

Dopamine increases protein synthesis in hippocampal neurons enabling dopamine-dependent LTP

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This manuscript addresses a mechanism by which dopamine (DA) regulates synaptic plasticity. The authors build upon their previous finding that DA applied after a timing pattern that ordinarily induces long-term depression (LTD) now induces long-term potentiation (LTP). The new findings that this "DA-dependent LTP" involves de novo protein synthesis, a cyclicAMP signalling pathway, and calcium-permeable AMPA receptors (CP-AMPA) are of **valuable** significance. The conclusions are **convincing** and largely supported by the evidence provided.

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Abstract

The reward and novelty related neuromodulator dopamine plays an important role in hippocampal long-term memory, which is thought to involve protein synthesis-dependent synaptic plasticity. However, the direct effects of dopamine on protein synthesis, and the functional implications of newly synthesized proteins for synaptic plasticity, have not yet been investigated. We have previously reported that timing-dependent synaptic depression (t-LTD) can be converted into potentiation by dopamine application during synaptic stimulation (Brzosko et al., 2015) or postsynaptic burst activation (Fuchsberger et al., 2022). Here we show that dopamine increases protein synthesis in mouse hippocampal CA1 neurons, enabling dopamine-dependent long-term potentiation (DA-LTP), which is mediated via the Ca^{2+} -sensitive adenylate cyclase (AC) subtypes 1/8, cAMP, and cAMP-dependent protein kinase (PKA). We found that neuronal activity is required for the dopamine-induced increase in protein synthesis. Furthermore, dopamine induced a protein synthesis-dependent increase in the AMPA receptor subunit GluA1, but not GluA2. We found that DA-LTP is absent in GluA1 knock-out mice and that it requires calcium-permeable AMPA receptors. Taken together, our results suggest that dopamine together with neuronal activity controls synthesis of plasticity-related proteins, including GluA1, which enable DA-LTP via a signalling pathway distinct from that of conventional LTP.

Introduction

An important role of dopamine in hippocampal long-term memory has long been recognised (O'Carroll et al., 2006 [↗](#); Rossato et al., 2009 [↗](#); Shohamy and Adcock., 2010 [↗](#); Takeuchi et al., 2016 [↗](#)). Long-term memory is thought to be encoded by synaptic plasticity, in particular long-term potentiation (LTP; Bliss and Lømo, 1973) which, based on its duration, has been divided into an early phase and a protein synthesis-dependent late phase (Frey et al., 1988 [↗](#)). Dopaminergic signalling has been implicated specifically in 'late-phase' LTP (Frey et al., 1990 [↗](#); Huang and Kandel, 1995 [↗](#); Matthies et al., 1997 [↗](#)). Dopamine acts on G-protein-coupled receptors which activate adenylyl cyclases (AC) to generate cAMP, which in turn activates several effectors, including the cAMP-dependent protein kinase (PKA). PKA has several downstream targets which ultimately regulate transcription and translation (Sassone-Corsi, 1995 [↗](#); Keibian and Calne, 1997; Mayr and Montminy, 2001 [↗](#); Smith et al., 2005 [↗](#)). Although the requirement of dopamine for protein synthesis-dependent LTP has been well established, the underlying cellular mechanisms of how dopamine modulates activated synapses via protein synthesis-dependent mechanisms remain poorly understood.

In order to selectively label newly synthesized proteins, we used a puromycin-based assay (Schmidt et al., 2009 [↗](#)), adapted for use in acute mouse hippocampal slices. We found that dopamine increases protein synthesis in hippocampal neurons. We then investigated whether protein synthesis is required for a recently described dopamine-dependent form of plasticity (DA-LTP) which, although being rapid in onset, otherwise shares properties with 'late-phase' LTP (Brzosko et al., 2015 [↗](#); Fuchsberger et al., 2022 [↗](#)). Whilst conventional 'early' LTP induced by a spike-timing-dependent plasticity protocol (t-LTP; Bi and Poo, 1998 [↗](#)) was unaffected, DA-LTP was completely abolished by either of two different protein synthesis inhibitors.

A previous study in primary cultured hippocampal neurons reported that dopaminergic signalling increases the surface expression of the AMPA receptor subunit GluA1 (Smith et al., 2005 [↗](#)), which has been widely studied in the context of LTP (Huganir and Nicoll, 2013 [↗](#); Diering and Huganir, 2018 [↗](#)). Whereas most hippocampal AMPA receptors are heteromeric, incorporating the edited form of the GluA2 subunit, rendering the receptor calcium-impermeable, GluA1 may form homomeric calcium-permeable AMPA receptors (CP-AMPA) (Burnashev et al., 1992 [↗](#); Wenthold et al., 1996 [↗](#)). As well as enabling calcium-permeability of the AMPAR, by lacking GluA2, the extracellular N-terminal domain of GluA1 in GluA1 homomers is highly flexible, which alters the gating of the receptor and hinders its synaptic anchoring (Zhang et al., 2023 [↗](#); Stockwell et al., 2024 [↗](#)). CP-AMPA have been implicated in some forms of LTP (Plant et al., 2006 [↗](#); Guire et al., 2008 [↗](#); Purkey et al., 2020), including a PKA-dependent form of plasticity (Park et al., 2021 [↗](#)).

We found that levels of the GluA1 receptor subunit, but not GluA2, were upregulated in response to dopamine in a protein synthesis-dependent manner. Moreover, DA-LTP was absent in a GluA1 genetic knock-out mouse model, while conventional t-LTP remained intact. These findings suggest that newly synthesized GluA1 receptor subunits mediate the expression of DA-LTP, possibly by forming GluA1 homomeric CP-AMPA. Indeed, while blocking CP-AMPA did not interfere with conventional t-LTP, they were required for DA-LTP.

Results

Dopamine increases protein synthesis in CA1, which is required for dopamine-dependent long-term potentiation (DA-LTP)

We hypothesized that application of dopamine induces synthesis of proteins that are required for the expression of DA-LTP. To address this, we first investigated whether dopamine directly affects protein synthesis in hippocampal neurons. In order to exclusively visualise newly synthesized proteins in hippocampal slices we used a puromycin (PMY)-based assay (Schmidt et al., 2009). Labelling was achieved by incubating acute hippocampal slices in artificial cerebrospinal fluid (aCSF) containing 3 μ M PMY for 30 minutes (Figure 1A). As expected, slices incubated with PMY showed a significantly higher PMY intensity than those without (control, 0.39 ± 0.02 a.u., $n = 9$, vs +PMY, 0.54 ± 0.03 , $t(16) = 4.0$, $p = 0.001$) (Figure 1B and C), confirming specificity of the antibody labelling approach. Furthermore, we tested whether PMY incorporation and labelling are specific to protein synthesis by adding the protein synthesis inhibitor cycloheximide (CHX) or anisomycin (AM) to the slices. We found that 30 minutes preincubation with 10 μ M CHX significantly reduced the PMY signal, confirming the specificity of the labelling approach (PMY, 1.8 ± 0.45 , $n = 7$ vs PMY+CHX, 0.4 ± 0.05 , $n = 8$, $t(6) = 3.1$, $p = 0.02$) (Figure 1—figure supplement S1A and B), as did 2 hr preincubation with 0.5 mM AM (PMY, 0.6 ± 0.14 , $n = 4$ vs PMY+AM, 0.16 ± 0.03 , $n = 5$, $t(3.2) = 3.1$, $p = 0.048$) (Figure 1—figure supplement S1C and D). All results are reported as mean $\pm$ S.E.M.

Strikingly, when applying 100 μ M dopamine the PMY signal was significantly increased compared to slices without dopamine (PMY, 0.54 ± 0.03 , $n = 9$ vs PMY+DA, 0.80 ± 0.019 , $n = 16$, $p < 0.001$) (Figure 1B and C). We next examined the effect of the D1/D5 receptor agonist SKF38393 (10 μ M), and found that it stimulates protein synthesis to a similar extent as dopamine, with no significant difference between those two conditions (PMY+DA, 0.80 ± 0.019 , $n = 16$, vs PMY+SKF, 0.79 ± 0.026 , $n = 9$, $p > 0.99$), and significantly different to PMY alone (PMY, 0.54 ± 0.03 , $n = 9$ vs PMY+SKF, 0.79 ± 0.026 , $n = 9$, $p < 0.001$) (Figure 1B and C). We next tested whether the dopamine-induced increase in protein synthesis could be prevented by application of D1/5 and D2 receptor antagonists. Using 10 μ M SCH23390, a selective antagonist at D1 ($K_i = 0.2$ nM) and D5 ($K_i = 0.3$ nM) receptor subtypes, and 50 μ M sulpiride, a D2 antagonist ($K_i = 8$ nM), we found that the dopamine-induced increase in PMY signal was prevented (PMY+DA, 0.80 ± 0.019 , $n = 16$, vs PMY+DA+SCH23390/sulpiride, 0.61 ± 0.029 , $n = 8$, $p < 0.001$; all p values reported above from post-hoc Tukey's HSD test, after confirming a statistically significant difference between at least two groups in one-way ANOVA; $F(4, 46) = 49.55$, $p < 0.001$) (Figure 1B and C).

To confirm the effect of dopamine on protein synthesis with an alternative quantification method, acute hippocampal slices were incubated as above and homogenates prepared for western blot analysis of the micro-dissected CA1 region. As expected, without PMY incubation the signal was virtually absent compared to samples with PMY (neg control, 5 ± 2 , $n = 5$, vs +PMY, 97 ± 21 , $n = 5$, $p = 0.01$). Dopamine application significantly increased the PMY signal (PMY, 97 ± 21 , $n = 5$, vs PMY+DA, 201 ± 25 , $n = 5$, $p = 0.006$; p values reported from post-hoc Tukey's HSD test after one-way ANOVA; $F(3, 16) = 19.85$, $p < 0.0001$) (Figure 1D and E).

We then tested whether newly synthesized proteins are required for DA-LTP induced by a spike timing-dependent long-term depression (t-LTD) pairing protocol followed by dopamine application during low-frequency afferent stimulation, which converts synaptic depression into potentiation (Brzosko et al., 2015). Conventional t-LTD induced by a post-before-pre pairing without dopamine application leads to synaptic depression, whereas a pre-before-post t-LTP protocol induces synaptic potentiation (Bi and Poo, 1998; Feldman, 2012). We confirmed that application of dopamine after the post-before-pre pairing protocol ($\Delta t = -20$ ms) induces robust synaptic potentiation ($138\% \pm 16\%$ vs 100% , $t(8) = 2.5$, $p = 0.039$, $n = 9$) (Figure 1F and G), while the same pairing protocol without dopamine application leads to robust input-specific synaptic depression (t-LTD $64\% \pm 9\%$ vs control $106\% \pm 8\%$, $t(6) = 5.8$, $p = 0.001$) (Figure 1—figure supplement S1E and F). Dopamine application alone had no effect on synapses that did not

