Long-term potentiation (LTP) at hippocampal CA1 synapses can be expressed by an increase either in the number (N) of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors or in their single channel conductance (γ). Here, we have established how these distinct synaptic processes contribute to the expression of LTP in hippocampal slices obtained from young adult rodents. LTP induced by compressed theta burst stimulation (TBS), with a 10 s inter-episode interval, involves purely an increase in N (LTP_N). In contrast, either a spaced TBS, with a 10 min inter-episode interval, or a single TBS, delivered when PKA is activated, results in LTP that is associated with a transient increase in γ (LTP_γ), caused by the insertion of calcium-permeable (CP)-AMPA receptors. Activation of CaMKII is necessary and sufficient for LTP_N whilst PKA is additionally required for LTP_γ. Thus, two mechanistically distinct forms of LTP co-exist at these synapses.
long-term potentiation (LTP) of synaptic function is considered to involve the major process underlying learning and memory, where it is involved in synaptic engram formation, yet the underlying cellular mechanisms remain incompletely understood. The best-characterized form of LTP occurs at the Schaffer collateral-commissural pathway (SCCP) in the hippocampus, where it is triggered by synaptic activation of NMDA (N-methyl-D-aspartate) receptors and is expressed as a persistent increase in AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor-mediated synaptic transmission. This modification is primarily due to a functional modulation of AMPA receptors (AMPARs), which may involve a change in the number of active channels (N) (termed LTPN) and/or their single-channel conductance (γ) properties (termed LTPγ) (e.g., 6–9). Whilst there is considerable evidence that LTPγ is triggered by activation of Ca2+/calmodulin-dependent kinase II (CaMKII)10,11 and involves exocytosis and lateral diffusion of AMPARs12–13, the mechanisms underlying LTPγ are largely unknown. The two most likely molecular mechanisms involve (i) CaMKII-mediated phosphorylation of Ser831 of GluA1, which can result in an increase in the time AMPARs dwell in higher conductance states14,15 or (ii) the insertion of calcium-permeable AMPA receptors (CP-AMPARs), which have a higher γ than their calcium-impermeable (CI) counterparts16,17.

In the present study, we have tested the hypothesis that LTPγ is due to the insertion of CP-AMPARs in young adult rodents using two theta burst stimulation (TBS) induction protocols that differed only in the timing between episodes, and applied peak-scaled non-stationary fluctuation analysis (NSFA)19–21 to estimate γ before and after the induction of LTP6,15,22–25. We found that the compressed TBS protocol (cTBS – inter-episode interval of 10 s) resulted exclusively in LTPγ, for which CaMKII was both necessary and sufficient. In contrast, a spaced TBS protocol (sTBS – inter-episode interval of 10 min) resulted in a transient increase in γ, lasting ~15 min, which was due to the insertion of CP-AMPARs and required both CaMKII and PKA. Insertion of CP-AMPARs mediates both the initial expression of LTPγ by enhancing the net synaptic unitary conductance, and helps trigger the processes that lead to a persistent increase in synaptic efficacy that outlasts the increase in γ. Since the PKA-dependent form of LTP also requires de novo protein synthesis and has stimulation features similar to spaced behavioural learning, LTPγ is likely to underlie the formation of synaptic engrams and long-term memory.

Results

An increase in γ is specifically triggered by a sTBS protocol. Simultaneous field excitatory postsynaptic potential (fEPSP) recordings from stratum radiatum and somatic whole-cell recordings were obtained in response to baseline stimulation of two independent SCCP inputs (Fig. 1a). TBS was delivered to one input (test), while the second input served as a control for stability and heterosynaptic effects (Fig. 1c, d). Synaptic potentiation was quantified and γ was estimated using NSFA (Fig. 1e, f), as described previously. To optimize the estimates of γ we used minimal stimulation and restricted our measurements to the first 20–30 min following TBS, since γ estimates are extremely sensitive to small fluctuations in series resistance. Thus, our study focused on the induction and initial expression mechanisms of LTP.

In the first series of experiments we delivered three episodes of TBS, with each episode comprising 5 shocks at 100 Hz delivered 5 times at 5 Hz (i.e. 75 stimuli in total; see Fig. 1b schematic); in interleaved experiments we either delivered these three episodes as a cTBS (10 s inter-episode interval) or as a sTBS (10 min inter-
episode interval). We referred to the resultant potentiation as cLTP (Fig. 2a–i) and sLTP (Fig. 2j–r), respectively. In response to cTBS there was a substantial cLTP (Fig. 2a), with EPSC amplitudes increasing to 212 ± 11% of baseline, averaged over the first 10 min after induction (Fig. 2b). For 22 neurons from 15 rats (n = 22/15), we obtained γ estimates in 10 min epochs and found it to be unaltered throughout (Fig. 2c–g). The γ values were 5.1 ± 0.3 pS (baseline), 5.3 ± 0.4 pS (first 10 min epoch post cTBS; LTP10γ; t21 = 1.23, p = 0.2327, vs baseline, paired Student’s t test) and 5.2 ± 0.4 pS (second 10 min epoch post cTBS; LTP20γ; t21 = 0.33, p = 0.7452; Fig. 2d). The control input was also stable throughout (4.9 ± 0.4 pS, 4.5 ± 0.3 pS and 4.8 ± 0.3 pS at the corresponding time points; Fig. 2d). The lack of change in γ was also clearly evident in the plots from individual experiments for control (Fig. 2e) and test inputs (Fig. 2f) and in the cumulative distribution plots (Fig. 2g). The lack of change in γ was observed over a wide range of cLTP magnitudes (Fig. 2h).

