and down.

Shank3 is a multidomain scaffold protein that is highly enriched at the postsynaptic density (Naisbitt et al., 1999) and interacts with a number of other scaffold and signaling proteins that are known to be important for synaptic scaling, such as Homer1 and the MAGUKs (Grabrucker et al., 2011; Jiang & Ehlers, 2013). Loss of function of human Shank3 is associated with autism spectrum disorders (ASDs), Phelan-McDermid syndrome, and

69 intellectual disability (Betancur & Buxbaum, 2013), indicating that Shank3 plays essential roles

70 within the central nervous system. We recently showed that a cell-autonomous reduction in

71 Shank3 is sufficient to completely abolish synaptic scaling up (Tatavarty et al., 2020), but how

72 exactly Shank3 facilitates activity-dependent homeostatic changes in synaptic glutamate

73 receptor abundance, and whether changes in phosphorylation state are important in this

74 process, are unknown.

75   Here we employed liquid chromatography, tandem mass spectrometry (LC-MS/MS) to

76 quantitatively profile changes in the synaptic phosphoproteome induced by scaling. We found

77 widespread and temporally complex changes at many phosphosites (>2000 for scaling up and

78 >3000 for scaling down), with strong enrichment for cytoskeletal and synapse-associated

79 proteins. Of these phosphosites, 424 (representing 332 distinct proteins) were bidirectionally

80 regulated during scaling up and down, including Shank3. Further analysis revealed two highly

81 conserved adjacent sites on Shank3 (rat S1615 and rat S1586) that were persistently

82 dephosphorylated during scaling up and were transiently hyperphosphorylated during scaling

83 down. Dephosphorylation of Shank3 during scaling up was maintained by PP2A phosphatase

84 activity, and reversing this dephosphorylation through PP2A inhibition also reversed synaptic

85 scaling up. Finally, we found that mutating S1615 and S1586 to mimic (DD) phosphorylation

86 blocked synaptic scaling up but not down, while mutating these sites to prevent (AA)

87 phosphorylation blocked scaling down but not up. Taken together, these data show that

88 hypophosphorylation of Shank3 through a PP2A-dependent process is essential for

89 maintaining increased postsynaptic strength during scaling up, and suggest that the

90 phosphorylation state of Shank3 can bias synapses toward upward or downward synaptic

91 scaling.

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RESULTS

Synaptic scaling protocols induce widespread and dynamic changes in the

phosphoproteome

While changes in phosphorylation of synaptic proteins such as glutamate receptors are

thought to play a role in the expression of homeostatic plasticity (Diering & Huganir, 2018;

Fernandes & Carvalho, 2016), the full range of activity-dependent phosphorylation changes

induced by synaptic scaling paradigms have not been characterized. We designed an LC-

MS/MS experiment to identify dynamic changes in the phosphoproteome during the prolonged

increases and decreases in activity that drive homeostatic plasticity. Cultured mouse

neocortical neurons were treated in biological duplicates with either tetrodotoxin (TTX, to block

action potential firing) or bicuculline (BIC, to enhance firing) for 5 min, 1 hr, 7 hr, or 24 hr, in

addition to a control untreated group. Following proteolytic digestion with trypsin and LysC, the

peptides were labeled with isobaric mass tag reagents (TMT) to enable sample multiplexing

and precise relative quantification. After mixing, samples were fractionated off-line and

enriched for phosphopeptides prior to on-line LC-MS/MS to increase the depth of coverage of

the proteome and phosphoproteome (Figure 1 – figure supplement 1A). A total of 31,840

phosphosites and 9,643 proteins were quantified in the TTX-treated experiment, and 32,635

phosphosites and 9,512 proteins were quantified in the BIC-treated experiment, altogether

showing the high quality of this proteomics resource. Although biological replicates showed

significant variance, the principal component analysis showed that replicate samples clustered

together and followed the temporal progression of the experimental design (Figure 1 – figure

supplement 1B, C).

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115   Differential abundance analysis was performed using a moderated F-test, identifying

116   proteins and phosphosites with a statistically significant response to BIC or TTX treatment

117   throughout the time course (FDR adjusted p-value < 0.10) (Figure 1 – table supplement 1A-

118   1D). No statistically significant changes in protein abundance were observed during TTX

119   treatment, while 27 proteins were significantly regulated during BIC treatment (Figure 1 –

120   figure supplement 1D, Figure 1 – table supplement 1A, C). These included transcriptional

121   regulators (e.g., Junb, Jund, and Fos) that increased at later time points (7 and 24 hrs),

122   suggesting long-term regulation of gene expression programs. In contrast to these modest

123   changes in protein levels, the phosphoproteome data revealed widespread changes in

124   phosphorylation, with 2,259 and 3,457 phosphosites regulated in response to TTX and BIC

125   treatments, respectively (Figure 1A, B; Figure 1 – table supplement 1B, D).

126   These changes in the phosphoproteome were complex and dynamic (Figure 1A-C).

127   Cluster analysis identified populations of phosphosites that increased or decreased during TTX

128   treatment, with a range of temporal profiles; the BIC cluster analysis revealed similar

129   complexity (Figure 1A, 1B, and 1C Top). To understand the biological processes regulated by

130   these temporal profiles, we performed pathway enrichment analysis followed by network

131   integration to summarize pathway enrichment results (Figure 1C Bottom, Figure 1 – table

132   supplement 2). Neurogenesis pathways were enriched in proteins hyperphosphorylated and

133   hypophosphorylated in response to TTX treatment (Figure 1C, Neurogenesis group), while

134   neuron projection morphogenesis functions (involved in axon and dendrite formation and

135   dynamics) were enriched in proteins hypophosphorylated in response to BIC (Figure 1C,

136   Neuron Projection Morphogenesis group). Numerous pathways associated with synaptic

137   signaling and the synaptic membrane were enriched in clusters that showed increases or

138   decreases during both TTX and BIC treatments (Figure 1C, Synaptic Signaling group).

139   Cytoskeletal pathways were enriched only in phosphosites that increased in response to TTX

140   (Cluster 3) and decreased in response to BIC (Cluster 4), suggesting that synaptic upscaling

141   involves hyperphosphorylation of cytoskeletal components, and vice versa (Figure 1C,

142   Cytoskeleton Organization group). Finally, splicing and RNA related processes were enriched

143   in phosphosites with an early and strong hyperphosphorylation response to BIC (Figure 1C,

144   Splicing and RNA Processing group), indicating gene expression regulation via

145   phosphorylation during downscaling. We also observed enrichment of several developmental

146   and morphogenesis pathways in BIC and TTX treatments. To facilitate visualization and

147   exploration of regulated phosphoproteins and pathways in our dataset, we have made

148   available web applications for browsing the TTX (https://proteomics.broadapps.org/HSP_TTX/)

149   and BIC (https://proteomics.broadapps.org/HSP_Bic/) proteomics data.

150   Synaptic scaling is a bidirectional process, with some overlap in the signaling pathways

151   that regulate up- and downscaling (Fernandes & Carvalho, 2016). We noticed a large overlap

152   of synapse-associated pathways across the BIC and TTX clusters. To gain more insight into

153   the processes underlying synaptic scaling, we identified the 424 phosphosites that were

154   bidirectionally regulated by TTX and BIC treatment when considering the average fold-change

155   across all time points (Figure 1D, Figure 1 – table supplement 1E). The majority of these (335)

156   were downregulated by TTX and upregulated by BIC, with a smaller number upregulated by

157   TTX and downregulated by BIC (89). We hypothesized that these phosphosites could be

158   prioritized for mechanistic characterization, as they may contribute to the bidirectional

159   processes that underlie homeostatic changes in synaptic strength. These bidirectionally

160   regulated phosphoproteins include the synaptic scaffold proteins Dbn1, Dlgap1, Dlgap4,

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161   Homer2, Shank2, and Shank3; a number of neurotransmitter receptors and auxiliary proteins,

162   including Grin3A, Grm5, Gabra2, Gabra5, and Shisa9; and several kinases important in

163   cytoskeletal and synaptic plasticity, including Camk1d and Camk2b (Figure 1 – table

164   supplement 1E). A number of motor, trafficking, and sorting proteins were also identified,

165   including the adaptor protein complex AP3 Beta2 subunit (Ap3b2); enhanced expression of the

166   AP3mu subunit was previously identified as an important factor in the routing of AMPAR to the

167   synaptic membrane during scaling up (Steinmetz et al., 2016). Finally, a number of

168   presynaptic proteins and ion channels were bidirectionally regulated during scaling (Figure 1 –

169   table supplement 1E). While several of these regulated phosphoproteins have been previously

170   implicated in synaptic scaling, one candidate in particular stood out to us: there were robust

171   bidirectional changes in the phosphorylation state of Shank3 – an autism-associated synaptic

172   scaffold protein known to be essential for synaptic scaling up - at S1539 (Figure 1E). These

173   phosphorylation changes occurred without significant changes in protein abundance (Figure 1

174   – table supplement 1A, C). We therefore prioritized Shank3 for in-depth characterization.

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176   **Shank3 is bidirectionally phosphorylated in cultured rat neocortical neurons**

177   Our phosphoproteome screen identified Shank3 as a major synaptic scaffold protein

178   that undergoes robust and bidirectional changes in phosphorylation during synaptic scaling

179   protocols. Shank3 is essential for synaptic scaling up (Tatavarty et al., 2020), but it is unknown

180   how Shank3 mediates synaptic plasticity, leading us to wonder whether activity-dependent

181   changes in Shank3 phosphorylation might be critical drivers of synaptic scaling.

182   To address this question, we first examined whether these activity-dependent

183   phosphorylation changes are conserved across two species (rats and mice) known to express

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184   robust synaptic scaling. We isolated Shank3 protein from neurons cultured from postnatal rat

185   visual cortex and analyzed by LC-MS/MS after 24 h TTX treatment or untreated controls

186   (Figure 2A). We found an almost six-fold reduction in phosphorylation at residue rat S1615

187   (Figure 2B). Sequence alignment analysis using Clustal Omega (Goujon et al., 2010;

188   McWilliam et al., 2013; Sievers et al., 2011) showed that Shank3 is highly conserved, with rat

189   Shank3 99.25% identical to its mouse homolog and human Shank3 94.62% and 95.66%

190   identical to its rat and mouse homologs, respectively. Notably, the sequence around

191   phosphosite S1615 is identical in rat and mouse, with S1615 corresponding to S1539 in mouse

192   Shank3 (Figure 2C Bottom). Thus TTX induces a hypophosphorylation at this conserved site in

193   both species. We identified a second residue, rat S1586, that was also hypophosphorylated by

194   TTX treatment, albeit to a lesser extent (Figure 2B). Of note, S1586 and S1615 reside in the

195   linker region between the proline-rich and the sterile-alpha-motif (SAM) domains of Shank3

196   (Figure 2C Top).

197   Given that synaptic scaling is a process that unfolds over a time scale of many hours,

198   we carefully explored the temporal dynamics of Shank3 phosphorylation. In mouse neocortical

199   cultures, there was robust hypophosphorylation of S1539 that was rapidly expressed (within 10

200   minutes) and persisted for up to 24 hours during continued TTX application (Figure 1E). In

201   contrast, BIC treatment induced transient hyperphosphorylation at S1539 that was evident

202   after 10 minutes but reversed within an hour (Figure 1E). A similar pattern was observed when

203   Shank3 phosphorylation was assessed in rat cultures using an antibody against

204   phosphorylated S1615 (pS1615, Figure 2 – figure supplement 1). Activity-blockade with TTX

205   induced Shank3 hypophosphorylation at 10 minutes and 24 hours, while raising activity with

206   picrotoxin (PTX, similar to BIC) induced transient hyperphosphorylation that reversed at 24 hr

207 (Figure 2D-G). Collectively, these data reveal activity-dependent, bidirectional modifications in

208 the phosphorylation state of Shank3, and show that the temporal dynamics of these

209 phosphorylation changes are conserved across species and culture conditions.

