Supplemental Material:

- Text: -Supplemental Results
- Supplemental Figures S1-S7
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- References for the Supplemental Information

Supplemental Results

Three plausible mechanisms for substrate-dependence of autonomy ruled out

Several possible mechanisms for substrate-dependence of autonomy were considered. The difference in autonomy toward AC2 and syntide2 could be caused by an effect on stimulated activity. Indeed, AC2 and syntide2 differed more in stimulated than in autonomous phosphorylation rates (Fig. 2A). However, two other substrates with similar or lower stimulated phosphorylation than AC2 showed the same autonomy as syntide2 (~25%)(Fig. 2B). Thus, stimulated activity was not a predictor of autonomy. The difference in autonomy could be caused by differential effects of pre-phosphorylation at T286 on stimulated maximal activities (increased for AC2 and/or decreased for syntide2). However, Fig. 2C demonstrates that this was not the case; this was further validated by a comparison of CaMKII wild type to the non-phosphorylatable T286A mutant (Fig. 2D). Importantly, the auto-phosphorylation conditions in Fig. 2C induced pre-phosphorylation of T286 but not T305, which would decrease stimulated activity by interfering with CaM binding (1-3). However, presence of AC2 could enhance T305 auto-phosphorylation during the activity assay, and thereby decrease stimulated activity and indirectly enhance autonomy. However, T305 phosphorylation under stimulated activity assay conditions was low and further decreased by both substrate peptides, and actually even more so by AC2 than by syntide2 (Fig. 2E,F). Thus, taken together, substrate-dependent autonomy was a function of autonomous, but not stimulated CaMKII activities. Elevated autonomy towards AC2

was found to depend on additional T-site interaction, while autonomy towards "regular" substrates was consistently low (Fig. 1).

Substrate-concentration dependence of CaMKII autonomy.

Substrate-concentration dependence of stimulated and autonomous CaMKII activity was assessed for syntide2 and AC2 (Fig. 5A) as well as hAKb-nn and th19 (Fig. S4), two peptides derived from human AKAP79 and tyrosine hydroxylase, respectively (see Table S2). Two results are shown in the traditional linear double-reciprocal Lineweaver-Burke plot (4)(Fig. 5A, S4). However, the calculation of Vmax and Km based on linear regression in a double-reciprocal plot is error prone. Thus, for the actual calculation of Vmax and Km, the more reliable method of non-linear regression in a non-reciprocal plot was used. The functions shown in Fig. 5A and S4 are based on these values (see also Table S2).

With known kinetic parameters for stimulated and autonomous activity (Table S2), CaMKII autonomy as function of substrate concentration (as in Fig. 5B) can be calculated based on the Michaelis-Menten equation:

$$V = V\max \cdot \left(\frac{[S]}{([S] + Km)} \right)$$

Then, autonomy (expressed as ratio instead of %) is:

$$Aut = \frac{Vaut}{Vstim} = \frac{V\max a}{V\max s} \cdot \frac{\left(\frac{[S]}{([S] + Kma)} \right)}{\left(\frac{[S]}{([S] + Kms)} \right)} = \frac{V\max a}{V\max s} \cdot \frac{([S] + Kms)}{([S] + Kma)} = rV\max \cdot \frac{([S] + Kms)}{([S] + (rKm \cdot Kms))}$$

(with $rV\max = \frac{V\max a}{V\max s}$ and $rKm = \frac{Kma}{Kms}$)

resulting in a sigmoid function between minimal and maximal asymptotes, defined as:

$$Aut\min = \frac{rV\max}{rKm} \qquad \text{and} \qquad Aut\max = rV\max$$

Thus, with a change in both Vmax and Km (Fig. 5A, S4), CaMKII autonomy is substrate concentration-dependent, but remains near constant both in the low and in the high range of substrate concentration, with a significant change only within a relatively narrow range between (Fig. 5B). It is noteworthy that the cellular concentration of virtually all substrates proteins falls into the low stable range observed here for all “regular” substrates. However, autonomy in the high stable range could occur within cells based on optimized co-localization of kinase and substrate.

