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Synaptic Plasticity and Spatial Memory

Running Title: *JIP1-regulated JNK and memory*

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1   **Abstract**

2   The c-Jun N-terminal kinase (JNK) signal transduction pathway is implicated in learning and

3   memory. Here, we examined the role of JNK activation mediated by the JIP1 scaffold protein.

4   We compared male wild-type mice with a mouse model harboring a point mutation in the *Jip1*

5   gene that selectively blocks JIP1-mediated JNK activation. These male mutant mice exhibited

6   increased NMDA receptor currents, increased NMDA receptor-mediated gene expression, and

7   a lower threshold for induction of hippocampal long-term potentiation. The JIP1 mutant mice

8   also displayed improved hippocampus-dependent spatial memory and enhanced associative

9   fear conditioning. These results were confirmed using a second JIP1 mutant mouse model that

10 suppresses JNK activity. Together, these observations establish that JIP1-mediated JNK

11 activation contributes to the regulation of hippocampus-dependent, NMDA receptor-mediated

12 synaptic plasticity and learning.

13

14 **Significance Statement**

15 The results of this study demonstrate that JNK activation induced by the JIP1 scaffold protein

16 negatively regulates the threshold for induction of long-term synaptic plasticity through the

17 NMDA-type glutamate receptor. This change in plasticity threshold influences learning. Indeed,

18 mice with defects in JIP1-mediated JNK activation display enhanced memory in hippocampus-

19 dependent tasks, such as contextual fear conditioning and Morris water maze, indicating that

20 JIP1-JNK constrains spatial memory. This study reports the identification of JIP1-mediated JNK

21 activation as a novel molecular pathway that negatively regulates NMDA receptor-dependent

22 synaptic plasticity and memory.

23

24 **Introduction**

25 Human genetic studies have demonstrated that mutations in genes underlying the cJun NH$_2$-

26 terminal kinase (JNK) signaling pathway are associated with neuropsychiatric, neurological and

27 neurodevelopmental disorders, including schizophrenia (*MAP2K7*) (Winchester et al., 2012),

28 epilepsy (*MAPK10*) (Shoichet et al., 2006), autism spectrum disorder (*MAPK8IP2* & *TAOK2*)

29 (Weiss et al., 2008; Giza et al., 2010; de Anda et al., 2012), and learning disability (MAPK10)

30 (Baptista et al., 2008; Kunde et al., 2013). These observations suggest that the JNK pathway

31 has an important normal function in the central nervous system (CNS). Indeed, recent studies

32 using *C. elegans* (Inoue et al., 2013) and murine (Sherrin et al., 2011) experimental models

33 showed that JNK-deficiency results in enhanced memory. This is exemplified by the

34 observations that JNK1-deficient mice exhibit enhanced associative learning, including

35 contextual fear conditioning (Sherrin et al., 2010) and altered synaptic plasticity (Li et al., 2007).

36 Complementary studies using pharmacological inhibition of JNK demonstrate increased long-

37 term depression (LTD) and loss of depotentiation (Yang et al., 2011). Moreover, JNK activation

38 has been implicated in stress-mediated inhibition of long-term potentiation (LTP) (Curran et al.,

39 2003; Wang et al., 2004). Mechanisms that contribute to JNK-regulated synaptic plasticity

40 include NMDA receptor-stimulated JNK activation (Mukherjee et al., 1999), AMPA receptor

41 internalization (Zhu et al., 2005), and synaptic recruitment of PSD95 (Kim et al., 2007).

42 Collectively, these data indicate that JNK plays a key role in the regulation of synaptic plasticity.

43 Although progress towards understanding the role of JNK in neuronal signaling has been

44 achieved, little is known about the mechanisms that regulate JNK during behavioral responses.

45 Previous studies have implicated roles for scaffold proteins in the control of MAP kinase

46 signaling cascades, including the JNK signaling pathway (Morrison and Davis, 2003). Indeed,

47 the JNK-interacting protein 1 (JIP1) scaffold protein can assemble a functional JNK signaling

48 pathway (Whitmarsh et al., 1998; Whitmarsh et al., 2001). JIP1 is required for JNK activation

49 caused by specific stimuli, including metabolic and excitotoxic stress (Whitmarsh et al., 2001;

50 Morel et al., 2010). However, JIP1 is not required for JNK activation caused by other stimuli,

51 including inflammatory cytokines (Whitmarsh et al., 2001).

52 JIP1 is highly expressed in the brain (Dickens et al., 1997; Whitmarsh et al., 1998) and

53 localizes to synapses (Pellet et al., 2000). Interestingly, mice with JIP1-deficiency exhibit

54 increased NMDA receptor signaling (Kennedy et al., 2007), implicating JIP1-mediated JNK

55 activation in the regulation of NMDA receptor activity. The purpose of this study was to examine

56 the impact of JIP1-regulated JNK activation on neuronal function and behavior. JIP1 may

57 contribute to multiple biological processes, including microtubule motor protein function and JNK

58 signaling (Morrison and Davis, 2003). The interpretation of studies using JIP1 knockout mice

59 (Whitmarsh et al., 2001) is therefore complicated by the presence of defects in JIP1-mediated

60 JNK activation and defects in other JIP1-mediated biochemical activities. Consequently, we

61 examined the effect of a point mutation in JIP1 ($Thr^{103}Ala$) that selectively prevents JIP1-

62 mediated JNK activation (Morel et al., 2010). This block in JNK activation is accounted for by a

63 required role of JIP1 phosphorylation on Thr^{103} for dynamic association with upstream MAP3K

64 components of the signaling cascade (Nihalani et al., 2003; Morel et al., 2010). Our results

65 demonstrated that JIP1-mediated JNK activation regulates hippocampus-dependent, NMDA

66 receptor-linked synaptic plasticity and memory. This conclusion was confirmed using a second

67 mouse model with point mutations that disrupt the JNK binding site on JIP1, which also prevents

68 JIP1-mediated JNK activation and alters hippocampus-dependent learning. Collectively, the

69 data demonstrate that the JIP1-JNK signaling axis negatively regulates synaptic plasticity and

70 spatial memory, possibly functioning to constrain and/or shape learning and memory under

71 specific contexts.

72

73 **Materials and Methods**

74

75 **Mice**

76 C57BL/6J mice (stock number 000664) were obtained from the Jackson Laboratory and were

77 estsablished as a colony in our facility. The *Jip1 Thr^{103}Ala* (JIP1^{TA}) mice have been described

78 previously (Morel et al., 2010). Mice with a defect in the JNK binding domain of JIP1

79 (replacement of Leu^{160}-Asn^{161}-Leu^{162} with Gly^{160}-Arg^{161}-Gly^{162}) were established by homologous

80 recombination in embryonic stem (ES) cells using standard methods. The mutated allele is

81 designated as *Jip1$^{\Delta JBD}$*. Briefly, a targeting vector was constructed that was designed to

82 introduce point mutations in exon 3 of the *Jip1* gene to create the ΔJBD mutation. TC1

83 embryonic stem cells (strain129svev) (RRID:CVCL_M350) were electroporated with this vector

84 and selected with 200 µg/ml G418 (Thermo Fisher Scientific, Cat# 10131035) and 2 µM

85 ganciclovir (Millipore, Cat# 345700). ES cell clones without (genotype *+/NeoR-Jip1^{WT}*) and with

86 (genotype *+/NeoR-Jip1$^{\Delta JBD}$*) the ΔJBD mutation in exon 3 were identified. These ES cells were

87 injected into C57BL/6J blastocysts to create chimeric mice that were bred to obtain germ-line

88 transmission of the targeted *Jip1* allele. The floxed *Neo^R* cassette was excised using Cre

89 recombinase. The full characterization of these mice has been described elsewhere (Kant et

90 al., 2017). All mice used in this study were backcrossed (ten generations) to the C57BL/6J

91 strain (Jackson Laboratories). All studies were performed using male mice. The mice were

92 housed in a facility accredited by the American Association for Laboratory Animal Care

93 (AALAC). The Institutional Animal Care and Use Committee (IACUC) of the University of

94 Massachusetts, University of Hawaii, and Morehouse School of Medicine approved all studies

95 using animals.

96

97 **Primary hippocampal neurons**

98 Embryonic day 16.5 mouse embryos were used for isolation of primary hippocampal neurons

99   (Whitmarsh et al., 2001). Briefly, hippocampi were placed in ice-cold Hank's buffered saline

100 solution containing 20 mM HEPES (pH 7.3; HBSS, Thermo Fisher Scientific, Cat# 15630080)

101 and digested with 1% Trypsin (Thermo Fisher Scientific, Cat# 17075029) in the presence of 1

102 mg/mL DNase I (Sigma, Cat# 11284932001). The trypsin solution was removed, and the

103 minced tissue was triturated in 1.0 mL of HBSS-20mM HEPES containing DNase I (1 mg/mL)

104 (Sigma, Cat# 11284932001) and soybean trypsin inhibitor (0.5 mg/mL) (Thermo Fisher

105 Scientific, Cat# 17075029) to obtain a single-cell suspension. Dissociated neurons were

106 centrifuged (180g, 10 min, 4°C) through a cushion of 4% bovine serum albumin (BSA) (Thermo

107 Fisher Scientific, Cat# B14) in HBSS. Hippocampal neurons were seeded in poly-D-

108 lysine/laminin-coated chamber slides (BD Biosciences, Cat# 354687) in Neurobasal medium

109 (Thermo Fisher Scientific, Cat# 21103049) containing B27 supplement (Thermo Fisher

110 Scientific, Cat# 17504044), 1% glutamine (Thermo Fisher Scientific, Cat# 21051024), and 1%

111 penicillin/streptomycin (Thermo Fisher Scientific, Cat# 15140122).

112   Gene expression studies were performed using hippocampal neurons cultured for 14

113 days *in vitro* (14DIV). The neurons were treated without or with 100 µM NMDA/10 µM Glycine

114 (Sigma, Cat# M3262, Cat# G5417) in complete Neurobasal media (Thermo Fisher Scientific,

115 Cat# 21103049). The expression of mRNA was measured by quantitative RT-PCR assays.

116   Immunofluorescence analysis was performed using hippocampal neurons cultured for 16

117 days *in vitro* (16DIV). Two different procedures were used to prepare neurons for

118 immunofluorescence analysis. First, permeabilized fixed neurons were prepared by incubation

119 with 4% (w/v) paraformaldehyde (Thermo Fisher Scientific, Cat# 28906) at room temperature

120 (20 min) followed by incubation with 0.1% Triton X-100 in PBS (5 min) (Thermo Fisher

121 Scientific, Cat# 85112), and then blocking buffer (1% BSA (Thermo Fisher Scientific, Cat# B14),

122 2% normal goat serum (Thermo Fisher Scientific, Cat# 31872), in PBS) for 1 h. Second, non-

123 permeabilized fixed neurons were prepared by incubation with 4% (w/v) paraformaldehyde / 4%

124 (w/v) sucrose (Thermo Fisher Scientific, Cat# 28906, Fisher Scientific, Cat# BP-2201) at room

125   temperature (8 min) followed by incubation (1 h) in blocking buffer. Neurons prepared by both

126   methods were incubated overnight with primary antibodies to GluN1 (1:100, Millipore, Cat# 05-

127   432, RRID: AB_390129) and β-Tubulin (1:500), Covance Research Products Inc, Cat# PRB-

128   433C-200, RRID: AB_291636) in blocking buffer and then washed. The primary antibodies

129   were detected by incubation with anti-mouse or anti-rabbit Ig conjugated to Alexa Fluor 488 or

130   633 (1:200, Molecular Probes, Cat# A-11094, RRID: AB_221544; Thermo Fisher Scientific,

131   Cat# A21100, RRID: AB_10374307). DNA was detected by staining with DAPI (Vectashield

132   with DAPI, Vector Laboratories, Cat# H-1200). Fluorescence was visualized using a Leica TCS

133   SP2 confocal microscope equipped with a 405-nm diode laser. The mean fluorescence

134   intensity was quantitated using ImageJ software (RRID:SCR_001775).

135

136   **Preparation of synaptosomes**

137   Hippocampi from 4 mice (age 8-12 weeks) were isolated and homogenized in Syn-PER buffer

138   (Thermo Fisher Scientific, Cat# 87793). Briefly, neuronal tissue was homogenized in Syn-PER

139   reagent, centrifuged following manufacturer's instruction, followed by suspension of the resulting

140   synaptosome pellet in Syn-PER reagent to yield 3-4μg/μl of synaptic protein. The synaptosomes

141   contain the complete presynaptic terminal, including mitochondria and synaptic vesicles, and

142   also the postsynaptic membrane and the postsynaptic density (Villasana et al., 2006).

143

144   **RNA analysis**

145   The expression of mRNA was examined by quantitative RT-PCR analysis using a 7500 Fast

146   real-time PCR machine. Taqman® assays were used to quantitate *cFos* (Mm00487425_m1),

147   *cJun* (Mm00495062_s1), *Bdnf* (Mm00432069_m1), *GluN1* (*Grin1*, Mm00433800_m1), *GluN2A*

148   (*Grin2a*, Mm00433802_m1) and *GluN2B* (*Grin2b*, Mm00433820_m1) (Thermo Fisher

149   Scientific). The relative mRNA expression was normalized by measurement of *Gapdh*

150   (4352339E-0904021; Thermo Fisher Scientific) in each sample using Taqman® assays.

151

152

153 **Immunoblot analysis**

154 Tissue extracts were prepared from snap-frozen brain regions from JIP1[WT] and JIP1[TA] adult

155 mice (8-12 week-old) using Triton lysis buffer (20 mM Tris-pH 7.4, 1% Triton-X100, 10%

156 glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 μM sodium orthovanadate,

157 1 μM PMSF, 10 μg/mL Leupeptin, and 10 μg/mL Aprotinin). Extracts (20-50 µg of protein) were

158 examined by protein immunoblot analysis by probing with antibodies to pSer[63]-cJun (1:1000,

159 Cell Signaling Technologies Cat# 2361, RRID: AB_490908), JNK (1:1000, Cell Signaling

160 Technologies Cat# 9252, RRID: AB_2250373), GAPDH (Cell Signaling Technologies Cat#

161 2118, RRID: AB_561053, GluN2B (1:1000, Cell Signaling Technologies Cat#4212S, RRID:

162 AB_2112463), SAP102 (1:1000, Cell Signaling Technologies Cat# 3730S, RRID: AB_2092180),

163 pERK1/2 (1:1000, Cell Signaling Technologies Cat# 5683P, RRID: AB_10841299), pSer[133]

164 CREB (1:1000, Cell Signaling Technologies Cat# 9198, RRID: AB_2561044) and CREB

165 (1:1000, Cell Signaling Technologies Cat#9197, RRID: AB_321277), ERK2 (1:1000, Santa Cruz

166 Biotechnology Cat# sc-81457, RRID: AB_1122619), JNK1/2 (Santa Cruz Biotechnology Cat#

167 sc-137019, RRID: AB_2140722), pJNK (Santa Cruz Biotechnology Cat# sc-6254, RRID:

168 AB_628232) and GluN2A (1:500, Millipore Cat# 07-632, RRID: AB_310837), pY[1472] GluN2B

169 (1:1000, Millipore Cat# AB5403, RRID: AB_11210694; GluN1 (Millipore Cat# 05-432, RRID:

170 AB390129), GluA1 (1:2000, Millipore Cat# 04-855, RRID: AB_1977216), GluA2 (1:1000,

171 Millipore Cat# 07-261, RRID: AB_2116167), Synapsin I (1:1000, Millipore Cat# AB 1543, RRID:

172 AB_11210367), β -Tubulin (1:5000, Covance Research Products Inc Cat# PRB-435P-100,

173 RRID: AB_291637), PSD-95 (1:2000, Sigma-Aldrich Cat# P246, RRID: AB_260911) and KIF17

174 (1:1000, Sigma-Aldrich Cat# K3638, RRID: AB_477148). Immunocomplexes were detected by

175 fluorescence using anti-mouse (1:5000, LI-COR Biosciences Cat# 926-32210, RRID:

176 AB_621842) and anti-rabbit secondary IRDye antibodies, (1:5000, LI-COR Biosciences Cat#

177 827-08365, RRID: 10796098) and quantitated using a LI-COR Imaging system.

178

Multiplexed ELISA

180 Quantitative analysis of pSer[63]-cJun (Bio-Rad, Cat#171-V50003M), cJun (Bio-Rad, Cat#171-

181 V60002M), pJNK (Bio-Rad, Cat#171-V50011M), JNK (Bio-Rad, Cat#171-V60007M), pERK

182 (Bio-Rad, Cat#171-V50006M) and ERK1/2 (Bio-Rad, Cat#171-V60003M) was performed using

183 Bio-Plex Pro Cell Signaling Reagent kit (Bio-Rad, Cat#171-304006M) and a Luminex 200

184 instrument (Millipore-Sigma).

