The role of calsenilin/DREAM/KChIP3 in contextual fear conditioning

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Potassium channel interacting proteins (KChIPs) are members of a family of calcium binding proteins that interact with Kv4 potassium (K+) channel primary subunits and also act as transcription factors. The Kv4 subunit is a primary K+ channel pore-forming subunit, which contributes to the somatic and dendritic A-type currents throughout the nervous system. These A-type currents play a key role in the regulation of neuronal excitability and dendritic processing of incoming synaptic information. KChIP3 is also known as calsenilin and as the transcription factor, downstream regulatory element antagonist modulator (DREAM), which regulates a number of genes including prodynorphin. KChIP3 and Kv4 primary channel subunits are highly expressed in hippocampus, an area of the brain important for learning and memory. Through its various functions, KChIP3 may play a role in the regulation of synaptic plasticity and learning and memory. We evaluated the role of KChIP3 in a hippocampus-dependent memory task, contextual fear conditioning. Male KChIP3 knockout (KO) mice showed significantly enhanced memory 24 hours after training as measured by percent freezing. In addition, we found that membrane association and interaction with Kv4.2 of KChIP3 protein was significantly decreased and nuclear KChIP3 expression was increased six hours after the fear conditioning training paradigm with no significant change in KChIP3 mRNA. In addition, prodynorphin mRNA expression was significantly decreased six hours after fear conditioning training in wild-type (WT) but not in KO animals. These data suggest a role for regulation of gene expression by KChIP3/DREAM/calsenilin in consolidation of contextual fear conditioning memories.

Theories of Hebbian-type synaptic plasticity propose that strengthening of synaptic connections is dependent on coincident activity in pre- and postsynaptic neurons. Research has focused on regulation of transmitter release and receptor function at the synapse; however, changes in neuronal excitability during learning processes have received less attention. Modulation of postsynaptic excitability, via regulation of ion channels, is another possible mechanism for modification of the threshold for induction of synaptic plasticity. Transient outward or A-type currents are generally responsible for regulation of both neuronal excitability and the inter-spike interval in neurons in both vertebrate and invertebrate systems. A-type currents control action potential broadening, back-propagating action potentials in CA1 pyramidal cells, and are regulated by neuromodulators and long-term potentiation (LTP) (Hoffman et al. 1997; Hoffman and Johnston 1998; Yuan et al. 2002; Frick et al. 2004; Kim et al. 2005).

Modulation of back-propagating action potentials by the A-type current is a mechanism that could have dramatic effects on regulation of synaptic strength, particularly during associative plasticity. Recent studies demonstrated that the A-type current in CA1 pyramidal cell dendrites is mediated by the Kv4.2 subunit (Chen et al. 2006) and that Kv4.2 surface expression is decreased by LTP-inducing stimuli (Kim et al. 2007). This suggests that rapid remodeling of the subunits that underlie the A-type current during activity-dependent processes can enhance the induction of plasticity.

Kv4 surface expression and currents are modulated by KChIP (potassium channel interacting protein) and DPPX expression (Birnbaum et al. 2004; Jerng et al. 2005). Four subtypes of KChIPs (1–4) are described to date, and 12 splice variants currently exist (An et al. 2000; Holmqvist et al. 2002; Morohashi et al. 2002; Boland et al. 2003; Van Hoorick et al. 2003). These proteins bind specifically to the N-terminal of Kv4 primary subunits (Scannevin et al. 2004; Zhou et al. 2004) and form octomeric structures with four primary subunits (Kv4) and four KChIP subunits forming a channel (Kim et al. 2004). The KChIPs co-localize and co-immunoprecipitate with brain Kv4 subunits and thus are integral components of the native A-type current complexes (An et al. 2000). Co-expression of the KChIPs and Kv4 subunits in heterologous cells increases the current density, slows inactivation kinetics, and increases the rate of recovery from inactivation of the Kv4 channels (An et al. 2000; Rahm et al. 2001; Holmqvist et al. 2002; Patel et al. 2002, 2004; Schrader et al. 2002; Shibata et al. 2003). The KChIPs also possess four EF-hand-like domains, three of which bind calcium ions (Burgoyne and Weiss 2001). The Ca2+-binding properties of the KChIPs make them intriguing subjects to study with reference to activity-dependent plasticity.
as in most cases an increase in calcium in the postsynaptic cell is an initial step in changes of synaptic efficacy. KChIP3 was originally isolated as a presenilin (P5)-interacting protein and is also known as calsenilin (Buxbaum et al. 1998; Zaidi et al. 2002) and later described with 99% homology with DREAM (downstream regulatory element antagonist modulator), a Ca"+-regulated transcriptional repressor (Carrion et al. 1999). KChIP3 is strongly expressed in the hippocampus, specifically the dentate gyrus (DG), and its expression overlaps at the cellular and subcellular localization with Kv4 subunits and presenilin (Lillicook et al. 2003; Rhodes et al. 2004). Moreover, the dentate granule cells of KChIP3 knockout (KO) mice exhibit slightly reduced A-type current; consistent with this observation LTP magnitude at perforant path–dentate granule cell synapses is enhanced in KChIP3 KO mice (Lillicook et al. 2003). Together, these data suggest that KChIP3 may play a role in learning and memory. In this study, we investigated the role of KChIP3 in hippocampus-dependent contextual fear memory using a mouse genetic engineering approach.

