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A local sensitivity analysis of Ca^{2+}-calmodulin binding and its influence over PP1 activity

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Abstract: Synapses are the site of signal transmission between neurons (neurotransmission). Long term synaptic plasticity refers to synaptic efficacy being modulated in response to neurotransmission events. This change can last from minutes to years. In postsynaptic excitatory synapses, increasing synaptic efficacy (known as long term potentiation (LTP)) is a molecular mechanism of memory formation, while decreasing efficacy (long term depression (LTD)) induces memory loss. Postsynaptic LTP and LTD are modulated when Ca^{2+} fluxes into postsynaptic neurons and binds to the signal transducer protein called calmodulin (CaM). Upon binding four Ca^{2+} ions, CaM becomes active (as CaM_4) and integrates the signal. That is, CaM_4 can bind and activate proteins that pertain LTP (kinases), and also protein phosphatases which induce LTD. The ratio of kinase: phosphatase activity determines the direction of synaptic plasticity. Interestingly, activity of the key LTD- inducing protein, "protein phosphatase 1" (PP1) is coordinated by other CaM_4- sensitive proteins. PP1 is inactive when bound to protein inhibitor 1 (i-1). Phosphorylation of i-1 prevents it from dissociating from PP1, thereby blocking its activity. At basal Ca^{2+}, i-1 is phosphorylated due to kinase activity, meaning PP1 activity gets blocked. Synaptic activity can then cause intracellular Ca^{2+} influx to the cell which alters the phosphorylation state of i-1. This is because Ca^{2+} binds CaM, then CaM_4 activates "protein kinase A" (PKA) and "protein phosphatase 2B" (PP2B) which phosphorylate and dephosphorylate i-1 respectively. These proteins thereby modulate PP1 activity by collectively controlling the phosphorylation status of i-1. When Ca^{2+} levels begin to rise, PP2B gets preferential activation due to having a faster CaM_4 binding rates than PKA, resulting in net dephosphorylation of i-1 and subsequent activation of PP1. If CaM_4 levels escalates further, more PKA gets activated which has greater catalytic activity than PP2B. This means increased phosphorylation of i-1 and subsequent suppression of PP1. PP1 activity can therefore be modelled as a function of CaM_4 formation, meaning its activation is affected by CaM binding to Ca^{2+}. CaM has two separate lobes (the N- and C-lobes) which have different mechanisms of binding cooperativity to Ca^{2+}. In the current study, mathematical modelling is used to determine to influence each of these CaM cooperativities/binding rates. Here, a detailed Ca^{2+}-CaM binding model based on Mass Action fed into simplified Hill equations of PKA/PP2B regulation over PP1 activity were used. To determine the influence of each lobe, Ca^{2+} binding/unbinding rates to CaM, a local sensitivity analysis (LSA) was performed over a range of Ca^{2+} concentrations. Since PP1 activity is dependent on CaM_4 formation, PP1 activation was used as an output for the LSA. From the analysis, it is evident that C-lobe has more stable binding than the N-lobe across all Ca^{2+} levels.

Keywords: *Calcium, Calmodulin, signal detection, computational biology, local sensitivity analysis*

1. INTRODUCTION

NMDAR- dependent synaptic plasticity refers to increasing (long term potentiation (LTP)) or decreasing (long term depression (LTD) efficacy of neurotransmission in response to Ca^{2+} flux into neurons through NMDAR channels. Upon entry into the postsynaptic neuron, four Ca^{2+} ions can bind and activate the secondary signalling protein, calmodulin (CaM) to form CaM_4. CaM_4 can bind to and activate proteins which induce synaptic plasticity (Xia & Storm, 2005; He *et al.,* 2014). The direction of synaptic plasticity is determined by the binding partners of active CaM_4. That is, a relatively low level of CaM_4 will bind and preferentially activate LTD inducing proteins called phosphatases since they have faster binding rates (Török, 2002; He *et al.,* 2016; Xia & Storm, 2005). Higher level of active CaM_4 will activate kinases which have a lower binding rate but increased activity and affinity (lower dissociation rates). The direction of synaptic plasticity depends upon the relative activation of LTD verses LTP inducing proteins which is a function of CaM_4 concentration, meaning CaM binding dynamics with Ca^{2+} is central to understanding synaptic plasticity signalling in neurons (Xia & Storm, 2005).