210

211 **Phosphorylation state modulates homeostatic changes in the synaptic enrichment of**

212 **Shank3**

213 Shank3 is highly enriched at the postsynaptic density, where it interacts with a number

214 of synaptic scaffolding and signaling proteins that are important mediators of synaptic scaling

215 (Gainey et al., 2015; Grabrucker et al., 2011; Hu et al., 2010; Jiang & Ehlers, 2013; Shin et al.,

216 2012; Sun & Turrigiano, 2011). We asked whether synaptic scaling protocols might regulate

217 the synaptic abundance of Shank3 by altering its phosphorylation state. Rat visual cortical

218 cultures (used for all subsequent experiments) were treated with TTX or PTX for 24 hours,

219 then fixed and labeled using antibodies against Shank3 and a surface epitope of the

220 postsynaptic AMPA-type glutamate receptor GluA2 (sGluA2, Figure 3A), or the presynaptic

221 glutamate transporter VGluT1 (Figure 3D). Sites where Shank3 was colocalized with these

222 postsynaptic (sGluA2) or presynaptic (VGluT1) markers of excitatory synapses were identified,

223 and the density of these sites along apical-like pyramidal neuron dendrites, as well as the

224 intensity of the signals at these colocalized sites, were determined. Synaptic scaling protocols

225 induced bidirectional changes in the synaptic accumulation of sGluA2, as expected (Figure 3A,

226 B, Gainey et al., 2015; Ibata et al., 2008; Tatavarty et al., 2013); interestingly, activity blockade

227 with TTX also increased, while enhancing activity with PTX reduced, the synaptic intensity of

228 Shank3 (Figure 3A, C). Thus bidirectional changes in Shank3 synaptic abundance occur in

229 tandem with the changes in AMPAR abundance that underlie synaptic scaling.

230   To evaluate whether the phosphorylation state of Shank3 influences its synaptic

231   abundance, we generated expression constructs for Shank3 with double point mutations at

232   residues S1586 and S1615, designed to mimic (DD mutants, where serine, S, was replaced

233   with aspartic acid, D) or prevent (AA, where serine was replaced with alanine) phosphorylation

234   at these sites (Figure 3D). We then expressed GFP-tagged wild-type, DD, or AA Shank3 at low

235   efficiency in rat cultures, immunolabeled against VGluT1, and quantified the synaptic intensity

236   of the GFP signal in pyramidal neuron apical-like dendrites. The density of colocalized puncta

237   was not different between wildtype Shank3 and Shank3 mutants (Figure 3F). In contrast, there

238   was a significant reduction in the accumulation of Shank3 DD at these colocalized sites

239   relative to wildtype Shank3, while the AA mutant was slightly but not significantly increased

240   (Figure 3E). These results show that the phosphomimetic Shank3 mutant accumulates less

241   efficiently at synaptic sites and suggests that TTX-induced hypophosphorylation may

242   contribute to the enhanced synaptic abundance of Shank3 during scaling up.

243

244   **Increased PP2A activity maintains TTX-induced Shank3 hypophosphorylation.**

245   Neuronal activity could alter Shank3 phosphorylation by modulating kinase and/or

246   phosphatase activity. Since scaling up is accompanied by robust and persistent

247   hypophosphorylation of Shank3, we probed for the role of activity-dependent phosphatase

248   activity in this hypophosphorylation. Protein phosphatase 2A (PP2A) plays an important role in

249   some forms of synaptic plasticity (Colbran, 2004; Launey et al., 2004; Mauna et al., 2011;

250   Winder & Sweatt, 2001; Woolfrey & Dell'Acqua, 2015); therefore, it seemed a likely candidate

251   to dephosphorylate Shank3. We immunoprecipitated the catalytic unit of PP2A (PP2Ac) from

252   cells treated with TTX and subjected it to an *in vitro* PP2A activity assay to detect any changes

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253   in the activity of PP2A during scaling up. In comparison with untreated samples, we first

254   observed a reduction in PP2A activity after one-hour TTX treatment (Figure 4A), which shifted

255   to an increase after 24 hr treatment (Figure 4B).

256   These biphasic changes in phosphatase activity led us to hypothesize that PP2A may

257   have a critical role in maintaining, rather than inducing, Shank3 hypophosphorylation during

258   the late phase of scaling up. To test this idea, we treated cells with TTX for 1 or 24 hr, and

259   during the last hour of the regimen, we applied okadaic acid (OKA), a phosphatase inhibitor

260   that preferentially blocks PP2A activity at a low concentration (50 nM, Bialojan and Takai,

261   1988; Cohen et al., 1989; Ishihara et al., 1989; Pribiag and Stellwagen, 2013). We then

262   immunoprecipitated Shank3 and assessed the phosphorylation state of Shank3. As expected,

263   inhibition of PP2A did not alter Shank3 hypophosphorylation in the early phase of scaling (one-

264   hour TTX, Figure 4C, E); in contrast, the hypophosphorylation was reversed to baseline when

265   OKA was introduced during the last hour of a 24-hour treatment with TTX (Figure 4D, F). We

266   also observed a second band of phosphorylated Shank3 after OKA treatment, suggesting that

267   there may be other phosphosites regulated by the OKA treatment that collectively induce a

268   shift in molecule weight. OKA treatment alone did not alter the baseline phosphorylation level

269   of Shank3, indicating that the role of PP2A in dephosphorylating Shank3 only manifested

270   during prolonged neuronal inactivity (Figure 4E, F). We wondered whether other phosphatases

271   such as protein phosphatase 1 (PP1) could also be involved in Shank3 phosphorylation, so we

272   repeated this experiment using a higher concentration of OKA known to inhibit both PP2A and

273   PP1 (500 nM, duBell et al., 2002; Ishihara et al., 1989; Pribiag and Stellwagen, 2013). Unlike

274   PP2A, inhibition of PP1 resulted in significant hyperphosphorylation of Shank3 under baseline

275   conditions (Figure 4 – figure supplement 1A, B). Taken together, these data suggest that PP1

276 controls baseline Shank3 phosphorylation, while PP2A undergoes an activity-dependent

277 biphasic change in activity that maintains the hypophosphorylated state of Shank3 during

278 activity blockade.

279

280 **PP2A is required for TTX-induced synaptic enrichment of Shank3.**

281 We next assessed whether PP2A activity influences the synaptic enrichment of Shank3

282 during scaling up, using the more specific PP2A inhibitor fostriecin (FST, Walsh et al., 1997).

283 Neurons were treated with TTX for 24 hours, and 10 nM FST was added during the final hour.

284 Consistent with our previous dataset (Figure 3C), TTX treatment increased the intensity and

285 density of synaptic Shank3 puncta (Figure 5A-C). Inhibition of PP2A prevented the increase in

286 Shank3 puncta density (Figure 5C) and reduced (but did not eliminate) the increase in Shank3

287 intensity at remaining Shank3 puncta (Figure 5B). FST treatment alone did not impact the

288 baseline synaptic intensity or density of Shank3 puncta (Figure 5B, C), supporting the view that

289 PP2A regulates phosphorylation and synaptic Shank3 localization only during activity

290 blockade. We repeated this experiment with OKA and confirmed that PP2A inhibition

291 diminished synaptic enrichment of Shank3 during activity blockade (Figure 5 – figure

292 supplement 1A, B).

293 Because PP1 regulates Shank3 phosphorylation under basal conditions, we also tested

294 whether PP1 inhibition influences synaptic Shank3 clustering by applying tautomycetin

295 (TAUT), a specific PP1 inhibitor (10 nM, Mitsuhashi et al., 2001). TAUT treatment alone

296 reduced the baseline intensity of synaptic Shank3 without disrupting the density of synaptic

297 Shank3 puncta (Figure 5D-F). Taken together with our direct measurements of Shank3

298 phosphorylation (Figure 4C-F and Figure 4 – figure supplement 1), these data suggest that

299   PP1 influences baseline synaptic Shank3 clustering, while PP2A is recruited during activity

300   deprivation to dephosphorylate Shank3 and promotes its enrichment at synapses.

301

302   **The phosphorylation state of Shank3 is critical for enabling bidirectional synaptic**

303   **scaling.**

304   Shank3 is necessary for synaptic scaling up (Tatavarty et al., 2020), but whether

305   changes in its phosphorylation state are critical for this (or indeed any other) function of

306   Shank3 is unknown. To test this, we transfected neurons with our phosphomimetic (DD)

307   mutant of Shank3 to determine whether preventing hypophosphorylation would block synaptic

308   scaling up. We performed whole-cell patch-clamp recordings from transfected neurons and

309   measured AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), a

310   physiological measure of postsynaptic strength. In neurons overexpressing Shank3 WT, TTX

311   induced the normal increase in the mEPSC amplitude that is the classic measure of scaling up

312   (Figure 6A, C). Strikingly, overexpression of the DD mutant completely blocked scaling up

313   (Figure 6B, D), suggesting that dephosphorylation of Shank3 at these sites is essential for its

314   induction.

315   While a normal complement of Shank3 is essential for scaling up (Tatavarty et al.,

316   2020), whether it is also required for scaling down is unknown. To investigate this, we

317   expressed a short-hairpin RNA at low efficiency to reduce synaptic Shank3 by ~50% in

318   transfected pyramidal neurons (Tatavarty et al., 2020). Treatment of cultures with PTX for 24

319   hr induced the expected reduction in mean mEPSC amplitude and a shift in the cumulative

320   probability distribution of amplitudes toward smaller values (Figure 6 – figure supplement 1A,

321   B), indicative of synaptic scaling down. Knockdown of Shank3 completely prevented scaling

322   down in transfected neurons (Figure 6 – figure supplement 1A, B), indicating that Shank3 is

323   necessary for both directions of synaptic scaling. Because elevated neuronal activity induces

324   transient hyperphosphorylation of S1615 (Figure 2D-G), we next wondered if this

325   hyperphosphorylation was necessary for scaling down. Indeed, although scaling down was

326   intact in neurons expressing WT Shank3, it was absent in neurons expressing the

327   phosphodeficient AA mutant (Figure 6E-H).

328   Our data show that hypophosphorylation of Shank3 is necessary for scaling up, while

329   hyperphosphorylation is necessary for scaling down. This suggests that the phosphorylation

330   state of Shank3 acts as a switch to enable up or downscaling; if so, then scaling up should be

331   intact in the AA (non-phosphorylatable) mutant, and scaling down should be intact in the DD

332   (phosphomimetic) mutant. To test this, we transfected neurons with the AA mutant and treated

333   them with TTX, or the DD mutant and treated with BIC, and then quantified synaptic GluA2 to

334   measure synaptic scaling. We found that scaling up was indeed preserved in the AA mutant;

335   likewise, scaling down was preserved in the DD mutant (Figure 6 – figure supplement 2);

336   notably, expression of these mutants does not by itself drive scaling up or down, as baseline

337   mEPSC amplitude was unaffected by AA or DD expression (Figure 6). Thus, phosphorylation

338   of Shank3 at residues S1586 and S1615 blocks scaling up but is permissive for scaling down,

339   while dephosphorylation at these same residues blocks scaling down but is permissive for

340   scaling up.

341

342   **PP2A sustains scaling up through hypophosphorylation of Shank3.**

343   If the ability of PP2A inhibition to reverse scaling up is dependent upon enabling

344   phosphorylation at S1586 and S1615 (Figure 4F), then it should be prevented by expression of

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345   the AA mutant, which cannot be phosphorylated at these sites. To test this, we transfected

346   neurons with either WT or AA Shank3, treated cultures with TTX for 24 hours, added FST

347   during the last hour of treatment, and then measured synaptic scaling by quantifying the

348   synaptic accumulation of GluA2. Compared with TTX treatment alone, we found that FST

349   treatment significantly reduced synaptic sGluA2 intensity in cells expressing Shank3 WT

350   (Figure 7A, B). In contrast, FST treatment was unable to reduce GluA2 accumulation in

351   neurons expressing the AA mutant (Figure 7A, B). Similar to endogenous Shank3 (Figure 5B),

352   the synaptic abundance of Shank3 WT was reduced upon FST treatment, while that of the AA

353   mutant was not (Figure 7C). Taken together, these data demonstrate that PP2A maintains the

354   expression of synaptic scaling up by dephosphorylating Shank3 at S1586 and S1615.

