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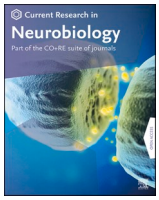

Rb deficiency, neuronal survival and neurodegeneration: In search of the perfect mouse model

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ABSTRACT

Three decades following the introduction of the first Rb knockout (KO) mouse model, the role of this critical protein in regulating brain development during embryogenesis and beyond remains a major scientific interest. Rb is a tumor suppressor gene known as the master regulator of the G1/S checkpoint and control of cell cycle progression in stem and progenitor cells, but also their differentiated progeny. Here, we review the recent literature about the various Rb conditional Knockout (cKO) and inducible Knockout (iKO) models studied thus far, highlighting how findings should always be interpreted in light of the model and context under inquiry especially when studying the role of Rb in neuronal survival. There is indeed evidence of age-specific, cell type-specific and region-specific effects following Rb KO in the embryonic and the adult mouse brain. In terms of modeling neurodegenerative processes in human diseases, we discuss cell cycle re-entry (CCE) as a candidate mechanism underlying the increased vulnerability of Rb-deficient neurons to cell death. Notably, mouse models may limit the extent to which CCE due to Rb inactivation can mimic the pathological course of these disorders, such as Alzheimer's disease. These remarks ought to be considered in future research when studying the consequences of Rb inactivation on neuronal generation and survival in rodents and their corresponding clinical significance in humans.

1. Introduction

The Retinoblastoma protein, Rb, has been regarded as an 'enigma' in that it is implicated in the regulation of many cellular processes in various types of body tissues beyond its central role in cell cycle control, including mitochondrial function, chromosome stability, metabolic flux and autophagy (Dyson, 2016). Having been the first tumor suppressor gene to be discovered, Rb was involved in cancer formation and progression, especially in the retina, and has been modeled by several mouse lines. Less recognized, however, is its critical role in maintaining the postmitotic state of differentiated neurons. This review aims to highlight the recent literature on this topic, which relies to a large extent on distinct Rb knockout (KO) mouse models generated over the past two decades.

While we focus here on the mouse Central Nervous System (CNS) – the telencephalon in particular, cell death mechanisms have also been described in the Peripheral Nervous System (PNS) in the context of Rb inactivation. For instance, Rb siRNA knockdown in adult peripheral sensory nerves promotes their plasticity by increasing neurite outgrowth after axotomy without affecting cell survival (Christie et al., 2014), in contrast to what is seen for newborn neurons in the adult brain (refer to next sections). This discrepancy in CNS and PNS requirements for Rb might indeed be mirrored in partially overlapping or non-overlapping cell death pathways, as $Rb^{-/-}E2f1^{-/-}$, $Rb^{-/-}Trp53^{-/-}$ and $Rb^{-/-}Apaf1^{-/-}$ mice show either partial or complete reduction in apoptosis following loss of Rb in the embryonic CNS but not the PNS. In contrast, $Rb^{-/-}$Caspase-$3^{-/-}$ embryos rescue Rb-deficient neurons from apoptosis in the PNS but not CNS (reviewed in (Chau and Wang, 2003)). These findings might have been the first clue that the Rb pathway does not act uniformly across different neural tissues to maintain neuronal survival.

2. Rb deficiency: a genealogy of mouse models

2.1. Tissue-specific Rb deletion using conditional KO (cKO) models

The reason why several Rb KO mouse models are currently available can be traced back to the severe defective phenotype, primarily massive

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apoptosis, that the first germline $Rb^{-/-}$ lines brought about in extraembryonic lineages that form the placenta, which then led to developmental lethality between E14.5–15.5 (de Bruin et al., 2003). As such, numerous conditional KO (cKO) models targeted at distinct neural tissues were introduced, which has allowed Rb-deficient embryos to survive to full term, and therefore be able to assess their phenotype at late developmental stages. These include: Foxg1-Cre mice (targeting cortical progenitors in the telecephalon), Nestin-Cre mice (stem and progenitor cells and their progeny in the CNS), Pax6-Cre mice (retinal progenitor cells) and Chx10-Cre mice (retinal progenitor cells) (reviewed in (Ajioka, 2014)).