CaM contains the two lobes (N- and C-lobe) which each binds to two Ca^{2+} ions independent of each other. When Ca^{2+} binds to a lobe, the neighbouring Ca^{2+} binding site of the lobe switches state from a tense (T) to relaxed (R) state (Faas *et al.,* 2011). This switch in state gives rise to positive cooperativity. For the N-lobe, the R-state has approximately 40-fold increase in Ca^{2+} binding (one of the highest known binding rates found in biomolecules); while the dissociation rate of the R state is similar to that of T-state. By contrast, for the C-lobe, the binding rate of Ca^{2+} to the R state changes little; its dissociation rate however decreases by ~ 400 fold. This means when C-lobe contains two Ca^{2+} ions, Ca^{2+} binding more stable on the lobe. The N-lobe, although less stable, can bind to Ca^{2+} rapidly once one of its binding sites are bound to Ca^{2+} provided there is an adequate concentration of Ca^{2+} in the postsynaptic synapse to occupy the site.

A 9-state Ca^{2+}/CaM binding model involving all C- and N-lobe Ca^{2+} bound states was used to determine which was fed into a simplified CaM_4- dependent protein kinase A (PKA)/ protein phosphatase 2B (PP2B) feedback regulation loop over PP1 was used. PKA and PP2B are both controlled by CaM_4- PP2B is directly controlled by CaM; while PKA is regulated by cAMP, which in-turn is controlled by CaM-sensitive AC1/8 (Tang & Gilman, 1992).The role of active PKA and PP2B is to control the phosphorylation and dephosphorylation of protein inhibitor 1 (i-1) respectively (Nairn & Shenolikar, 1992). When i-1 is phosphorylated by PKA, PP1 activity is shielded as it binds to it with high affinity and blocks its activity whereas dephosphorylation if i-1 by PP2B allows for PP1 to dissociate from i-1 and become active in the cell.

The purpose of the study was to determine how the N- and C- lobe binding/unbinding rates influence CaM_4 formation at a range of Ca^{2+} levels; and determine how each lobe differentially binds to Ca^{2+} depending on Ca^{2+} concentration. A local sensitivity analysis was used to determine which kinetic rates of the Ca^{2+}-CaM binding model have the most influence on CaM_4 and therefore PP1 activation. The main conclusion of the study was that the N-lobe is more sensitive to perturbation than does the C-lobe. Specifically, N-lobe binding which is most rate-limiting in CaM_4 formation. Evidently, the C-lobe preferentially binds Ca^{2+} to the N-lobe across all Ca^{2+} levels.

2. METHODS

The current study includes a 9-state Ca^{2+}/CaM binding model involving all C- and N-lobe Ca^{2+} bound states. The model kinetic parameters of the model are based on mass action kinetics and the rates are taken from Faas *et al.* (2011). In the model, binding of Ca^{2+} to a lobe changes the neighbouring site from the T to the R state which has different binding kinetics. CaM_4 was then fed into a simplified model of the key LTD-inducing protein, protein phosphatase 1 (PP1) (see Figure 1 for a schematic).

2.1.1. First Ca^{2+} binding/unbinding (to the tense (T) state):

$$2 \bullet kon\_(N/C)T \bullet [T_{N/C}\ T_N] \bullet [Ca^{2+}] \rightarrow [Ca_{N/C}\ R_{N/C}]\ (\textit{binding}) \quad (1)$$

$$koff\_(N/C)T \bullet [Ca_{N/C}\ R_{N/C}] \rightarrow [T_{N/C}\ T_{N/C}]\ (\textit{unbinding}) \quad (2)$$

