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Títol del treball:

Genotyping of a *Dyrk1a* conditional mutant mice

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ABSTRACT

DYRK1A is a protein kinase involved in multiple cellular functions through its interaction with different substrates, such as transcription factors, splicing machinery components, RNA polymerase II, chromatin regulators, Cyclin D, Caspase-9 or RNF169. This kinase regulates brain growth and several evidences indicate that DYRK1A may control neuron production, physiological apoptosis in differentiating neurons and neuron differentiation. Previous experiments of the group showed that *Dyrk1a* null mutant mice (*Dyrk1a*$^{-/-}$) are embryonic lethal. *Dyrk1a*$^{-/-}$ embryos showed developmental delay, growth retardation and defects in the maturation of the neural tube. These mutant embryos die at the beginning of neurogenesis, around embryonic day (E) 11.5, thus precluding the use of this model to study the role of DYRK1A in brain development. Mice with only a functional *Dyrk1a* allele (*Dyrk1a*$^{+/-}$) were viable but also showed affectations in the nervous system. They had a small brain and thinner retinas that correlates with an enhanced developmental apoptosis and defects in the division mode of the neural progenitors. *Dyrk1a*$^{+/-}$ mice are a phenocopy of patients with *DYRK1A* haploinsufficiency syndrome, which is caused by loss-of-function mutations in the *DYRK1A* gene. Patients with this syndrome present microcephaly, intellectual disability, growth retardation, speech problems, developmental delay and behavioural problems. Human *DYRK1A* gene is in chromosome 21 and studies in transgenic mouse models carrying 3 copies of *Dyrk1a* have shown that the overexpression of DYRK1A causes a reduction in neuronal cell production in the developing cerebral cortex that are similar to those reported in Down syndrome brains. Therefore, *Dyrk1a*$^{+/-}$ mice and *Dyrk1a* transgenic mice are good models to study the effect of *DYRK1A* dosage imbalance in human syndromes. However, little is known about the different functions/activities regulated by DYRK1A during neurogenesis. In this work we present the crossings and genotyping in order to generate a conditional *Dyrk1a* knockout mouse. In this data we provide how to perform a mutant mouse with *Dyrk1a* gene truncated in all neural progenitors by following a specific mating strategy understanding how alleles are segregated and a mice selection with a genotyping analysis. This deletion takes place before the onset of neurogenesis, allowing the study of the effect of a *Dyrk1a* null mutation in neural progenitor cells.

RESUMEN

DYRK1A es una proteína quinasa involucrada en múltiples funciones celulares a través de su interacción con diferentes sustratos, como por ejemplo factores de transcripción, componentes de la maquinaria de splicing, RNA polimerasa II, reguladores de cromatina, Ciclina D, Caspasa-9 o RNF169. Esta quinasa regula el crecimiento cerebral y varias evidencias indican que DYRK1A controla la formación de neuronas, la apoptosis en neuronas en diferenciación y la diferenciación neuronal. Experimentos previos del equipo han observado que el ratón mutante nulo para *Dyrk1a* (*Dyrk1a*$^{-/-}$) es letal embrionario. Los embriones *Dyrk1a*$^{-/-}$ presentaban un retraso en el desarrollo y en el crecimiento, i defectos en la maduración del tubo neural. Estos embriones mutantes morían al comenzar la neurogénesis, alrededor de los 11.5 días embrionarios (E), excluyendo así la utilización de este modelo para estudiar la función de DYRK1A en la formación del cerebro. Los ratones con una única copia funcional del alelo *Dyrk1a* (*Dyrk1a*$^{+/-}$) eran viables, pero también mostraban afectaciones en el sistema nervioso. Presentaban un cerebro pequeño y unes retinas finas, relacionado con una mayor muerte apoptótica durante el desarrollo y con defectos en el patrón de división de los progenitores neurales. Los ratones *Dyrk1a*$^{+/-}$ son una fenocopia de los pacientes con síndrome de haploinsuficiencia para *DYRK1A*, el cual es causado por una mutación de perdida de función en el gen *DYRK1A*. Los pacientes con este síndrome presentan microcefalia, discapacidad intelectual, retraso en el crecimiento y en el desarrollo, problemas de comunicación y problemas de conducta. El gen *DYRK1A* humano se encuentra en el cromosoma 21 y estudios con un ratón transgénico con 3 copias de *Dyrk1a* han demostrado que una sobreexpresión de DYRK1A provoca una reducción en la producción de células neuronales durante el desarrollo del córtex, que es similar a la descrita en cerebros con síndrome de Down. Por lo tanto, los ratones *Dyrk1a*$^{+/-}$ y los ratones transgénicos para *Dyrk1a* son buenos modelos para estudiar los efectos del desequilibrio en la dosis de *DYRK1A* en síndromes humanos. Sin embrago, se sabe muy poco sobre las diferentes funciones/actividades que son reguladas por DYRK1A durante la neurogénesis. En este proyecto presentamos los cruces y los genotipados necesarios para generar un ratón mutante knockout condicional para *Dyrk1a*. Estos datos proporcionan la manera de crear un ratón mutante con el gen *Dyrk1a* truncado en todos los progenitores neurales siguiendo una estrategia de apareamientos específica comprendiendo como se segregan los alelos de interés y una selección de ratones con un análisis del genotipando. Esta deleción tiene lugar antes de que comience la neurogénesis, permitiendo el estudio del efecto de una mutación nula para *Dyrk1a* en células progenitoras neurales.

RESUM

DYRK1A es una proteïna quinasa involucrada en multiples funcions cel·lulars a través de la interacció amb diferents substrats, com per exemple factors de transcripció, components de la maquinària de splicing, RNA polimerasa II, reguladors de cromatina, Ciclina D, Caspasa-9 o RNF169. Aquesta quinasa regula el creixement cerebral i vàries evidències, indiquen que DYRK1A controla la formació de neurones, l'apoptosi en neurones en diferenciació i la diferenciació neuronal. Experiments previs del grup han observat que el ratolí mutant nul per *Dyrk1a* (*Dyrk1a*$^{-/-}$) és letal embrionari. Els embrions *Dyrk1a*$^{-/-}$ presentaven retard en el desenvolupament i en el creixement, i defectes en la maduració del tub neural. Aquests embrions mutants morien en començar la neurogènesi, al voltant dels 11.5 dies embrionaris (E), excloent així la utilització d'aquest model per estudiar la funció de DYRK1A en la formació del cervell. Els ratolins amb una única còpia funcional de l'al·lel *Dyrk1a* (*Dyrk1a*$^{+/-}$) eren viables, però també mostraven afectacions en el sistema nerviós. Presentaven un cervell petit i unes retines primes, relacionat amb una major mort apoptòtica durant el desenvolupament i amb defectes en el patró de divisió dels progenitors neurals. Els ratolins *Dyrk1a*$^{+/-}$ són una fenocòpia dels pacients amb síndrome d'haploinsuficiència per *DYRK1A*, el qual es causat per una mutació de pèrdua de funció en el gen *DYRK1A*. Els pacients amb aquest síndrome presenten microcefàlia, discapacitat intel·lectual, retard en el creixement i en el desenvolupament, problemes de comunicació i problemes de conducta. El gen *DYRK1A* humà es troba en el cromosoma 21 i estudis amb un ratolí transgènic amb 3 còpies de *Dyrk1a* han demostrat que una sobreexpressió de DYRK1A provoca una reducció en la producció de cèl·lules neuronals durant el desenvolupament del còrtex, que és similar a la descrita en cervells amb síndrome de Down. Per tant, els ratolins *Dyrk1a*$^{+/-}$ i els ratolins transgènics per Dyrk1a són bons models per estudiar els efectes del desequilibri en la dosi de *DYRK1A* en síndromes humans. Malgrat això, se sap poc sobre les diferents funcions/activitats que són regulades per DYRK1A durant la neurogènesi. En aquest projecte presentem els encreuaments i els genotipatges necessaris per generar un ratolí mutant knockout condicional per *Dyrk1a*. Aquestes dades proporcionen la manera de crear un ratolí mutant amb el gen *Dyrk1a* truncat en tots els progenitors neurals seguint una estratègia específica d'aparellaments entenent com són segregats els al·lels d'interès i una selecció de ratolins amb un anàlisi del genotipatge. Aquesta deleció té lloc abans de que comenci la neurogènesi, permetent l'estudi de l'efecte d'una mutació nul·la per *Dyrk1A* en cèl·lules progenitores neurals.