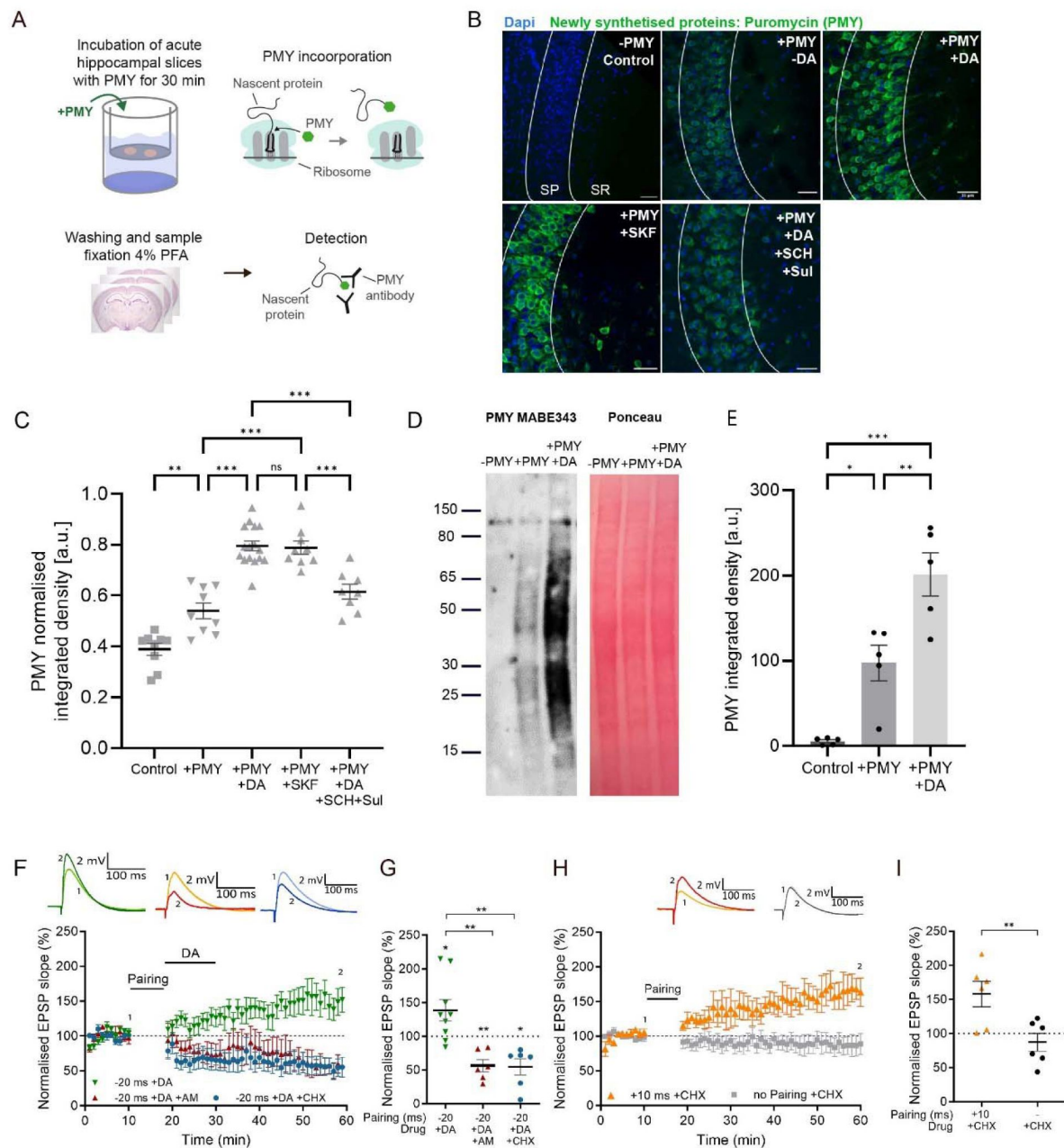


Figure 1.

Dopamine increases protein synthesis in CA1, required for DA-LTP, but not for conventional t-LTP. **(A)**, Experimental workflow for protein synthesis labelling in acute hippocampal slices using a puromycylation assay. PMY = puromycin. **(B)**, Representative images of the CA1 region of the hippocampus (SP: stratum pyramidale, SR: stratum radiatum) of puromycin-labelled proteins (green) and Dapi (blue) in the following conditions: negative control (no PMY), PMY alone, PMY + dopamine (DA), PMY + SKF38393 (SKF), and PMY + DA + SCH23390 (SCH) + sulpiride (Sul). The slices show an increase in protein synthesis after DA or SKF38393 application, which is blocked by DA receptor antagonists SCH23390 and sulpiride. Scale bar, 30 μ m. **(C)**, Summary of results. One-way ANOVA followed by Tukey's HSD test, **, $p < 0.01$; ***, $p < 0.001$; ns, not significant. **(D)**, Representative western blot of newly synthesised proteins detected by PMY Mabe343 antibody shows no signal in negative control (-PMY), and increased PMY signal in the presence of dopamine (+DA). Ponceau stain confirms equal loading of total protein. **(E)**, Summary of results. One-way ANOVA followed by Tukey's HSD test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **(F)**, Dopamine application (DA) after a post-before-pre pairing protocol (Pairing, $\Delta t = -20$ ms) leads to synaptic potentiation (green trace), which is blocked by postsynaptically applied anisomycin (AM) (red trace) or cycloheximide (CHX) (blue trace). **(G)**, Summary of results. t -test, **, $p < 0.01$. **(H)**, Intact conventional t-LTP ($\Delta t = +10$ ms, orange trace) in the presence of cycloheximide (CHX). **(I)**, Summary of results. t -test, **, $p < 0.01$. Traces show an EPSP before (1) and 40 minutes after (2) pairing. Plots show averages of normalised EPSP slopes $\pm$ SEM.

undergo prior pairing ($102\% \pm 14\%$ vs 100% , $t(8) = 0.13$, $p = 0.89$, $n = 9$) (**Figure 1** [—figure supplement S1G](#) [and I](#)). When delivering the protein synthesis inhibitor anisomycin (AM) to the postsynaptic cell through the recording pipette, DA-LTP was fully blocked, leaving synaptic depression instead (DA+AM, $56\% \pm 9\%$ vs 100% , $t(5) = 4.8$, $p = 0.0047$, $n = 6$), which was significantly different from dopamine without AM (DA $138\% \pm 16\%$ vs DA+AM $56\% \pm 9\%$, $t(13) = 3.9$, $p = 0.0016$) (**Figure 1F and G**). To exclude the possibility that the effect of AM on blocking DA-LTP is mediated via other signalling pathways affected by AM (Croons et al., 2009 [; Hazzalin et al., 1998](#) [; Kyriakis et al., 1994](#) [\) rather than by specific inhibition of protein synthesis, we used cycloheximide \(CHX\), an alternative blocker of protein synthesis. We found that loading CHX into the postsynaptic cell through the recording pipette also completely blocked DA-LTP, leaving synaptic depression instead \(DA+CHX, \$55\% \pm 12\%\$ vs \$100\%\$, \$t\(5\) = 3.7\$, \$p = 0.013\$, \$n = 6\$ \) \(**Figure 1F** \[\\), which was significantly different from dopamine without CHX \\(DA \\$138\% \pm 16\%\\$ vs DA+CHX \\$55\% \pm 12\%\\$, \\$t\\(13\\) = 3.9\\$, \\$p = 0.002\\$ \\) \\(**Figure 1F and G**\\). We also confirmed that postsynaptically applied AM or CHX had no effect on baseline synaptic transmission throughout the 60-minute duration of the experiment in the control pathway \\(no pairing +AM, \\$104 \pm 17\%\\$ vs \\$100\%\\$, \\$t\\(5\\) = 0.23\\$, \\$p = 0.83\\$, \\$n = 6\\$; and no pairing +CHX, \\$98 \pm 6\%\\$ vs \\$100\%\\$, \\$t\\(5\\) = 0.32\\$, \\$p = 0.76\\$, \\$n = 6\\$ \\) \\(**Figure 1** \\[—figure supplement S1H\\]\\(#\\) \\[and I\\]\\(#\\)\\).\]\(#\)](#)

In contrast, when we used a t-LTP protocol ($\Delta t = +10$ ms) while CHX was loaded into the postsynaptic cell, we still observed robust potentiation (t-LTP + CHX, $158\% \pm 19\%$ vs 100% , $t(5) = 3.11$, $p = 0.026$, $n = 6$, **Figure 1H** [\), and, as expected, there was no significant effect of CHX on the control pathway \(CHX, \$87\% \pm 13\%\$ vs \$100\%\$, \$t\(5\) = 0.97\$, \$p = 0.37\$, \$n = 6\$, **Figure 1H** \[\\). These results demonstrate that DA-LTP, but not conventional t-LTP, requires postsynaptic protein synthesis, revealing two different signalling pathways for LTP, one of which requires dopamine and protein synthesis, while the other one does not.\]\(#\)](#)

Dopamine and neuronal activity mediate increase in protein synthesis

DA-LTP requires neuronal activation during dopamine application either via subthreshold synaptic stimulation (Brzosko et al., 2015 [\) or postsynaptic bursts \(Fuchsberger et al., 2022](#) [\). Thus, we investigated whether neuronal activity is also required for the dopamine-induced increase in protein synthesis.](#)

To test this, acute hippocampal slices were treated with $1 \mu\text{M}$ tetrodotoxin (TTX), a voltage-gated sodium channel blocker, during PMY incubations (**Figure 2A** [\). We confirmed using whole-cell patch clamp recordings that application of TTX inhibits spontaneous EPSPs in CA1 pyramidal neurons in our preparations \(**Figure 2B** \[\\). Using the PMY labelling assay, we found that TTX incubation alone did not significantly alter PMY labelling, suggesting that the baseline level of protein synthesis is not affected by blocking neuronal activity for 30 minutes \\(control, \\$0.55 \pm 0.033\\$, \\$n = 8\\$, vs TTX, \\$0.53 \pm 0.026\\$, \\$n = 8\\$, \\$p = 0.90\\$ \\) \\(**Figure 2A and C** \\[\\\). However, TTX significantly diminished the dopamine-induced increase in protein synthesis \\\(DA, \\\$0.83 \pm 0.024\\\$, \\\$n = 9\\\$, vs DA+TTX, \\\$0.65 \pm 0.017\\\$, \\\$n = 9\\\$, \\\$p < 0.001\\\$ \\\). Nevertheless, the dopamine-induced increase in protein synthesis was not fully blocked by TTX, as it remained significantly higher in the presence of dopamine and TTX compared to TTX alone \\\(TTX+DA, \\\$0.65 \pm 0.017\\\$, \\\$n = 9\\\$, vs TTX \\\$0.53 \pm 0.026\\\$, \\\$n = 8\\\$, \\\$p = 0.005\\\$; \\\$p\\\$ values reported from post-hoc Tukey's HSD test after one-way ANOVA; \\\$F\\\(3, 30\\\) = 30.57\\\$, \\\$p < 0.001\\\$ \\\) \\\(**Figure 2A and C** \\\[\\\\).\\\]\\\(#\\\)\\]\\(#\\)\]\(#\)](#)

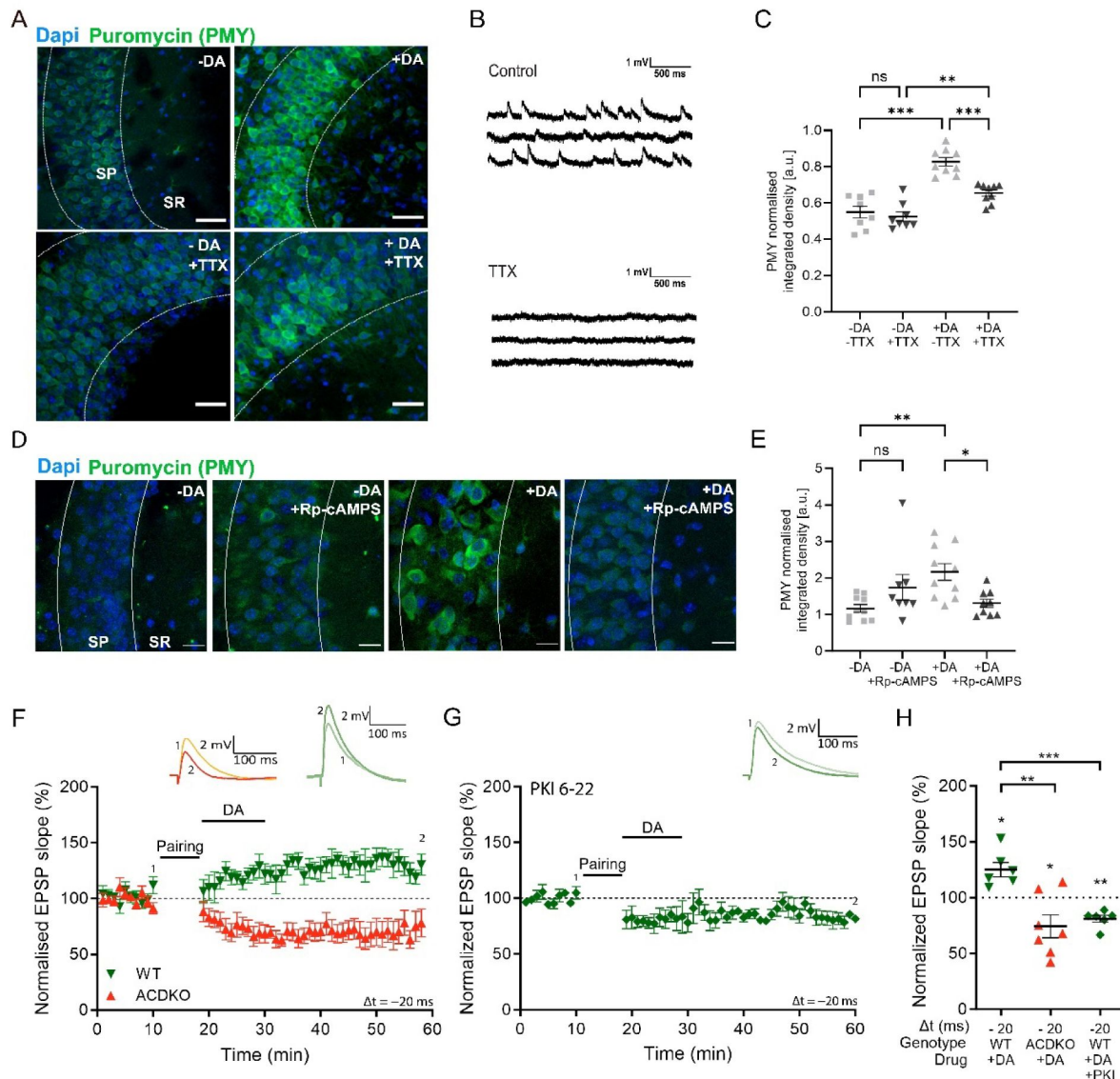


Figure 2.

