Cholinergic Synaptic Homeostasis Is Tuned by an NFAT-Mediated α7 nAChR-Kv4/Shal Coupled Regulatory System

Abdunaser Eadaim1, Eu-Teum Hahm1, Elizabeth D. Justice1, Susan Tsunoda1,2,*

1Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA
2Lead Contact

SUMMARY

Homeostatic synaptic plasticity (HSP) involves compensatory mechanisms employed by neurons and circuits to preserve signaling when confronted with global changes in activity that may occur during physiological and pathological conditions. Cholinergic neurons, which are especially affected in some pathologies, have recently been shown to exhibit HSP mediated by nicotinic acetylcholine receptors (nAChRs). In Drosophila central neurons, pharmacological blockade of activity induces a homeostatic response mediated by the Drosophila α7 (Dα7) nAChR, which is tuned by a subsequent increase in expression of the voltage-dependent Kv4/Shal channel. Here, we show that an in vivo reduction of cholinergic signaling induces HSP mediated by Dα7 nAChRs, and this upregulation of Dα7 itself is sufficient to trigger transcriptional activation, mediated by nuclear factor of activated T cells (NFAT), of the Kv4/Shal gene, revealing a receptor-ion channel system coupled for homeostatic tuning in cholinergic neurons.

In Brief

Eadaim et al. show that in vivo reduction of cholinergic signaling in Drosophila neurons induces synaptic homeostasis mediated by Dα7 nAChRs. This upregulation of Dα7 induces Kv4/Shal gene expression mediated by nuclear factor of activated T cells (NFAT), revealing a receptor-ion channel system coupled for homeostatic tuning in cholinergic neurons.

Graphical Abstract
INTRODUCTION

Homeostatic synaptic plasticity (HSP) is thought to function as an adaptive mechanism counterbalancing changes in global neural activity (Davis, 2006; Marder and Goaillard, 2006; Nelson and Turrigiano, 2008; Pozo and Goda, 2010; Turrigiano, 2011, 2008). In general, increasing overall neural activity triggers HSP mechanisms that reduce synaptic strength, while suppressing activity leads to HSP mechanisms that boost synaptic strength. These homeostatic responses have been proposed to come into play during physiological processes, such as learning/memory and development, as well as in response to pathological conditions. Although decades of studies on HSP have focused on glutamatergic synapses, HSP mediated by nicotinic acetylcholine receptors (nAChRs) has also been implicated in numerous pathologies. For example, in patients and models of Alzheimer’s disease (AD), nAChRs and cholinergic neurotransmission are increased and decreased at different stages of the disease (Albuquerque et al., 2009; DeKosky et al., 2002; Dineley et al., 2001; Frölich, 2002; Nordberg, 2001; Small and Fodero, 2002), and these changes have been suggested to be homeostatically related (Hahm et al., 2018; Hernandez et al., 2010; Pratt et al., 2011; Small, 2004, 2007). Nicotine dependence has also been shown to involve changes in the expression/function of nAChRs (De Biasi and Dani, 2011; Picciotto et al., 2008), and some studies have suggested that these changes may involve a homeostatic response to the desensitization of nAChRs (Fenster et al., 1999; Orteils and Barrantes, 2010).
Recent studies have verified that nAChRs can indeed mediate HSP (Hahm et al., 2018; Ping and Tsunoda, 2011; Wang et al., 2018). In a previous study, we showed that pharmacological blockade of nAChR activity in primary neurons cultured from Drosophila resulted in an increase in synaptic strength that was explained by a selective upregulation of the Drosophila α7 (Da7) nAChR (Ping and Tsunoda, 2011). And, more recently, we showed that early hyperactivity in a Drosophila AD model triggers endogenous Da7-dependent HSP mechanisms that contribute to eventual synaptic depression (Hahm et al., 2018). α7-mediated HSP is intriguing because α7 receptors are among the most abundant and widespread of nAChRs in the mammalian brain and have been implicated in AD, nicotine addiction, nicotine-induced seizure, and schizophrenia (Albuquerque et al., 2009; Steinlein and Bertrand, 2008).

Accurate tuning of HSP, however, is likely to be a critical determinant of whether a homeostatic response is adaptive or maladaptive. For example, if a homeostatic response were to “overshoot” its target activity range, this would result in additional aberrant signaling rather than restoration of effective signaling. We previously showed that Da7-mediated HSP triggers a tuning mechanism: the upregulation of voltage-gated Kv4/Shal channels, which prevents such an overshoot of the homeostatic response (Ping and Tsunoda, 2011). Whether this occurs when HSP is induced in vivo has not been addressed. Little is also known about how inactivity actually leads to the regulation of Kv4/Shal expression. Here, we use genetic mutants to reduce activity in vivo and investigate the mechanisms underlying inactivity-induced Kv4/Shal expression. We show that Kv4/Shal expression is responsive to, and dependent on, changing levels of Da7 nAChRs. We also show that it is indeed Kv4/Shal mRNA levels that are dynamic, and this regulation is dependent on the transcriptional activator, nuclear factor of activated T cells (NFAT). In addition, we find that increased expression of Da7 nAChRs, or the Da7 regulator NACHO, is sufficient to upregulate Kv4/Shal expression, revealing a receptor-ion channel system, coupled for homeostatic tuning.

RESULTS

Chafts2-Induced Activity Loss Induces a Homeostatic Response

Previously, we showed that prolonged pharmacological blockade of neural activity in cultured Drosophila neurons results in a homeostatic increase in synaptic activity that triggers a subsequent upregulation of Kv4/Shal channel levels, as a mechanism for tuning the homeostatic response (Ping and Tsunoda, 2011). Here, we set out to examine whether we could apply an in vivo blockade of activity and detect a similar homeostatic response and upregulation of Kv4/Shal. Since the predominant excitatory neurotransmitter in the Drosophila CNS is acetylcholine (ACh), we used temperature-sensitive mutants of the cholineacetyl transferase (ChAT) gene, referred to as Chafts alleles (Greenspan et al., 1980). Chafts mutations reduce/disable enzyme activity when exposed to elevated temperatures. For example, homozygous Chafts2 mutants have been shown to display a ~75% reduction in ChAT activity at 30°C (Kitamoto et al., 2000; Salvaterra and McCaman, 1985). However, since homozygous Chafts2 mutants exhibit significantly reduced levels of ChAT activity even at permissive temperatures (18°C) (Kitamoto et al., 2000; Salvaterra and McCaman, 1985),
we used heterozygous \textit{Chd}^{ts2}/+ mutants, which exhibit ChAT activity more similar to wild type (WT) when grown at 18\degree C. \textit{Chd}^{ts2}/+ heterozygotes raised at 30\degree C have been reported to exhibit \textasciitilde35\% less ChAT activity compared to WT (Greenspan et al., 1980).

We first tested if inhibition of ACh synthesis in \textit{Chd}^{ts2}/+ mutants could trigger a homeostatic upregulation of miniature excitatory postsynaptic currents (mEPSCs), a standard assessment of synaptic strength. We grew \textit{Chd}^{ts2}/+ primary cultures for 8 days \textit{in vitro} (DIV) at 18\degree C, then shifted cultures to 30\degree C for increasing durations of time and recorded mEPSCs at room temperature over the next 60 min. With increasing durations of heat treatment (HT), neurons from \textit{Chd}^{ts2}/+ cultures exhibited mEPSCs that were progressively less frequent, displaying significantly longer interevent times (Figures 1A and 1C; no HT, 750.19 \pm 57.42 ms; 2/3 h HT, 1192. \pm 76.34 ms; 4/5 h HT, 3364.76 \pm 195.26 ms; \( n = 7\)–8 cells). The reduction in mEPSC frequency is consistent with a progressive decline in availability of ACh from 2 to 5 h. After 2–3 h of HT, however, we also observed a 34\% increase in the amplitude of mEPSCs (Figures 1B and 1C; no HT, 6.12 \pm 0.05 pA; 2/3 h HT, 9.26 \pm 0.19 pA; 4/5 h HT, 7.0 \pm 0.16 pA; \( n = 7\)–8 cells), demonstrating a postsynaptic compensatory change. These results show that \textit{Chd}^{ts2}-induced activity reduction induces a physiologically detectable homeostatic response and suggest that HSP mechanisms involve a postsynaptic change.