2.1.2. Second Ca^{2+} binding/unbinding (relaxed (R) lobe state):

$$kon\_\ R\,(N/C) \bullet [Ca^{2+}] \bullet [Ca_{N/C}\ R_{N/C}] \rightarrow Ca_{N/C}\ Ca_{N/C} \quad (3)$$

$$2 \bullet koff\_R(N/C) \bullet [Ca_{N/C}\ Ca_{N/C}] \rightarrow [Ca_{N/C}\ R_{N/C}] \quad (4)$$

In equations 1-4, kon and koff represents Ca^{2+} binding and dissociation respectively; the N/C represent either the N or C lobes of CaM; $Ca_{N/C}$ represent Ca^{2+}-bound sites of CaM. T and R represent the tense and relaxed states of the unoccupied site. The two lobes are assumed to bind Ca^{2+} independent of each other. The output of the Ca^{2+}-CaM model is that every site is Ca^{2+}-bound (as CaM_4) (see Figure 1).

2.2. PP1 regulation model

The output of the Ca^{2+}-CaM model, CaM_4, fed into Hill equations of PKA/PP2B regulation over PP1 activity (Graupner & Brunel, 207). This regulation is modelled using simplifications of PP1 activation (Graupner & Brunel, 2007). In the model, PP2B requires less CaM_4 than PKA to get activated (Graupner & Brunel, 2012). As CaM_4 concentration increases further, more PKA gets activated. Since PKA has a higher catalytic activity than PP2B, there is net phosphorylation of i-1 and subsequent suppression of PP1. Essentially the phosphorylation status is determined by PP2B: PKA activity ratio, and that ratio is determined by CaM_4 concentration. PP1 activation therefore has a parabolic response as a function of CaM_4 concentration (see Figure 2).

The Ca^{2+} binding model acts as an input for the PP1 activation loop (Graupner & Brunel, 2007). Here, Hills equations are used to represent the phosphorylation and dephosphorylation of i-1 in response to CaM_4 concentration. Upon dephosphorylation of i-1, mass action kinetics are used to model PP1 dissociation from i-1. Dissociation from i-1 causes PP1 to become active. The phosphorylation and dephosphorylation of i-1 by PP2B and PKA was modelled by the following equations:

$$V_{PP2B} = basal_{PP2B} + \frac{PP2B_{max}(\frac{[CaM_4]}{KPP2B})^{nPP2B}}{1+(\frac{CaM_4}{KPP2B})^{nPP2B}} \quad (5)$$

$$V_{PKA} = basal_{PKA} + \frac{PKA_{max}(\frac{[CaM_4]}{KPKA})^{nPKA}}{1+(\frac{[CaM_4]}{KPP2B})^{nPKA}} \quad (6)$$

Here V_{PP2B} represents PP2B total activity; $basal_{PP2B}$ and $basal_{PKA}$ are the basal rates of PP2B and PKA respectively; KPP2B and KPKA are the half activation energy for PP2B and PKA as a function of CaM_4 concentration (which is represented by CaM_4..nPP2B and nPKA are the Hill coefficients. he full differential equation for i-1 phosphorylation and subsequent PP1 activation is as follows:

$$\frac{d[I1_p]}{dt} = -kon_{PP1} \bullet [I1_p][PP1_{act}] + koff_{PP1}[PP1_{act}] - V_{PP2B} \bullet [i1_p] + V_{PKA} \bullet [i1] \quad (7)$$

$$\frac{d[PP1_{act}]}{dt} = kon_{PP1} \bullet [I1_p][PP1_{act}] - koff_{PP1} \bullet [PP1_{act}] \quad (8)$$

Here [i1p] represents phosphorylated i-1 concentration, and [PP1act] is active PP1 concentration. Kon_{PP1} and $koff_{PP1}$ are binding and unbinding of between PP1 and i-1.