2.2. Stage-specific Rb deletion by Cre plasmid electroporation

To study the role of Rb along the same cortical lineage but at different stages of embryonic neurogenesis, Oshikawa et al. electroporated $Rb^{flox/flox}$ embryos with pCAG-Cre plasmid targeted at cortical progenitor cells, whereas pMAP2-Cre was used to induce Rb deletion in differentiated cortical neurons (Oshikawa et al., 2013). This approach uncovered a distinct requirement for Rb in neuronal survival, that is before versus after neuronal differentiation, which could not be revealed with the cKO models currently used (refer to next section for detail). While all lines established a critical requirement for Rb in regulating neural progenitor proliferation, neuronal survival (post-differentiation) was only a requirement for Rb in the forebrain but not retinal lineages (Ajioka, 2014) possibly due to the diencephalic origin of retinal cells. Consequently, as expected, Rb loss in retinal cells causes retinoblastoma formation in mice (Chen et al., 2004).

2.3. Rb deletion in postnatal and adult brain using inducible KO (iKO) models

All Rb cKO mouse models contributed significantly to the characterization of the embryonic roles of Rb in CNS development; however, mice could not survive postnatally (for instance, due to respiratory defects in Foxg1-Cre mice (Ferguson et al., 2002)). This hindered further analysis of the requirement of the Rb pathway in the young adult brain as well as the mid-aged and the old-aged brain. The study of the role of Rb in the adult brain was made possible with inducible KO models (iKO), which can trigger Rb inactivation at desired timepoints by tamoxifen administration. Such approach combines both spatial and temporal control of gene deletion, hence eliminating ectopic and unwanted carry-over effects usually observed with KO and cKO models. Two such inducible models are the Nestin-$CreER^{T2}$ mice (targeting adult neural stem and progenitor cells and their progeny) used to study the role of Rb during adult neurogenesis (Fong et al., 2022; Naser et al., 2016; Vandenbosch et al., 2016), and CamK$CreER^{T2}$ mice targeting post-mitotic cortical neurons (Andrusiak et al., 2012). The temporal and regional specifications of each Rb KO line are outlined in Fig. 1.

3. Contextual neuronal survival following Rb KO: which, when and where?

Bearing in mind the different backgrounds of Rb KO models, comparisons can still be made between various Rb-deficient mouse lines as to suggest that neuronal survival in the developing and adult brain is highly contextual on at least three levels: age, cell-type (or stage) and lineage (or brain region) (Fig. 2).

3.1. Age-dependent effects

In the telencephalon, neuronal progenitors seem most dependent on Rb during mid-development up until birth given their ectopic proliferation and later differentiation and migration defects following Rb KO (Ferguson et al., 2002; Ghanem et al., 2012). However, during young adulthood i.e. at P90, Rb inactivation in the adult subventricular zone (aSVZ) leads to a more controlled boost in progenitors' proliferation without affecting subsequent migration and differentiation of