In response to sTBS the results were strikingly different. For this set of experiments, whole-cell recordings were obtained shortly after delivery of the second TBS episode and the effects of the third TBS were evaluated (Fig. 2i). This method was necessary because of the rapid wash-out of LTP with low access whole-cell recordings. In response to the third TBS there was a substantial additional LTP, with EPSC amplitudes increasing to 177 ± 9% of baseline, averaged over the first 10 min after induction (Fig. 2k). The estimate of γ upon break in was significantly higher (6.9 ± 0.4 pS) compared to the control input (4.9 ± 0.4 pS; Fig. 2n–o; t22 = 3.22, p = 0.0039, paired Student’s t test) and this was further increased in response to the third episode of TBS to 8.4 ± 0.4 pS (LTP30γ; t22 = 3.75, p = 0.0011, Fig. 2l, m, o, p; n = 23/17). However, when we quantified γ at 10–20 min after the last TBS, the value (5.5 ± 0.3 pS) was no longer significantly different from the control input (LTP20γ; t22 = 2.01, p = 0.0570, paired Student’s t test; Fig. 2m). In contrast to the test input, sTBS did not result in a significant γ change in the control input (4.9 ± 0.4 pS, 5.4 ± 0.4 pS and 4.6 ± 0.3 pS at the corresponding time points; Fig. 2m, n). Thus, the increase in γ is specifically related to sLTP. Furthermore, this increase in γ positively correlated with the magnitude of sLTP (Fig. 2q).

Since sLTP, but not cLTP, is associated with the insertion of CP-AMPARs26,27 these results suggest that CP-AMPARs may account for the increase in γ. CP-AMPARs have slightly faster decay kinetics (τdecay) than CI-AMPARs25,28, which can be detected using single exponential fits to EPSC decays. We found that cLTP was not associated with an alteration in τdecay (Fig. 2i, Supplementary Table 1; t21 = 0.66, p = 0.5146, paired Student’s t test), whereas sLTP was associated with a highly significant decrease in τdecay (Supplementary Table 1; p = 0.0051, t22 = 3.11, paired Student’s t test). A regression analysis showed a trend for the τdecay to be inversely related with the increase in γ (Fig. 2r; p = 0.0712, F(1,21) = 3.61). Therefore, the kinetic analysis provides additional support for the notion that insertion of CP-AMPARs occurs during the induction of LTP in response to a sTBS.

The role of PKA in LTPγ. It is established that elevating cAMP by, for example, use of the phosphodiesterase 4 inhibitor rolipram, enables a weak stimulus to generate an enhanced PKA-dependent form of LTP29. Previously, we found that in the presence of rolipram a weak TBS, comprising one episode of TBS, generated an LTP that is largely dependent on the insertion of CP-AMPARs. Here we used this same method as an independent means to investigate whether insertion of CP-AMPARs are responsible for the increase in γ. Since only one TBS is required to induce the PKA-dependent form of LTP in the presence of rolipram we could make γ measurements before and after the full...
induction of LTP. As illustrated in Fig. 3a, a single episode of TBS (wTBS; comprising 25 stimuli), when delivered in the presence of rolipram (1 µM), generated a robust LTP (234 ± 14% of baseline for test vs. 121 ± 6% for control input). We found that this LTP was also associated with a transient increase in \( \gamma \) (baseline = 4.9 ± 0.4 pS, \( LTP_{10} = 8.0 \pm 0.6 \) pS; \( t_{20} = 5.90, p < 0.0001 \)) that returned to baseline by the second 10 min epoch (\( LTP_{20} = 5.4 \pm 0.3 \) pS; \( t_{20} = 1.39, p = 0.1810, \) paired Student’s t test) following the wTBS (\( n = 21/15; \) Fig. 3c, d, f, g). This potentiation required the wTBS since the control input was largely unaffected (5.1 ± 0.3 pS, 5.4 ± 0.5 pS and 4.8 ± 0.3 pS at the corresponding time points; Fig. 3d, e) and since the baseline \( \gamma \) values in the presence of rolipram were not significantly different to the baseline \( \gamma \) values in its absence (Fig. 3d–f; Supplementary Table 1). As was the case with the sLTP, the size of the change in \( \gamma \) correlated with the magnitude of LTP (\( p = 0.004, F_{(1,19)} = 12.77; \) Fig. 3h). Additionally, there was an associated reduction in \( \tau_{\text{decay}} \) (\( p = 0.0007, t_{20} = 3.99, \) paired Student’s t test; Supplementary Table 1) that also negatively correlated with the increased \( \gamma \) (\( p = 0.0199, F_{(1,19)} = 6.46; \) Fig. 3i). These results...
Fig. 2 Increased AMPA receptor unitary conductance ($\gamma$) during sLTP, but not cLTP. a A representative LTP experiment with sample traces for baseline and post TBS – the mean of selected records for analysis, superimposed with peak-scaled individual traces (10 successive sweeps, thin lines; baseline = grey, LTP = black). Scaled trace is from the baseline normalized to the LTP. Scale bars: 20 pA and 10 ms. Two inputs were stimulated alternately and cTBS (3 x TBS with an inter-episode interval of 10 s; blue arrows) delivered to one input (filled symbols) with the second input (open symbols) serving as a control (Con). b Levels of cTBS-induced LTP (cLTP) for control and test inputs, quantified during the 10 min epoch after the induction (mean ± SEM, $n = 22$ neurons from 15 animals; $t_{21} = 8.545$, $p < 0.0001$, two-sided paired Student’s $t$ test). c Corresponding current–variance relationship of the EPSCs for the test input. The unitary channel conductance ($\gamma$) of AMPA receptors was estimated during baseline (grey) and after the induction of LTP ($\text{LTP}_{10}$; black). d Grouped comparison of control and test input $\gamma$ estimates for baseline and the initial 10 min epoch ($\text{LTP}_{10}$) and the subsequent 10 min epoch ($\text{LTP}_{20}$). $n = 22$ neurons from 15 animals. e, f Summary plot for the $\gamma$ at baseline (left) and $\text{LTP}_{10}$ (right) for control (e) and test (f) inputs. Individual values from each neuron are connected by lines. Circles indicate mean values. g Cumulative distribution of the same data set for $\text{LTP}_{20}$. Dotted lines indicate the mean values for each input. h, i Analysis of the relationships of $\gamma$ with LTP ($p = 0.6517$, $F_{(1,20)} = 0.2101$, F-test) (h) and EPSC decay time ($p = 0.9521$, $F_{(1,20)} = 0.0037$, F-test) (i). Linear regression with 95% confidence intervals (shaded) for the amount of cLTP and the corresponding level of $\gamma$. j–r Equivalent analysis for the LTP induced by sTBS (3 x TBS at inter-episode interval of 10 min; see arrows). The whole-cell recordings were obtained after the second TBS. This was necessary due to the lability of LTP washout. k Levels of sTBS-induced LTP (sLTP) for control and test inputs, quantified during the 10 min epoch after the induction ($n = 23$ neurons from 17 animals; $t_{22} = 5.238$, $p < 0.0001$, two-sided paired Student’s $t$ test). m–o Statistical analysis between control and test pathways ($t_{22} = 3.220$, $p = 0.0039$ for baseline and $t_{22} = 6.123$, $p < 0.0001$ for $\text{LTP}_{10}$, two-sided paired Student’s $t$ test) (m) and within pathway analysis for control ($t_{22} = 1.065$, $p = 0.2986$, two-sided paired Student’s $t$ test) (n) and test ($t_{22} = 3.753$, $p = 0.0011$, two-sided paired Student’s $t$ test) (o) pathway reveals a time- and pathway-dependent increase in $\gamma$. Note that higher conductance was observed in the test input (o) compared to the control (n) under the “baseline” state, suggesting that the first + second TBS were sufficient to increase $\gamma$. The third TBS triggered a small but discernible further increase in $\gamma$. q, r Analysis of the relationships of $\gamma$ with LTP ($p = 0.0225$, $F_{(1,21)} = 6.066$, F-test) (q) and decay time of EPSCs ($p = 0.0712$, $F_{(1,20)} = 3.612$, F-test) (r). Data are presented as mean ± SEM. Source data are provided as a Source Data file.
further support the idea that insertion of CP-AMPARs mediates LTPγ.