2.3 Local sensitivity analysis:

PP1 activation was used as an output for CaM_4 formation since it is controlled by PP2B and PKA (see Figure 1). This is because PP1 activity is determined by the phosphorylation status of i-1 which is controlled by CaM_4 concentration due to activation of PP2B and PKA (as per the PP1 activation equation model). To determine the sensitivity of the Ca^{2+}-CaM binding/unbinding rates as a function of Ca2+ concentration, an LSA was performed. Here, all Ca^{2+}-CaM parameters was perturbed to two values, 10% and 190% of its standard value, one at a time. This was done to determine maximum and minimum ranges of each parameter. For each perturbation, the model was run at until steady state at a range of Ca^{2+} values to determine the relationship between CaM_4 formation and Ca^{2+} concentration and determine how binding kinetics affect Ca^{2+} binding stability. By performing an LSA of PP1 activation over a range of Ca^{2+} concentrations, the effects that each kinetic rate has over CaM_4 formation and therefore PP1 activity were explored. To quantify the influence of each parameter perturbation had on PP1 activation, equation (9) was used:

$$V_j = \frac{\frac{(active\ PP1^{max}_{new}) - (active\ PP1^{max}_{standard})}{(active\ PP1^{max}_{standard})}}{\frac{(k_{new} - k_{standard})}{(k_{standard})}} \quad (9)$$

Where $(active\ PP1^{max}_{new})$ represents the Ca^{2+} concentration at which PP1 activation peaked when parameters were changed; $(active\ PP1^{max}_{standard})$ represents Ca^{2+} when peak active PP1 occurs under standard conditions;

(k_{new})is the value of the changed parameter; and $k_{standard}$is the value of the parameter of interest under standard conditions. The LSA results were then ranked according to V_j.

The transition from unbound CaM to the formation of CaM_4 depends upon the stability of Ca^{2+} binding to each site. This is determined by the ratio of binding: unbinding kinetic rates of CaM as a function of Ca^{2+} concentration. PP1 activation is key for the induction of LTD so was used as an output to demonstrate a functional consequence of Ca^{2+}-CaM binding. A consequence of increased Ca^{2+} sensitivity results in PKA activation at lower concentrations, leading to PP1 suppression at lower Ca^{2+} ranges. By performing an LSA of PP1 activation the effects that each kinetic rate has over CaM_4 formation and therefore PP1 activity were explored was a function of CaM_4 formation.

3. RESULTS

3.1. Comparison of N- and C-lobe binding using simplified model

Since every Ca^{2+} binding site must be occupied for CaM_4 to form, cooperativity occurs in the sense that whichever binding site is most limiting will be most sensitive to perturbation. Before performing an LSA, a simplified version of lobe binding was used. In this model, the N- and C- lobes were treated as separate molecules which bound to Ca^{2+} independently. Here the lobes would effectively compete for Ca^{2+}. This was done to determine which lobe preferentially bound Ca^{2+} and therefore which lobe limits CaM_4 forming. The analysis revealed that the C-lobe binds to more Ca^{2+} than the N-lobe across all Ca^{2+} levels (see Figure 3). This suggests that N-lobe parameters can be expected to be most sensitive.