Since previous studies had shown that pharmacological activity blockade results in a concurrent increase in the Da7 nAChR (Ping and Tsunoda, 2011), we investigated whether inhibition of cholinergic activity in the \textit{Chd}^{ts2}/+ mutant also resulted in an increase in Da7 nAChRs. Unfortunately, anti-Da7 antibodies have proven to be problematic when used for immunoblot analysis. However, since \textit{in vitro} studies suggested that homeostatic changes in Da7 are translationally regulated (Ping and Tsunoda, 2011), we reasoned that a transgenically expressed EGFP-tagged Da7 protein would likely represent the changes that the endogenous Da7 protein undergoes. We used the pan-neuronal \textit{elav-Gal4} transgene to drive expression of Da7-EGFP under control of the upstream activating sequence (\textit{UAS-Da7-EGFP}). We used flies expressing \textit{Da7-EGFP} in WT and \textit{Chd}^{ts2}/+ backgrounds (\textit{elav-Gal4} \( \rightarrow \) \textit{UAS-Da7-EGFP}/+ and \textit{elav-Gal4} \( \rightarrow \) \textit{UAS-Da7-EGFP/Chd}^{ts2}, respectively). In this way, a similar basal level of Da7-EGFP is constitutively transcribed in both genotypes. Flies were grown at 18\degree C, collected at \textless 24 h after eclosion (AE), and subjected to either no HT or 3 h HT at 30\degree C to test if \textit{Chd}^{ts2}-induced inactivity homeostatically enhanced Da7-EGFP protein levels. We found that levels of Da7-EGFP were indeed enhanced by \textasciitilde23\% in response to \textit{Chd}^{ts2}-induced activity inhibition, compared to untreated flies; in contrast, similar HT induced no change in Da7-EGFP levels in the absence of the \textit{Chd}^{ts2} allele (Figure 1D). To examine \textit{Chd}^{ts2}-induced upregulation of Da7 by another approach, we immunostained whole \textit{Chd}^{ts2}/+ brains after either no HT or HT. We used an anti-Da7 antibody (kindly provided by Dr. Hugo Bellen) that we were able to validate for immunocytochemistry, although unfortunately not for immunoblot analysis. We found that anti-Da7 signal, which should represent endogenous Da7, was indeed also increased in HT \textit{Chd}^{ts2}/+ brains (Figure S1). Altogether, our results suggest that reducing cholinergic activity using the \textit{Chd}^{ts2} allele induces a homeostatic increase in mEPSC amplitude that is mediated by an increase in Da7 nAChRs, consistent with previous \textit{in vitro} findings (Ping and Tsunoda, 2011).
Although mammalian studies have reported α7 nAChRs both pre- and postsynaptically ([Duffy et al., 2009; Fabian-Fine et al., 2001; Jones et al., 2004; Jones and Wonnacott, 2004; Levy and Aoki, 2002; Lubin et al., 1999; Pakkanen et al., 2005]), less is known about the subcellular localization of nAChRs in *Drosophila*. Da7 has been localized to dendrites in multiple neurons in the CNS, including the giant fiber neurons that mediate the escape reflex, Kenyon cells (KCs) intrinsic to the mushroom bodies, and motion-sensitive lobula plate tangential cells (Fayyazuddin et al., 2006; Raghu et al., 2009); it is unclear, however, whether Da7 is also trafficked to any axonal/presynaptic regions of neurons like mammalian α7 receptors.

We first examined anti-Da7 immunostaining in the brain, which showed widespread expression, albeit with variable intensity, throughout much of the neuropil (Figure S2). Since neuropil regions contain both axonal projections and dendritic arbors, we used the UAS/Gal4 system to drive expression of UAS-Da7-EGFP in neuronal sub-populations, in which these subcellular compartments are easily distinguished by their anatomy in the intact brain. Previous studies have also shown that expression of UAS-Da7-EGFP results in localization similar to the anti-Da7 antibody (Fayyazuddin et al., 2006; Leiss et al., 2009). We used the GH146-Gal4 and 201Y-Gal4 transgenes to drive expression of UAS-Da7-EGFP and UAS-DsRed in projection neurons (PNs) of the antennal lobe (AL) or a subset of KCs, respectively. Young adult brains were dissected, immunostained, and imaged by confocal microscopy. In PNs, Da7-EGFP signal was found in cell bodies (CBs), in the dendrites of glomeruli in the AL, and in the proximal region of the major inner antennocerebral tract (iACT) of axons and throughout axons of the medial ACT (mACT) (Figures 2A and 2B); DsRed signal was seen in all subcellular compartments of the PNs, as expected (Figures 2A and 2B). A recent study has similarly found Da7-EGFP localization in the proximal iACT in brains from young flies (Hussain et al., 2018). To reduce overall overexpression of Da7 and the chance of mis-expression, we also performed these experiments in a Da7ΔEY6 null mutant background and found a similar localization pattern (Figure S3).

In KCs, Da7-EGFP signal was also found in CBs, which are clustered in a layer just above the calyx (CX), a neuropil structure into which KCs extend their dendrites, as well as in the CX itself (Figures 2C and 2D). KC axons fasciculate into an anteriorly projecting structure called the peduncle (PED) that then branches into five distinct lobes of the MBs; the 201Y-Gal4 transgene drives expression in KCs that extend axons into the α/β and γ lobes. We found Da7-EGFP signal clearly in the PED (Figures 2C and 2D) but less prominently and perhaps absent from more distal axonal regions that extend into in the mushroom body (MB) lobes (data not shown); a similar localization pattern was also observed in a Da7ΔEY6 null mutant background (Figure S3).

Altogether, we suggest that Da7 is localized to somato-dendritic compartments, as well as proximal regions of axonal tracts. Interestingly, when Cha2/+ mutants were subjected to longer HTs (e.g., 6–7 h), not only were mEPSC amplitudes enhanced, but mEPSC interevent times were also significantly decreased (Figure S4). These results suggest that there may be a later stage of HSP mediated on the presynaptic side of synapses, perhaps involving these axonally localized Da7 nAChRs.
**In Vivo Activity Blockade Induces an Increase in Kv4/Shal Channel Protein That Is Dependent on Dα7**

To test if *in vivo* activity inhibition using the *Chd*<sup>s2</sup> allele also induces an upregulation of Kv<sub>4</sub>/Shal channels, newly eclosed *Chd*<sup>s2</sup>/+ and WT flies were collected and subjected to HT at 30ºC. We tested different durations of HT, followed by a 3-h recovery period at 18ºC. We found that *Chd*<sup>s2</sup>/+ flies displayed a ~27% increase in Kv<sub>4</sub> protein with a 3- or 6-h HT (Figure 3A); we refer to the 3 or 6 h of HT, followed by 3 h of recovery, as 3/3 or 6/3-protocols, respectively. Similarly treated WT flies showed no change in relative Kv<sub>4</sub>/Shal protein levels (Figure 3B). Because *in vitro* studies had shown that the upregulation of Kv<sub>4</sub>/Shal was not apparent immediately following cholinergic blockade (3/0-protocol) but required recovery of synaptic transmission, we subjected *Chd*<sup>s2</sup>/+ flies to HT for 3 h with different durations of recovery. Indeed, we found that no increase in Kv<sub>4</sub>/Shal was observed immediately following activity inhibition and required a 3-h recovery period (Figure 3C).

For further confirmation that HT of *Chd* was a reliable method of inducing HSP, we tested another *Chd* allele, *Chd*<sup>s3</sup> (Kitamoto et al., 2000). Heterozygous *Chd*<sup>s3</sup>/+ flies subjected to the 3/3-protocol also exhibited ~29% increase in Kv<sub>4</sub>/Shal protein (Figure 3D).

We also inhibited cholinergic activity by another approach, using a transgenic line that expresses tetanus toxin light chain (TnT), which cleaves n-synaptobrevin and has been shown to completely block evoked neurotransmitter release and reduce spontaneous release by 50%–75% (Deitcher et al., 1998; Sweeney et al., 1995). We used a *Chat-Gal4* line, which drives expression of Gal4 in ChAT-expressing neurons, to induce expression of *UAS-TnT*. To induce expression transiently, we included expression of the temperature-sensitive Gal80 protein (Gal80<sup>ts</sup>) to negatively regulate the function of Gal4. At 18ºC, Gal80<sup>ts</sup> inhibits Gal4; at 30ºC, Gal80<sup>ts</sup> is no longer functional, and Gal4 promotes transcription of the *UAS-TnT* transgene in cholinergic neurons. We raised *Chat-Gal4/tub-Gal80<sup>ts</sup> >> UAS-TnT* and WT lines at 18ºC to allow them to develop normally, without expression of UAS-TnT, then AE, young adult flies were subjected to HT at 30ºC for 3 or 6 h. After HT, we allowed flies to recover at 18ºC for 3 h. Fly heads were analyzed by immunoblot analysis for steady-state levels of Kv<sub>4</sub>/Shal normalized to a loading control protein. We found that levels of Kv<sub>4</sub>/Shal were increased by 42% after a 6/3-protocol (Figure 3E).