To more specifically test the requirement of PKA for driving alterations in γ, we included the catalytic subunit of PKA (PKA Ca; 300 U/mL) in the patch solution (Fig. 4). This treatment had little effect on the control input that did not receive any wTBS (Fig. 4a), suggesting that PKA alone has minimal effect on synaptic transmission. However, as was the case with rolipram, the wTBS in the presence of PKA Ca generated a robust potentiation (Fig. 4a) that was associated with an increase in γ (Fig. 4b, c). The levels quantified during baseline and 10 min post wTBS (LTP10) were 5.2 ± 0.5 pS and 7.8 ± 0.8 pS (t16 = 5.80, p <
are responsible for the increase in the transient insertion of CP-AMPARs and that these receptors, in the presence of the catalytic subunit of PKA, results in LTP during (i) a sTBS, (ii) a wTBS in the presence of rolipram or (iii) a phase of LTP.

The correlations between the change in \( \gamma \) and either the magnitude of LTP (Fig. 5f; \( p = 0.2813, F(1,13) = 1.26 \)) or \( t_{\text{decay}} \) (Fig. 5k; \( p = 0.2918, F(1,13) = 0.84 \)) were not statistically significant, as the increase in \( \gamma \) was transient, as the increase returned to baseline levels within 20–30 min of whole-cell recording (Fig. 6e).

| 0.2918 | 0.0002, paired Student \( t \)-test; \( n = 16/13; \) also see Supplementary Table 1). The respective \( \gamma \) values for baseline (i.e. first 5 min of recording) and 10–15 min of whole-cell recording were 4.7 ± 0.6 pS and 4.3 ± 0.5 pS (\( t_{16} = 0.74, p = 0.4740 \), paired Student’s \( t \)-test; \( n = 15/12; \) Fig. 5g). There was no correlation between \( \gamma \) change and either the magnitude of LTP (Fig. 5j; \( p = 0.2265, F(1,13) = 1.61 \)) or \( t_{\text{decay}} \) (Fig. 5k; \( p = 0.2813, F(1,13) = 1.26 \)). We can conclude, therefore, that CaMKII alone can generate substantial potentiation that does not involve any alteration in \( \gamma \).

**Activation of CaMKII and PKA are both necessary and sufficient for LTP**. Since neither PKA alone nor CaMKII alone affected \( \gamma \), we explored whether the combination of the two kinases may be sufficient for the effect. We, therefore, patch loaded PKA Ca (300 U/mL) with either the active or inactive forms of CaMKII (250 U/mL). In interleaved experiments, we found that PKA Ca + active CaMKII produced a robust potentiation of synaptic responses, specifically 178 ± 10% of baseline when quantified 15 min after whole-cell (Fig. 6a, b, f). In this case, the effect was also associated with an increase in \( \gamma \) (Fig. 6d, e, g). The levels of conductance for the baseline and potentiation (calculated between 10 to 15 min of recording) were 4.6 ± 0.4 pS and 6.5 ± 0.4 pS, respectively (\( t_{15} = 5.38, p = 0.0002, \) paired Student’s \( t \)-test; \( n = 18/15 \)). Again, this effect was only transient, as the \( \gamma \) returned to baseline levels within 20–30 min of whole-cell recording (Fig. 6e).

In contrast, inactive CaMKII plus PKA Ca, had no significant effect on synaptic transmission (112 ± 9%; Fig. 6b, f) or on \( \gamma \) (4.2 ± 0.4 pS vs. 4.2 ± 0.4 pS, \( n = 16/14; \) Fig. 6e, g). These results suggest that (i) both CaMKII and PKA are required for and (ii) their combined activity is sufficient for LTP \( \gamma \) at these synapses.

In additional interleaved experiments, the sensitivity to IEM was tested on the potentiation produced by CaMKII plus PKA Ca. Consistent with the involvement of CP-AMPARs, there was a reduced level of potentiation (Fig. 6b, f) and no change in \( \gamma \) in the presence of IEM (Fig. 6e, g). The respective amounts, quantified after 10–15 min of whole-cell recording, were 128 ± 9% of baseline (\( p = 0.0003 \) vs. CaMKII + PKA Ca, one-way ANOVA with Bonferroni’s correction) and 4.1 ± 0.4 pS (\( p = 0.0009 \) vs. CaMKII + PKA Ca, one-way ANOVA with Bonferroni’s correction; \( n = 20/15 \)). The correlations between the change in \( \gamma \) with the level of LTP and decay times for these experiments are summarized in Fig. 6h–k. There was a substantial correlation between the increase in \( \gamma \) and the level of potentiation (Fig. 6h) and the decrease in \( t_{\text{decay}} \) (Fig. 6i) with CaMKII plus PKA Ca but no such correlation was found in the presence of IEM (Fig. 6j, k).