Results

Expression of related proteins in KChIP3 KO animals

All mice used in this study were genotyped using polymerase chain reaction (PCR), and KO genotype was confirmed by Western blot analysis, i.e., lack of a KChIP3 band in the hippocampus (Fig. 1). In previous experiments Kv4.2 KO mice showed cell-specific decreased expression of KChIPs 1–3 in the hippocampus (Chen et al. 2006; Menegola and Trimmer 2006), therefore we determined if there was a reciprocal regulation of Kv4 or other KChIPs in the KChIP3 KO mice. Western blot analysis of total hippocampus with the respective antibodies (see Materials and Methods) showed that the levels of these proteins were not significantly altered (Fig. 1B). We found no significant difference in the levels of KChIP1 (KO n = 4, 118 ± 27% of WT n = 5, 100 ± 32%; t(5) = 0.44, P = 0.68) or KChIP2 (KO n = 6, 79 ± 13% of WT n = 5, 100 ± 12%; t(9) = 1.2, P = 0.28) as well as Kv4.2 (KO n = 10, 119 ± 14% of WT n = 8, 100 ± 10%; t(17) = 0.97, P = 0.35) and Kv4.3 (KO n = 5, 83 ± 13% of WT n = 5, 100 ± 16%; t(9) = 0.73, P = 0.49). This suggests that lack of the KChIP3 gene does not cause significantly altered regulation of protein expression of Kv4.2, Kv4.3, KChIP2, or KChIP1 in the hippocampus and that these mice exhibit normal levels of these proteins.

Behavioral analysis

We performed several behavioral tests to assess locomotor activity, foot shock response, and anxiety in the male KChIP3 KO vs. WT mice. KChIP3 KO mice showed no difference from WT mice in the accelerating rotarod task (Fig. 2A). Mice were tested over 2 d with four trials per day. There was no significant effect of genotype over trials (F1,147 = 1.24; P = 0.28; KO n = 15; WT n = 8; Repeated Measures ANOVA). This suggests that KChIP3 KO mice perform comparable to WT mice on a test of motor coordination and motor learning.

A previous report showed that KChIP3 KO mice exhibit decreased sensitivity to pain (Cheng et al. 2002). Since the fear-conditioning test uses a mild foot shock as the unconditioned stimulus, we compared foot shock sensitivity in WT and KO mice. Mice were tested for foot shock response at differing intensities to be sure that any alterations in memory were not due to changes in sensitivity to the foot shock (see Materials and Methods for details). There was no significant difference in the response of the KO mice compared to the WT mice at any of the intensity levels tested (0.1–0.7 mA) (F1,185) = 0.02, P = 0.88; WT n = 10; KO n = 10; Repeated Measures ANOVA; Fig. 2B). This suggests that any behavioral effects in the fear-conditioning paradigm are not due to a differential sensitivity to shock threshold in the KChIP3 KO mice. A lack of difference in mild foot shock sensitivity in KChIP3 KO mice is not surprising even though these animals were reported to have alterations in pain sensitivity (Cheng et al. 2002) because the foot shock intensities utilized in fear-conditioning paradigms are deliberately chosen to be aversive yet not painful in order to assure learning.

The KChIP3 KO mice were also tested in the open field assay (OFA) (Weiss et al. 2000) and elevated plus maze (EPM) to determine baseline locomotor activity and anxiety levels (Itoh et al. 1991; Kulkarni and Sharma 1991). The total exploratory activity in the OFA exhibited by the KChIP3 KO mice was decreased but not significantly different compared to WT mice (WT, n = 16, 1416 ± 184 cm; KO, n = 11, 1041 ± 245 cm, t(25) = 1.3, P = 0.22; Fig. 3A). Furthermore, the center distance exploration was decreased but not significantly different between the WT and KO mice (WT, 208 ± 42 cm vs. KO, 105 ± 31 cm; t(25) = 1.8, P = 0.08; Fig. 3A). Similarly, the center/total distance ratio was decreased in KChIP3 KO mice as compared to WT, but the difference was not significant (WT 0.13 ± 0.02 vs. KO 0.09 ± 0.01; t(25) = 1.6, P = 0.13; Fig. 3B). We also tested a subset of these animals in the EPM (Fig. 3C). There was no significant difference between WT and KChIP3 KO mice in percent time spent in the open arms of the EPM (Fig. 3C; KO, n = 6; 8 ± 7%; WT, n = 10; 9 ± 3%; t(14) = 0.09, P = 0.93) or crossings into the open arms (Fig. 3D; KO, n = 6; 2.8 ± 1.4; WT, n = 10; 6.4 ± 1.8, t(14) = 1.4, P = 0.18). Overall, these data suggest...
The mice were also tested in the contextual fear-conditioning paradigm. In the contextual fear-conditioning paradigm, KChIP3 KO mice showed a significant enhancement of freezing behavior when tested at 24 h after training (Fig. 4C; WT 28 ± 5%, n = 11; vs. KO 60 ± 7%, n = 10, t19 = 3.54, P = 0.002). This effect was long-lasting as KChIP3 KO mice re-exposed to the box one month after training also showed enhanced freezing compared to WT mice (WT 11 ± 4%, n = 7; KO 31 ± 6%, n = 5, t15 = 3.2323, P = 0.009).

