Research

Possible novel features of synaptic regulation during long-term facilitation in *Aplysia*

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Most studies of molecular mechanisms of synaptic plasticity have focused on the sequence of changes either at individual synapses or in the cell nucleus. However, studies of long-term facilitation at *Aplysia* sensory neuron–motor neuron synapses in isolated cell culture suggest two additional features of facilitation. First, that there is also regulation of the number of synaptic contacts between two neurons, which may occur at the level of cell pair-specific branch points in the neuronal arbor. Branch points contain many molecules that are involved in protein synthesis-dependent long-term facilitation including neurotrophins and the RNA binding protein CPEB. Second, the regulation involves homeostatic feedback and tends to keep the total number of contacts between two neurons at a fairly constant level both at rest and following facilitation. That raises the question of how facilitation and homeostasis can coexist. A possible answer is suggested by the findings that they both involve spontaneous transmission and postsynaptic Ca^{2+}, which can have bidirectional effects similar to LTP and LTD in hippocampus. In addition, long-term facilitation can involve a change in the set point of homeostasis, which could be encoded by plasticity molecules such as CPEB and/or PKM. A computational model based on these ideas can qualitatively simulate the basic features of both facilitation and homeostasis of the number of contacts.

Synaptic plasticity is a change in strength of the synaptic connection (postsynaptic potential or PSP) between neurons and includes increases during facilitation and decreases during depression. Plasticity is thought to underlie circuit formation during development and learning and memory in adults, and correspondingly to be defective in neurodevelopmental disorders including autism, ADHD, and schizophrenia as well as learning and memory disorders including Alzheimer's, age-related memory loss, and drug addiction (Hawkins 2013; Hawkins et al. 2017). Most studies of molecular mechanisms of synaptic plasticity have focused on either changes at individual synapses or gene regulation in the cell nucleus. However, studies of long-term facilitation at *Aplysia* sensory neuron–motor neuron (SN–MN) synapses in isolated cell culture (Glanzman et al. 1990), sensitization in the intact animal (Wainwright et al. 2004), and long-term potentiation in hippocampal neurons (Antonova et al. 2001, 2009) have shown that there are also changes in the number of contacts between presynaptic varicosities and the postsynaptic neuron. We refer to these as synaptic contacts although not all of them are functional synapses (Kim et al. 2003). The number of contacts is thought to be an important determinant of the strength of the PSP (Zhang et al. 2003) and to be different for different neuron pairs. It also increases during long-term facilitation of the PSP and is thought to be a major determinant of the time course of the facilitation (Bailey and Chen 1989).

As in other systems (Antonova et al. 2001, 2009; Holtmaat and Svoboda 2009), the contacts are dynamic and are continually being formed and eliminated, but the total number and the PSP remain fairly constant both at rest and during long-term facilitation (Miniaci et al. 2008; Chen et al. 2014). Furthermore, the number of contacts and the PSP return to baseline when maintenance of the facilitation is blocked, but the individual contacts are not all the same as they were before facilitation. These results have led some to suggest that memories are not stored at individual synaptic contacts, as is often supposed, but rather are stored in the nucleus (Chen et al. 2014). However, most of the previous experiments have involved a single SN and a single MN, so it has not been possible to examine the synapse specificity of the effects. Experiments with one SN and two MNs (Martin et al. 1997) or two SNs and 1 MN (Schacher et al. 1997) have shown that facilitation of the number of synaptic contacts and the PSP is specific to the stimulated synaptic pair (e.g., SN–MN1) and does not occur for the other pair (e.g., SN–MN2). These results should generalize to multiple pre- and postsynaptic partners and suggest two novel features of synaptic regulation during plasticity: (1) that the number of synaptic contacts between two neurons is regulated, and (2) that the regulation is homeostatic. We first describe those features and some of the evidence supporting them, then propose a model that could account for them and present computational modeling to illustrate the plausibility of the model.

Results

Proposed novel features of synaptic regulation

First, in addition to regulation of individual synapses and gene expression we propose that the number of synaptic contacts between two neurons is also regulated, and that regulation involves plasticity molecules at cell pair-specific branch points in the neuronal arbor (Fig. 1). Plasticity molecules at branch points are in a unique position to receive intracellular signals about the number of downstream synaptic contacts and in turn regulate the formation and elimination of branches and contacts. Here we focus on the

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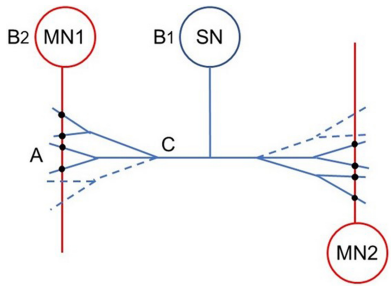

Figure 1. Cartoon of a culture with one bifurcated SN synapsing on two MNs. The number of synaptic contacts (filled circles) between two neurons could be regulated at the contacts (A), the SN (B1), or MN (B2) cell body, or cell pair-specific branch points in the synaptic arbor (C).

presynaptic arbor, but similar regulation could also occur in the postsynaptic arbor.

LTF and formation of new branches and contacts in *Aplysia* require plasticity molecules including CPEB and ApNT as well as protein synthesis (Martin et al. 1997; Miniaci et al. 2008; Kassabov et al. 2013). To begin to examine whether those molecules are present at branch points we performed immunocytochemistry for ApCPEB with a specific antibody against an epitope at the C terminus. In agreement with Si et al. (2010) we found that there is punctate staining for CPEB throughout the arbor but it is clearly present specifically at branch points, where it could contribute to local synthesis of proteins including ApNT (Fig. 2A). There was labeling for CPEB in 68% ± 3% of 126 branch points that we examined in six sensory neurons, and 77% ± 2% of 182 branch points in six motor neurons. Similarly, in experiments in which we overexpressed ApNT-GFP in sensory neurons in cocultures (Kassabov et al. 2013) we found that it can also be observed at branch points that give rise to new processes and varicosities (Fig. 2B). In addition we used fluorescent tagged gapmer oligonucleotides to label ApNT transcripts, and found that they are present at branch points as well (Fig. 2C). These results illustrate that plasticity molecules are present at branch points.