INTRODUCCION

DYRK1A (dual-specificity tyrosine phosphorylation regulated kinase 1A) belongs to the DYRK family of protein kinases. These kinases autophosphorylate a particular tyrosine residue (Tyr312 in DYRK1A) in the catalytic domain for self-activation and phosphorylate serine/threonine residues on exogenous substrates (Becker & Joost, 1999).

In addition to the catalytic domain, all DYRK proteins present in their amino-terminus a highly conserved domain (DH domain). DYRK1A presents two nuclear localization signals, a PEST domain that is possible involved in the degradation of the protein and in its carboxyl-terminus a domain rich in histidines, important for interacting with other proteins, and a region rich in serine and threonine residues with an unknown function (Becker et al., 1998; Figure 1). DYRK1A localizes in the cytoplasm and in the nucleus and regulates cytoplasmic and nuclear activities through its interaction with several substrates (Arbones et al., 2018).

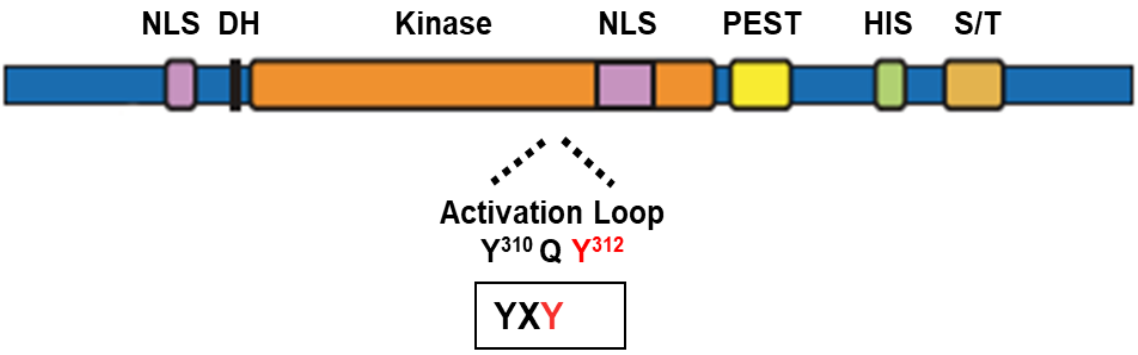

Figure 1. **DRK1A domain.** Scheme of DYRK1A protein domain structure. NLS: nuclear localization signal. DH: DYRK homology domain. PEST: domain enriched in proline, glutamic acid, serine, and threonine residues. His: histidine repeats. S/T: serine and threonine-rich region.

As shown in Figure 2, DYRK1A is involved in different cellular and molecular processes. It regulates gene expression by interacting with splicing machinery components, chromatin regulators, transcription factors and RNA polymerase II (Arbones et al., 2018). DYRK1A regulates cell cycle progression by controlling the protein levels of the G1 to S phase transition regulators Cyclin D (Najas et al., 2015) and p27kip1 (Hämmerle et al., 2011) and apoptosis by phosphorylating Caspase-9 in a threonine residue that prevents its cleavage and the subsequent activation of the intrinsic cell death pathway (Laguna et al., 2008). DYRK1A is also involved in DNA damage repair by phosphorylating RNF169 (Roewenstrunk et al., 2019; Menon et al., 2019) and in vesicle recycling by interacting with proteins (Synaptojanin 1, Dynamin 1 and Amphiphysin 1) of the endocytic protein complex machinery (Tejedor & Hämmerle, 2011).

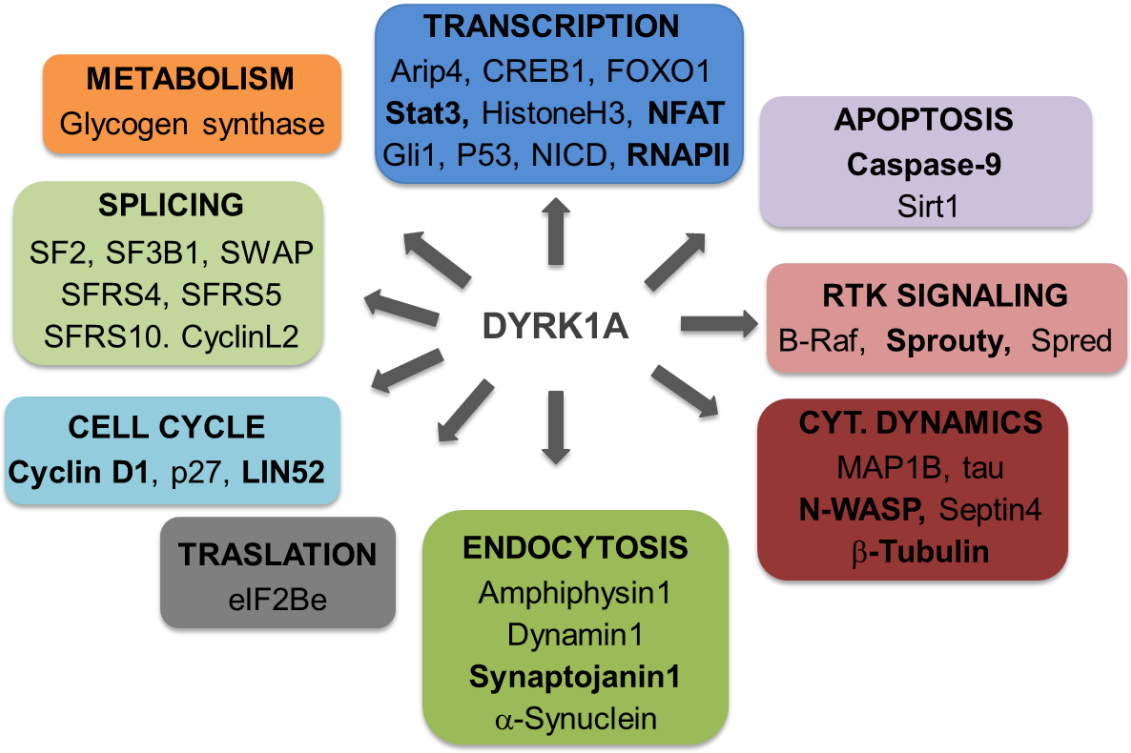

Figure 2. **Functions regulated by DYRK1A.** Representation of DYRK1A functions with different substrates and protein interactions.