The proportion of synthetically incorporated CP-AMPARs during LTP \( \gamma \). Together, the previous experiments provide multiple lines of evidence that LTP \( \gamma \) is due to the insertion of CP-AMPARs...
into synapses that contain CI-AMPARs. In order to determine the relative proportions of each it was necessary to measure \( \gamma \) for synapses containing either just CI-AMPARs or just CP-AMPARs, under our recording conditions. To achieve this, we used lentivirus-driven CRISPR/Cas9 expression to delete GluA2 in a fraction of neurons in vivo, allowing a direct comparison between a knock-out (KO) and a wild-type (WT) neuron within each adult brain slice (Fig. 7a). When compared with uninfected neighbouring neurons, the KO cells showed a reduced AMPAR synaptic transmission (Fig. 7b) and an inwardly rectifying current–voltage relationship (Fig. 4).

**Fig. 4** wTBS with PKA C\(\alpha\) transiently increases \( \gamma \) via CP-AMPAR insertion. a–b A wTBS in the presence of intracellular PKA C\(\alpha\) (300 U/mL) transiently increased \( \gamma \) (n = 17 neurons from 13 animals, mean ± SEM, \( F_{(1,31),27.04} = 52.89 \) for EPSCs and \( F_{(1,83),26.08} = 12.59 \) for \( \gamma \), one-way repeated measures ANOVA followed by Bonferroni’s multiple comparisons test; \( * \)p < 0.05, \( ** \)p < 0.01 and \( *** \)p < 0.001 vs. baseline). EPSCs (a) and \( \gamma \) (b) were analyzed in 10-min bins. A single episode of TBS (at time marked by an arrow) was delivered to one input (filled symbols) with the second input (open symbols) serving as a control; base = baseline. c A representative current-variance plot for PKA C\(\alpha\) plus wTBS for baseline, the first 10 min (LTP10) and the last 10 min of LTP (LTP30). Sample traces were obtained from baseline and LTP30. Scale bars: 10 pA and 10 ms. d–f Equivalent experiments in the presence of IEM-1460 (IEM, 30 \( \mu \)M; n = 16 neurons from 13 animals, \( F_{(1,85),13.14} = 25.66 \) for EPSCs and \( F_{(2,184),26.21} = 0.2547 \) for \( \gamma \), one-way repeated measures ANOVA followed by Bonferroni’s multiple comparisons test; \( * \)p < 0.05, \( ** \)p < 0.01 and \( *** \)p < 0.001 vs. baseline). g, h Quantification of the levels of LTP (t\( _{31} = 3.006, \) p = 0.0052, two-sided unpaired Student’s t test) (g) and (t\( _{31} = 3.544, \) p = 0.0013, two-sided unpaired Student’s t test) (h) measured during the 10 min after wTBS with cumulative distributions (right). n = 17 neurons from 13 animals (PKA C\(\alpha\) + wTBS) and 16 neurons from 13 animals (PKA C\(\alpha\) + wTBS + IEM). i, j Analysis of the relationships between \( \gamma \) and LTP for PKA C\(\alpha\) + wTBS (p = 0.0021, \( F_{(1,15)} = 13.72, \) F-test) (i) and PKA C\(\alpha\) + wTBS + IEM (p = 0.9090, \( F_{(1,14)} = 0.0136, \) F-test) (j). k, l Analysis of the relationships between \( \gamma \) and EPSC decay time for PKA C\(\alpha\) + wTBS (p = 0.0117, \( F_{(1,15)} = 8.243, \) F-test) (k) and PKA C\(\alpha\) + wTBS + IEM (p = 0.3931, \( F_{(1,14)} = 0.7764, \) F-test) (l). Data are presented as mean ± SEM. Source data are provided as a Source Data file.
The level of $\gamma$ in KO neurons was significantly higher at 17.3 ± 1.2 pS ($n = 16$) compared to 4.6 ± 0.4 pS for WT neurons ($n = 17$; $t_{31} = 10.09$, $p < 0.0001$, unpaired Student’s $t$ test, data from 12 animals; Fig. 7e–h). This increase in $\gamma$ in these neurons was associated with a decreased $\tau_{\text{decay}}$ from 6.8 ± 0.4 ms in WT to 5.3 ± 0.4 ms in KO neurons ($t_{31} = 2.85$, $p = 0.0026$, unpaired Student’s $t$ test, Fig. 7i). Assuming that these EPSCs were comprised of 100 and 0 % CP-AMPARs, respectively, then the increase in $\gamma$ that we observed during sLTP can be explained by CP-AMPARs comprising ~30% of the synaptic current during the first 10 min following LTP induction.

### Discussion
NMDA receptor-dependent LTP has been extensively studied as the primary mechanisms utilized are crucial for the formation of long-term memories. Despite many molecules being discovered...
and different aspects of their regulation being uncovered, there are crucial gaps in our knowledge. One relates to the fact that long-term memory requires de novo protein synthesis yet most of our mechanistic understanding of LTP has been obtained from the study of a protein synthesis-independent form of LTP. A second pertains to the fact that much of this understanding has been derived from the study of juvenile animals, where technical issues have permitted more in-depth analysis, whereas most studies of learning and memory are conducted in adult animals. In the present study, we have addressed these issues by studying LTP at CA1 synapses in young adult rodents and have compared induction protocols that are known to activate the protein synthesis-independent (cTBS) and protein synthesis-dependent (sTBS, rolipram + tTBS) forms. Using a cTBS protocol, LTP involved the insertion of additional CI-AMPARs, for which activation of CaMKII is both necessary and sufficient. The increase in the number of AMPARs without a change in their multiple conductance states and/or by the insertion of CP-AMPARs. In terms of signalling cascades, we found that transient insertion of CP-AMPARs, for which activation of CaMKII and PKA are both necessary and, in combination, sufficient. The insertion of CP-AMPARs increases AMPA receptor γ and this underlies the initial expression of this form of LTP, which we have termed LTPγ. The insertion of CP-AMPARs is transient and is replaced by a persistent increase in the number of CI-AMPARs.