185

Kainate-induced excitotoxicity

187 JIP1[WT] and JIP1[TA] mice (8-12 week-old) were injected intraperitoneally with 30 mg/kg kainic acid

188 (Tocris, Cat# 0222) (Yang et al., 1997). At 2h post-treatment, the mice were perfused with 4%

189 paraformaldehyde. Brains were harvested and fixed for an additional 24h in 4%

190 paraformaldehyde, then dehydrated and embedded in paraffin. Coronal sections (5 µm) were

191 cut, rehydrated and subjected to heat-induced antigen retrieval (Vector Laboratories, cat# H-

192 3301). Sections were blocked for 1h at room temperature (1% BSA, 2% normal goat serum,

193 0.4% Triton-X100 in PBS) and incubated overnight with primary antibodies to pSer[63]-cJun

194 (1:100, Cell Signaling Technologies Cat# 2361, RRID: AB_490908), or cFos (1:200, Cell

195 Signaling Technologies Cat# 4384S, RRID: AB_10698737) or cJun (1:100, Santa Cruz

196 Biotechnology Biotechnology Cat# sc-1694, RRID:AB_631263). The primary antibodies were

197 detected by incubation with anti-rabbit Ig conjugated to Alexa Fluor 488 (1:200, Thermo Fisher

198 Scientific Cat# A11008, RRID: AB_143165). DNA was detected by staining with DAPI

199 (Vectashield with DAPI, Vector Laboratories, Cat# H-1200). Fluorescence was visualized using

200 a Leica TCS SP2 confocal microscope equipped with a 405-nm diode laser.

201

Surgery and infusions

Mice were anesthetized with 1.2% Avertin (2, 2, 2-Tribromoethanol, Sigma-Aldrich, #T4, 840-2,

15.5 ml tert-amyl alcohol (2-methyl-2-butanol), Fisher, #A730-1) and implanted bilaterally with

26 gauge guide cannulae (Plastics One) into the dorsal hippocampus (anteroposterior (AP) - 0.5

mm, lateral 1 mm, depth 1.3 mm). Mice were allowed to recover for at least 7 days prior to

behavioral experiments. Intrahippocampal infusions were made using custom 28 gauge

injectors (Plastics One, Cat# C232I/SPC) that extended 1 mm beyond the tips of the guide

cannulae. On the day of the experiment, bilateral injections were performed using an infusion

pump (CMA/100, CMA/Microdialysis) at a constant rate of 0.5 µl/min (final volume: 0.25 µl/side).

The competitive NMDA receptor antagonist, D,L-2-amino-5-phosphonovaleric acid (APV; 10

µg/ml; Sigma, Cat# A5282), was dissolved in artificial cerebrospinal fluid (aCSF) (130 mM NaCl,

3.5 mM KCl, 10 mM glucose, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 24 mM

$NaHCO_3$). Controls received equal volumes of aCSF infused at the same rate. All infusions

were made 15 minutes before contextual fear conditioning.

Analysis of dendritic spine density and morphology

Golgi staining was performed using the FD Rapid Golgi Stain Kit (FD Neurotechnologies, Cat#

PK401) following the manufacturer's guidelines. Coronal sections (150 µm) were obtained

using a microtome (Leica VT1000S). Spines examined were apical (*stratum radiatum*) and

basal (*stratum oriens*) dendrites of CA1 pyramidal neurons. CA1 pyramidal neurons were

traced using a Zeiss Axioskop 2 Plus microscope with a 100x oil immersion objective. Only

pyramidal neurons that exhibited complete impregnation and not obscured by other neurons or

artifacts were examined. Five neurons per animal were three-dimensionally reconstructed using

NeuroLucida Software (MicroBrightField) (RRID:SCR_001775). At least three apical (>50 µm

from soma) and three basal (>30 µm from soma) dendritic segments (>25 µm length) were

227   quantified in each neuron. Spine densities were calculated as mean numbers of spines per 10

228   μm per dendrite per neuron in individual mice. Dendritic arborization was carried out using a

229   Sholl analysis of the apical and basilar dendrites of these neurons. Briefly, a series of

230   increasingly large concentric circles centered at the cell body and separated by 10 μm radius

231   intervals were superimposed upon traces of apical and basilar dendrites; the number of

232   dendritic intersections with each concentric circle was recorded. On the basis of morphology,

233   spines were classified into the following categories: thin, mushroom, and stubby (Korobova and

234   Svitkina, 2010).

235

236   **Analysis of tissue sections**

237   Paraformaldehyde-fixed brains were cryoprotected in 30% sucrose in 0.1 M PBS for 48 h at

238   4°C. Immunohistochemical analysis was performed on free-floating sections cut at 30 μm on a

239   cryostat. The sections were washed once with PBS and blocked with 5% goat serum (Vector

240   Laboratories, Cat# S-1000) in PBST (PBS + 0.3% Triton X-100) for 1 h. The sections were then

241   incubated overnight with primary antibodies to JIP1 (1:500, BD Biosciences, Cat# 611890,

242   RRID: AB_399370), anti-NeuN antibody (1:500, Millipore, Cat# MAB377, RRID: AB_2298772),

243   MAP2 antibody (1: 500, Millipore, Cat# AB5622, RRID: AB_91939), GAD67 antibody (1: 5000,

244   Millipore, Cat# MAB5406 RRID: AB_2278725), or GFAP antibody (1:150, Promega, Cat#

245   G5601, RRID:AB_430855) at 4°C. The sections were washed in PBST and incubated (1h) with

246   anti-mouse Ig or anti-rabbit Ig conjugated to Alexa Fluor 488 (Thermo Fisher Scientific Cat#

247   S11223 RRID: AB_2336881) or 546 (Thermo Fisher Scientific Cat# S11225 RRID:

248   AB_2532130). Nuclei were stained using DAPI (Vectashield with DAPI, Vector Laboratories,

249   Cat# H-1200). Images were obtained with a Zeiss Axio Imager 2 microscope at 10X and 20X

250   magnification. The mean fluorescence intensity was quantitated using ImageJ (RRID:

251   SCR_003070) software. Sections were also examined using Nissl stain (Thermo Fisher

252   Scientific, Cat# N21479).

253

Fear conditioning and extinction

Context-dependent and tone-dependent fear conditioning were performed using a computer-

controlled fear conditioning system (TSE, Bad Homburg, Germany) (Todorovic et al., 2007).

The fear conditioning was performed in a Plexiglas cage (36 x 21 x 20 cm) within a fear

conditioning box. The training (conditioning) consisted of a single trial. The mouse was exposed

to the conditioning context (180 s), followed by a tone [conditioned stimulus (CS), 30 s, 10 kHz,

75 dB SPL, pulsed 5 Hz]. After termination of the tone, a foot shock [unconditioned stimulus

(US), 0.8 mA or 0.4 mA, 2 s, constant current] was delivered through a stainless steel grid floor.

Under these conditions, the context served as background stimulus. Background contextual fear

conditioning but not foreground contextual fear conditioning, in which the tone is omitted during

training, has been shown to involve the hippocampus (Phillips and LeDoux, 1992). A

loudspeaker provided constant background noise. Contextual memory was tested in the fear-

conditioning box for 180 s without CS or US presentation (with background noise), 24 h after

contextual fear conditioning. The context-dependent extinction trials were performed at 24 h

intervals and consisted of non-reinforced, 3-min exposures (absence of a foot shock) to the

same context. For fear response extinction, the aforementioned protocol was used to acquire

contextual fear memory. Tone-dependent memory test was performed in a novel context

(context 2), 24 h after cued fear conditioning. Context 2 represented an identically sized cage

with a plain floor in a light-surrounding environment (350-500 lux) outside the fear-conditioning

box. No background noise was provided in context 2. During tone-dependent memory test, a

180-s pause without stimulation (pre-CS phase) preceded a 180-s period of auditory stimulation.

Freezing, defined as a lack of movement except for respiration was recorded every 10 sec by a

trained observer for a total of 18 sampling intervals. The mean number of observations

indicating freezing was expressed as a percentage of the total number of observations. The

exploration of the fear conditioning box during the training and activity burst produced by electric

279   foot-shock were automatically detected by an infrared beam system and analyzed using TSE

280   software.

281

282   **Morris water maze**

283   The water maze paradigm (Morris et al., 1982) was performed in a circular tank (diameter 180

284   cm; height 75 cm). It was located in a room with various distal cues. The tank was filled with

285   water (40 cm depth) maintained at 23°C, which was made opaque by the addition of a nontoxic

286   white paint. Inside the pool was a removable, circular (12 cm in diameter) Plexiglas platform 0.5

287   cm below the surface of the water. On the first two days, each mouse received visible platform

288   training that consisted of four consecutive trials of climbing onto the visible platform (with a black

289   plastic brick placed above it) until each subject was able to climb without help. For the hidden

290   platform task, the mice were given four consecutive trials per day starting from four different

291   pseudo-randomized start locations, with a 15 min inter-trial interval. Mice were allowed to

292   search for the hidden platform for 60 s. If the mice did not find the platform within 60 s, they

293   were guided to it. Mice were allowed to rest on the platform for 15 s after each trial. The hidden

294   platform task was composed of two phases: (1) 10 days (acquisition phase-days 3-13) with a

295   hidden platform in located in the center of the target quadrant; (2) reversal phase (day 14) with

296   the hidden platform located in the center of the quadrant opposite to the original target quadrant.

297   Reversal platform training was conducted without changing any distal visual cues. Probe trials

298   in which the escape platform was removed from the pool were conducted on days 10 (target

299   quadrant), 13 (target quadrant), and 15 (opposite quadrant). During the memory test (probe

300   trials), the platform was removed from the tank, and the mice were allowed to swim in the maze

301   for 60 s. The swimming path of the mice was recorded by a video camera and analyzed with the

302   computer-based tracking software Videomot 2 (TSE Systems, RRID:SCR_014334). The

303   percentage of swim distance spent in the platform quadrant and the latency to find the platform

304   were analyzed.

305

Rotarod test

Motor coordination and skill learning were assessed using an accelerating Rotarod (Stoelting).

Starting speed for the Rotarod began at 4 rpm and increased to 40 rpm over a 5-min period.

Mice were tested 4 times daily for 2 consecutive days with an inter-trial interval of 1 h between

tests. The latency to fall off the rod was measured for each trial.

311

Elevated plus maze test

The elevated plus-maze test for anxiety-related behaviors was performed as previously

described (Todorovic et al., 2007). Briefly, mice were placed in the center platform of the

elevated plus maze and allowed to explore for 5 min. Animal behavior was recorded by a video

camera connected to a PC and analyzed by videotracking software (VideoMot 2, TSE Systems,

RRID:SCR_014334). The percentage of time spent in the open and closed arms were

recorded. Shift of preference from the open to the closed arms was interpreted as an increase

of anxiety-like behavior. Locomotor activity was determined with this test by the distance

traveled.

321

Open field test

General exploratory activity and anxiety were assessed in an open field test. Mice were placed

in the center of an open field apparatus (50 x 50 cm) protected with 10 cm high opaque walls

and allowed to explore for 5 min. The field was divided into 16 equal squares (12.5 cm x 12.5

cm), consisting of 12 outer squares and 4 inner squares. Animal behavior was recorded by a

video camera connected to a PC and analyzed by videotracking software (VideoMot 2, TSE

Systems, RRID:SCR_014334). The amount of time spent in the inner and outer squares and

the total distance traveled was measured.

330

331

Acoustic startle and prepulse inhibition

Acoustic startle and prepulse inhibition test were performed as previously described (Pitts et al.,

2012). Mice were placed in the startle chamber (Responder-X, Columbus Instruments) and

allowed a 5 min acclimation period with the background noise (70 dB) continuously present.

Following the acclimation period, two blocks of trials were administered to assess the acoustic

startle response and prepulse inhibition, respectively. The first block of trials consisted of 8 sets

of 4 types of trials that were randomly distributed. Startle stimuli (40 ms) of varying intensities

were administered, with an interstimulus interval of 15 s. The stimulus intensities were 80, 90,

100, and 110 dB. Baseline activity was assessed by a set of no-stimulus trials. The startle

amplitude was defined as the peak response during a 100 ms sampling window beginning with

the onset of the startle stimulus. Mean startle amplitudes were derived by subtracting the

average startle amplitudes of stimulus intensities employed (80–110 dB) from the no-stimulus

trial (70 dB). The second block of trials consisted of 8 sets of 5 trial types, distributed randomly

and separated by 20 s interstimulus intervals. The trial types were (1) no-stimulus/background

noise (70 dB); (2) 40 ms, 110 dB startle alone; (3–5) 110 dB startle preceded 100 ms by one of

three 20 ms prepulses at the following intensities: 74, 80, 86 dB. The startle amplitude for each

subject at each of the different prepulse intensities was calculated using the following formula:

prepulse inhibition (PPI)=100−100×[response amplitude for prepulse stimulus paired with startle

stimulus/response amplitude for startle stimulus alone].

Electrophysiology

Extracellular recordings were performed as described previously (Lawrence et al., 2014).

Hippocampi of wild-type or mutant mice (8-12 weeks) were rapidly removed and briefly chilled in

ice-cold artificial CSF (aCSF) (consisting of the following: 130 mM NaCl, 3.5 mM KCl, 10 mM

glucose, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 24 mM $NaHCO_3$ (equilibrated

357 with 95% O_2/5% CO_2, pH 7.4). Transverse slices 350 µm thick were prepared with a Vibratome

358 (Leica; VT1200S) and maintained at least 1 h in a holding chamber containing aCSF. The

359 slices were then transferred to a recording chamber and perfused (3 mL/min) with aCSF at

360 32°C. CA1 field EPSPs (fEPSPs) were recorded with a glass electrode filled with 3 M NaCl

361 (resistance 1-1.5 MΩ) by stimulating the Schaffer collateral fibers through a bipolar stimulating

362 electrode. The slope of the initial rising phase (20–60% of the peak amplitude) of the fEPSP

363 was used as a measure of the postsynaptic response. Basal synaptic neurotransmission was

364 studied by plotting stimulus strength or fiber volley against fEPSP slope to generate input–

365 output relationships. Paired-pulse facilitation (PPF) was defined as the second fEPSP slope

366 divided by the first at various inter-stimulus intervals (10, 50, 90, 130, 170, 210 and 250 ms).

367 For the LTP and long-term depression (LTD) measurements a minimum of 20 min of baseline

368 stimulation (0.05 Hz) was recorded every minute at an intensity that evoked a response 40% of

369 the maximum response. The strong tetanic LTP induction protocol consisted of two 100-Hz

370 tetani (1 s each), with an interval of 20 s between tetani. The weak LTP induction protocol

371 consisted of 900 pulses given at 10-Hz. To induce NMDA receptor-dependent LTD, a 1-Hz and

372 0.5-Hz single pulse stimuli were delivered for 15 and 30 min, respectively (900 stimuli). To

373 induce mGluR-dependent LTD, slices were incubated with 100 µM (S) −3,5-

374 dihydroxyphenylglycine (DHPG) (Torcis, Cat# 0805) for 5 min. For the depotentiation study LTP

375 was evoked by HFS with two trains of 100 Hz (20 s between tetani). Ten minutes after LTP

376 induction, depotentiation was induced by low frequency stimulation consisting of 900 pulses

377 delivered at 1 Hz for 15 min. fEPSP responses were recorded using a computer with WinLTP

378 data acquisition software (WinLTP Ltd, Bristol, UK). For pharmacological studies, JNK-in-8

379 (Selleckchem, Cat# S 4901) (6µM in aCSF with 10% DMSO) or vehicle were applied for 20 min

380 prior to testing and were maintained throughout the recording period.

381   For excitatory post-synaptic current (EPSC) measurements, mice (6 – 10 weeks old)

382 were anesthetized with isoflurane (Sigma Aldrich, Cat# 792632) and perfused with ice cold

383   sucrose solution containing: 200 mM sucrose, 20 mM glucose, 5 mM KCl, 1.2 mM NaH_2PO_4, 25

384   mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.3 mM ascorbic acid and 2.4 mM sodium pyruvate.