355

356   **DISCUSSION**

357   Homeostatic synaptic scaling is a bidirectional process that modifies the accumulation

358   of synaptic glutamate receptors through a complex remodeling of postsynaptic scaffolding,

359   trafficking, and signaling networks (Gainey et al., 2015; Hu et al., 2010; Louros et al., 2018;

360   Steinmetz et al., 2016; Sun & Turrigiano, 2011; Tatavarty et al., 2020; Venkatesan et al.,

361   2020). Notably, scaling up and down have been reported to support the therapeutic effects of

362   ketamine and lithium on depression and bipolar disorder, respectively; thus, a complete

363   understanding of how synaptic scaling is regulated bidirectionally could potentially help

364   advance targeted treatments of these mood disorders (Kavalali & Monteggia, 2020). While

365   transcriptional and translational regulation of this process has been intensively studied, it

366   remains unclear which causal role(s) posttranslational modifications play. Here we analyzed

367   the temporal dynamics of the phosphoproteome during the prolonged changes in activity that

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368 drive synaptic scaling up and down and found widespread and dynamic regulation of

369 phosphorylation that was especially enriched in pathways related to synaptic scaffolding and

370 signaling. We then focused on Shank3, a synaptic scaffold protein known to be essential for

371 synaptic scaling (Tatavarty et al., 2020), and which exhibited robust and bidirectional changes

372 in phosphorylation during scaling protocols. We found that Shank3 is dephosphorylated at two

373 sites (S1586 and S1615) during scaling up and hyperphosphorylated during scaling down.

374 These changes in phosphorylation modified the synaptic localization of Shank3 during scaling

375 and were necessary for its expression. Finally, we found that dephosphorylation of these sites

376 via PP2A activity was essential for the maintenance of synaptic scaling up. These data show

377 that Shank3 undergoes an activity-dependent switch between hypo- and hyperphosphorylation

378 at S1586/ S1615 that is necessary to enable scaling up or down, respectively. More broadly,

379 widespread changes in the phosphoproteome are likely to be instrumental in reconfiguring pre-

380 and postsynaptic scaffold and signaling pathways during homeostatic plasticity.

381   Mass spectrometry-based proteomics has been successful in elucidating

382 phosphorylation signaling and adaptation mechanisms in various forms of synaptic plasticity

383 (Guan et al., 2011; Hwang et al., 2021; Li et al., 2016). In this work, we began by exploring

384 changes induced by homeostatic scaling protocols, using a quantitative proteomic and

385 phosphoproteomic methodology. The multiplexing strategy and extensive peptide fractionation

386 provided deep coverage of proteins and phosphosites while reducing technical variability. We

387 identified over 5200 temporally regulated phosphosites (FDR adjusted p-value < 0.10), with

388 only subtle changes in protein levels: only 27 proteins in the BIC treatment condition showed

389 differential abundance changes with our statistical significance criteria. Dörrbaum et al.

390 previously characterized the proteome synthesis, degradation, and turnover during

391   homeostatic scaling (Dörrbaum et al., 2020). Their work identified hundreds of proteins with

392   differential abundance during 7 days of treatment; the longer treatment conditions and higher

393   number of replicates they used may be necessary to capture these relatively modest

394   abundance changes. Nonetheless, our work shows that extensive phosphorylation events with

395   large effect sizes occur at a point in time (24 hr) when there is robust synaptic scaling and

396   relatively small changes in protein abundance. Importantly, 424 of these phosphosites –

397   including Shank3 – exhibited bidirectional changes in phosphorylation during scaling up and

398   down. The global pattern of change in the phosphoproteome during synaptic scaling up and

399   down, and the enrichment in pathways related to synaptic scaffolding and signaling, is

400   consistent with a recent study that used a label-free MS approach (Desch et al., 2021). Thus

401   synaptic scaling results in widespread bidirectional regulation of the phosphoproteome, and

402   these changes may contribute to the regulation of a wide range of cell-biological processes

403   that contribute to the expression of homeostatic plasticity.

404   In addition to synaptic scaling of excitatory synapses, prolonged changes in activity

405   produce a wide range of homeostatic network adaptations that include changes in intrinsic

406   excitability and inhibition (Turrigiano, 2011). Proteomics revealed a number of voltage-gated

407   ion channel subunits that are differentially phosphorylated during scaling up and down

408   protocols that are candidate contributors to these intrinsic excitability changes (Figure 1 – table

409   supplement 1). These ion channels include hyperpolarization-activated cyclic nucleotide-gated

410   (HCN) channels (Hcn1 and Hcn2), delayed rectifier and inwardly rectifying potassium channel

411   subunits (Kcnb1 and Kcnj3), and L and T type calcium channel subunits (Cacna1c and

412   Cacna1i). The function of phosphorylation at the sites identified on these channel subunits will

413   be an interesting avenue of exploration for understanding the mechanisms of intrinsic

18

414   homeostatic plasticity. Similarly, we identified changes in phosphorylation in a network of

415   presynaptic scaffold and release proteins including Bassoon (Bsn), Piccolo (Pclo), Synapsin 2

416   and 3 (Syn2 and Syn3), and Synaptotagmin 11 and 17 (Syt11 and Syt17), that may contribute

417   to presynaptic adaptations. Coupled with the widespread changes in postsynaptic scaffolding

418   and signaling proteins (including Dlgap1 and 4, Homer2, Shank2, Shank3, and Shisa9), these

419   data support the notion of coordinated pre- and postsynaptic adaptations during synaptic

420   scaling. Finally, we identified bidirectional changes in phosphorylation of a number of

421   neurotransmitter receptors known to be important for inhibitory and excitatory homeostatic

422   plasticity, including the GABA$_A$R subunits Gabra2 and Gabra5, the NMDAR subunit Grin3A,

423   and the mGluR5 subunit Grm5. In contrast, although bidirectional changes in phosphorylation

424   of GluA2 at Y876 has been reported after 48 hr of activity manipulation (Yong et al., 2020), we

425   failed to detect such changes; this may reflect the shorter treatment times used here (24 rather

426   than 48 hr) and underscores the dynamic nature of activity-dependent phosphorylation events.

427   Although we have focused our attention here on sites that undergo bidirectional

428   phosphorylation, it is important to note that the machinery that drives scaling up and down (and

429   possibly other forms of homeostatic plasticity) are not entirely overlapping (for scaling up

430   specific examples, see Stellwagen & Malenka, 2006; Tan et al., 2015; for scaling down

431   examples, see Sun & Turrigiano, 2011; Wang et al., 2017), and so some of the large number

432   of unidirectionally affected phosphosites we identified may also prove to be mechanistically

433   important.

434   Changes in the phosphorylation state of Shank3 were of particular interest to us since

435   Shank3 is critical for synaptic scaling up (Tatavarty et al., 2020), interacts with many synaptic

436   partners also known to be important for homeostatic plasticity (Gainey et al., 2015; Hu et al.,

19

437   2010; Shin et al., 2012), and is strongly associated with ASDs and intellectual disability

438   (Betancur & Buxbaum, 2013). We found that Shank3 is bidirectionally phosphorylated during

439   scaling on two conserved sites in the linker region between the proline-rich and SAM domains

440   in both mouse and rat neocortical neurons and that these phosphorylation changes showed

441   similar dynamics between species, with immediate and prolonged dephosphorylation induced

442   by activity-deprivation, and transient early hyperphosphorylation when activity is raised. One of

443   these sites (reported as S1511, corresponding to rat S1586 in our dataset) was also identified

444   in Desch et al.'s report, while rat S1615 was not (Desch et al., 2021). This likely reflects more

445   comprehensive coverage of Shank3 in our assays. These changes in phosphorylation state

446   gate changes in the synaptic abundance of Shank3 during scaling and are essential for the

447   expression of synaptic scaling up and down (respectively). The different time courses of

448   Shank3 phosphorylation changes during scaling up and down are intriguing and suggest that

449   the temporal dynamics of the synaptic scaling machinery are more complex than previously

450   appreciated. In particular, while the transient hyperphosphorylation of Shank3 is necessary to

451   initiate scaling down, it need not be maintained for the subsequent slow removal of synaptic

452   AMPAR that underlies the reduction in synaptic strength. In contrast, upscaling requires the

453   continuous and active hypophosphorylation of Shank3, as transiently reversing this

454   hypophosphorylation after 24 hr of scaling by inhibiting PP2A rapidly reverses scaling up.

455   Activity-dependent changes in Shank3 phosphorylation could be achieved through the

456   altered activity of kinases, phosphatases, or both. Several activity-dependent kinases are

457   known to regulate Shank3: ERK2 and its downstream target ribosome S6 kinase (RSK)

458   phosphorylate Shank3 at S1134/S1163/S1253 (Wang et al., 2020a) and S1648 (Thomas et al.,

459   2005), respectively, while CaMKII can target S782 (Jeong et al., 2021) and S1586 (Dosemeci

460 & Jaffe, 2010). Moreover, both CaMKII and PKA can phosphorylate S685 (Perfitt et al., 2020;

461 Wang et al., 2020b), highlighting the complexity of the kinase network that could potentially

462 regulate Shank3 phosphorylation. In addition to being a target of multiple kinases, Shank3

463 dephosphorylation is actively maintained by phosphatases: we found that PP1 modulates

464 basal levels of Shank3 phosphorylation, while PP2A undergoes a delayed increase in activity

465 upon TTX treatment that is required to maintain the hypophosphorylated state of Shank3

466 during late scaling. One model that could explain these temporal dynamics during scaling up

467 is that reduced kinase (such as ERK2) activity may account for the early stage of Shank3

468 hypophosphorylation, which is then maintained by the delayed activation of PP2A.

469   How does the phosphorylation state of Shank3 enable scaling up and down? Our

470 observation that phosphorylation at S1586/ S1615 influences synaptic enrichment of Shank3

471 suggests that this phosphorylation contributes to the recruitment and/or stability of Shank3 at

472 the synapse. This connection between Shank3 phosphorylation and synaptic clustering is

473 supported by the recent observation that dephosphorylation at S782 increases the synaptic

474 enrichment of Shank3 (Jeong et al., 2021). S1586 and S1615 are located in the linker region

475 between the proline-rich domain and the SAM domain. The SAM domain promotes synaptic

476 targeting (Boeckers et al., 2005), oligomerization, and stabilization of Shank3 (Baron et al.,

477 2006; Hayashi et al., 2009; Naisbitt et al., 1999), suggesting that phosphorylation at these sites

478 might influence synaptic Shank3 enrichment by regulating the function of the SAM domain.

479 The proximity of S1586 and S1615 to the upstream proline-rich domain, which interacts with

480 important cytoskeletal and signaling elements including cortactin and Homer1 (Naisbitt et al.,

481 1999), also raises the possibility that the phosphorylation state of these sites modulates the

482 interaction of Shank3 with local signaling pathways essential for synaptic plasticity.

483   Shank3 is a complex, multiply phosphorylated protein that is highly enriched in the

484   postsynaptic density and interacts both directly and indirectly with cytoskeletal elements,

485   synaptic scaffold proteins, glutamate receptors, and synaptic signaling elements (Grabrucker

486   et al., 2011; Jiang & Ehlers, 2013). Shank3 is thus perfectly placed to act as a signaling and

487   scaffolding hub that can coordinate the activity of the multiple cell-biological processes

488   required to drive homeostatic increases and decreases in synaptic strength. Taken together,

489   our data show that bidirectional and temporally complex changes in Shank3 phosphorylation

490   are necessary for synaptic scaling, and suggest that the function of Shank3 within the

491   postsynaptic density is dynamically modulated by its phosphorylation state to switch it from a

492   configuration that promotes scaling up to one that promotes scaling down. This has important

493   implications for our understanding of bidirectional synaptic plasticity, and how loss of Shank3

494   contributes to synaptic and circuit dysfunction.

