Research

The anaphase promoting complex is required for memory function in mice

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Learning and memory processes critically involve the orchestrated regulation of de novo protein synthesis. On the other hand it has become clear that regulated protein degradation also plays a major role in neuronal plasticity and learning behavior. One of the key pathways mediating protein degradation is proteosomal protein destruction. The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that targets proteins for proteosomal degradation by the 26S proteasome. While the APC/C is essential for cell cycle progression it is also expressed in postmitotic neurons where it has been implicated with axonal outgrowth and neuronal survival. In this study we addressed the role of APC/C in learning and memory function by generating mice that lack the essential subunit APC2 from excitatory neurons of the adult forebrain. Those animals are viable but exhibit a severe impairment in the ability to extinct fear memories, a process critical for the treatment of anxiety diseases such as phobia or post-traumatic stress disorder. Since deregulated protein degradation and APC/C activity has been implicated with neurodegeneration we also analyzed the effect of *Apc2* deletion in a mouse model for Alzheimer's disease. In our experimental setting loss of APC2 from principle forebrain neurons did not affect the course of pathology in an Alzheimer's disease mouse model. In conclusion, our data provides genetic evidence that APC/C activity in the adult forebrain is required for cognitive function.

[Supplemental material is available for this article.]

Memory consolidation is a complex process and the underlying molecular and cellular processes are only beginning to emerge. A better understanding of the mechanisms underlying memory formation is especially important since cognitive dysfunction is a key feature of neuropsychiatric diseases such as Morbus Alzheimer that constitute an increasing emotional and economical burden to our societies (Sananbenesi and Fischer 2009).

A unanimous finding across species is that the formation of long-term memories involves the orchestrated regulation of gene expression. As such it is now commonly accepted that learning initiates differential gene expression and critically depends on de novo protein synthesis (Kandel 2001; Barco et al. 2008; Sananbenesi and Fischer 2009; Peleg et al. 2010). On the other hand, a number of studies clearly demonstrated the importance of protein degradation during cognitive function (Lopez-Salon et al. 2001; Merlo and Romano 2007; Artinian et al. 2008; Mabb and Ehlers 2010). A key pathway of protein degradation in eukaryotic cells is ubiquitin-mediated proteosomal degradation (Ciechanover 2005). The final step of ubiquitination, the attachment of ubiquitin to target proteins, is performed by E3-ubiquitin ligases. The anaphase-promoting complex/cyclosome (APC/C) is an E3-ligase consisting of 12 subunits (Fig. 1A) of which APC2 and APC11 form the catalytic core that is essential for ubiquitination (Peters 2006). APC/C activity is controlled via interaction with the coactivator proteins CDH1 or CDC20 and is essential for cell cycle progression (Peters 2006; Pesin and

Orr-Weaver 2008). It was therefore surprising when APC/C was detected in postmitotic neurons (Gieffers et al. 1999). Subsequent studies demonstrated a role for APC/C activity in neurodevelopmental processes such as axonal outgrowth in cerebellar granular neurons (Konishi et al. 2004; Yang et al. 2009) and data obtained in *Drosophila* and *C. elegans* suggested a role of APC/C in synaptic plasticity (Juo and Kaplan 2004; van Roessel et al. 2004). Interestingly, deregulation of APC/C via down-regulation of CDH1 protein has been implicated with neuronal cell death and its contribution to Alzheimer's disease (Almeida et al. 2005; Maestre et al. 2008) and cognitive decline (Li et al. 2008) has been suggested. As such, there is emerging evidence that APC/C activity is critically involved in cognitive processes and that its dysfunction may also contribute to the pathogenesis of neurological diseases.

In this study we tested the role of APC/C in learning and memory processes directly. To this end, we generated mice that lack the essential APC/C subunit APC2 from excitatory forebrain neurons (*Apc2* conditional knockout mice; *Apc2* cKO). We show that *Apc2* cKO mice develop normally and exhibit no alterations in basal anxiety, explorative and depressive-like behavior or motor coordination. However, the ability to form spatial memories was reduced in *Apc2* cKO mice. In addition, *Apc2* cKO were severely impaired in the extinction of fear memories. Since deregulated APC/C activity has been implicated with neuronal cell death, we also tested a role of APC/C in the pathogenesis of Alzheimer's disease by crossing *Apc2* cKO mice to an animal model for amyloid pathology. Loss of APC2 in this model did not affect memory function or amyloid-beta-plaque pathology. In summary, our data shows that APC/C function in postmitotic