Two distinct postsynaptic forms of LTP at CA1 synapses. The division of NMDA receptor-dependent LTP into multiple components was made on the basis of sensitivity to various pharmacological agents and substantiated by genetic studies. In particular, when a single train (tetanus or TBS) is employed, the resultant LTP may be independent of both PKA activation and de novo protein synthesis; this is commonly referred to as LTP1 or LTPN and LTP2 (LTP and LTPN, respectively). In contrast, when multiple trains are delivered, with an interval in the order of minutes, then there is often the generation of a new PKA and AMPAR protein synthesis-dependent component of LTP, which is commonly referred to as LTP2 or L-LTP2. LTP2 is generally assumed to underlie long-term memory formation, that also requires de novo protein synthesis.

NMDA receptor-dependent LTP has also been divided into two distinct postsynaptic mechanisms of expression, one involving an increase in the number of AMPARs without a change in γ (LTPN) and the other involving an increase in γ (LTPγ). Here, one of our goals was to determine whether these separate expression mechanisms specifically relate to LTP1 and LTP2. We found that LTP1 never involved an alteration in γ whereas LTP2 invariably did. The increase in γ was transient, lasting between 10 and 20 min and could be fully explained by the insertion of CP-AMPARs. In terms of signalling cascades, we found that CaMKII was both necessary and sufficient for LTP1 whereas both CaMKII and PKA were required, and in combination were sufficient, for LTP2 (see model in Fig. 8). Our findings do not conflict with a large body of literature regarding alterations in AMPARs underlying LTP at these synapses and the roles of both CaMKII and PKA.

The roles of CP-AMPARs and alterations in γ in LTP have been controversial. However, these controversies can now be reconciled on the basis of the type of LTP under investigation. Under the conditions of our study, we could readily switch between forms of LTP that do not (LTP1) or do (LTP2) involve a CP-AMPAR component by simply altering the timing between TBS episodes. We saw similar effects when we compared whole-cell recordings, using minimal stimulation, with field potential recordings, which provide an average measure of synaptic transmission across a wide range of release probabilities P(r). Therefore, we do not expect that our observations are dependent on the P(r) of the synapse under investigation. However, the extent to which CP-AMPARs are involved in synaptic plasticity is likely to involve additional factors, such as the developmental stage of the animal, the level of stress experienced prior to euthanasia and the precise experimental conditions used, including the stimulus parameters employed.

We can conclude that LTP1 equates to LTPN and LTP2 with LTPγ. It is important to note, however, that although a compressed induction protocol (cTBS) will ordinarily result in just LTP1/LTPN, a spaced protocol will comprise a mixture of LTP1 and LTP2 (LTPγ), since the initial train will induce LTP1 upon which subsequent trains will add LTP2 under our experimental conditions. The relative proportion of these two components will depend on a variety of conditions, including the interval between the trains, with ~10 min being optimal for the induction of LTP2.

On the mechanism of LTPγ. The increase in γ is regulated by the c-terminal tail of GluA1 and could result from a CaMKII-dependent phosphorylation of Ser831 of GluA1 to directly modulate their multiple conductance states and/or by the insertion of CP-AMPARs, since these have a higher single channel conductance than CI-AMPARs. Our findings have demonstrated that LTPγ can be explained exclusively by the latter mechanism, since all changes in γ were eliminated by IEM. Furthermore, we found that activation of PKA plus CaMKII increased γ whereas...
CaMKII alone did not, despite leading to a substantial potentiation. The failure of CaMKII alone to increase $\gamma$, which is contrary to some previous studies\cite{14,15}, could be explained on the basis of the native AMPAR configuration since $\gamma$ alterations are affected by the subunit combination and accessory protein composition of AMPA receptors\cite{16,18}. It is worth noting, however, that whilst activation of CaMKII alone was not sufficient to induce LTP$_{\gamma}$ its activation was necessary. It is possible, therefore, that phosphorylation of Ser831 of GluA1 is a necessary step for LTP$_{\gamma}$. Such a mechanism would involve dual phosphorylation of GluA1 on Ser831 and Ser845, which is known to occur\cite{49}. One scenario is that GluA1 is firstly phosphorylated on Ser845 to drive CP-AMPARs to peri-synaptic sites, for which considerable evidence already exists\cite{30,41,42,49-51}. From here, they are next
phosphorylated on Ser831 to drive them into the synapse. In this model, both phosphorylation steps are required for synaptic y to increase because they are regulating different trafficking steps on route to the synapse. In which case, CaMKII should not be able to increase y further in neurons lacking GluA2 because CP-AMPARs are already synaptically expressed. Future work could address this and other aspects of the temporal sequence and consequences of PKA and CaMKII-dependent phosphorylation of GluA1 for LTP.

Our data are compatible with an exchange of a subset of CI-AMPARs for CP-AMPARs. The latter could be explained by a mechanism involving the Ca$^{2+}$ sensor PICK1$^{52,53}$, which has been shown to bind and internalize GluA2-containing AMPARs to enable the insertion of CP-AMPARs during LTP$^{54}$. The next step involves the replacement of the newly inserted CP-AMPARs with CI-AMPARs, a process that requires baseline (low frequency) synaptic activation$^{26,43}$ and probably involves Ca$^{2+}$ permeation through the CP-AMPARs themselves$^{35}$. The rapid replacement of CP-AMPARs with CI-AMPARs was originally described at excitatory synapses onto cerebellar stellate neurons from P18–P20 rats$^{56}$. At this synapse, high-frequency stimulation (tetanus) induces CP-AMPARs to be replaced with the equivalent number of CI forms resulting in a reduction in the synaptic current by a third, reflecting lower y of the latter form. We observed an initial reduction in EPSC amplitude following the tetanus, which might be explained, in part, by a one-to-one exchange of CP-AMPARs for CI-AMPARs. Additionally, the transient expression of CP-AMPARs could trigger an increase in the number of AMPAR slots at synapses that enables an increase during LTP1.
AMPARs to maintain the enhanced synaptic response? One possibility is that the expression of CP-AMPARs at these synapses needs to be restricted in time due to potential excitotoxicity. Therefore, they can only provide a transient mechanism of expression whilst triggering the more persistent switch resulting in a larger number of CI-AMPARs.