495

496   **MATERIALS AND METHODS**

497   All animal procedures were performed according to NIH guidelines and were approved

498   by the Broad Institute of MIT and Harvard IACUC (mouse cultures) or the Brandeis University

499   IACUC (rat cultures).

500

501   **Key Resources Table**

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (Rattus norvegicus)	Long-Evans	Charles River Laboratories	Strain: 006; RRID: RGD_2308852	
strain, strain background (Mus musculus)	C57BL/6	Charles River Laboratories	Strain:027; RRID: IMSR_CRL:27	
antibody	Guinea pig anti-Shank3 (polyclonal)	Synaptic Systems	Cat#: 162 304; RRID: AB_2619863	IF (1:1000); WB (1:1000)

22

antibody	Rabbit anti-pS1615 (polyclonal)	this paper		WB (1:1000)
antibody	Rabbit anti-HA (monoclonal), C29F4	Cell Signaling Technology	Cat#:3724S; RRID: AB_1549585	IP (1:500)
antibody	Chicken anti-HA (polyclonal)	Aves Labs	Cat#: ET-HA100; RRID: AB_2313511	WB (1:1000)
antibody	Donkey anti-rabbit IRDye 680RD	LI-COR	Cat#: 925-68073; RRID: AB_2716687	WB (1:5000)
antibody	Donkey anti-guinea pig IRDye 800CW	LI-COR	Cat#: 925-32411; RRID: AB_2814905	WB (1:5000)
antibody	Donkey anti-chicken IRDye 800CW	LI-COR	Cat#: 925-32218; RRID: AB_2814922	WB(1:5000)
antibody	Chicken anti-GFP (polyclonal)	Aves Labs	Cat#: GFP-1020; RRID: AB_2307313	IF (1:1000)
antibody	Guinea pig anti-VGluT1 (polyclonal)	Synaptic Systems	Cat#: 135 304; RRID: AB_887878	IF (1:1000)
antibody	Rabbit anti-VGluT1 (polyclonal)	Synaptic Systems	Cat#: 135 302; RRID: AB_887877	IF (1:1000)
antibody	Mouse anti-GluA2 (monoclonal)	gift from Eric Gouaux, OHSU		IF (1:1000)
antibody	Goat anti-chicken Alexa 488 (polyclonal)	Thermo Fisher Scientific	Cat#: A-11039; RRID: AB_142924	IF (1:400)
antibody	Goat anti-mouse Alexa 555 (polyclonal)	Thermo Fisher Scientific	Cat#: A-21424; RRID: AB_141780	IF (1:400)
antibody	Goat anti-guinea pig Alexa 555 (polyclonal)	Thermo Fisher Scientific	Cat#: A-21435; RRID: AB_2535856	IF (1:400)
antibody	Goat anti-guinea pig Alexa 647 (polyclonal)	Thermo Fisher Scientific	Cat#: A-21450; RRID: AB_2735091	IF (1:400)
antibody	Goat anti-rabbit Alexa 647 (polyclonal)	Thermo Fisher Scientific	Cat#: A-21245; RRID: AB_2535813	IF (1:400)
recombinant DNA reagent	Shank3 Short Hairpin (PVLTHM)	gift from Chiara Verpelli		Verpelli et al., 2011
recombinant DNA reagent	pDEST53-CMV-Cycle3GFP-Shank3 WT (short-hairpin insensitive)	gift from Chiara Verpelli		Verpelli et al., 2011
recombinant DNA reagent	pDEST53-CMV-Cycle3GFP-Shank3 S1586A/S1615A	this paper		5'*gca*ttgggggaggaaccagttggtggcctggg tagcctgctggaccctgctaagaagtcgcccattg cagcagctcggctcttcagc*gca*3'
recombinant DNA reagent	pDEST53-CMV-Cycle3GFP-Shank3 S1586D/S1615D	this paper		5'*gac*ttgggggaggaaccagttggtggcctggg tagcctgctggaccctgctaagaagtcgcccattg cagcagctcggctcttcagc*gac*3'
recombinant DNA reagent	pDEST53-CMV-HA-Shank3 WT	this paper		
recombinant DNA reagent	pDEST53-CMV-HA-Shank3 S1586A/S1615A	this paper		
recombinant DNA reagent	pAAV-CMV-PI-EGFP-WPRE-bGH	gift from James M. Wilson	Addgene#: 105530; RRID: Addgene_105530	
commercial assay or kit	Lipofectamine 2000	Thermo Fisher Scientific	Cat#: 11668-027	
commercial assay or kit	Gibson Assembly Master Mix	New England Biolabs	Cat#: E2611S	
commercial assay or kit	Lambda protein phosphatase	New England Biolabs	Cat#: P0753S	
commercial assay or kit	BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#: 23227	
commercial assay or kit	Protein-G Magnetic Beads	Thermo Fisher Scientific	Cat#: 88847	

commercial assay or kit	SimplyBlue SafeStain	Thermo Fisher Scientific	Cat#: LC6060	
commercial assay or kit	PP2A Immunoprecipitation Phosphatase Assay Kit	Millipore	Cat#: 17-313	
commercial assay or kit	Ni-NTA Superflow Agarose Beads	Qiagen	Cat#: 30410	
chemical compound, drug	Tetrodotoxin	Tocris	Cat#: 1069	
chemical compound, drug	Bicuculline methobromide	Tocris	Cat#: 0109	
chemical compound, drug	Picrotoxin	Sigma-Aldrich	Cat#: P1675	
chemical compound, drug	Okadaic acid	Santa Cruz	Cat#: sc-3513	
chemical compound, drug	Tautomycetin	Tocris	Cat#: 2305	
chemical compound, drug	Fostriecin	Tocris	Cat#: 1840	
chemical compound, drug	Sequencing-grade trypsin	Promega	Cat#: V5111	
chemical compound, drug	Tandem Mass Tag (TMT) 10plex	Thermo Fisher Scientific	Cat#: 90110	
software, algorithm	Image Lab Software	Bio-Rad	RRID:SCR_014210	https://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z&source_wt=imagelabsoftware_surl
software, algorithm	ZEN Black	Zeiss	RRID:SCR_018163	https://www.zeiss.com
software, algorithm	Metamorph	Molecular Devices	RRID:SCR_002368	http://www.moleculardevices.com/Products/Software/Meta-Imaging-Series/MetaMorph.html
software, algorithm	FIJI	FIJI	RRID:SCR_002285	http://fiji.sc
software, algorithm	GraphPad Prism	GraphPad	RRID:SCR_002798	http://www.graphpad.com/
software, algorithm	IGOR pro	Wavemetrics	RRID:SCR_000325	https://www.wavemetrics.com/products/igorpro/igorpro.htm
software, algorithm	Spectrum mill v.7.00.208	Agilent Technologies		
software, algorithm	R v 4.0	The R Foundation	RRID:SCR_001905	https://www.R-project.org/
software, algorithm	Cytoscape v 3.8.2	Cytoscape	RRID:SCR_003032	http://cytoscape.org
software, algorithm	EnrichmentMap	EnrichmentMap	RRID:SCR_016052	http://baderlab.org/Software/EnrichmentMap
other	Odyssey Blocking Buffer	LI-COR	Cat#: 927-40000	
other	DAPI-Fluoromount-G	SouthernBiotech	Cat#: 0100-20	

502

503 **Neuronal Cultures, Transfections and Drug Treatments**

504   *Rat cultures:* Timed-pregnant Long-Evans rats were obtained from Charles River.

505 Primary neuronal cultures were dissociated from the visual cortex of newborn pups (postnatal

506 day1-3) and plated onto glass-bottomed dishes pre-seeded with glial feeders as previously

507 described (Gainey et al., 2015; Tatavarty et al., 2020). All the experiments were performed

508 from 7 to 10 days in vitro (DIV), during which neurons were sparsely transfected with the

509 following constructs using lipofectamine 2000 (Thermo Fisher Scientific). To exogenously

510 express Shank3 phospho-mutants, Shank3 constructs (2500 ng per dish) were transfected. To

511 knock down endogenous Shank3, an shRNA targeting Shank3 (Tatavarty et al., 2020; Verpelli

512 et al., 2011) was used. For better visualization of neurons during recording, an empty GFP

513 vector was co-transfected in both conditions. In the imaging experiments that measured

514 endogenous Shank3, an empty GFP vector (500 ng per dish) was transfected to delineate the

515 neurons. To induce scaling up, 6 hrs (Figure 6 – figure supplement 2) or 24 hrs after

516 transfection (the remaining experiments) neurons were treated with tetrodotoxin (TTX, 5 μM,

517 Tocris) for ~16 hours. To induce scaling down, picrotoxin (PTX, 100 μM, Sigma-Aldrich) or

518 bicuculline (BIC, 20 μM, Tocris) were used for the same duration. In the experiments where

519 phosphatases were inhibited, okadaic acid (50 nM or 500 nM, Santa Cruz), fostriecin (10 nM,

520 Tocris), or tautomycetin (10 nM, Tocris) were introduced during the last hour of the scaling-

521 inducing regimen. Because the phosphatase inhibitors were dissolved in DMSO, the same

522 volume of DMSO was added to matched sister cultures for the same duration as controls. For

523 western blotting and mass spectrometry, dissociated neurons were plated onto 10-cm plates

524 without the glial feeders and underwent the same treatments described above.

525   *Mouse cultures:* Timed pregnant C57BL/6 mice were acquired from Charles River.

526   Tissue collection was performed at E17. Cortex was dissected in ice-cold Hibernate E medium

527   (Thermo Fisher Scientific) supplemented with 2% B27 supplement (Thermo Fisher Scientific)

528   and 1% Pen/Strep (Thermo Fisher Scientific). Brain tissues were digested in Hibernate E

529   containing 20 U/mL papain, 1 mM L-cysteine, 0.5 mM EDTA (Worthington Biochem kit), and

530   0.01% DNase (Sigma-Aldrich) for 10 min. Neurons were dissociated and plated at a density of

531   6×10^6 /dish onto poly-D-lysine coated 100 mm plates (Biocoat, Corning). Cortical neurons

532   were seeded and maintained in NbActiv1 (BrainBits Inc, Springfield, IL) and grown at 37 °C in

533   95% air with 5% CO2 humidified incubator for 16 days. Cortical neurons were left untreated for

534   control or treated with 1 µM TTX or 20 µM Bicuculline for 5 min, 1 hr, 7 hr, and 24 hr before

535   collection for proteomics and phosphoproteomics analysis.

536

Proteomic Profiling of Mouse Neuronal Cultures

538   *In-solution digestion:* Neuronal cell pellets containing ~6.6×10^6 cells were lysed for 30

539   min at 4 °C in urea lysis buffer (8M urea, 50 mM Tris-HCl pH 8.0, 75 mM NaCl, 1 mM EDTA, 2

540   µg/µl aprotinin (Sigma-Aldrich), 10 µg/µl leupeptin (Roche), 1 mM phenylmethylsulfonyl fluoride

541   (PMSF) (Sigma-Aldrich), 10mM NaF, and 1:100 phosphatase inhibitor cocktails 2 and 3

542   (Sigma-Aldrich)) and cleared by centrifugation at 20,000 x g. Samples were reduced with 5

543   mM dithiothreitol (DTT) for 1 h at 25°C, followed by alkylation with 10 mM iodoacetamide for 45

544   min at 25°C. Samples were diluted with 50 mM Tris-HCl pH 8.0 to a final urea concentration of

545   2 M preceding enzymatic digestion. Proteins were digested with endoproteinase LysC (Wako

546   Laboratories) for 2 h at 25 °C followed by overnight digest with sequencing-grade trypsin

547   (Promega) at 25 °C (enzyme-to-substrate ratios of 1:50). Following digestion, samples were

548   acidified to a concentration of 1% formic acid (FA) and cleared by centrifugation at 20,000 rcf.

549   Remaining soluble peptides were desalted using a reverse phase tC18 SepPak cartridge

550   (Waters). Cartridges were conditioned with 1 ml 100% acetonitrile (MeCN) and 1 ml 50%

551   MeCN/0.1% FA, then equilibrated with 4X 1 ml 0.1% trifluoroacetic acid (TFA). Samples were

552   loaded onto the cartridge and washed 3X with 1 ml 0.1% TFA and 1X with 1 ml 1% FA, then

553   eluted with 2X 600 µl 50% MeCN/0.1% FA. Samples were dried down by vacuum

554   centrifugation, then reconstituted, and their concentrations were measured by BCA assay. 400

555   µg aliquots were made based on the peptide level concentration for TMT labeling.