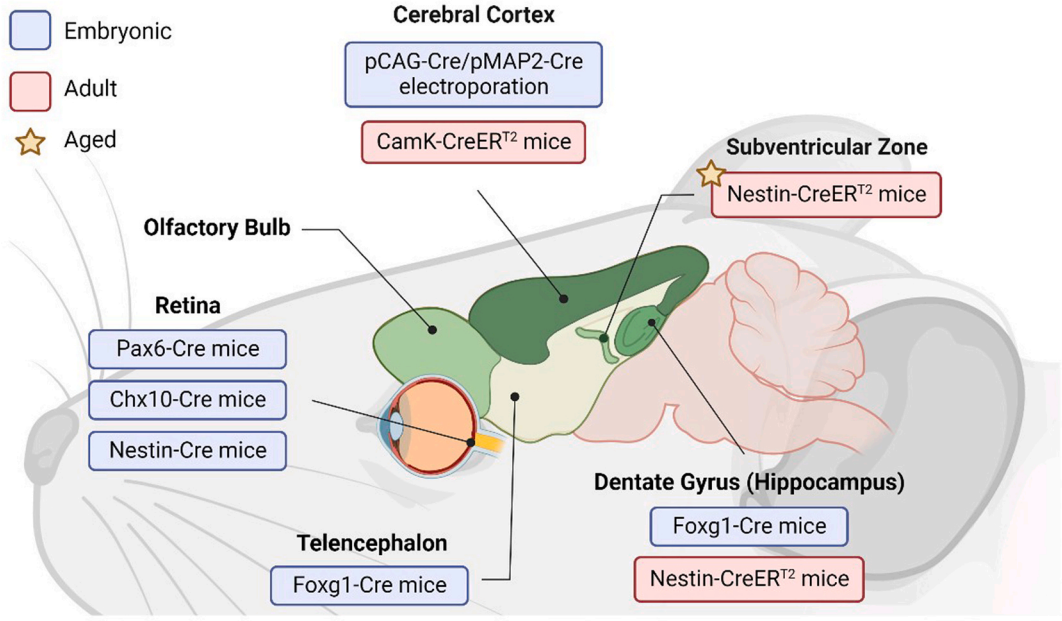

Fig. 1. Rb knockout mouse models target specific regions and different developmental stages in the embryonic and the adult mouse brain. Rb conditional KO (cKO) mouse models limit the effect of gene inactivation to desired neuronal populations expressing a defined marker, which restricts Cre expression to specific region(s) and help overcome embryonic lethality as seen with Rb KOs. Pax6-Cre, Chx10-Cre and Nestin-Cre mice were used to study the loss of Rb during retinal development (specifically in relation to Retinoblastoma tumorigenesis), while Foxg1-Cre mice targeted cortical progenitors in the embryonic telencephalon including the dentate gyrus of the hippocampus. In addition, *in utero* plasmid electroporation of pCAG-Cre and pMAP2-Cre targeted cortical progenitors and differentiated neurons respectively during embryonic development. As for the temporal control of Cre activation, tamoxifen is administered to young adult mice of Nestin-$CreER^{T2}$ and CamK-$CreER^{T2}$ lines, targeting adult neural stem and progenitor cells and post-mitotic cortical neurons, respectively. Of these two Rb inducible KO (iKO) systems, only the former was used to assess the role of Rb during neurogeneis in mid-aged (12m) and old-aged (20m) mice. Refer to text for references.

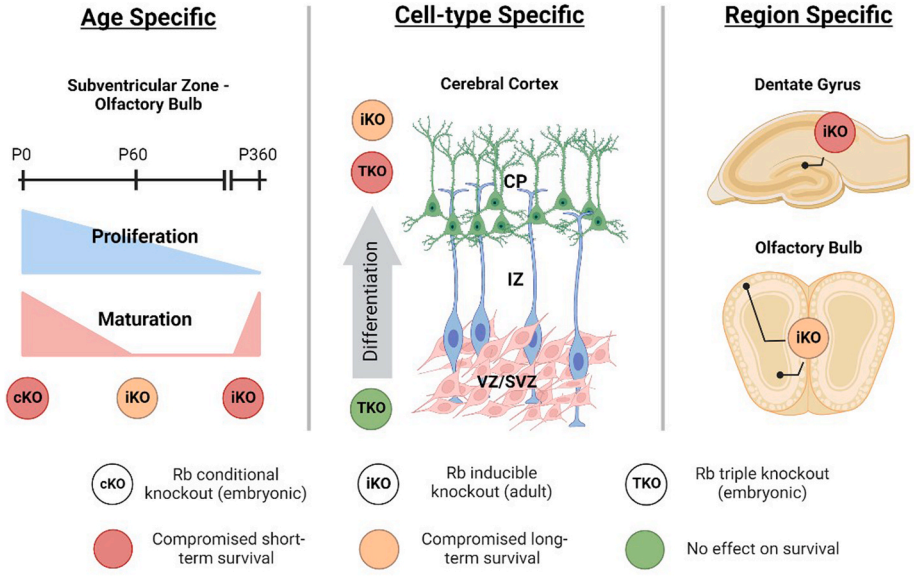

Fig. 2. The effects of Rb inactivation on neuronal survival in the telencephalon is dependent on mouse age, neural stage/cell-type and targeted brain region. *Left*: With age, the subventricular zone-olfactory bulb axis witnesses a steady decline in Rb-dependent regulation of progenitors' proliferation but a non-monotonic requirement for Rb in controlling neuronal maturation (absent at P60 but present at P0 and P360). This translates to a less compromised short-term survival in adult-born post-mitotic neurons of young-aged mice. *Middle*: In the developing cerebral cortex, the combined knockout of all three pocket proteins (Rb TKO) in VZ-SVZ progenitors does not affect downstream neuronal survival post-differentiation. In contrast, Rb TKO in differentiated cortical neurons is significantly lethal (see Fig. 3 for detail), as well as a single Rb iKO in adult differentiated cortical neurons – albeit less vulnerable to neuronal death in the short-term. *Right*: The Rb iKO (NestinCreER^{T2}) mouse model causes severe loss of immature adult-born neurons prior to maturation in the dentate gyrus inside the hippocampus; however, it does not affect maturation but only long-term survival of adult-born neurons in the olfactory bulb. P: postnatal day. VZ/SVZ: ventricular zone/subventricular zone, IZ: intermediate zone, CP: cortical plate. Refer to text for references.