As described above, the results from the anxiety tests showed that the KChIP3 KO mice exhibited slightly enhanced anxiety relative to WT animals. Increased anxiety could be a possible explanation for the enhanced freezing behavior in the context test after the foot shock. Two points argue against this possibility: (1) The KChIP3 KO and WT mice exhibit similar baseline activity on training day at all time points: before the tone, during tone exposure, and after the foot shock (Fig. 4A); and (2) there was no significant difference in the cued test between WT and KO mice.

To be certain that the increased anxiety did not play a role in the post-shock enhanced performance in the contextual test in the KChIP3 KO mice, we tested another group of animals 1 h after training on both the cued and contextual tests (Fig. 5). One hour after training, KChIP3 KO mice froze at the same level as the WT animals in both the contextual (Fig. 5A; KChIP3 KO, n = 4, 35 ± 16% vs. WT n = 8, 36 ± 11%; t10 = 0.04, P = 0.9725) and cued fear-conditioning tests (Fig. 5B; KChIP3 KO, n = 4, 73 ± 18% vs. WT n = 8, 77 ± 10%; t10 = 0.9, P = 0.85). Together with the 24-h contextual test, these data suggest that KChIP3 may reduce consolidation of hippocampus-dependent contextual memory. Furthermore, this effect was long-lasting as the KO mice exhibited enhanced freezing when re-exposed to the training context one month later.

**Mechanism of enhanced contextual memory**

We wished to further investigate the role that KChIP3 plays in fear conditioning. We hypothesized that KChIP3 may act through its role as a Kv4 channel interacting protein (An et al. 2000) or through its role as a transcriptional repressor (Carrion et al. 1999). Therefore, we used Western blot analysis to study changes in KChIP3 localization (membrane association or localization in the nucleus) in the hippocampus after fear-conditioning training. We determined the membrane association and nuclear expression of KChIP3 and membrane expression of Kv4.2 in the hippocampus at different time points after fear-conditioning training (Fig. 6). KChIP3 expression was investigated in the cytosolic fraction; however, no detectable KChIP3 immunoreactivity was found in this fraction, therefore we did not investigate the cytosolic fraction further. This suggests that detectable KChIP3 protein is either localized in membrane or nuclear fractions. Furthermore, the immunoreactivity for KChIP3 in the membrane fraction was much higher than the nuclear expression making relative comparison amounts inaccurate. Therefore, we did not compare membrane and nuclear expression levels.

Circadian fluctuations in the expression of KChIP3 were previously reported in the retina and pineal gland (Link et al. 2004). For this reason, we tested three groups of animals at each time point. These groups included: fear-conditioning trained animals (FC), animals that were placed in the same context for the same amount of time but not trained (CXT), and animals that received latent inhibition training (LI) (see Materials and Methods). LI-trained animals do not learn to associate the context with the foot shock (Levenson et al. 2004), and therefore serve as a control for the associative learning. At each time point (1, 6, and 24 h after removal from the chamber) FC and LI were normalized to CXT as the control.

Figure 6A–C and Table 1 show the results of these experiments. One hour after training, KChIP3 membrane association in

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**Figure 2.** KChIP3 KO mice exhibit normal locomotor activity and shock sensitivity. (A) The accelerating rotarod task was used to assess motor learning and coordination in KChIP3 KO and WT littermate mice. Mice were trained four trials a day for 2 d for a total of eight trials, and time spent on the rod per trial was averaged across genotype and plotted per trial. No significant effect of genotype over trials was observed (KO n = 15; WT n = 8; Repeated Measures ANOVA; P = 0.28). (B) Response to foot shock in WT and KO mice. KChIP3 KO (n = 10) and WT littermate mice (n = 10) were tested for foot shock responses (see Materials and Methods) at increasing intensities (0.1–0.7 mA in 0.1 increments). There were no significant differences in the responses of the KChIP3 KO mice compared to WT littermates (P > 0.05, Repeated Measures ANOVA).
point (and anxiety-like behavior. WT littermate mice were tested in the open field assay (OFA) to determine baseline locomotor activity

Figure 3. Open field and elevated plus maze (EPM) performance in KChIP3 KO mice. KChIP3 KO and WT littermate mice were tested in the open field assay (OFA) to determine baseline locomotor activity and anxiety-like behavior. (A) Summary bar graph of exploratory activity of KChIP3 KO vs. WT mice in the OFA. Total exploratory activity and exploration of the center in the OFA exhibited by the KChIP3 KO mice (n = 11) was decreased but not significantly different compared to WT mice (n = 16; P > 0.05, t-test). (B) Bar graph representing the center/total distance ratio decreased in KChIP3 KO mice as compared to WT but not significantly different (P > 0.05; t-test). (C) KChIP3 KO and WT littermate mice were also tested in the EPM. KChIP3 KO mice (n = 6) spent approximately the same amount of time in the open arms as did the WT littermates (n = 10). (D) Crossings into the open arms by KO mice were decreased compared to the WT animals, but the differences were not significant (P > 0.05; t-test).