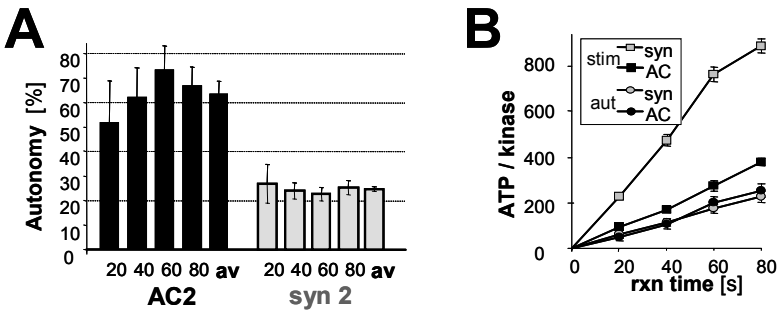

FIGURE S1. **CaMKII autonomy is substrate dependent**.
A, Degree of CaMKII autonomy towards AC2 and syntide2 at different reaction times, from 20 to 80 seconds (compare Fig. 1D).
B, Phosphorylation rates of AC2 and syntide by stimulated and autonomous CaMKII in the timecourse shown in panel A and Fig. 1D. Error bars indicate s.e.m in all panels.

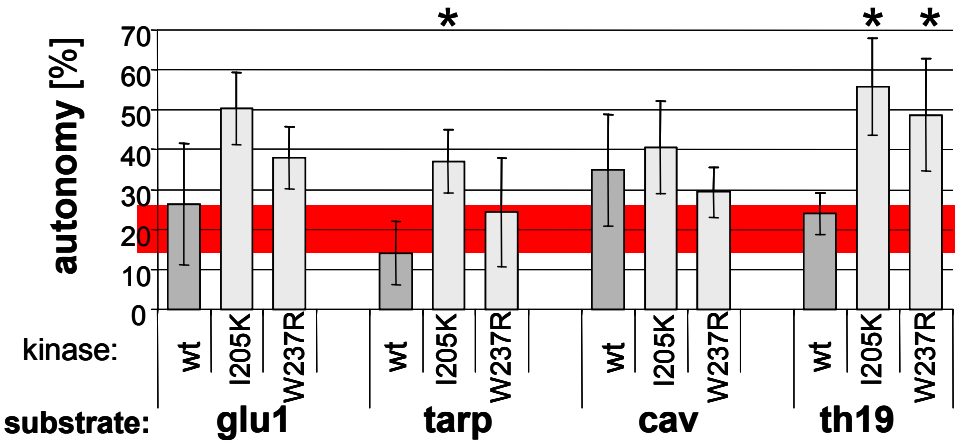

FIGURE S2. **The T-site mutations I205K and W237 did not reduce autonomy of mGFP-CaMKIIα** (10 nM) **towards several "regular" substrates** (75 μM). This is in contrast to the reduction seen for AC2 substrate, but similar to syntide2, another regular substrate (see Fig. 3D). For regular substrates, there was a general trend towards increased autonomy by the mutations, which became statistically significant in some cases (asterisks; $p<0.05$). If any, this further enhances the conclusion regarding T-site dependence of the high autonomy towards AC2. The reverse effect on autonomy towards regular substrates by the T-site mutations is consistent with reduced inhibitory interaction with the region around T286 (which binds to the T-site in the basal state). Details on the substrate peptides examined in this Figure are provided in Table S1. The autonomy range of 15-25% expected for regular substrates is indicated by a red bar. Compared to AC2, rate of stimulated phosphorylation of these peptides was 20-40 fold lower.