Dopamine and neuronal activity mediate increase in protein synthesis via AC1/8 and PKA enabling DA-LTP. **(A)**, Representative images of the CA1 region (SR: stratum oriens, SP: stratum pyramidale) of the hippocampus of puromycin-labelled proteins in the following conditions: PMY alone (-DA), PMY+dopamine (+DA), PMY+TTX (-DA+TTX), PMY+dopamine+TTX (+DA+TTX). Images show that the dopamine-induced increase in protein synthesis is reduced in the presence of TTX. Scale bar, 30 μ m. **(B)**, TTX abolishes spontaneous activity shown in traces of whole-cell patch clamp recording. **(C)**, Summary of results. One-way ANOVA followed by Tukey's HSD test, **, $p < 0.01$; ***, $p < 0.001$; ns, not significant. **(D)**, Representative images of the CA1 region (SR: stratum oriens, SP: stratum pyramidale) of the hippocampus of puromycin-labelled proteins in the following conditions: PMY alone (-DA), PMY+Rp-cAMPS (-DA+Rp-cAMPS), PMY+dopamine (+DA), and PMY+dopamine+Rp-cAMPS (+DA+Rp-cAMPS). Images show that the dopamine-induced increase in protein synthesis is prevented by Rp-cAMPS. **(E)**, Summary of results. One-way ANOVA followed by Tukey's HSD test, *, $p < 0.05$; **, $p < 0.01$; ns, not significant. **(F)**, Dopamine application (DA) after a post-before-pre pairing protocol (Pairing, $\Delta t = -20$ ms) leads to synaptic potentiation in WT (green trace), but not in ACDKO mice (red trace). **(G)**, Postsynaptically applied PKA inhibitor PKI6-22 blocks DA-LTP (green trace), leaving synaptic depression instead. **(H)**, Summary of results. One-way ANOVA followed by Tukey's HSD test, **, $p < 0.01$; ***, $p < 0.001$. Traces show an EPSP before (1) and 40 minutes after (2) pairing. Plots show averages of normalised EPSP slopes $\pm$ SEM.

Dopamine-induced increase in protein synthesis is mediated via AC1/8 and PKA

We then sought to identify the signalling pathway that mediates the changes in protein synthesis in response to dopamine and neuronal activity. D1 and D5 dopamine receptors are known to activate adenylate cyclases (AC), which increase cyclic adenosine monophosphate (cAMP) activating the cAMP-dependent protein kinase (PKA) (Sassone-Corsi, 2012). We used the PMY incubation assay to assess the protein synthesis level in the presence of Rp-cAMPS, a cell-permeable cAMP analogue which acts as an inhibitor of PKA. We found that application of 30 μ M Rp-cAMPS had no significant effect on protein synthesis in control conditions (control, 1.2 ± 0.10 , $n = 10$, vs Rp-cAMPS 1.7 ± 0.4 , $n = 8$, $p = 0.25$), but completely blocked the dopamine-induced increase in PMY levels (DA, 2.2 ± 0.2 , $n = 10$, vs DA+Rp-cAMPS, 1.3 ± 0.1 , $n = 9$, $p = 0.029$; p values reported from post-hoc Tukey's HSD test after one-way ANOVA; $F(3, 33) = 4.912$, $p = 0.0062$) (Figure 2D and E).

AC subtypes 1 and 8 are additionally activated by Ca^{2+} , which would make them attractive candidates to mediate activity-dependent dopamine signalling. We therefore tested whether DA-LTP requires these adenylate cyclases using an AC1/AC8 double knock-out (DKO) mouse model (Wang et al., 2003). We found that DA-LTP was completely absent in AC1/AC8 DKO mice, leaving synaptic depression instead (AC DKO $74\% \pm 10\%$ vs 100% , $t(6) = 2.6$, $p = 0.04$, $n = 7$), which was significantly different to DA-LTP in WT mice (WT $125\% \pm 16\%$, $n = 6$, vs AC DKO $74\% \pm 10\%$, $n = 7$, $t(11) = 4.2$, $p = 0.0016$) (Figure 2F and H). To test whether PKA signalling is required for DA-LTP, we loaded 1 μ M of the PKA blocker, protein kinase inhibitor-(6-22)-amide (PKI), into the postsynaptic cell through the recording pipette. Dopamine application after the t-LTD priming protocol followed by subthreshold synaptic stimulation failed to convert depression into potentiation in the presence of PKI (DA+PKI $81\% \pm 3\%$ vs 100% , $t(5) = 6.1$, $p = 0.0017$, $n = 6$), which was significantly different to the effect of dopamine application without PKI (DA+PKI $81\% \pm 3\%$ vs DA $125\% \pm 16\%$, $t(10) = 6.2$, $p = 0.0001$) (Figure 2G and H).

Dopamine increases GluA1, which is required for DA-LTP

It has been reported in hippocampal primary neuronal culture that dopaminergic stimulation enhances surface expression of the AMPA receptor subunit GluA1 (Smith et al., 2005). To confirm whether this finding holds in an acute slice preparation, we tested the effect of dopamine on GluA1 levels in the micro-dissected CA1 region. Using western blot analysis, we found that dopamine application induces a significant increase in the GluA1 receptor subunit compared to control conditions (control, 0.43 ± 0.069 a.u., $n = 7$, vs DA, 0.84 ± 0.07 , $n = 7$, $F(1.8, 10.8) = 12.04$, $p = 0.013$) (Figure 3A and B). Importantly, when we blocked protein synthesis with cycloheximide, the GluA1 increase was prevented (DA, 0.84 ± 0.07 , $n = 7$, vs DA+CHX, 0.38 ± 0.05 , $n = 7$, $F(1.8, 10.8) = 12.04$, $p = 0.037$) (Figure 3A and B) showing that the dopamine-induced increase in GluA1 is protein synthesis-dependent. In contrast, when we measured the levels of the GluA2 subunit under the same conditions, we could not detect significant differences between dopamine-treated and control CA1 (control, 0.8 ± 0.2 , $n = 6$, vs DA, 0.7 ± 0.14 , $n = 6$, $F(1.4, 6.88) = 0.86$, $p = 0.46$) (Figure 3C and D), nor in the presence of CHX (DA 0.7 ± 0.14 , $n = 6$, vs DA+CHX, 0.76 ± 0.19 , $n = 6$, $F(1.4, 6.88) = 0.86$, $p = 0.87$) (Figure 3C and D). Whole western blot images are shown in Figure 3—figure supplement S3.

We next investigated the functional implications of GluA1 for DA-LTP using a GluA1 knock-out (KO) mouse model. GluA1 plays an important role in synaptic plasticity (Zamanillo et al., 1999; Granger et al., 2013; Park et al., 2019; Purkey et al., 2020), but is not required for all forms of LTP (Hoffman et al., 2002; Romberg et al., 2009; Frey et al., 2009). Thus, we first tested whether we could induce conventional t-LTP and t-LTD in hippocampal slices from GluA1 KO mice. We found that a t-LTP protocol ($\Delta t = +10$ ms) led to synaptic potentiation, albeit of somewhat

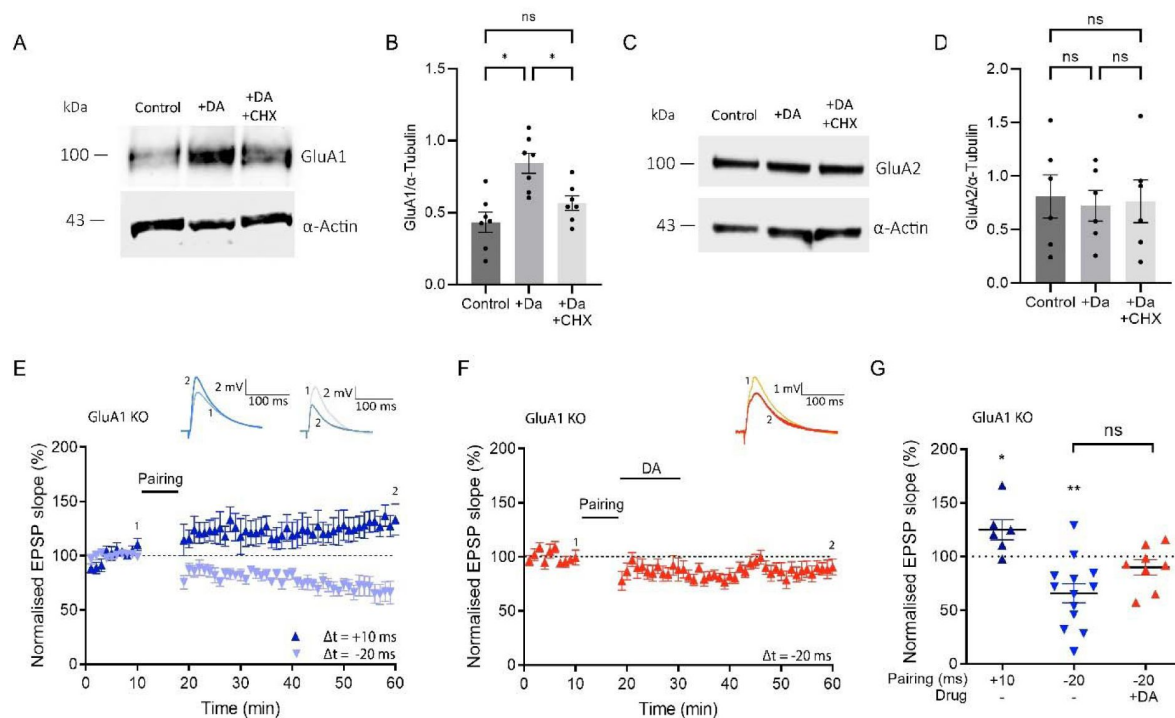


Figure 3.

Dopamine increases GluA1 in a protein synthesis-dependent manner, which is required for DA-LTP. (A), Western blot images from tissue homogenates of the hippocampal CA1 region shows increase of GluA1 upon dopamine application (+DA), which is abolished in the presence of cycloheximide (+DA +CHX). α -Actin was used as loading control. (B) Summary of results. One-way ANOVA followed by Tukey's HSD test, *, $p < 0.05$; ns, not significant. (C) Western blot shows unchanged GluA2 following dopamine application (DA) and no change with cycloheximide (+DA +CHX). (D), Summary of results. One-way ANOVA followed by Tukey's HSD test, ns, not significant. (E), A t-LTP pairing protocol ($\Delta t = +10$ ms) induces potentiation (dark blue trace), and a t-LTD protocol ($\Delta t = -20$ ms) induces depression (light blue trace) in GluA1 KO mice, (F) No DA-LTP in GluA1 KO mice. (G), Summary of results. All traces show an EPSP before (1) and 40 minutes after (2) pairing. Plots show averages of normalised EPSP slopes $\pm$ SEM. t-test, *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

reduced magnitude (t-LTP GluA1 KO, $125\% \pm 9\%$ vs 100% , $t(5) = 2.6$, $p = 0.046$, $n = 6$) (**Figure 3E and G**), while a t-LTD protocol ($\Delta t = -20$ ms) induced synaptic depression (t-LTD GluA1 KO, $66\% \pm 9\%$ vs 100% , $t(12) = 3.9$, $p = 0.002$, $n = 13$) (**Figure 3E and G**). Strikingly, however, in the GluA1 KO mice, application of dopamine failed to convert t-LTD into LTP (DA-LTP GluA1 KO, $90\% \pm 7\%$, $n = 8$; **Figure 3F**) and the resulting depression was not significantly different from t-LTD without dopamine (t-LTD vs DA-LTP, $t(19) = 1.905$, $p = 0.07$) (**Figure 3G**). Taken together, these results suggest that newly synthesized GluA1 subunits are required for expression of DA-LTP.