We next tested if the upregulation of Kv<sub>4</sub>/Shal induced by *in vivo* activity blockade requires the Da.7 nAChR. 0/0- and 3/3-protocols were applied to *Chd*<sup>s2</sup>/+ flies in a Da.7<sup>PΔEY6</sup> null mutant background (*Da.7<sup>PΔEY6</sup>;*Cha*<sup>s2</sup>+/+). We found that in the absence of Da.7 nAChRs, *Chd*<sup>s2</sup>-induced upregulation of Kv<sub>4</sub>/Shal was indeed inhibited (Figure 3F), suggesting a requirement for the Da.7 nAChR. Together, our results suggest that *in vivo* inhibition of cholinergic activity induces a homeostatic upregulation of Da.7 nAChRs that is required for the subsequent increase in Kv<sub>4</sub>/Shal protein.

**Activity Inhibition Induces Upregulation of Kv<sub>4</sub>/Shal Current**

We then tested whether *Chd*<sup>s2</sup>-mediated activity inhibition would also induce a detectable increase in Kv<sub>4</sub>/Shal current in neurons. Primary cultures were grown at 18ºC for 8 DIV, then subjected to HT at 30ºC for 6 h followed by 2–3 h of recovery at 18ºC. Previous studies have identified Kv<sub>4</sub>/Shal currents in these neurons as the predominant A-type K<sup>+</sup> current.
present, one that can easily be isolated from the delayed rectifier (DR) currents encoded by 
$K_{v}2(Shab)$ and $K_{v}3(Shaw)$ (Tsunoda and Salkoff, 1995a, 1995b). Because of the 
hyperpolarized closed-state inactivation properties of $K_{v}4/Shal$ channels, a 500-ms prepulse 
at $-45 \text{ mV}$ specifically inactivates $K_{v}4/Shal$ channels in these neurons, and the $K_{v}4/Shal$
current can be isolated by subtracting the DR component from the whole-cell current (Ping 
and Tsunoda, 2011; Ping et al., 2011a; Tsunoda and Salkoff, 1995a, 1995b). Indeed, $K_{v}4/$
Shal current density in HT $Cha^{52}/+$ neurons was 67% and 88% greater than in non-HT $Cha^{52}/+$ neurons or in HT background control neurons, respectively (Figure 4); HT alone 
did not induce a change in $K_{v}4/Shal$ current density in genetic background control neurons 
(Figure 4).

In Vivo Activity Blockade Also Induces a $D_{\alpha}7$-Dependent Increase in $K_{v}4/Shal$ mRNA

Although inactivity-induced upregulation of $K_{v}4/Shal$ protein was previously shown to be 
blocked by transcriptional inhibitors (Ping and Tsunoda, 2011), it has been unclear whether 
this was due to a transcriptional block of $K_{v}4/Shal$ itself since mRNA levels of $K_{v}4/Shal$
could not be examined in that preparation. Our in vivo $Cha^{52}$ and $ChatGal4/tub-
GAL80^{ts}>>UAS-TnT$ models, however, allowed for sufficient mRNA to be isolated for 
quantitative reverse transcriptase polymerase chain reactions (qRT-PCR). We examined if 
mRNA levels of $K_{v}4/Shal$ are indeed elevated following activity blockade. To this end, we 
validated universal probes with corresponding PCR primers for efficient amplification and 
optimized RNA preparation, RT reactions, and cDNA dilution factors for reliable qRT-PCR 
(see Method Details). For every experimental genotype and genetic background tested, the 
stability of each of the reference genes, ribosomal protein S20 ($RpS20$) and eukaryotic 
initiation factor 1A (eIF1A), was validated before use (see Method Details).

We tested both the $Chat-Gal4/tub-Gal80^{ts}>>UAS-TnT$ and $Cha^{52}/+$ line immediately after 
inhibiting activity with a 6-h or 3-h HT at 30ºC, respectively, each of which leads to an 
upregulation of $K_{v}4/Shal$ protein that is detectable after a subsequent 3 h of recovery 
(Figures 3A and 3E). We quantified $K_{v}4/Shal$ mRNA levels relative to reference gene 
expression without HT (0/0) and after 6 or 3 h HT (6/0 or 3/0, respectively). In WT, $K_{v}4/$
Shal mRNA levels showed no change with HT (Figures 5A and 5B, left). In contrast, when 
HT was applied to $Chat-Gal4/tub-Gal80^{ts}>>UAS-TnT$ or $Cha^{52}/+$ lines, $K_{v}4/Shal$ mRNA 
levels were significantly elevated by 27% and 23% compared to untreated controls, 
respectively (Figures 5A and 5B, right). Interestingly, the rise in $K_{v}4/Shal$ mRNA was rather 
short-lived when examined in $Cha^{52}$ flies, returning to baseline levels after 3 h of recovery 
at 18ºC (3/3-protocol; Figure 5B, right), suggesting a transient and dynamic regulation of 
$K_{v}4/Shal$ mRNA.

We next tested whether the inactivity-induced upregulation of $K_{v}4/Shal$ mRNA is dependent 
on $D_{\alpha}7$ nAChRs, as was the increase in $K_{v}4/Shal$ protein (see Figure 3F). We examined relative 
$K_{v}4/Shal$ mRNA levels in $Da^{7PAEY6};Cha^{52}/+$ flies. We found that the 3/0-protocol 
that induced an increase in $K_{v}4/Shal$ mRNA in $Cha^{52}/+$ mutants resulted in no significant 
increase in $K_{v}4/Shal$ mRNA in the $Da^{7PAEY6}$ null background (Figure 5C, right); as an 
additional control, we tested $Da^{7PAEY6}$ null mutants alone and found no change in $K_{v}4/Shal$
mRNA levels with HT (Figure 5C, left). Together, our results show that cholinergic activity
blockade/inhibition induces an increase in $K_v 4/Shal$ mRNA, and this upregulation in $K_v 4/Shal$ mRNA is dependent on Da7 nAChRs.

**Increased Expression of Da7 is Sufficient to Upregulate $K_v 4/Shal$ Channel Protein and mRNA**

We next tested if upregulation of Da7-EGFP alone is sufficient to induce an increase in $K_v 4/Shal$ channel protein and mRNA. To transiently induce expression of UAS-Da7-EGFP, we generated elav-Gal4;tub-Gal80$^{ts}$>>UAS-Da7-EGFP/+ lines. We raised elav-Gal4;tub-Gal80$^{ts}$>>UAS-Da7-EGFP/+ and background control lines at 18°C to allow them to develop normally, without overexpression of Da7-EGFP, and then AE, young adult flies were subjected to HT at 30°C for 4 days to induce expression of Da7-EGFP (Figure 6A). We then tested whether this post-eclosion overexpression of Da7-EGFP induced an increase in steady-state level of $K_v 4/Shal$ protein. Indeed, we found that induced expression of Da7-EGFP resulted in $K_v 4/Shal$ protein levels that were elevated by ~14% (Figure 6A, left). Similar upregulation of $K_v 4/Shal$ was also observed when we used a Da7-Gal4 driver (Figure S5).

To further test the Da7-$K_v 4/Shal$ relationship, we set out to increase the expression of endogenous Da7 and test for effects on $K_v 4/Shal$. Recent studies have identified NACHO as a transmembrane endoplasmic reticulum resident protein that promotes biogenesis and surface expression of nAChRs (Matta et al., 2017) and, in particular, a7 nAChRs (Gu et al., 2016). Thus, we aimed to overexpress the Drosophila ortholog of NACHO in neurons, as a way of increasing the expression/trafficking of endogenous Da7 nAChRs, and then test if this results in a consequent increase in $K_v 4/Shal$ expression. We used an UAS-NACHO-3xHA transgenic line in combination with elav-Gal4 and tub-Gal80$^{ts}$ transgenes to conditionally overexpress UAS-NACHO-3xHA in the nervous system of adult flies. The 3xHA epitope tag was used to confirm expression of NACHO-3xHA. We raised elav-Gal4;tub-Gal80$^{ts}$>>UAS-NACHO-3xHA and background control lines at 18°C, and then AE, flies were subjected to HT at 30°C. Interestingly, elav-Gal4;tub-Gal80$^{ts}$>>UAS-NACHO-3xHA flies, which were homozygous for the UAS-NACHO-3xHA insertion, were only viable for 12–15 h at 30°C. As such, we assayed $K_v 4/Shal$ protein at 12 h and found that $K_v 4/Shal$ protein levels were indeed elevated in HT elav-Gal4;tub-Gal80$^{ts}$>>UAS-NACHO-3xHA flies by ~33%, while no change in $K_v 4/Shal$ was observed in similarly treated background control lines (Figure 6B).