Mechanisms of decreased levels of KChIP3 protein
We were interested to determine whether changes in transcription of KChIP3 mRNA may contribute to the decrease of KChIP3 protein associated with the membrane. Therefore, we performed real-time PCR experiments to study KChIP3 mRNA expression. Similar to the protein expression, we normalized mRNA levels in LI and FC to CXT at each time point. There were no differences in KChIP3 mRNA expression at any of the time points between CXT, FC, and LI (Fig. 6D); 1-h time point (F(2,7) = 1.78, P = 0.24; FC1, n = 3, 147 ± 35%; LI1, n = 3, 138 ± 15%; 6-h time point (F(2,11) = 0.53; P = 0.6; FC6, 100 ± 30%; n = 5; LI6, n = 5, 73 ± 18%), and 24-h time point (F(2,7) = 1.47, P = 0.29; FC24, n = 3, 77 ± 20%; LI24, n = 3, 79 ± 9%). These data suggest that the changes in membrane KChIP3 protein expression are not mediated by changes in KChIP3 mRNA levels.

Kv4.2/KChIP3 interaction
This result led us to the hypothesis that the reduction of KChIP3 membrane association may reduce the Kv4.2/KChIP3 interaction. Therefore, we performed co-immunoprecipitation experiments (co-IP) using the KChIP3 antibody to investigate interactions with Kv4.2 (Fig. 7). As expected, there was no significant difference in Kv4.2 membrane expression between CXT and FC trained at the 6-h time point (FC6, 98 ± 15% of CXT6, n = 3; input and bar graph in Fig. 7B); however, the Kv4.2 interaction with KChIP3 in the FC6-trained tissue was absent compared to the CXT6-trained tissue (IP KChIP3). We did not find any interaction of KChIP3 and Kv4.3 when the blots were probed with the Kv4.3 antibody, but we should note that we found less Kv4.3 antibody immunoreactivity in hippocampus. This suggests that the functional down-regulation of KChIP3 membrane association is related to a decreased interaction of Kv4.2 and KChIP3.

Nuclear localization
Since KChIP3 acts as a transcription factor, another possibility is that it may also translocate from the membrane to the nucleus at the 6-h time point. Therefore, we assayed nuclear fractions for the presence of KChIP3 antibody in the nucleus. We found that the KChIP3 antibody recognized a specific band in the nuclear fraction that was slightly smaller than the membrane KChIP3 band (~25 kDa) and was not present in the KO nuclear fraction (data not shown). This band may be the large portion of the cleavage product of KChIP3 when cleaved by caspase 3 (Choi et al. 2003). This band increased in the nucleus at the 6-h time point in the FC-trained animals relative to CXT, but not in the LI animals (F(2,6) = 16.1, LI n = 3, 59 ± 37%; FC n = 3, 198 ± 20%, P < 0.01; Fig. 8). To be sure that the nuclear fraction was pure, we probed the nuclear fraction with Kv4.2 and enolase antibodies (data not shown). These antibodies did not recognize any bands in the nuclear fraction, suggesting that the nuclear fraction was not contaminated with membrane and cytosol, respectively. This suggests that this increase in nuclear expression of KChIP3 may be specific to the fear-conditioned animals and may play a transcriptional role after fear conditioning.

Role as transcription factor
Dynorphin, a prodynorphin-derived peptide, and activation of the δ opioid receptors can modulate learning and memory. Since prodynorphin is regulated by KChIP3 in its function as the transcriptional repressor DREAM (Carrión et al. 1999), we wished to determine if prodynorphin mRNA expression was regulated after the fear-conditioning paradigm. The increased nuclear KChIP3 expression at the 6-h time point suggested that KChIP3 might act as a transcriptional repressor, eliciting a decrease in prodynorphin expression. Indeed prodynorphin
mRNA was significantly decreased at the 6-h time point compared to CXT6 in both FC6 and LI6 hippocampus ($F_{(2,14)} = 6.9, P = 0.012$; FC6, $n = 3, 142 \pm 15\%$, $P < 0.05$; LI6, $n = 3, 151 \pm 21\%$ $P < 0.05$) and prodynorphin mRNA was decreased in both FC and LI at the 24-h time point, although not significantly ($F_{(2,8)} = 1.29$, $P = 0.3279$, FC24, $n = 3, 71 \pm 25\%$, LI24, $n = 3, 69 \pm 25\%$).

Many factors regulate prodynorphin mRNA expression; therefore, we wished to determine whether KChIP3 regulates prodynorphin mRNA expression in the hippocampus after training. We assessed whether prodynorphin mRNA is regulated in KChIP3 KO mice 6 h after fear conditioning. Prodynorphin mRNA was increased rather than decreased in the fear-conditioned KO hippocampus ($150 \pm 28\%$, $n = 6$) compared to context-trained KO hippocampus ($P > 0.05$; Fig. 9). Prodynorphin mRNA was slightly, but not significantly, decreased in the context-trained KO relative to WT context-trained ($75 \pm 15\%$). Overall, these observations suggest that KChIP3 translocation away from the membrane and into the nucleus serves to regulate prodynorphin expression in long-term memory formation.

