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Notch intracellular domain (NICD) suppresses long-term memory formation in adult *Drosophila* flies

Jiabin Zhang^{1,2}, Jerry C.P. Yin^{1,2,3}, and Cedric S. Wesley²

¹Neuroscience Training Program, University of Wisconsin-Madison, Madison, Wisconsin 53706

²Departments of Genetics and Medical Genetics, University of Wisconsin-Madison, Madison, Wisconsin 53706

³Department of Neurology, University of Wisconsin-Madison, Madison, Wisconsin 53706

Abstract

Notch receptor signaling is evolutionarily conserved and well known for its roles in animal development. Many studies in *Drosophila* have shown that Notch also performs important functions in memory formation in adult flies. An intriguing observation is that increased expression of the full-length Notch receptor (Nfull) triggers long-term memory (LTM) formation even after very weak training (single training). Canonical Notch signaling is mediated by Notch intracellular domain (NICD), but it is not known whether increased expression of NICD recapitulates the LTM enhancement induced by increased Nfull expression. Here we report that increased NICD expression either has no impact on LTM formation or suppresses it. Furthermore, it either has no impact or decreases both the levels and activity of cAMP response element binding protein (CREB), a key factor supporting LTM. These results indicate that NICD signaling is not sufficient to explain Nfull-induced LTM enhancement. Our findings may also shed light on the molecular mechanisms of memory loss in neurological diseases associated with increased NICD expression and canonical Notch signaling.

Keywords

Notch; NICD; CREB; LTM; memory

1. Introduction

Notch is an evolutionarily conserved cell surface receptor that is activated when bound by cognate ligands expressed on neighboring cells. The best-known Notch signaling mechanism is based on the transcriptional activity of the Notch intracellular domain (NICD) and Suppressor of Hairless [Su(H), the *Drosophila* homologue of mammalian RBP-j]. Besides its well-known roles in development, Notch also performs functions in adult brain, for example, regulation of memory formation (Ables et al. 2011).

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Despite some controversies (Dias et al. 2014; Zheng et al. 2012), multiple studies in mice indicate Notch functions in memory formation (Alberi et al. 2011; Costa et al. 2003; Sargin et al. 2013). *Drosophila* studies using conditional Notch alleles and inducible transgenic flies clearly demonstrate the significance of Notch in long-term memory (LTM) formation (Ge et al. 2004; Matsuno et al. 2009; Presente et al. 2004). Amazingly, increased expression of the full-length Notch receptor (Nfull) prior to training was found to reduce the training sessions required for LTM induction, from ten to one (Ge et al. 2004).

Although NICD is the immediate downstream effector of Nfull for many Notch functions, it is unknown whether increased NICD expression recapitulates the LTM enhancement observed with increased Nfull expression. In this study, we found a negative effect of increased NICD expression on LTM and CREB function.

2. Materials and Methods

Fly stocks

hs-NICD and hs-N (described previously in (Lieber et al. 1993; Struhl et al. 1993)), CRE-luciferase reporter fly (described previously in (Belvin et al. 1999)) and *yw* (from Dr. Amita Sehgal, University of Pennsylvania) were used.

Pavlovian olfactory conditioning

Procedures used to train and test flies for LTM formation have been previously described (Drier et al. 2002). For single training, a group of flies (~100) was sequentially exposed to two different odors (3-octanol and 4-methylcyclohexanol) and electric shock was paired with one of the odors (that was alternated). Weak training involved a single trial of training. For more intense training, we utilized 5× spaced training protocol (5 training cycles with a 15 min interval in-between). LTM was measured 24 hours later by allowing the flies to choose between the two odors presented without electric shock. To test immediate memory, flies were forced to choose between the odors right after a single trial of training.