Mouse DYRK1A is expressed in most adult tissues (Tejedor & Hämmerle, 2011) and is highly expressed in the brain. DYRK1A in the nervous system is expressed mainly in the cytoplasm in progenitors and in differentiating and mature neurons (Arbones et al., 2018; Figure 3).

Minibrain (mnb) was the first DYRK1A protein discovered in *Drosophila melanogaster* and it was proposed as a larval neurogenesis regulator. Less expression of *Mnb* implied a reduction of central brain hemispheres and adult optics lobes volumes due to neurogenesis defects (Tejedor et al., 1995).

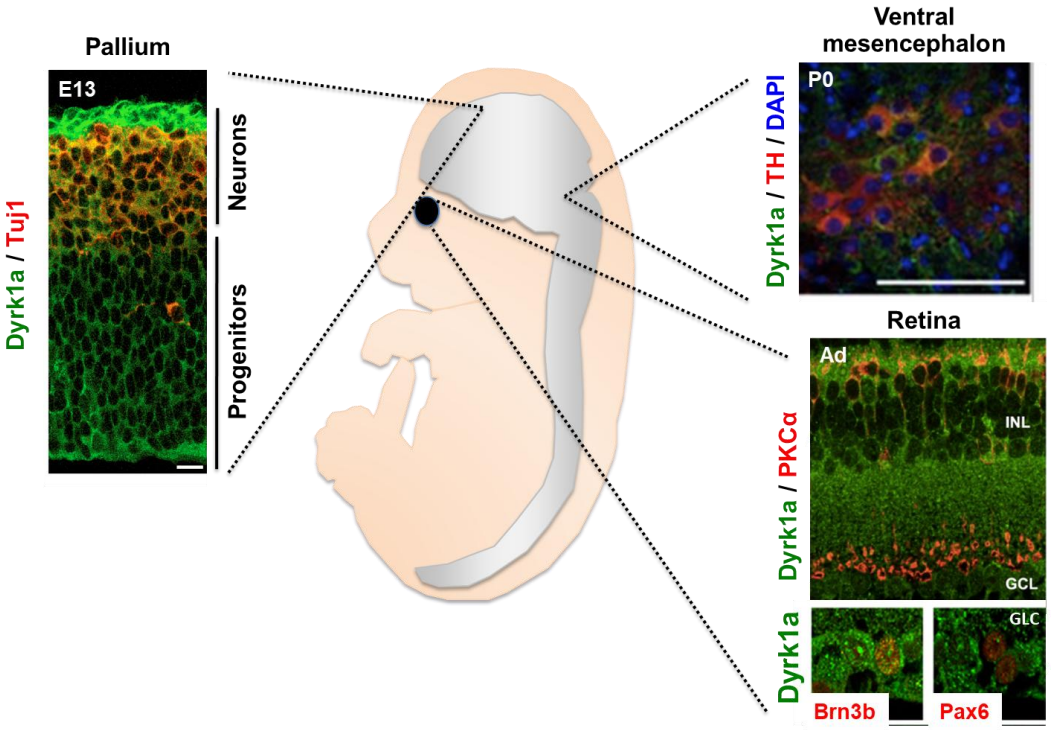

Figure 3. **DYRK1A expression in nervous tissues.** Representation of mouse DYRK1A expression in the dorsal telencephalon (pallium) of a E13 embryo, ventral mesencephalon of a new-born mouse (P0) and in the adult (Ad) retina. Sections were immunostained with a DYRK1A antibody. DAPI: nuclear marker. Pax6 and Brn3b are ganglion cell (GCL) markers: PKCα: interneuron marker. TH: dopaminergic marker. Tuj1: pan-neuron marker.

Loss-of-function mutations in the mouse *Dyrk1a* gene (*Dyrk1a*$^{+/-}$ mice) cause phenotype that is similar to mnb flies. *Dyrk1a*$^{+/-}$ heterozygous mutant with one copy of the targeted allele presented a significant reduction of the size of the brain (Figure 4) and growth defects. These mutants showed defects in neuron cellularity in different parts of the retina and the brains including the cerebral cortex. These defects were in part due to the role of DYRK1A regulating neurogenesis and Caspase-9 developmental cell death (Fotaki et al., 2002, Laguna et al., 2008, Guedj et al., 2012 and Najas et al., 2015). The phenotype of *mnb* flies and *Dyrk1a*$^{+/-}$ indicated that DYRK1A kinase plays a key role in neuronal development that is conserved across evolution (Tejedor & Hämmerle, 2011).

Dyrk1a$^{-/-}$ null mutant mouse embryos, with any copy of *Dyrk1a* gene functional, showed developmental delay, growth retardation and a significant reduction of body size (almost have of the size of their wild-type littermates). Null mutants were embryonic lethal showing that this kinase has an essential role. *Dyrk1a*$^{-/-}$ embryos died from E10.5 to E12.5 and presented defects in the maturation of the nervous system and a significant decrease in the number of postmitotic neural cells (Fotaki et al., 2002).

The human *DYRK1A* gene is located in chromosome 21, in the Down Syndrome (DS) critical region in 21q22.2 (Becker & Joost, 1999; Arbones et al., 2018). Different studies performed in transgenic mouse models that overexpressed DYRK1A indicated that overexpression of the gene contribute to the intellectual disability and other neurological deficits associated to Down syndrome (Arbones et al., 2019). Contrary to what has been shown in trisomic mouse model of this syndrome, transgenic mice with 3 copies of *Dyrk1a* present macrocephaly (Figure 4). However, the study of the development of the cerebral cortex in trisomic mice (around 60 genes in trisomy including *Dyrk1a*) and in mice with only *Dyrk1a* in trisomy showed that the overexpression of DYRK1A causes a deficit in neurons of the cerebral cortex that is due to alterations in the levels of the cell cycle regulator Cyclin D1 (Najas et al., 2015). In addition, multiple evidences indicate that the overexpression DYRK1A contributes to the Alzheimer's like disease that suffer most of Down syndrome patients older than 35 years. Thus, DYRK1A has been proposed to be an excellent therapeutic target to ameliorate the cognitive deficits in people with this syndrome and different DYRK inhibitors have been generated and tested for this purpose (Arbones et al., 2019).

Chromosomal mutations affecting DYRK1A gene (Chettouh et al., 1995) and point mutations within this gene cause a syndrome, named *DYRK1A* haploinsufficiency syndrome or MRD7 (mental retardation dominant 7). Patients with this syndrome present microcephaly, intellectual disability, growth retardation, speech problems, developmental delay and behavioural problems. The *Dyrk1a*$^{+/-}$ mouse model presents most of the characteristic phenotypes of *DYRK1A* haploinsufficiency syndrome and therefore is a good model to study the pathology associated with this syndrome.

Figure 4. Brains with different *Dyrk1a* dosage. Coronal mouse brain sections showing the size reduction of the brain directly correlated with *Dyrk1a* dosage. *Dyrk1a*$^{+/-}$: monosomic mouse brain for *Dyrk1a*. Wild-type: euploid mouse brain for *Dyrk1a*. mBACTg*Dyrk1a*: trisomic mouse brain for *Dyrk1a*. D3V: dorsal 3rd ventricle. hp: hippocampus. LV: lateral ventricle.