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Figure 1. Deletion of *Apc2* from the adult forebrain. (A) Model of the APC/C (Passmore 2005). APC2 and APC11 form the catalytic core of the APC/C and are therefore essential for its function. (B) The *Apc2* gene is localized on chromosome (chr) 2. In the mice employed in this study exon 2–4 were flanked by loxP sites. The genomic location of the primers used for qPCR analysis is indicated. (C) The *Apc2* mRNA levels are reduced in the forebrain of *Apc2* cKO mice (* $P < 0.01$ vs. control). (D) APC2 protein levels are reduced in the forebrain of *Apc2* cKO mice (* $P < 0.0001$ vs. control). (Left panel) Representative immunoblot; (right panel) quantification. (E) Elevated Cdh1 levels in the forebrain of *Apc2* cKO mice (* $P < 0.001$ vs. control). (Left panel) Representative immunoblot; (right panel) quantification. Cx, cortex; Hip, hippocampus; C, control; bp, base pair; AU, arbitrary units. $n = 4$ –6/group. Error bars indicate SEM.

forebrain neurons is critical for spatial memory and the extinction of fear memories in mice.

Results

Mice lacking APC2 from principal forebrain neurons are viable and show normal brain morphology

To better understand the role of the APC/C during learning and memory function we decided to delete the essential APC/C subunit APC2 from postmitotic excitatory forebrain neurons of mice (Fig. 1A). To this end we employed animals in which exon 2–4 of the *Apc2* gene are flanked by loxP sites (Fig. 1B; Wirth et al. 2004) and crossed them to mice expressing *cre* recombinase under the control of the *CamKII α* promoter (Minichiello et al. 1999). As a result we obtained mice bearing the floxed *Apc2* allele (*Apc2^{f/f}*) in homozygosity and the transgene of *CamKII α -Cre*, which drives the expression of *cre* recombinase in postmitotic neurons of the adult forebrain, hereafter referred to as *Apc2* conditional knockout mice (*Apc2* cKO). Since previous work demonstrated that *CamKII α -cre* mice are indistinguishable to wild-type littermates, we employed *Apc2^{f/f}* mice as control groups (Minichiello et al. 1999; Wirth et al. 2004). When compared to control littermates quantitative real time PCR (qPCR) analysis revealed a significant

reduction of *Apc2* mRNA levels in the cortex and hippocampus of adult *Apc2* cKO mice. The reduction of *Apc2* mRNA was not complete since the *CamKII α -CRE* mediated deletion is specific to excitatory neurons and does not affect other cell types such as astrocytes that also express a functional APC/C (Herrero-Mendez et al. 2009). Consistent with the expression pattern of *cre* recombinase in *CamKII α -CRE* mice (Minichiello et al. 1999) *Apc2* expression in the cerebellum was not affected (Fig. 1C). The reduced gene-expression of *Apc2* in the adult forebrain resulted in reduced APC2 protein levels in the hippocampus and cortex of *Apc2* cKO mice while protein levels in the cerebellum were similar among groups (Fig. 1D). In line with these data we detected a significant up-regulation of CDH1 protein levels in cortical and hippocampal lysates of *Apc2* cKO mice (Fig. 1E). CDH1 is a coactivator of the APC/C that was shown to be itself an APC/C substrate (Listovsky et al. 2004). Thus, elevated CDH1 levels in *Apc2* cKO mice are likely due to a failure in CDH1 degradation and are therefore indicative for impaired APC/C activity. In line with this CDH1 levels in the cerebellum were not altered (Fig. 1E).

Importantly, while deletion of *Apc2* during development is lethal in mice (Wirth et al. 2004), *Apc2* cKO mice were indistinguishable from control littermates and showed normal home cage behavior (Fig. 2A). Brain weight was similar among *Apc2*

Figure 2. Normal brain morphology in *Apc2* cKO mice. (A) Image of an *Apc2* cKO mice and a control littermate. (B) Representative image of a brain from an *Apc2* cKO and a control mouse (right panel). Quantitative analysis of brain weight (left panel). (C) Representative images showing MAP2 and NEUN immunoreactivity in *Apc2* cKO and control mice. Scale bar: 100 μ m. (D) Representative immunoblot images showing equal levels of PSD-95, GLUR1, and GLUR2 levels in *Apc2* cKO and control mice (left panel). Quantification $n = 4-6$ /group (right panel). CA1, CA3, hippocampal subfields Ca1 and Ca3; DG, dentate gyrus. Error bars indicate SEM.