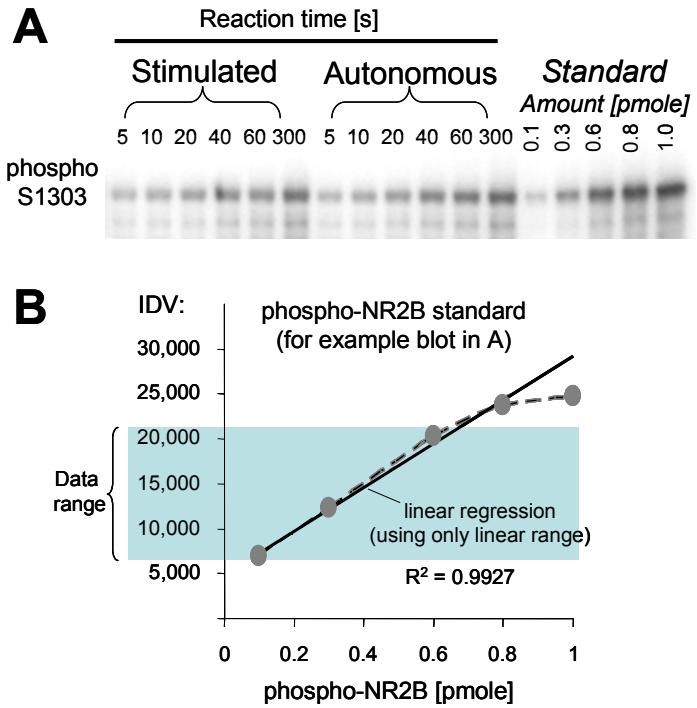

FIGURE S3. **NR2B Phospho-S1303 detection was in the linear range of the Western blot analyses,** demonstrating that it was indeed the reaction, not the detection, that was not in the linear range in Fig. 3.

A, Phosphorylation of GST-NR2B-c (a GST fusion protein with cytoplasmic C-terminus of NR2B; 0.5 μM) at S1303 by stimulated and autonomous CaMKII (5 nM) activity after different reaction times, as determined by Western-blot analysis with a phospho-S1303 specific antibody. For each reaction, 0.6 pmole NR2B was loaded. The standard (NR2B phosphorylated for 30 min at 30°C with CaMKII) covered 0.1-1.0 pmole, and allowed comparison between blots and quantification.

B, The phopho-NR2B standard was in the linear range between 0.1 and 0.8 pmole. In order to determine the phospho-NR2B immuno detection value (IDV), the background immunoreaction with 0.6 pmole un-phosphorylated NR2B (~1/3 of the raw IDV of the 5 s reaction time) was subtracted for each sample; for each standard, a weighted background (according to amount in the standard lane) was subtracted. As all data values were below the 0.8 pmole standard, the 1.0 pmole was not included in the calculation of the linear regression used to calculate the phopho-NR2B pmole equivalents in the samples.

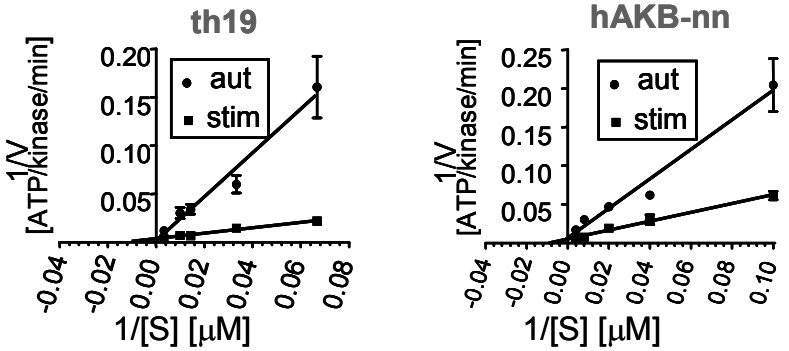

FIGURE S4. **Double reciprocal plot of phosphorylation rate** as function of substrate concentration for stimulated and autonomous CaMKII activity, for the substrates hAKb-nn (derived from human AKAP79) and th19 (derived from tyrosine hydroxylase; see also Table S1). As for the regular substrate syntide2, in both cases autonomous CaMKII activity showed lower Vmax and higher Km than stimulated activity (compare also Fig. 5 and Table S2).

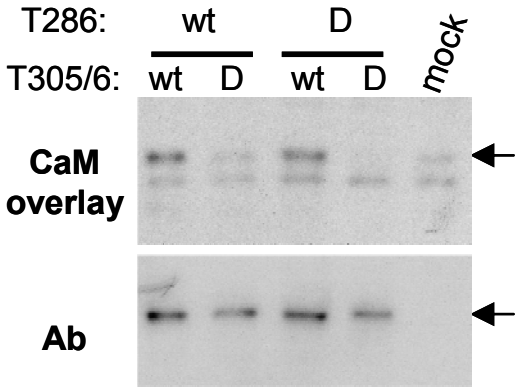

FIGURE S5**. CaM binding to CaMKII is impaired by T305/306D mutation,** as assessed in an overlay assay with biotinylated CaM (upper panel). Extracts from mock-transfected HEK cells were loaded as negative control. Equal amount of GFP-CaMKII (arrow) were measured by GFP fluorescence in the extracts, and verified by be re-probing the blot with an anti-GFP antibody (Ab; lower panel).