As mention before, DYRK1A is expressed in neural progenitors before neurogenesis, but the early lethality of the *Dyrk1a*$^{-/-}$ null mutant precludes the use of this mutant model to assess the effect of a *Dyrk1a* null mutation in neurogenesis. To circumvent this problem the laboratory has made a conditional knockout (CKO) mouse model.

CKO mice allow the study of a protein by mutating the target gene in a tissue controlled manner using the loxP/Cre-recombinase system (Liu, Jenkins & Copeland, 2003).

The *Dyrk1a* CKO mouse model was made by crossing two mutant lines. The *NesCre8* transgenic line (Petersen et al., 2002) and the *Dyrk1a*$^{f/f}$ line (Thompson et al., 2015).

The targeted *Dyrk1a* floxed allele (see Figure 6) was constructed as described previously (Liu, Jenkins & Copeland, 2003). This targeted *Dyrk1a* allele was inserted by homologous recombination in mouse embryonic stem cells. These cells were injected into blastocyst to generate *Dyrk1a*$^{f/f}$ mice (Thompson et al., 2015).

The *NesCre8* transgene consists in the *Nestin* enhancer fused to the Cre-recombinase gene. Nestin is a protein expressed in all neural progenitors. Studies using a reporter mouse line have shown that Cre-recombinase in the *NesCre8* transgenic line is active in neural progenitor cells and somites by E8.5 and that the deletion of the floxed allele is complete by E11.5 (Petersen et al., 2002). Thus, it is expected that the deletion of the *Dyrk1a* floxed sequences in neural progenitors happens before the onset of neurogenesis.

OBJECTIVES

The main objective of this work was to achieve a CKO mutant mouse to be used in the future to assess the function of DYRK1A in neurogenesis. The two objectives of this work were:

- Design the mating strategy to generate the CKO *Dyrk1a* mouse model from the parental lines *NesCre8* and *Dyrk1a*$^{f/f}$.

- Genotype the progeny of the different crosses needed for the generation of the CKO *Dyrk1a* model.

METHODS

Mice

Dyrk1a conditional knockout mice (*Dyrk1a*$^{\Delta/\Delta}$) were generated by the mating strategy represented in Figure 5. NesCre8 transgenic mice (*NesCre8*$^{+}$) were constructed integrating a plasmid that contained the Cre-recombinase gene next to the *Nestin* promoter randomly into the mouse genome and a subsequent line selection of mouse candidates (Petersen et al. 2002).

Maintenance of the selected transgenic *NesCre8* line was done by mating *NesCre8*$^{+}$ males with wild-type (*NesCre8*$^{-}$) females (cross 1) in order to ensure that the transgene was always in heterozygosis to avoid possible errors with the random integration. Only *NesCre8*$^{+}$ males were selected for later matings, reducing significantly the number of mice used obtaining enough information to make clear conclusions applying the three R's principle (Russell & Burch, 1959).

Dyrk1a$^{f/f}$ were homozygous mice for *Dyrk1a* gene with loxP sites and were performed with the construction of a targeting vector with loxP sites flanking exons 5 and 6 of the targeted *Dyrk1a* allele as represented in Figure 6. After stem cell electroporation of the vector, clones with the targeted allele introduced by homologous recombination were screened by PCR and selected to finally have a *Dyrk1a* floxed allele in homozygosity (Thompson et al., 2015).

Maintenance of the *Dyrk1a*$^{f/f}$ line was done by mating *Dyrk1a*$^{f/f}$ females with *Dyrk1a*$^{f/f}$ males (cross 1) to avoid unnecessary genotyping and reducing the number of mice used without the performing of the *Dyrk1a*$^{f/f}$ mice every time.

The crossing of *Dyrk1a*$^{f/f}$ females with *NesCre8*$^{+}$ males generated mice with only one floxed *Dyrk1a* allele (*Dyrk1a*$^{f/wt}$) (cross 2).

Progeny from cross 2 was genotyped and only mice that expressed the *NesCre8* transgene were selected (*Dyrk1a*$^{\Delta/wt}$) and backcrossed with *Dyrk1a*$^{f/f}$ (cross 3) in order to obtain mice in which one (*Dyrk1a*$^{\Delta/wt}$) or both *Dyrk1a* alleles (*Dyrk1a*$^{\Delta/\Delta}$) have been deleted by Cre-recombinase expression. The littermates of this cross that don't expressed Cre-recombinase (*Dyrk1a*$^{f/wt}$ and *Dyrk1a*$^{f/f}$) can be used as controls (wild-type phenotype) for future experiments.

Ethical considerations

All procedures were approved by the institutional animal care committee from CSIC-IBMB. As described above we tried to reduce in each of the experiments the number of animals per group using specific crosses. In addition, male mice were used because the collection of semen allows us to obtain many more samples from a single specimen.