cKO and control mice (Fig. 2B). Immunoreactivity of microtubule-associated protein 2 (Map2) and Neuronal N (NeuN), two well-established markers for neuronal integrity (Fischer et al. 2007; Peleg et al. 2010), was similar among *Apc2* cKO and control mice (Fig. 2C). As such, our data suggest that gross brain morphology and neuronal integrity is normal in *Apc2* cKO mice. In line with this immunoblot analysis of hippocampal lysates revealed that proteins levels of markers for neuronal integrity such as postsynaptic density-95 (PSD95), synaptophysin (SVP), or Glutamate receptor 1 (GLUR1) or SH3 and multiple ankyrin repeat domains protein 1 (SHANK1) were similar among groups (Fig. 2D). While we could confirm significant up-regulation of CDH-1 levels in *Apc2* cKO mice, we did not observe differences in the levels of the APC/C target SnoN (Fig. 2D). In summary, this data indicates that loss of APC2 from excitatory neurons of the adult forebrain of mice does not cause an overt phenotype.

Basal anxiety, motor-coordination, explorative and depressive-like behavior is normal in *Apc2* cKO mice

Next, we exposed male *Apc2* cKO and control mice to the open field paradigm, a simple test for basal anxiety, explorative behavior, and locomotion. We did not observe a significant difference in the distance traveled during the test period among groups, indicating that explorative and locomotor behavior in a novel context is normal in *Apc2* cKO mice (Fig. 3A,B). No difference was observed when we specifically analyzed the time spent in the center vs. the border of the open field arena, a common measure of anxiety, suggesting that basal anxiety was similar among groups (Fig. 3C). To investigate this in greater detail we subjected a group of male *Apc2* cKO and control mice to the elevated plus maze test, another paradigm that allows the assessment of anxiety levels in rodents. Both groups spent similar times in the closed vs. open arms of the maze (Fig. 3D). In conclusion this data

demonstrates that loss of APC2 in the forebrain of mice does not affect explorative behavior and basal anxiety. In addition we did not observe significant group differences when male *Apc2* cKO and control mice were exposed to the porsolt forced swim test (Fig. 3E) and well-established paradigm to analyze depressive like behavior. We also examined the performance of male *Apc2* cKO mice and control littermates in the accelerated Rotarod test, a motor coordination task, but did not observe significant differences among groups (Fig. 3F). Similarly, basal anxiety, explorative and depressive-like behavior as well as motor coordination were similar amongst female *Apc2* cKO and control mice (data not shown).

Apc2 cKO mice display impaired spatial memory

To test learning and memory function *Apc2* cKO ($n = 16$) and control mice ($n = 17$) were subjected to the Morris water maze test, a hippocampal-dependent assay for spatial memory performance. All groups improved in their ability to find the hidden platform. There was no significant gender effect in control ($P = 0.9223$, $F = 0.3969$) or *Apc2* cKO mice ($P = 0.1677$, $F = 1.462$). However, *Apc2* cKO displayed significantly impaired escape latency on training days 3–4 and 6–10 when compared to the control group. In line with this, repeated measures ANOVA showed that the escape latency throughout the 10 training days, an indicator of spatial learning, was significantly impaired ($P < 0.0001$, $F = 55.01$ vs. control) in *Apc2* cKO mice when compared to the corresponding control group (Fig. 4A). Since groups did not differ in finding a visible platform (Fig. 4A) or the swimming speed during the training (Fig. 4B) our data suggests that *Apc2* cKO mice are impaired in spatial learning ability. Consistently, when compared to the control group, *Apc2* cKO spent significantly less time in the target quadrant during a probe test performed after 10 d of training (Fig. 4C). Similarly, the number of platform crossings was reduced in *Apc2* cKO mice (Fig. 4D), which is indicative of impaired spatial

Figure 3. Basal anxiety, explorative and depressive-like behavior, and motor coordination in *Apc2* cKO mice. (A) Representative image showing the explorative behavior of *Apc2* cKO and control mice in an open field. (B) Distance traveled during 5 min exposure to an open field. (C) Time spent in the center vs. the corner regions of the open field during a 5 min test session. (D) *Apc2* cKO and control mice were subjected to the rotarod test. No significant difference was observed among groups. (E) When exposed to the elevated plus maze test *Apc2* cKO and control mice spent similar times in the center, the closed or the open arms. (F) *Apc2* cKO and control mice were subjected to the Porsolt forced swim test. The time spent immobile was similar among groups. $n = 8-12/\text{group}$. Error bars indicate SEM.

memory. When exposed to a reversal-learning paradigm in which the platform was moved to a new location, *Apc2* cKO also performed significantly worse than the corresponding control group (Fig. 4E). In conclusion this data shows that spatial memory performance is impaired in *Apc2* cKO mice.