385   Transverse sections (350 μm) of the hippocampus were made and then incubated in aCSF at

386   34°C (20 min) before storing at room temperature. The aCSF for these experiments was

387   composed of: 125 mM NaCl, 10 mM glucose, 2.8 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2

388   mM $CaCl_2$, 1.5 mM $MgSO_4$. Whole-cell voltage clamp recordings were done at 32°C utilizing an

389   EPC-10 amplifier and Patchmaster acquisition software (HEKA Instruments Inc., Bellmore, NY,

390   RRID: SCR_000034). Data acquisition and analysis was performed on a MacPro computer

391   (Apple Inc., Cupertino, CA). Bicuculline (30 μM) (Tocris, Cat# 0130) and CGP-55845 (1 μM)

392   (Tocris, Cat# 1248) were added to the artificial aCSF to block $GABA_A$ and $GABA_B$ responses,

393   respectfully. Both sucrose and artificial CSF solutions were continually bubbled with

394   95%O_2/5%CO_2. Patch pipettes were filled with an intracellular solution containing: 130 mM Cs-

395   gluconate, 5 mM NaCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 5 mM Cs_4BAPTA, 2 mM

396   MgATP, 0.3 mM Na_2GTP. The intracellular solution was titrated to pH 7.2 and 280 mOsm. CA1

397   principal cells were held at either -70 or +50 mV to measure the AMPA and NMDA receptor

398   components of EPSCs, respectively. Stimulation of the Schaffer collaterals synapsing onto the

399   CA1 cells was performed using an A360 stimulus isolation unit (World Precision Instruments,

400   Sarasota, FL) with a monopolar platinum iridium electrode placed in the *stratum radiatum*

401   approximately 350 μm from the cell body. The stimulation intensity was adjusted to be

402   approximately 75% of the threshold for firing an action potential. Peak amplitudes were

403   analyzed utilizing Igor Pro v.6.3 (RRID: SCR000325).

404

405   **Experimental design and statistical analysis**

406   Data were statistically analyzed using StatView (SAS Institute, Cary, NC) and GraphPad

407   Prism 6 software (GraphPad, San Diego, CA). Student's t-test was used for comparing two

408   conditions, and ANOVA was used with Bonferroni post hoc test for comparing more than two

409 conditions. All data are expressed as means ± SEM. The accepted level of significance was

410 p ≤ 0.05, indicated by an asterisk; p values ≤0.01 are indicated by double asterisks, while p

411 values ≤ 0.001 are indicated by triple asterisks.

412

413 **Results**

414 The aim of this study was to examine the impact of JIP1-regulated JNK activation on neuronal

415 function and behavior. To accomplish this aim we generated a novel mouse model harboring a

416 T103A point mutation in the *Jip1* gene (also known as *Mapk8ip1*). These mutant

417 *Mapk8ip1$^{T103A/T103A}$* mice (JIP1^{TA}) exhibit a profound defect in JIP1-mediated JNK activation

418 compared to control JIP1^{WT} animals. We therefore used this mutant mouse to determine the

419 contribution of JIP1-mediated JNK activation to NMDA-dependent receptor signaling, memory

420 and synaptic plasticity.

421 ***JIP1 expression in hippocampal neurons***

422 Our initial studies were designed to examine the expression of JIP1 protein in the hippocampus.

423 Immunohistochemical staining of tissue sections demonstrated that JIP1 is expressed in the

424 CA1, CA3, and the dentate gyrus (DG) regions of the hippocampus (**Fig. 1A**). JIP1 was

425 primarily found on dendrites (colocalization with MAP2) and to a lesser extent on cell bodies

426 (colocalization with NeuN) of the CA1/CA3 pyramidal and DG granule neurons (**Fig. 1A**).

427 We next examined the hippocampal architecture of JIP1^{TA} and JIP1^{WT} mice. Qualitative

428 analysis of Nissl- (**Fig. 1 B,C**) and Golgi-stained (**Fig. 2A**) coronal sections did not reveal

429 differences in hippocampus morphology between JIP1^{TA} and JIP1^{WT} mice. Moreover, analysis

430 of basal and apical dendrites from Golgi-stained CA1 pyramidal neurons revealed no significant

431 differences in dendritic branching, spine density, or spine type (**Fig. 2A-E**). In addition, the

432 intensity and distribution of the neuronal marker NeuN ($t_{(6)} = 0.89$, p=0.41; two-tailed unpaired

433 Student's t-test), the dendritic marker MAP2 ($t_{(6)} = 0.71$, p=0.51; two-tailed unpaired Student's t-

434 test), the GABAergic interneuron marker GAD67 ($t_{(6)}= 0.27$, p=0.79; two-tailed unpaired

435 Student's t-test), and the glial marker GFAP ($t_{(6)} = 0.44$, p=0.67; two-tailed unpaired Student's t-

436 test), were similar in JIP1^{TA} and JIP1^{WT} mice (**Fig. 1B-D**). Collectively, these data indicate that

437 the Thr[103]Ala mutation in JIP1 did not cause detectable changes in basic hippocampus

438 morphology.

439 ***JIP1-mediated JNK activation in the hippocampus***

440 We have previously demonstrated that JIP1[TA] mice exhibit defects in metabolic stress-induced

441 JNK activation in adipose tissue (Morel et al., 2010). To test whether the Thr[103]Ala mutation in

442 JIP1 also caused defects in JNK activation in neural tissue, we examined a model of JIP1-

443 dependent JNK activation (kainate excitotoxicity) in the hippocampus. Examination of the DG

444 following short-term exposure to kainate caused JIP1-dependent JNK activation and

445 phosphorylation of the JNK substrate cJun and JIP1-independent increased expression of cJun

446 and cFos (Whitmarsh et al., 2001). We found that kainate caused a similar increase in cJun

447 and cFos expression in JIP1[TA] and JIP1[WT] mice (**Fig. 3B,C**). In contrast, kainate caused

448 increased cJun phosphorylation in JIP1[WT], but not JIP1[TA] mice (**Fig. 3A**). These data

449 demonstrate that the Thr[103]Ala mutation suppresses JIP1-mediated JNK activation in the

450 hippocampus. Time-course analysis confirmed that cJun phosphorylation in the hippocampus

451 of kainate-stimulated JIP1[WT] mice was strongly suppressed in JIP1[TA] mice after exposure to

452 kainate (two-way ANOVA: genotype $F_{(1,32)}$= 15.11, p<0.001; time $F_{(3,32)}$= 34.26, p<0.001;

453 genotype x time $F_{(3,32)}$ = 5.05, p=0.005) (**Fig. 3D,E**). These data extend our previous finding that

454 JIP1[TA] mice exhibit defects in JIP1-dependent JNK activation in adipose tissue (Morel et al.,

455 2010) to demonstrate that JIP1[TA] mice also exhibit profound defects in JIP1-mediated JNK

456 activation in the hippocampus.

457   To test how JIP1-mediated JNK activation contributes to a physiologically relevant

458 response, we examined the effect of contextual fear conditioning, which was previously shown

459 to cause transient JNK activation in the dorsal hippocampus (Sherrin et al., 2010). Immunoblot

460 analysis using antibodies to JNK and phospho-JNK was performed to assess changes in JNK

461 activation in dorsal hippocampal extracts from JIP1[WT] and JIP1[TA] mice that were subjected to

462 single-trial contextual fear conditioning. Extracts were prepared at various times following the

463 single-trial to correspond to the consolidation phase of contextual fear (Igaz et al., 2002). JNK

464 immunoblot analysis detects the 46 kDa (JNK1α1 & JNK1β1) and 54 kDa isoforms (JNK2α2,

465 JNK2β2, and JNK3α2) in brain (Davis, 2000). We found that contextual fear conditioning

466 caused an increase in 46-kDa phospho-JNK in JIP1^{WT} mice at 30 min (176 ± 12% of naïve

467 control mice; Bonferroni post hoc test, p<0.001) and 60 min (127 ± 8% of naïve; Bonferroni post

468 hoc test, p= 0.09) (**Fig. 4**). In contrast, we found that JIP1^{TA} mice exhibited decreased 46-kDa

469 phospho-JNK at 30 min (75 ± 11% of naïve; Bonferroni post hoc test, p=0.011), 60 min (53 ±

470 7% of naïve; p<0.001) and 180 min (79 ± 6% of naïve; p=0.009) after contextual fear

471 conditioning (two-way ANOVA: genotype $F_{(1,50)}$= 15.11, p<0.001; time $F_{(4,50)}$= 10.42, p<0.001;

472 genotype x time $F_{(4,50)}$ = 22.11, p<0.001) (**Fig. 4**). These data demonstrate that the JIP1

473 Thr^{103}Ala mutation suppresses JNK activation caused by contextual fear conditioning.

474

475 ***JIP1-mediated JNK activation suppresses NMDA-dependent synaptic plasticity***

476 JNK activation is associated with mechanistic changes in synaptic plasticity in the hippocampus

477 (Wang et al., 2004; Chen et al., 2005; Zhu et al., 2005; Li et al., 2007; Yang et al., 2011).

478 Consequently, synaptic plasticity may be altered by JIP1-dependent JNK activation in the

479 context of fear conditioning (**Fig. 4**). We therefore examined synaptic transmission in JIP1^{WT}

480 and JIP1^{TA} mice.

481 Basal synaptic transmission at Schaffer collateral-CA1 synapses in JIP1^{WT} and JIP1^{TA}

482 mice was characterized through the input-output (I/O) relationship of field excitatory

483 postsynaptic potentials (fEPSPs). Analysis revealed that the fEPSP slopes, plotted against the

484 stimulation strength, were comparable in slices from JIP1^{WT} and JIP1^{TA} mice (two-way repeated

485 measures ANOVA: genotype $F_{(1,420)}$ = 0.23, p= 0.63; amplitude $F_{(14,442)}$= 79.27, p<0.001;

486 genotype x amplitude $F_{(14,420)}$= 0.35, p=0.63) (**Fig. 5A**). Similarly, when the fEPSPs slopes were

487   plotted as a function of increasing fiber volley amplitudes, no differences between JIP1^{WT} and

488   JIP1^{TA} mice were detected (**Fig. 5B**). Moreover, paired-pulse facilitation, indicative of

489   presynaptic plasticity (Zucker and Regehr, 2002), at Schaffer collateral-CA1 synapses from

490   JIP1^{TA} mice was similar to JIP1^{WT} mice at several inter-pulse intervals (two-way repeated

491   measures ANOVA: genotype $F_{(1,223)}$ = 0.08, p= 0.78); interval $F_{(6,223)}$= 13.56, p<0.001; genotype

492   x interval $F_{(8,208)}$= 0.15, p=0.98) (**Fig. 5C**). These data demonstrate similar basal synaptic

493   transmission in the hippocampi of JIP1^{TA} and JIP1^{WT} mice.

494   To determine whether the JIP1 Thr^{103}Ala mutation affects NMDA receptor-dependent

495   forms of synaptic plasticity (Citri and Malenka, 2008), LTP was measured using a high-

496   frequency tetanic stimulation protocol (HFS: 2 trains of 1 sec 100-Hz, separated by 20 sec).

497   Similar potentiation was produced in both JIP1^{WT} and JIP1^{TA} mice (**Fig. 5D**; fEPSPs were

498   potentiated to 145 ± 7% for JIP1^{WT} and 146 ± 4% for JIP1^{TA}) ($t_{(18)}$ = 0.12, p=0.89; two-tailed

499   unpaired Student's t-test), indicating that the ability to induce LTP with strong stimulation was

500   unimpaired in JIP1^{TA} mice. We next examined whether the threshold stimulation for induction of

501   LTP (Hu et al., 2007) differed between JIP1^{WT} and JIP1^{TA} mice. A 900-pulse train of 10 Hz

502   stimulation resulted in a modest, but detectable, degree of LTP in slices obtained from JIP1^{TA}

503   mice (**Fig. 5E**; 122 ± 4% of baseline at 50-60 min after LTP induction), though with minimal

504   post-tetanic potentiation, whereas fEPSPs in JIP1^{WT} slices were not potentiated (**Fig. 5E**; 92.1 ±

505   5.5 % of baseline) ($t_{(18)}$ = 7.61, p<0.001; two-tailed unpaired Student's t-test), with a trend toward

506   depression. These data demonstrate that LTP could be induced at lower frequencies in JIP1^{TA}

507   mice compared with JIP1^{WT} mice.

508   We also examined the induction of NMDA receptor-dependent LTD (Collingridge et al.,

509   2010) in JIP1^{WT} and JIP1^{TA} mice. We found that low-frequency stimulation (LFS), consisting of

510   900 single pulses at 1 Hz for 15 min, generated robust LTD in JIP1^{WT} mice, but LTD was absent

511   in JIP1^{TA} mice (**Fig. 5F**) (JIP1^{WT} mice, 72 ± 2%; JIP1^{TA} mice, 98 ± 3%; p<0.01) ($t_{(18)}$ = 7.05,

512   p<0.001; two-tailed unpaired Student's t-test). In view of the similar post-tetanic depression

513 [initial dip] between JIP1^{WT} and JIP1^{TA} brain slices, we determined whether JIP1^{TA} mice exhibit

514 other forms of LTD. We therefore induced LTD by delivering the same number of single pulses

515 (900) at 0.5 Hz for 30 min (**Fig. 5G**).   This form of LTD also relies on NMDA receptors (Dudek

516 and Bear, 1992), but depends to a greater degree on release of calcium from intracellular stores

517 (Nakano et al., 2004). This LTD protocol produced equivalent depression in both JIP1^{TA} and

518 JIP1^{WT} mice (**Fig. 5G**; JIP1^{WT}, 68% ± 3%; JIP1^{TA}, 66% ± 2%) ($t_{(26)}$ = 0.55, p=0.58; two-tailed

519 unpaired Student's t-test). These data indicate that disruption of JIP1-mediated JNK activation

520 reduces the optimal frequency stimulation for LTD, while enabling LTP at lower stimulation

521 frequencies at Schaffer collateral-CA1 synapses (**Fig. 5H**).

522   Two different forms of LTD coexist at Schaffer collateral-CA1 synapses in the

523 hippocampus, one dependent on NMDA receptors and another dependent on the activity of

524 metabotropic glutamate receptors (mGluRs), specifically mGluR5 (Huber et al., 2001). Thus, we

525 next examined a possible effect of the JIP1^{TA} mutation on mGluR-dependent LTD. We found

526 that a 5-min bath application of 100 μM DHPG (a group I mGluR agonist) to acute slices

527 resulted in a persistent depression of Schaffer collateral-evoked responses that was

528 comparable between JIP1^{TA} and JIP1^{WT} slices (**Fig. 5I**; 62% ± 1% of baseline at 60 min post-

529 drug application for JIP1^{TA} versus 65% ± 8% for JIP1^{WT}) ($t_{(18)}$ = 0.68, p=0.49; two-tailed unpaired

530 Student's t-test). These results suggest that whereas the JIP1^{TA} mutation blocks the NMDAR-

531 dependent forms of LTD, it does not affect the mGluR-dependent form.

532   Another important aspect of synaptic modification is synaptic depotentiation, namely that

533 the synaptic potentiation could be subsequently reversed by LFS. In view of the finding that two

534 trains of HFS at 100 Hz induced significant LTP in response to a 20-s inter-tetanus interval in

535 both groups of mice **(Fig. 5J)**, we used this HFS protocol followed by LFS 10 min later (900

536 pulses at 1 Hz) to induce depotentiation. One hour after the LFS, the evoked responses in both

537 JIP1^{WT} and JIP1^{TA} mice dropped to the baseline level (100% ± 4% compared with baseline

538 before the HFS for JIP1^{WT}, and 101% ± 3% for JIP1^{TA}) (t$_{(18)}$ = 0.23, p=0.82; two-tailed unpaired

539 Student's t-test), indicating effective LFS-induced depotentiation in both genotypes.

540   We next tested whether application of a selective inhibitor of JNK would mimic effects of

541 the JIP1 Thr^{103}Ala mutation on NMDA-receptor dependent forms of LTP and LTD. Slices were

542 obtained from wild-type mice and exposed either to the selective JNK inhibitor JNK-in-8 (6 µM)

543 or solvent (aCSF) for 20 min prior to stimulation protocols. The drug application was maintained

544 throughout the recording period. Basal synaptic transmission was not affected by JNK-in-8 as

545 indicated by the unchanged input–output relation (two-way repeated measures ANOVA:

546 genotype F$_{(1,308)}$ = 0.88, p= 0.35); amplitude F$_{(14,308)}$= 61.73, p<0.001; genotype x amplitude

547 F$_{(14,308)}$= 0.35, p=0.63) and unchanged paired-pulse facilitation (two-way repeated measures

548 ANOVA: genotype F$_{(1,132)}$ = 0.46, p= 0.56); interval F$_{(6,132)}$= 42.12, p<0.001; genotype x interval

549 F$_{(6,132)}$= 1.17, p=0.11) (**Fig. 6A, B**). In addition, inhibiting JNK activation in slices did not affect

550 HFS LTP (**Fig. 6C**, 60 min after 2X100Hz tetanization: 127 ± 6% of baseline for vehicle-treated

551 slices vs. 125 ± 5% for JNK-in-8-treated slices) (t$_{(22)}$ = 0.25, p=0.81; two-tailed unpaired

552 Student's t-test). However, JNK inhibition prevented the induction of LTD using the 1 Hz

553 stimulation protocol (**Fig. 6D** - vehicle-treated slices, 77 ± 3%; JNK-in-8-treated slices, 101 ±

554 4%; p<0.05) (t$_{(18)}$ = 4.81, p<0.001; two-tailed unpaired Student's t-test). The latter result closely

555 parallels the result obtained for the JIP1^{TA} slices (cf. **Fig. 5F**). Thus, these data confirm that

556 JIP1-mediated JNK activation plays an essential, but differential, role in certain forms NMDA-

557 receptor dependent synaptic plasticity.