556   ***TMT labeling of peptides***: TMT labeling was performed as previously described

557   (Zecha et al., 2019). Briefly, 400 µg of peptides per sample were resuspended in 50 mM

558   HEPES pH 8.5 at a concentration of 5 mg/ml. Dried Tandem Mass Tag (TMT) 10-plex reagent

559   (Thermo Fisher Scientific) was reconstituted at 20 µg/µl in 100% anhydrous MeCN and added

560   to samples at a 1:1 TMT to peptide mass ratio. The reaction was incubated for 1 hr at 25 °C

561   while shaking and quenched with 5% hydroxylamine to a final concentration of 0.2% for 15 min

562   at 25 °C while shaking. The TMT-labeled samples were then combined, dried to completion by

563   vacuum centrifugation, reconstituted in 1 ml 0.1% FA, and desalted with a 100 mg SepPak

564   cartridge as described above.

565   ***Basic Reverse Phase (bRP) fractionation:*** TMT-labeled peptides were fractionated

566   via offline basic reverse-phase (bRP) chromatography as previously described (Mertins et al.,

567   2018). Chromatography was performed with a Zorbax 300 Extend-C18 column (4.6 x 250 mm,

568   3.5 µm, Agilent) on an Agilent 1100 high pressure liquid chromatography (HPLC) system.

569   Samples were reconstituted in 900 µl of bRP solvent A (5 mM ammonium formate, pH 10.0 in

570   2% vol/vol MeCN). Peptides were separated at a flow rate of 1ml/min in a 96 min gradient with

571   the following concentrations of solvent B (5 mM ammonium formate, pH 10.0 in 90% vol/vol

572   MeCN) 16%B at 13 min, 40%B at 73 min, 44%B at 77 min, 60%B at 82 min, 60%B at 96 min.

573   A total of 96 fractions were collected and concatenated non-sequentially into 24 fractions. A

574   total of 5% of each of the 24 fractions was reserved for global proteome analysis. The

575   remaining 95% of each fraction were concatenated into 13 fractions for metal affinity

576   chromatography and phosphoproteome analysis.

577   *Metal affinity chromatography*: Ni-NTA Superflow Agarose Beads (Qiagen) were

578   prepared for metal affinity enrichment by performing 3x washes in HPLC water, 1x 30 min

579   incubation in 100 mM EDTA, 3x washes in HPLC water, 1x 30 min incubation in 10 mM $FeCl_3$

580   in water (Sigma), and 3x washes in HPLC water. The beads were centrifuged to remove

581   supernatant and resuspended in 1:1:1 Acetonitrile: Methanol: 0.01% Acetic Acid solvent prior

582   to incubation with peptides. Dried peptides were resuspended in 80% MeCN/0.1% TFA at a

583   concentration of 0.5 µg/µL and incubated with 10 µL of beads for 30 min at room temperature

584   with gentle end-over-end mixing and then centrifuged briefly to remove the flowthrough. The

585   beads with phosphopeptides bound were transferred on top of a stage tip containing 2X C18

586   (Empore) discs and washed 3x with 100 µL of 80%MeCN/0.1%TFA and 1x with 50 µl 1% FA

587   by centrifugation. Peptides were eluted from the beads and bound to the C18 discs using 225

588   µL IMAC elution buffer (500 mM K2HPO4, pH 7). Stage-tip desalting was performed with 1x

589   100 µL 1%FA in water and eluted with 50 µL of 50%ACN/0.1%FA in water. Peptides were

590   dried by vacuum centrifugation.

591   *Liquid chromatography and mass spectrometry*: Dried fractions were reconstituted

592   in 3% MeCN/0.1% FA to an estimated peptide concentration of 1 µg/µl for global proteome or

593   by adding 8 µL per fraction for phosphoproteome fractions. Peptides were analyzed via

28

594   coupled nanoflow liquid chromatography and tandem mass spectrometry (LC-MS/MS) using a

595   Proxeon Easy-nLC 1000 (Thermo Fisher Scientific) coupled to an Orbitrap Q-Exactive Plus

596   Mass Spectrometer (Thermo Fisher Scientific). A sample load of 1 µg (global proteome) or half

597   of the available sample (phosphoproteome) for each fraction was separated on a capillary

598   column (360 x 75 µm, 50 °C) containing an integrated emitter tip packed to a length of

599   approximately 25 cm with ReproSil-Pur C18-AQ 1.9 µm beads (Dr. Maisch GmbH).

600   Chromatography was performed with a 110 min gradient of solvent A (3% MeCN/0.1% FA)

601   and solvent B (90% MeCN/0.1% FA). The gradient profile, described as min:% solvent B, was

602   0:2, 1:6, 85:30, 94:60, 95:90, 100:90, 101:50, 110:50. Ion acquisition was performed in data-

603   dependent acquisition mode with the following relevant parameters: MS1 orbitrap acquisition

604   (70,000 resolution, 3E6 AGC target, 5ms max injection time) and MS2 orbitrap acquisition (top

605   12, 1.6m/z isolation window, 30% HCD collision energy, 35,000 resolution, 5E4 AGC target,

606   120 ms max injection time, 2.1E4 intensity threshold, 20s dynamic exclusion). The original

607   mass spectra and the protein sequence databases used for searches have been deposited in

608   the public proteomics repository MassIVE (http://massive.ucsd.edu) and are accessible at

609   ftp://MSV000087926@massive.ucsd.edu.

610   ***MS Data Processing****:* MS/MS data was analyzed using Spectrum Mill v.7.00.208

611   (Agilent Technologies). MS2 spectra were extracted from RAW files and merged if originating

612   from the same precursor, or within a retention time window of +/- 60 s and m/z range of +/- 1.4,

613   followed by filtering for precursor mass range of 750-6000 Da and sequence tag length > 0.

614   MS/MS search was performed against the mouse UniProt protein database downloaded on

615   April 2021 and common contaminants, with digestion enzyme conditions set to "Trypsin allow

616   P", <5 missed cleavages, fixed modifications (cysteine carbamidomethylation and TMT10 on

617 N-term and internal lysine), and variable modifications (oxidized methionine, acetylation of the

618 protein N-terminus, pyroglutamic acid on N-term Q, and pyro carbamidomethyl on N-term C).

619 For phosphoproteome analysis, phosphorylation of S, T, and Y were added to the variable

620 modifications. Matching criteria included a 30% minimum matched peak intensity and a

621 precursor and product mass tolerance of +/- 20 ppm. Peptide-level matches were validated at

622 a 0.8% false discovery rate (FDR) threshold and within a precursor charge range of 2-6. A

623 second round of validation was then performed for protein-level matches, requiring a minimum

624 protein score of 13 for the global proteome dataset. TMT10 reporter ion intensities were

625 corrected for isotopic impurities in the Spectrum Mill protein/peptide summary module using

626 the afRICA correction method which implements determinant calculations according to

627 Cramer's Rule (Shadforth et al., 2005) and correction factors obtained from the reagent

628 manufacturer's certificate of analysis for lot number SE240163. For global proteome analysis,

629 protein-centric data, including TMT intensity values divided by the corresponding replicate

630 control, were summarized in a table, which was further filtered to remove non-mouse

631 contaminants and proteins with less than 2 unique peptides. For phosphoproteome analysis,

632 peptide-centric data, including TMT intensity values divided by the corresponding replicate

633 control, were summarized and filtered to remove non-mouse peptides.

634

635 **Immunoprecipitation and Western Blotting**

636 Shank3 protein was enriched by immunoprecipitation before analysis by western

637 blotting. At the end of scaling induction, neurons were lysed in RIPA buffer (150 mM NaCl, 50

638 mM Tris, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA) containing

639 cocktails of protease inhibitors (cOmplete, Sigma-Aldrich) and phosphatase inhibitors

640   (PhosSTOP, Sigma-Aldrich), incubated on a rotating rocker at 4°C for 20 minutes, and

641   centrifuged at 13,000 rpm for 15 minutes. The supernatants were then collected, and the

642   protein concentration was measured using a commercial BCA assay (Thermo Fisher

643   Scientific). To enrich the Shank3 protein, cell lysates (~800 ng) were incubated with guinea pig

644   anti-Shank3 antibodies (1 μg, 162304, Synaptic Systems) on the rotating rocker at 4°C

645   overnight. On the next day, the protein-antibody mixtures were incubated with magnetic

646   protein-G beads (20 μl, Thermo Fisher Scientific) for another hour. After being washed

647   thoroughly with RIPA buffer, the protein-antibody-bead mixtures were resuspended directly in

648   the SDS-containing loading buffer (30 μl, LI-COR) and the Shank3 proteins were eluted into

649   the buffer by heating at 70°C for 10 minutes. Once cooled down on the ice, the eluates were

650   loaded into the 7% NuPAGE tris-acetate gel (Thermo Fisher Scientific), electrophoresed until

651   well separated, and then slowly transferred to a PVDF membrane at 4°C overnight. Afterward,

652   the membranes were incubated with the Odyssey blocking buffer (LI-COR) at room

653   temperature for one hour and probed for Shank3 phosphorylation with a rabbit anti-pS1615

654   antibody (1:1000, Broad Institute) at 4°C overnight, followed by one-hour incubation with the

655   donkey anti-rabbit IRDye 680RD antibody (1:5000, LI-COR) at room temperature. After

656   thorough washes, the membranes were imaged on a GelDoc imager (Bio-Rad). To measure

657   total Shank3, the membranes were subject to a second round of staining where the guinea pig

658   anti-Shank3 antibody (1:1000) and a donkey anti-guinea pig IRDye 800CW antibody were

659   used. All the bands were visualized and quantified using the Image Lab Software (Bio-Rad).

660   Data were collected from at least four independent experiments, in which all conditions were

661   run in parallel with and normalized to the untreated control.

662

31

663   **Quantitative Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis**

664   **of Rat Shank3 Phosphorylation**

665   *Sample preparation:* The immunoprecipitation method described above was used to

666   extract the Shank3 protein for mass spectrometry with the following modifications: neurons

667   from two 10-cm plates were pooled for each replicate. Two replicates were prepared for each

668   condition (untreated and TTX-treated) and were subject to the electrophoresis protocol

669   described above. Once separated, the bands containing Shank3 were visualized on the gel

670   with the Coomassie Blue SafeStain (Thermo Fisher Scientific), cut out with a clean blade, and

671   sent to Taplin Mass Spectrometry Facility at Harvard for further processing and analysis.

672   *In-gel digestion:* Excised gel bands were cut into approximately 1 mm^3 pieces. The

673   samples were reduced with 1 mM dithiothreitol for 30 minutes at 60ºC and then alkylated with

674   5 mM iodoacetamide for 15 minutes in the dark at room temperature. Gel pieces were then

675   subjected to a modified in-gel trypsin digestion procedure (Shevchenko et al., 1996). Gel

676   pieces were washed and dehydrated with acetonitrile for 10 min, followed by removal of

677   acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces

678   was with 50 mM ammonium bicarbonate solution containing 12.5 ng/µl modified sequencing-

679   grade trypsin (Promega, Madison, WI) at 4ºC.   Samples were then placed in a 37ºC room

680   overnight. Peptides were later extracted by removing the ammonium bicarbonate solution,

681   followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The

682   extracts were then dried in a speed-vac (~1 hr).

683   *TMT labeling:* Samples were re-suspended in 20 µl 200 mM HEPES buffer along with 6

684   µl of acetonitrile; 2 µl of TMT0 or TMT Super Heavy were added as light and heavy labels to

685   each set of samples.   After one hour 2 µl of a 5% hydroxylamine was added for 15 minutes

686   followed by the addition of 10 µl of 20% formic acid. Samples were mixed and then dried.

687   Desalting of the samples was performed with an in-house de-salting tube using reverse phase

688   C18 Empore™ SPE Disks (3M, Eagan, MN)

689   ***Mass spectrometry analysis:*** On the day of analysis, the samples were reconstituted

690   in 10 µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase

691   HPLC capillary column was created by packing 2.6 µm C18 spherical silica beads into a fused

692   silica capillary (100 µm inner diameter x ~30 cm length) with a flame-drawn tip (Peng & Gygi,

693   2001). After equilibrating the column, each sample was loaded via a Famos auto sampler (LC

694   Packings, San Francisco CA) onto the column. A gradient was formed and peptides were

695   eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). As

696   each peptide was eluted, they were subjected to electrospray ionization, and then they entered

697   into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, San

698   Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem

699   mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence

700   protein identity) were determined by matching protein or translated nucleotide databases with

701   the acquired fragmentation pattern by the software program, Sequest (ThermoFinnigan, San

702   Jose, CA) (Eng et al., 1994). The static modifications of 224.1525 mass units were set for

703   lysine and the N-terminal of peptides (light label), along with 57.0215 mass units on cysteine

704   (iodoacetamide). Differential modifications of 11.0243 mass units were set for lysine and the

705   N-terminal of peptides (heavy label) along with 79.9663 mass units to serine, threonine, and

706   tyrosine was included in the database searches to determine phosphopeptides.