Interestingly, $K_v 4/Shal$ protein levels were increased by ~33% with NACHO overexpression but were increased by only ~14% with Da7-EGFP overexpression. One possibility is that endogenous NACHO is limiting when Da7-EGFP is overexpressed, preventing maximal Da7/Da7-EGFP surface expression and thereby more limited upregulation of $K_v 4/Shal$. To address this, we conditionally overexpressed both UAS-NACHO-3xHA and UAS-Da7-EGFP, using the elav-Gal4;tub-Gal80$^{ts}$ driver line. To allow for more balanced expression of NACHO-3xHA and Da7-EGFP, experimental and background control lines used were heterozygous for each insertion. Both lines were raised at 18°C during development, and then AE, flies were subjected to HT at 30°C. With co-expression of UAS-NACHO-3xHA/UAS-Da7-EGFP, flies lived longer than those overexpressing two copies of UAS-

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NACHO-3x-HA alone, allowing us to apply HT for days. We then examined how co-overexpression of NACHO-3xHA and Dα7-EGFP affected Kv4/Shal protein levels. We found that Kv4/Shal protein levels more than tripled (Figure 6C). To confirm that overexpression of the NACHO-3xHA fusion protein does indeed result in increased Dα7/DA7-EGFP levels, we compared transgenic lines expressing Dα7-EGFP with and without NACHO-3xHA expression. We found that, indeed, Dα7-EGFP levels were elevated by ~45% when NACHO-3x-HA was expressed (Figure 6D). Interestingly, we also found that RNAi knockdown of NACHO prevented any Cha^{ts2}-induced increase in Kv4/Shal (Figure 6E), suggesting that NACHO is required for the inactivity-induced upregulation of Kv4/Shal.

We next tested if induced expression of Dα7-EGFP and/or NACHO results in an elevation in Kv4/Shal mRNA. We used the elav-Gal4 driver in combination with the temperature-sensitive Gal80 protein, as performed when examining Kv4/Shal protein expression. For each genotype, we assayed for Kv4/Shal mRNA levels at times prior to detected elevations in Kv4/Shal protein (see Figures 3, 5, and 6). Indeed, Kv4/Shal mRNA levels were enhanced by Dα7-EGFP or NACHO overexpression (Figures 6A and 6B, right) and similar to induced Kv4/Shal protein levels, even more so by overexpression of Dα7-EGFP and NACHO (Figure 6C, right). Together, our results suggest that an increase in Dα7-EGFP alone is sufficient to trigger an upregulation of Kv4/Shal mRNA and protein expression.

**Cha^{ts2}-Induced Inactivity, or Overexpression of Dα7, Activates the NFAT-Based CaLexA Reporter**

With mounting evidence that Kv4/Shal is dynamically and transcriptionally regulated downstream of changing neural activity and Dα7 nAChR levels, we set out to identify transcriptional regulators involved. Previously, we demonstrated that the inactivity-induced increase in Kv4/Shal was dependent on intracellular Ca^{2+}, likely enhanced by the increased number of Ca^{2+}-permeable Dα7 nAChRs (Ping and Tsunoda, 2011). We considered Ca^{2+}-dependent transcriptional regulators, including NFATs, which are activated by the Ca^{2+}-dependent protein phosphatase 2b, calcineurin (CaN). CaN has been shown to trigger translocation of mammalian NFATs into the nucleus, where they lead to increases or decreases in transcription (Macián et al., 2001; Mognol et al., 2016; Rao et al., 1997). To test if NFAT might be a Ca^{2+}-dependent transcriptional regulator involved in the inactivity-induced upregulation of Kv4/Shal, we first examined if inactivity induced with the Cha^{ts2} allele would activate an in vivo NFAT-based reporter system, the Ca^{2+}-dependent nuclear import of LexA (CaLexA) system (Masuyama et al., 2012). In the CaLexA system, a transgene containing the regulatory domain of human NFATc1 is fused to the mutant bacterial DNA-binding protein mLexA and the VP16 activation domain, UAS-mLexA-VP16-NFAT. When the mLexA-VP16-NFAT protein is expressed, it is cytoplasmic, and if the NFAT regulatory domain is activated, the fusion protein translocates to the nucleus, where it promotes transcription from a LexAop sequence upstream of a reporter gene. This modified human NFAT has been shown to successfully translocate to the nucleus and drive expression of LexAop-CD2/8-GFP in the fly CNS and has been used as a Ca^{2+} reporter (Masuyama et al., 2012).
We used elav-Gal4 to drive neuronal expression of UAS-mLexA-VP16-NFAT, in a Chd\textsuperscript{s2}/+ mutant background, which also contained two reporter insertions, LexAop-CD8-GFP and LexAop-CD2-GFP. These flies were subjected to 0/0 and 3/0 HT (30ºC) protocols. We then tested for CD8/CD2-GFP expression by immunoblot analysis, as an indicator for CaLexA activation. Indeed, we found that CD8/CD2-GFP expression was increased by ~49% with the 3/0-protocol, compared with age-matched flies not subjected to HT (0/0) (Figure 7A, left), and levels of CD8/CD2-GFP continue to rise with additional recovery time after HT (Figure S6). In contrast, elav-Gal4>UAS-mLexA-VP16-NFAT/LexAop-CD8-GFP;LexAop-CD2-GFP/+ lines, without the Cha\textsuperscript{s2} allele, showed no increase in CD2/8-GFP with the same HT. These results suggest that inactivity induced by Chd\textsuperscript{s2} does indeed activate an NFAT-based reporter system in vivo.

We additionally tested whether overexpression of Da7-EGFP alone was sufficient to activate the CaLexA reporter. We raised elav-Gal4;tub-Gal80\textsuperscript{ts}>>UAS-Da7-EGFP/UAS-mLexA-VP16-NFATLexAop-CD8-GFP;LexAop-CD2-GFP/+ flies at 18ºC to allow for normal development, and then AE, young adult flies were subjected to HT at 30ºC to induce expression of Da7-EGFP. Because HT on the order of days was required to observe sufficient overexpression of Da7-EGFP using the elav-Gal4;tub-Gal80\textsuperscript{ts}-inducible driver to induce \(K_v4/Shal\) expression (Figure 6A), we expected that activation of the CaLexA reporter would require at least 24 h HT to induce sufficient overexpression of Da7-EGFP. We found that CD8/CD2-GFP reporter expression more than doubled after 24 h of heat-induced Da7-EGFP expression compared to HT of the background control line, elav-Gal4;tub-Gal80\textsuperscript{ts}>>UAS-mLexA-VP16-NFATLexAop-CD8-GFP/+;LexAop-CD2-GFP/+ (Figure 7A, right). Our results demonstrate that inactivity, which homeostatically upregulates Da7, or even direct overexpression of Da7, is sufficient to activate an NFAT-based reporter system and possibly endogenous NFAT itself.

**NFAT Is Required for Inactivity-Induced Upregulation of \(K_v4/Shal\)**

We next investigated whether NFAT might be involved in the regulation of \(K_v4/Shal\) expression. In *Drosophila*, there is only a single NFAT gene (Keyser et al., 2007) that is 53%–64% similar to mammalian NFATs (Freeman et al., 2011). *Drosophila* NFAT has two predicted splice forms, \(\text{--A and --B}\), generated from two different promoter sites (Keyser et al., 2007). We obtained mutants that have been generated and verified to eliminate both NFAT splice forms (NFAT\textsuperscript{ΔAB}), NFAT-A only (NFAT\textsuperscript{ΔA}), or NFAT-B only (NFAT\textsuperscript{ΔB}) (Freeman et al., 2011; Keyser et al., 2007). First, we tested flies that were null mutants for either or both NFAT-A and/or NFAT-B and compared them to WT controls. We found no significant difference in steady-state \(K_v4/Shal\) protein or mRNA levels (Figure 7B), suggesting that NFAT does not regulate basal levels of \(K_v4/Shal\) expression.

Next, we tested if NFAT is required for the inactivity-induced upregulation of \(K_v4/Shal\). We crossed NFAT mutant alleles into the Chd\textsuperscript{s2}/+ mutant background and assayed whether inhibition of activity could still induce an upregulation of \(K_v4/Shal\) protein in these flies. We subjected mutant combinations of Chd\textsuperscript{s2} with NFAT\textsuperscript{ΔA}, NFAT\textsuperscript{ΔB}, or NFAT\textsuperscript{ΔAB}, as well as background control lines, to the 0/0- and 3/3-protocols that upregulate \(K_v4/Shal\) expression in Chd\textsuperscript{s2}/+ mutants. We found that Chd\textsuperscript{s2}-induced inactivity was unable to elicit an increase in CD8/CD2-GFP expression.
in Kv4/Shal protein in the absence of NFAT-A and/or NFAT-B (Figures 7C–7E, left); NFAT mutant alleles alone also showed no difference in Kv4/Shal levels with HT.