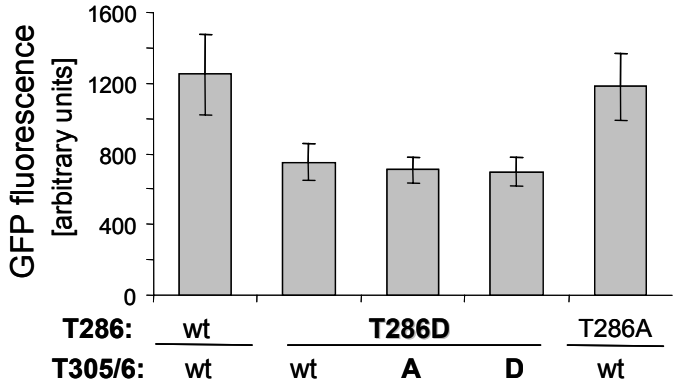

FIGURE S6**. Relative expression levels of GFP-CaMKIIα mutants in differentiated PC12 cells,** as assessed by average GFP fluorescence intensities in individual transfected cells (n=20 to 23; see Fig. 7A for example picture). The three constructs containing the T286D mutant show identical expression levels, but ~1/3 less than CaMKII wild type and the T286A mutant (ANOVA with Bonferroni post-hoc analysis). Error bars show s.e.m.