Western blotting

6% or 10% SDS-PAGE gels were used for western blots. PO4-CREB, CREB monoclonal, and Notch antibodies used have been described previously (Belvin et al. 1999; LeComte et al. 2006; Zhang et al. 2013a). Luciferase antibody used was a mouse monoclonal antibody from Santa Cruz Biotechnology (sc-57604). Protein extracts were prepared by homogenizing four fly heads in 40 µl of Laemmli buffer or four male flies in 80 µl of Laemmli buffer and 40 µl was loaded in each lane. The heads were collected individually by placing flies in a perforated microfuge tube and flash freezing them by dipping into liquid nitrogen for a couple of minutes. Live flies were transferred to microfuge tubes, without freezing or CO₂ anesthesia, tapped down, and crushed.

In vivo luciferase assay

The assays were performed as previously described (Belvin et al. 1999). Briefly, CREB reporter flies were entrained on a circadian cycle for at least 5 days before loading them into a black 96-well microplate containing luciferin media: 1% agar and 5% sucrose food

containing D-Luciferin (Gold Bio Technology). For the experiment shown in Fig. 3B, *in vivo* luciferase activity was measured at room temperature and at different time points following the heat shock treatment.

3. Results

3.1 Increased expression of NICD does not promote LTM

Heat shock promoter driven NICD expression (hs-NICD) is commonly used in developmental studies for more than two decades to test the involvement of canonical Notch signaling or suppress the effects of loss of this signaling. It is particularly useful for studies in adults as transgenic hs-NICD expression can be specifically induced at adult stage to avoid any developmental defects. We used this tool to probe the question whether hs-NICD could mimic the LTM enhancement generated by hs-N. The training protocol we used (30 min heat shock at 37°C followed by a 3-hour recovery before training) is the same one used previously in Nfull experiments (Ge et al. 2004). Results of our experiments showed that NICD induction does not enhance LTM formation (Fig. 1A, left). Since NICD is produced by proteolytic cleavages following ligand binding of Nfull, and we were directly expressing NICD, we reasoned that hs-NICD flies might respond much more rapidly than hs-N flies. Therefore, we decided to train hs-NICD flies immediately after the heat shock treatment (omitting the 3-hour recovery time). We still did not observe enhanced LTM formation (Fig. 1A, right). To examine whether NICD improves LTM after a more intense training, we did similar experiments as above but subjected the flies to the 5× spaced training protocol. Depending on the timing of training, LTM was either not significantly changed (Fig. 1B, left) or blocked (Fig. 1B, middle). The NICD-induced LTM impairment was surprising, and we further tested immediate memory to confirm that it was not due to learning defects or any abnormal “task-relevant” sensorimotor responses (Fig. 1B, right). Thus, our results show that increased NICD expression does not promote LTM formation, which is different from the results observed with increased Nfull expression. Besides, increased NICD expression was found to even impair LTM formation following LTM-inducing training.

3.2 Increased expression of NICD decreases CREB levels

Our previous study has shown that increased expression of Nfull leads to a higher level of hyper-PO4 CREB, a isoform that is critical for LTM formation (Zhang et al. 2013a). Our finding that increased NICD expression impairs LTM formation (Fig. 1) prompted us to examine its impact on CREB. We incubated hs-NICD and its genetic background control flies (*yellow white, yw*) at 37°C for 30 minutes, extracted total proteins from four fly heads for each group at different times following heat shock treatment. After NICD induction, we found that the levels of phosphorylated CREB isoforms (hyper-PO4 and hypo-PO4 CREB) were decreased in hs-NICD flies compared with the levels in *yw* control flies (Fig. 2A, top two panels). To examine if the decrease in phosphorylated CREB isoforms is due to less phosphorylation or decrease in CREB protein level, the same blot was re-probed with a monoclonal CREB antibody that recognizes both phosphorylated and unphosphorylated CREB isoforms. The results showed that the levels of almost all CREB isoforms were suppressed in association with increased NICD expression (Fig. 2A, first and third panel). We confirmed the results by including wild type, hs-N and hs-NICD groups in the same set