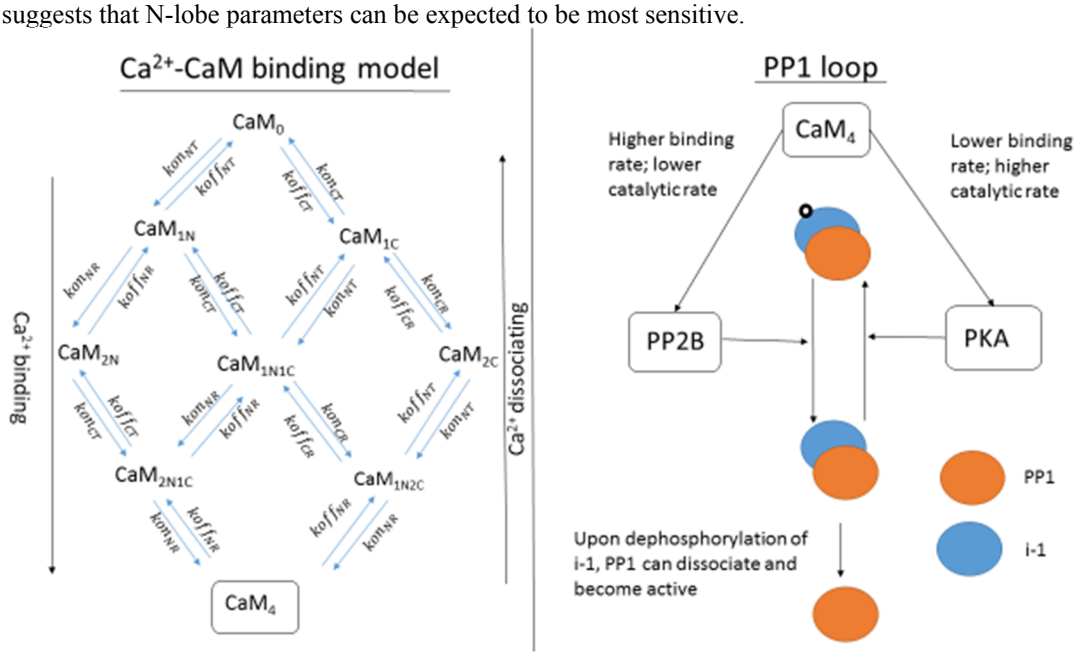

Figure 1. A schematic diagram of the reactions of the model used. On the left is the Ca^{2+}-CaM binding model. Here CaM_o represents CaM bound to no Ca^{2+}; $CaM_{1N/2N/1C/2C}$ represent 1 and 2 Ca^{2+} ions bound to the N- or C- lobe, while CaM_4, the output of the model represents fully bound CaM. kon and koff represents Ca^{2+} binding and dissociation respectively, the subscripts after represent either the T or R state in which the Ca^{2+} is binding or unbinding. CaM_4 then feeds into the PP1 model. Here at lower concentrations, CaM4 activates PP2B which dephosphorylates i-1. As CaM_4 increases further, PKA becomes activated which phosphorylates i-1. When i-1 is phosphorylated, it binds to and inhibits PP1. Dephosphorylation of i-1 destabilises its interaction with PP1, leading to PP1 becoming available/active.

Table 1. List of parameters and reaction rates

Parameters values of the Ca2+-CaM and PP1 loop models			
Ca^{2+}-CaM binding model parameters[1]		PP1 loop parameters[2]	
kon_NT	770 (μM^{-1} s^{-1})	KPP2B	0.0053 (μM)
koff_NT	160000 (s^{-1})	nPP2B	3
kon_NR	32000 (μM^{-1} s^{-1})	koff_PP1	0.1 (s^{-1})
koff_NR	22000 (s^{-1})	kon_PP1	500 (μM^{-1} s^{-1})
kon_CT	84 (μM^{-1} s^{-1})	$basal_{PKA}$	0.00359 (s^{-1})

koff_CT	2600 (s^{-1})	PKA_{max}	100 (s^{-1})
kon_CR	25 (μM^{-1} s^{-1})	KPKA	0.11 (μM)
[1]Faas *et al.* (2011) [2]Graupner & Brunel (2012).		nPKA	8
		KPKA	0.11 (μM)
		$basal_{PP2B}$	0.1 (s^{-1})
		$PP2B_{max}$	18 (s^{-1})