707   Phosphorylation assignments were determined by the Ascore algorithm (Beausoleil et al.,

33

708   2006). All databases include a reversed version of all the sequences and the data were filtered

709   to between a one and two percent peptide false discovery rate.

710

711   **Expression Constructs and Generation of Shank3 Mutants**

712   The construct expressing wild-type rat Shank3 (AJ133120.1) with an N-terminal GFP

713   tag was obtained from Chiara Verpelli (Verpelli et al., 2011). Constructs expressing GFP-

714   tagged or HA-tagged Shank3 phospho-mutants were generated using the Gibson Assembly kit

715   (NEB) with the wild-type Shank3 as the template. For the double phospho-mimetic mutant,

716   residues 1586 (TCC) and 1615 (AGC) were mutated from serine to aspartic acid (GAC). For

717   the double phospho-deficient mutant, the same residues were mutated from serine to alanine

718   (GCA). The coding regions of all constructs were fully sequenced to ensure that no unwanted

719   random mutations were generated during cloning.

720

721   **Generation and Validation of Phosphospecific Shank3 Antibody**

722   Polyclonal antibodies targeting pS1615 Shank3 were generated in rabbits using the

723   immunogen peptide AARLFS[pS]LGELSTI and purified against the phosphopeptide and

724   counter selected against the non-phospho peptide using affinity chromatography (21st Century

725   Biochemicals, Marlborough MA). We then conducted two experiments to validate the

726   specificity of the antibody. In the first experiment, we transfected HEK293 cells with either HA-

727   tagged wild-type Shank3 or the S1586A/S1615A mutant, and 48 hours later performed

728   immunoprecipitation and immunoblotting as described above (Figure 2 – figure supplement

729   1A). In the second experiment, protein lysates containing wild-type HA-Shank3 were subjected

730   to the same western blotting protocol except that after being transferred to the PVDF

34

731 membrane, one set of the replicates were treated with lambda phosphatase (NEB) overnight at

732 25°C before incubation with the pS1615 antibody (Figure 2 – figure supplement 1B).

733

734 **Protein Phosphatase 2A (PP2A) Activity Assay**

735   Dissociated cortical neurons were prepared as described above. After being treated

736 with TTX for 1 hr or 24 hrs, cells were lysed and sonicated in phosphatase extraction buffer (20

737 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0 with cocktails of protease inhibitors and

738 phosphatase inhibitors). Untreated sister cultures were prepared in parallel and served as

739 controls. After centrifugation, protein concentrations of the supernatants were determined

740 using the BCA assay and diluted to 500 µg/mL per sample. The same amounts of cell lysates

741 were then subject to a commercial colorimetric PP2A activity assay (Millipore).

742

743 **Immunocytochemistry**

744   24 or 48 hours after transfection, neurons were fixed with 4% paraformaldehyde for 15

745 minutes and subject to the established staining protocol (Gainey et al., 2015; Tatavarty et al.,

746 2020). To probe endogenous or exogenously expressed Shank3, cells were permeabilized

747 with the blocking buffer (0.1% Triton X-100/10% goat serum in PBS) at room temperature for

748 45 minutes. They were then incubated with the dilution buffer (5% goat serum in PBS)

749 containing the following primary antibodies at 4°C overnight: chicken anti-GFP (1:1000, Aves

750 Labs), guinea pig anti-Shank3 (1:1000, Synaptic Systems), guinea pig or rabbit anti-VGluT1

751 (1:1000, Synaptic Systems). To stain surface GluA2, the protocol was modified such that prior

752 to permeabilization, cells were first incubated with mouse anti-GluA2 (1:1000, gift from Eric

753 Gouaux, Vollum Institute, Oregon Health & Science University, Portland, Oregon) diluted in the

35

754   blocking buffer without Triton X-100 for 1 hr at room temperature. On the next day, neurons

755   were washed three times with PBS and incubated with the dilution buffer containing the

756   following secondary antibodies at room temperature for one hour: goat anti-chicken Alexa-488,

757   goat anti-mouse Alexa-555, goat anti-guinea pig Alexa-555, goat anti-guinea pig Alexa-647,

758   and goat anti-rabbit Alexa-647 (1:400, Thermo Fisher Scientific). After 3 more washes with

759   PBS, the glass bottoms containing stained neurons were detached from the dishes, mounted

760   onto the slides using DAPI-Fluoromount-G mounting medium (SouthernBiotech), and sealed

761   with nail polish.

762

763   **Image Acquisition and Analysis**

764   All the images were acquired using a 63X oil immersion objective on a laser-scanning

765   confocal microscope (LSM880, Zeiss) using ZEN Black acquisition software. For all

766   experiments, acquisition settings including laser power, gain/offset, and pinhole size were kept

767   consistent. During image acquisition, pyramidal neurons were identified by their typical

768   teardrop-shaped somata and apical-like dendrite. For each neuron, ~12 stacked images (step

769   size: 0.33 μm) were obtained to include the apical dendrites and their dendritic branches, and

770   then subject to maximum intensity projection using ZEN Black. To quantify the colocalization of

771   synaptic proteins and their signal intensities, images were analyzed using the MetaMorph

772   software (Molecular Devices). In all cases, GFP expression was first thresholded to create a

773   mask that outlined the neuron. A region of interest was then manually drawn to include the

774   dendritic branches distal to the primary branch point of apical-like dendrite. A threshold was set

775   for the signal intensity in each channel to exclude background noise and was kept consistent

776   across experimental groups. The granularity function in MetaMorph was then used to threshold

36

777 puncta in each channel (puncta size: 0.5 – 5 μm). Binary images were generated to outline

778 identified puncta in each channel, and the colocalized puncta were determined using the

779 Logical AND operation. A synapse was defined as a punctum double-labeled with GFP and

780 VGluT1(Figure 3D, Figure 5, Figure 5 – figure supplement 1) or triple-labeled with GFP,

781 sGluA2, and VGluT1 (Figure 7, Figure 6 – figure supplement 2). Total puncta intensity for each

782 channel at colocalized sites was then measured. The experimental conditions were always run

783 in parallel with the control condition on sister cultures from the same dissociation, and the total

784 puncta intensity measured in the experimental conditions was normalized to the mean total

785 puncta intensity of control in sister cultures unless described otherwise.

786

787 **mEPSC Recordings**

788 Recordings were performed in whole-cell voltage clamp at room temperature; holding

789 potential was -70 mV. Neurons with pyramidal morphology were targeted by visual inspection.

790 Bath solution was ACSF containing (in mM): 126 NaCl, 5.5 KCl, 2 MgSO4, 1 NaH2PO4, 25

791 NaHCO3, 2 CaCl2, 14 dextrose; and 25 μM picrotoxin, 25 μM D-amino-5-phosphovaleric acid

792 (AP5), and 0.1 μM TTX to isolate AMPA-mediated mEPSCs. Internal solution composition (in

793 mM) was: 120 KMeSO$_4$, 10 KCl, 2 MgSO$_4$, 10 K-Hepes, 0.5 EGTA, 3 K$_2$ATP, 0.3 NaGTP, and

794 10 Na$_2$phosphocreatine. Dextrose was added to adjust osmolarity to 320-330 mOsm. Neurons

795 were excluded if resting membrane potential (Vm) was > −55 mV, series resistance (Rs) was >

796 20 MΩ, input resistance (Rin) was < 100 MΩ, Rin or Vm changed by > 30%, or < 25 mEPSCs

797 were obtained. MEPSCs were detected and analyzed using in-house software (see (Pacheco

798 et al., 2021); detection criteria included amplitudes > 5 pA and rise times < 3 ms. To construct

799 cumulative histograms, the first 25 events for each neuron were included.

800

Statistical Analysis

Western blotting, PP2A activity assay, imaging, and electrophysiology

experiments: GraphPad Prism software was used to conduct statistical analyses. For each

experiment, data distribution in individual condition was tested for normality using the

Anderson-Darling test. If all experimental conditions passed the normality test, a t-test, paired

t-test, or two-way ANOVA was used where appropriate. If one or more conditions failed the

normality test, a Mann-Whitney test or Kruskal-Wallis test was used as indicated. Significant

Kruskal-Wallis tests were then subject to a Dunn's post-hoc test for multiple comparisons. The

significance levels were marked by asterisks (*): *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: P

< 0.0001.

Differential abundance analysis of proteomics data: Statistical analysis was

performed in the R environment for statistical computing. Sample log2 TMT ratios were

median-MAD normalized. Proteins and phosphosites with more than 50% missing values were

removed. In order to identify proteins and phosphopeptides that respond to TTX or BIC

treatment, a linear model with time groups as factors was fitted and a moderated F-test was

performed on all coefficients using the limma package (Ritchie et al., 2015). Multiple

hypothesis testing correction was performed using the BH method. Bidirectionally regulated

phosphosites across TTX and BIC treatment were those that showed significant regulation by

F-test in both treatments, as well as opposite signs when calculating the mean logFC across

all time points for each treatment.

Pathway enrichment and network visualization: Proteins and phosphosites showing

differential abundance in response to TTX or BIC treatment were clustered using the k-means

823   methods (k=4 for BIC and k=3 for TTX). The optimal number of clusters was calculated using

824   the elbow method using the total within sums of squares. Pathway enrichment analysis was

825   performed for each cluster with the g:profiler tool (Raudvere et al., 2019). The background list

826   of proteins was set to all detected in the proteomics analysis, and the databases used for

827   annotation were Gene Ontology, KEGG, Wikipathways, and Reactome. The list of enriched

828   pathways and genes contained in each pathway were exported to Cytoscape (Shannon et al.,

829   2003). The EnrichmentMap app was used to generate a network of enriched pathways with the

830   following parameters (min pathway p-value = 0.01; overlap index = 1) (Merico et al., 2010).

831   ***Interactive visualization of proteomic, phosphoproteomic data, and derived***

832   ***pathway activity scores:*** All proteome and phosphoproteome data can be explored as

833   interactive R Markdown documents at https://proteomics.broadapps.org/HSP_TTX/ and

834   https://proteomics.broadapps.org/HSP_Bic/. In addition to TTX/Bic-induced temporal changes

835   on protein and phophosite-level, the apps enable the user to explore pathway-level changes

836   across time points. To project protein and phosphosite expression matrices

837   onto MSigDB canonical pathways (c2.cp v7.4) single sample Gene Set Enrichment Analysis

838   (ssGSEA) was used. TMT ratios of phosphosites and proteins mapping to the same gene

839   symbol were combined by median expression prior to ssGSEA. The resulting normalized

840   enrichment scores (NES) can be interpreted as *pathway activity scores* and served as input for

841   the longitudinal analysis described above.

842

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848

849   **COMPETING INTERESTS**

850   The authors declare no competing interests.

851

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1105

1106 **FIGURE LEGENDS**

1107 **Figure 1. The temporal phosphoproteome response induced by synaptic scaling**

1108 **protocols.** (A) Heatmap showing the abundance (log2 TMT ratios to control) of phosphosites

1109 with a significant response to TTX treatment (F-test, adj. p < 0.1). Hierarchical clustering

1110 shows three major clusters with different temporal profiles. The number of significant

1111 phosphosites in each cluster is indicated. (B) Heatmap showing the abundance (log2 TMT

1112 ratios to control) of phosphosites with a significant response to BIC treatment (F-test, adj. p <

1113 0.1). Hierarchical clustering shows four major clusters with different temporal profiles. The

1114 number of significant phosphosites in each cluster is indicated. (C) Network representation of

1115 pathway enrichment results from phosphosites showing a significant response to TTX or BIC

1116   treatment. (Top) Temporal profiles are shown for the 3 TTX clusters and 4 BIC clusters; thin

1117   lines represent individual phosphosites, while thick lines represent the cluster mean. (Bottom)

1118   Network showing pathways significantly enriched in each of the TTX/BIC clusters. Pathways

1119   are shown as nodes with colors of each node indicating the associated cluster or clusters.