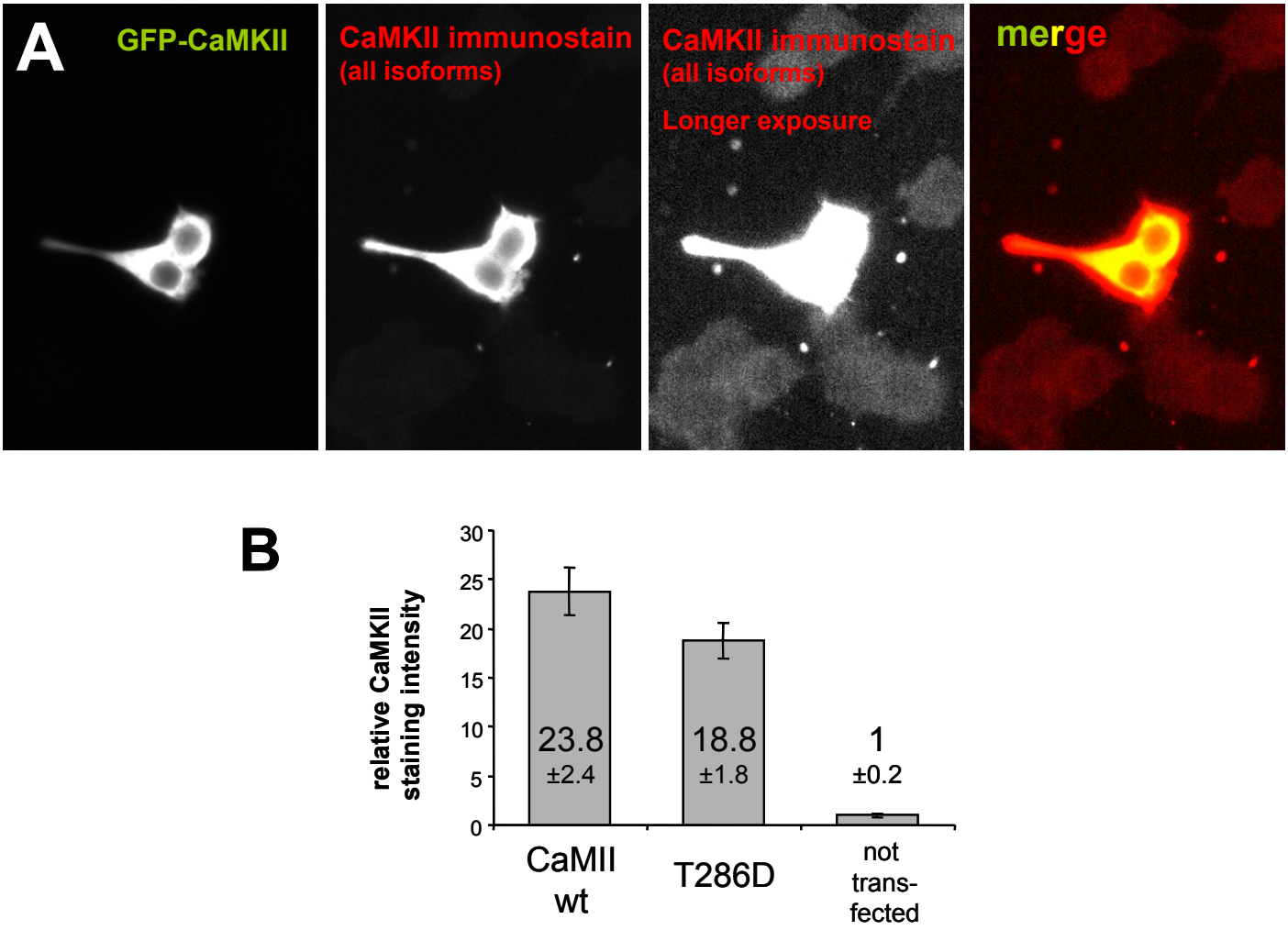

FIGURE S7**. Levels of overexpressed versus endogenous CaMKII in differentiated PC12 cells,** as assessed by immunocytochemistry of transfected cells compared to their untransfected neighbors.

A, Example micrographs. Transfected PC12 were identified by GFP fluorescence (left panel). PC12 cells were stained with an antibody against all CaMKII isoforms (BD Biosciences; middle panels). Visualization of untransfected cells required longer exposures (right panels).

B, Quantification of CaMKII immunostaining from experiments as shown in panel A showed that transfection of PC12 resulted in ~20fold overexpression of GFP-CaMKII over endogenous CaMKII expression.

TABLE S1. **The CaMKII substrates used in this study, and their functions.**

Peptide	Sequence	Protein site	Protein Function	Phosphorylation Function	References
AC2	KKALRRQE**T**VDAL	CaMKII T286	Protein kinase	Generates CaMKII autonomy	(5-10)
syn2	PLARTL**S**VAGLPGKK	GS S8	Glycogen synthase	Inhibits activity	(11,12)
NR2B	(190 kDa protein)	S1303	NMDA-receptor subunit	Regulation of channel desensitization	(13,14)
MAP2	(200 kDa protein)	> 10 sites	Microtubule regulation	Regulation of microtubule stability	(15-17)
hAKb-wt	A***W***AS***L***KRLV**T**RRKRSE**S**SK	AKAP79 T87	PKA, PKC, PP2B targeting	Interferes with CaM and F-actin binding	(18-21)
-“ - -nn	A***N***AS***N***KRLV**T**RRKRSE**S**SK	-“ -			
-“ - -np	A***N***AS***P***KRLV**T**RRKRSE**S**SK	-“ -			
glu1	LIPQQ**S**INEAI	GluR1 S831	AMPA-receptor subunit	Increases single channel conductance	(22,23)
tarp	TEASPSRDA**S**PVGL	Tarp γ-4 S259	AMPA-receptor regulator	Increase of synaptic strength	(24)
cav	YLTRDW**S**ILGP	CaV1.2 S439	L-type Ca^{2+} channel	Mode 2 gating; Timothy Syndrome	(25,26)
th19	AKGFRRAV**S**ELDA	TH S19	Tyrosine hydroxylase	Increases activity, the rate-limiting step in dopamine synthesis	(27,28)
Consensus CaMKII site:	*h*x R *n* x **S**/**T** *h* *h*: hydrophobic *n*: non-basic (not R or K) x: any				

TABLE S2. **Kinetic parameters used for the plot of CaMKII autonomy** as a function of substrate concentration (in Fig. 5B)

Parameter	Substrate:			
	Syntide-2	**AC2**	**th19**	**hAKb-nn**
*rV*max	0.449	0.722	0.506	0.426
rKm	1.991	1.245	2.995	1.524
Km,stim	26.3 μM	8.9 μM	126 μM	151 μM

Km,stim represents the Km of the subtrate for Ca^{2+}/CaM-stimulated CaMKII activity. The prefix *r* indicates the ratio of the autonomous to the stimulated value. The values for Syntide2 are based on four independent experiments. While the values for the autonomous and stimulated Vmax varied between experimental days, their ratio (*rV*max) was very consistent.

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