DA-LTP requires CP-AMPA

GluA1 homomers may form CP-AMPA in the hippocampus (Wenthold et al., 1996). A transient increase in CP-AMPA has been reported in some forms of LTP (Plant et al., 2006; Guire et al., 2008; Park et al., 2019; Purkey et al., 2020), including a PKA-dependent form of plasticity (Park et al., 2021). Since we observed a protein synthesis-dependent increase in the GluA1, but not GluA2, AMPA receptor subunit after dopamine application, we hypothesised that CP-AMPA receptors might be involved in DA-LTP.

We compared the effect of NASPM ($100 \mu\text{M}$), a selective CP-AMPA antagonist, on conventional t-LTP ($\Delta t = +10$ ms) to the effect of NASPM on DA-LTP. We found that robust t-LTP was elicited in the presence of extracellular NASPM ($139\% \pm 11\%$ vs 100% , $t(5) = 3.6$, $p = 0.015$, $n = 6$) (**Figure 4A and C**). In contrast, when applying NASPM during a DA-LTP protocol, potentiation was completely prevented ($75\% \pm 15\%$ vs 100% , $t(5) = 1.66$, $p = 0.16$, $n = 6$) (**Figure 4B and C**). This shows that CP-AMPA receptors are indeed required for DA-LTP. The selective increase of GluA1 receptor subunit levels seen in response to dopamine (**Figure 3A-D**) suggests that GluA1-containing CP-AMPA receptors are required for expression of DA-LTP.

We have recently reported that, in addition to synaptic activation, postsynaptic burst stimulation can also induce DA-LTP, which is mediated via the same AC-PKA signalling pathway and also requires protein synthesis (Fuchsberger et al., 2022). Synaptically induced DA-LTP develops gradually, while burst-induced DA-LTP shows rapid potentiation. We therefore used burst-induced DA-LTP to test whether a transient increase in CP-AMPA receptors may be required for potentiation. We applied NASPM during or shortly after the plasticity protocol to test whether this affects the expression of DA-LTP. We found that application of NASPM during burst stimulation completely prevented synaptic potentiation ($78\% \pm 11\%$ vs 100% , $t(6) = 2.01$, $p = 0.091$, $n = 7$) (**Figure 4D and F**). When applying NASPM 7 minutes after burst stimulation, we observed an initial potentiation ($118\% \pm 4\%$ vs 100% , $t(6) = 4.52$, $p = 0.0063$, $n = 6$), which gradually returned to baseline in the presence of NASPM ($91\% \pm 11\%$ vs 100% , $t(5) = 0.82$, $p = 0.45$, $n = 6$) (**Figure 4E and F**), suggesting that expression of DA-LTP is mediated by CP-AMPA receptors.

To confirm these results with an alternative CP-AMPA blocker we applied $10 \mu\text{M}$ IEM1460 extracellularly. Conventional t-LTP ($\Delta t = +10$ ms) was compared to DA-LTP in the presence of IEM1460 throughout the recording, which were significantly different (t-LTP+IEM1460, $142\% \pm 21\%$, $n = 7$, vs DA-LTP+IEM1460, $80\% \pm 8\%$, $n = 7$, $t(12) = 2.75$, $p = 0.02$) (**Figure 4—figure supplement S4A, B and D**). When applied immediately after the pairing protocol, IEM1460 still prevented DA-LTP and left a synaptic depression instead (DA-LTP+IEM after, $68\% \pm 12\%$ vs 100% , $t(8) = 2.68$, $p = 0.028$, $n = 9$) (**Figure 4—figure supplement S4C and D**). Taken together, these results suggest that DA-LTP, but not conventional t-LTP, requires CP-AMPA receptors for expression of synaptic potentiation.

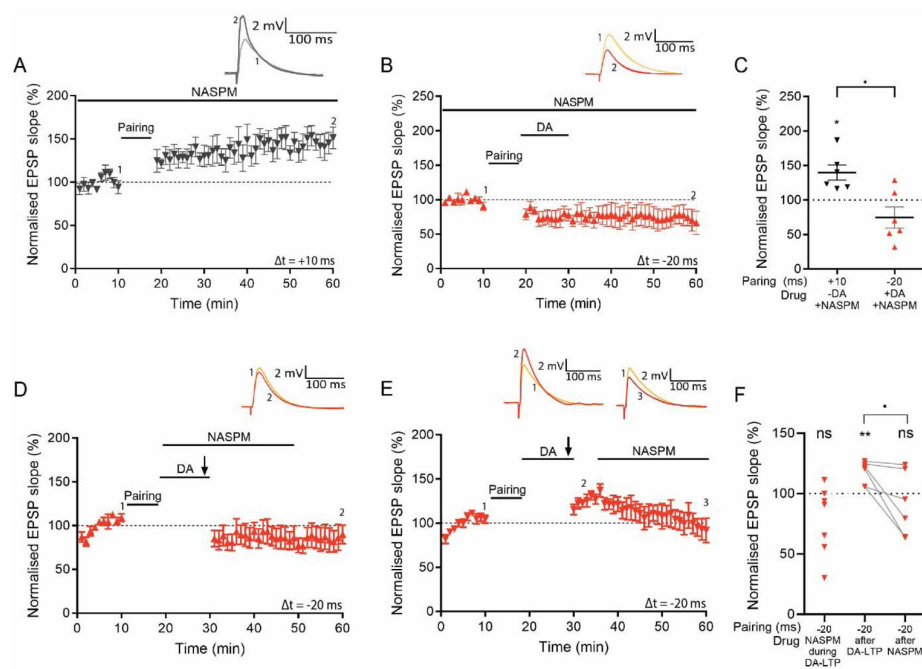


Figure 4.

CP-AMPA receptors are required for DA-LTP but not for conventional t-LTP. **(A)**, A t-LTP pairing protocol ($\Delta t = +10$ ms) induces synaptic potentiation in the presence of extracellularly applied NASPM. **(B)**, NASPM blocks DA-LTP. **(C)**, Summary of results. *t*-test, *, $p < 0.05$. **(D)**, Burst-induced DA-LTP is blocked by NASPM. **(E)** Burst-induced DA-LTP potentiation decreases when NASPM is applied 7 minutes afterwards. **(F)**, Summary of results. *t*-test, *, $p < 0.05$; **, $p < 0.01$; ns, not significant. Traces in **(A, B and D)** show an EPSP before (1) and 40 minutes after pairing (2). Traces in **(E)** show an EPSP before (1), 5 minutes after DA and burst stimulation (2), and 40 minutes after pairing (3). Plots show averages of normalised EPSP slopes $\pm$ SEM.

Discussion

In summary, we investigated the effect of dopamine on protein synthesis in hippocampal neurons and how protein synthesis enables dopamine-dependent long-term potentiation (DA-LTP). We report four main findings: (1) Dopamine increases protein synthesis in an activity-dependent manner through the activation of PKA. (2) Dopamine enables a rapid onset protein synthesis-dependent form of synaptic potentiation (DA-LTP). (3) Dopamine increases the level of GluA1 but not GluA2 subunit of AMPA receptors. (4) Expression of DA-LTP requires GluA1 AMPA receptor subunit and Ca^{2+} -permeable (CP)-AMPA receptors, whereas t-LTP does not, suggesting the existence of two distinct forms of LTP.

Recent developments of protein synthesis labelling techniques enabled us to directly monitor protein synthesis in neurons in response to dopamine. We validated our puromycin-based approach for labelling protein synthesis using protein synthesis inhibitors anisomycin (AM) and cycloheximide (CHX) in acute hippocampal slices. We report that dopamine increases protein synthesis in pyramidal neurons in CA1 of acute hippocampal slices within minutes. This is consistent with previous reports from hippocampal and cortical neuronal cell culture systems, which showed that dopamine receptor agonist SKF-38393 enhances protein synthesis (Smith et al., 2005 [↗](#); David et al., 2020 [↗](#)). We found that the dopamine-induced increase in protein synthesis is mediated by dopamine receptors via the AC-cAMP-PKA pathway (Mayr et al., 2001; Sassone-Corsi, 1995 [↗](#)), which is also consistent with previously reported hippocampal cell culture results which showed that application of Sp-cAMPS, an activator of PKA, was sufficient to induce an increase in protein synthesis (Smith et al., 2005 [↗](#)). In addition, the AC-cAMP-PKA pathway induced by dopamine may affect several downstream targets and interact with other signalling pathways that could enhance protein synthesis. It has been reported in cortical primary neuronal cultures that D1 receptors, but not D2 receptors, increase protein synthesis via the mTOR-ERK pathway resulting in dephosphorylation of eEF2 (David et al., 2020 [↗](#)).

Consistent with our previous results that burst-induced potentiation in the presence of dopamine requires postsynaptic PKA and protein synthesis (Fuchsberger et al., 2022 [↗](#)), we found here that synaptic potentiation induced by low frequency synaptic stimulation in the presence of dopamine following a priming protocol, which would otherwise induce synaptic depression (Brzosko et al., 2015 [↗](#)), also requires postsynaptic PKA and new protein synthesis. While conventional t-LTP remained intact, blocking protein synthesis in the postsynaptic neurons completely prevented DA-LTP, suggesting that dopamine induces the synthesis of plasticity-related proteins required for converting synaptic depression into potentiation.

DA-LTP shares properties with 'late-phase' LTP (L-LTP), which also requires dopamine signalling (Frey et al., 1990 [↗](#)), PKA (Frey et al., 1993 [↗](#)) and protein synthesis (Frey et al., 1988 [↗](#)), and it was reported that protein synthesis was required hours after LTP induction for the maintenance of synaptic strength (Frey et al., 1988 [↗](#)). In contrast, for the form of dopamine-dependent LTP studied here, protein synthesis was required ahead of or within the first few minutes of the induction protocol. Moreover, while previous studies have used extracellular application of protein synthesis inhibitors, here we loaded the protein synthesis inhibitors into the postsynaptic neuron via the patch pipette, suggesting it is specifically postsynaptic protein synthesis that is required for DA-LTP.

We found that neuronal activity was also required for dopamine to increase protein synthesis. Moreover, we identified the Ca^{2+} -dependent AC subtypes AC1/AC8 to be involved in the induction of DA-LTP. This coincidence detector is stimulated by Gs-coupled dopamine D1/D5 receptor activation together with Ca^{2+} influx (Wayman et al., 1994 [↗](#); Ferguson and Storm, 2004 [↗](#)). Our

results show that the AC1/AC8 subtypes and PKA are required for DA-LTP. These results suggest that dopamine application during neuronal activity induces the synthesis of plasticity-related proteins that enable synaptic potentiation.

Western blots showed that dopamine increased the levels of the GluA1 AMPA receptor subunit in a protein synthesis-dependent manner, but that the GluA2 subunit remained unchanged. This is consistent with a previous study in hippocampal primary cultured neurons which reported that dopaminergic signalling increases the surface expression of AMPA receptor subunit GluA1 (Smith et al., 2005 [DOI](#)). The selective increase of the GluA1 over GluA2 subunit is interesting because of the important role ascribed to the GluA1 subunit in LTP (Huganir and Nicoll, 2013 [DOI](#); Diering and Huganir, 2018 [DOI](#)).

We report here that although conventional t-LTP could still be induced in a GluA1 KO mouse model, GluA1 was required for DA-LTP. These observations revealed a possible mechanism through which dopamine can modulate synaptic strength. Furthermore, the findings indicated another intriguing possibility, namely that GluA1 homomeric AMPA receptors, which are calcium-permeable, mediate the expression of DA-LTP. CP-AMPA receptors appear to play a role in some forms of LTP (Plant et al., 2006 [DOI](#); Guire et al., 2008 [DOI](#); Frey et al., 2009 [DOI](#); Wakazono et al., 2024 [DOI](#)), while other studies found no involvement (Adesnik and Nicoll, 2007 [DOI](#); Gray et al., 2007 [DOI](#)).