Second, we propose that regulation of synaptic contacts is homeostatic, and tends to keep the total number of contacts between two neurons at a fairly constant level both at rest and following facilitation. Plasticity and homeostasis are both essential for long-term memory storage but would seem to be mutually exclusive. How can they coexist at the same synapses? A possible answer is suggested by the findings that in *Aplysia* and other systems homeostasis and facilitation both involve spontaneous transmitter release (Jin et al. 2012a,b,c) and many of the same molecular mechanisms including postsynaptic Ca^{2+} (Li et al. 2005; Sutton et al. 2006; Jin et al. 2011). We previously found that spontaneous transmitter release is critical for the induction of long-term facilitation in *Aplysia* (Jin et al. 2012a,b) and acts cooperatively with other factors including neurotrophins (Jin et al. 2018). Increased spontaneous release during short-term facilitation by 5HT acts as an anterograde signal to recruit postsynaptic mechanisms of intermediate- and long-term facilitation including postsynaptic protein synthesis, in part by stimulating mGluR5 receptors linked to the production of IP3 and increased postsynaptic Ca^{2+}. Spontaneous release also acts through mGluR5 receptors to stimulate membrane insertion and recruitment of clusters of AMPA-like receptors.

Analysis of the pretest control data from those experiments suggests that spontaneous release also contributes to homeostasis in *Aplysia*, but with the opposite sign of action as facilitation: A decrease in spontaneous transmission (which includes both presynaptic release and activation of postsynaptic metabotropic receptors) leads to a compensatory increase in the pretest (baseline) synaptic strength (Fig. 3). Thus, presynaptic injection of BoTx D, which decreases spontaneous release, produced an increase in the baseline evoked EPSP ($F_{(1,52)} = 4.37$, $P < 0.05$ compared with vehicle). Similarly, the mGluR5 inhibitor MPEP, which blocks receptors activated by spontaneous release, increased the baseline EPSP ($F_{(1,45)} = 7.76$, $P < 0.01$ compared with control), as did postsynaptic injection of an antisense oligonucleotide for ApmGluR5 ($F_{(1,73)} = 4.20$, $P < 0.05$

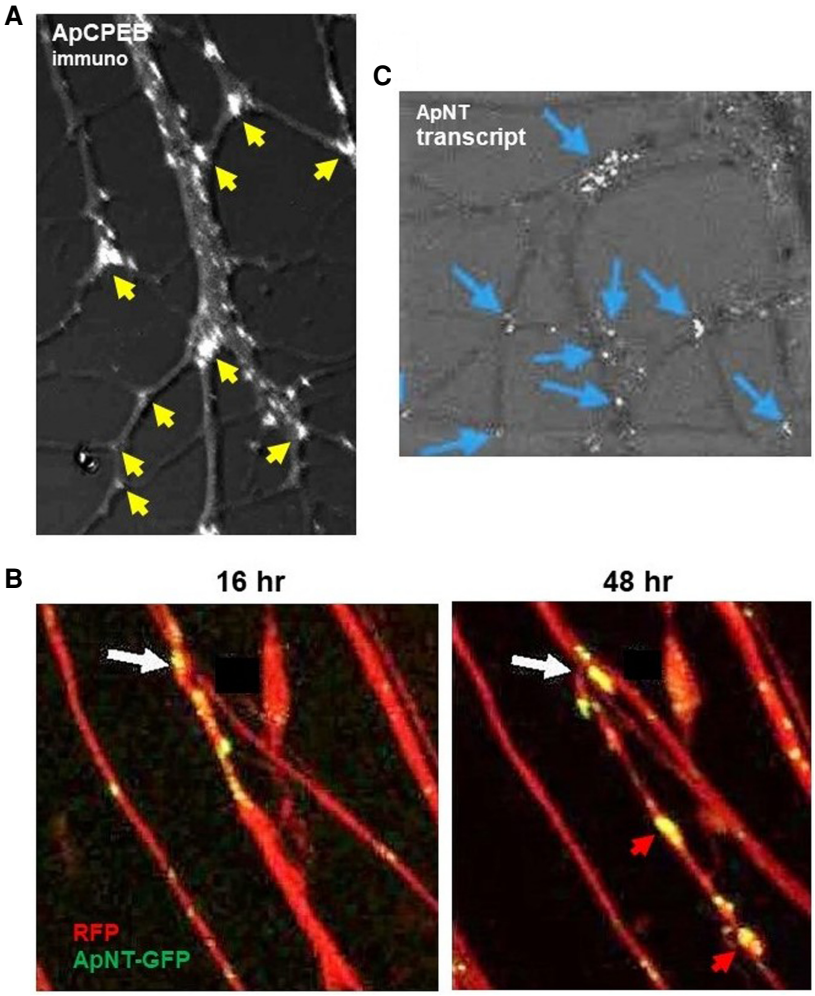

Figure 2. Examples of plasticity molecules at branch points. (*A*) Immunoreactivity for ApCPEB at SN branch points. (*B*) Overexpressed ApNT-GFP at a SN branch point (white arrow) that gives rise to a process where it stimulates new varicosities (red arrows). (*C*) Labeling of ApNT transcripts with fluorescent-tagged gapmer oligonucleotides at MN branch points.

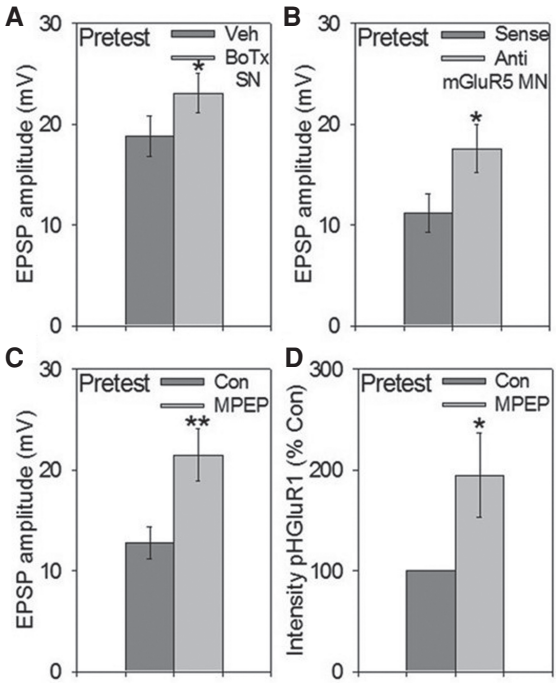

Figure 3. Spontaneous transmission contributes to homeostasis. Manipulations that decrease spontaneous transmission including injection of the light chain of botulinum toxin D into the SN (*A*), injection of an antisense oligonucleotide for ApmGluR5 into the MN (*B*), or application of the mGluR5 inhibitor MPEP in the bath (*C*,*D*) increased the amplitude of the baseline (pretest) EPSP between the SN and MN and the intensity of puncta of ApGluR1-pHluorin indicative of membrane insertion of AMPA-like receptors in the MN (preliminary results were reported in abstract form in Jin et al. 2012c). (*) $P<0.05$, (**) $P<0.01$ compared with controls.