We then tested these same genotypes for inactivity-induced upregulation of Kv4/Shal mRNA. We subjected flies to the 0/0- and 3/0-protocols in which an upregulation in Kv4/Shal mRNA is detected following inactivity. Similar to Kv4/Shal protein analyses, we found that a loss of NFAT-A and/or NFAT-B blocked the upregulation of Kv4/Shal mRNA (Figures 7C–7E, right); HT of NFAT alleles alone also had no effect on Kv4/Shal mRNA levels. Altogether, our results suggest that while NFAT does not affect basal levels of Kv4/Shal mRNA or protein, NFAT is required for the transient upregulation of Kv4/Shal mRNA and protein induced by cholinergic activity blockade.

**DISCUSSION**

While HSP has been widely studied at glutamatergic synapses, less is known about these mechanisms at cholinergic synapses. Proper tuning of HSP at any synapse is critical for neuroprotection since over-/under-compensation is likely to be maladaptive. Here, we inhibit activity that evokes a homeostatic increase in mEPSC amplitude that correlates with an increase in Da7 nAChR protein as well as a subsequent increase in Kv4/Shal channel expression that is dependent on Da7 receptors. We show that regulation of Kv4/Shal is at the mRNA level and is dependent on the transcriptional regulator, NFAT. We also show that upregulation of Kv4/Shal can be induced by an increase in Da7 nAChRs alone, demonstrated by overexpressing Da7-EGFP and/or the recently identified α7 chaperone protein, NACHO, and suggesting that there is a homeostatic Da7-Kv4/Shal regulatory pathway that has evolved to prevent over-excitation whenever there is an over-production of Da7 nAChRs.

α7 nAChRs are known to be highly abundant in the mammalian brain, and their Ca2+-permeability makes them ideal candidates for mediating different forms of plasticity. As such, preventing over-activity of these receptors is likely to be important, and the “coupling” of Kv4/Shal expression to levels of Da7 receptors is a mechanism that would act as a protective “brake.” Because Kv4/Shal channels have been shown to open at subthreshold potentials and regulate mEPSCs as well as the time to first action potential firing (Ping and Tsunoda, 2011; Ping et al., 2011a), they make good modulators for tuning subthreshold membrane potentials. Indeed, regulation of Kv4/Shal channels has been shown to be involved in other forms of synaptic plasticity, including long-term-potentiation (LTP) (Chen et al., 2006; Jung and Hoffman, 2009; Kim et al., 2007). Future studies will need to investigate if newly upregulated Kv4/Shal channels are trafficked to specific synapses, perhaps to sites where Da7 nAChRs have been especially enhanced, for local modulation of membrane potential.

Our study also shows that in vivo induction of NACHO-3xHA results in an upregulation of total Da7-EGFP protein and Kv4 channel protein. With two copies of UAS-NACHO-3xHA, and likely stronger induction of NACHO-3xHA, the increase in Kv4 occurs within 12 h. It is unclear whether, in this rather short amount of time, this is due to an increased steady-state level of Da7 protein and/or facilitated Da7 release from the endoplasmatic reticulum (ER)
that results in increased surface expression. Future studies will need additional approaches to study how NACHO functions in the trafficking and/or stabilization of Da7 nAChRs.

Finally, we show that the Ca\(^{2+}\)/CaN-dependent transcriptional regulator, NFAT, is involved in the inactivity/D\(\alpha\)7-induced increase in \(K_{v4}/Shal\) expression. Other studies have also implicated NFATs in the regulation of ion channels, especially in the cardiovascular system. For example, in arterial smooth muscle, the vasoactive peptide, angiotensin II, induces an increase in Ca\(^{2+}\) through L-type Ca\(^{2+}\) channels that subsequently leads to a CaN-NFATc3-dependent downregulation of \(K_{v2.1}\) channel expression (Amberg et al., 2004). During hypertension, NFATc3 has also been implicated in the downregulation of the \(\beta1\) subunit of large/big conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels (Nieves-Cintrón et al., 2007). In cardiomyocytes, NFATc3 is reported to decrease \(K_{v4.2}\) expression in response to myocardial infarction (Rosow et al., 2004) and to increase \(K_{v4.2}\) expression during cardiac hypertrophy (Gong et al., 2006). NFATc3 has also been reported to contribute to the gradient of \(K_{v4}\) expression across the mouse left ventricular free wall (Rosow et al., 2006). In neurons of the rat superior cervical ganglion (SCG), increases in activity were shown to induce activation of NFATc1/c2, which leads to an increase in \(K_{v7}\) channel expression (Zhang and Shapiro, 2012). In cerebellum granule neurons, Ca\(^{2+}\)/CaN-NFATc4 signaling underlies the upregulation of \(K_{v4.2}\) expression in response to the neurotrophin, neuritin (Yao et al., 2016). In Drosophila, overexpression of one splice form of the single \(NFAT\) gene has been shown to result in the suppression of excitability in motor neurons (Freeman et al., 2011), although the ion channels involved have not been identified. We used the same overexpression line but found no significant increase in \(K_{v4}/Shal\). It is possible that activated NFAT is required to induce HSP and upregulation of \(K_{v4}/Shal\), rather than solely an increase in basal levels of NFAT. Future studies will also need to address whether activated NFAT acts directly on the \(K_{v4}\) locus.

Uncovering how HSP mechanisms are tuned to accurately protect neurons from over-/under-activity is critically important to understanding how an adaptive HSP response can become maladaptive. For example, HSP has been reported to be impaired or maladaptive in mouse and Drosophila AD models (Gilbert et al., 2016; Hahm et al., 2018; Jang and Chung, 2016). Interestingly, \(K_{v4}/Shal\) channels examined in this study, which tune HSP in cholinergic neurons, have been shown to be especially affected in various models of AD, and genetic restoration of \(K_{v4}/Shal\) channels ameliorates downstream pathologies (Hall et al., 2015; Ping et al., 2015; Scala et al., 2015), supporting the idea that tuning HSP by \(K_{v4}/Shal\) channels is critically important. With a better understanding of how HSP is accurately tuned, these molecules and mechanisms may be identified as potential targets for treatment when HSP becomes maladaptive.

**STAR METHODS**

**RESOURCE AVAILABILITY**

**Lead Contact**—Further information and requests should be directed to, and will be fulfilled by the Lead Contact, Susan Tsunoda (susan.tsunoda@colostate.edu).

**Materials Availability**—This study did not generate new unique reagents.
Data and Code Availability—This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

_Drosophila_ Stocks—_w^{1118}_ or genetic background strains were used as control lines in this study. We used previously generated mutant and transgenic lines: _UAS-TnT_ (Deitcher et al., 1998; Sweeney et al., 1995); _Chats2_ and _Chats3_ alleles (Salvaterra and McCaman, 1985); _UAS-Da7-EGFP_ (Leiss et al., 2009) and _Da7^{PΔEY6}_ (Fayyazuddin et al., 2006); _UAS-NACHO-3xHA_ (Bischof et al., 2013); _UAS-mLexA-VP16-NFAT, LexAop-CD8-GFP_, and _LexAop-CD2-GFP_ transgenes (Masuyama et al., 2012); _NFAT^{ΔA}, NFAT^{ΔB},_ and _NFAT^{ΔAB}_ alleles (Keyser et al., 2007); _elav-GAL4 (elav-GAL4^{c155})_ and _tub-GAL80^{P}_ (Bloomington Drosophila Stock Center, Indiana University); _201Y-GAL4_ (O’Dell et al., 1995; Yang et al., 1995); _GHI46-GAL4_ (Stocker et al., 1997); _UAS-RNAi-NACHO_ (VDRC, Vienna, Austria), _Da7-GAL4_ (Fayyazuddin et al., 2006). All _Drosophila_ stocks were raised at 18–25°C as specified in text, on standard fly food medium.

METHOD DETAILS

Reverse Transcription-Quantitative Polymerase Chain Reaction (qRT-PCR)

RNA isolation, Reverse Transcription, and qPCR: Total RNA was extracted from 10 fly heads using TRizol reagent, treated with DNase I (Thermo Scientific) to remove potential genomic DNA contamination. The integrity of the representative RNA samples was assessed using gel electrophoresis. Total RNA concentration was measured in duplicate using NanoDrop Lite Spectrophotometer (Thermo Scientific) and the purity of the samples was estimated by the OD ratios (A260/A280, ranging within 1.9–2.0). cDNA was synthesized from 700 ng of DNA-free total RNA in a 20 μl reaction volume using SuperScript II RT (Invitrogen) and Oligo (dT) as reverse transcription primers. cDNA samples were diluted 1:5 for qPCR reactions. Gene-specific transcription levels were determined in a 20-μl reaction volume in triplicate using a probe from the Universal Probe Library (UPL) and Light Cycler® 480 (Roche) following the manufacturer’s instructions; under the following conditions: incubation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s and 72°C for 1 s.