The role of CP-AMPA receptors in synaptic plasticity remains controversial. We report here that they are required for DA-LTP, but not for conventional t-LTP. Importantly, even blocking CP-AMPA receptors after the pairing paradigm reversed DA-LTP, which supports the possibility that they are required for expression of DA-LTP. Whether they also contribute to the induction of the dopamine-dependent potentiation induced by low-frequency afferent stimulation following a t-LTD priming protocol (Brzosko et al., 2015 [DOI](#)) remains unresolved. Furthermore, it should be noted that using western blotting we observed an increase in total GluA1 levels, but we cannot conclude whether DA increases surface GluA1 or CP-AMPA receptors from our experiments. While we show a requirement for CP-AMPA receptors for DA-LTP, it remains to be confirmed whether the pairing protocol together with dopamine indeed induces an increase in CP-AMPA receptors at the synapse. There are currently no suitable imaging techniques available to unambiguously quantify specific AMPA receptor subunit compositions at the synapse in acute slices.

Interestingly, previous studies have reported two forms of NMDA receptor-dependent LTP, induced by spaced and compressed theta burst stimuli (TBS), that can be distinguished at hippocampal CA1 synapses based on their dependence on PKA (Park et al., 2016 [DOI](#)). Additionally, it was shown that the PKA-dependent form of LTP triggered by spaced stimulation leads to a transient increase in single-channel conductance, probably mediated by the insertion of calcium-permeable (CP)-AMPA receptors (Park et al., 2021 [DOI](#)). The signaling pathway identified in our study overlaps with this pathway and, together, these findings suggest that two mechanistically distinct forms of LTP coexist at these synapses. It is possible that plasticity protocols that use extracellular electrodes also trigger release of dopamine, and it would be of interest to investigate whether LTP induced by spaced TBS requires dopamine receptor activation and protein synthesis. In summary, our findings suggest a possible mechanism for how the reward-related neuromodulator dopamine may contribute to protein synthesis-dependent synaptic plasticity facilitating hippocampal long-term memory. Signalling mechanisms underlying dopaminergic control of protein synthesis and synaptic weights may also be important for pathophysiological processes. Dysregulation in dopamine systems has long been implicated in drug addiction (for review see Dalley and Everitt, 2009 [DOI](#)), schizophrenia (for review see Kahn et al., 2015 [DOI](#)) and neurodegenerative diseases such as Parkinson's disease (for review see Poewe et al., 2017 [DOI](#)), and, more recently, also Alzheimer's disease (Nobili et al., 2017 [DOI](#)). Understanding signalling pathways underlying dopamine-dependent synaptic plasticity may help guide future research into additional treatment options.

Methods

Animals

Mice used for this study were housed at the Combined Animal Facility, Cambridge University. They were held on a 12-hr light/dark cycle at 19–23 °C and were provided with water and food *ad libitum*. Experiments were carried out using wildtype C57BL/6 J mice (Charles River Laboratories, UK), and the transgenic mouse lines AC1 cyclase (AC) subtypes 1 and 8 double knockout (AC1/AC8 DKO) mice and GluA1 knockout mice (GluA1 KO). The AC1/AC8 DKO mice have the genes for both AC1 and AC8 deleted globally. The mouse line was generated as described previously (Wang, et al., 2003 [DOI](#)) and was imported from Michigan State University, MI, US. The GluA1 KO mouse line was generated as described previously (Zamanillo et al., 1999 [DOI](#)) and was imported from the MRC Laboratory of Molecular Biology, Cambridge, UK.

All procedures were performed in accordance with the animal care guidelines of the UK Home Office regulations of the UK Animals (Scientific Procedures) Act 1986, and Amendment Regulations 2012, following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). Animal procedures were authorised under Personal and Project licences held by the authors.

Preparation of acute hippocampal slices

Male and female mice at postnatal days 12–19 were briefly anaesthetised with isoflurane (4% isoflurane in oxygen) and decapitated. The brain was rapidly removed into ice-cold artificial cerebrospinal fluid (aCSF) (10 mM glucose, 26.4 mM NaH₂CO₃, 126 mM NaCl, 1.25 mM NaH₂PO₄, 3 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂) bubbled with carbogen gas (95% O₂/5% CO₂) (pH 7.2, 270–290 mOsm/L) and glued to a vibrating microtome stage. Horizontal slices (350 µm) were sectioned with a vibratome (Leica VT 1200S, Leica Biosystems, Wetzlar, Germany) and were subsequently submerged in aCSF for at least 1 hr at room temperature (RT) in a storage chamber. Slices were used 1–6 hr after preparation.

Electrophysiology

Whole-cell recordings and synaptic plasticity

Individual slices were transferred to a submersion-type chamber for recordings, and superfused with aCSF (2 ml/min) at RT (24–26 °C). Neurons were visualised with an infrared differential interference contrast (DIC) microscope using a 40x water-immersion objective. Hippocampal subfields were identified and whole-cell patch-clamp recordings were performed on CA1 pyramidal neurons. Monopolar stimulation electrodes were placed in stratum radiatum for stimulation of Schaffer collaterals. Electrodes for test and control pathways were placed at the same distance (>100 µm) from either side of the recorded neuron. Patch pipettes (pipette resistance 4–7 MΩ) were pulled from borosilicate glass capillaries (0.68 mm inner diameter, 1.2 mm outer diameter) using a P-97 Flaming/Brown micropipette puller (Sutter Instruments Co., Novato, California, USA). Pipettes were filled with intracellular solution containing: 110 mM potassium gluconate, 4 mM NaCl, 40 mM HEPES, 2 mM ATP-Mg, 0.3 mM GTP (pH 7.2–7.3, 270–285 mOsm/L). The liquid junction potential was not corrected.

All experiments were performed in current-clamp mode. Cells were accepted for the experiment if their resting membrane potential was between –55 and –70 mV. Throughout the recording the membrane potential was held at –70 mV by direct current application via the recording electrode.

All cells were tested for regular spiking responses to positive current steps (20 pA, 800 ms) characteristic of pyramidal neurons before the start of each recording.

TTX experiments were performed measuring spontaneous EPSPs for 5 minutes before and after adding 1 μ M TTX to superfusing aCSF for 15 minutes.

Plasticity recordings were carried out as described previously (Brzosko et al., 2015 [↗](#)). Briefly, excitatory postsynaptic potentials (EPSPs) were evoked alternately in two input pathways (test and control) by direct current pulses at 0.2 Hz (stimulus duration 50 μ s) through metal stimulation electrodes. The stimulation intensity was adjusted (100 μ A – 500 μ A) to evoke an EPSP with peak amplitude between 3 and 8 mV. After a stable EPSP baseline period of at least 10 minutes, spike-timing-dependent plasticity (STDP) was induced in the test pathway by repeated pairings (100 times at 0.2 Hz) of single evoked EPSPs and single postsynaptic action potentials elicited with the minimum somatic current pulse (1 – 1.8 nA, 3 ms) via the recording electrode. Spike-timing intervals (Δt in ms) were measured between the onset of the EPSP and the onset of the action potential. Alternate stimulation of test and control EPSPs was resumed immediately after the pairing protocol and monitored for at least 40 minutes, except when the burst stimulation protocol was used for plasticity induction. In that case, stimulation of EPSPs was not resumed for an additional 10 minutes in the test pathway, and at the end of that period, five bursts, each of five action potentials at 50 Hz, were elicited with an inter-burst interval of 0.1 Hz by somatic current pulses (1.8 nA, 10 ms) via the recording electrode. Immediately after the bursts, stimulation of EPSPs was resumed and monitored for at least 30 minutes. Presynaptic stimulation frequency to evoke EPSPs remained constant throughout the experiment. The unpaired control pathway served as a stability control.

Drug application

Drugs were bath-applied to the whole slice through the perfusion system by dilution of concentrated stock solutions in aCSF, or by adding the drugs to the patch pipette solution when it was applied intracellularly to the postsynaptic cell only. For each set of recordings, interleaved control and drug conditions were carried out and were pseudo-randomly chosen. The following drugs were used in this study: 100 μ M dopamine hydrochloride (Sigma–Aldrich, Dorset, United Kingdom), 10 μ M cycloheximide (Tocris Bioscience), 0.5 mM anisomycin (stock solution in EtOH; Tocris Bioscience), 1 μ M PKA inhibitor fragment (6-22) amide (Tocris Bioscience), 100 μ M 1-naphthyl acetyl spermine trihydrochloride (NASPM trihydrochloride; Tocris Bioscience), 10 μ M N,N,H-trimethyl-5-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylmethyl)amino]- 1-pentanaminiumbromide hydrobromide (IEM1460; Tocris Bioscience).

Data acquisition and data analysis of slice recordings

Data were collected using an Axon Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California, USA). Data were filtered at 2 kHz and were acquired and digitised at 5 kHz using an Instrutech ITC-18 A/D interface board (Instrutech, Port Washington, New York, USA) and custom-made acquisition procedures in Igor Pro (WaveMetrics, Lake Oswego, Oregon, USA).

All experiments were carried out in current clamp mode, and only cells with an initial series resistance from 9 – 16 M Ω were included. Series resistance was compensated for by adjusting the bridge balance, and recordings were discarded if series resistance changed by more than 30%. Offline analyses of plasticity recordings were done using custom-made procedures in Igor Pro. EPSP slopes were measured on the rising phase of the EPSP as a linear fit between the time points corresponding to 25–30% and 70–75% of the peak amplitude.

For quantifications, the mean EPSP slope per minute of the recording was calculated from 12 consecutive sweeps and normalised to the baseline (each data point in source data files is the mean of 12 averaged EPSPs). Normalised EPSP slopes from the last 5 minutes of the baseline

(immediately before pairing) and from the last 5 minutes of the recording were averaged. The magnitude of plasticity, as an indicator of change in synaptic weights, was defined as the average EPSP slope 40 minutes after the plasticity protocol expressed as a percentage of the average EPSP slope during baseline. For the burst-induction protocol, the change in synaptic weights was defined as the average EPSP slope 30 minutes after the plasticity protocol expressed as a percentage of the average EPSP slope during baseline.

Statistical analysis of slice recordings

All data are presented as mean $\pm$ S.E.M. Statistical comparisons were performed using one-sample two-tailed, paired two-tailed, or unpaired two-tailed Student's *t*-test, with a significance level of $\alpha = 0.05$. Significance level used was $\alpha = 0.05$ and *p*-values are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Protein synthesis labelling in acute hippocampal slices

Incubation chambers were set up to submerge slices in oxygenated (95% O₂, 5% CO₂) aCSF containing a selection from the following drugs: 3 μ M puromycin dihydrochloride (Sigma P8833), 100 μ M dopamine hydrochloride (Sigma H8502), 10 μ M SKF38393 (Sigma D047), 10 μ M SCH23390 hydrochloride (Sigma D054), 50 μ M sulpiride (Sigma S8010), 1 μ M TTX (Tocris Bioscience), 10 μ M cycloheximide (Tocris Bioscience), 0.5 mM anisomycin (stock solution in EtOH; Tocris Bioscience), 30 μ M Rp-cAMPS (Tocris Bioscience).

Protein synthesis was measured using a puromycin-based labelling assay adapted to acute hippocampal slices, similar to that described previously (Schmidt et al., 2009). Briefly, 3 μ M puromycin was used to incorporate into proteins during the elongation phase of synthesis (Figure 1A). After 30 minutes of incubation with puromycin, samples were washed in phosphate-buffered saline (PBS), and further processed for immunohistochemistry or western blotting as described below. The specificity of the conjugated puromycin monoclonal antibody was confirmed with incubations of no-puromycin controls and protein synthesis inhibitors.