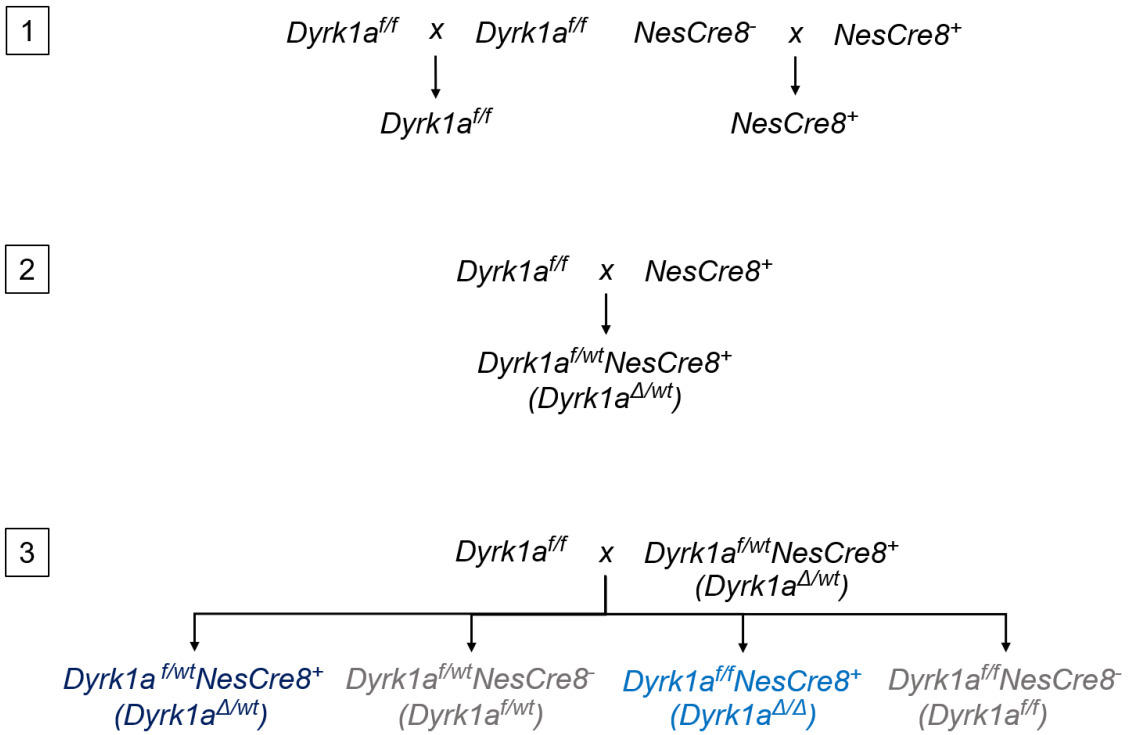

Figure 5. Representation of mice mating strategy. (1) The first two crosses were for maintenance of the *Dyrk1a*$^{f/f}$ line and NesCre8 line. (2) The second cross was for creating mice with *Dyrk1a* floxed allele in heterozygosity and *NesCre8* transgene. (3) The third cross was to finally have *Dyrk1a* CKO mice (*Dyrk1a*$^{\Delta/\Delta}$), with a *Dyrk1a* deletion in both alleles, conditional heterozygous mutants (*Dyrk1a*$^{\Delta/wt}$: *Dyrk1a* deletion in one allele) and two genotypes that were *NesCre8*$^{-}$ (*Dyrk1a*$^{f/wt}$; *Dyrk1a*$^{f/f}$) that can be used as controls for phenotyping the mutants.

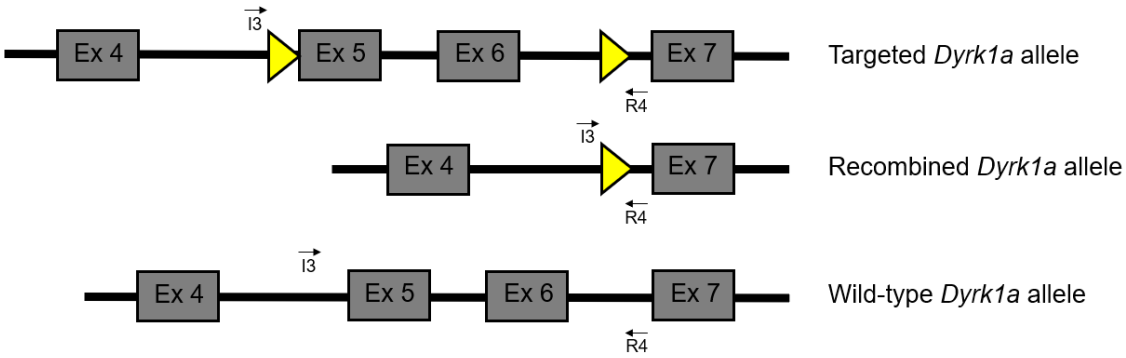

Figure 6. Different *Dyrk1a* alleles studied. *Dyrk1a* floxed allele with exons 5 and 6 flanked by loxP sites (▷). This allows Cre-recombinase mediated deletion of *Dryk1a* sequences resulting a null allele (recombined *Dyrk1a* allele). Wild-type allele had all exons for the correct expression of Dyrk1a. Primers for assessing deletion of the *Dyrk1a* floxed sequence are represented as I3 and R4.

NesCre8 genotyping

Adult male mice generated by mating *NesCre8*$^{+}$ with wild-type mice (cross 1) and mice generated by mating *Dyrk1a*$^{f/f}$ with *NesCre8*$^{+}$ mice (cross 2) were genotyped by PCR analysis using tail genomic DNA to asses for the presence of the *NesCre8* transgene in heterozygosity (*NesCre8*$^{+}$). For the progeny of cross 3, DNA samples were obtained from the non-nervous embryonic structures.

DNA samples were prepared by incubating the tissue sample at 95ºC for 30 min with 70 µl of Hotshot reagent (25 mM NaOH, 0,2 mM EDTA, ddH_2O, pH 12). Then 70 µl of neutralising reagent (40 mM Tris-HCl, ddH_2O, pH 5) were added to neutralize the basic solution.

1 µl of the DNA preparation was amplified by PCR with the program described in Table 1. The pair of primers used to amplify the *NesCre8* transgene and the primers used as positive PCR controls are shown in Table 2.

A volume of 10 µl of each PCR product was analysed with a 2% agarose gel containing with 2 µl of RedSafe. To visualize the PCR products, were added 5 µl of loading buffer (0,5 M EDTA, glycerol, orange G (Sigma 03756), H_2O) in each sample. Electrophoresis was run in TBE (TBE 10X, ddH_2O) in a constant current of 120 V during 40 min.

Dyrk1a floxed genotyping

Progeny resulting by mating *Dyrk1a*$^{f/f}$ with *Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$ (cross 3) were genotyped by PCR analysis of genomic DNA prepared from limb samples to check for the presence of the wild-type *Dyrk1a* allele and the target *Dyrk1a* allele with loxP sites in heterozygosis or homozygosis (*Dyrk1a*$^{f/wt}$ or *Dyrk1a*$^{f/f}$).

Extraction of DNA was performed as mentioned above by adding 100 µl of Hotshot and 100 µl of neutralising reagent to each tissue sample.

The PCR program used to amplify 1 µl of each DNA extraction is shown in Table 1. The sequences of the pair of primers used for PCR amplification of the target allele are described in Table 2.

To detect the *NesCre8* allele, the analysis of the PCR products was done by electrophoresis in 1,5% agarose gels instead of 2% agarose.

Dyrk1a recombined genotyping

Mice embryos resulting from cross 3 (*Dyrk1a*$^{f/f}$ x *Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$) were also genotyped to check for the presence of the Cre-recombinase transgene and the *Dyrk1a* floxed gene. Genotyping was done by PCR analysis using DNA extractions from nervous tissue samples.

DNA samples were prepared as mentioned previously by incubating the tissue in 50 µl of Hotshot reagent and neutralizing the solution by adding 70 µl of neutralising reagent.

Again 1 µl of each reaction was amplified with the PCR program shown in Table 1. The pair of primers used to amplify the recombined allele (see Figure 6) is described in Table 2.

Analysis of each reaction was separating PCR products by electrophoresis in an agarose gel with the same procedure as in *Dyrk1a* floxed genotyping.

Table 1. PCR programs for each genotyping amplification procedure.

Genotyping	Temperature (ºC)	Time	Cycles
NesCre8	95	2 min	1
	95	30 s	35
	51	30 s	
	72	30 s	
	72	10 min	1
	15	∞	1
Dyrk1a flox	95	2 min	1
	95	30 s	35
	58	30 s	
	72	45 s	
	72	10 min	1
	15	∞	1
Dyrk1a recombined	95	2 min	1
	95	30 s	35
	58	30 s	
	72	50 s	
	72	10 min	1
	15	∞	1

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Name	Direction	Sequence (5'-3')	Genotyping	Annealing temperature (ºC)	Amplicon size (bp)
CreF	Forward	GCGGTCTGGCAGTAAAAACTATC	*NesCre8*	51	228
CreR	Reverse	CTAGGCCACGAATTGAAAGATCT			
CtrlR	Forward	CTAGGCCACGAATTGAAAGATCT			320
CtrlF	Reverse	GTAGGTGGAAATTCTAGCATCATCC			
I3	Forward	ATTACCTGGAGAAGAGGGCAAG	*Dyrk1a* recombined	58	750
			Dyrk1a floxed		594 and 500
E4	Reverse	TTCTTATGACTGGAATCGTCCC	*Dyrk1a* floxed		594 and 500
R4	Reverse	CACCGGCTTTGATGAATGTA	*Dyrk1a* recombined		750

RESULTS

To generate a mutant mouse with conditional inactivation of *Dyrk1a* we started mating mice with *Dyrk1a* alleles floxed with mice with the *NesCre8* transgene and subsequent specific crosses; to finally have a CKO mutant mice with *Dyrk1a* recombined allele mediated by Cre-recombined expression. We analysed the genotyping of mice for mating and the CKO mutant mice candidates for future studies.