compared with sense). MPEP also produced an increase in the intensity of puncta of ApGluR1-pHluorin, indicative of membrane insertion of AMPA-like receptors ($F_{(1,14)}=5.01$, $P<0.05$). The same experimental manipulations reduced 5HT-induced facilitation of the evoked EPSP and AMPAR insertion (Jin et al. 2012a,b). Moreover, although the increases in pretest levels could contribute to the reductions in facilitation through occlusion or ceiling effects those reductions were still significant ($P<0.05$ in each case) when the pretest levels were factored out in ANCOVAs, suggesting that the reductions were not due solely to the differences in pretest levels. These results suggest that homeostasis and facilitation at *Aplysia* sensory–motor neuron synapses involve many of the same molecular processes including spontaneous transmission, but with opposite signs of action.

A cellular model that may begin to unite plasticity and homeostasis in a common framework

More generally, these results support a novel cellular model based on known molecular pathways in *Aplysia* that may begin to unite long-term plasticity and homeostasis in a common framework (Figs. 4–6). In this model they both involve transynaptic feedback loops that include spontaneous release, mGluR5, and AMPARs. The model also includes ApCPEB and ApPKM, which are important for the long-term maintenance of facilitation and therefore could contribute to homeostasis as well. Figures 4A and 5A illustrate two components of the model that occur together but are first shown separately for simplicity. In this model growth of branches and synaptic contacts is regulated at branch points, but similar mechanisms could occur at synapses. The first component (Fig. 4A) involves regulation of growth by presynaptic CPEB (throughout this paper, we use "CPEB" as shorthand for aggregated CPEB) (Miniaci et al. 2008), and the second component (Fig. 5A) involves regulation of both AMPARs and growth by postsynaptic PKM (Villareal et al. 2009; Cai et al. 2011).

The model embodies two additional hypotheses. The first is that spontaneous release stimulates an increase in postsynaptic Ca^{2+}, which can have bidirectional effects during plasticity and homeostasis, similar to LTP and LTD in hippocampus (Bear and Malenka 1994). During plasticity brief 5HT produces a relatively brief, large increase in the frequency of spontaneous release that stimulates a brief, large increase in postsynaptic Ca^{2+} (Hi Ca^{2+}). Elevated postsynaptic Ca^{2+} in turn produces increases in local protein synthesis of a retrograde messenger that may stimulate growth (Fig. 4A; Villareal et al. 2007; Hu et al. 2010; Choi et al. 2011; Jin et al. 2011; Kassabov et al. 2013) and/or calpain-dependent proteolysis of PKC to PKM, leading to increased AMPA receptor insertion (Fig. 5A; Sutton et al. 2004b; Bougie et al. 2009, 2012; Villareal et al. 2009). At rest, a constant, low level of spontaneous release stimulates a constant, low level of postsynaptic Ca^{2+} (Low Ca^{2+}), which activates proteasomes or phosphatases with relatively high Ca^{2+} affinities (Friedrich 2004; Djakovic et al. 2009), resulting in low levels of the retrograde messenger and/or AMPA receptors (Ezzeddine

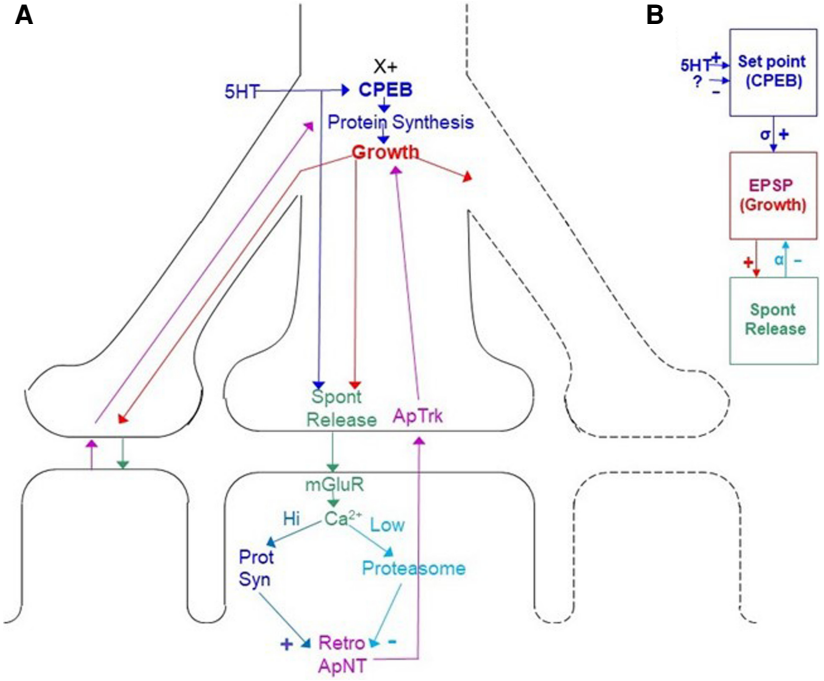

Figure 4. Model of mechanisms that could contribute to homeostasis as well as facilitation at *Aplysia* SN–MN synapses, focusing on the possible role of CPEB as a set point for the growth of contacts. The arrows in the *left* branch indicate that its contact is regulated similarly to the *central* branch, and the dashed lines in the *right* branch indicate potential growth of a new contact.