558

559 ***JIP1^{TA} mice exhibit increased NMDA receptor signaling***

560 It is established that JIP scaffold proteins influence NMDA receptor activity (Kennedy et al.,

561 2007). The induction of synaptic plasticity at lower stimulation frequencies detected in JIP1^{TA}

562 mice compared with JIP1^{WT} mice (**Fig. 6**) may therefore be mediated by changes in NMDA

563 receptor signaling. Indeed, we found increased expression of NMDA receptor subunits GluN1

564   ($t_{(8)}$ = 2.66, p=0.029; two-tailed unpaired Student's t-test), GluN2A ($t_{(8)}$ = 2.85, p=0.037; two-

565   tailed unpaired Student's t-test), and GluN2B proteins ($t_{(8)}$ = 2.65, p=0.027; two-tailed unpaired

566   Student's t-test) (but not *mRNA*) in the whole hippocampus of JIP1^{TA} mice compared to JIP1^{WT}

567   mice (**Fig. 7A-C**). Control studies detected no differences in the expression of the AMPA

568   receptor subunits GluA1 and GluA2 (**Fig. 7A**). The increased expression of NMDA receptor

569   subunits was also detected in hippocampal synaptoneurosomes (**Fig. 7D**) and by increased cell

570   surface detection of GluN1 by immunofluorescence analysis of hippocampal neurons ($t_{(16)}$ =

571   2.85, p=0.012; two-tailed unpaired Student's t-test), (**Fig. 7E,F**) cultured from JIP1^{TA} mice

572   compared with JIP1^{WT} mice. These changes were associated with biochemical evidence of

573   increased NMDA receptor activity through assessment of downstream signaling changes,

574   including increased GluN2B Tyr^{1472} phosphorylation (Salter and Kalia, 2004) (**Fig. 7A**),

575   increased *cFos* mRNA expression ($t_{(9)}$ = 3.31, p=0.009; two-tailed unpaired Student's t-test),

576   (Bading et al., 1993) (**Fig. 7G**), ERK phosphorylation ($t_{(8)}$ = 3.11, p=0.017; two-tailed unpaired

577   Student's t-test) (Xia et al., 1996) (**Fig. 7H**), and CREB phosphorylation ($t_{(8)}$ = 5.25, p=0.008;

578   two-tailed unpaired Student's t-test) (Ginty et al., 1993) (**Fig. 7H**). Control experiments

579   demonstrated that the expression of KIF17, a microtubule motor that transports GluN2B

580   vesicles and regulates synaptic plasticity (Yin et al., 2011; Yin et al., 2012), was similar in

581   JIP1^{WT} and JIP1^{TA} mice (**Fig. 7H**). Together, these data indicate that JIP1^{TA} mice may exhibit

582   increased NMDA receptor signaling in the hippocampus compared with JIP1^{WT} mice.

583   To functionally test whether NMDA receptor signaling was increased in JIP1^{TA} mice

584   compared with JIP1^{WT} mice, we examined excitatory post-synaptic currents (EPSCs) via whole-

585   cell recordings of CA1 pyramidal cells in acute hippocampus slices. For CA1 pyramidal cells

586   held under voltage clamp at -70 mV, the response to Schaffer collateral stimulation is primarily

587   mediated by AMPA receptors. The NMDA receptor-mediated component of EPSCs is readily

588   distinguished from the AMPA receptor component by measuring current responses at a

589   membrane potential of +50 mV approximately 30 ms after stimulation when approximately 90%

590   of the AMPA receptor response has decayed (Liao et al., 1995; Myme et al., 2003). We found

591   that the average NMDA receptor-mediated peak EPSC was 1.6-fold larger in JIP1^{TA} mice

592   compared with JIP1^{WT} mice ($t_{(31)}$ = 2.41, p=0.022; two-tailed unpaired Student's t-test) (**Fig.**

593   **8A,B**), while the average peak amplitude of the AMPA receptor-mediated EPSCs were similar in

594   JIP1^{TA} and JIP1^{WT} mice ($t_{(31)}$ = 0.28, p=0.77; two-tailed unpaired Student's t-test) (**Fig. 8A,C**).

595   These findings were reflected in the NMDA/AMPA peak current ratio calculated for each cell

596   tested. JIP1^{WT} mice had a NMDA/AMPA receptor ratio of 37 ± 4%, whereas JIP1^{TA} mice yielded

597   a significantly larger ratio of 50 ± 4% ($t_{(31)}$ = 2.21, p=0.038; two-tailed unpaired Student's t-test)

598   (**Fig. 8D**). This analysis demonstrated that NMDA receptor signaling is increased in JIP1^{TA}

599   hippocampal neurons compared with those of JIP1^{WT} mice. To confirm this conclusion, we

600   examined NMDA receptor-stimulated gene expression in primary JIP1^{TA} and JIP1^{WT}

601   hippocampal neurons. We found that NMDA-induced expression of *brain-derived neurotrophic*

602   *factor* (*Bdnf*) mRNA (Ghosh et al., 1994; Tabuchi et al., 2000) and *cFos* mRNA (Bading et al.,

603   1993; Xia et al., 1996) by JIP1^{TA} neurons were increased when compared with JIP1^{WT} neurons

604   ([two-way ANOVA: genotype $F_{(1,29)}$= 70.26, p<0.001; time $F_{(2,29)}$= 39.7, p<0.001; genotype x time

605    $F_{(2,29)}$= 14.91, p<0.001; for *Bdnf* mRNA]; [two-way ANOVA: genotype $F_{(1,30)}$= 17.67, p<0.001;

606   time $F_{(2,30)}$= 182.1, p<0.001; genotype x time $F_{(2,30)}$= 7,98, p<0.001; for *cFos* mRNA]) (**Fig.**

607   **8E,F**). Together, these data indicate that the increased NMDA-mediated signaling detected in

608   the JIP1^{TA} hippocampus is due to enhanced NMDA receptor activity in individual neurons.

609

610   ***JIP1-mediated JNK activation in locomotor, sensory and emotional responses***

611   To address the possibility that increased NMDA receptor signaling in JIP1^{TA} mice compared

612   with JIP1^{WT} mice may cause behavioral changes, we first performed a battery of basic

613   behavioral tests of CNS function on JIP1^{WT} and JIP1^{TA} mice. We found that anxiety-related

614   behavior was increased in JIP1^{TA} mice. For example, JIP1^{TA} mice in an elevated plus maze

615   spent less time in the open arms, compared with JIP1^{WT} mice ($t_{(18)}$ = 2.79, p= 0.012; two-tailed

616 unpaired Student's t-test), with no changes in locomotor activity ($t_{(18)}$ = 0.76, p= 0.45; two-tailed

617 unpaired Student's t-test) (**Fig. 9, A-C**). Moreover, JIP1^{TA} mice spent significantly less time in

618 the center ($t_{(18)}$ = 5.40, p< 0.001; two-tailed unpaired Student's t-test) and more in the periphery

619 ($t_{(18)}$ = 6.46, p< 0.001; two-tailed unpaired Student's t-test) during an open field test compared to

620 JIP1^{WT} animals (**Fig. 9, D-E**). Consistent with previous reports (Grillon et al., 1998), elevated

621 anxiety in JIP1^{TA} mice was accompanied by an enhancement of the startle response to strong

622 acoustic stimuli (two-way repeated measures ANOVA: genotype $F_{(1,42)}$= 5.42, p= 0.035; stimulus

623 $F_{(3,42)}$ = 105.42, p< 0.001; genotype x stimulus= 9.25, p< 0.001) (**Fig. 9F**). No changes in

624 sensorimotor gating (pre-pulse inhibition) were observed (two-way repeated measures ANOVA:

625 genotype $F_{(1, 28)}$= 0.13, p= 0.722) (**Fig. 9G**). We also found that "fast" improvement in motor

626 coordination on the accelerating Rotarod was comparable between JIP1^{WT} and JIP1^{TA} mice.

627 However, during the second day of Rotarod training JIP1^{TA} mice did not display improved "slow"

628 skill learning, unlike JIP1^{WT} mice, as indicated by significant genotype x trial interaction value

629 (two-way repeated measures ANOVA: genotype $F_{(1,98)}$=4.84, p=0.045), trial $F_{(7,98)}$= 7.73,

630 p<0.001; genotype x trial $F_{(7,98)}$=3.34, p= 0.003) (**Fig. 9H**). Taken together, these data

631 demonstrate that JIP1^{TA} mice have normal sensory and motor activity, and attention function.

632 However, JIP1^{TA} mice displayed increased levels of anxiety-related behaviors and altered skill

633 learning.

634 ***Improved extinction-resistant contextual fear memory in JIP1^{TA} mice***

635 Contextual fear conditioning triggers JIP1-dependent JNK activation in the hippocampus (**Fig.**

636 **4**). Moreover, JNK activation in the hippocampus is implicated in some forms of hippocampus-

637 dependent memory (Sherrin et al., 2011). We therefore compared JIP1^{WT} and JIP1^{TA} mice to

638 determine if JIP1-mediated JNK activation is required for the behavioral response to contextual

639 fear conditioning. The mice received either "weak" (0.4 mA shock) or "strong" (0.8 mA shock)

640 training and were then re-exposed to the chamber 24 h later to assess long-term fear memory

641 (Shalin et al., 2006). The two training procedures were employed to adjust for nociceptive

642 sensitivity and also to test whether a "weak" stimulation protocol, similar to one that facilitated

643 synaptic plasticity **(**cf. **Fig. 5E),** results in enhanced hippocampal memory in JIP1^{TA} mice. JIP1^{TA}

644 and JIP1^{WT} mice showed no differences in freezing or activity during the exploration period

645 before the foot-shock (data not shown), or after the 0.8mA foot-shock delivery ($t_{(19)}$ = 0.46,

646 p=0.65; two-tailed unpaired Student's t-test). (**Fig. 10A**, right panel). Significantly enhanced

647 contextual freezing of JIP1^{TA} mice compared with JIP1^{WT} controls was found 24 h after "strong"

648 training (**Fig. 10A**, left panel, mean percentage freezing: JIP1^{TA} = 85 ± 3%; JIP1^{WT}= 59 ± 4%)

649 ($t_{(19)}$ = 5.11, p<0.001; two-tailed unpaired Student's t-test). This enhancement of contextual fear

650 was more pronounced in JIP1^{TA} mice when the "weak" paradigm was used (**Fig. 10B**, mean

651 percentage freezing: JIP1^{TA} = 81 ± 4%; JIP1^{WT} = 44 ± 5%) ($t_{(26)}$ = 5.26, p<0.001; two-tailed

652 unpaired Student's t-test). We next investigated short-term memory by testing JIP1^{TA} and

653 JIP1^{WT} mice immediately or 1 hr after the "strong" conditioning paradigm. We found that JIP1^{TA}

654 and JIP1^{WT} mice displayed similar responses in the both contextual tests ($t_{(20)}$ = 0.18, p=0.85; 0

655 hr post-conditioning, two-tailed unpaired Student's t-test) ($t_{(26)}$ = 0.11, p= 0.75; 1hr post-

656 conditioning, two-tailed unpaired Student's t-test) (**Fig. 10A**, left panel). Thus, JIP1^{TA} and JIP1^{WT}

657 mice did not differ in exploration activity, nociceptive reaction to electric shock, post-shock

658 freezing response, or short-term fear memory. These findings also indicate that the increased

659 anxiety phenotype observed with the JIP1^{TA} mice does not appear to fundamentally affect

660 shock-induced and post-shock behaviors, including contextual fear conditioning and learning

661 (see also **Fig. 11**). Nevertheless, JIP1^{TA} mice exhibited increased long-term fear memory

662 compared with JIP1^{WT} mice (**Fig. 10A,B**).

663 We then tested whether contextual fear memory is differentially affected in JIP1^{TA} and

664 JIP1^{WT} mice following antagonism of the NMDA receptors. JIP1^{TA} and JIP1^{WT} mice were

665 implanted with cannulae into the dorsal hippocampus and subjected to contextual fear

666 conditioning. A selective NMDA receptor antagonist, D,L-2-amino-5-phosphonovaleric acid

667 (APV; 10 µg/ml; 0.5 ml) or solvent (aCSF), was infused into the dorsal hippocampus (i.h.) 15

668 min prior to training. Both JIP1^{TA} and JIP1^{WT} mice given APV showed substantially reduced

669 freezing to context compared to mice that received aCSF prior to conditioning (two-way

670 ANOVA: genotype $F_{(1,36)}$= 8.76, p= 0.0054; treatment $F_{(1,36)}$= 143.06, p< 0.001; genotype x

671 treatment= 5.29, p=0.027) (**Fig. 10C)**. However, no differences between APV-treated JIP1^{TA}

672 mice and APV-treated JIP1^{WT} mice were observed (Bonferroni post hoc test: p= 0.62) (**Fig.**

673 **10C)**, indicating that memory facilitating effects observed in JIP1^{TA} were mediated via activation

674 of NMDA receptors. Taken together, these experiments show that blockade of JIP1-mediated

675 JNK activation can improve contextual learning induced by a relatively mild learning protocol

676 and that enhanced NMDA receptor function is required for this facilitatory effect of JIP1^{TA}

677 mutation on learning.

678   Previous studies have demonstrated that activation of hippocampal NMDA receptor

679 regulates fear extinction (Szapiro et al., 2003; Suzuki et al., 2004; Leaderbrand et al., 2014),

680 and that NMDA receptor blockade can prevent fear extinction in a variety of fear-related tasks

681 (Santini et al., 2001; Sotres-Bayon et al., 2007). Thus, we extended our cognitive evaluation of

682 JIP1^{TA} mice by assessing contextual fear extinction to further assess the link of JIP1-JNK

683 activation through NMDA receptors. Conditioned JIP1^{TA} and JIP1^{WT} mice were subjected to

684 brief (3-min) daily extinction trials (E1-E7) by non-reinforced placement in the training context for

685 7 days after contextual conditioning. During the extinction trials, JIP1^{TA} mice exhibited almost

686 unchanged freezing responses, indicating nearly fully impaired extinction compared to JIP1^{WT}

687 mice (repeated measure ANOVA: genotype $F_{(1,108)}$= 388.76, p= p< 0.001; time $F_{(6,108)}$= 36.26, p<

688 0.001; $F_{(6,108)}$ genotype x time= 25.35, p< 0.001) (**Fig. 10D)**. Overall, these results indicate that

689 the enhanced contextual fear observed in JIP1^{TA} mice is strongly resistant to extinction.

690   Finally, cue-dependent fear conditioning was assessed in JIP1^{TA} mice and JIP1^{WT}

691 controls. As with contextual fear conditioning, mice received either a "strong" or a "weak"

692 training protocol, both consisting of a single pairing of tone and shock at 0.8mA and 0.4mA,

693 respectively. In both protocols, JIP1^{TA} mice, compared to JIP1^{WT} controls, displayed significantly

694 more freezing when re-exposed to the tone 24 h post-training (**Fig. 10E**, left panel, mean

695 percentage freezing: "weak" cued test- JIP1^{TA} = 66 ± 3%; JIP1^{WT}= 44 ± 4%) (t$_{(20)}$ = 3.71, p=0.01;

696 two-tailed unpaired Student's t-test) (**Fig. 10E**, right panel, mean percentage freezing: "strong"

697 cued test- JIP1^{TA} = 70 ± 3%; JIP1^{WT}= 55 ± 4%) (t$_{(20)}$ = 2.65, p=0.015; two-tailed unpaired

698 Student's t-test), indicating that JIP1^{TA} mice display memory enhancement in the fear learning

699 tasks in a manner involving both amygdala- and hippocampus-dependent memory systems.

700

701 ***Improved spatial learning in JIP1^{TA} mice***

702 We next tested JIP1^{TA} and JIP1^{WT} mice using the Morris water maze (MWM) protocol, a

703 hippocampus-dependent spatial memory task (Morris et al., 1982). During the hippocampus-

704 independent visible portion of the training, both JIP1^{TA} and JIP1^{WT} mice readily learned to find

705 the marked platform (**Fig. 11A**). Mice were then trained to swim to a hidden platform located in

706 a fixed location of the pool (target quadrant, T, **Fig. 11A,D**). JIP1^{TA} mice had significantly

707 decreased escape latencies compared to JIP1^{WT} mice on days 6-9 and day 12 of training (two-

708 way repeated measures ANOVA: genotype F$_{(1,208)}$ = 11.03, p= 0.0049); time F$_{(8,208)}$= 21.01,

709 p<0.001; genotype x time F$_{(8,208)}$= 0.74, p=0.65) (**Fig. 11A**). However, JIP1^{TA} and JIP1^{WT} mice

710 did not differ in the time spent in the platform-containing quadrant during probe trials on day 10

711 (**Fig. 11B**, 36 ± 3% of time in quadrant T for JIP1^{TA}; 35 ± 2% JIP1^{WT}) (t$_{(26)}$ = 0.27, p=0.783; two-

712 tailed unpaired Student's t-test) and day 13 (**Fig. 11C**, 38 ± 5% of time in quadrant T for JIP1^{TA}

713 mice; 36 ± 3% JIP1^{WT} mice) (t$_{(26)}$ = 0.34, p=0.734; two-tailed unpaired Student's t-test).