NesCre8 genotyping

Littermates of *NesCre8* line (cross 1) were genotyped for checking for the two possible genotypes: *NesCre8*$^{+}$ or *NesCre8*$^{-}$.

NesCre8^{+} male mice were selected for crossing with wild-type mice for the maintenance of the transgenic line or were crossed with *Dyrk1a*$^{f/f}$ females to generate *Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$ mice (cross 2).

The expected PCR bands are shown in Figure 8 (A). *NesCre8*$^{+}$ mice showed a band of 228 bp corresponding to the amplification of the *NesCre8* transgene with primers CreF and CreR and a band of 320 bp that correspond to the amplification of an un-related locus with primers CtrlR and CtrlF (see Table 2). Ctrl primers were used as a PCR positive control. *NesCre8*$^{-}$ mice showed only the control amplicon band. Results interpretation of *NesCre8* genotyping is represented in Table 3.

Table 3. *NesCre8* genotyping results.

Mouse	*NesCre8*	Genotype

Nes 13.1	+	*NesCre8*$^{+}$
Nes 13.2	+	*NesCre8*$^{+}$
Nes 13.3	-	*NesCre8*$^{-}$
Nes 13.4	-	*NesCre8*$^{-}$
Nes 14.1	+	*NesCre8*$^{+}$
Nes 14.2	-	*NesCre8*$^{-}$
Nes 14.3	+	*NesCre8*$^{+}$
Nes 14.4	+	*NesCre8*$^{+}$
Nes 14.5	-	*NesCre8*$^{-}$
Nes 14.6	-	*NesCre8*$^{-}$
Nes 14.7	+	*NesCre8*$^{+}$
Nes 14.8	-	*NesCre8*$^{-}$
Nes 14.9	-	*NesCre8*$^{-}$
Nes 14.10	-	*NesCre8*$^{-}$
Nes 14.11	+	*NesCre8*$^{+}$

There were assessed P45 (P: postembryonic age) mice from two different litters: Nes 13 and Nes 14. Presence and absence of the corresponding band for the *NesCre8* transgene is indicated with + and – respectively.

Progeny resulting by mating *Dyrk1a*$^{f/f}$ females with *NesCre8*$^{+}$ males (cross 2) could had the two following genotypes: *Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$ or *Dyrk1a*$^{f/wt}$*NesCre8*$^{-}$.

We genotyped these adult mice to check for the presence of the *NesCre8* transgene again. The males with *Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$ genotype were used for mating with *Dyrk1a*$^{f/f}$ mice to generate conditional null mutant animals.

Genotyping analysis was as previously described with the same expected band pattern as in Figure 8. (A). Electrophoresis results of *NesCre8* genotyping are represented in Table 4.

Table 4. *NesCre8* genotyping results.

Mouse	*NesCre8*	Genotype
NesDF 22.1	+	*Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$
NesDF 22.2	-	*Dyrk1a*$^{f/wt}$*NesCre8*$^{-}$
NesDF 22.3	-	*Dyrk1a*$^{f/wt}$*NesCre8*$^{-}$

DNA samples assessed were from P30 mice of the same litter: NesDF 22. Presence and absence of the corresponding band for the *NesCre8* transgene is indicated with + and – respectively.

***NesCre8*, *Dyrk1a* floxed and *Dyrk1a* recombined genotyping**

Embryos resulting from cross 3 (*Dyrk1a*$^{f/f}$ x *Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$) were genotyped to select and classify each of the four possible genotypes shown in Figure 7.

We assess for the presence of the transgene *NesCre8*, the *Dyrk1a* floxed allele and the *Dyrk1a* recombined allele to study the three different genotypes: CKO mutant mice (*Dyrk1a*$^{f/f}$*NesCre8*$^{+}$), heterozygous conditional mutant mice (*Dyrk1a*$^{f/wt}$*NesCre8*$^{+}$) and wild-type mice (*Dyrk1a*$^{f/wt}$*NesCre8*$^{-}$*; Dyrk1a*$^{f/f}$*NesCre8*$^{-}$).

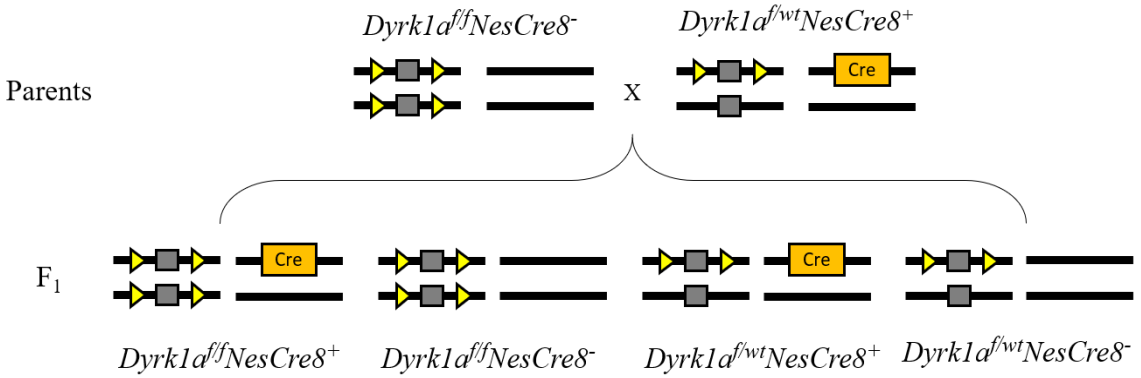

Figure 7. Allelic scheme. Representation of *Dyrk1a* floxed, *Dyrk1a* wild-type and *NesCre8* alleles resulting from cross 3.

The size of the PCR bands separated by electrophoresis for the genotypes *NesCre8*, *Dyrk1a* floxed and *Dyrk1a* recombined are represented in Figure 8.

Genotyping of the *Dyrk1a* floxed allele was done to assess mice with one or two *Dyrk1a* floxed alleles (*Dyrk1a*$^{f/wt}$ or *Dyrk1a*$^{f/f}$). *Dyrk1a*$^{f/wt}$ mice showed two bands, one of 500 bp that corresponds to the *Dyrk1a* wild-type allele and a second one of 594 bp that correspond to the *Dyrk1a* floxed allele. This difference in size is due to the inserted loxP sites (94 bp) into the targeted gene (Liu, Jenkins & Copeland, 2003). *Dyrk1a*$^{f/f}$ mice showed one unique band of 500 bp that corresponds to the two *Dyrk1a* floxed alleles.