Developmental regulation of the expression mechanisms of LTP. There is strong evidence that the expression mechanisms of LTP are developmentally regulated. The co-existence of two mechanisms involving the insertion of CI-AMPARs and CP-AMPARs can account for the LTP at P14 and in young adults, as observed herein. However, at around P7, LTP is associated with a decrease in γ, which is most likely explained by the replacement of CP-AMPARs with a larger number of CI-AMPARs. Early in development there is also the initial insertion of CP-AMPARs into synapses that appear to lack AMPARs altogether; so-called “silent” synapses. A potential scenario is as follows: first synapses acquire CP-AMPARs, next these are replaced by more CI-AMPARs. Thereafter LTP can increase the number of these CI-AMPARs via two mechanisms, one of which involves the transient insertion of CP-AMPARs and one that does not.

There have been few studies regarding the mechanisms of synaptic plasticity in tissues obtained from adult animals compared to juvenile animals, although most learning and memory studies are conducted in adult animals. This is a concern when attempting to relate mechanisms of synaptic plasticity to learning and memory. Our present study, conducted exclusively in tissue from young adult animals, shows that two distinct forms of synaptic plasticity can be readily induced simply by altering the patterns of activation. Our result that a cTBS protocol induces LTP that does not involve an alteration in γ is consistent with another study in adult animals. Our finding that a sTBS induces an additional component of LTP that involves an increase in γ is the first evidence that such a process occurs beyond early developmental stages.

Functional significance of two forms of LTP. This raises the question as to why there are two distinct mechanisms to increase the synaptic complement of CI-AMPARs. Previous work has shown that the insertion of CP-AMPARs is specifically associated with the PKA and protein synthesis component of LTP.

![Fig. 8 Schematic outlining the induction of two mechanistically distinct forms of LTP. 1 Under baseline conditions synaptic transmission is mediated by GluA2-containing, calcium-impermeable (CI)-AMPARs, two shown for simplicity. 2 The first theta-burst stimulation (TBS) activates NMDA receptors (NMDARs) and this drives more CI-AMPARs into the synapse by lateral diffusion from a peri-synaptic pool, via a process that involves CaMKII. PKA is also activated (via adenyl cyclase, not shown) and this induces the process of inserting GluA2-lacking calcium-permeable (CP)-AMPARs into peri-synaptic sites on the plasma membrane. 3 LTP is expressed by the increase in number of CI-AMPARs (LTPγ) but synapses also become primed for LTP by the availability of peri-synaptic CP-AMPARs. 4 Within ~1h, the peri-synaptic CP-AMPARs are removed and, presumably, degraded. 5 If a second TBS is delivered whilst the synapses are still primed then NMDAR activation drives the peri-synaptically located CP-AMPARs into the synapse, via a CaMKII-dependent process. This might involve an exchange of CP-AMPARs for CI-AMPARs, which are removed from the synapse via a mechanism triggered by PICK1. 7 These CP-AMPARs increase synaptic strength due to their higher single channel conductance (LTP). However, their dwell time in the synapses is quite short (~15 min) before they are removed. If synapses remain active, such as by basal stimulation, activation of the transiently available, synaptic CP-AMPARs triggers protein synthesis and the insertion of more CI-AMPARs (8), which can extend the expression of LTP for long periods.](Image)
Methods

LTP1 and LTP2. This latter form of LTP involves the transient both necessary and sufficient way in explaining many of the controversies that have plagued the existence of these two distinct LTP mechanisms goes a long way in explaining many of the controversies that have plagued the field. LTP1 can be induced by a cTBS and involves the insertion of CI-AMPARs, and for this to occur activation of CaMKII is both necessary and sufficient. A sTBS, however, triggers both LTP1 and LTP2. This latter form of LTP involves the transient insertion of CI-AMPARs and this requires activation of PKA in addition to CaMKII.

Concluding remarks

We have identified the molecular basis of two independent forms of LTP that co-exist at hippocampal synapses in young adult animals, the occurrence of which is controlled by the patterns of synaptic activation during induction. The existence of these two distinct LTP mechanisms goes a long way in explaining many of the controversies that have plagued the field. LTP1 can be induced by a cTBS and involves the insertion of CI-AMPARs, and for this to occur activation of CaMKII is both necessary and sufficient. A sTBS, however, triggers both LTP1 and LTP2. This latter form of LTP involves the transient insertion of CI-AMPARs and this requires activation of PKA in addition to CaMKII.

Field excitatory postsynaptic potential (fEPSP) recordings. The extracellular electrophysiology was performed in both interface and submerged type chambers maintained at 32 °C, and continuously perfused at 2–4 mL/min with oxygenated ACSF. The slope of evoked fEPSPs (V/τ) was measured in the CA1 region of hippocampal slices, where stimulating electrodes were used at a constant voltage intensity (0.1 ms pulse width) throughout the experiments. Signals were amplified using Axopatch 1D (Molecular Devices) and digitized with BNC-2110 (National Instruments) A/D board at a sampling rate of 20 kHz. Recordings were monitored and analyzed using WinLTP v2.3.19. Each specific experiment was conducted on a single slice from an animal, so the n-value reflects both the number of slices and animals used. Two independent Schaffer collateral-commissural pathways (SCCPs) were stimulated alternately to obtain the evoked synaptic responses, each at a constant baseline frequency of between 0.033 and 0.1 Hz. Following a stable baseline period of at least 20 min, LTP was induced using theta-burst stimulation (TBS) delivered at the same basal stimulus intensity. An episode of TBS comprises 5 bursts at 5 Hz, with each burst composed of 5 pulses at 100 Hz. For LTP induced by compressed TBS (cTBS), three TBS episodes were delivered with an inter-episode interval (IEI) set to obtain a sporadic observation of transmission failures but high enough to obtain a reliable estimate of y.