Apc2 cKO mice exhibit severely impaired extinction of fear memories

To further assess the role of *Apc2* in memory function mice were subjected to the Pavlovian fear conditioning paradigm, a standardized test to measure associative learning in rodents (Fischer et al. 2007). Male *Apc2* cKO and control mice were subjected to contextual fear conditioning. The activity during the training and the response to the electric footshock was similar among groups (Fig. 5A). When freezing behavior was analyzed in a memory test performed 24 h later no difference was observed among groups (Fig. 5B). Similar results were obtained for tone-dependent fear conditioning (Fig. 5C) or when mice were subjected to a weaker training protocol (Supplemental Fig. 1). We conducted

the same experiments in female *Apc2* cKO and control mice. While explorative behavior during the training and the response to the footshock was similar among groups (Fig. 5D), *Apc2* cKO mice displayed significantly more freezing behavior during a contextual memory test performed 24 h after the training (Fig. 5E). Similar data were observed during tone-dependent fear conditioning (Fig. 5F). Interestingly, this data indicates that the consolidation of fear memories is normal in male and even enhanced in female mice that lack APC2 in the adult forebrain.

A strong association between a fear-eliciting and an otherwise neutral stimulus, such as the electric foot shock and the novel context in the fear conditioning paradigm, is often the basis for anxiety diseases such as phobia or post-traumatic stress disorder (Myers and Davis 2007; Sananbenesi et al. 2007). Treatment of such disorders involves repeated exposure to the frightening stimulus in the absence of the aversive event, which gradually leads to the reduction of the fear memory response, a process named fear extinction. Fear extinction is often impaired in patients suffering from anxiety diseases. While the mechanisms underlying fear extinction are not well understood, a recent study implicated proteasome activity with the extinction of fear memories (Lee et al. 2008). To this end we set out experiments to analyze whether APC2 plays a role in fear extinction. Male *Apc2* cKO mice and a corresponding control group were subjected to contextual fear conditioning and subsequently exposed to extinction training, consisting of reexposure to the conditioning context on consecutive days in the absence of the footshock (Fischer et al. 2004; Sananbenesi et al. 2007; Tronson

et al. 2009). While freezing behavior declined throughout extinction training in the control group, *Apc2* cKO displayed high freezing on all extinction trials (Fig. 6A). Similar data was obtained in female mice (Fig. 6B). However, as shown before (see Fig. 5E) female *Apc2* cKO mice already displayed elevated freezing behavior on extinction trial 1 (E1), which may compromise the observed effect. Nevertheless, impaired fear extinction in female *Apc2* cKO mice was still obvious when freezing levels on E1 were normalized to allow a direct comparison among groups (Fig. 6C). This data demonstrates that fear extinction is severely impaired in male and female *Apc2* cKO mice.

Loss of *Apc2* from the adult forebrain does not influence disease progression in a mouse model for amyloid pathology

Recent *in vitro* studies implicated deregulated APC/C function with neuronal cell death and it has been suggested that loss of APC/C activity might play a role in the pathogenesis of Alzheimer's disease (Almeida et al. 2005; Maestre et al. 2008). To

Figure 4. Impaired spatial memory formation in *Apc2* cKO mice. (A) When compared to the control group ($n = 16$), the escape latency throughout the training was significantly impaired in *Apc2* cKO mice ($n = 17$; $P < 0.0001$, $F = 55.01$ vs. control). Groups did not significantly differ in finding a visible platform (VP). (B) Swimming speed during the days of training was similar among groups. (C) After 10 d of training *Apc2* cKO and control mice were subjected to a probe trial. The time spent in the target quadrant was significantly impaired in *Apc2* cKO mice when compared to the control group ($P < 0.0001$). The right panel shows representative swim path of *Apc2* cKO and control mice during the probe test. (D) During the probe test the number of visits to the target quadrant was significantly reduced in *Apc2* cKO mice ($P < 0.0001$ vs. control). (E) Reversal learning was impaired in *Apc2* cKO mice when compared to control mice. (Left panel) Escape latency during the reversal training ($P < 0.05$; $F = 5.699$ vs. control); (right panel) time spent in the new target quadrant during the probe test ($P < 0.05$ vs. control). T, target quadrant; Q1, quadrant 1; Q2, quadrant 2; Q3, quadrant 3; Q4, quadrant 4. Error bars indicate SEM.