Mice assessed in *Dyrk1a* recombined genotyping with prepared of DNA nervous tissue extractions showed presence or absence of *Dyrk1a* recombined allele. Mice carrying the *NesCre8* transgene expressed Cre-recombinase in neuronal progenitor cells thanks to the *Nestin* promoter. These *NesCre8*$^{+}$ mice display the deletion of one or two *Dyrk1a* floxed allele mediated by the Cre-loxP system leading to the presence of one or two *Dyrk1a* recombined alleles. PCR amplification of *Dyrk1a* recombined allele with primers I3 and R4 (see Figure 6) generated a band of 750 bp. The results of the PCR reactions and the genotype of the embryos are summarized in Table 5.

Table 5. *NesCre8*, *Dyrk1a* floxed and *Dyrk1a* recombined genotyping results.

Mouse	*NesCre8*	*Dyrk1a* floxed	*Dyrk1a* wt	*Dyrk1a* recombined	Genotype

ENDF 52.1	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 52.2	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 52.3	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 52.4	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 52.5	-	+	-	-	*Dyrk1a*$^{f/f}$
ENDF 52.6	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 52.7	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 52.8	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 52.9	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 52.10	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 52.11	-	+	-	-	*Dyrk1a*$^{f/f}$
ENDF 52.12	-	+	+	-	*Dyrk1a*$^{f/wt}$
Mouse	*NesCre8*	*Dyrk1a* floxed	*Dyrk1a* wt	*Dyrk1a* recombined	Genotype
ENDF 52.13	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 53.1	-	+	-	-	*Dyrk1a*$^{f/f}$
ENDF 53.2	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 53.3	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 53.4	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 53.5	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 53.6	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 53.7	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 53.8	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 53.9	-	+	-	-	*Dyrk1a*$^{f/f}$
ENDF 53.10	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$

(continuation of Table 5)

ENDF 53.11	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 53.12	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$
ENDF 54.1	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 54.3	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 54.4	-	+	-	+	*Dyrk1a*$^{f/f}$
ENDF 54.5	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 54.6	-	+	-	+	*Dyrk1a*$^{f/f}$
ENDF 54.7	-	+	+	+	*Dyrk1a*$^{f/wt}$
ENDF 54.8	+	+	-	+	*Dyrk1a*$^{\Delta/\Delta}$
ENDF 54.9	-	+	+	-	*Dyrk1a*$^{f/wt}$
ENDF 54.10	+	+	+	+	*Dyrk1a*$^{\Delta/wt}$

PCRs were performed with DNA samples obtained from E11 embryos (litters ENDF 52 and ENDF 53) and E15 embryos (litter ENDF 54). Presence and absence of the corresponding band for each allele is indicated with + and – respectively.

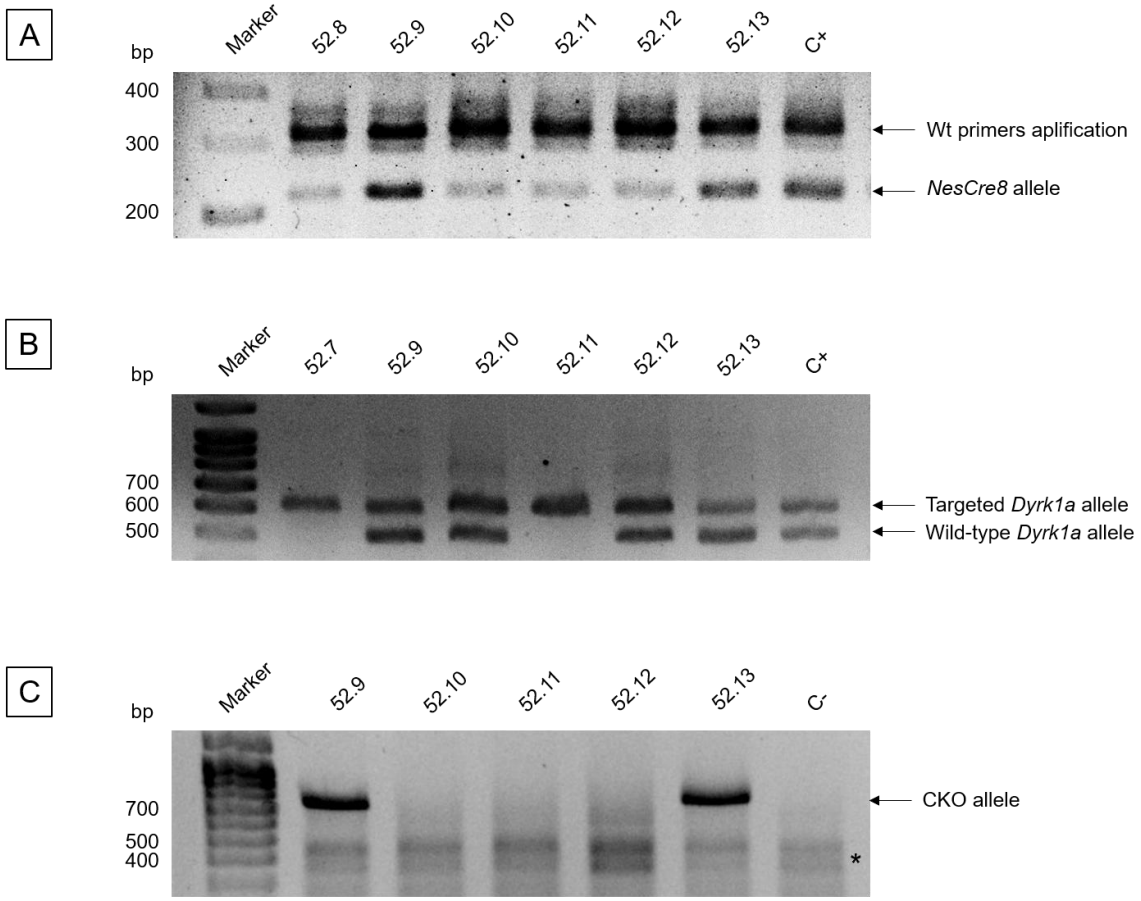

Figure 8. Agarose electrophoresis gels of PCR products. PCRs were performed with the primers indicated in Table 2. The molecular size marker used to visualize bands indicated with bp (base pairs) was Ladder VII. Samples were from litter ENDF 52. C+ and C- were positive and negative PCR controls. (A) PCR products from *NesCre8* genotyping to assess for the presence or the absence of the Nes*Cre8* allele. (B) PCR products from *Dyrk1a* floxed genotyping to assess for the presence of the *Dyrk1a* floxed allele and the *Dyrk1a* wild-type allele. (C) PCR products from *Dyrk1a* recombined genotyping to check for the presence or absence of *Dyrk1a* recombined allele. *: nonspecific amplification bands.

NesCre8, *Dyrk1a* floxed and *Dyrk1a* wild-type allele heredity

We studied the proportion of embryos with the 4 distinct genotypes resulting from cross 3. *NesCre8* and *Dyrk1a* floxed allele were segregated independently following Mendel's First Law (Castle, 1903), the inheritance of the *NesCre8* transgene was independent of the inheritance of the *Dyrk1a* floxed and of the *Dyrk1a* wild-type allele.

Different allele segregations in parental and maternal gametes are described in Figure 9. The four genotypes had the same expected percentage, 25%. However, there was a 50% probability that mice were wild-type against 50% probability that mice had one or two *Dyrk1a* alleles deleted. The expected percentage of embryos for each of the 4 possible genotypes are in Table 6.