Primer design and verification—Common sequence from multiple mRNA transcript variants (predicted in Fly Base) were used for PCR primer design. Probe finder version 2.35 and intron spanning assay (Roche) were used to find a proper probe and design primers; Primer3 software was used with the following settings: melting temperatures between 59°C and 61°C, GC content between 40 and 60% and amplicon length limited to 60–200 base pairs. The maximum self-complementarity of the primers was set at 8 and the maximum 3’ complementarity at 3. The PCR primer sets specificity were verified by Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the _Drosophila_ transcriptome. Probe 66 was used for _Kv4/Shal_ primers (Left, GCTAACGAAAGGAGGAACG; Right, TGAACCTATTGCTGTTCATTTTGC) and _RPS20_ primers (Left, CGACCCAGGAAATTTGCTAAA; Right, CGACATGGGGCTTCTCAATA); Probe 147 was used for _eIF1A_ primers (Left, TCG TCT GGA GGC AAT GTG; Right, GCC CTG GTT AAT CCA CAC C). Real-time products were extracted for sequencing and PCR efficiency.
was calculated from 10-fold serial dilutions of cDNA samples; only PCR efficiencies of 1.9–2.0 were accepted. Similarity in GOI and Reference gene amplification efficiencies were verified, which allowed us to use $2^{-\Delta\Delta Ct}$ method for data analysis. Ribosomal Protein S 20 (RpS20) and Eukaryotic Initiation Factor 1 A (eIF1A) were selected as reference genes based on their stability across experimental conditions.

**Data Analysis**—After completing each real-time PCR run, outlier C_t values among each triplicate were identified and excluded manually using the Q-test. Individual $\Delta\Delta C_t$ values were calculated as the difference between $\Delta C_t$ (Experimental or Control) and the average $\Delta C_t$ (Control) value; $\Delta C_t$ is the difference between averaged triplicate C_t values of the GOI and triplicate C_t values of the reference gene. $\Delta\Delta C_t$ values were used to calculate $2^{-\Delta\Delta Ct}$ values, which represent relative fold-changes of the GOI in the experimental group relative to the GOI in the control group.

**Digital Drop PCR**—RNA isolation, RT reactions, primers and probes for Kv4/Shal and reference gene amplification are as described for RT-qPCR. To generate droplets, the 20ul PCR reaction mix and 60ul droplet generation oil were added to wells in a DG8 Cartridge for the QX200 Droplet Generator (Bio-Rad Laboratories). After automated droplet generation, droplets were transferred to a 96-well plate. The plate was sealed with foil using the PX1 PCR Plate Sealer (Bio-Rad Laboratories), and PCR amplification was performed (C1000 Touch Thermal Cycler, Bio-Rad Laboratories). The following thermal cycling protocol was used: 95°C for 10 minutes (one cycle), 94°C for 30 s (40 Cycles) and then 60°C for 1 minute (40 cycles), 98°C for 1 minutes (one cycle), hold at 4°C. The ramp rate was set at 2°C/s, the sample volume at 40 mL, and the heated lid at 105°C. After PCR amplification, plate was read in the QX200 Droplet Reader (Bio-Rad Laboratories). Absolute template expression in copies per microliter were quantified using QuantaSoft software (Bio-Rad Laboratories); number of Kv4/Shal copies/ul were normalized to the number of RpS20 copies/ul from the same RNA sample.

**Immunoblot Analysis**—For each sample, five adult *Drosophila* heads were sonicated in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 10% SDS, glycerol, Dithiothreitol (DTT), bromophenol blue); N refers to the number of samples tested. Proteins were separated on a 10% acrylamide gel. Nitrocellulose blots were probed with primary antibodies overnight at room temperature: anti-Kv4/Shal 1:100, anti-actin 1:10000 (EMD Millipore), anti-GFP 1:10000 (Torrey Pines Biolabs); anti-HA 1:500 (Covance Research Products), and anti-syntaxin 1:50 (Developmental Hybridoma Studies Bank). Anti-Kv4/Shal antibodies were generated as previously described (Diao et al., 2010, 2009). Blots were incubated with peroxidase-conjugated secondary antibodies (1:1000; Jackson ImmunoResearch Laboratories) for one hour at room temperature, developed using Supersignal Signal™ West Pico PLUS (Thermo Scientific). Kv4/Shal, GFP, and HA signal densities were normalized to densities from loading control signals from the same lane.

**Electrophysiological Recordings**—For neuronal cultures, single embryos aged 5–6 hours (at room temperature) were dissociated in drop cultures of 20 μL culture medium, as previously described (Ping and Tsunoda, 2011; Tsunoda and Salkoff, 1995a, 1995b).

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Cultures were grown for up to 2 weeks in a humidified chamber at room temperature. Whole-cell recordings were performed in perforated patch-clamp configuration by adding 400 – 800 μg/ml Amphotericin-B (Sigma-Aldrich) in the pipette, as described previously (Ping and Tsunoda, 2011; Ping et al., 2011a). We used external solution (in mM): NaCl, 140; KCl, 2; HEPES, 5; CaCl$_2$, 1.5; MgCl$_2$, 6; pH 7.2. For mEPSC recordings, TTX (1 μM) was added to the external solution to block voltage-dependent Na$^+$ channels. Electrodes were filled with internal solution (in mM): K-gluconate, 120; KCl, 20; HEPES, 10; EGTA, 1.1; MgCl$_2$, 2; CaCl$_2$, 0.1; ATP-Mg, 4; pH 7.2. All recordings were performed at room temperature. Gigaohm seals were obtained for whole-cell recordings. Data was acquired and analyzed using an Axopatch 200B amplifier, Axon Digidata 1550 and pClamp 10 software (Molecular Devices Corp.). Recordings were digitized at 5 kHz and filtered at 2 kHz, using a lowpass Bessel filter.

**Immunostaining, Confocal Microscopy, and Image Processing**—Fixation and staining of 1–3 day old adult fly brains dissected in 1X PBS on ice was performed as previously described (Daubert and Condron, 2007). Primary antibodies used were chicken anti-GFP (1:2000, Aves Labs), rabbit anti-RFP (1:2000, Rockland Immunochemicals Inc.), and rat anti-D$_{a7}$ (1:2000, a gift from Dr. Hugo Bellen; Fayyazuddin et al., 2006). Alexa Fluorophores, anti-chicken 488, anti-rabbit 568, and anti-rat 488 and 568 from Invitrogen were used at a 1:2000 dilution. Fluorescent imaging was performed with Zeiss LSM 710 or LSM 800 confocal microscope, and images were analyzed in ImageJ. Images of D$_{a7}$-GFP expression were processed using the “Despeckle” noise filter in ImageJ before maximum intensity Z-projections were generated.

**Quantification of D$_{a7}$ labeling**—< 24 hour old adult male Cha$^{ts2}$/+ flies were either kept at 18°C for 9 hours (0/0) or heat-shocked at 30°C for 6 hours followed by 3 hours recovery at 18°C (6/3). Brains from both groups were then dissected and fixed onto the same glass coverslip in 4% PFA for 7 minutes to ensure identical treatment. This short fixation time was previously found to generate the best labeling with the rat anti-D$_{a7}$ antibody (Fayyazuddin et al., 2006; Raghu et al., 2009). Subsequent immunohistochemical steps were as described. Brains from each independent experiment were imaged on the same day with the same settings using a Zeiss LSM 710 confocal microscope.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All experiments were conducted multiple times with multiple samples per condition, as indicated. Q-tests were run to identify outliers, and an upaired Student’s t test was used to compare populations. Averaged data presented as mean ± SEM, p < 0.05 (Student’s t test). Additional specifics to particular techniques are given below.

**RT-qPCR**—After completing each real-time PCR run, outlier C$_t$ values among each triplicate were identified and excluded manually using the Q-test. Individual ΔΔC$_t$ values were calculated as the difference between ΔC$_t$ (Experimental or Control) and the average ΔC$_t$ (Control) value; ΔC$_t$ is the difference between averaged triplicate Ct values of the GOI and averaged triplicate Ct values of the reference gene. ΔΔC$_t$ values were used to calculate...
$2^{-\Delta \Delta ct}$ values, which represent relative fold-changes of the GOI in the experimental group relative to the GOI in the control group.

**Electrophysiology**—Analysis and presentation software used for electrophysiological data include: Mini Analysis (Synaptosoft, Inc.), Origin (Microcal Software), GraphPad Prism (GraphPad Software), and Photoshop (Adobe) and Illustrator (Adobe).