Whole-cell patch clamp recording. Whole-cell recording was made with AFS that contained 50 µM miconorin (Abcam) and 20 µM (+)-bicuculline (Hello Bio) to prevent GABA_A receptor mediated contribution. CA1 pyramidal cells were visualized with IR-DIC optics (Zeiss). The whole-cell solution comprised (mM): 124 NaCl, 3 KCl, 1.25 NaHCO3, 10 MgSO4, 10 D-glucose and 1 CaCl2, saturated with 95% O2 and 5% CO2. The hippocampal slices were transferred to an incubation chamber that contained the recording solution (artificial cerebrospinal fluid, ACSF; mM): 124 NaCl, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 10 MgSO4, 10 D-glucose and 2 CaCl2 (carbonated with 95% O2 and 5% CO2). Slices were allowed to recover at 32–34 °C for 30 min, and then maintained at 26–28 °C for a minimum of 1 h before recordings were made.

Plasmid vectors and lentivirus production. The following oligonucleotide sequences were used to generate single guide RNA (sgRNA) for GluA2 knockout: forward (5’ to 3’) CAGC G taacagatcagtgatggtaa (reverse (5’ to 3’) AACAC Gctatcgtactctcgtag C64. These were annealed and ligated into the lentivirus backbone developed by the Zhang lab26. The construct was modified and used with the CaMKIIa promoter for Cas9-P2A-EGFP expression. Lentivirus was produced by transfecting Lentri-X293 T cells (Takara Bio) with pMD2.G, psPAX2 and lentCRISPRe29. The 293 T cells were maintained in serum-free ULTRACULTURE media (supplemented with 4 mM L-glutamine, 2 mM Glutamate-X, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 1x penicillin/streptomycin). Three days after transfection, the supernatant was filter sterilized (0.45 µm pore membrane, Millipore) and ultracentrifuged at 110,000 × g (Beckman Coulter) with an additional sucrose filtration. The lentivirus pellet was resuspended in Dulbecco’s PBS and kept at −80 °C.

In vivo stereotactic injections and dual whole-cell recordings. The surgical procedure was performed under sterile conditions in accordance with the Institutional Animal Care and Use Committee of Seoul National University. Male C57BL/6 mice (2–3 months of age) were anesthetized by intraperitoneal injection of a ketamine (130 mg/kg body weight) and xylazine (10 mg/kg) mixture. The anesthetized mice were immobilized on a stereotactic apparatus and the lentiviral vector (80 µL of a single injection) was infused into the lateral ventricle using a 2.5 mm guide cannula stereotaxically positioned as follows: 1.7 mm anterior to bregma, 1.7 mm lateral to the sagittal suture. The guide cannula was then removed, and the injection site was thrombinized (0.05 Muhammad, Millipore) and imaged on a confocal microscope (Leica SP8).
Compounds. Drugs were prepared as frozen stock solutions (stored below −20 °C). Compounds were as follows: N,N,N-Trimethyl-5-[(tricyclo[3.3.1.37,10]octa-4,7,9-trienyl)methyl]amino|1-pentanaminium bromide hydrobromide (IEM-1460; Hello Bio); 4-(3-cyclopentoyl-4-methoxyphenyl)pyrrolidin-2-one (rolipram; Abcam); 4-[(2S)-2-[[5-isouquinolinylsulfonyl]methyl]amino]-3-etoxy-3-(4-phenyl-1-piperazinyl)propyl] phenyl isouquinolinesulfonic acid ester (KN-62; Tocris and Hello Bio); D-AP5 (Hello Bio); L-689,560 (Tocris); a catalytic subunit of protein kinase A (PKA Cat; New England Biolabs); Ca4+/calmodulin-dependent protein kinase II (CaMkII, New England Biolabs).

Statistical analysis. All treatment groups were interleaved with control experiments. Data are presented as mean ± SEM (standard error of the mean). Responses were normalized to the baseline prior to LTP induction unless otherwise stated. Statistical significance was assessed using two-tailed paired or unpaired Student’s t tests or one-way ANOVA as appropriate using Graphpad Prism 8. Adjustments were made for multiple comparisons using Bonferroni’s correction. The level of significance is denoted on the figures as follows: *p < 0.05, **p < 0.01 and ***p < 0.001.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All original data are available upon reasonable request from the authors; the values for data underlying the Figures are provided as a Source Data file. Source data are provided with this paper.

Received: 2 July 2020; Accepted: 8 December 2020; Published online: 18 January 2021

References
1. Bliss, T. V. P. & Collingridge, G. L. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39 (1993).
2. Choi, J.-H. et al. Interregional synaptic maps among engram cells underlie memory formation. Science 399, 1130–1134 (2020).
3. Josselyn, S. A. & Tonegawa, S. Memory engrams: recalling the past and predicting the future. Neuron 72, 533–540 (2012).
4. Sanderson, J. L., Gorski, J. A. & Dell’Orco, M. L. NMDA receptor-dependent LTD requires transient synaptic incorporation of Ca2+-permeable AMPARs mediated by AKAP150-anchored PKA and calcineurin. Neuron 89, 1000–1015 (2016).
5. McGlade-McCulloh, E., Yamamoto, H., Tan, S.-E., Brickey, D. A. & Soderling, T. R. Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. Nature 562, 640–642 (1993).
6. Herring, B. E. & Nicoll, R. A. Long-term potentiation: from CaMKII to AMPA receptor gating. Neuron 99, 607–620 (2019).
7. Shi, S. H. et al. Rapid spine delivery and redistribution of AMPA receptors with this paper.
8. Weisz, P. et al. Adenylyl cyclase anchoring by a kinase anchor protein AKAP150 is important for postsynaptic β-adrenergic signaling. J. Biol. Chem. 281, 19718–19731 (2006).
9. Huganir, R. L. & Nicoll, R. A. AMPARs and synaptic plasticity: the last 25 years. Neuron 80, 704–717 (2015).
10. Lisman, J., Yasuda, R. & Raghavachari, S. Mechanisms of CaMkII action in long-term potentiation. Nat. Rev. Neurosci. 13, 169–182 (2012).
11. Benke, T. A., Lüthi, A., Isaac, J. T. & Collingridge, G. L. Formation of AMPA receptor unitary conductance by synaptic activity. Nature 393, 793–797 (1998).
12. Shi, S. H. et al. Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science 284, 1811–1816 (1999).
13. Collingridge, G. L., Isaac, J. T. & Wang, Y. T. Receptor trafficking and synaptic plasticity. Nat. Rev. Neurosci. 5, 952–962 (2004).
14. Derkach, V., Barria, A. & Soderling, T. R. Ca2+-/calmodulin kinase II enhances channel conductance of alpha-amin-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc. Natl Acad. Sci. USA 96, 3269–3274 (1999).
15. Ponce, J.-C., Esteban, J. A. & Malinow, R. Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha-Ca2+/calmodulin-dependent protein kinase II. J. Neurosci. 22, 4406–4411 (2002).
16. El-Husseini, A. et al. Mechanism of Ca2+/calmodulin-dependent kinase II regulation of AMPA receptor gating. Nat. Neurosci. 14, 727–735 (2011).
17. Verdoorn, T. A., Berthelot, E., Monyer, H., Seeburg, P. H. & Sakmann, B. Structural determinants of ion flow through recombinant glutamate receptor channels. Science 252, 1715–1718 (1991).