**Discussion**

The evidence presented here using KChIP3 KO mice indicates that KChIP3/calsenilin/DREAM plays a role in hippocampus-dependent memory. KChIP3 is highly expressed in the hippocampus, specifically the dentate gyrus (Lilliehook et al. 2003; Rhodes et al. 2004). It is a multifunctional protein that acts as an interacting protein for Kv4 channels (An et al. 2000), a transcriptional repressor (Carrion et al. 1999), and a protein that interacts with...
presenilin (Buxbaum et al. 1998). The role of KChIP3 in learning and memory could utilize any one or any combination of these mechanisms. In this study, we investigated its roles as a transcriptional repressor and K+ channel interacting protein. We suggest that KChIP3 plays a role in consolidation of hippocampus-dependent fear memories through dissociation from the membrane, translocation to the nucleus, and regulation of gene transcription (Fig. 10).

We assessed the KChIP3 KO animals in terms of their baseline expression of other proteins known to interact with KChIP3 in its role as a K+ channel interacting protein. Previous reports demonstrated that KChIP3 and KChIP2 expression levels are decreased in Kv4.2 KO mice (Chen et al. 2006; Menegola and Trimmer 2006), but was significantly different from CXT1 (n = 5) at 1 h after training, but was significantly higher than LI1 (**P < 0.01, n = 3), which was significantly different from context, *P < 0.05; One-Way ANOVA with post-hoc Tukey’s test). KChIP3 membrane association was significantly decreased (P < 0.05) 6 h after FC training (n = 8) relative to CXT6 (n = 8). No significant differences between CXT24 (n = 3) and FC24 (n = 3) or LI24 (n = 3) were found at the 24-h time point. (C) Membrane Kv4.2 expression in the hippocampus at different time points after FC and LI training. There was no significant effect on immunoreactivity for Kv4.2 membrane expression at the 1-h time point (P > 0.05; FC1, n = 3, LI1, n = 3, CXT1, n = 3), 6-h time point (P > 0.05; FC6, n = 5, LI6, n = 5, CXT6, n = 5), or 24-h time point (P > 0.05; FC24, n = 4, LI24, n = 2, CXT24, n = 4). (D) There was no difference in KChIP3 mRNA expression at any of the time points; 1-h time point (P > 0.05; FC1, n = 3, LI1, n = 3, CXT1, n = 3), 6-h time point (P > 0.05; FC6, n = 5, LI6, n = 5), and 24-h time point (P > 0.05; FC24, n = 3, LI24, n = 3).

This information suggests that KChIP3 does not regulate the total expression levels of these proteins in vivo, or that compensation by other KChIPs of Kv4 protein expression can substitute for the loss of KChIP3.

Interestingly, we found that KChIP3 KO mice exhibited enhanced memory in a contextual fear-conditioning paradigm that was long-lasting and present upon reactivation. When animals were tested again 1 mo later, the KO mice still showed enhanced freezing in the context. This is in contrast to no significant difference between the WT and KO mice on the cued fear-conditioning task. Furthermore, KO animals that were tested an hour after the training protocol froze at a similar level compared to WT mice. This suggests that there is no post-shock enhancement of anxiety in the KChIP3 KO mice and that KChIP3 does not play a role in memory acquisition. Cued fear conditioning is dependent on the amygdala, while the hippocampus is also important for contextual fear conditioning (Phillips and LeDoux 1992). This data do suggest that the KChIP3 KO mice exhibit enhanced memory in a hippocampus-dependent task and that KChIP3 may play a role in hippocampus-dependent memory consolidation.

| Table 1. KChIP3 protein expression (as percentage of context) |
|-----------------|-----------------|-----------------|-----------------|
|                 | 1 h             | 6 h             | 24 h            |
| **LI**          | 73 ± 4%; n = 3  | 56 ± 16%; n = 4 | 81 ± 41%; n = 3 |
| **FC**          | 111 ± 8%; n = 5 | 58 ± 7%; n = 8  | 113 ± 27%; n = 3|
| **ANOVA**       | F(2,9) = 10.35, P = 0.005 | F(2,17) = 5.2, P = 0.02 | F(2,10) = 0.48, P = 0.64 |