**Immunostaining quantification**—Brains from each independent experiment were imaged on the same day with the same settings using a Zeiss LSM 710 confocal microscope. Comparisons were only made between brains from the same coverslip. Equivalent summed Z stacks surrounding the anatomical regions containing prominent Da7 immunoreactivity were generated. ROIs were generated around the central brain and total fluorescence was measured in ImageJ.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Inhibiting activity induces an increase in mEPSCs and Dα7 nAChR protein
- Inhibiting activity induces upregulation of Kv4/Shal mRNA, protein, and current
- Increasing Dα7 and/or NACHO is sufficient to induce upregulation of Kv4/Shal
- Inactivity-induced upregulation of Kv4/Shal requires transcription factor NFAT
Figure 1. Inhibition of Cholinergic Activity in Cha\textsuperscript{+/-} Neurons Induces a Homeostatic Increase in mEPSC Amplitudes and DA\textsubscript{7} Protein

(A–C) Cha\textsuperscript{+/-} primary cultures 8 DIV, grown at 18\textdegree C, were heat treated (HT) at 30\textdegree C for indicated times before mEPSCs were recorded. Comparisons are shown of mEPSC inter-event time (A) and amplitude (B) between Cha\textsuperscript{+/-} cultures without HT and Cha\textsuperscript{+/-} cultures with HT for indicated times. Note that mEPSCs from Cha\textsuperscript{+/-} neurons show progressively decreased activity with significantly enhanced inter-event times with increasing HT (no HT, 750.19 ± 57.42 ms; 2/3 h HT, 1192.85 ± 76.34 ms; 4/5 h HT, 3364.76 ± 195.26 ms; N = 7–8 cells), and consequently, homeostatically enhanced amplitudes (no
HT, 6.12 ± 0.05 pA; 2/3 h HT, 9.26 ± 0.19 pA; 4/5 h HT, 6.95 ± 0.16 pA; N = 7–8 cells). (C) Representative traces showing synaptic activity from $Cha^{n2}/+$ control and experimental cells. Scale bars, 500 ms/5 pA.

(D) Quantification and representative immunoblots of $elav-Gal4>>UAS-Da7-EGFP/+$ and $elav-Gal4>>UAS-Da7-EGFP/Cha^{n2}$ flies grown at 18°C, then subjected to 0 or 3 h HT and 3 h recovery at 18°C (0/0 or 3/3, respectively). All immunoblots were run with five heads per sample per lane. Anti-GFP band intensities were normalized to those of anti-actin, which were used as a loading control; for each condition. N = 24–46 samples per condition. All data are presented as mean ± SEM; *p < 0.05, Student’s t test.
Figure 2. Dα7 nAChRs Are Localized to Somato-Dendritic and Axonal Compartments

Representative images showing Dα7-EGFP localization when UAS-Dα7-EGFP is driven by GH146-Gal4 (A and B) or 201Y-Gal4 (C and D). DsRed and EGFP signals were enhanced with antibody labeling and are shown in magenta and green, respectively, in (B) and (D).

(A) Z-projection of confocal sections of the left side of an GH146-Gal4>> UAS-DsRed adult fly brain, showing the anatomy of the projection neurons (PNs; midline is right of image). Scale bar, 20 μm.

(B) Top: Z-projection of three confocal sections spanning 1 μm showing DsRed labeling of the PN cell bodies (CBs) (CB and arrows indicate an example cluster) and antennal lobe (AL) with example glomeruli indicated (arrowhead). Dα7-EGFP is clearly observed in PN CBs and in the glomeruli of the AL (center). Scale bar, 20 μm. Inset: a magnified view of the boxed region demonstrating membrane localization of Dα7-EGFP in CBs (scale bar, 5 μm).

Bottom: Z-projection of three confocal sections spanning 1.5 μm showing DsRed (left) and Dα7-EGFP (center) localization on proximal axonal regions of the iACT (arrowhead) and mACT (arrow). Scale bar, 10 μm.

(C) 3D rendering of GFP signal in 201Y-Gal4>>UAS-mCD8-GFP adult brains, demonstrating cellular compartments of the mushroom body. The blue plane indicates the approximate location of the cross-section shown in bottom row of (D). Image is rotated as indicated by the axes. m, medial; a, anterior; v, ventral.

(D) Top: Z-projection of three confocal sections spanning 3 μm through the CBs and calyx (CX) of the mushroom body (left). Dα7-EGFP is observed on CBs and within the neuropil of the CX (center). Scale bar, 20 μm. Inset: a magnified view of the boxed region demonstrating membrane localization of Dα7-EGFP in CBs (scale bar, 5 μm).

Bottom: Single confocal cross-section of the peduncle (PED) showing DsRed (left) and Dα7-EGFP (center) localization on these axonal structures of the mushroom body. Scale bar, 10 μm. The
“despeckle” median noise filter in ImageJ was applied to all images shown from the GFP channel.
Figure 3. Blocking Neural Activity In Vivo Results in an Upregulation of Kv4/Shal Protein

(A–D) Quantification of relative Kv4/Shal protein levels and representative immunoblots from Chaps2/+ (A and C), wild type (WT; B), and Chaps3/+ (D) after HT protocols, indicated as hours of HT at 37°C/h of recovery at 18°C (e.g., 3/3, 6/3; 0/0 indicates flies kept at 18°C with no HT). Note that 3–6 h HT of Chaps2/+ induces an increase in Kv4/Shal protein.

(E) Quantification of relative Kv4/Shal protein levels and representative immunoblots from Chat-Gal4/tub-Gal80ts>>UAS-TnT flies after indicated HT shows a similar increase in Kv4/Shal levels after 6 h HT.

(F) Quantification and representative immunoblots of Da7PΔEY6; Chaps2/+ and Da7PΔEY6 samples after indicated HT protocols show no significant change in Kv4/Shal in the absence of Da7. All immunoblots were run with 5 male heads per lane; for each condition, number of samples (N) = 15–46. Anti-Kv4/Shal band intensities were normalized to those of anti-syntaxin (syn), which was used as a loading control.

Data are presented as mean ± SEM; *p < 0.05, Student’s t test.
Figure 4. Inhibition of Cholinergic Activity in Cha<sup>F2/+</sup> Neurons Induces an Increase in K<sub>v</sub>4/Shal Current Density

Primary cultures from genetic background controls (Ctrl) and Cha<sup>F2/+</sup> mutants (Cha<sup>F</sup>) were grown at 18ºC for 8 DIV, then either not HT (−HT) or HT for 6 h at 30ºC (+HT) followed by 2–3 h recovery at 18ºC. K<sub>v</sub>4/Shal currents were separated from delayed rectifier currents as described in the text. Peak K<sub>v</sub>4/Shal current densities were significantly increased in +HT Cha<sup>F2/+</sup> mutants compared to −HT Cha<sup>F2/+</sup> mutants or −HT and +HT controls (Ctrl−HT, 18.18 ± 3.15 pA/pF, N = 7; Ctrl+HT, 18.88 ± 3.93 pA/pF, N = 8; Cha−HT, 21.26 ± 3.59 pA/pF, N = 14; Cha<sup>F2+HT</sup>, 35.60 ± 5.99 pA/pF, N = 6). Data are presented as mean ± SEM; *p < 0.05, Student’s t test. Scale bars, 100 pA/10 ms.
Figure 5. Blocking Neural Activity In Vivo Induces an Increase in $K_v4/Shal$ mRNA, and This Increase Is Dependent on $\alpha_7$ nAChRs

qRT-PCR analyses of $K_v4/Shal$ mRNA levels normalized to reference gene expression, expressed as “fold-change” (see Method Details for analyses and calculation).

(A) Comparison of WT and Chat-Gal4>>UAS-TnT male flies subjected to HT at 30°C and recovery (HT/R; hours at 30°C/h of recovery at 18°C) protocols, as indicated. Note that 0/0 indicates no HT.

(B) Comparison of WT and Chat$^{ko2}$/+ flies subjected to HT/R protocols, as indicated.

(C) Comparisons of Da$^{P\Delta EY6}$ null mutants and Da$^{P\Delta EY6}$/; Chat$^{ko2}$/+ flies subjected to HT/R, as indicated.