committed neuroblasts into mature olfactory bulb interneurons (Naser et al., 2016). Of interest, we recently showed that in the aSVZ of mid-aged (P360) and old-aged (P600) mice, Rb becomes almost completely dispensable in regulating division of neural stem and progenitor cells; it is, however, once again required for the early maturation of migrating neuroblasts and their subsequent survival (Omais et al., 2022). Therefore, with age and in the absence of Rb, the resulting neuronal progeny is least compromised in young adult mice in contrast to embryonic and aged mice (Fig. 2). Altogether, the above findings highlight the presence of age-specific effects linked to a non-monotonic requirement played by Rb during neurogenesis.

3.2. Cell type-dependent effects

Rb inactivation by Cre plasmid electroporation targeting cortical progenitors versus differentiated neurons revealed striking cell-type specific effects, that is in pre-versus post-differentiation stages during development (Oshikawa et al., 2013). To rule out compensatory effects of the other two members of the Rb family of pocket proteins, p107 and p130, the same study generated a triple KO of all Rb family members (Rb-TKO) in targeted cells. While Rb-TKO cortical progenitors could mature and survive well and even divide following differentiation, the triggered Rb-TKO in differentiated neurons quickly prompted apoptosis (Oshikawa et al., 2013). This is due to the fact that differentiating neurons derived from Rb-TKO progenitors are able to activate the DNA double-strand break (DSB) repair pathway, which is essential for complete cell division. Interestingly, other mouse models limit the extent of generalizing a cell type-specific effect of Rb KO, especially when age is considered as a contributing factor. For instance, unlike Rb-TKO embryonic progenitors, Rb iKO alone in activated stem and progenitor cells of the aSVZ induced severe long-term loss of adult-born neurons in the OB (1–3 months post-differentiation) (Naser et al., 2016). On the other hand, Rb iKO in adult cortical neurons compromised their survival much like their embryonic counterparts, yet this was a long-term effect in the adult cortex (by 4 weeks but not 1 week following Rb iKO) (Andrusiak et al., 2012) (Fig. 2).

3.3. Region-specific effects

In addition to age- and cell-type specific effects, we and others have shown the existence of region-specific effects following Rb iKO as seen in the two neurogenic sites of the adult brain. Using the same Rb iKO mouse model, we reported apoptotic death of neuroblasts associated with a severe decline in the number of mature neurons generated at 1 month post-Rb deletion in the hippocampal dentate gyrus (DG) but not in the OB where neuronal maturation was not affected (Naser et al., 2016; Vandenbosch et al., 2016). Moreover, we recently generated a Nestin-CreER^{T2} TKO of the Rb family of proteins in both neurogenic sites in young mice (Rb iKO-p130 iKO-p107 germline deletion). In the DG, TKO-NSCs displayed a striking exit from quiescence marked by fast activation and expansion of progenitors leading to niche depletion and massive cell death after 2 months. A comparable phenotype was detected inside the SVZ of the same TKO mice and manifested by a significant reduction in niche density but without NSCs' depletion at least after 2 months (Fong et al., 2022). In fact, the two neurogenic zones are well known to have distinct developmental dynamics as well as circuitry requirements. For instance, Nestin is expressed in both quiescent and activated stem cells and their progeny inside the DG's subgranular zone, whereas it only labels activated stem cells and their derived lineages in the SVZ. Furthermore, newborn neurons are glutamatergic in the DG but GABAergic in the OB, and there exists distinct temporal requirements for these neurons to receive inputs from the local circuit (early in the DG versus late in the OB) (Lepousez et al., 2015). Therefore, both cell autonomous and non-autonomous functions of Rb may be involved in the lineage-specific effects described above (Fig. 2).