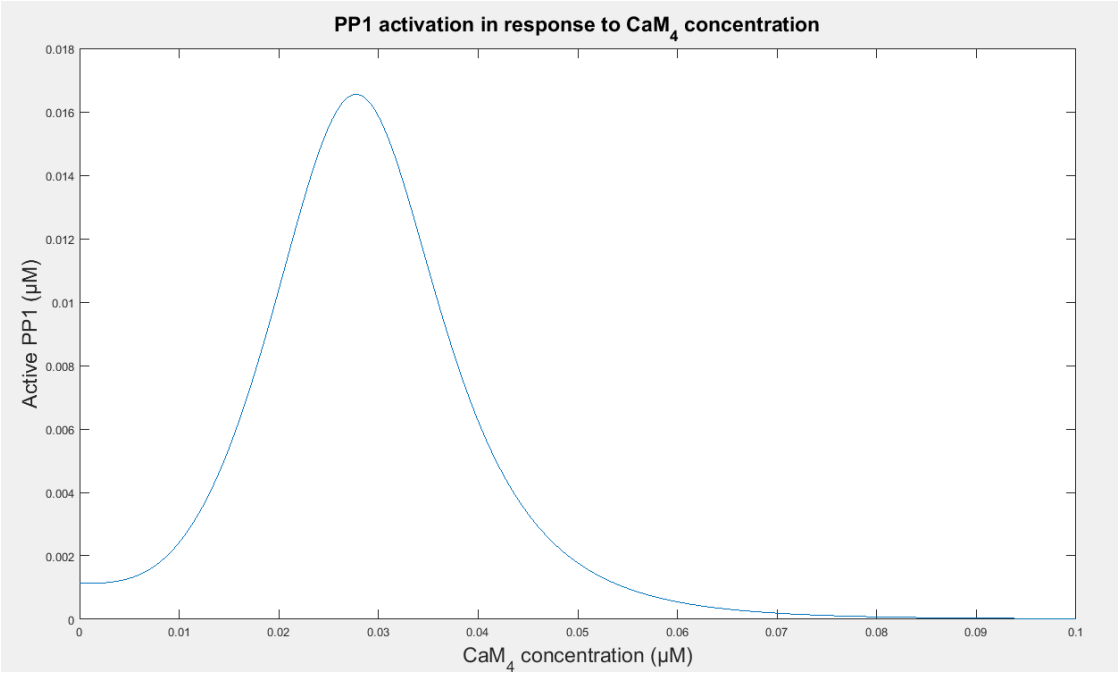

Figure 2. PP1 activation over a range of CaM_4 concentrations. At lower CaM_4 concentrations, basal PKA activity suppresses activity. As CaM_4 concentration rises, PP2B gets activated which increases concentration of active PP1. Once CaM_4 reaches a threshold, PKA then gets preferentially activated which explains why PP1 activity decreases as CaM_4 increases further.

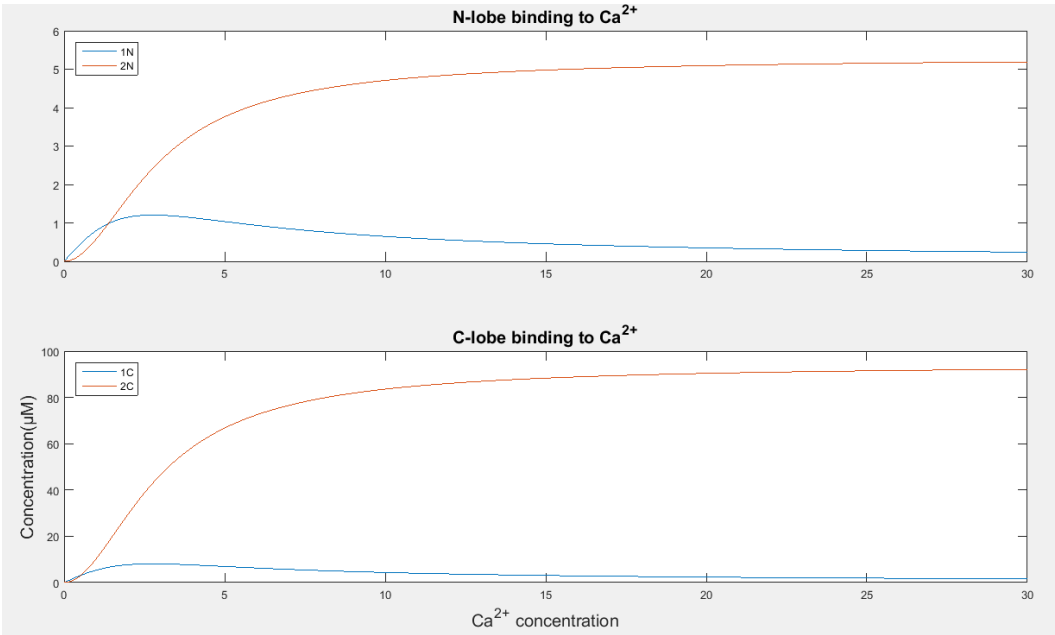

Figure 3. Results of a simplified model of Ca^{2+} binding model. Evidently, the C-lobe preferentially binds to Ca^{2+} compared to the N-lobe across all Ca^{2+} concentrations.