714 Although JIP1^{TA} mice did not display preference for the target quadrant during probe trials, the

715 decrease in escape latency during training suggests that the presence of the JIP1 Thr^{103}Ala

716 mutation appears to moderately affect gradual learning of the hidden platform water maze task.

717   To test whether JIP1^{TA} mice display enhanced fast spatial learning, we performed a

718 reversal of the hidden platform in the MWM. The hidden platform was moved to the opposite

719   quadrant of the pool (new target quadrant (NT)) and the mice were trained to swim to this new

720   location for four trials separated by 15-20 min (**Fig. 11D**). JIP1^{TA} mice learned significantly faster

721   to swim to the new platform location compared to control mice (two-way repeated measures

722   ANOVA: genotype $F_{(1,78)}$ = 16.37, p= 0.0012; time $F_{(3,78)}$= 1.98, p=0.13; genotype x time $F_{(3,78)}$=

723   1.86, p=0.15) (**Fig. 11E**). Moreover, the JIP1^{TA} mice displayed increased preference for the NT

724   quadrant during a probe trial given on probe day 15 (**Fig. 11F**; time spent in NT quadrant: 41 ±

725   2% for JIP1^{TA}; 23 ± 4% for JIP1^{WT}) ($t_{(26)}$ = 4.02, p<0.001; two-tailed unpaired Student's t-test).

726   This enhancement in spatial reversal learning of the new platform location was accompanied by

727   a decrease in the percent of time JIP1^{TA} mice spent searching for the platform in the previously

728   correct quadrant (O) ($t_{(26)}$= 2.34, p=0.028; two-tailed unpaired Student's t-test) (**Fig. 11F**).

729   These data indicate that the Thr^{103}Ala mutation in JIP1 allows for improved fast spatial learning.

730   ***JNK binding is required for JIP1-mediated NMDA receptor regulation of learning and***

731   ***memory***

732   To confirm that JIP1-mediated JNK activation plays a critical role in NMDA receptor-dependent,

733   hippocampus-dependent learning and memory, we tested an independent mouse model of

734   defective JIP1-mediated JNK activation: JIP1 lacking the JNK binding domain (JBD). To create

735   a mouse model lacking the JBD of JIP1 (JIP1$^{\Delta JBD}$ mice), the core of the JBD (Leu^{160}-Asn^{161}-

736   Leu^{162}) mediating a hydrophobic interaction of JIP1 with JNK (Whitmarsh et al., 2001; Heo et al.,

737   2004) was replaced with Gly^{160}-Arg^{161}-Gly^{162} in JIP$^{\Delta JBD}$ mice (**Fig. 12A-B**). Immunoblot analysis

738   of brain extracts from JIP1^{WT} and JIP1$^{\Delta JBD}$ mice indicated similar levels of JIP1 protein

739   expression (**Fig. 12C**). To establish that the ΔJBD mutation blocks JIP1-mediated JNK

740   activation *in vivo*, we compared kainate-induced excitotoxicity in the hippocampus of JIP1^{WT} and

741   JIP1$^{\Delta JBD}$ mice. This analysis demonstrated that kainate caused similar increases in cJun

742   expression in the dentate gyrus of JIP1^{WT} and JIP1$^{\Delta JBD}$ mice, but kainate-stimulated cJun

743   phosphorylation was markedly suppressed in JIP1$^{\Delta JBD}$ mice compared with JIP1^{WT} mice (**Fig.**

744 **12D,E**). These data confirm that JIP1$^{\Delta JBD}$ mice represent a model of defective JIP1-mediated

745 JNK signaling.

746 We then examined contextual and cued fear conditioning to assess the effect of the

747 JIP1$^{\Delta JBD}$ mutation on learning and memory. The JIP1$^{\Delta JBD}$ mice displayed a significantly

748 increased conditioned freezing response in both learning tasks compared to the JIP1^{WT} mice

749 when tested at 24 hours after training (**Fig. 13A left panel**: mean percentage contextual

750 freezing; JIP1^{WT} = 58 ± 3%; JIP1$^{\Delta JBD}$ = 89 ± 3%; $t_{(18)}$= 6.30, p<0.001; two-tailed unpaired

751 Student's t-test**; Fig. 13A right panel:** mean percentage cued freezing; JIP1^{WT} = 51 ± 1%;

752 JIP1$^{\Delta JBD}$ = 69 ± 6%; $t_{(20)}$= 2.97, p=0.007; two-tailed unpaired Student's t-test), a result very

753 similar to that observed for the JIP1^{TA} mice (**Fig. 10**). JIP1$^{\Delta JBD}$ mice exhibited enhanced

754 learning in the MWM (two-way repeated measures ANOVA: genotype $F_{(1,162)}$ = 26.04, p<0.001);

755 time $F_{(8,162)=}$ 126.7, p<0.001; genotype x time $_{(8,162)}$= 1.75, p= 0.84) , and spent significantly more

756 time in the target quadrant compared to control JIP1^{WT} mice when subjected to probe trials on

757 day 9 ($t_{(18)}$ = 4.02, p<0.001) (**Fig. 13C**, left panel; 41 ± 2% of time in quadrant T for JIP1$^{\Delta JBD}$; 32

758 ± 1% of time in quadrant T for JIP1^{WT}) and day 13 (**Fig. 13C**, right panel; 48 ± 4% of time in

759 quadrant T for JIP1$^{\Delta JBD}$; 36 ± 3% of time in quadrant T for JIP1^{WT}) ($t_{(18)}$ = 2.44, p=0.027; two-

760 tailed unpaired Student's t-test). This was an unexpected finding, as JIP1^{TA} mice did not show

761 enhanced memory retention in the hidden platform MWM task (**cf. Fig. 11B, C**). The larger

762 phenotype in the JIP1$^{\Delta JBD}$ mice in the MWM compared to the JIP1^{TA} mice may result from a

763 greater effect of the JNK binding site mutation on JIP1-mediated JNK activation than the

764 mutation of the T103 phosphorylation site on JIP1.

765 We then examined reversal learning by moving the hidden platform to the opposite

766 quadrant (**Fig. 13D**, left panel). Similar to JIP1^{TA} mice, the JIP1$^{\Delta JBD}$ mice showed shorter

767 latencies than control mice in finding the new platform location during a one-day trial period

768 (data not shown). Moreover, JIP1$^{\Delta JBD}$ mice spent significantly more time in the new target

769 quadrant compared to JIP1^{WT} control mice (**Fig. 13D**, right panel; mean percentage of time

770   spent in new target quadrant [NT] was 49 ± 3%, for JIP1$^{\Delta JBD}$; 28 ± 4% for JIP1^{WT}) ($t_{(18)}$ = 4.21,

771   p<0.001; two-tailed unpaired Student's t-test). Furthermore, like JIP1^{TA} mice, we found

772   significantly increased expression of GluN1, GluN2A, and GluN2B in the hippocampus of

773   JIP1$^{\Delta JBD}$ mice as compared to hippocampi from JIP1^{WT} mice (**Fig. 13E**) and increased ERK

774   activation ($t_{(8)}$ = 2.57, p=0.033; two-tailed unpaired Student's t-test) (**Fig. 13F**).

775   Together, these analyses of JIP1^{TA} and JIP1$^{\Delta JBD}$ mice confirm that loss of JIP1-mediated

776   JNK activation enhances NMDA-dependent hippocampus-dependent memory.

777

778 **Discussion**

779 The JIP1 scaffold protein can assemble a functional JNK signaling module formed by members

780 of the mixed-lineage protein kinase family of MAP3K, the MAP2K family member MKK7, and

781 JNK (Whitmarsh et al., 1998). However, JIP1 also functions as an adapter protein that

782 mediates transport by microtubule motor proteins (Fu and Holzbaur, 2014), including kinesin-1

783 (Verhey et al., 2001; Whitmarsh et al., 2001) and dynein (Standen et al., 2009; Fu and

784 Holzbaur, 2013). These two functions of JIP1 compromise the interpretation of loss-of-function

785 studies focused on the analysis of JIP1 knockout mice (Whitmarsh et al., 2001; Kennedy et al.,

786 2007). To overcome this limitation, we studied two mouse models with germ-line mutations in

787 the *Jip1* gene that prevent JIP1-mediated JNK activation. First, a point mutation of the JIP1

788 phosphorylation site Thr^{103} (by replacement with Ala) in JIP1^{TA} mice suppresses JIP1-mediated

789 JNK activation **(Fig. 3)** by disrupting the regulated interaction of mixed-lineage protein kinases

790 with JIP1 (Morel et al., 2010). Second, a three-residue mutation of the JIP1 site that binds JNK

791 in JIP1$^{\Delta JBD}$ mice prevents JIP1-mediated JNK activation **(Fig. 12)**. These complementary

792 mouse models therefore provided an opportunity to selectively disrupt JIP1-mediated JNK

793 activation *in vivo*.

794   It is established that the JIP1 scaffold protein mediates JNK activation in the neuronal

795 response to excitotoxin (Whitmarsh et al., 2001) and in adipose tissue during metabolic stress

796 responses (Jaeschke et al., 2004; Morel et al., 2010). The results of the present study extend

797 these findings to neural functioning by demonstrating that JIP1-linked JNK activation in the

798 hippocampus regulates contextual fear conditioning in a NMDA receptor-dependent fashion.

799 This finding builds on the previous demonstration that JNK1-deficient mice exhibit enhanced

800 contextual fear conditioning (Sherrin et al., 2010) and altered synaptic plasticity (Li et al., 2007).

801   We further show that JIP1-mediated JNK activation regulates NMDA receptor signal

802   transduction associated with an altered threshold for LTP, decreased long-term fear memory,

803   and decreased spatial memory (**Figs. 5, 6, 8, 10, 11 & 13**). These observations are consistent

804   with the conclusion that JNK normally functions to negatively regulate mechanisms responsible

805   for learning and memory (Sherrin et al., 2011). We found that the enhanced learning in JIP1

806   mutant mice was associated with an increase in the NMDA receptor component of the synaptic

807   response, and enhanced activity of downstream pathways that facilitate induction of NMDA

808   receptor-dependent LTP. This is consistent with previous reports that have separately

809   implicated both JNK signaling (Sherrin et al., 2010) and CA1 hippocampal NMDA receptors in

810   contextual fear conditioning, spatial learning, and synaptic plasticity (Kutsuwada et al., 1996;

811   Tsien et al., 1996; Tang et al., 1999; Liu et al., 2004; Lau and Zukin, 2007; Yashiro and Philpot,

812   2008; Brigman et al., 2010). These data indicate that JIP1-mediated JNK activation may

813   constrain synaptic plasticity, learning and memory through attenuation of NMDA receptor

814   function. Furthermore, decreases in JIP1 level and/or localization affecting JNK activity, perhaps

815   resulting from distinct signaling pathways (e.g. glutamate-mediated down-regulation of JIP1

816   level in growth cones (Dajas-Bailador et al., 2014); Ca^{2+}-dependent degradation of JIP1

817   (Allaman-Pillet et al., 2003), would thus be predicted to reduce this constraint, leading to

818   enhanced learning and memory.

819   The increased NMDA receptor signaling caused by loss of JIP1-mediated JNK activation

820   in JIP1^{TA} mice is associated with increased expression of the NMDA receptor subunits GluN2A

821   and GluN2B (**Fig. 7**). This increase in the levels of NMDA receptor subunits is significant

822   because it is established that changes in GluN2A and GluN2B expression cause altered

823   plasticity and memory (Tang et al., 1999; Brigman et al., 2010; Chao et al., 2013). This may be

824   mediated by extending the integration time window for NMDA receptor signaling coincident with

825   pre- and postsynaptic activity, and decreasing the threshold for inducing long-term synaptic

826   changes. Indeed, a constraint by JIP1-JNK on plasticity thresholds may, in turn, regulate

827   information processing and learning (Kiyama et al., 1998; Hawasli et al., 2007; Hu et al., 2007).

828   This is consistent with the observation that loss of JIP1-mediated JNK activation in JIP1^{TA} mice

829   enables the establishment of LTP at lower stimulation frequencies (**Fig. 5H**), with the converse

830   being a requirement for higher stimulation frequencies needed in the presence of JIP1-JNK

831   activation. The mechanism of JIP1-dependent regulation of NMDA receptor subunit expression

832   (**Fig. 7A,B**) remains to be determined and may include changes in NMDA receptor membrane

833   insertion, internalization, or lateral movement into synapses (**Fig 14**). Additionally, JIP1 may

834   regulate NMDA receptor subunit expression through a post-transcriptional mechanism (**Fig. 14**).

835   Indeed, it is known that GluN1, GluN2 and GluN2B protein expression can be regulated by

836   CPEB3 (Chao et al., 2013), that GluN2A protein expression can be regulated by CPEB1

837   (Udagawa et al., 2012; Swanger et al., 2013), and that GluN2B expression can be regulated by

838   a microRNA (Harraz et al., 2012). Strikingly, the learning and memory phenotypes of *Cpeb3$^{-/-}$*

839   mice associated with increased NMDA receptor expression (Chao et al., 2013) are similar to the

840   phenotypes of the mice with defects in JIP1 function studied in the present study (JIP1^{TA} and

841   JIP1$^{\Delta JBD}$ mice).

842   It is possible that increased expression of NMDA receptor subunits only partially

843   accounts for the learning and memory phenotypes of JIP^{TA} and JIP1$^{\Delta JBD}$ mice. Indeed, it has

844   been shown that cFyn mediates phosphorylation of the NMDA receptor subunit GluN2B on

845   Y^{1472}, resulting in increased NMDA receptor activity (Salter and Kalia, 2004) by attenuating

846   NMDA receptor internalization (Roche et al., 2001; Prybylowski et al., 2005), increasing the

847   proper localization of the GluN2B NMDA receptors at synapses (Nakazawa et al., 2006), and

848   enhancing GluN2B NMDA receptor-mediated currents at CA1 synapses (Yang et al., 2012). In

849   the present study, increased GluN2B Y^{1472} phosphorylation resulted from disruption of JIP1-

850   mediated JNK activation in JIP1^{TA} mice (**Fig. 7A**), also perhaps contributing to the observed

851   increase in NMDA receptor signaling. This change in GluN2B Y^{1472} phosphorylation may be

852   caused by JIP1-mediated recruitment of cFyn (Kennedy et al., 2007) or by JNK-mediated

853   recruitment of PSD-95/Fyn complex (Kim et al., 2007). Another potential contributing factor may

854   be the binding of JIP1 to LRP8, a protein that regulates NMDAR signaling (Stockinger et al.,

855   2000; Beffert et al., 2005). Finally, it is possible that JIP1-mediated interactions with the

856   exchange factors Ras-GRF1 and Tiam1 may contribute to increased NMDA receptor dependent

857   activation of the ERK pathway and activity-dependent actin remodeling critical for synaptic

858   plasticity and memory (Buchsbaum et al., 2002; Krapivinsky et al., 2003; Tolias et al., 2007).

859   Contextual-fear learning recruits both the hippocampus and amygdala, while cued-fear

860   learning relies on the amygdala (Phillips and LeDoux, 1992). JIP^{TA} and $JIP1^{\triangle JBD}$ mice displayed

861   enhancement in both contextual and cued fear conditioning (**Figs. 10, 13**). As such, these

862   learning enhancements suggest that JIP1-mediated JNK activation is also important in the

863   amygdala. Although JIP1 is expressed in amygdala (preliminary observations), JNK signaling

864   and the importance of JIP1 in the amygdala have yet to be thoroughly investigated. We

865   hypothesize that the observed contextual-learning enhancement is, at least, in part due to a lack

866   of hippocampal JIP1-mediated JNK signaling. Not only is signal transduction altered in this

867   region, but JIP^{TA} and $JIP1^{\triangle JBD}$ mice also demonstrate an improved spatial water maze memory,

868   which classically relies on the hippocampus.