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-20523-3 | www.nature.com/naturecommunications
44. Adesnik, H. & Nicoll, R. A. Conservation of glutamate receptor 2-containing AMPA receptors during long-term potentiation. J. Neurosci. 27, 4598–4602 (2007).
45. Gray, E. E., Fink, A. E., Sarinana, J., Vissel, B. & O’Dell, T. J. Long-term potentiation in the hippocampal CA1 region does not require insertion and activation of GluR2-lacking AMPA receptors. J. Neurophysiol. 98, 2488–2492 (2007).
46. Lu, Y. et al. Age-dependent requirement of AKAP150-anchored PKA and GluR2-lacking AMPA receptors in LTP. EMBO J. 26, 4879–4890 (2007).
47. Cizeron, M. et al. A brainwide atlas of synapses across the mouse life span. Science 369, 270–275 (2020).
48. Zhou, Z. et al. The C-terminal tails of endogenous GluA1 and GluA2 differentially contribute to hippocampal synaptic plasticity and learning. Nat. Neurosci. 21, 50–62 (2018).
49. Diering, G. H. & Huganir, R. L. The AMPA receptor code of synaptic plasticity. Neuron 100, 314–329 (2018).
50. He, K. et al. Stabilization of Ca2+-permeable AMPA receptors at perisynaptic sites by GluR1-S845 phosphorylation. Proc. Natl Acad. Sci. 106, 20033–20038 (2009).
51. Hell, J. W. How Ca2+-permeable AMPA receptors, the kinase PKA, and the phosphatase PP2B are intertwined in synaptic LTP and LTD. Sci. Signal. 9, pe2 (2016).
52. Gardner, S. M. et al. Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF. Neuron 45, 903–915 (2005).
53. Jaafari, N., Henley, J. M. & Hanley, J. G. PICK1 mediates transient synaptic expression of GluA2-lacking AMPA receptors during glycine-induced AMPA receptor trafficking. J. Neurosci. 32, 11618–11630 (2012).
54. Terashima, A. et al. Regulation of synaptic strength and AMPA receptor subunit composition by PICK1. J. Neurosci. 24, 5381–5390 (2004).
55. Morita, D., Rah, J.-C. & Isaac, J. T. R. Incorporation of inwardly rectifying AMPA receptors at silent synapses during hippocampal long-term potentiation. Philos. Trans. R. Soc. B 369, 20130156 (2014).
56. Liu, S. Q. & Cull-Candy, S. G. Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Proc. Natl Acad. Sci. USA 102, 12230–12235 (2005).
57. Noh, K.-M. et al. Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. Proc. Natl Acad. Sci. USA 102, 3881–3886 (2005).
58. Isaac, J. T., Nicoll, R. A. & Malenka, R. C. Evidence for silent synapses: implications for the expression of LTP. Neuron 15, 427–434 (1995).
59. Liao, D., Hessler, N. A. & Malinow, R. Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature 375, 400–404 (1995).
60. Nonaka, M. et al. Everyday memory: towards a translationally effective method of modelling the encoding, forgetting and enhancement of memory. Eur. J. Neurosci. 46, 1937–1953 (2017).
61. Hu, H. et al. Emotion enhances learning via noradrenaline regulation of AMPA receptor trafficking. Cell 131, 160–173 (2007).
62. Takeuchi, T. et al. Locus coeruleus and dopaminergic consolidation of everyday memory. Nature 537, 357–362 (2016).
63. Anderson, W. W. & Collingridge, G. L. Capabilities of the WntLTP data acquisition program extending beyond basic LTP experimental functions. J. Neurosci. Methods 162, 346–356 (2007).
64. Incontro, S., Asensio, C. S., Edwards, R. H. & Nicoll, R. A. Efficient, complete deletion of synaptic proteins using CRISPR. Neuron 83, 1051–1057 (2014).
65. Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. Nat. Methods 11, 783–784 (2014).

Acknowledgements

This work was supported by the MRC, ERC, CIHR Foundation Grant #154276 (G.L.C.) and Brain Canada Foundation (G.L.C.) through the Canada Brain Research Fund, with the financial support of Health Canada. G.L.C. is the holder of the Krembil Family Chair in Alzheimer’s Research. This work was also supported by The National Honor Scientist Program (NRF-2012R1A3A1050385) of Korea (B.-K.K.).

Author contributions

P.P. and G.L.C. conceived the study. P.P. carried out the experimental work. K.-H.K. and J.-i. K. helped with CRISPR experiments. T.M.S., Z.A.B., H.K., M.Z. and B.-K.K. provided advice and technical and/or financial support. P.P., J.G., C.A.B. and G.L.C. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-20523-3.

Correspondence and requests for materials should be addressed to G.L.C.

Peer review information Nature Communications thanks Graham Diering, Johannes Hell, and other, anonymous, reviewers for their contributions to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021