Figure 6. Changes in the immunoreactivity of hippocampus membrane expression levels of KChIP3, Kv4.2, and KChIP3 mRNA following fear conditioning in WT mice. Western blotting and real-time PCR were used to study expression of KChIP3 and Kv4.2 protein immunoreactivity as well as KChIP3 mRNA in the hippocampus at different time points (1, 6, and 24 h) following fear-conditioning training. Three groups of WT mice were trained as described in Materials and Methods: a context-trained group (CXT), a fear-conditioned group (FC), and a latent inhibition group (LI). (A) Representative Western blots of membrane KChIP3 and Kv4.2 protein levels at 1- and 6-h time points in all three groups. (B) Bar graph summarizing immunoreactivity for KChIP3 membrane association at different time points after FC and LI training. KChIP3 membrane association in FC1 (n = 5) hippocampus was not significantly different from CXT1 (n = 5) at 1 h after training, but was significantly higher than LI1 (**P < 0.01, n = 3), which was significantly different from context, *P < 0.05; One-Way ANOVA with post-hoc Tukey’s test). KChIP3 membrane association was significantly decreased (P < 0.05) 6 h after FC training (n = 8) relative to CXT6 (n = 8). No significant differences between CXT24 (n = 3) and FC24 (n = 3) or LI24 (n = 3) were found at the 24-h time point. (C) Membrane Kv4.2 expression in the hippocampus at different time points after FC and LI training. There was no significant effect on immunoreactivity for Kv4.2 membrane expression at the 1-h time point (P > 0.05; FC1, n = 3, LI1, n = 3, CXT1, n = 3), 6-h time point (P > 0.05; FC6, n = 5, LI6, n = 5, CXT6, n = 5), or 24-h time point (P > 0.05; FC24, n = 4, LI24, n = 2, CXT24, n = 4). (D) There was no difference in KChIP3 mRNA expression at any of the time points; 1-h time point (P > 0.05; FC1, n = 3, LI1, n = 3, CXT1, n = 3), 6-h time point (P > 0.05; FC6, n = 5, LI6, n = 5), and 24-h time point (P > 0.05; FC24, n = 3, LI24, n = 3).
In our behavioral studies we found that the KChIP3 KO animals are slightly more anxious than their WT littermates, based on altered behavior in anxiety levels in the open field assessment. This increased anxiety, however, does not appear to underlie the enhancement of contextual fear conditioning for two reasons: (1) Prior to shock in the initial training period, KChIP3 KO and WT mice exhibited similar exploratory behavior; (2) a control experiment ruled out the possibility of a post-shock enhancement of freezing behavior as we tested a second group of animals 1 h after training in order to test for a post-shock anxiety enhancement. We found no differences between WT and KO in the cued or contextual tests 1 h after training. These data have several implications: First, KChIP3 KO animals exhibit similar exploratory behavior and post-shock motor behavior to WT animals, and secondly, the increased anxiety phenotype in the KO animal does not appear to affect fear conditioning. Furthermore, this effect was not due to an enhanced sensation of the foot shock as KO animals tested in the shock threshold paradigm did not differ from WT animals. Together, these data suggest that KChIP3 plays a role in associative contextual memory per se as opposed to altering baseline behaviors or altering training-associated sensory perception.

These experiments are consistent with previous data showing a decrease in the A-type current in dentate granule cells and enhanced LTP at the perforant path–dentate granule cell synapse in another strain of KChIP3 KO mice (Lilliehook et al. 2003). The enhanced memory of the context in the KChIP3 KO mice suggests that KChIP3 may play an inhibitory role in consolidation of fear memories. Therefore, we investigated possible mechanisms for KChIP3’s role in contextual fear conditioning. Since KChIP3 is a multifunctional protein, several possibilities existed, including regulation of A-type currents and transcriptional regulation. In an attempt to determine KChIP3’s role, we first investigated changes in membrane and nuclear KChIP3 localization after the fear-conditioning training protocol.

Our data suggest a post-training regulation of Kv4.2 and KChIP3 association and that translocation to the nucleus may contribute to the role of KChIP3 in the hippocampus. Interestingly, a significant difference in KChIP3 membrane association 1 h after training was observed between FC and LI. This suggests a specific role for an increase in membrane KChIP3 association 1 h after FC training compared to LI. Furthermore, membrane KChIP3 expression is decreased 6 h after training (Fig. 6) and the KChIP3/Kv4.2 interaction is diminished (Fig. 7), while nuclear KChIP3 is increased (Fig. 8). The membrane-associated KChIP3 decreased by approximately one-half (50%) in both LI6 and FC6 compared to context. This suggests that membrane KChIP3 is decreased after both LI and FC training and a general role for down-regulation of membrane KChIP3 function. Furthermore, the nuclear expression of KChIP3 doubled at the 6-h time point after FC. This suggests that the membrane KChIP3 translocated to the nucleus. On the other hand, nuclear KChIP3 decreased by ~50% in the LI-treated animals. This suggests an overall down-regulation and degradation of the KChIP3 protein in the LI-treated animals after training. To support this idea, KChIP3 mRNA is decreased, although not significantly, at the 6-h time point after LI, but remains stable in the FC-trained (Fig. 6D). Certainly, memories are formed in all situations including the context exploration, and the general decrease in membrane KChIP3 in FC-trained and LI-trained suggests that it is not specific to the associative FC-trained memory. The specific presence of KChIP3 in the nucleus after FC training does suggest a particular role for the nuclear translocation of KChIP3 in associative FC memory.

The function of the decrease in membrane KChIP3 and in the Kv4.2 and KChIP3 interaction is unknown but is expected to regulate A-type current and modulate cell excitability, possibly contributing to changes in plasticity. Depending on the role of KChIP3 in modulation of the A-type currents, this could either enhance or decrease cell excitability, and several possibilities exist. KChIPs are known to enhance A-type currents (for review, see Birnbaum et al. 2004) and a simple explanation is that less KChIP3 at the membrane would be expected to decrease A-type currents and enhance cell excitability. On the other hand, this decrease in KChIP3 at the membrane may actually decrease cell excitability. Recent data suggest that direct phosphorylation of Kv4.2 subunits by PKA causes internalization of the Kv4.2 channels (Hammond et al. 2008), which would result in a decrease in A-type current. We have shown that KChIP3 association is necessary for functional modulation of the current by PKA (Schrader et al. 2002); therefore, it is possible that, in the absence of association with KChIP3, PKA may not internalize the channels and hence increase A-type currents and decrease cell excitability. Yet another possibility exists with the discovery of the transmembrane KChIPs that may mediate differential effects on retention in the endoplasmic reticulum (Jerg and Pfaffinger 2008). In the future it will be interesting to determine whether the changes in KChIP3 membrane association at the

| Table 2. Kv4.2 protein expression (as percentage of context) |
|-------------|-------------|-------------|
|             | 1 h         | 6 h         | 24 h        |
| LI          | 84 ± 8%; n = 3 | 122 ± 22%; n = 5 | 122 ± 62%; n = 2 |
| FC          | 106 ± 19%; n = 3 | 120 ± 20%; n = 5 | 125 ± 18%; n = 4 |
| ANOVA       | F(2,9) = 1.0, P = 0.42 | F(2,12) = 0.52, P = 0.61 | F(2,7) = 0.44, P = 0.66 |