1120   Edges connecting the nodes indicate a significant gene overlap between pathways (Jaccard

1121   Index > 0.5). Related pathways are clustered and the overall function is summarized in text.

1122   The Synaptic Signaling, Neurogenesis, Cell Membrane, Neuronal Cell Body, and Synapse

1123   Organization clusters show enrichment in response to TTX and BIC treatment in both

1124   upregulate and downregulated phosphosites. (D) Upset plot showing the number of unique

1125   and overlapping regulated phosphosites across BIC and TTX datasets with directionality.

1126   Groups highlighted in red represent a total of 424 phosphosites showing regulation in both BIC

1127   and TTX datasets, with opposite directionality. (E) Temporal profile of mouse Shank3 S1539

1128   phosphosite abundance in response to TTX (left) and BIC (right), displaying opposite response

1129   to these treatments; different colors represent two biological replicates.

1130

1131   **Figure 2. Neuronal activity bidirectionally modulates the phosphorylation state of**

1132   **Shank3.** (A) The experiment protocol for extraction of Shank3 from rat cultured neocortical

1133   neurons for further quantitative mass spectrometry (MS) or western blot analyses. (B) Volcano

1134   plot of quantitative MS data showing Shank3 residues that were differentially phosphorylated in

1135   TTX-treated samples compared to untreated controls. The log2 values of fold changes, if

1136   below zero, indicated hypophosphorylation (paired t test: S1586, adjusted p = 0.034142,

1137   S1614/5, 0.014444). (C) Top: diagram showing the location of S1586 and S1615 within the rat

1138   Shank3 protein. Functional domains: ANK = ankyrin repeat; SH3 = SRC homology 3; PDZ =

1139   PSD-95/Disc Large/ZO-1; Pro-rich = proline-rich; SAM = sterile alpha motif. Bottom: homology

1140   comparison of sequences flanking rat S1586 and S1615 (matching mouse S1539) across

1141   species (human Shank3: NP_001358973.1; rat Shank3: NP_067708.2; mouse Shank3:

1142   UniprotKB: Q4ACU6.3). Phosphosites of interest are labeled in red; the only residue not

1143   conserved is shown in blue. (D, E) Representative western blot using an antibody specific for

1144   phosphorylated S1615, showing changes in Shank3 phosphorylation after 10-min (D) or 24-hr

1145   (E) treatment with TTX or PTX. (F) Quantification of the fold-change of Shank3 S1615

1146   phosphorylation in (D). Dashed line indicated the baseline untreated control (one-sample t test:

1147   TTX, $p = 0.0005$ (***), PTX, $p = 0.0675$, n = 5 and 5 biological replicates, respectively). (G)

1148   Quantification of the fold-change of Shank3 S1615 phosphorylation in (E) (one-sample t test:

1149   TTX, $p < 0.0001$ (****), PTX, $p = 0.6336$, n = 7 and 7 biological replicates, respectively). Solid

1150   colored horizontal lines indicate the mean, and error bars represent S.E.M.

1151

1152   **Figure 3. Phosphorylation state modulates activity-dependent changes in the synaptic**

1153   **enrichment of Shank3.** (A) Representative images of synaptic puncta colocalized with

1154   surface GluA2 (sGluA2) and Shank3 in neuron dendrites ± TTX (scale bar = 5 μm). (B)

1155   Quantification of synaptic sGluA2 intensity changes induced by scaling up and down protocols

1156   (number of neurons: untreated, n = 77, TTX, n = 40, PTX, n = 29; Kruskal-Wallis test with post-

1157   hoc Dunn's multiple comparison tests: Un vs. TTX, $p = 0.0034$ (**), Un vs. PTX, $p = 0.0408$ (*),

1158   TTX vs. PTX, $p < 0.0001$ (****)). (C) Quantification of synaptic Shank3 intensity during scaling

1159   up and down protocols (Kruskal-Wallis test with post-hoc Dunn's tests: Un vs. TTX, $p = 0.0155$

1160   (*), Un vs. PTX, $p = 0.0205$ (*), TTX vs. PTX, $p < 0.0001$ (****)). (D) Representative images of

1161   synaptic localization of wild-type Shank3 and Shank3 phospho-mutants (scale bar = 5 μm). (E)

1162 Quantification of synaptic intensity of Shank3 phospho-mutants (number of neurons: WT, n =

1163 33, AA, n = 30, DD, n = 24; Kruskal-Wallis test with post-hoc Dunn's tests: WT vs. AA, p >

1164 0.9999, WT vs. DD, p = 0.0395 (*), AA vs. DD, p = 0.0039 (**)). (F) Quantification of the

1165 density of synaptic puncta containing Shank3 phospho-mutants (number of neurons: WT, n =

1166 32, AA, n = 30, DD, n = 24; Kruskal-Wallis test: p = 0.2814). For imaging experiments here and

1167 below, each data point represents a single pyramidal neuron, and data were collected from at

1168 least four independent experiments.

1169

1170 **Figure 4. Increased PP2A activity maintains TTX-induced Shank3 hypophosphorylation.**

1171 (A) Quantification of PP2A activity after 1-hr TTX treatment (Un, n = 5, TTX, n = 5; paired t

1172 test: p = 0.0018 (**)). (B) Quantification of PP2A activity after 24-hr TTX treatment (Un, n = 7,

1173 TTX, n = 7; paired t test: p = 0.0129 (*)). (C, D) Western blot analyses showing changes in

1174 S1615 phosphorylation after 1-hr (C) or 24-hr (D) TTX treatment, with inhibition of PP2A by

1175 okadaic acid (OKA, 50 nM) during the last hour of treatment. (E) Quantification of S1615

1176 phosphorylation in (C) (Two-way ANOVA with post-hoc Tukey's test: Un vs. OKA, 0.1723, Un

1177 vs. TTX, p = 0.0076 (**), Un vs. TTX/OKA, p = 0.0311 (*), TTX vs. TTX/OKA, p = 0.8942, n = 5

1178 biological replicates). Dashed lines indicated the baseline untreated control. (F) Quantification

1179 of S1615 phosphorylation in (D). (Two-way ANOVA test with post-hoc Tukey's test: Un vs.

1180 OKA, = 0.9979, Un vs. TTX, p = 0.0503, Un vs. TTX/OKA, p > 0.9999, TTX vs. TTX/OKA, p =

1181 0.0531, n = 8 biological replicates).

1182

1183 **Figure 5. PP2A activity is required for TTX-induced synaptic enrichment of Shank3.** (A)

1184 Representative images of synaptic enrichment of endogenous Shank3 upon treatment with

54

1185   TTX and PP2A inhibitor fostriecin (FST) (scale bar = 10 μm). (B) Quantification of synaptic

1186   Shank3 intensity in (A) (number of neurons: DMSO, n = 26, FST, n = 28, TTX, n = 28,

1187   TTX/FST, n = 29; Kruskal-Wallis test with post-hoc Dunn's tests: Un vs. FST, p > 0.9999,

1188   DMSO vs. TTX, p = 0.0002 (***), FST vs. TTX/FST, p = 0.1259, TTX vs. TTX/FST, p =

1189   0.1292). (C) Quantification of density of synapses containing Shank3 in (A) (Kruskal-Wallis test

1190   with post-hoc Dunn's tests: Un vs. FST, p = 0.9458, Un vs. TTX, p = 0.0051 (**), FST vs.

1191   TTX/FST, p = 0.2446, TTX vs. TTX/FST, p = 0.0273 (*)). (D) Representative images of

1192   synaptic enrichment of endogenous Shank3 upon treatment with TTX and PP1 inhibitor

1193   tautomycetin (TAUT) (scale bar = 10 μm). (E) Quantification of synaptic Shank3 intensity in (D)

1194   (number of neurons: DMSO, n = 26, TAUT, n = 21, TTX, n = 28, TTX/TAUT, n = 32; Kruskal-

1195   Wallis test with post-hoc Dunn's tests: DMSO vs. TAUT, p= 0.0315 (*), DMSO vs. TTX, p =

1196   0.0006 (***), TAUT vs. TTX/TAUT, p = 0.0002 (***), TTX vs. TTX/TAUT, p = 0.0392 (*)). (F)

1197   Quantification of density of synapses containing Shank3 in (D) (Kruskal-Wallis test with post-

1198   hoc Dunn's tests: DMSO vs. TAUT, p = 0.2450, DMSO vs. TTX, p = 0.0116 (*), TAUT vs.

1199   TTX/TAUT, p = 0.6552, TTX vs. TTX/TAUT, p = 0.0007 (***)).

1200

1201   **Figure 6. Changes in the phosphorylation state of Shank3 are crucial for bidirectional**

1202   **synaptic scaling.** (A, B) Representative mEPSC recordings from neurons overexpressing

1203   Shank3 WT (A) or DD mutant (B) during scaling up. (C) Quantification of average mEPSC

1204   amplitude in (A) (WT, n = 8, WT+TTX, n = 9; unpaired two-tailed t test: p = 0.0074 (**)). (D)

1205   Quantification of average mEPSC amplitude in (B) (number of neurons: DD, n = 12, DD+TTX,

1206   n = 14; unpaired two-tailed t test: p = 0.5708). (E, F) Representative traces of mEPSCs

1207   recorded from neurons overexpressing Shank3 WT (E) or AA mutant (F) during scaling down.

1208   (G) Quantification of average mEPSC amplitude in (E) (number of neurons: WT, n = 8,

1209   WT+BIC, n = 8; Mann-Whitney test: p = 0.0148 (*)). (H) Quantification of average mEPSC

1210   amplitude in (F) (AA, n = 9, AA+BIC, n = 14; unpaired two-tailed t test: p = 0.8612).

1211

1212   **Figure 7. Brief PP2A inactivation reverses scaling up.** (A) Representative images showing

1213   the effects of 1 hr FST treatment on synaptic sGluA2 intensity in neurons expressing Shank3

1214   WT or AA, after 24 hr of TTX to scale up synaptic strengths (scale bar = 10 µm). (B)

1215   Quantification of synaptic sGluA2 intensity in (A) (number of cells: WT/TTX, n = 22,

1216   WT/TTX/FST, n = 23, AA/TTX, n = 26, AA/TTX/FST, n = 25; Mann-Whitney test: WT/TTX vs.

1217   WT/TTX/FST, p = 0.0007 (***), AA/TTX vs. AA/TTX/FST, p = 0.6337). (C) Quantification of

1218   synaptic Shank3 intensity in (A) (Mann-Whitney test: WT/TTX vs. WT/TTX/FST, p = 0.0058

1219   (**), AA/TTX vs. AA/TTX/FST, p = 0.6204). In this experiment, WT/TTX/FST and AA/TTX/FST

1220   were normalized to WT/TTX and AA/TTX, respectively.

1221

1222   **SUPPLEMENTARY FIGURE LEGENDS**

1223   **Figure 1 – Figure Supplement 1. Proteome and phosphoproteome dynamics in synaptic**

1224   **signaling.** (A) Experimental workflow for deep proteome and phosphoproteome analysis of

1225   cortical neuron explants treated with TTX or BIC. (B) Principal component analysis of the TTX

1226   phosphoproteome dataset. (Left) Percent of variance explained by experimental factors in

1227   each principal component. Most of the variance is explained by the biological replicate,

1228   followed by the treatment time point. (Right) PCA plot shows grouping of replicates by

1229   timepoint in dimensions 2 and 3. (C) Principal component analysis of the BIC

1230   phosphoproteome dataset. (Left) Percent a variance explained by experimental factors in each

1231   principal component. Most of the variance is explained by the biological replicate, followed by

1232   the treatment time point. (Right) PCA plot shows grouping of replicates by timepoint in

1233   dimensions 2 and 3. (D) Heatmap showing the abundance (log2 TMT ratios) of proteins with a

1234   significant response to BIC treatment (F-test, adj. p < 0.1). The gene symbols of the 27

1235   regulated proteins are shown next to the heatmap.