869   Genetic anomalies on the JNK pathway have also been associated with a subset of

870   other psychiatric disorders (Coffey, 2014). However, the degree to which and the mechanism

871   by which JNK is involved is unknown. $JIP1^{TA}$ mutant mice display a range of behaviors

872   including exaggerated fear responses to cues associated with the danger, difficulty suppressing

873   fear behavior even when these cues no longer predict danger, elevated acoustic startle

874   response, and anxiety-like behaviors that may represent rodent homologues of the symptoms

875   that are diagnostic for trauma- and stressor-related disorders, such as posttraumatic stress

876 disorder (PTSD) (Shalev et al., 2017). These responses may be regulated by JIP1-mediated

877 JNK signaling in the hippocampus, the amygdala, or in various cortical regions that interconnect

878 to form the neural circuits that promote adaptation to stress and fear conditioning. Interestingly,

879 we have shown that intrahippocampal infusion with a JNK inhibitor prevents stress-induced

880 changes in fear conditioning (Sherrin et al, 2010). Thus, it is possible that drugs that target

881 the function of JIP1 to positively regulate JNK activity or NMDA receptor function (Myers and

882 Davis, 2007; Feder et al., 2014; Ori et al., 2015; Mataix-Cols et al., 2017) may therefore be

883 useful for the treatment of PTSD or anxiety disorders marked by abnormal fear learning and

884 maladaptive processing of information related to threat. Our study provides a proof-of-concept

885 that validates this approach using a model organism. An exciting future possibility is the

886 application of this strategy to the treatment of human fear and anxiety.

887   Overall, the results of this study suggest that JNK activation caused by the JIP1 scaffold

888 protein constrains learning and memory in a NMDA receptor-dependent fashion. This role of

889 JIP1 starkly differs from the related protein JIP2 that acts to promote NMDA receptor signaling

890 by a JNK-independent mechanism (Kennedy et al., 2007). Our studies of JIP1 therefore

891 establish a role for the JIP1-JNK pathway in NMDA receptor-dependent regulation of memory

892 acquisition, consolidation, and retention.

893

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1266 **Legends**

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1268 **Figure 1. Analysis of JIP1 expression in the hippocampus.**

1269 (**A**) Fluorescent immunohistochemistry of the CA1, CA3, and DG regions of the hippocampus indicates
1270 that JIP1 (green) predominantly co-localizes with MAP2 (red) in wild-type brain sections (*upper panels*),
1271 suggesting relative enrichment of the JIP1 protein in neuronal processes. Co-localization between JIP1
1272 (green) and neuron-specific nuclear protein (NeuN) (red) is sparse in the hippocampal subfields (*lower*
1273 *panels*). Abbreviations: CA1, Cornu Ammonis 1; CA3, Cornu Ammonis 3; DG, dentate gyrus; grDG,
1274 granular layer of DG; poDG, polymorphic layer of DG; s.l., stratum lucidem; s.l.m., stratum lacunosum
1275 moleculare; s.p., stratum pyramidale; s.r., stratum radiatum. Scale bar = 50 µm.

1276 (**B**) Nissl stain and (NeuN) stain of JIP1^{WT} and JIP1^{TA} coronal hippocampal sections. Scale bar = 200 µm.

1277 (**C,D**) Pyramidal cells of the CA1 region of JIP1^{WT} and JIP1^{TA} were stained with Nissl, NeuN, the dendritic
1278 marker MAP2, the astrocytic marker GFAP, and the inhibitory GABAergic marker GAD67 (C). The
1279 staining was quantitated (D) (mean ± SEM; n=4; p>0.05; Student's t–test). Scale bar = 50 µm.

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1283 **Figure 2. Neuronal spine density and dendritic arborization of CA1 pyramidal neurons are similar**
1284 **in JIP1^{WT} and JIP1^{TA} mice.**

1285 (**A**) Representative images of apical and basal dendrites spine morphology in JIP1^{TA} mice and JIP1^{WT}
1286 littermates. Scale bar = 10 µm.
1287 (**B**) Quantitation of basal and apical dendritic spine density (mean ± SEM; n= 5 slices from 5 mice per
1288 genotype; p>0.05, Student's t-test).
1289 (**C**) Quantitation of different spine types in basal and apical dendrites (mean ± SEM, n= 5 slices from 5
1290 mice per genotype; p>0.05, Student's t-test).

1291 (**D,E**) Sholl analysis of dendritic arborization of CA1 pyramidal neurons. Values on the x-axis represent
1292 increasing distance from the soma of the pyramidal cells. Basal and apical dendrites of pyramidal cells
1293 from n= 5 slices from 5 mice per genotype were examined (mean ± SEM; p>0.05, Student's t-test).

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1297 **Figure 3. JIP1-dependent JNK activation in the hippocampus is suppressed in JIP1^{TA} mice.**

1298 (**A-C**) JIP1^{WT} and JIP1^{TA} mice were treated by systemic injection of kainate. At 2 h post-treatment,
1299 sections of the brain were prepared and stained (green) with antibodies to pSer^{63} cJun (A), cJun (B), or
1300 cFos (C). DNA was stained with DAPI (red). Representative sections of the dentate gyrus of the
1301 hippocampus are presented. Scale bar = 75 µm.

1302 (**D,E**) Extracts prepared from the hippocampus of JIP1^{WT} and JIP1^{TA} mice treated with kainate (0 – 60
1303 mins) were examined by multiplexed ELISA to measure the amount of pSer^{63}-cJun (D) and cJun (E)
1304 normalized to the amount of JNK. The data presented are the mean ± SEM (n=5; **, p<0.01, two-way
1305 ANOVA followed by Bonferroni's post-hoc test).

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1309 **Figure 4. JIP1^{TA} mice exhibit reduced JNK activation in the dorsal hippocampus following**
1310 **contextual fear conditioning.**

1311 Dorsal hippocampal tissue was isolated from naïve mice and from mice at different times after contextual
1312 fear conditioning (FC) and examined by immunoblot analysis by probing with antibodies to phospho-JNK,
1313 JNK, and GAPDH. The amount of 46-kDa and 54-kDa phospho-JNK was quantitated and normalized to
1314 the amount of JNK in each sample (mean ± SEM; n=5; ***, $p<0.001$, for JIP1^{TA} compared with JIP1^{WT}
1315 mice; #, $p<0.01$, ##, $p<0.001$ compared with the naïve control, two-way ANOVA, followed by Bonferroni's
1316 post-hoc test

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1320 **Figure 5. The threshold for LTP induction is reduced in JIP1^{TA} mice.**

1321 (**A,B**) Basal synaptic transmission at Schaffer collateral-CA1 synapses, as assessed by measuring the
1322 fEPSP input-output relationship (A) and the fEPSP slope to fiber volley relationship (B), was similar in
1323 JIP1^{TA} slices (n = 16 slices, 13 mice) compared with slices obtained from JIP1^{WT} littermates (n = 16
1324 slices, 12 mice). No statistically significant differences were found ($p>0.05$, two-way repeated measures
1325 ANOVA).

1326 (**C**) fEPSPs from JIP1^{TA} (n = 16 slices, 13 mice) and JIP1^{WT} (n = 16 slices, 12 mice) slices exhibited
1327 similar paired pulse facilitation. No statistically significant differences were found ($p>0.05$, two-way
1328 repeated measures ANOVA).

1329 (**D**) High-frequency stimulation LTP was induced by two trains of 100 Hz stimulation (separated by a 20
1330 sec interval) to the Shaffer collaterals in slices from JIP1^{TA} and JIP1^{WT} mice (n = 10 slices, 8
1331 mice/genotype). Stimulation was delivered at time 0 (arrow). No statistically significant differences were
1332 found ($p>0.05$, Student's t–test).

1333 (**E**) An intermediate-stimulation LTP protocol involved 900 pulses of 10 Hz stimuli delivered at time 0.
1334 LTP induced at intermediate frequencies was significantly facilitated in slices taken from JIP1^{TA} mice
1335 when compared to JIP1^{WT} controls n= 10 slices, 9 mice/genotype). Statistically significant differences are
1336 indicated (***, $p<0.001$, Student's t–test).

1337 (**F**) LTD induced by low-frequency (1 Hz, 900 pulses, 0-15 min time) stimulation was significantly reduced
1338 in JIP1^{TA} slices compared to JIP1^{WT} slices (n= 10 slices, 10 mice/genotype). Statistically significant
1339 differences are indicated (***, $p<0.0001$, Student's t–test).

1340 (**G**) LTD induced by 0.5 Hz stimulation (0.5 Hz, 900 pulses, 0-30 min time) was similar in JIP1^{WT} and
1341 JIP1^{TA} slices (n = 14 slices, 11 mice/genotype). No statistically significant differences were found
1342 ($p>0.05$, Student's t–test).

1343 (**H**) Frequency-response function in JIP1^{TA} and JIP1^{WT} mice. The percentage change in synaptic
1344 strength from baseline in JIP1^{TA} and JIP1^{WT} mice at 50-60 min following stimulation at the indicated
1345 frequency is presented. Values are mean ± SEM. Magnitudes of LTP/LTD were calculated as the ratio of
1346 the average fEPSPs between 50-60 min and average baseline fEPSPs between -20 min -0 min.
1347 Statistically significant differences are indicated (***, $p<0.001$, Student's t–test).

1348 (**I**) mGluR-dependent LTP in hippocampal slices from JIP1^{TA} and JIP1^{WT} mice. mGluR-LTD was induced
1349 by incubation of JIP1^{TA} and JIP1^{WT} slices with DHPG (100 μM) for 5 min (n= 10 slices, 10 mice/genotype).
1350 Basal fEPSPs were recorded prior to LTD induction with DHPG. No statistically significant differences
1351 were found ($p>0.05$, Student's t–test).

1352 (**J**) Depotentiation is not affected in JIP1^{TA} mice. High-frequency stimulation (100 Hz twice for 1 s with 20
1353 s interval), followed with 1 Hz (15 min) stimulation 10 min later to the Shaffer collaterals produced similar
1354 depotentiation in slices from JIP1^{TA} and JIP1^{WT} mice (n = 10 slices, 10 mice/genotype). No statistically
1355 significant differences were found ($p>0.05$, Student's t–test).

1356 The insets in D-J show representative fEPSP responses obtained before and after LTP, LTD and
1357 depotentiation inducing stimuli. (Calibration: 0.2 mV/10 ms).

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Figure 6. Inhibition of JNK signaling mimics the effect of JIP1 (Thr^{103}Ala) mutation on NMDA receptor-dependent LTD.

(**A, B**) JNK-in-8 treatment did not affect baseline synaptic transmission or paired-pulse facilitation in wild-type slices. Input–output curves, as assessed by the fEPSP slope to fiber volley relationship, were similar in vehicle-treated slices (n = 12 slices) compared with wild-type slices treated with JNK-in-8 (n = 12 slices) (A). fEPSPs from vehicle-treated slices (n = 12 slices) and JNK-in-8 treated (n = 12 slices) slices exhibited similar paired pulse facilitation (PPF) (B) (p>0.05, two-way repeated measures ANOVA).

(**C**) High-frequency stimulation (HFS)-LTP was induced with a pair of 100 Hz tetani in the presence of either vehicle (n = 12) or 6 μM JNK–in-8 (n = 12). LTP was unaffected by JNK inhibition. (p> 0.05, Student's t–test).

(**D**) Low-frequency stimulation (LFS)-LTD was induced (1 Hz, 900 pulses) in the presence of either vehicle (n = 10) or 6 μM JNK–in-8 (n = 10). LTD was impaired by JNK inhibition (***, p<0.001, Student's t–test).

The insets in C and D show representative fEPSP responses obtained before and after LTP/LTD inducing stimuli. (Calibration: 0.2 mV/10 ms).

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Figure 7. The JIP1^{TA} mutation promotes increased NMDA receptor expression and activity.

(**A-C**) Lysates prepared from the hippocampi of JIP1^{TA} and JIP1^{WT} mice were examined by immunoblot analysis by probing with antibodies to NMDA and AMPA receptor subunits, SAP102, JIP1, and β-tubulin (A). The amount of NMDA receptor subunits in the hippocampus was quantitated and normalized to the amount of β-tubulin in each sample (B, mean ± SEM, n = 5; **, p<0.01, Student's t-test). The amount of NMDA receptor subunit mRNA in the hippocampus was measured by quantitative RT-PCR and normalized to the amount of *Gapdh* mRNA in each sample (C, mean ± SEM, n = 5; p>0.05, Student's t-test).

(**D**) Enrichment of NMDA receptor subunits in the synaptoneurosome fraction of the hippocampus of JIP1^{WT} and JIP1^{TA} mice was examined by immunoblot analysis.

(**E,F**) Primary JIP1^{WT} and JIP1^{TA} hippocampal neurons were fixed and processed for immunofluorescence analysis under non-permeabilized (*left panels*) and permeabilized (*right panels*) conditions. GluN1 surface and intracellular expression was examined by confocal microscopy (E). Quantitation of the cell surface expression of GluN1 in JIP1^{TA} and JIP1^{WT} hippocampal neurons was performed using ImageJ software (F). The data presented are the mean ± SEM; n = 8~10; **, p<0.01, Student's t-test).

(**G**) The expression of c*Jun* and c*Fos* mRNA in the hippocampus of JIP1^{WT} and JIP1^{TA} mice was normalized to the amount of *Gapdh* in each sample (mean ± SEM, n = 5~6). Statistically significant differences are indicated (**, p<0.01, Student's t test).

(**H**) Lysates prepared from the hippocampus of JIP1^{WT} and JIP1^{TA} mice were examined by immunoblot analysis with antibodies to phospho-ERK, ERK, phospho-CREB, CREB, KIF17, and β-Tubulin. The amount of phospho-ERK and phospho-CREB was quantitated (mean ± SEM, n = 5). Statistically significant differences are indicated (**, p<0.01, Student's t-test).

Figure 8. Increased synaptic NMDA receptor activity in hippocampal slices from JIP1^{TA} mice.

(**A**) Whole-cell voltage clamp traces depicting typical EPSCs elicited by stimulating the Schaffer collaterals while recording CA1 pyramidal cells from JIP1^{WT} and JIP1^{TA} hippocampal slices. Strong blockade of NMDA receptors at a holding potential of -70 mV by magnesium isolates the AMPA

1406   component (*lower traces*). The +50 mV upper traces primarily represent the NMDA component because
1407   the traces were recorded 30-50 msec after stimulation when approximately 90% of the AMPA receptor
1408   response had decayed. The traces show the increase in the NMDA receptor-mediated component for the
1409   JIP1^{TA} group relative to JIP1^{WT}, while AMPA receptor mediated responses were not different. Traces
1410   depicted are averages of 20 sweeps for both NMDA and AMPA receptor-mediated components of JIP1^{WT}
1411   recorded from the same pyramidal neuron; 10 and 15 sweeps were used to produce the averages
1412   depicted for the NMDA and AMPA receptor-mediated components recorded from a JIP1^{TA} pyramidal
1413   neuron.

1414   (**B,C**) Comparison of average NMDA and AMPA receptor currents. NMDA receptor-mediated currents
1415   (B) were significantly greater in the JIP1^{TA} group in comparison to the JIP1^{WT} group (mean ± SEM; n = 16
1416   ~ 17 cells; *, $p<0.05$, Student's t-test) while average AMPA receptor-mediated currents (C) did not differ.

1417   (**D**) NMDA to AMPA receptor current ratios were evaluated on a cell-by-cell basis. The JIP1^{TA} ratios
1418   (I_{NMDA} / I_{AMPA}) were larger than ratios measured from neurons in the JIP1^{WT} group (mean ± SEM; n = 16 ~
1419   17 cells; *, $p<0.05$, Student's t-test).

1420   (**E,F**) NMDA-stimulated gene expression in primary cultures of JIP1^{WT} and JIP1^{TA} hippocampal neurons
1421   was studied by treating neurons with 100 µM NMDA plus 10 µM glycine. The expression of *Bdnf* (E) and
1422   *cFos* (F) mRNA was quantitated by RT-PCR and normalized to *Gapdh* (mean ± SEM; n= 5~6; ***,
1423 $p<0.001$, two-way ANOVA followed by Bonferroni's post-hoc test).

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1427   **Figure 9. JIP1^{TA} mice display normal locomotor function, motor coordination, elevated anxiety-**
1428   **like behavior and increased acoustic startle response.**

1429   **(A-C)** Elevated Plus Maze. JIP1^{TA} mice show decreased time spent in open arms (A), increased time
1430   spent in closed arms (B) relative to wild-type mice, indicative of elevated anxiety-like behaviors. In
1431   addition, JIP1^{TA} mice show normal activity as measured by total distance traveled (C). The data are
1432   presented as the mean ± SEM (n = 10; **, $p<0.01$, Student's t–test).

1433   **(D,E)** Open Field Test. JIP1^{TA} mice show increased anxiety-like behavior in an open field test. Mice
1434   were allowed to explore an open field for 5 min. JIP1^{TA} mice spent more time in the periphery (D) and less
1435   time in the center region of the open field (E), both indicators of increased levels of anxiety-like behavior
1436   in this test. The data are presented as the mean ± SEM (n = 10; ***, $p<0.001$, Student's t–test).