further test this possibility we crossed *Apc2* cKO mice with APP/PS1 mice, a mouse model for severe amyloid pathology (Radde et al. 2006). We analyzed learning behavior in 6-mo-old female *Apc2* cKO/APP/PS1 mice, because that age marks the onset of memory disturbances and thus should allow us to observe either an enhancement or an impairment of learning abilities (Fig. 7A). When trained in the Morris water maze test APP/PS1 and *Apc2* cKO/APP/PS1 mice showed no significant difference with respect to the escape latency (Fig. 7B,C). Similarly, when a probe test was performed after 10 d of training, the performance of *Apc2* cKO and *Apc2* cKO APP/PS1 mice was similar and both groups showed a nonsignificant preference for the target quadrant (Fig. 7D). When trained in the contextual fear conditioning paradigm no significant difference was observed among groups (Fig. 7E). This data suggests that loss of APC2 does not affect memory function in APP/PS1 mice. APP/PS1 mice are characterized by a very aggressive amyloid pathology and A β plaques are already observed in 2-mo-old mice (Radde et al. 2006). When we analyzed the A β -plaque load in the hippocampal region of APP/PS1 and *Apc2* cKO/APP/PS1 mice, both groups exhibited similar amyloid pathology (Fig. 7F). In line with this data, no difference in NeuN or MAP2 levels was observed when analyzed in the hippocampal region by quantitative immunohistochemistry (Fig. 7G). This data suggests that in our experimental setting loss of *Apc2* from the adult forebrain does not affect hippocampus-dependent learning behavior and amyloid-plaque load in APP/PS1 mice.

Discussion

In this work we investigated the role of APC/C activity during learning and memory by specifically deleting the essential

APC/C subunit APC2 from postmitotic excitatory neurons of the adult forebrain in mice. To this end we crossed *Apc2*^{n/n} mice with transgenic animals expressing *cre*-recombinase under control of the *CamKII α* promoter. As expected, we observed a significant reduction of *Apc2* mRNA levels in the hippocampus and cortex of *Apc2* cKO mice whereas *Apc2* mRNA levels in the cerebellum were not affected. The reduction of *Apc2* mRNA in the cortex and hippocampus was however not 100% since the genetic manipulation eliminates *Apc2* expression only in excitatory neurons, whereas glial cells, interneurons and other neural types maintain normal expression (Herrero-Mendez et al. 2009). Similarly, we observed a significant, but not complete reduction of APC2 protein in hippocampus and cortex of *Apc2* cKO mice. In line with this data, we detected a significant up-regulation of CDH1 protein levels in hippocampal and cortical, but not in cerebellar lysates. CDH1 associates with APC/C as an activator but at the same time is targeted for degradation in an APC/C dependent manner (Peters 2006). Therefore, elevated CDH1 protein levels are indicative of reduced APC/C activity in *Apc2* cKO mice. Loss of APC2 activity during development is lethal in mice (Wirth et al. 2004). Moreover, deletion of *Apc2* from hepatocytes leads to an unscheduled entry into cell cycle causing a severe liver failure (Wirth et al. 2004). In contrast, when we postnatally deleted *Apc2* from principal forebrain neurons mice were viable and did not show any overt phenotype. We also did not observe brain atrophy or gross changes in neuronal integrity suggesting that APC2 is not critical for neuronal survival in adult mice. In addition explorative behavior in a novel context and motor coordination was indistinguishable in *Apc2* cKO and control mice. *Apc2* cKO mice also exhibited normal basal anxiety and no change in depressive-like behavior. However, when tested in the Morris water maze paradigm, *Apc2* cKO displayed impaired spatial

Figure 5. Acquisition of fear memories in *Apc2* cKO mice. (A) The activity in response to the footshock increased to a similar degree in male *Apc2* cKO and control animals ($P < 0.0001$ training vs. ES). (B) Freezing behavior in the contextual memory test was similar in male *Apc2* cKO mice and control littermates. (C) Freezing behavior in the cued memory test was similar in male *Apc2* cKO mice and control littermates. (D) The activity in response to the footshock increased to a similar degree in female *Apc2* cKO and control animals ($P < 0.0001$ training vs. ES). (E) Freezing behavior in the contextual memory test was significantly higher in female *Apc2* cKO mice when compared to control littermates ($P < 0.05$). (F) Freezing behavior in the cued memory test was significantly higher in female *Apc2* cKO mice when compared to control littermates ($P < 0.05$). $n = 9$ /group; ES, electric footshock. Error bars indicate SEM.