Figure 7. KChIP3 and Kv4.2 interaction is decreased 6 h after FC training relative to CXT. (A) Co-immunoprecipitation with KChIP3 antibody and probed with the Kv4.2 antibody shows that the Kv4.2 and KChIP3 interaction is diminished in the FC-trained animals relative to the CXT trained animals (n = 3). The exposure time for the Kv4.2 input was shorter than the exposure time for IP. (B) Summary bar graph showing that there was no difference in immunoreactivity of Kv4.2 expression measured in the input.
Role of dynorphin in learning and memory

The prodynorphin-derived peptide, dynorphin, and activation of the \( \kappa \) opioid receptors both play a role in learning and memory. Dynorphin is thought to play a role in activity-dependent processes, including epilepsy (Simonton and Romualdi 1996) and learning and memory. Specifically, injection of dynorphin into the hippocampus of rats impaired spatial memory (McDaniel et al. 1990; Sandin et al. 1998). Dynorphin was increased in the hippocampus of aged animals and this increase is related to poor performance in spatial learning tasks in aged animals (Jiang et al. 1989). Moreover, aged mice lacking the prodynorphin gene showed improved spatial learning compared to WT. These data suggest that high levels of dynorphin in the hippocampus may play a role in encoding sensory inputs and spatial pattern separation as well as encoding spatial information and sending that information to CA3. The place fields of dentate granule cells are small and highly reliable (Jung and McNaughton 1993), which indicates the granule cells may play a role in separation of distinct environments and unique feature detection. Behavioral lesion studies have defined a role for the dentate gyrus (DG) in detecting novel environment but not object information (Kesner et al. 2004; Lee and Kesner 2004b; Lee et al. 2005), as well as retrieval of contextual fear-conditioning information (Lee and Kesner 2004a). Since KChIP3 is highly expressed in the dentate granule cells, the dentate is most likely where the effects are occurring. We demonstrated that KChIP3 disassociates from the membrane and translocates to the nucleus to regulate gene expression after fear conditioning. These studies implicate KChIP3 as an important molecular component of memory-associated processes operating in the dentate gyrus.

Specific role of dentate gyrus in memory

KChIP3 is highly expressed in the dentate gyrus (Lilliehook et al. 2003; Rhodes et al. 2004). Furthermore, dentate granule cells from KChIP3 KO mice show decreased A-type current and enhanced LTP induction at the perforant path–dentate granule cell synapse (Lilliehook et al. 2003). We showed here that KChIP3 plays a role in hippocampus-dependent contextual fear conditioning.

Computational models of hippocampal function (Rolls and Kesner 2004), and now experimental data (McHugh et al. 2007), suggest that the dentate gyrus plays a role in encoding sensory information and spatial pattern separation as well as encoding spatial information and sending that information to CA3. The place fields of dentate granule cells are small and highly reliable (Jung and McNaughton 1993), which indicates the granule cells may play a role in separation of distinct environments and unique feature detection. Behavioral lesion studies have defined a role for the dentate gyrus (DG) in detecting novel environment but not object information (Kesner et al. 2004; Lee and Kesner 2004b; Lee et al. 2005), as well as retrieval of contextual fear-conditioning information (Lee and Kesner 2004a). Since KChIP3 is highly expressed in the dentate granule cells, the dentate is most likely where the effects are occurring. We demonstrated that KChIP3 disassociates from the membrane and translocates to the nucleus to regulate gene expression after fear conditioning. These studies implicate KChIP3 as an important molecular component of memory-associated processes operating in the dentate gyrus.

Materials and Methods

Generation of KChIP3 KO mice

The KChIP3 KO mice were developed by Dr. W. Frank An through Lexicon. These animals were generated by targeted disruption of the prodynorphin gene in the mouse embryo. We show here that prodynorphin mRNA is regulated after fear-conditioning training by KChIP3. Interestingly, acute stressors increased dynorphin immunoreactivity in the hippocampus and nucleus accumbens (Shirayama et al. 2004), suggesting up-regulation of the dynorphin protein. The increase in prodynorphin mRNA at the 1-h time point after LI and FC that we observed (Fig. 9) may be related to the stress-induced enhancement of dynorphin.

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immunoblotting with compartment specific antibodies (enolase: cytosol; Kv4.2: DTT) was added, and the samples were stored at –20 °C. Westerns were performed using Biorad’s IQ SYBR Green Supermix in an ICycler from Biorad. Each well contained (in milliliters) Sybr Green Supermix (12.5), forward primers (10 mM) (1), DEPC-treated H2O (12.5), and cDNA (200 ng/μL) (1). All reactions were performed in triplicate. The specific primers used to amplify prodynorphin from cDNA were (F): 5′-AGTGAGATCCCTGCCAA-3′, (R): 5′-AGAGCTCTGTCGAGTGAGA-3′; KChIP3 (F): 5′-AGTGGAGATCCCCTGCCAATGGCGCACCGTGATTGTC-3′, (R): 5′-ATGGCGCACCGTGATTGTC-3′. Genes of interest (KChIP3 and prodynorphin) were normalized to 18S rRNA for all samples reported. Changes in the fear-conditioned and latent inhibition time points are normalized to the context trained at the same time point.