3.3 LSA results

The LSA results showed disparities between increasing and decreasing rates. For perturbation of Ca^{2+} binding rates, each parameter was more sensitive to decrease than to increase of its rate. For example, kon_NR had a Vj of -2.35 when its rate was 10% of its standard rate, compared to Vj of 0.3876 when 190% of its standard value. The reason for this disparity is that when binding rates are increased, other binding rates become limiting since Ca^{2+} must occupy all CaM binding sites to become active.

For the dissociation rates there were disparities between increasing and decreasing rates. Here increasing the dissociation rates were more sensitive than decreasing them. Like the binding rates, this is because increasing dissociation rates destabilises of the binding/unbinding ratio for Ca^{2+}, thus limiting CaM_4 formation. Meanwhile, decreasing dissociation rates leads to other rates becoming limiting in the formation of fully bound CaM_4. One exception to this rule was the dissociation of Ca^{2+} from the T state of the C-lobe it showed to have the same sensitivity to perturbation in both directions showing equal robustness to both increase and decrease of its rate.

In all instances the N-lobe was more sensitive to perturbation than the C-lobe of increasing and decreasing rates of respective T and R states. The four most sensitive parameters in the model were decreasing N-lobe binding and dissociation rates. Specifically, the decreasing the binding rates of the N-lobe had the highest V_j scores (see Table 2 and Figure 3). The C-lobe on the other hand, proved to be robust against perturbation, especially its dissociation rates, which were the least sensitive parameters in the model when they were decreased.