memory performance. The water maze test is hippocampus-dependent suggesting that hippocampal APC2 is required for the consolidation of spatial memories. This data is in line with previous findings that reported impaired spatial or associative learning in mice that were injected intrahippocampally with a protein degradation inhibitor (Lopez-Salon et al. 2001; Artinian et al. 2008). Impaired hippocampus dependent memory formation was also observed in mice that exhibited reduced CDH1 levels, a critical APC/C coactivator. In this study complete *Cdh1* knock out mice were used and while homozygosity was lethal, heterozygous animals were viable and displayed impaired hippocampus-dependent memory formation (Li et al. 2008). It is therefore interesting that hippocampal and cortical CDH1 levels were elevated in our *Apc2* cKO mice. However, this is most likely due to impaired APC/C function, which normally targets CDH1 for degradation (Listovsky et al. 2004). As such the elevated *Cdh1* levels in *Apc2* cKO mice reflect a failure in *Cdh1* degradation. Importantly, hippocampal levels of other potential of known APC/C targets such as SnoN or SHANK1, which was shown to be regulated via ubiquitination (Lee et al. 2008), were not altered in *Apc2* cKO mice. This data indicates that different APC/C substrates play a role in APC/C mediated memory function. Moreover, the APC/C can interact with different E2 ligases

and it is possible that such interactions are rather dynamic and induced via specific environmental stimuli. Thus, the possibility remains that known and unknown APC/C substrates are deregulated in *Apc2* cKO mice only in response to specific environmental stimuli.

Interestingly Pavlovian fear conditioning that is commonly used to measure associative fear memories in rodents was not affected in male *Apc2* cKO mice and even enhanced in female *Apc2* cKO mice. Although spatial memory in the water maze paradigm and contextual fear conditioning are hippocampus dependent, the fear conditioning paradigm specifically tests for the acquisition of fear memories. It is therefore important to reiterate that excessive fear memory is often the basis for neuropsychiatric disorders such as phobia or post-traumatic stress disorder (PTSD), which severely affect the life of patients and are an increasing burden to our societies (Sotres-Bayon et al. 2006; Myers and Davis 2007; Bremner et al. 2008; Fischer and Tsai 2008; Hartley and Phelps 2010). Therapeutic strategies to inhibit excess fear are therefore central to the treatment of anxiety and mood disorders. Fear inhibition can be achieved by repeated reexposure to the fear-eliciting stimulus in the absence of any aversive event. This normally leads to a gradual reduction of fear, a process named fear extinction. Interestingly, we observed that male and female *Apc2* cKO mice exhibited severely impaired fear extinction. As such, the aversive freezing behavior did not decline throughout our extinction training protocol and was still significantly higher than in control mice when tested 28 d after the last

extinction trial. This data indicates that APC2 function is of particular importance for the extinction of fear memories and indicates that the APC/C may play a role in the pathogenesis of neuropsychiatric diseases. In line with this view a recent study demonstrated that proteasomal protein degradation in the hippocampus is required for fear extinction in mice (Lee et al. 2008). Moreover, while the partial NMDA receptor agonist D-cycloserine (DCS) facilitates fear extinction in rodents and also exhibits beneficial effects in patients suffering from phobia (Ressler et al. 2004; Davis et al. 2006) the effect of DCS injection was blocked when coadministered with proteasome inhibitors (Mao et al. 2008). While proteasome inhibitors are rather unselective our data strongly suggest that the APC/C is one of the players mediating the effect of proteasomal protein degradation during the extinction of fear memories. While the precise mechanisms affected by APC/C during fear extinction remain to be elucidated it is interesting to note that cell culture studies provided substantial evidence that proteasomal protein degradation is critical for synaptic plasticity, especially the formation and dynamics of dendritic spines (Mabb and Ehlers 2010). As such, morphological or functional reorganization of particular hippocampal synapses could be a likely downstream mechanism of APC/C activity during fear extinction.