of experiments. As expected, hs-N increased the level of hyper-PO4 CREB, while hs-NICD decreased it (Fig. 2B, left). We also examined the effects of a weaker induction. We tried two conditions, lowering the temperature of heat shock to 30°C (Fig. 2B, right) and halving the period of heat shock (Fig. 2C). At 30°C, we saw no significant decrease of hyper-PO4 CREB in the hs-NICD group, though NICD is clearly induced, but a significant increase of hyper-PO4 CREB in the hs-N group (Fig. 2B, right). At the shorter period of heat shock at 37°C, we only observed a mild decrease of hyper-PO4 CREB in the hs-NICD group at 1 hour after the heat shock treatment (Fig. 2C). We also studied hs-NICD induction at 32°C and found results similar to the 30°C experiment (data not shown). An interesting observation in the 30°C experiments was that NICD level in hs-N group was even lower than the level in the wild type group. This finding was confirmed by running a 6% SDS-PAGE gel by which we could compare both Nfull and NICD levels (Fig. 2D). Together, our experiments with hs-NICD and hs-N flies (Fig. 2A, 2B and 2D) showed that the higher level of hyper-PO4 is positively associated with Nfull not NICD. Thus, our data suggest that increased NICD expression has either no effect or a suppression effect on CREB levels. Since in the 15 minutes at 30°C experiment hyper-PO4 CREB level in hs-NICD group is unaffected (instead of increased) before reduction at the 1 hour time point, we think the suppression is unlikely to be due to a dominant-negative effect. Our data showing that NICD impairs LTM formation but not learning ability (Fig. 1B) also argues against non-specific toxic effects.

3.3 Increased expression of NICD impairs CREB activity

To probe the effect of increased NICD expression on CREB-dependent transcription, we utilized a Luciferase-based CREB reporter, which has been widely used to study CREB transcriptional activity (Belvin et al. 1999). The CREB reporter flies (CRE-luci) were crossed with either hs-NICD or its genetic control *yw* flies. Both groups were subjected to the same heat shock and protein extraction procedure described above, and the western blots were probed with a Luciferase antibody. Increased expression of NICD was found to dramatically decrease the level of Luciferase expression (Fig. 3A). We further found that the same response would also occur in live, freely behaving animals (Fig. 3B). These results indicate that increased NICD expression negatively regulates CREB activity.

4. Discussion

Our results are consistent with several mammalian studies that raised questions about the positive role of NICD (canonical Notch signaling) in supporting synaptic plasticity or memory. One study in pyramidal neurons of the mouse visual cortex has shown that increased expression of NICD suppressed spine density and impaired long-term potentiation (Dahlhaus et al. 2008). Another recent study using *RBPj* postnatal knockout mice failed to find any learning and memory deficits (Sato et al. 2012). In *Drosophila*, Su(H) is found to be required specifically for LTM (Song et al. 2009). However, it is notable that Nfull expression has been shown to be decreased in Su(H) mutants (Wesley and Mok 2003), making it difficult to determine whether the LTM defect in Su(H) mutant is due to impaired canonical Notch signaling [mediated by Su(H) and NICD] or non-canonical Notch function (mediated by Nfull). Interestingly, the same study (Song et al. 2009) also showed that

increased expression of Su(H) [hs-Su(H)] in a wild type background specifically impairs LTM (Song et al. 2009), which is very similar to our observation in hs-NICD flies. It is possible that Su(H) plays a role in LTM formation through regulation of Nfull expression or some other feed back regulation.

While our paper was under review, a similar study using mammalian cultured cell system became available online (Hallaq et al. 2014). The data in this paper together with ours raise strong doubts regarding the involvement of canonical Notch signaling in the up-regulation of hyper-PO4 CREB by Nfull. It is possible that a non-canonical Notch signaling mechanism is involved (Andersen et al. 2012; Le Gall et al. 2008; Wesley et al. 2011; Zhang et al. 2013b). Our previous study has revealed the ultradian oscillation of hyper-PO4 CREB involved in *Drosophila* LTM, which is fascinating but poorly understood (Zhang et al. 2013a). It is possible that Nfull and NICD contribute to the generation of the peaks and the troughs, respectively, of the oscillation (Zhang et al. 2013a; Zhang et al. 2013b). On the other hand, NICD could be involved indirectly through regulation of Nfull expression. A simple model incorporating these possibilities is shown in Figure 3C.