1437   (**F**) JIP1^{TA} mice showed an increased acoustic startle response for the 110 dB acoustic startle stimulus
1438   compared with JIP1^{WT} mice (mean ± SEM; n = 8; *, $p<0.05$, two-way repeated measures ANOVA
1439   followed by Bonferroni's post-hoc comparisons tests).

1440   (**G**) No significant differences in prepulse inhibition for the 74, 80 and 86 dB pre-pulse sound levels
1441   followed by a 110 dB startle stimulus were observed between JIP1^{TA} and JIP1^{WT} mice (mean ± SEM; n =
1442   8; $p>0.05$, two-way repeated measures ANOVA followed by Bonferroni's post-hoc test.

1443   (**H**) JIP1^{TA} mice have normal balance and motor coordination, but impaired skill learning on the rotarod.
1444   Mice received four trials on day 1 (Trials 1-4) and day 2 (Trials 5-8). The duration of balance or latency to
1445   fall (4–40 rpm over 5 min) was recorded. Mice were trained on day 1 to establish baseline performance,
1446   and retested 24 hours later to measure skill learning. Both JIP1^{TA} and JIP1^{WT} mice exhibited increased
1447   skill in maintaining balance on the rotarod over the first four trials on day 1. On day 2, JIP1^{TA} mice failed
1448   to display motor coordination achieved after the day 1, indicative of impaired motor learning in the rotarod
1449   task. Data are presented as mean ± SEM; n = 8; *, $p<0.05$, two-way repeated measures ANOVA
1450   followed by Bonferroni's post-hoc test.

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1454 **Figure 10. JIP1^{TA} mice display enhanced contextual fear and impaired fear extinction.**

1455 (**A**) "Strong" (0.8 mA electric shock) training demonstrated that JIP1^{TA} and JIP1^{WT} littermate mice
1456 exhibited similar contextual freezing when tested immediately after training, or 1 h later, but the JIP1^{TA}
1457 mice froze more than JIP1^{WT} mice at 24 h following training (left panel). Foot-shock reactivity during fear
1458 conditioning training did not significantly differ between JIP1^{TA} and JIP1^{WT} mice (right panel). The data
1459 presented are the mean ± SEM (n = 10 ~ 11; ***, p<0.001, Student's t-test).

1460 (**B**) "Weak" (0.4 mA electric shock) training demonstrated that JIP1^{TA} mice (n = 14) exhibited contextual
1461 freezing that was similar to the "strong" training schedule, but JIP1^{WT} mice (n = 14) displayed significantly
1462 less contextual fear conditioning at 24 h following training (mean ± SEM; n = 14; ***, p<0.001, Student's t-
1463 test).

1464 (**C**) JIP1^{TA} and JIP1^{WT} littermate mice were infused with vehicle or the selective NMDA receptor
1465 antagonist APV (10 µg/ml) before "strong" (0.8 mA) contextual fear conditioning. The following day, there
1466 was a similar impairment in both genotypes in freezing levels to the conditioning context (mean ± SEM; n
1467 = 10; ***, p<0.001 vs. JIP1$^{WT+ veh}$; not significant (ns) JIP1$^{WT+ APV}$ vs. JIP1$^{TA+ APV}$; two-way ANOVA followed
1468 by Bonferroni's post-hoc test).
1469
1470 (**D**) JIP1^{TA} and JIP1^{WT} littermate mice were trained by "strong" (0.8 mA electric shock) contextual fear
1471 conditioning. Extinction began 24 h later and consisted of daily 3-min re-exposures of mice to the
1472 conditioning context in the absence of shock. When compared with JIP1^{WT} littermates, JIP1^{TA} mice
1473 showed increased freezing behavior throughout extinction days 1-7 (E1-E7), indicating impaired
1474 extinction process in JIP1^{TA} mice (mean ± SEM; n = 10; *, p<0.05, ***, p<0.001; two-way repeated
1475 measures ANOVA followed by Bonferroni's post-hoc test).

1476 (**E**) "Weak" (0.4 mA electric shock; left panel) and "strong" (0.8 mA; right panel) cued fear training,
1477 consisting of a single pair of cue (tone) and shock, demonstrated that JIP1^{TA} and JIP1^{WT} mice exhibited
1478 enhanced conditioned freezing to a cue, tone, when tested 24 h following training (mean ± SEM; n = 11;
1479 *, p<0.05, **, p<0.01, Student's t-test).

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1483 **Figure 11. JIP1^{TA} mice exhibit enhanced acquisition and reversal learning in the Morris water**
1484 **maze test.**

1485 (**A-C**) JIP1^{TA} and JIP1^{WT} littermate mice learned the visible platform task (day 1 & 2), as indicated by
1486 reductions in escape time during training. The mice were then trained to find a hidden platform during the
1487 next seven days. JIP1^{TA} mice showed faster escape latencies at days 6-9 training compared with JIP1^{WT}
1488 littermates (A). A first probe test (day 10) was conducted 24 h after the completion of training. No
1489 significant differences in percentage time spent in the target quadrant (T) between JIP1^{TA} and JIP1^{WT}
1490 mice were observed (B). The mice were then subjected to 2 days of additional training (days 11-12), and
1491 a second probe trial was performed 24 h later. No significant differences between JIP1^{TA} and JIP1^{WT}
1492 mice were observed during second probe trial (C). Data presented are the mean ± SEM (n = 14; *,
1493 p<0.05, two-way repeated measures ANOVA, followed by Bonferroni's post-hoc test).

1494 (**D-F**) Twenty-four hours after the second probe test, the platform was moved to the opposite quadrant in
1495 the pool and mice were trained for four trials (day 14, reversal learning). In this new setting, JIP1^{TA} mice
1496 displayed shorter escape time to find newly placed platform (NT) compared with JIP1^{WT} littermate mice
1497 (E). The probe test for reversal training was conducted 24 h after the completion of new platform training
1498 (day 15). Analysis of the time spent in the quadrants revealed that JIP1^{TA} mice spent significantly more
1499 time in the new target quadrant than JIP1^{WT} mice (F). Data presented are the mean ± SEM (n = 14; *,
1500 p<0.05, ***, p<0.001, two-way repeated measures ANOVA (E) and two-way ANOVA (F) followed by
1501 Bonferroni's post-hoc tests).

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Figure 12. Suppression of kainate-induced JNK activity in the hippocampus of JIP1$^{\Delta \text{JBD}}$ mice.

(**A,B**) A targeting vector was designed to replace JIP1 residues Leu^{160}-Asn^{161}-Leu^{162} with Gly^{160}-Arg^{161}-Gly^{162} in exon 3 of the *Jip1* gene by homologous recombination in ES cells. The *floxed Neo^R* cassette inserted in intron 3 and used for selection was deleted with *Cre* recombinase. H, HindIII.

(**C**) Lysates prepared from the cerebral cortex of *Jip1$^{+/+}$* (WT) and *Jip1$^{\Delta JBD/\Delta JBD}$* (ΔJBD) mice were examined by immunoblot analysis using antibodies to JIP1 and β-Tubulin.

(**D,E**) JIP1^{WT} and JIP$^{\Delta JBD}$ mice were treated without and with kainate. Representative sections of the dentate gyrus stained (green) with antibodies to phospho-cJun (D) or cJun (E) are presented. DNA was stained with DAPI (red). Scale bar = 75 μm.

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Figure 13. Disruption of the JNK binding domain (ΔJBD) on JIP1 causes enhanced associative learning.
(**A**) Contextual and cued fear conditioning of JIP1$^{\Delta JBD}$ and JIP1^{WT} mice consisted of one exposure to cue [context + tone] and 0.8mA shock (mean ± SEM; n = 10~11; **, p<0.01, Student's t-test ***, p<0.001, Student's t-test).
(**B,C**) Morris water maze tests of mean latencies to escape to a visible (days 1-2) or a hidden platform (days 3-12) are presented for JIP1$^{\Delta JBD}$ or JIP1^{WT} mice (B). Probe trials were performed on days 9 and 13 of water maze training (C). JIP1$^{\Delta JBD}$ mice spent significantly longer time in the target quadrant compared to JIP1^{WT} littermates (mean ± SEM; n = 10; *, p<0.05; **, p<0.01; ***, p<0.001, two-way repeated measures ANOVA (B) and two-way ANOVA (C) followed by Bonferroni's post-hoc tests).
(**D**) The water maze platform was moved to the opposite quadrant in the pool and mice were trained for four trials (day 14, reversal learning). The probe test for reversal training was conducted 24 h after the completion of new platform training (day 15). Analysis of the time spent in the quadrants during the probe trial revealed that JIP1$^{\Delta JBD}$ mice spent significantly more time in the new target quadrant (NT) than JIP1^{WT} mice (mean ± SEM; n = 10; ***, p<0.001, two-way ANOVA followed by Bonferroni's post-hoc test).
(**E**) Hippocampus lysates of JIP1^{WT}, JIP1^{TA} and JIP1$^{\Delta JBD}$ mice were examined by immunoblot analysis by probing with antibodies to NMDA receptor subunits and β-Tubulin.
(**F**) The amount of phospho-ERK in hippocampus lysates of naïve JIP1^{WT} and JIP1$^{\Delta JBD}$ mice was quantified by multiplexed ELISA and normalized to the amount of ERK2 in each sample (mean ± SEM; n = 5; *, p<0.05, Student's t-test).

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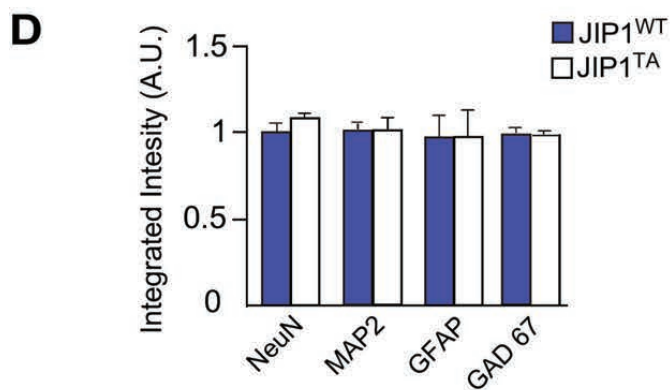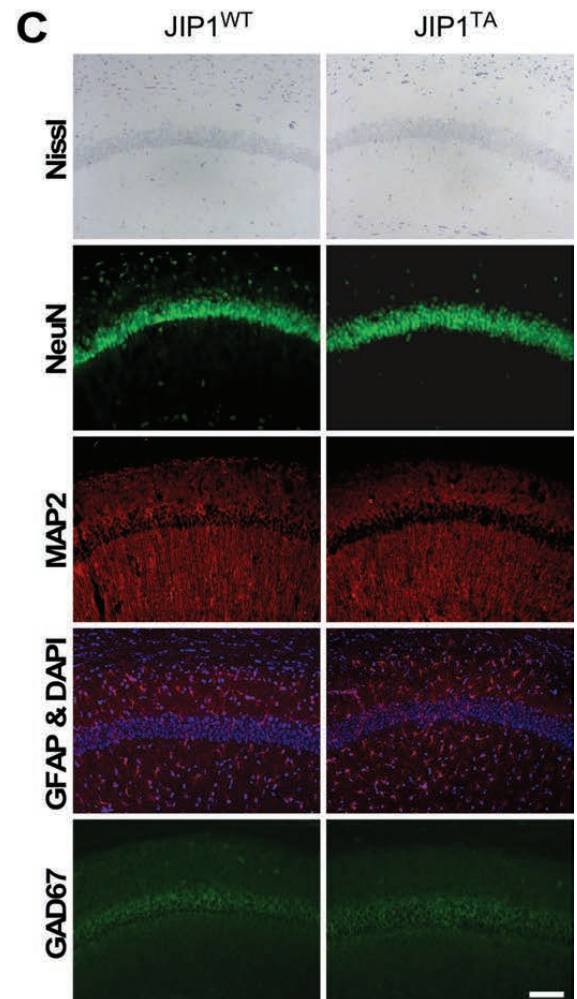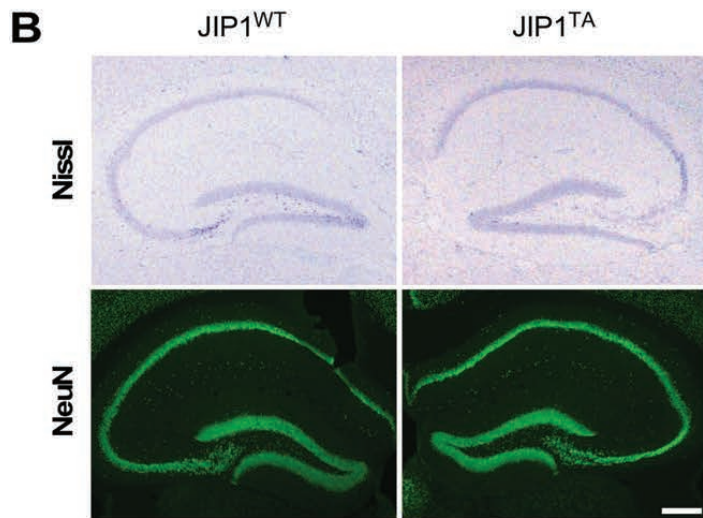1540

Figure 14. A model of how JIP1-mediated JNK signaling regulates synaptic NMDA receptor expression. JIP1-dependent JNK activation by the NMDA receptor (NMDAR) may suppress translation of NMDA receptor subunit mRNA (*Grin1, Grin2a, Grin2b*). Alternatively, the same pathway regulates cell surface insertion or retrieval of NMDA receptors and/or lateral diffusion of extrasynaptic NMDA receptors into synaptic sites.

A

CA1

CA3

DG

JIP1 MAP2

CA1

CA3

DG

JIP1 NeuN

s.p.

s.r.

s.l.m.

s.p.

s.l.

grDG

poDG

B

JIP1^WT

JIP1^TA

Nissl

NeuN

C

JIP1^WT

JIP1^TA

Nissl

NeuN

MAP2

GFAP & DAPI

GAD67

D

A

Basal dendrites

Apical dendrites

JIP1^{WT}

JIP1^{TA}

B Spine Density

Spine number per 10 µm

JIP1^{WT}
JIP1^{TA}

Basal   Apical

C Spine Type

% of total spines

Mushroom
Stubby
Thin

Basal
JIP1^{WT} Basal
JIP1^{TA} Apical
JIP1^{WT} Apical
JIP1^{TA}

D Basal dendrites

Dendritic intersections

JIP1^{WT}
JIP1^{TA}

Distance from cell body (µm)

E Apical dendrites

Dendritic intersections

JIP1^{WT}
JIP1^{TA}

Distance from cell body (µm)

A Phospho-cJun & DAPI

Control   Kainate

JIP1^WT

JIP1^TA

B cJun & DAPI

Control   Kainate

C cFos & DAPI

Control   Kainate

D

JIP1^WT
JIP1^TA

Relative cJun phosphorylation

6
5
4
3
2
1
0

0   15   30   60

Time after kainate (min)

E

JIP1^WT
JIP1^TA

Relative cJun expression

1.8
1.5
1.2
0.9
0.6
0.3
0

0   15   30   60

Time after kainate (min)

Time after FC (min)

Naive 10 30 60 180
WT TA WT TA WT TA WT TA WT TA

pJNK

JNK

GAPDH

46kDa

JNK phosphorylation (pJNK/GAPDH)

JIP1^{WT}
JIP1^{TA}

*** *** ***
##

Naive 10 30 60 180
Time after FC (min)

54kDa

JNK phosphorylation (pJNK/GAPDH)

JIP1^{WT}
JIP1^{TA}

#

Naive 10 30 60 180
Time after FC (min)

A Input/Output Curve

B Input/Output Curve
JIP1^{WT}
JIP1^{TA}

C Paired-pulse facilitation
JIP1^{WT}
JIP1^{TA}

D JIP1^{WT}
JIP1^{TA}
JIP1^{WT} JIP1^{TA}
100Hz

E JIP1^{WT} JIP1^{TA}
JIP1^{WT}
JIP1^{TA}
***
10HzX900

F JIP1^{WT} JIP1^{TA}
JIP1^{WT}
JIP1^{TA}
***
1HzX900

G JIP1^{WT} JIP1^{TA}
JIP1^{WT}
JIP1^{TA}
0.5HzX900

H Frequency Response function
JIP1^{WT}
JIP1^{TA}
***
***
LTP
LTD

I JIP1^{WT} JIP1^{TA}
JIP1^{WT}
JIP1^{TA}
DHPG

J JIP1^{WT} JIP1^{TA}
JIP1^{WT}
JIP1^{TA}
100Hz 1Hz

A Input/Output Curve

B PPF

C

D

A

GluN2B
pY1472
GluN2A
GluN1
GluA1
GluA2
SAP102
JIP1
β-Tubulin

B

C

D

GluN2A
GluN2B
GluN1
JIP1
PSD-95
Synapsin I

E

Non-permeabilized   Permeabilized

JIP1^{WT} JIP1^{TA} JIP1^{WT} JIP1^{TA}

GluN1
β-Tubulin
DAPI
Merge

F

G

H

p-ERK
ERK2
p-CREB
CREB
KIF17
β-Tub.