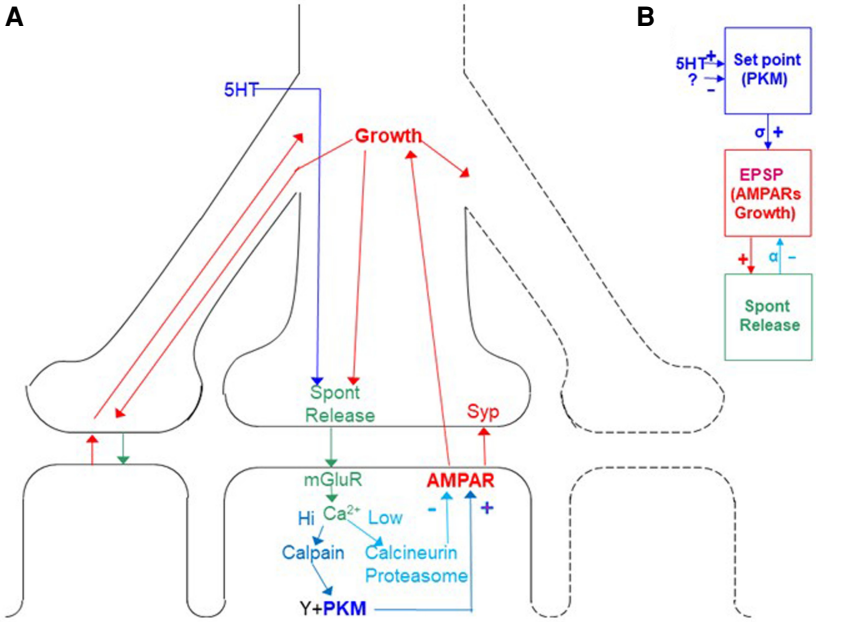

Figure 5. Model of mechanisms that could contribute to homeostasis as well as facilitation at *Aplysia* SN–MN synapses, focusing on the possible role of PKM as a set point for AMPAR insertion and growth.

and Glanzman 2003; Sharma et al. 2003; Zhao et al. 2003; Esdin et al. 2010). During homeostasis a reduction in spontaneous transmission produces a further decrease in baseline Ca^{2+} and removes activation of the proteasomes and/or phosphatases, resulting in compensatory increases in the retrograde messenger and AMPA receptors.

The second additional hypothesis is that plasticity and homeostasis are not contradictory, but rather long-term plasticity can involve a change in the set point of homeostasis, similar to a change in the set point of a thermostat (Figs. 4B, 5B). That idea could explain how synaptic plasticity and memory can be maintained at fairly constant levels for days, months, or years. However, it begs the question of what could be the neuronal representation of the set point. Research in the last decade has suggested that two molecules in *Aplysia*, CPEB and PKM, might play that role (Si et al. 2003a,b; Bougie et al. 2009, 2012; Cai et al. 2011). Presynaptic CPEB regulates growth (Miniaci et al. 2008) and postsynaptic PKM is thought to regulate both AMPAR insertion and growth (Villareal et al. 2009; Cai et al. 2011), so they may operate on somewhat different spatial and temporal scales. Inhibiting CPEB or PKM does not affect the baseline EPSP, suggesting that the baseline is regulated by some other molecules (X in Fig. 4A and Y in Fig. 5A) that may act as set points during development in a homeostatic hierarchy (Schacher and Hu 2014). However, inhibiting CPEB or PKM blocks maintenance of long-term facilitation (Si et al. 2003b; Cai et al. 2011), suggesting that either or both of them could act as a set point for homeostasis during long-term plasticity. Although these are probably the best current candidates there are questions about each individually, which might be resolved by the idea that they act cooperatively.

According to this hypothesis, under resting conditions (low postsynaptic Ca^{2+}) spontaneous release is part of a negative feedback loop that acts to maintain growth, AMPARs, and synaptic transmission at constant levels. Growth and AMPARs are stimulated by CPEB and PKM, and come to equilibrium when that stimulation is balanced by the negative feedback. During plasticity, relatively brief 5HT is thought to produce long lasting increases in the levels of CPEB and PKM, leading to higher equilibrium levels for growth and AMPARs and increased synaptic transmission. According to that idea, the levels of CPEB and PKM correspond to the set point on a thermostat, growth and AMPAR insertion correspond to the furnace, and spontaneous release corresponds to the room temperature. Spontaneous release may serve as a proxy for the evoked EPSP as an indicator of synaptic strength because it provides continuous feedback for ongoing adjustments, whereas evoked EPSPs are intermittent and therefore do not. 5HT produces an increase in the frequency of spontaneous release at the stimulated synapses, leading to an increase in postsynaptic Ca^{2+} that stimulates a retrograde signal back to plasticity

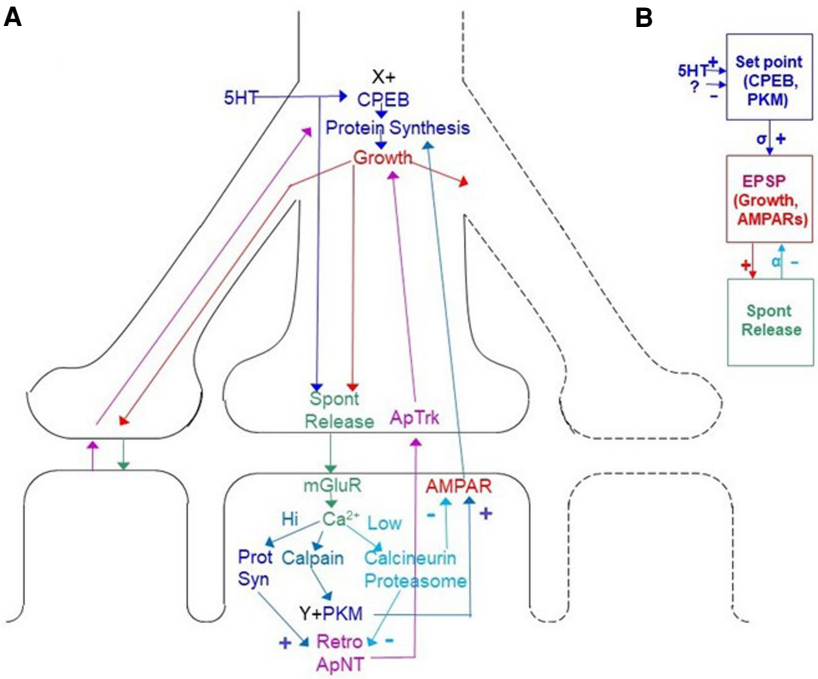

Figure 6. Combined model of mechanisms that could contribute to homeostasis as well as facilitation at Aplysia SN–MN synapses. In this model CPEB and PKM act cooperatively to stimulate presynaptic protein synthesis, which acts as a joint set point for growth, and PKM also acts as a set point for AMPAR insertion.

molecules at the presynaptic branch point and growth of new branches and contacts. In addition, 5HT produces an increase in the set point for homeostasis of the contacts.