Some studies have indicated an increase of NICD activity (canonical Notch signaling) in neurological diseases like Down's syndrome (Fischer et al. 2005) and sporadic Alzheimer's disease (Leal et al. 2012). It is possible that the connections we have identified between increased NICD, CREB dysfunction, and memory deficits may contribute to the memory loss in these disease conditions.

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Figure 1. Effects of increased expression of NICD on LTM in adult flies

(A) Induction of NICD expression either 3 hours prior to (left) or immediately before (right) the single training trial does not enhance LTM. $n=8$ PIs per group, mean $\pm$ SEM, Student's t -test ($p<0.05$). (B) Induction of NICD expression 3 hours prior to $5\times$ spaced training does not enhance LTM (left), while NICD induction immediately before $5\times$ spaced training impairs LTM (middle) but not immediate memory (right). $n=6$ or 8 PIs per group, mean $\pm$ SEM, Student's t -test ($p<0.05$).

Figure 2. Effects of increased NICD expression on CREB protein levels

(A) Increased expression of NICD decreases the levels of different CREB isoforms. *yw* (+) flies were used as control, as *hs-NICD* was generated in *yw* background. A Notch antibody was used to reveal the expression of NICD (first blot), PO4-CREB antibody to reveal the expression of phosphorylated CREB isoforms (second blot), and CREB monoclonal antibody to reveal the expression of all CREB isoforms (third blot). The same blot was probed with an antibody against heat shock protein 70 (Hsp70) to assess relative total protein levels in the extracts. (B) *hs-N* and *hs-NICD* have different effects on hyper-PO4 CREB at both high and low induction regimens. At high regimen (37°C) *hs-N* increases hyper-PO4 CREB while *hs-NICD* decreases CREB levels. At low regimen (30°C), *hs-N* increases the level of hyper-PO4 CREB but *hs-NICD* has no significant impact on it. Note that NICD level in *hs-NICD* samples is higher relative to NICD in wild type and *hs-N* flies. Extracts using four heads were loaded in each lane and the same blots were sequentially probed with PO4 CREB and Notch antibodies. (C) At the lower 15 minute at 37°C heat shock induction regimen, NICD has no significant impact on CREB at the two earlier time points but negatively impacts CREB at the one-hour time point. (D) Up-regulation of hyper-PO4 CREB in *hs-N* flies in the 30°C induction regimen is correlated to the level of Nfull and not to the level of NICD-like molecules. For each lane, proteins were extracted from four male flies and half the sample was loaded onto a 6% gel (to assess the level of Nfull that migrates at > 350 kDa range) and the other half onto a 10% gel to assess the level of hyper-PO4 CREB. We used male whole flies (to avoid variation due to eggs/embryos in females) since the level of Nfull in head samples of wild type flies is below the reliable detection level (hyper-PO4 CREB behaves similarly in heads and whole bodies, (Zhang et al. 2013a)).

Figure 3. Effect of increased NICD expression on CREB activity

(A) Increased expression of NICD impairs CREB-dependent transcription *ex vivo*. hs-NICD flies and the genetic background control (yw, +) were crossed with CRE-luciferase reporter flies, respectively. Total proteins extracted from four heads of the progeny were loaded in each lane. The blot was probed with a Luciferase antibody. Hsp70 served as a loading control. (B) Increased expression of NICD impairs CREB-dependent transcription in live, behaving animals. The luciferase activity was measured before and at different time points after heat shock treatment. N=48 for each group, data are presented as mean $\pm$ SEM, Student's *t*-test (* $p < 0.05$). (C) A simple hypothetical model for the roles of different Notch signaling activities in LTM formation. Pkc and Nfull activities are involved in the up-regulation of hyper-PO4 CREB and LTM enhancement in adult flies (Zhang et al. 2013a). γ -secretase (Presenilin complex) is involved in the generation of NICD.