Immunohistochemistry

Immediately after incubations with puromycin, slices were washed in PBS and fixed in 4% paraformaldehyde (PFA) in 1x PBS for 24 hr at 4 °C. Slices were subsequently washed 3 x 5 minutes in PBST 0.01% (DPBS ThermoFisher, Tween P1379 Sigma), permeabilised for 15 minutes in PBST 0.5% and washed in PBST 0.01% a further three times. Slices were incubated for 2 hr at room temperature in a blocking solution of 5% goat serum in PBST 0.01% (SigmaAldrich G9023) followed by an incubation overnight at 4 °C in blocking solution with anti-puromycin antibody (clone 12D10, MABE343; 1:500) AlexaFluor 488 or AlexaFluor 647 (MABE343-AF488 or MABE343-AF647; Merck). On the following day, slices were left on a shaker for 2 hr at RT, followed by 3 x 5 minutes washes in PBST 0.01%. Finally, slices were mounted onto microscope slides (ThermoScientific J1810AMNZ), allowed to dry for at least 2 hr at RT, then covered with Fluoroshield with Dapi mounting medium for nuclear staining (Sigma F6057) and sealed with coverslip sealant (Biotium 23005).

Imaging and image analysis

Immunohistochemical preparations were visualised using a confocal laser-scanning microscope (Leica TCS SP8). Z-stacks of hippocampal CA1 region were taken using an HC PL APO 20x/0.75 CS2 objective using the same exposure settings for all conditions that were compared to each other. AlexaFluor 647 was excited at 638 nm and emission detected at 671 nm; AlexaFluor488 was excited at 495 nm and emission detected at 519 nm, Dapi was excited at 359 nm, emission detected at 461 nm. Images were analysed using ImageJ. To obtain normalised integrated density, the maximum intensity Z-projection of slices were extracted, from which the integrated density was

measured and normalised to the corresponding nuclear staining (Dapi) Z-projection measurement. Normalised integrated density was plotted and all data are presented as mean $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA and post-hoc Tukey's HSD test, following adherence to tests for normality and equality of variance. Significance level used was $\alpha = 0.05$ and p-values indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Western blot analysis

Acute hippocampal slices were prepared and incubated with various compounds as described above. After 30 minutes incubation, slices were rapidly dissected in aCSF to obtain the CA1 region, which were pooled for slices from each condition and flash frozen in liquid N₂. Protein extraction buffer (150 mM NaCl, 1% Triton x-100, 50 mM TrisHCl pH 7.4 with protease inhibitor (11836170001 Roche)), at a volume adjusted to the tissue weight for each sample, was added to lyse the tissue for 1 hr at 4 °C with frequent vortexing. Samples were centrifuged at 16,000 x g for 30 minutes at 4 °C and the supernatant was retained. Lysate was mixed with LDS sample buffer (Invitrogen NP0007) and boiled at 95 °C for 5 min, after which it was loaded onto a polyacrylamide gel (Bolt 4-12% Bis-Tris NW04127BOX). Loading was adjusted to achieve comparable actin signal. Proteins were transferred onto a nitrocellulose membrane using Trans-Blot Turbo Transfer (BioRad 1704158) and membranes were then blocked for 30 minutes at room temperature with 5% milk in TBST. Membranes were incubated with primary antibodies (anti-actin MAB1501R, anti-puromycin MABE343, anti-GluA1 AB1504, anti-GluA2 AF1050), washed 3 times in TBST, incubated with horseradish peroxidase (HRP)-coupled secondary antibody (Sigma) and washed a further 3 times. Membranes were developed using enhanced chemiluminescence and imaged with a ChemiDoc MP Imaging System (Biorad). Quantification of antibody staining was made using ImageJ. Integrated density of GluA1/2 bands was normalised to integrated density of actin in the corresponding lane. All data are presented as mean $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA and post-hoc Tukey's HSD test, following adherence to tests for normality and equality of variance. Significance level used was $\alpha = 0.05$ and p-values indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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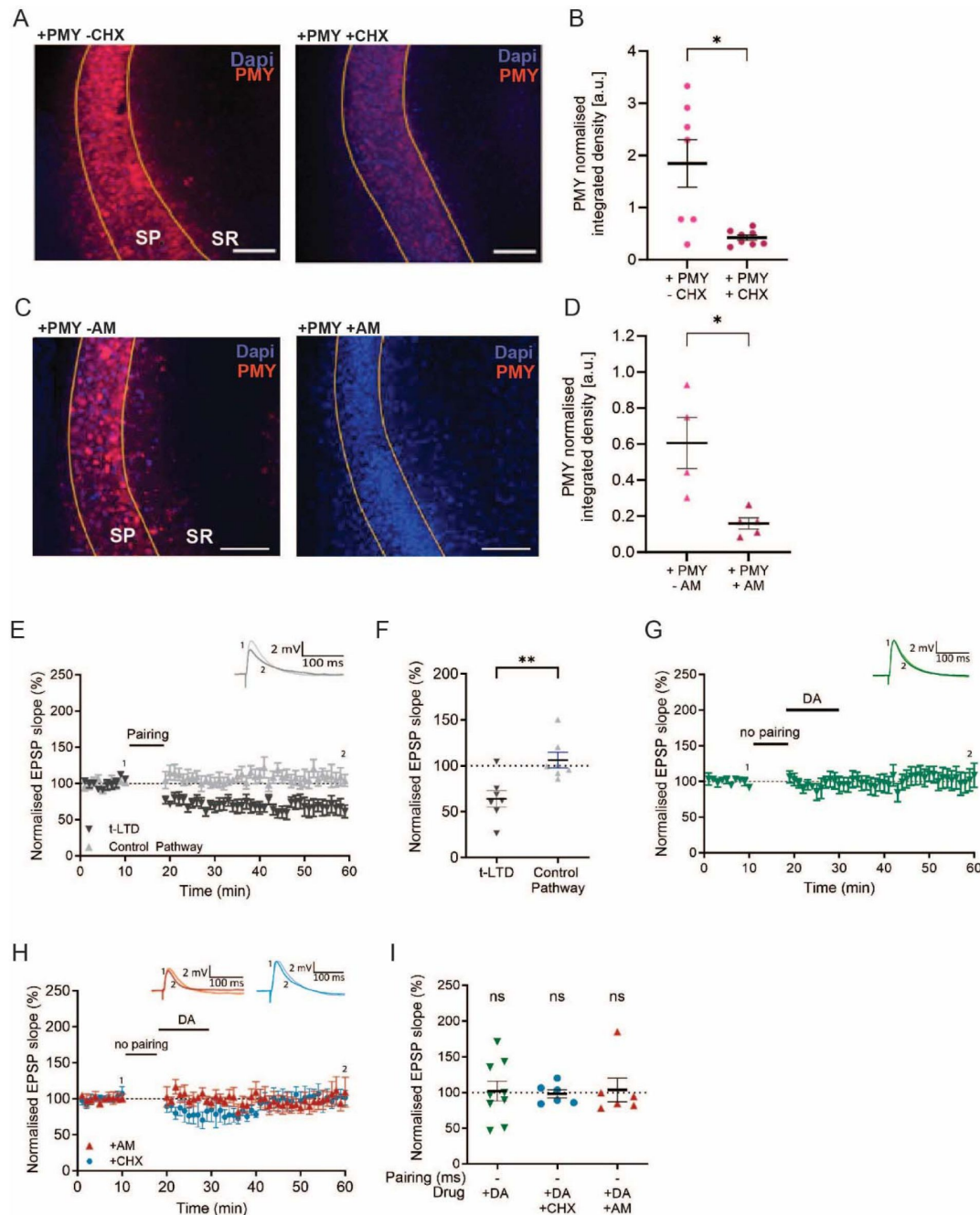


Figure S1.

Puromycin assay is specific to protein synthesis and protein synthesis inhibitors do not affect baseline synaptic transmission within 60 minutes of recording. (**A**, **C**), Representative images of the CA1 region of the hippocampus (SP: stratum pyramidale, SR: stratum radiatum) of puromycin-labelled proteins (PMY, red) and Dapi (blue). Puromycin incorporation is significantly reduced in the presence of the protein synthesis inhibitors (**A**) cycloheximide (CHX) or (**C**) anisomycin (AM). Scale bar, 80 μ m (**B**, **D**), Summary of results. t-test, *, $p < 0.05$ (**E**), Post-before pre-pairing protocol leads to input-specific synaptic depression. Pairing protocol ($\Delta t = -20$ ms) induces t-LTD (black trace) and does not affect synaptic weights in control pathway (gray trace). (**F**), Summary of results. (**G**), Dopamine application alone, without prior pairing, does not affect synaptic weights in control pathway (green trace). (**H**), Postsynaptically applied anisomycin (AM) (red trace) or cycloheximide (CHX) (blue trace) does not affect synaptic weights throughout recording. Traces show an EPSP before (1) and 40 minutes after (2) pairing. Plots show averages of normalised EPSP slopes $\pm$ SEM. (**I**), Summary of results. t-test, ns, not significant.

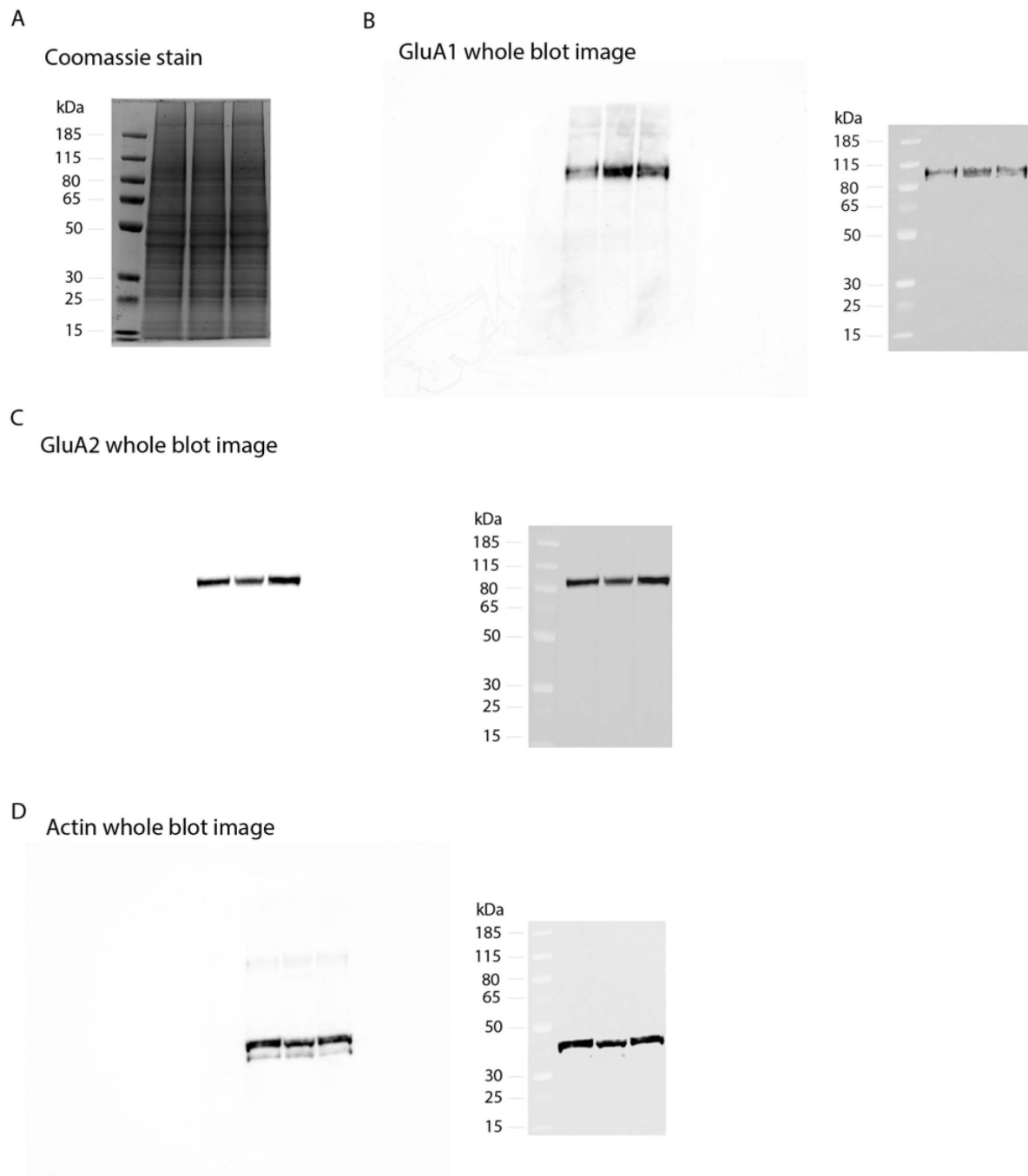


Figure S3.