Computational modeling of plasticity and homeostasis

To test the feasibility of these ideas we have combined the two components in Figure 6A and performed computational modeling of the simplified scheme illustrated in Figure 6B. This scheme and the computational model are focused on the possible roles of CPEB, PKM, and spontaneous release in the coexistence of plasticity and homeostasis, and do not include other elements illustrated in Figure 6A. In this model, $E(t+1) = E(t) + [\mathrm{Sp}]\sigma - E(t)\alpha$, where $E(t)$ is the EPSP (or growth or AMPARs) at time t, Sp is the set point (CPEB or PKM), $E(t)\alpha$ is spontaneous transmission ("minis" in the figures), which is proportional to the EPSP, and α and σ are scaling factors that are constant in all of the simulations except homeostasis (see below). This system is at equilibrium when $E = [\mathrm{Sp}]\sigma/\alpha$. As shown in Figure 7A (facilitation), a step increase in the set point (Sp) causes a gradual increase in the EPSP (or growth or AMPARs) to a new equilibrium that depends positively on σ and negatively on α. As shown in Figure 7B (homeostasis), a step decrease in spontaneous transmission (modeled by a step decrease in α) also causes a gradual increase in the EPSP to a new equilibrium, with no change in CPEB or PKM. This model thus successfully simulates the basic features of both plasticity and homeostasis. It also predicts homeostasis from the new equilibrium level following facilitation (Fig. 7C) or vice versa.

In addition, the model can qualitatively simulate many of the published results on CPEB and PKM. Experimentally inhibition of either CPEB or PKM blocks the maintenance of facilitation with no effect on the baseline EPSP (Si et al. 2003b, 2010; Miniaci et al. 2008; Villareal et al. 2009; Cai et al. 2011; Chen et al. 2014). However, although CPEB and PKM are probably the best current candidates for set points during facilitation, there are questions about each. Experiments in mammals have shown that inhibiting PKM blocks potentiation but knockout does not (Lee et al. 2013; Volk et al. 2013; but see Tsokas et al. 2016). Furthermore, although increasing PKM produces an increase in the EPSP in mammals and *Aplysia* (Ling et al. 2002, 2006; Yao et al. 2008; Ferguson et al. 2019), consistent with a role as a set point, increasing CPEB in *Aplysia* does not (Si et al. 2010). In fact, overexpression of CPEB blocks subsequent facilitation by 5HT, suggesting that it may act as a dominant negative (Si et al. 2010). Alternatively, however, all of these results might be accounted for by an additional feature of the model, which is that presynaptic CPEB and postsynaptic PKM are not independent, but rather act cooperatively through retrograde signaling to stimulate presynaptic protein synthesis, which acts as a joint set point for growth of contacts (Fig. 6). This feature of the model can simulate the results produced by decreases or increases of PKM or CPEB (Figs. 8A–D). Thus, in the model, (1) the increase in the EPSP during facilitation depends on increases in AMPA receptors and/or growth, (2) the increase in AMPA receptors depends on PKM but not CPEB or protein synthesis, and (3) the increase in growth depends on protein synthesis, which in turn depends on both CPEB and PKM that act cooperatively as indicated by a multiplicative relation in the model (to a first approximation Δ Prot Syn = Δ CPEB × PKM). Therefore, blocking either CPEB or PKM blocks the increases in protein synthesis and growth, and blocking PKM also blocks the increase in AMPA receptors. Increasing CPEB alone does not produce an increase in protein synthesis, growth, or AMPA receptors but increasing PKM alone produces an increase in AMPA receptors, and increasing both CPEB and PKM produces increases in all three during facilitation by 5HT.

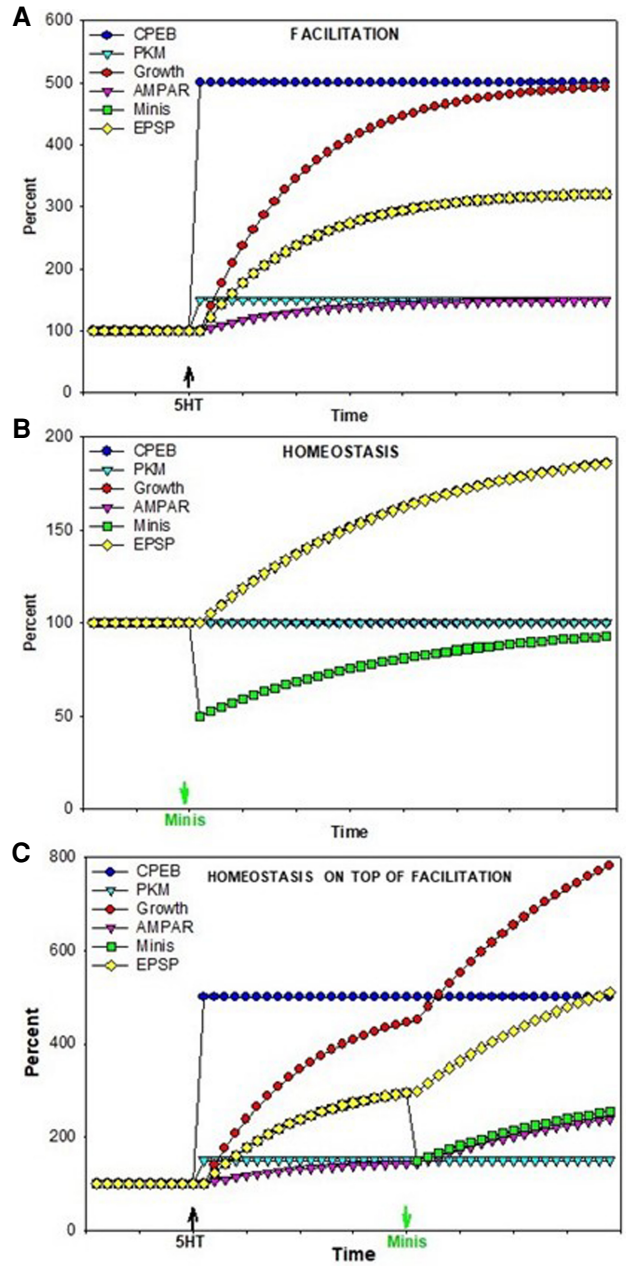

Figure 7. Computational modeling of facilitation (*A*), homeostasis (*B*), and homeostasis on top of facilitation (*C*) based on the combined model illustrated in Figure 6B. During facilitation 5HT produces step increases in CPEB and PKM that lead to gradual increases in the growth of contacts and AMPAR insertion, resulting in increases in the EPSP and spontaneous transmission ("minis" in the figures). During homeostasis a step decrease in spontaneous transmission leads to compensatory increases in growth and AMPARs, resulting in gradual increases in the EPSP and spontaneous transmission with no change in CPEB or PKM, which remain at 100. In *A* "minis" and the EPSP overlap, and in *B* Growth, AMPAR, and the EPSP overlap.