**Fear-conditioning protocol**

The experimenter was blinded to the genotype of animals during all behavioral experiments. KChIP3 WT and KChIP3 KO littermate controls were tested on cued and contextual fear-conditioning paradigms. Both naive mice (mice that had not previously been tested in a behavioral test) and mice that had previously experienced other tests (OFA, elevated plus, and rotarod—several days to one week between tests) were tested. There was no significant difference in the results, therefore the data were combined. Mice were trained in a single-shock protocol. An animal was placed in a chamber and allowed to freely explore the chamber. After 120 sec an 80 dB white noise cue commenced for 30 sec. The cue was terminated with a single mild foot shock (0.50 mA) for 2 sec. The mouse was left to explore the chamber for an additional 90 sec. Different mice were tested 1 h or 24 h following training on the cued and contextual tests. Contextual testing consisted of analysis of freezing time in the same context for 240 sec. For the cued test the mice were placed in a unique context and allowed to freely explore the new context. After 180 sec the white noise cue commenced. Freezing was measured during the 180 sec of the cue. The percent freezing to both the context and cued paradigms was analyzed for each mouse via Med Associates software. Some animals were independently observed by a blinded scorer.

**Rotarod protocol**

Mice were placed onto a horizontal rod, –3 cm in diameter. The rotarod accelerated from 4 to 40 rpm during the course of the 5-min trial. The amount of time that an animal spent on the rod before drop (~12 cm onto a rest platform) to a maximum of 5 min, was determined. The animal was then returned to the home cage, and rod and rest platforms were cleaned with ethanol and water. Each animal was tested for four trials per day for two consecutive days. The animals rested in their home cages for 0.5–1 h between each trial. Total time spent on the rod was determined for each trial. Time spent on the rod per trial was averaged across genotype and plotted per trial.

**Open field protocol**

A single mouse was placed in the center of a clear, plexiglass chamber measuring 43 × 43 × 18 cm. The animal was left in the
chamber for 15 min to explore the novel environment, and was monitored by a computer program (Versamex). After 15 min, the animal was removed from the chamber and returned to its home cage. The plexiglass chamber was then wiped clean with ethanol and water before the next trial. Total movements as well as center/total distance ratio were reported.

Elevated plus maze protocol

The elevated plus maze (EPM) consisted of four arms (5 cm × 30 cm) arranged perpendicularly in a plus shape and elevated 38 cm off of the ground. Two arms were enclosed by 15.5-cm dark plexiglass walls and two arms were open. The subject animal was placed in the center of the elevated maze. The mouse was free to move into open or closed arms. An observer, blinded to genotype, recorded activity and time spent in the enclosed arms and the time required for the mouse to move to the open arm of the maze. At the end of the experimental trial (10 min), the animal was lifted from the maze and returned into its home cage. Each mouse was tested once.

Shock threshold

Mice were tested in a range of foot shocks (0.1–0.7 mA in 0.1 increments) to determine sensitivity to the foot shock. Animals were scored on their response, including running, jumping, and vocalization. The response was assigned a numerical score by a blinded observer. The following scale was used: 0, no response; 1, move; 2, vocalize; 3, run; 4, jump; 5, maximum response (vocalize, jump, and run).

Fear-conditioning protocol for biochemistry

WT male mice were trained using the same fear-conditioning protocol as before. Three groups of mice, a context-trained group (CXT), a fear-conditioned group (FC), and a latent inhibition group (LI) were sacrificed at 1, 6, and 24 h later and their hippocampi removed and frozen for biochemistry at a later date. Data were normalized to the levels of expression of the given protein or mRNA in the animals that only experienced context at each time point. Results at each time point were compared between CXT, LI, and FC using a one-way ANOVA and post-hoc Tukey’s test.

Co-immunoprecipitation

Co-immunoprecipitation reactions were performed at room temperature (RT) using the normalized membrane fraction from the Chemicon fractionation kit (as described above) from the whole hippocampus (CXT6 and FC6). Affinity-purified mouse monoclonal antibody specific for KChIP3 (Neuromab) were added into the reaction tube and incubated at RT for 2 h on a rocker. A 50% slurry of protein A agarose (Pierce) was added to the tube, and the samples were incubated at RT for another 2 h on a rocker. After incubation, protein A agarose was centrifuged at 2500g for 3 min, and the resulting pellets were washed by resuspension and centrifuged three times with IP buffer (Pierce). The final pellets were resuspended in 30 μL of elution buffer, incubated for 5 min, and centrifuged at 2500g for 5 min. Supernatant was saved and the step was repeated. Supernatants were combined in the same tube. For neutralization, 6 μL 1 M Tris- HCl pH 7.5 was added. Finally, samples were prepared for Western blots by adding 100 mM DTT and 15 μL 5× nonreducing sample buffer and heated at 50°C for 10 min.

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