Figure 6. Impaired extinction of fear memories in *Apc2* cKO mice. (A) The extinction of contextual fear memories was significantly impaired in male *Apc2* cKO mice when compared to a control group ($***P < 0.0001$, repeated measurements ANOVA $P < 0.0001$, $F = 19.84$). (B) The extinction of contextual fear memories was significantly impaired in female *Apc2* cKO mice when compared to a control group ($***P < 0.0001$, repeated-measurements ANOVA $P < 0.0001$, $F = 5.85$). (C) The data shown in B was normalized so that freezing on extinction trial 1 was similar among groups. $n = 9/\text{group}$. Error bars indicate SEM.

Dysfunction of proteasomal degradation has been implicated with the pathogenesis of various neurodegenerative diseases including Alzheimer's disease (Oddo 2008). Recent evidence specifically points to a role of deregulated APC/C function during AD-like neurodegeneration. For example, RNA interference mediated down-regulation of CDH1 in cultured hippocampal neurons induced apoptotic cell death, most likely due to cell cycle reentry (Almeida et al. 2005). Moreover, CDH1 is a target of the cyclin-dependent kinase 5 (Cdk5) which, when activated by the p25 protein, has been implicated with neurodegeneration and learning impairments (Fischer et al. 2005, 2007; Maestre et al. 2008). To test a function of APC/C in the pathogenesis of Alzheimer's disease we crossed *Apc2* cKO mice to APP/PS1 mice, which develop severe amyloid pathology and memory deficits (Radde et al. 2006). However, in our experimental settings spatial and associative memory function was indistinguishable between *Apc2* cKO and *Apc2* cKO/APP/PS1 mice. In addition, amyloid pathology, as measured as A β -plaque load, was indistinguishable among groups. This data indicates that APC/C activity has no influence on the amyloid pathology and the associated memory impairment in the employed mouse model. The fact that loss of APC2 does not aggravate memory deficits in APP/PS1 mice may also suggest that the mechanisms by which amyloid- β pathology and loss of APC2 affect memory function might share similar mechanism of action. However, amyloid pathology is only one pathological hallmark of Alzheimer's disease and it is important to note that neuronal cell death is usually not detectable at significant levels in mouse models for amyloid pathology. As such, the effect of APC/C activity on Alzheimer's related neuronal cell death or other pathological features such as Tau pathology remains to be investigated in future studies.

In conclusion, our study shows that APC/C activity in principle forebrain neurons is essential for cognitive function. At least in our experimental settings APC/C seems to have only limited impact on neuronal survival in adulthood and Alzheimer's disease related amyloid-pathology. Interestingly, our data indicates that loss of APC2 recapitulate some, but not all effects on learning behavior that has been observed with pharmacological inhibition proteasome activity. As such, while the acquisition of fear memories was not affected or even enhanced in female *Apc2* cKO mice, APC/C activity seems to be of particular importance for the extinction of fear memories. Thus, our data indicate that APC/C activity in the adult forebrain may play a role in the pathogenesis of anxiety diseases.

Materials and Methods

Animals

Mice were housed under standard conditions with free access to food and water. All experiments were carried out in accordance with the animal protection law and were approved by the District Government of Germany. The genetic background of all mice was C57Bl/6J. The generation of *Apc2*^{fl/fl}, CamKII α -cre, and APP/PS1 mice has been described previously (Minichiello et al. 1999; Wirth et al. 2004; Radde et al. 2006).

Behavioral analysis

Behavior testing was performed as described previously (Fischer et al. 2004; Park et al. 2005; Peleg et al. 2010). In brief, mice were single housed and habituated to the testing room at least 3 d before behavior experiments. For the open field test mice were placed in the center of a plastic arena (length 1 m; width 1 m; side walls 20 cm height) for 5 min. The explorative behavior was recorded by a camera and analyzed using the VideoMot2 software (TSE Systems). For elevated plus maze analysis mice were placed in the center region of the elevated maze facing the open arm. The behavior was recorded for 5 min using the VideoMot2 system (TSE systems). The Porsolt forced swim test was conducted by exposing mice for 5 min to a container (diameter of 20 cm) that was filled with water. An observer that was blind to the genotype measured immobility. Fear conditioning training was performed using the TSE fear conditioning system. The procedure consisted of exposing mice to the conditioning context (3 min) followed by a single electric footshock (0.7 mA, constant current, 2 sec). Afterward, mice were left in the conditioning box for 15 sec before being returned to their home cage. Freezing was analyzed 24 h later during reexposure to the conditioning context. To control for potential ceiling effects in male *Apc2* cKO mice an additional fear conditioning test was performed using lower shock intensity of 0.5 mA (constant current, 2 sec). For cued-dependent fear conditioning, a tone (10 kHz, 75 db) was presented for 30 sec prior to the footshock and tone-dependent memory was tested by placing the mice into a novel context for 1 min followed by 3 min exposure to the conditioning tone. For fear extinction, mice were subjected to extinction trials (E) on consecutive days. Unless otherwise stated, contextual and cued-conditioning was combined as one training session. In this case mice were first tested for contextual fear memory after 24 h and then tested for cued conditioning another 24 h later. Each extinction trial consisted of 3 min reexposure to the conditioning context. The water maze training was performed in a circular tank (diameter 1.2 m) filled with opaque water. A platform (11 $\times$ 11 cm) was submerged