In addition the model makes the predictions that (1) an increase in CPEB can produce an increase in the EPSP if it is preceded by a small increase in PKM (Fig. 8E), and (2) CPEB alone might also produce an increase in the EPSP when PKM is functionally deleted, if CPEB compensates for the loss of PKM by becoming independent of it (Fig. 8F). In that case PKM is removed from the equations, and

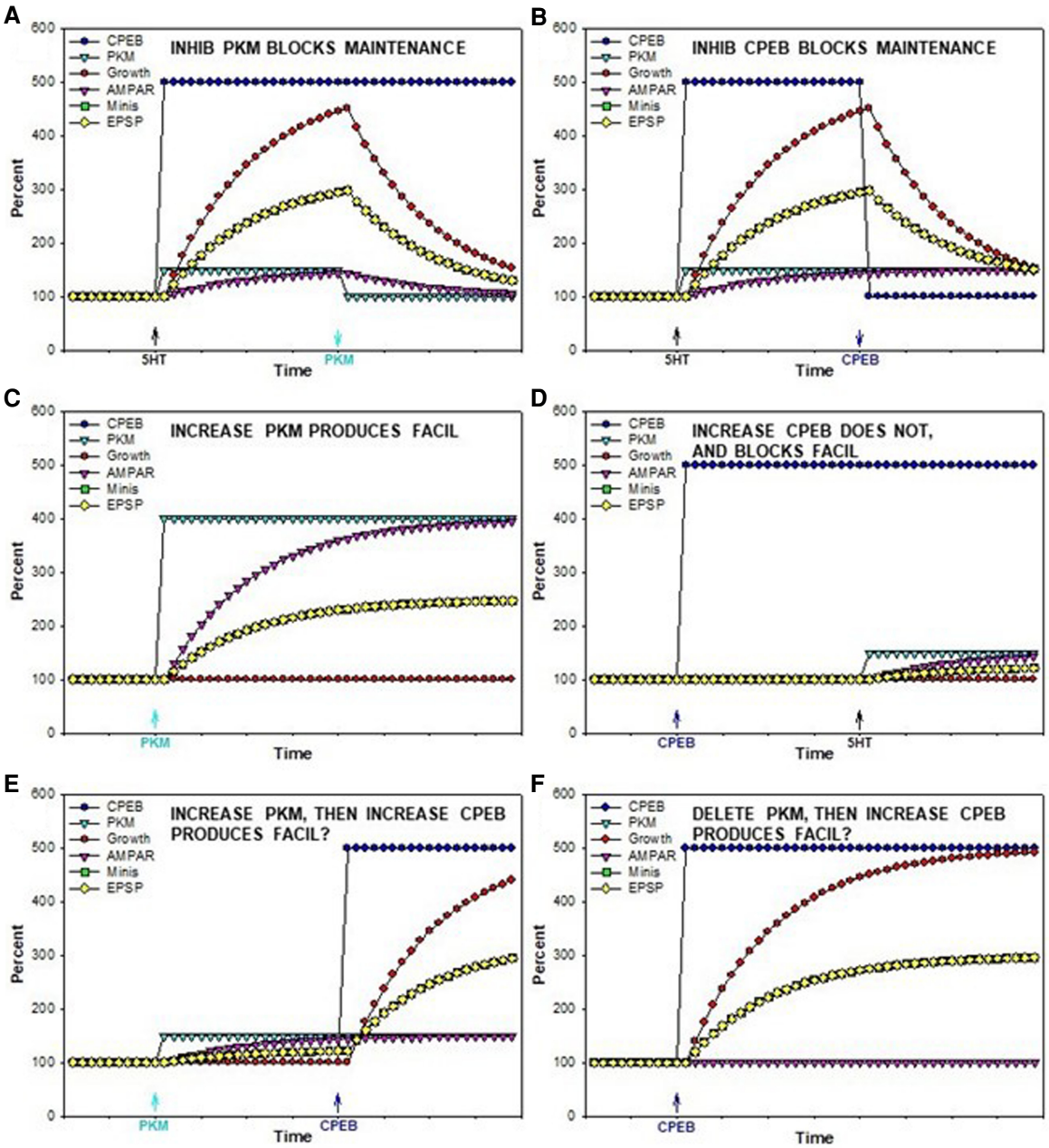

Figure 8. Computational modeling of PKM and CPEB. The model simulates the findings that inhibiting either PKM (*A*) or CPEB (*B*) blocks the maintenance of facilitation, and increasing PKM (*C*) but not CPEB produces facilitation. (*D*) Instead, increasing CPEB blocks subsequent facilitation by 5HT. The model also predicts that increasing CPEB will produce facilitation if it is preceded by either a small increase in PKM (*E*) or functional deletion of PKM (*F*). In *C* CPEB and Growth remain at 100, in *D* Growth remains at 100, and in all of the figures "minis" and the EPSP overlap.

the increase in growth depends on CPEB alone. These predictions remain to be tested experimentally. However, the computational model illustrates the feasibility of our ideas for the coexistence of plasticity and homeostasis. Furthermore, some of the key molecules in the model are found at branch points, where they could regulate plasticity and homeostasis of the number of synaptic contacts.

Discussion

These results support the idea that the number of synaptic contacts between two neurons is regulated during long-term plasticity. However, that regulation cannot occur at the level of individual contacts ("A" in Fig. 1) because they cannot "know" and regulate how many other contacts there are. Rather, regulation has been suggested to occur at the level of the cell nucleus ("B") (Chen et al. 2014), but the nucleus cannot "know" and regulate how many contacts there are for each cell pair if there is more than one pair (e.g., SN–MN1 and SN–MN2). The stimulated synaptic pair could be "tagged" for capture of plasticity molecules or those molecules could be "mailed" from the nucleus to specific synapses, but those processes seem unlikely because they would have to be quantitative and cell pair-specific if there were multiple cell pairs (Frey and Morris 1998; Smolen et al. 2019).

Instead, we propose that the number of contacts could be regulated at the level of cell pair-specific branch points in the neuronal arbor ("C").