Data are presented as mean fold-change ± SEM (means are from N = 10–17 independent RNA extraction and qRT-PCR); note that fold-changes are calculated in comparison to the corresponding 0/0 condition shown. *p < 0.05, Student’s t test.
Figure 6. Increase in Da7-EGFP and/or NACHO Is Sufficient to Induce an Increase in K<sub>v</sub>4/Shal Protein and mRNA

(A–D) Representative immunoblots and quantitative analyses of steady-state protein levels (left) and digital droplet PCR (ddPCR) analyses (right) of samples from (A) UAS-Da7-EGFP/+ (UAS) and elav-Gal4;tub-Gal80<sup>ts</sup> &gt;&gt; UAS-Da7-EGFP/+ (Da7), (B) UAS-NACHO-3xHA (UAS) and elav-Gal4;tub-Gal80<sup>ts</sup> &gt;&gt; UAS-NACHO-3xHA (NACHO), (C) UAS-Da7-EGFP/UAS-NACHO-3xHA (UAS/UAS) and elav-Gal4;tub-Gal80<sup>ts</sup> &gt;&gt; UAS-Da7-EGFP/UAS-NACHO-3xHA (Da7/NACHO), and (D) elav-Gal4;tub-Gal80<sup>ts</sup> &gt;&gt; UAS-Da7-EGFP/UAS-NACHO-3xHA (Da7/NACHO) and elav-Gal4;tub-Gal80<sup>ts</sup> &gt;&gt; UAS-Da7-EGFP/UAS-NACHO-3xHA (Da7/NACHO). All fly lines were grown at 18°C, allowing them to develop normally, then subjected to HT at 30°C for the indicated times. All immunoblots (left) were run with five male heads per lane. Anti-K<sub>v</sub>4/Shal or anti-GFP band intensities were normalized to those of anti-syntaxin (syn), which was used as a loading control. Experimental means were then normalized to similarly treated genetic background.
control (UAS) means on the same immunoblots; for each condition, N = 17–23, data are represented as mean ± SEM; *p < 0.05, Student’s t test. For ddPCR (right), data are presented as mean copy number per ng cDNA ± SEM (means are from N = 10–21 independent RNA extractions and RTs), normalized to mean copy number/ng cDNA from similarly treated genetic background control (UAS) values.

(E) Representative immunoblots and quantitative analyses of steady-state Kv4/Shal protein levels from heads of Chat-Gal4/UAS-Dcr2>>UAS-NACHO-RNAi/ChatΔs2 flies grown at 18°C, then either not HT (0/0) or subjected to HT at 30°C for 3 h followed by recovery at 18°C for another 3 h (3/3). Immunoblot analyses are as described for (A)–(D); N = 15. Note that no significant increase in Kv4/Shal is observed when expression of NACHO is inhibited, in contrast to samples from similarly treated ChatΔs2/+ flies (left; data here are the same as shown in Figure 3). *p < 0.05, Student’s t test.
Figure 7. NFAT Is Required for the Cha$^{ts2}$-Induced Increase in Kv4/Shal Protein and mRNA
(A) Left: representative immunoblots and quantitative analyses for CD8/CD2-GFP expression in elav-Gal4>>LexAop-CD8-GFP/+;UAS-mLexA-VP16-NFAT,LexAop-CD2-GFP/+;UAS-mLexA-VP16-NFAT,LexAop-CD2-GFP/Cha$^{ts2}$ flies subjected to 0/0 and 3/0 HT protocols (HT/R; hours at 30ºC/h of recovery at 18ºC), as indicated. Anti-GFP signals were normalized to anti-syntaxin (syn) signals as a loading control, then normalized to control (0/0) samples on the same immunoblots; N = 11–12 samples for elav>>CaLexA, N = 18 for elav>>Cha$^{ts2}$.
elav>>CaLexA/Ca^{b2}. Note that the CD8/2-GFP levels responding to activation of the CaLexA reporter are elevated with HT of Chd^{b2}. Right: representative immunoblots and quantitative analyses for CD8/CD2-GFP expression in heads from elav-Gal4,tub-GAL80^{ts}>>UAS-mLexA-VP16,NFAT,LexAop-CD2-GFP/UAS-Da7-EGFP; LexAop-CD8-GFP/+ flies were grown at 18°C, then either not HT (–HT) or HT at 30°C for 24 h (+HT). Quantification of GFP, normalized to anti-syntaxin, was performed as described above; N = 12–14 samples. Data are presented as mean ± SEM. Note that the CD8/2-GFP levels responding to activation of the CaLexA reporter are elevated with HT to induce overexpression of Da7-EGFP.

(B–E) Shown are representative immunoblots and quantitative analysis of relative K_{v4}/Shal protein levels (left) and qRT-PCR analyses for K_{v4}/Shal mRNA, expressed as fold-change (right) comparing (B) WT and NFAT^{dab} mutants and (C) NFAT^{dab} mutants and NFAT^{dab};Chd^{b2}/+, (D) NFAT^{da} and NFAT^{da};Chd^{b2}/+, and (E) NFAT^{hb} and NFAT^{hb};Chd^{b2}/+ lines, subjected to HT/R (hours at 30°C/h of recovery at 18°C) protocols, as indicated. All immunoblots were run with five heads per lane. Anti-K_{v4}/Shal or anti-GFP band intensities were normalized to those of anti-syntaxin (syn), which was used as a loading control; for each condition; N = 15–25; data are represented as mean ± SEM. For qRT-PCR, data are presented as mean fold-change ± SEM (means are from N = 9–14 independent RNA extraction and qRT-PCR); note that fold-changes are calculated in comparison to the corresponding 0/0 condition shown. *p < 0.05, Student’s t test.
## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-D<sub>a</sub>7 | Dr. Hugo Bellen | (Fayyazuddin et al., 2006) |
| anti-K<sub>v</sub>4/Shal | Dr. Susan Tsunoda | (Diao et al., 2009) |
| anti-actin          | EMD Millipore | MAP1501 |
| rabbit anti-GFP     | Torrey Pines Biolabs | TP401; RRID:AB_10013661 |
| anti-syntaxin       | Developmental Hybridoma Studies Bank | SC3-S |
| anti-HA             | Biolegend | 901501; RRID:AB_2565006 |
| chicken anti-GFP    | Aves Labs | GFP-1020; RRID:AB_10000240 |
| anti-RFP            | Rockland Immunochemicals Inc. | 600–401-379; RRID:AB_2209751 |
| **Chemicals, Peptides and Recombinant Proteins** |        |            |
| qRT-PCR Probe #66 for <i>Kv4</i>/Shal and <i>RPS20</i> | Universal Probe Library (UPL), Roche Molecular Systems | 04688651001 |
| qRT-PCR Probe #147 for <i>eIF1A</i> | Universal Probe Library (UPL), Roche Molecular Systems | 0469433001 |
| **Experimental Models: Organisms/Strains** |        |            |
| <i>w<sub>1118</sub></i> | Bloomington Drosophila Stock Center | FlyBase: FBal0018186 |
| UAS-TnT             | Dr. Mark Frye | (Deitcher et al., 1998; Sweeney et al., 1995) |
| Cha<sup>4</sup> alleles | Dr. Paul Salvaterra | (Salvaterra and McCaman, 1985) |
| UAS-<i>D<sub>a</sub>7-EGFP</i> | Dr. Steven Sigrist | (Leiss et al., 2009) |
| <i>D<sub>a</sub>7<sup>ΔPΔEY6</sup></i> | Dr. Hugo Bellen | (Fayyazuddin et al., 2006) |
| UAS-NACHO-3xHA      | FlyORF | FlyORF: F002996 (Bischof et al., 2013) |
| <i>UAS-mLexA-VP16-NFAT, LexAop-CD8-GFP, LexAop-CD2-GFP</i> | Bloomington Drosophila Stock Center | BDSC: 66542 (Masuyama et al., 2012) |
| <i>NFAT<sup>ΔA</sup>, NFAT<sup>Δb</sup>, and NFAT<sup>ΔB</sup> alleles</i> | Bloomington Drosophila Stock Center | BDSC: 32652, 33593, 36269 (Keyser et al., 2007) |
| elav-GAL4<sup>135</sup> | Bloomington Drosophila Stock Center | BDSC: 458 |
| 201Y-GAL4           | Bloomington Drosophila Stock Center | BDSC: 4440 (O’Dell et al., 1995; Yang et al., 1995) |
| GH146-GAL4          | Bloomington Drosophila Stock Center | BDSC: 30026 (Stocker et al., 1997) |
| UAS-RNAi-NACHO      | Vienna Drosophila Resource Center | VDRC: 46993 |
| **Oligonucleotides** |        |            |
| <i>Kv4/Shal</i> primers (Left, GCTAACGAAAGGAGGAACG; Right, TGAACCTATTGTGCTGTTTTC) | This paper | N/A |
| <i>RPS20</i> primers (Left, CGACCAGGAAATTGCTAAA; Right, CGACATGGGGGCTTCTCAATA) | This paper | N/A |
| <i>eIF1A</i> primers (Left, TCG TCT GGA GOC AAT GTG; Right, GCC CTG GTT AAT CCA CAC C) | This paper | N/A |
| REAGENT or RESOURCE Antibodies | SOURCE | IDENTIFIER |
|-------------------------------|--------|------------|
| Software and Algorithms       |        |            |
| pClamp10                      | Molecular Devices Corp | N/A |
| ImageJ                        | Open Source | [https://imagej.net/](https://imagej.net/) |
| Mini Analysis                 | Synaptosoft, Inc. | N/A |
| Origin                        | Microcal Software | N/A |
| GraphPad Prism                | GraphPad Software | N/A |
| Photoshop                     | Adobe   | N/A |
| Illustrator                   | Adobe   | N/A |