Figure 7. Loss of *Apc2* in principal forebrain neurons does not affect pathology in APP/PS1 mice. (A) Experimental design. (B) The escape latency during the water maze training was similar among *Apc2* cKO and APP/PS1 mice. (C) The escape latency was analyzed on individual training days. No significant changes were observed among groups. (D) Time spent in the target quadrant was similar among groups. Both groups showed a nonsignificant preference for the target quadrant. (E) Contextual freezing behavior was similar among groups. (F) Aβ-plaque load in the hippocampus; (left panel) representative images showing Aβ-plaque load in the hippocampus; (right panel) quantification. (G) Neuronal integrity as measured by NeuN and MAP-2 staining was not different among groups. (Left panel) Representative images; (right panel) quantification. $n = 10$ /group for behavior experiments, $n = 3$ /group for immunohistochemistry; T, target quadrant; O, other quadrants. Error bars indicate SEM.

below the water's surface in the center of the target quadrant. The swimming path of the mice was recorded by a video camera and analyzed by the Videomot 2 software (TSE). For each training session, the mice were placed into the maze subsequently from four random points of the tank and were allowed to search for the platform for 60 sec. If the mice did not find the platform within 60 sec, they were gently guided to it. Mice were allowed to remain on the platform for 15 sec. Mice were subjected to one memory test (probe trial) 24 h after the last training session. During the probe test the platform was removed from the tank and the mice were allowed to swim in the maze for 60 sec. For the visual Morris water maze test, mice were habituated to the pool for two consecutive days. During the visual probe test the platform was placed in the pool and made visible by an elevated cube. For reversal learning the platform was transferred to a new quadrant. The Rotarod test was conducted using the Rota Rod V4.02 System from TSE Systems. Mice were first habituated to the procedure and then subjected to an accelerated paradigm for 4 min on two consecutive days (10–40 rpm).

Immunoblot analysis and immunohistochemistry

Brain tissue was homogenized in TX buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, and protease inhibitors) incubated for 15 min at 4°C and centrifuged for 10 min (10,000 rpm). The supernatant was used for immunoblotting. Immunoblots were performed using fluorescent secondary antibodies and data were quantified using an Odyssey Imager (Licor). Antibodies were diluted either in 0.5% milk PBT or 0.5% milk TBT, respectively. The following antibodies were commercially purchased and used in the mentioned concentrations: β-Actin (Ac-15) 1:1000, Santa Cruz Biotechnology; GAPDH, 1: 5000, Chemicon; GluR1 1:1000, Synaptic Systems; Synaptophysin (Syp38) 1:1000, Sigma-Aldrich, Map2, 1:1000; Synaptic Systems, NeuN 1:1000, Chemicon. The antibody against APC2 was a gift from J.M. Peters. APC2 hybridoma cell supernatant was used in a 1:200 dilution. The antibody used to detect Cdh1 was a gift by J. Gannon. In this case, 66 μl of the hybridoma cell supernatant were dissolved in 2% BSA in TBT. Immunostaining was performed as described previously (Fischer et al. 2007) and analyzed using a Leica SP2 confocal microscope. Microtubule associated protein 2 (Map2) antibody was from Synaptic Systems (1:200) and β-amyloid, 17–24 (4G8) antibody was from Conconvance (1:1000).

Quantitative real time PCR (qPCR)

qPCR was performed using a Roche 480 light cycler. cDNA was generated using

the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and qPCR for individual genes were performed using the Roche Universal probe library (UPL). Data was normalized to the house-keeping gene hypoxanthine phosphoribosyltransferase (Hprt).

Statistical analysis

The data was analyzed by unpaired student's *t*-test and one-way or two-way ANOVA (ANALYSIS OF VARIANCE) when appropriate. Errors are displayed as standard error of the mean (SEM).

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