Two ideas have previously been proposed for how new contacts are formed: either (1) splitting of old branches and contacts (Bailey et al. 1992; Lüscher et al. 2000; but see Harris et al. 2003) or (2) outgrowth from old contacts (Hatada et al. 2000). Either mechanism would create new contacts between the same cell pair as the old contacts and would be consistent with our proposal that the number of contacts is regulated at cell pair-specific branch points. In vertebrates, a number of molecules involved in protein synthesis-dependent plasticity are enriched at axonal branch points including mRNA transcripts for actin and actin depolymerizing factor (ADF), miR-124 and RNAi machinery, and mitochondria, which are controlled by CPEB and provide energy for the plasticity (Lee and Hollenbeck 2003; Oruganty-Das et al. 2012; Gershoni-Emek et al. 2018). We have found that molecules involved in long-term plasticity in Aplysia including the RNA binding protein ApCPEB, the neurotrophin ApNT, and ApNT transcript are also present at branch points (Fig. 2), where they could regulate the number of contacts.

These results also support the idea that regulation of synaptic contacts during plasticity is homeostatic. Like plasticity, the word homeostasis is used to refer to a number of different phenomena. For the purposes of this paper, homeostasis refers to the local maintenance of synaptic strength at some desired level, and can range from synapse-specific up to more global "scaling" of strength (Turrigiano 2008). Plasticity and homeostasis both involve many of the same molecular mechanisms including spontaneous transmitter release, mGluR5 receptors, and postsynaptic Ca^{2+} as well as protein synthesis (Sutton et al. 2006; Villareal et al. 2007; Jin et al. 2011), AMPA receptor insertion (Sutton et al. 2004a, 2006; Li et al. 2005, 2009), and modulation of the presynaptic probability of release (Frank et al. 2006, 2009; Dickman and Davis 2009; Hawkins et al. 2017). Spontaneous release is critical for the induction of long-term facilitation and is engaged quite early, during an intermediate-term stage that is the first stage to involve postsynaptic as well as presynaptic molecular mechanisms (Jin et al. 2011). Spontaneous release from the presynaptic neuron recruits postsynaptic mechanisms of intermediate-term facilitation that may be first steps in synaptic growth during long-term facilitation (Jin et al. 2012b). These results suggest that the different stages of facilitation involve a cascade of pre- and postsynaptic mechanisms, which is initiated by spontaneous transmission and may culminate in synaptic growth.

We have found that spontaneous transmission also contributes to homeostasis in *Aplysia*, but with the opposite sign of action as facilitation (Fig. 3). These results suggest a novel cellular model that may begin to unite mechanisms of long-term plasticity and homeostasis (Fig. 6). In the model, during plasticity a brief, large increase in postsynaptic Ca^{2+} produces increases in local protein synthesis of a retrograde messenger and/or calpain-dependent formation of AMPARs. During homeostasis a decrease in baseline Ca^{2+} removes activation of proteasomes and/or phosphatases, resulting in compensatory increases in the retrograde messenger and AMPA receptors. In support of that idea, previous experiments suggest that homeostasis could involve postsynaptic proteasomes and/or phosphatases including calcineurin. Inhibition of the proteasome in either the presynaptic or postsynaptic neuron produces an increase in the EPSP, as well as increases in the number of presynaptic processes or postsynaptic AMPARs (Zhao et al. 2003), consistent with a role in homeostasis. Likewise, inhibition of calcineurin enhances intermediate-term and long-term sensitization (Sharma et al. 2003) and blocks long-term habituation (Esdin et al. 2010), perhaps by blocking AMPAR removal (Ezzeddine and Glanzman 2003; Malenka and Bear 2004).

In addition, long-term plasticity can involve a change in the set point of homeostasis. However, what is the neuronal representation of the set point? Such a molecule should have several unusual properties: (1) It should be very stable, (2) it should undergo a stable increase (or decrease) that outlasts the stimulus during long-term plasticity, (3) changes in its level should lead to comparable changes in synaptic strength, (4) its level should also determine the target for homeostatic regulation of strength, and (5) its level should not change during homeostatic regulation. Research in the last decade has suggested that two molecules in *Aplysia* that are important for the long-term maintenance of facilitation, CPEB and PKM, might play this role.

Aplysia cytoplasmic polyadenylation element binding protein (CPEB) is a regulator of local mRNA translation that has prion-like properties, which means that it exists in two conformations, one of which forms self-perpetuating aggregates (Si et al. 2003a, 2010). 5HT up-regulates the active, self-perpetuating form, which plays a critical role in the stable maintenance of long-term facilitation beyond 24 h (Si et al. 2003b, 2010). CPEB is also necessary for the stable maintenance of new presynaptic varicosities that form during long-term facilitation (Miniaci et al. 2008). CPEB proteins with similar properties have been described in *Drosophila* (Majumdar et al. 2012) and mammals (Pavlopoulos et al. 2011; Darnell and Richter 2012) as well. PKMζ, the constitutively active catalytic fragment of PKCζ, is thought to play a critical role in the stable maintenance of LTP in vertebrates (Sacktor 2008). In *Aplysia*, calpain-dependent proteolysis of atypical PKC *Apl* III forms a similar PKM fragment (Bougie et al. 2009, 2012; Farah et al. 2016). Inhibiting ApPKM blocks stable maintenance of long-term facilitation of sensory–motor neuron EPSPs beyond 24 h (Cai et al. 2011; Hu et al. 2017) and stable maintenance of new presynaptic varicosities that form during long-term facilitation (Chen et al. 2014). It also blocks changes in AMPA receptors during intermediate-term facilitation (Chitwood et al. 2001; Villareal et al. 2009; Bougie et al. 2012), and PKM is believed to regulate AMPARs in mammals as well (Ling et al. 2006; Yao et al. 2008).

The model we propose has two components that involve regulation of growth by presynaptic CPEB (Fig. 4) and regulation of both AMPARs and growth by postsynaptic PKM (Fig. 5). Because changes in AMPARs occur more rapidly than growth, these two components may operate on somewhat different time scales. Furthermore, because of transsynaptic signaling either molecule could have actions on both sides of the synapse. A number of other variations are also possible (e.g., postsynaptic CPEB or presynaptic PKM), but the ones shown probably have the most current support. According to our hypothesis presynaptic CPEB and postsynaptic PKM are not independent but rather act cooperatively through retrograde signaling to stimulate presynaptic protein synthesis, which acts as a joint set point for growth of contacts (Fig. 6). Several studies suggest that presynaptic protein synthesis and growth of contacts require a retrograde signal that depends on postsynaptic Ca^{2+} and protein synthesis (Cai et al. 2008; Wang et al. 2009; Hu et al. 2010; Kim and Martin 2015; Jin et al. 2018), but it is not yet known whether that signal depends on postsynaptic PKM. However, computational modeling based on these ideas can qualitatively simulate many of the published results on facilitation, homeostasis, and the roles of CPEB and PKM in those processes (Figs. 7, 8), supporting the feasibility of the ideas.