(A) Total protein stain of whole polyacrylamide gel stained with coomassie blue as loading control. Western blot of this gel was probed for (B) GluA1, (C) GluA2 and (D) actin.

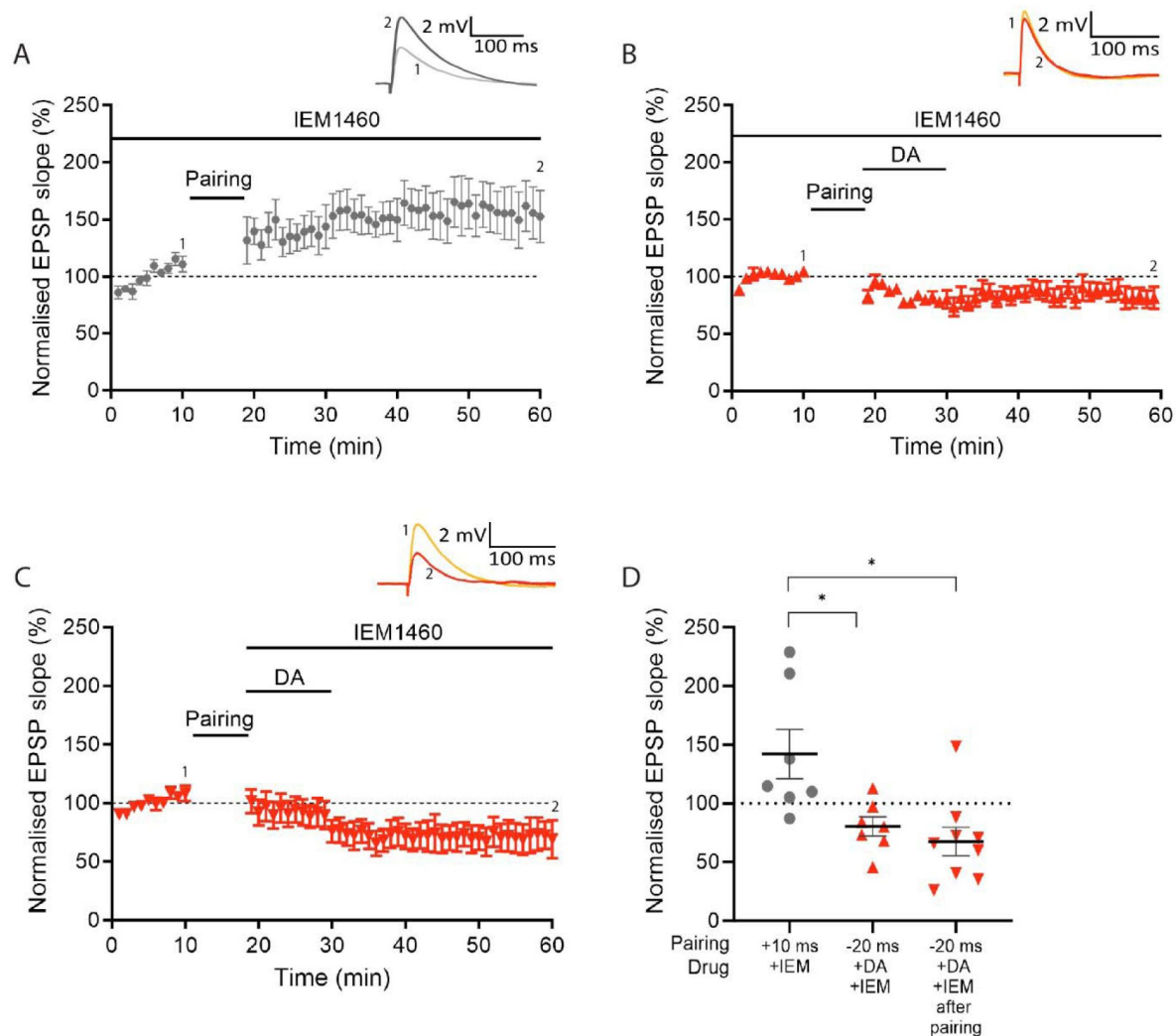


Figure S4.

CP-AMPA receptor blocker IEM-1460 confirms that CP-AMPA receptors are required for DA-LTP but not for conventional t-LTP. **(A)**, A t-LTP pairing protocol ($\Delta t = +10$ ms) induces synaptic potentiation in the presence of extracellularly applied IEM-1460. **(B, C)**, IEM-1460 blocks DA-LTP, whether applied throughout **(B)** or after the pairing protocol **(C)**. **(D)**, Summary of results. t -test, *, $p < 0.05$. Traces show an EPSP before (1) and 40 minutes after pairing (2). Plots show averages of normalized EPSP slopes $\pm$ SEM.

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Reviewer #1 (Public review):

Summary:

In this manuscript, Fuchsberger et al. demonstrate a set of experiments which ultimately identifies the de novo synthesis of GluA1-, but not GluA2-containing Ca²⁺ permeable AMPA receptors as a key driver of dopamine-dependent LTP (DA-LTP) during conventional post-before-pre spike-timing dependent (t-LTD) induction. The authors further identify adenylate cyclase 1/8, cAMP, and PKA as the crucial mitigators of these actions. While some comments have been identified below, the experiments presented are thorough and address the aims of the manuscript, figures are presented clearly (with minor comments), and experimental samples sizes and statistical analyses are suitable. Suitable controls have been utilized to confirm the role of Ca²⁺ permeable AMPAR. This work provides a valuable step forward built on convincing data towards understanding the underlying mechanisms of spike-timing dependent plasticity and dopamine.

Strengths:

Appropriate controls were used.

The flow of data presented is logical and easy to follow.

The quality of the data is solid.

Weaknesses:

Our concerns raised within the first round of review have been appropriately addressed by the authors.

<https://doi.org/10.7554/eLife.100822.2.sa3>

Reviewer #2 (Public review):**Summary:**

The aim was to identify the mechanisms that underlie a form of long-term potentiation (LTP) that requires activation of dopamine (DA).

Strengths:

The authors have provided multiple lines of evidence that supports their conclusions; namely that this pathway involves activation of a cAMP / PKA pathway that leads to the insertion of calcium permeable AMPA receptors.

Weaknesses:

Some of the experiments could have been conducted in a more convincing manner.

<https://doi.org/10.7554/eLife.100822.2.sa2>

Reviewer #3 (Public review):

The manuscript of Fuchsberger et al. investigates the cellular mechanisms underlying dopamine-dependent long-term potentiation (DA-LTP) in mouse hippocampal CA1 neurons. The authors conducted a series of experiments to measure the effect of dopamine on the protein synthesis rate in hippocampal neurons and its role in enabling DA-LTP. The key results indicate that protein synthesis is increased in response to dopamine and neuronal activity in the pyramidal neurons of the CA1 hippocampal area, mediated via the activation of adenylate cyclases subtypes 1 and 8 (AC1/8) and the cAMP-dependent protein kinase (PKA) pathway. Additionally, the authors show that postsynaptic DA-induced increases in protein synthesis are required to express DA-LTP, while not required for conventional t-LTP.

The increased expression of the newly synthesized GluA1 receptor subunit in response to DA supports the formation of homomeric calcium-permeable AMPA receptors (CP-AMPA). This evidence aligns well with data showing that DA-LTP expression requires the GluA1 AMPA subunit and CP-AMPA, as DA-LTP is absent in the hippocampus of a GluA1 genetic knock-out mouse model.

Comments on revisions:

The authors addressed adequately all my comments.

<https://doi.org/10.7554/eLife.100822.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:**Reviewer #1 (Public Review):****Summary:**

In this manuscript, Fuchsberger et al. demonstrate a set of experiments that ultimately identifies the de novo synthesis of GluA1-, but not GluA2-containing Ca²⁺ permeable AMPA receptors as a key driver of dopamine-dependent LTP (DA-LTP) during conventional

post-before-pre spike-timing dependent (t-LTD) induction. The authors further identify adenylate cyclase 1/8, cAMP, and PKA as the crucial mitigators of these actions. While some comments have been identified below, the experiments presented are thorough and address the aims of the manuscript, figures are presented clearly (with minor comments), and experimental sample sizes and statistical analyses are suitable. Suitable controls have been utilized to confirm the role of Ca²⁺ permeable AMPAR. This work provides a valuable step forward built on convincing data toward understanding the underlying mechanisms of spike-timing-dependent plasticity and dopamine.

Strengths:

Appropriate controls were used.

The flow of data presented is logical and easy to follow.

The quality of the data, except for a few minor issues, is solid.

Weaknesses:

The drug treatment duration of anisomycin is longer than the standard 30-45 minute duration (as is the 500uM vs 40uM concentration) typically used in the field. Given the toxicity of these kinds of drugs long term it's unclear why the authors used such a long and intense drug treatment.

In an initial set of control experiments (Figure S 1C-D) we wanted to ensure that protein synthesis was definitely blocked and therefore used a relatively high concentration of anisomycin and a relatively long pre-incubation period. We agree with the Reviewer that we cannot exclude the possibility that this treatment could compromise cell health in addition to the protein synthesis block. Therefore, we carried out an additional experiment with an alternative protein synthesis inhibitor cycloheximide at a lower standard concentration (10 μ M) which confirmed a significant reduction in the puromycin signal (Figure S 1A-B). Together these results support the conclusion that puromycin signal is specific to protein synthesis in our labelling assay.

Furthermore, in the electrophysiology experiments, we used 500 μ M anisomycin in the patch pipette solution. Under these conditions, we recorded a stable EPSP baseline for 60 minutes, indicating that the treatment did not cause toxic effects to the cell (Figure S1F). This high concentration would ensure an effective block of local translation at dendritic sites. Nevertheless, we also carried out this experiment with cycloheximide at a lower standard concentration (10 μ M) and observed a similar result with both protein synthesis inhibitors (Figure 1F).

With some of the normalizations (such as those in S1) there are dramatic differences in the baseline "untreated" puromycin intensities - raising some questions about the overall health of slices used in the experiments.

We agree with the Reviewer that there is a large variability in the normalised puromycin signal which might be due to variability in the health of slices. However, we assume that the same variability would be present in the treated slices, which showed, despite the variability, a significant inhibition of protein synthesis. To avoid any bias by excluding slices with low puromycin signal in the control condition, we present the full dataset.

The large set of electrophysiology experiments carried out in our study (all recorded cells were evaluated for healthy resting membrane potential, action potential firing, and synaptic responses) confirmed that, generally, the vast majority of our slices were indeed healthy.

Reviewer #2 (Public Review):*Summary:*

The aim was to identify the mechanisms that underlie a form of long-term potentiation (LTP) that requires the activation of dopamine (DA).

Strengths:

The authors have provided multiple lines of evidence that support their conclusions; namely that this pathway involves the activation of a cAMP / PKA pathway that leads to the insertion of calcium-permeable AMPA receptors.

Weaknesses:

Some of the experiments could have been conducted in a more convincing manner.

We carried out additional control experiments and analyses to address the specific points that were raised.

Reviewer #3 (Public Review):

The manuscript of Fuchsberger et al. investigates the cellular mechanisms underlying dopamine-dependent long-term potentiation (DA-LTP) in mouse hippocampal CA1 neurons. The authors conducted a series of experiments to measure the effect of dopamine on the protein synthesis rate in hippocampal neurons and its role in enabling DA-LTP. The key results indicate that protein synthesis is increased in response to dopamine and neuronal activity in the pyramidal neurons of the CA1 hippocampal area, mediated via the activation of adenylate cyclases subtypes 1 and 8 (AC1/8) and the cAMP-dependent protein kinase (PKA) pathway. Additionally, the authors show that postsynaptic DA-induced increases in protein synthesis are required to express DA-LTP, while not required for conventional t-LTP.