1236

1237   **Figure 2 – Figure Supplement 1. Validation of the pS1615 antibody.** (A) Western blot

1238   image showing the intensity of pS1615 in HA-tagged wild-type Shank3 or the S1586A/S1615A

1239   (AA) mutant. One set of replicates were treated with the phosphatase inhibitor, okadaic acid

1240   (OKA, 500 nM) for 30 minutes before lysis. Untr., untransfected control; n = 3 biological

1241   replicates. (B) Western blot image showing the intensity of pS1615 in HA-tagged wild-type

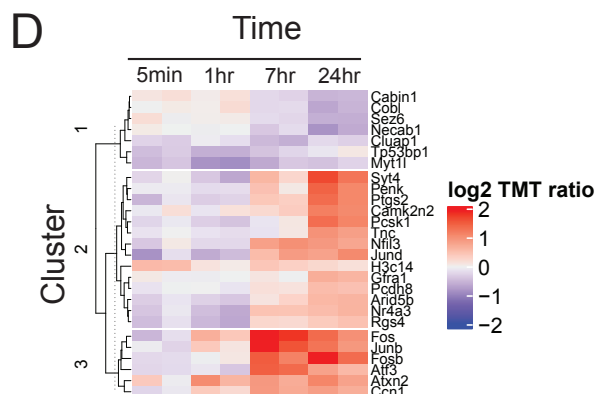1242   Shank3 with and without the treatment of lambda phosphatase (n= 4 biological replicates).

1243

1244   **Figure 4 – Figure Supplement 1. PP1 regulates baseline phosphorylation of Shank3.** (A)

1245   Western blot analysis showing changes in S1615 phosphorylation after 24-hr TTX treatment,

1246   with PP1 inhibition by okadaic acid (OKA, 500 nM) during the last hour of the regimen. (B)

1247   Quantification of S1615 phosphorylation in (A).

1248

1249   **Figure 5 – Figure Supplement 1. Inhibition of PP2A by OKA reverses TTX-induced**

1250   **synaptic enrichment of Shank3.** (A) Representative images of synaptic enrichment of

1251   endogenous Shank3 upon treatment with TTX and PP2A inhibitor OKA (50 nM, scale bar = 10

1252   μm). (B) Quantification of synaptic Shank3 intensity in (A) (Un, n = 31, OKA, n = 29, TTX, n =

1253   30, TTX/OKA, n = 26; Kruskal-Wallis test with post-hoc Dunn's tests: Un vs. OKA, p = 0.3286,

1254   Un vs. TTX, p = 0.0002 (***), OKA vs. TTX/OKA, p > 0.9999, TTX vs. TTX/OKA, p = 0.0036

1255   (**)). (C) Quantification of density of synapses containing Shank3 in (A) (Kruskal-Wallis test

1256   with post-hoc Dunn's tests: Un vs. OKA, p < 0.0001 (****), Un vs. TTX, p = 0.5125, OKA vs.

1257   TTX/OKA, p > 0.9999, TTX vs. TTX/OKA, p < 0.0001 (****)).

1258

1259   **Figure 6 – Figure Supplement 1. Shank3 is required for synaptic scaling down.** (A)

1260   Quantification of average mEPSC amplitudes recorded from neurons depleted of Shank3.

1261   Scaling down was induced by 24-hr treatment with PTX (CTNL, n = 19, PTX, n = 19, KD, n =

1262   18, KD/PTX, n = 16; Mann-Whitney test: CTNL vs. PTX, p = 0.0233 (*), KD vs. KD/PTX, p =

1263   0.8515). (B) Cumulative histogram of mEPSC amplitudes (two-sample Kolmogorov-Smirnov

1264   test: CTNL vs. PTX, p = 0.0017 (**), PTX vs. KD/PTX, p = 0.0624, CTNL vs. KD/PTX, p =

1265   0.0945).

1266

1267   **Figure 6 – Figure Supplement 2. Scaling up and down remain intact in neurons**

1268   **overexpressing Shank3 AA and DD, respectively.** (A) Representative images of changes in

1269   synaptic sGluA2 intensity in neurons overexpressing Shank3 WT and AA during scaling up

1270   (scale bar = 10 µM). (B) Quantification of average synaptic sGluA2 intensity in (A) (WT, n = 28,

1271   WT/TTX, n = 30, AA, n = 34, AA/TTX, n = 28; Mann-Whitney test: WT vs. WT/TTX, p = 0.0009

1272   (***), AA vs. AA/TTX, p = 0.0013 (**)). (C) Representative images of changes in synaptic

1273   sGluA2 intensity in neurons overexpressing Shank3 WT and DD during scaling down (scale

1274   bar = 10 µM). (D) Quantification of average synaptic sGluA2 intensity in (C) (WT, n = 41,

1275   WT/BIC, n = 45, DD, n = 29, DD/BIC, n = 30; Mann-Whitney test: WT vs. WT/BIC, p = 0.0230

1276   (*), DD vs. DD/BIC, p = 0.0300 (*)).

Figure 1

A. Significant phosphosites in TTX treatment
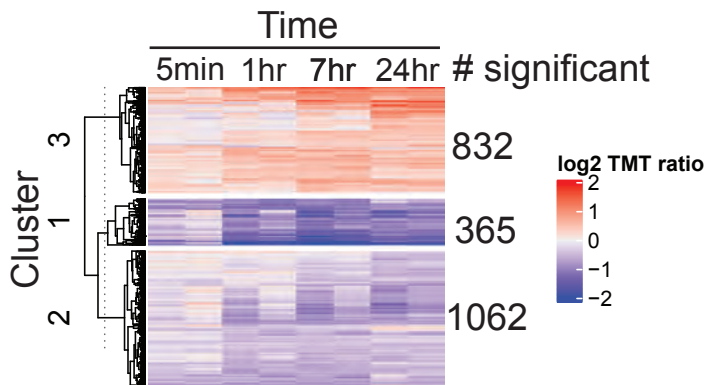
B. Significant phosphosites in BIC treatment
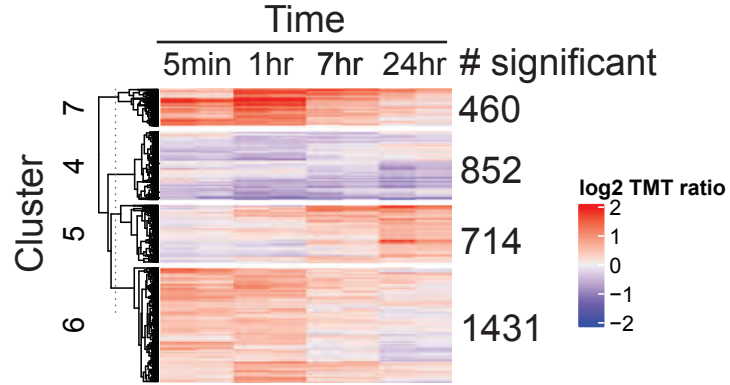
C. TTX phospho cluster profile / BIC phospho cluster profile
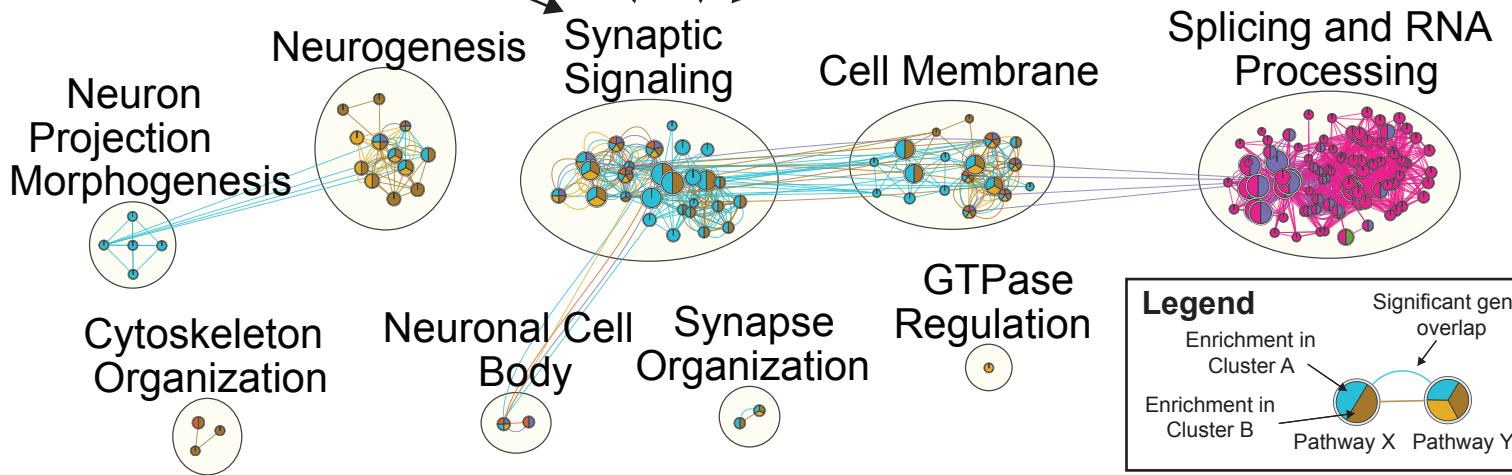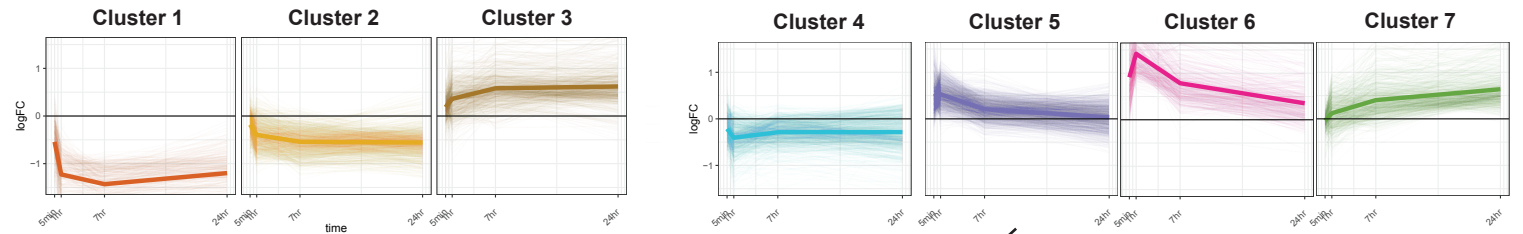
D. Sig. phosphosites TTX and BIC

E. TTX treatment Shank3 pS1539 / BIC treatment Shank3 pS1539

Figure 2

A. Rat cortical neurons, Shank3 pull-down. Untreated (UN), Treated with TTX or PTX (24 hr), lysis. Quantitative LC-MS/MS or Western blotting.

B. TTX vs. UN (rat Shank3). Adjusted P value (-log10) vs. Fold change (log2). pS1614/5, pS1586.

C. ANK, SH3, PDZ, Pro-rich, SAM. rS1586, rS1615.

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Human  LPGPEDDKPTVISELSSRLQQLNKDTRSLGEEPVGGLGS  1596
Rat    LPGPEDDKPTVISELSSRLQQLNKDTRSLGEEPVGGLGS  1597
Mouse  LPGPEDDKPTVISELSSRLQQLNKDTRSLGEEPVGGLGS  1521

Human  LLDPAKKSPIAAARLFSSLGELSSISAQRSPGGPGGGAS  1635
Rat    LLDPAKKSPIAAARLFSSLGELSTISAQRSPGGPGGGAS  1636
Mouse  LLDPAKKSPIAAARLFSSLGELSTISAQRSPGGPGGGAS  1560
```

D. 10 min. Un TTX PTX. IP: Shank3, IB: anti-pS1615, 250kD. IB: anti-Shank3, 250kD.

E. 24 hr. Un TTX PTX. IP: Shank3, IB: anti-pS1615, 250kD. IB: anti-Shank3, 250kD.

F. 10 min. Fold change. TTX (***), PTX (ns).

G. 24 hr. Fold change. TTX (****), PTX (ns).

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 1 — figure supplement 1

Figure 2 — figure supplement 1

Figure 4 — figure supplement 1

A

B

Figure 5 — figure supplement 1

Figure 6 — figure supplement 1

A

B

Figure 6 — figure supplement 2