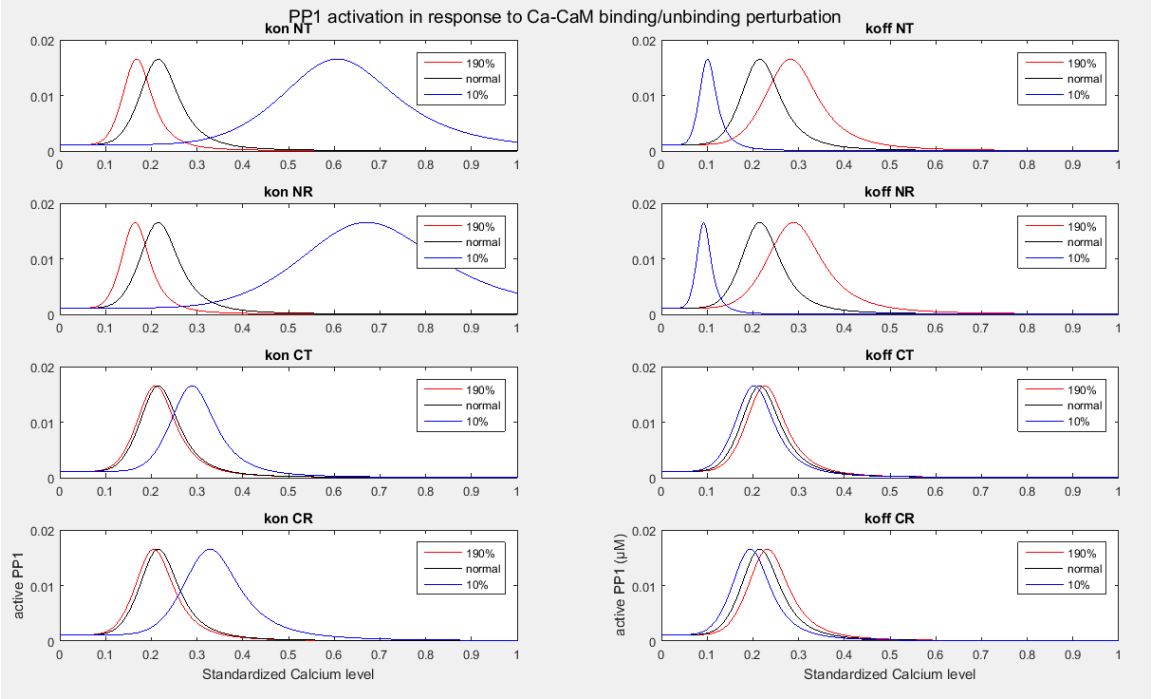

Figure 1. LSA results. Here each parameter is labelled above each plot and is varied to 190% and 10% of its basal value. Each of the curves represent PP1 activation and how that changes upon altering each parameter. A shift of the peak to the left implies CaM_4 formation occurs at lower concentrations, while a shift to the right implies CaM requires more Ca^{2+} to reach the CaM_4 concentration required for PP1 peak activity.

Table 2. LSA variation results with respect to PP1 activation ('=' means ranks are equal). LSA rank refers to the ranking of Vj score.

Parameter name (10% of standard value)	V_j	LSA rank	Parameter name (190% of standard value)	V_j	LSA rank
kon_NR	-2.35142	1	koff_NR (+90%)	0.3876	6=
kon_NT	-2.02842	2	koff_NT (+90%)	0.348837	8
koff_NR	0.633075	3	kon_NR (+90%)	-0.2584	9
koff_NT	0.594315	4	kon_NT (+90%)	-0.24548	10

kon_CR	-0.5814	5	koff_CR (+90%)	0.090439	11
kon_CT	-0.3876	6=	koff_CT (+90%)	0.064599	13=
koff_CR	0.103359	11	kon_CR (+90%)	-0.05168	15
koff_CT	0.064599	13=	kon_CT (+90%)	-0.02584	16

4. DISCUSSION

The key determinant of CaM_4 formation is the kinetic ratio of binding: unbinding rate of Ca^{2+} to each site. The results in this paper collectively show the mechanisms by which CaM_4 forms over a range of Ca^{2+} values. The binding: unbinding ratio of the C-lobe leads to favourable binding of Ca^{2+} across all Ca^{2+} concentrations compared to the N-lobe. This was demonstrated in the simplified 5 state model as well as in the LSA. This is despite the N-lobe R state having some of the highest binding rate of any biomolecule that have been found experimentally (Faas *et al.,* 2011). Evidently, this is offset by having high Ca^{2+} dissociation rates. This means that a higher Ca^{2+} concentration is required for the N-lobe to have stabilised interactions with Ca^{2+} compared with the C-lobe.

The differences in lobe cooperativity suggests that although the N-lobe has high positive binding cooperativity, higher Ca^{2+} levels are required to facilitate this binding. The C-lobe however, has cooperativity in the sense that binding facilitates stability for Ca^{2+} once bound. For this reason, the C-lobe preferentially binds to Ca^{2+} which then primes CaM to become fully bound readily when adequate Ca^{2+} is present for N-lobe binding. This explains why the N-lobe parameters are more sensitive than the C-lobe.

Ca^{2+} signal detection determines the direction of synaptic plasticity. Given that CaM is the central regulator of both LTP and LTD, its activation is important to understand (Xia & Storm, 2005). In a true system, elevations of Ca^{2+} are transient, while the current model studies a range of Ca^{2+} concentrations until each level reaches steady state to see how stable Ca2+ binds as at function of Ca2+ concentrations. To understand Ca^{2+}-CaM binding stability is to understand the mechanisms by which Ca^{2+} binds CaM and therefore understand how CaM binds Ca^{2+} as it fluxes into a synapse.

In a more complete system, LTD-inducing PP1, and LTP- inducing CaM dependent kinase II (CaMKII) work in concert to regulate the direction of synaptic plasticity. That is, the balance between the relative activities of PP1 and CaMKII are key components of synaptic plasticity direction (Nicoll & Roche, 2013; Xia & Storm, 2005). CaMKII has a slower binding rate than phosphatases but lower dissociation rate, meaning a higher CaM_4 concentration is required to activate CaMKII verses PP1. This illustrates why CaM activation and its sensitivity to Ca^{2+} levels are important for modelling synaptic plasticity.

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