Materials and Methods

Cell culture preparation

Aplysia cocultures consisting of an L7 gill motor neuron and a pleural sensory neuron were prepared as described previously and used 4–6 d after plating (Schacher 1985; Bao et al. 1997). L7 gill motor

neurons were isolated from juvenile (1- to 3-g) animals and pleural sensory neurons were isolated from adults (70–120 g). The animals were purchased from the Howard Hughes Medical Institute Mariculture Facility. All experiments were performed at room temperature (20°C–23°C) and the culture dish was continuously perfused at a rate of 0.5 mL/min with 50% filtered hemolymph and 50% L15 medium supplemented with salts.

EPSP recording

All recordings were performed as described previously (Bao et al. 1997). Briefly, the L7 motor neuron was impaled with an intracellular recording electrode (10–20 MΩ) filled with 2.5 M KCl and held at −50 mV in current clamp mode, and evoked EPSPS were produced by brief stimulation of the SN with an intracellular or extracellular electrode. MPEP (50 μM, EMD Bioscience) or saline control was added to the bath 30 min before the recording session. The light chain of botulinum toxin D (1 μM in the electrode; List Biological Laboratories) or vehicle control was pressure injected into the SN 30 min before the recording session, and antisense mGluR5 (50 μg/mL in the electrode) or sense control was injected into the MN 4 h before the recording session. The injection electrode (3–6 MΩ) was filled with 0.5 M potassium acetate, 10 mM Tris-HCl to adjust the pH to 7.5, 0.2% fast green to visualize the injection, and the reagent. The electrode was then removed and replaced with a stimulating (SN) or recording (MN) electrode. Antisense mGluR5 was constructed based on the *Aplysia* sequence, and the reagents have been characterized previously in *Aplysia* (Jin et al. 2011, 2012b).

Immunocytochemistry and image analysis

Immunocytochemistry was performed as previously described (Martin et al. 1997). The ApCPEB antibody was raised against an epitope at the C terminus. The preparations were incubated with a primary antibody (diluted 1:100) overnight at 4°C and with a fluorescent secondary antibody (diluted 1:1000; Invitrogen) at room temperature for 1 h, and viewed with an Olympus FV1000 scanning unit coupled to an IX81 inverted microscope. Branch points in the neuronal arbor were identified based on having a Y configuration, often with a larger branch splitting into two smaller ones, and were considered positive for ApCPEB if there was a punctum of immunofluorescence in or immediately adjacent to the branch point. We did not attempt a similar quantitative analysis of ApNT-GFP or ApNT gapmer oligonucleotides because expression of those constructs tended to alter the branching of the neurons.

Expression of fluorescent fusion proteins

The constructs for the fluorescent fusion proteins ApNT-GFP and ApGluR1-pHluorin (the *Aplysia* homolog of the AMPA receptor subunit GluR1 tagged with a pH-dependent variant of GFP that increases its fluorescence intensity during exocytosis) were cloned into the *Aplysia* expression vector pNEX3 (Kaang 1996). Purified plasmid DNA was microinjected into the sensory or motor neuron, which was examined 1–2 d later with the imaging system described above. ApGluR1-pHluorin fluorescence intensity was measured in the initial segment area of the motor neuron.

Fluorescent-tagged ApNT gapmer oligonucleotides

Locked nucleic acid (LNA) oligonucleotides modified with a 3′ 6-flourescein amidate (6FAM) fluorescent tag targeting all ApNT isoforms as well as random sequence controls were designed and synthesized by Exicon, Inc., at 1 μM scale and purified by HPLC and Ion exchange chromatography. The LNA oligos were added directly to the media (1 μM) and imaging was performed 48–72 h later. The LNA oligo sequences used were as follows: ApNT: 5′ +C + G+T + GA*A*C*G*G*G*C*G + A+G + A+T-3′(6FAM) and Neg Control: 5′+A + A+C + AC*G*T*C*T*A*T + A+C + G+C-3′(6FAM), where + indicates LNA nucleotide and * indicates phosphorothioate bond.

Statistical analysis

For the data on EPSP amplitude drug conditions were compared with interleaved saline, vehicle, or sense control conditions. For the data on intensity of pHGluR1 results with MPEP were normalized to saline controls from the same day. Those data were log transformed for statistical analysis because they were highly skewed and many points were >500%. The data are presented as mean ± SEM and were analyzed with ANOVAs. $P < 0.05$ is considered significant.

Computational modeling

The EPSP and spontaneous transmission are proportional to the average of Growth and AMPARs, each of which is governed by a scheme similar to that illustrated in Figure 6B $\{E(t+1) = E(t) + [Sp]\sigma - E(t)\alpha\}$ with its own set point and scaling factors. Thus, $AMPAR(t+1) = AMPAR(t) + [PKM(t) \times \sigma 1(t)] - [AMPAR(t) \times \alpha 1(t)]$. Similarly, $Growth(t+1) = Growth(t) + [ProtSyn(t) \times \sigma 2(t)] - [Growth(t) \times \alpha 2(t)]$. However, in this case the set point is based on protein synthesis, which is determined by a normalized combination of PKM and CPEB. Roughly, $\Delta ProtSyn = \Delta CPEB \times PKM$. More specifically, $ProtSyn(t+1) = [CPEB(t+1) - CPEB(t) + ProtSyn(t) - 100] \times \{[PKM(t+1) - 100]/50\} + 100$. The same model was used for all of the examples shown in Figures 7, 8, and the starting parameters were PKM = 100, CPEB = 100, ProtSyn = 100, and σ1, α1, σ2, and α2 all = 0.1. For facilitation (Fig. 7A,C) PKM was increased to 150 and CPEB was increased to 500 at the arrow, and for homeostasis (Fig. 7B,C) $\alpha 1(t)$ and $\alpha 2(t)$ were decreased to 0.05 at the arrow. Otherwise α and σ were constant. For experimental manipulations of PKM and CPEB (Fig. 8A–F) they were increased or decreased as indicated at the arrows. The parameters were chosen to qualitatively but not quantitatively simulate experimental results, and therefore are somewhat arbitrary. The pattern of results for facilitation was not sensitive to twofold changes in CPEB, α, or σ, but was more sensitive to PKM, and homeostasis was not sensitive to σ or σ but the baseline was.

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