A

JIP1^WT   JIP1^TA

V_H = +50 mV

V_H = -70 mV

0.1 nA

50 ms

B

■ JIP1^WT
□ JIP1^TA

*

NMDA Current (pA)

120
100
80
60
40
20
0

C

■ JIP1^WT
□ JIP1^TA

AMPA Current (pA)

-200
-150
-100
-50
0

D

■ JIP1^WT
□ JIP1^TA

*

I_{NMDA} / I_{AMPA} (%)

60
50
40
30
20
10
0

E   *Bdnf*

■ JIP1^WT
□ JIP1^TA

***

***

Relative mRNA expression

25
20
15
10
5
0

0   1h   2h
NMDA/Gly

F   *cFos*

■ JIP1^WT
□ JIP1^TA

***

***

Relative mRNA expression

200
160
120
80
40
0

0   1h   2h
NMDA/Gly

Elevated Plus Maze

Open field Test

Startle Response

Rotarod

A JIP1^{WT} JIP1^{TA} Probe 1 (d10) Probe 2 (d13); Latency (sec); d1 Visible platform; d3 d9 d12 Hidden platform

B JIP1^{WT} JIP1^{TA}; % of time spent in quadrants; L O R T; Probe Trial 1 (d10)

C JIP1^{WT} JIP1^{TA}; % of time spent in quadrants; L O R T; Probe Trial 2 (d13)

D Opp / Left / Right / Target → day 14 → New Target / Left / Right / Opp

E Reversal training-d14; JIP1^{WT} JIP1^{TA}; Latency (sec); *** * *; Trial 1 2 3 4

F JIP1^{WT} JIP1^{TA}; % of time spent in quadrants; L O R NT; *** *; Reversal probe trial (d15)

A

11.9 kb

H 2 3 4 5 6 7 8 9 10 11 12 H *Jip1*

Homologous Recombination

Targeting vector 3 Neo 4 5 6 7 8 9 10 11 12 TK

6.6 kb

H 2 3 H Neo 4 5 6 7 8 9 10 11 12 H

Cre Recombinase
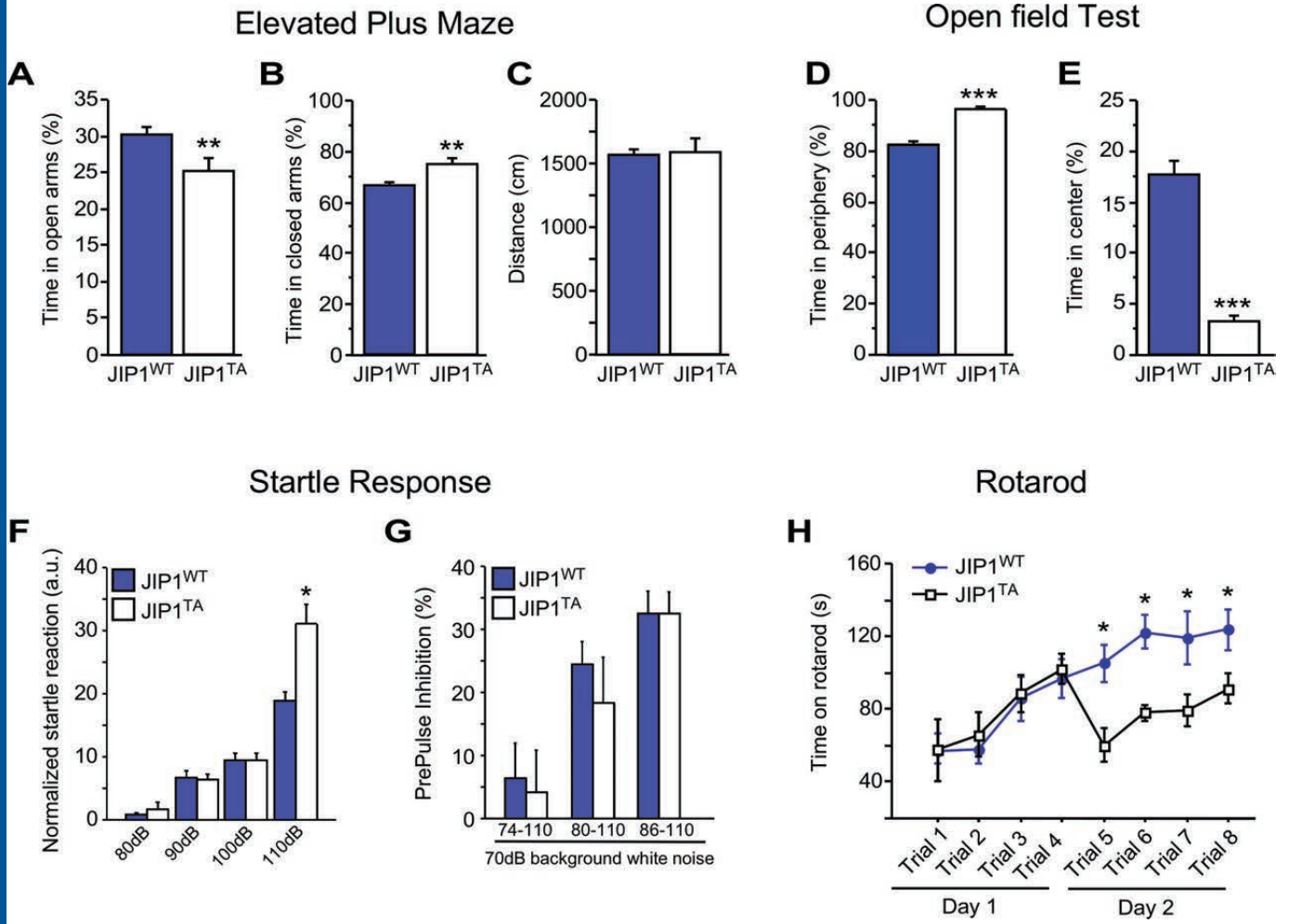
H 2 3 4 5 6 7 8 9 10 11 12 H *Jip1^ΔJBD*
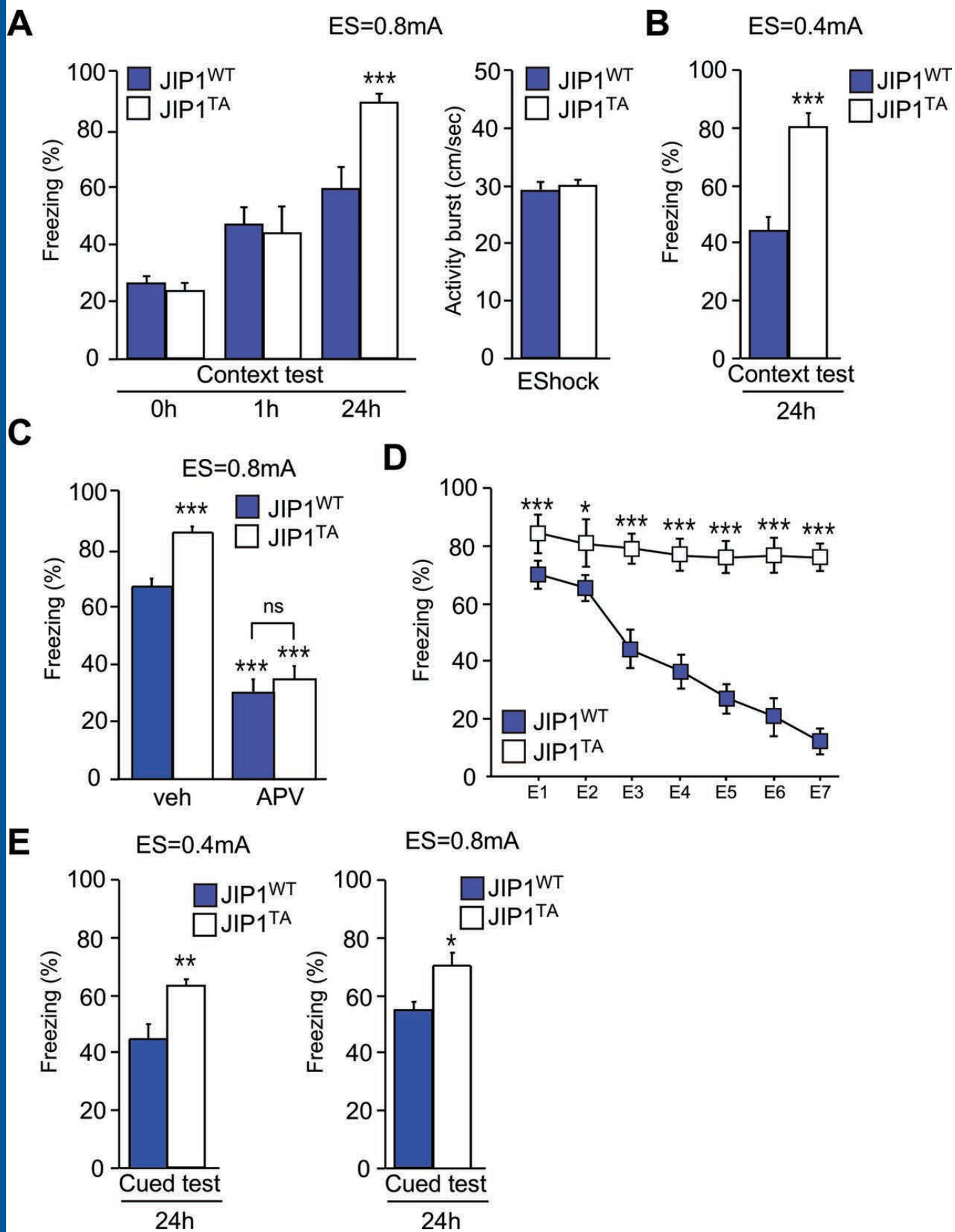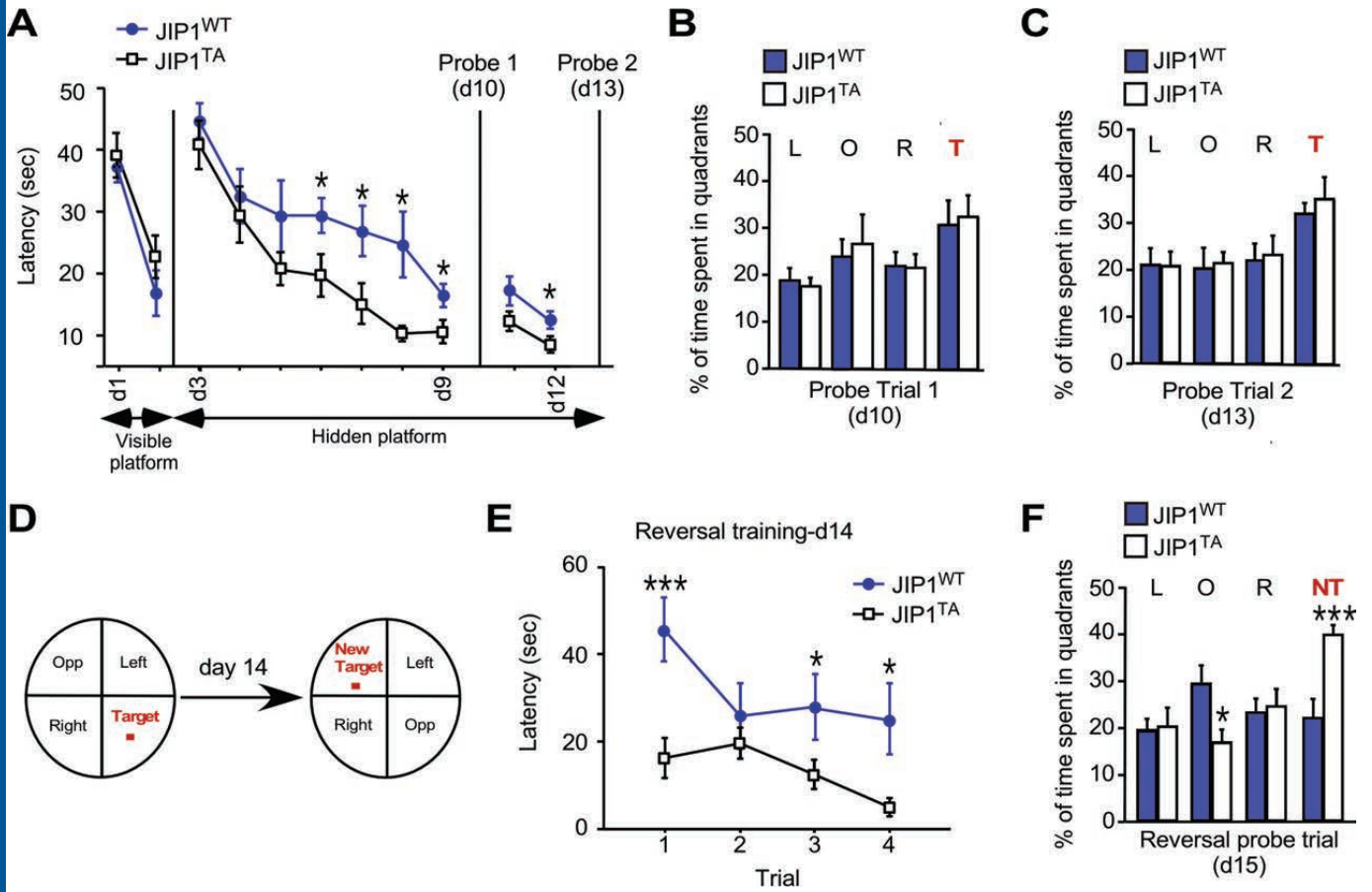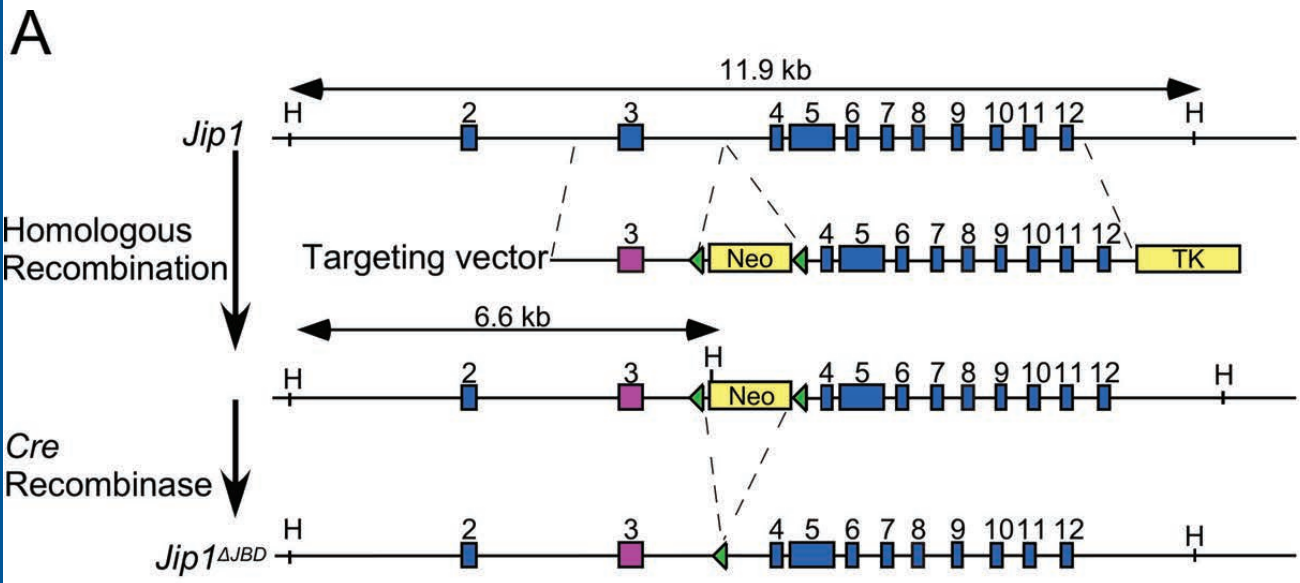
B

Exon III

AGGCCTACCACGCTCAACCTTTTCCCGCAG *Jip1*
R   P   T   T   L   N   L   F   P   Q

--- --- --- AC CGGCCGCGGT --- --- --- *Jip1^ΔJBD*
                G   R   G

C

Cortex
WT ΔJBD
]JIP1
β-Tubulin

D

Kainate
−   +

p-cJun

JIP1^WT

JIP1^ΔJBD

E

Kainate
−   +

cJun

JIP1^WT

JIP1^ΔJBD