The increased expression of the newly synthesized GluA1 receptor subunit in response to DA supports the formation of homomeric calcium-permeable AMPA receptors (CP-AMPA receptors). This evidence aligns well with data showing that DA-LTP expression requires the GluA1 AMPA subunit and CP-AMPA receptors, as DA-LTP is absent in the hippocampus of a GluA1 genetic knock-out mouse model. Overall, the study is solid, and the evidence provided is compelling. The authors clearly and concisely explain the research objectives, methodologies, and findings. The study is scientifically robust, and the writing is engaging. The authors' conclusions and interpretation of the results are insightful and align well with the literature. The discussion effectively places the findings in a meaningful context, highlighting a possible mechanism for dopamine's role in the modulation of protein-synthesis-dependent hippocampal synaptic plasticity and its implications for the field. Although the study expands on previous works from the same laboratory, the findings are novel and provide valuable insights into the dynamics governing hippocampal synaptic plasticity.

The claim that GluA1 homomeric CP-AMPA receptors mediate the expression of DA-LTP is fascinating, and although the electrophysiology data on GluA1 knock-out mice are convincing, more evidence is needed to support this hypothesis. Western blotting provides useful information on the expression level of GluA1, which is not necessarily associated with cell surface expression of GluA1 and therefore CP-AMPA receptors. Validating this hypothesis by localizing the protein using immunofluorescence and confocal microscopy detection could strengthen the claim. The authors should briefly discuss the limitations of the study.

Although it would be possible to quantify the surface expression of GluA1 using immunofluorescence, it would not be possible to distinguish between GluA1 homomers and GluA1-containing heteromers. It would therefore not be informative as to whether these are indeed CP-AMPA receptors. This is an interesting problem, which we have briefly discussed in the Discussion section.

Additional comments to address:

(1) In Figure 2A, the representative image with PMY alone shows a very weak PMY signal. Consequently, the image with TTX alone seems to potentiate the PMY signal, suggesting a counterintuitive increase in protein synthesis.

We agree with the Reviewer that the original image was not representative and have replaced it with a more representative image.

(2) In Figures 3A-B, the Western blotting representative images have poor quality, especially regarding GluA1 and α -actin in Figure 3A. The quantification graph (Figure 3B) raises some concerns about a potential outlier in both the DA alone and DA+CHX groups. The authors should consider running a statistical test to detect outlier data. Full blot images, including ladder lines, should be added to the supplementary data.

We have replaced the western blot image in Figure 3A and have also presented full blot images including ladder lines in supplementary Figure S3.

Using the ROUT method (Q=1%) we identified one outlier in the DA+CHX group of the western blot quantification. The quantification for this blot was then removed from the dataset and the experiment was repeated to ensure a sufficient number of repeats.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) How the authors perform these experiments with puromycin, these are puromycylation experiments - not SuNSET. The SuNSET protocol (surface sensing of translation) specifically refers to the detection of newly synthesized proteins externally at the plasma membrane. I'd advise to update the terminology used.

We thank the Reviewer for pointing this out. We have updated this to 'puromycin-based labelling assay'.

(2) The legend presented in Figure 2F suggests WT is green and ACKO is orange, however, in Figure 2G the WT LTP trace is orange, consider changing this to green for consistency.

We thank the Reviewer for this suggestion and agree that a matching colour scheme makes the Figure clearer. This has been updated.

(3) In the results section, it is recommended to include units for the values presented at the first instance and only again when the units change thereafter.

The units of the electrophysiology data were [%], this is included in the Results section. Results of western blots and IHC images were presented as [a.u.]. While we included this in the Figures, we have not specifically added this to the text of individual results.

(4) Two hours pre-treatment with anisomycin vs 30 minutes pretreatment with cycloheximide seems hard to directly compare - as the pharmacokinetics of translational

inhibition should be similar for both drugs. What was the rationale for the extremely long anisomycin pretreatment? What controls were taken to assess slice health either prior to or following fixation? This is relevant to the below point (5).

In an initial set of control experiments (Figure S 1C-D) we wanted to ensure that protein synthesis was definitely blocked and therefore used a relatively high concentration of anisomycin and a relatively long pre-incubation period. We agree with the Reviewer that we cannot exclude the possibility that this treatment could compromise cell health in addition to the protein synthesis block. Therefore, we carried out an additional experiment with an alternative protein synthesis inhibitor cycloheximide at a lower standard concentration (10 μ M) which confirmed a significant reduction in the puromycin signal (Figure S1A-B). Together these results support the conclusion that puromycin signal is specific to protein synthesis in our labelling assay.

IHC slices were visually assessed for health. The large set of electrophysiology experiments carried out in our study (all recorded cells were evaluated for healthy resting membrane potential, action potential firing, and synaptic responses) also confirmed that, generally, the vast majority of our slices were indeed healthy.

(5) In Supplementary Figure 1, there is a dramatic difference in the a.u. intensities across CHX (B) and AM (D), please explain the reason for this. It is understood these are normalised values to nuclear staining, please clarify if this is a nuclear area.

We agree with the Reviewer that there is a large variability in normalised puromycin signal which may be due to variability in the health of the slices. However, we assume that the same variability would be present in the treated slices, which showed, despite the variability, a significant effect of protein synthesis inhibition. To prevent introducing bias by excluding slices with low puromycin signal in the control condition, we present the full dataset.

The CA1 region of the hippocampus contains of a dense layer of neuronal somata (pyramidal cell layer). We normalized against the nuclear area as it provides a reliable estimate of the number of neurons present in the image. This approach minimizes bias by accounting for variation in the number of neurons within the visual field, ensuring consistency and accuracy in our analysis.

(6) Please clarify the decision to average both the last 5 minutes of baseline recordings and the last 5 minutes of the recording for the normalisation of EPSP slopes.

The baseline usually stabilises after a few minutes of recording, thus the last 5 minutes were used for baseline measurement, which are the most relevant datapoints to compare synaptic weight change to. After induction of STDP, potentiation or depression of synaptic weights develops gradually. Based on previous results, evaluating the EPSP slopes at 30-40 minutes after the induction protocol gives a reliable estimate of the amount of plasticity.

Reviewer #2 (Recommendations For The Authors):

The concentration of anisomycin used (0.5 mM) is very high.

As described above, in an initial set of control experiments (Figure S 1C-D) we wanted to ensure that protein synthesis was definitely blocked and therefore used a relatively high concentration of anisomycin and a relatively long pre-incubation period. We agree with the Reviewer that this is higher than the standard concentration used for this drug and we cannot exclude the possibility that this treatment could compromise cell health in addition to the protein synthesis block. Therefore, we carried out an additional experiment with an alternative protein synthesis inhibitor cycloheximide at a lower standard concentration (10

μM) which confirmed a significant reduction in the puromycin signal (Figure S1A-B). Together these results support the conclusion that puromycin signal is specific to protein synthesis in our labelling assay.

Furthermore, in the electrophysiology experiments, we also used 500 μM anisomycin in the patch pipette solution. Under these conditions, we recorded a stable EPSP baseline for 60 minutes, indicating that the treatment did not cause toxic effects to the cell (Figure S1F). This high concentration would ensure an effective block of local translation at dendritic sites. Nevertheless, we also carried out this experiment with cycloheximide at a lower standard concentration (10 μM) and observed a similar result with both protein synthesis inhibitors (Figure 1F).

The authors conclude that the effect of DA is mediated via D1/5 receptors, which based on previous work seems likely. But they cannot conclude this from their current study which used a combination of a D1/D5 and a D2 antagonist.

We thank the Reviewer for pointing this out. We agree and have updated this in the Discussion section to 'dopamine receptors', without specifying subtypes.

There is no mention that I can see that the KO experiments were conducted in a blinded manner (which I believe should be standard practice). Did they verify the KOs using Westerns?

Only a subset of the experiments was conducted in a blinded manner. However, the results were collected by two independent experimenters, who both observed significant effects in KO mice compared to WTs (TF and ZB).

We received the DKO mice from a former collaborator, who verified expression levels of the KO mice (Wang et al., 2003). We verified DKO upon arrival in our facility using genotyping.

Maybe I'm misunderstanding but it appears to me that in Figure 1F there is LTP prior to the addition of DA. (The first point after pairing is already elevated). I think the control of pairing without DA should be added.

We thank the Reviewer for pointing this out. Based on previous results (Brzosko et al., 2015) we would expect potentiation to develop over time once DA is added after pairing, however, it indeed appears in the Figure here as if there was an immediate increase in synaptic weights after pairing. It should be noted, however, that when comparing the first 5 minutes after pairing to the baseline, this increase was not significant ($t(9)=1.810$, $p=0.1037$). Nevertheless, we rechecked our data and noticed that this initial potentiation was biased by one cell with an increasing baseline, which had both the test and control pathway strongly elevated. We had mistakenly included this cell in the dataset, despite the unstable conditions (as stated in the Methods section, the unpaired control pathway served as a stability control). We apologise for the error and this has now been corrected (Figure 1F). In addition, we present the control pathway in Figure S1G and I.

We have also now included the control for post-before-pre pairing ($\Delta t = -20$ ms) without dopamine in a supplemental figure (Figure S1E and F).

The Westerns (Figure 3A) are fairly messy. Also, it is better to quantify with total protein. Surface biotinylation of GluA1 and GluA2 would be more informative.

We carried out more repeats of Western blots and have exchanged blots in Figure 3A.

We observed that DA increases protein synthesis, we therefore cannot exclude the possibility that application of DA could also affect total protein levels. Thus quantifying with total protein may not be the best choice here. Quantification with actin is standard practice.

While we agree with the Reviewer that surface biotinylation of GluA1 and GluA2 could in principle be more informative, we do not think it would work well in our experimental setup using acute slice preparation, as it strictly requires intact cells. Slicing generates damaged cells, which would take up the surface biotin reagents. This would cause unspecific biotinylation of the damaged cells, leading to a strong background signal in the assay.

In Figure 4 panels D and E the baselines are increasing substantially prior to induction. I appreciate that long stable baselines with timing-dependent plasticity may not be possible but it's hard to conclude what happened tens of minutes later when the baseline only appears stable for a minute or two. Panels A and B show that relatively stable baselines are achievable.

We agree with the Reviewer that the baselines are increasing, however, when looking at the baseline for 5 minutes prior to induction (5 last datapoints of the baseline), which is what we used for quantification, the baselines appeared stable. Unfortunately, longer baselines are not suitable for timing-dependent plasticity. In addition, all experiments were carried out with a control pathway which showed stable conditions throughout the recording.

In general, the discussion could be better integrated with the current literature. Their experiments are in line with a substantial body of literature that has identified two forms of LTP, based on these signalling cascades, using more conventional induction patterns.

We thank the Reviewer for this suggestion and have added more discussion of the two forms of LTP in the Discussion section.

It would be helpful to include the drug concentrations when first described in the results.

Drug concentration have now been included in the Results section.

It is now more common to include absolute t values (not just <0.05 etc).

While we indicate significance in Figures using asterisks when p values are below the indicated significance levels, we report absolute values of p and t values in the Results section.

Similarly full blots should be added to an appendix / made available.

We have now included full blot images in Supplementary Figure S3.

A 30% tolerance for series resistance seems generous to me. (10-20% would be more typical).

We thank the Reviewer for their suggestion, and will keep this in mind for future studies. However, the error introduced by the higher tolerance level is likely to be small and would not influence any of the qualitative conclusions of the manuscript.

Whereas series resistance is of course extremely important in voltage-clamp experiments, changes in series resistance would be less of a concern in current-clamp recordings of synaptic events. We use the amplifier as a voltage follower, and there are two problems with changes in the electrode, or access, resistance. First, there is the voltage drop across the

electrode resistance. Clearly this error is zero if no current is injected and is also negligible for the currents we use in our experiments to maintain the membrane voltage at -70 mV. For example, the voltage drop would be 0.2 mV for 20 pA current through a typical 10 MOhm electrode resistance, and a change in resistance of 30% would give less than 0.1 mV voltage change even if the resistance were not compensated. The second problem is distortion of the EPSP shape due to the low-pass filtering properties of the electrode set up by the pipette capacitance and series resistance (RC). This can be a significant problem for fast events, such as action potentials, but less of a problem for the relatively slow EPSPs recorded in pyramidal cells. Nevertheless, we take on board the advice provided by the Reviewer and will use the conventional tolerance of 20% in future experiments.

Reviewer #3 (Recommendations For The Authors):

In the references, the entry for Burnashev N et al. has a different font size. Please ensure that all references are formatted consistently.

We thank the Reviewer for spotting this and have updated the font size of this reference.

<https://doi.org/10.7554/eLife.100822.2.sa0>