Finally, the benefits and limitations of Cre-driven KO mouse models has been previously discussed (Enikolopov et al., 2015). However, it is noteworthy that in most KO models, Rb loss was commonly found to disrupt pre-differentiation stages of neurogenesis, especially during embryogenesis. Hence, a carryover KO burden could have contributed to the subsequent neuronal vulnerability to cell death observed at later stages. Induced Rb deletion aimed at post-differentiation stages, e.g. using pMAP2-Cre electroporation and CamKCreER^{T2} mice, is therefore ideal to avoid this confounder and likely to provide the most reliable assessment of Rb's involvement in postmitotic maintenance.

4. Rb inactivation and incomplete cell cycle re-entry: modeling neurodegeneration?

Rb deficiency may also be modeled to mimic the pathogenesis of

neurodegenerative diseases, known to involve Cell Cycle Re-Entry (CCE) in post-mitotic neurons (Arendt, 2012; Omais et al., 2018). For instance, the two-hit hypothesis of Alzheimer's disease (AD) etiology – which states that "both oxidative stress and mitogenic dysregulation are necessary and sufficient to cause the disease" – is extensively based on a temporary death resilience of Rb-deficient neurons as they undergo CCE (known as a 'mitotic steady state') (Zhu et al., 2007). A recent *in vitro* study further links CCE following Rb inhibition (by lipofection of SV40 large T antigen, TAg) with AD-like features such as delayed cell death, hyperploidy and synaptic dysfunction, although TAg also inhibits p53 and likely suppresses its downstream apoptotic signaling as well (Barrio-Alonso et al., 2018). As for the mouse models discussed above showing a critical requirement of Rb in maintaining neuronal survival, a unifying theme points to the initial escape of Rb-deficient postmitotic neurons from G0 quiescence followed by CCE (Andrusiak et al., 2013). However, Rb inactivation can only trigger an *incomplete* CCE as neurons succumb to cell death by the G2/M checkpoint, as summarized next for embryonic and adult-born neurons (Fig. 3).

First, following Cre plasmid electroporation in differentiated neurons of embryonic cortical explants, DNA content analysis and EdU labeling showed that Rb-TKO neurons attempt CCE but increasingly undergo apoptosis while transitioning from S to G2 phases. Remarkably, even in a p107-p130 double KO background, a single functional copy of Rb is sufficient to suppress CCE and later cell death seen in Rb-TKO neurons (Oshikawa et al., 2017). Of interest, the additional activation of Chk1/Atm pathway by Camptothecin – known to trigger DNA damage and subsequent apoptosis – allows Rb-TKO neurons to complete CCE and divide successfully thus rescuing them from cell death, just like Rb-TKO in cortical progenitors described earlier (Fig. 3). Furthermore, Chk1 pathway suppression can possibly be what distinguishes Rb-TKO effects in cortical compared with retinal lineages (Oshikawa et al., 2017). Second, in Rb-deficient adult-born OB neurons, immunostaining for endogenous cell cycle markers showed an increase in expression of PCNA (marker of interphase and mitosis) and Ki67 (S-G2-M) but not pH3 (G2-M) (Omais et al., 2022). Since this model triggered Rb deletion before differentiation, it was also essential to distinguish CCE in postmitotic neurons from a delayed cell cycle exit scenario upon differentiation. This was indeed established by coupling the Rb iKO model with BrdU birthdating, which labels a subset of postmitotic adult-born OB neurons (Omais et al., 2022).

Here again, the limitations of Rb KO models are worth highlighting. For instance, Tamoxifen is used to induce Cre recombinase activation in Rb iKO systems (Naser et al., 2016; Vandenbosch et al., 2016; Andrusiak et al., 2012; Omais et al., 2019, 2022). However, out of 1760 candidate compounds, Tamoxifen was the only drug identified with sufficient affinity to Cdk5 as to disrupt its interaction with p25 and inhibit downstream tau phosphorylation – a defining signature of AD pathophysiology (Corbel et al., 2015). This is critical, as it opposes the mechanism by which Rb inactivation likely causes AD-like neurodegenerative phenotypes along the same pathway: in rat primary cortical neurons, Rb expression is downregulated by the overexpression of miR-26b (seen in AD patients), leading to CCE and an increased kinase activity of Cdk5 by p25/p35 which then hyperphosphorylates Tau (Absalon et al., 2013). As such, the 'anti-neurodegenerative' role of Tamoxifen might partially explain why, 210- and 480-days following Rb iKO in adult-born neurons of the aged OB, we could not detect a significant tau hyperphosphorylation signal in these Rb-deficient neurons (Omais et al., 2022) (Fig. 3). Equally important, the link between Rb inactivation and tau hyperphosphorylation might be species-specific. Indeed, in the 3xTg-AD transgenic mouse model of AD, hyper-phosphorylated Rb (ppRb) was correlated with late but not early markers of tau hyperphosphorylation. While in human AD patients, ppRb co-localized with both early and late markers of tau alterations (Hradek et al., 2015).

In summary, CCE is a primary candidate mechanism underlying the increased vulnerability of Rb-deficient neurons to cell death as seen in KO/iKO models and some neurodegenerative diseases. However, Tamoxifen-inducible KOs may limit the extent to which CCE due to Rb inactivation can mimic the pathological course of specific disorders such as Alzheimer's disease.

5. Conclusion

We reviewed here the current knowledge of the role of Rb in maintaining neuronal survival as derived from various Rb KO mouse models. We highlighted the experimental and functional advantages associated with each model used as well as the contextual limitations, which nonetheless reflect the dynamic and specific roles that Rb can play in terms of developmental stage, cell type and brain region. We finally discussed the feasibility and extent of considering CCE associated with Rb inactivation as a potential link to neurodegeneration in mice, AD pathology in specific.

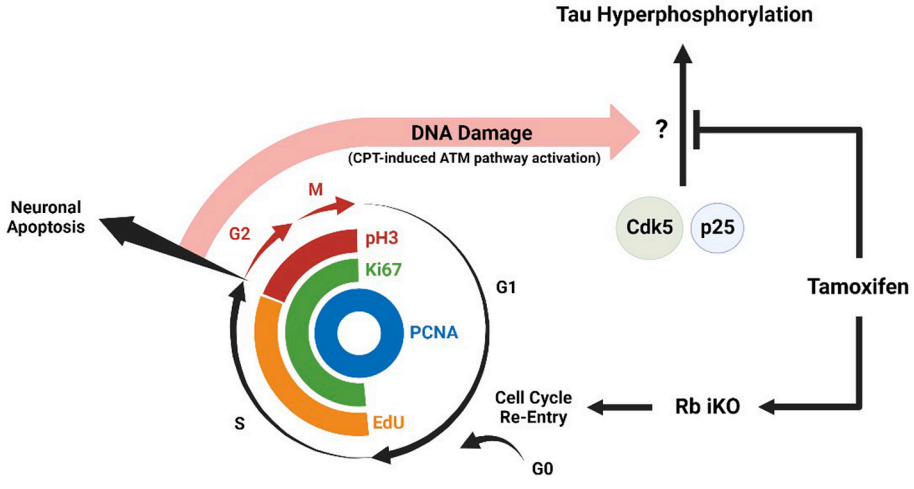

Fig. 3. Tamoxifen triggers neuronal cell cycle re-entry (CCE) following Rb iKO in adult mice, while also potentially inhibiting neurodegenerative phenotypes due to Rb inactivation. Tamoxifen is a commonly used drug in CreER^{T2} transgenic mice to trigger gene inactivation. Similar to other Rb KO models, Rb loss in tamoxifen-inducible KO (iKO) mice causes neuronal cell cycle re-entry (CCE) in the telencephalon, where Rb-deficient neurons exit postmitotic G0 phase, re-enter G1 and progress through S-phase. Although they then succumb to neuronal apoptosis, they might be able to resume cycling through G2 and M phases as seen with the additional activation of Chk1-ATM pathway by Camptothecin (marked by H2A.X phosphorylation) in pMAP2-Cre Rb TKO electroporated cortical neurons. A clinical consequence of Rb inactivation is likely the p25-enhanced kinase activity of Cdk5, which induces tau hyperphosphorylation, a hallmark of Alzheimer's disease pathology. This mechanism, however, was shown to be inhibited by Tamoxifen. Endogenous cell cycle markers can be used to label neurons: PCNA (interphase + mitosis), Ki67 (S-G2-M phases) and pH3 (G2-M phases). *EdU can be used to label S-phase when administered 2 h before animal sacrifice. Refer to text for references.

CRediT authorship contribution statement

Saad Omais: Conceptualization, Visualization, Data curation, Formal analysis, Writing - original draft. **Yara E. El Atie:** Data curation, Methodology. **Noël Ghanem:** Conceptualization, Supervision, Project administration, Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crneur.2023.100074.

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