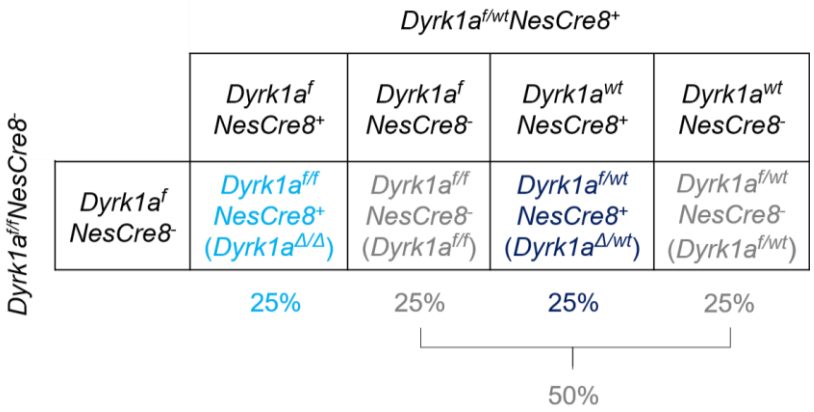

Figure 9. Punnett square of allele combinations in parental and maternal gametes in cross 3. The four possible genotypes are indicated with colours.

Table 6. Expected and observed percentage of the four possible genotypes in cross 3 progeny.

Litter	Genotype	Percentage of mice observed (%)	Pertantage of mice expected (%)
ENDF 52	*Dyrk1a*$^{\Delta/\Delta}$	23,08	25
	Dyrk1a$^{f/f}$	15,38	25
	Dyrk1a$^{\Delta/wt}$	30,79	25
	Dyrk1a$^{f/wt}$	30,79	25
ENDF 53	*Dyrk1a*$^{\Delta/\Delta}$	25	25
	Dyrk1a$^{f/f}$	16,67	25
	Dyrk1a$^{\Delta/wt}$	41,67	25
	Dyrk1a$^{f/wt}$	16,67	25
ENDF 54	*Dyrk1a*$^{\Delta/\Delta}$	33,33	25
	Dyrk1a$^{f/f}$	22,22	25
	Dyrk1a$^{\Delta/wt}$	11,11	25
	Dyrk1a$^{f/wt}$	33,33	25

Different litters studied are indicated: ENDF 52, ENDF 53 and ENDF.

Table 7. Percentage average for each genotype.

Genotype	% Average	Standard deviation
Dyrk1a$^{\Delta/\Delta}$	21,58	5,45
Dyrk1a$^{f/f}$	18,09	3,63
Dyrk1a$^{\Delta/wt}$	27,86	15,49
Dyrk1a$^{f/wt}$	26,93	8,97

DISCUSSION

In this work we report the strategy for generating a conditional *Dyrk1a* knockout mouse model that can be used to assess the role of the protein kinase DYRK1A in neurogenesis. We achieved this CKO mutant mouse for *Dyrk1a* by genotyping, selections and crosses of lines mice.

Genotyping of these mice showed that the three different alleles, *NesCre8*, *Dyrk1a* floxed and *Dyrk1a* wild-type allele, had different appearance rates. Heredity analysis shows the expected percentage of mice and embryos for each genotype was not met with Mendel's inheritance (Castle, 1903). However, the number of mice studied could contribute to this result, the percentage of mice observed could reach the expected value with more replications.

NesCre8 genotyping procedure assessed the presence of the transgene by using tail DNA extractions because *NesCre8* was present in all body tissues. *Dyrk1a* floxed genotyping was done using limb DNA extractions as it was found in every tissue. However, presence of *Dyrk1a* recombined allele was assessed by DNA extractions of nervous system tissue because there was only expression of Cre-recombinase in neural progenitor cells and somites promoted by *Nestin* (Petersen et al., 2002).

Genotyping results showed that E15 and even E11 mice embryos already had the *Dyk1a* truncated allele due to the expression of Cre-recombinase. This evidence correlates with previous experiments that show detection of Cre-recombinase at the early embryonic age of E8.5 (Petersen et al., 2002).

CKO mutant mouse displayed *Dyrk1a* loss of function just in neural progenitor cells, allowing the study of protein role in nervous system at the early age in which Cre-recombinase starts expressing. Contrary to null mutants (*Dyrk1a*$^{-/-}$), this CKO avoided early embryonic death and therefore can be used to study neurogenesis. However, CKO mutant mice were never recovered after birth because they start dying at E19.5, suggesting that DYRK1A plays an essential function in the nervous system.

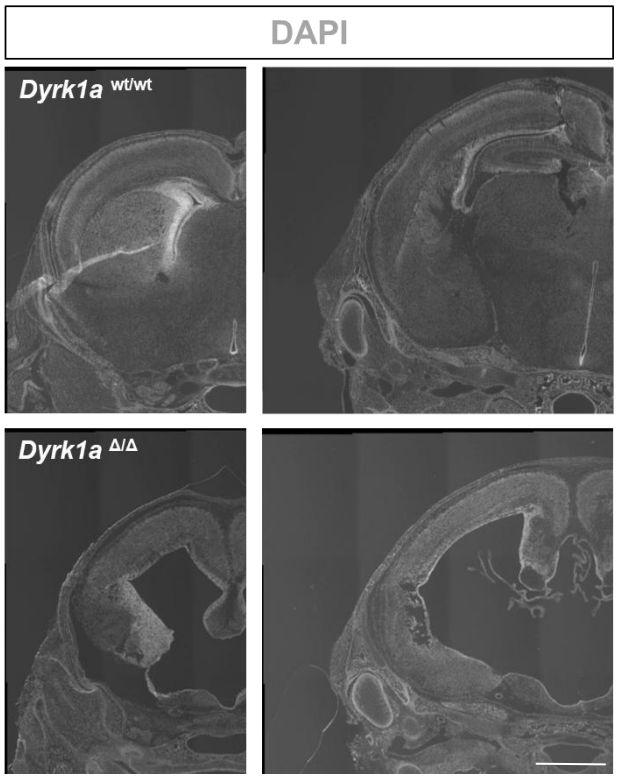

Figure 10. *Dyrk1a*$^{\Delta/\Delta}$ phenotype. E19.5 mouse embryo brain sections comparing wild-type mice (*Dyrk1a*$^{wt/wt}$) with CKO mutant mice (*Dyrk1a*$^{\Delta/\Delta}$) displaying a reduction in brain parenchyma. DAPI: nuclear marker. Scale bar: 1 mm.

Given that DYRK1A is involved in many important functions in the nervous system interacting with different proteins and substrates, it's suggested that *Dyrk1a* absence during the process of neurogenesis should cause a collapse in the brain.

The study of the targeted mouse (*Dyrk1a*$^{\Delta/\Delta}$) phenotype is shown in Figure 10. CKO mutant without any copy of *Dyrk1a* in the nervous system cells showed a significant reduction of brain parenchyma.

CONCLUSIONS

CKO mutant mouse model performance allows future studies to elucidate where DYRK1A is involved in the developmental cortex and what functions regulates there during neurogenesis.

As discussed above, DYRK1A takes an important role in neurodegenerative diseases such as DS and Alzheimer disease, where DYRK1A levels are higher than normal. This evidence explains all the effort applied to found DYRK1A inhibitors. DYRK1A therapeutic drugs can normalize prenatal development, improve cognition in patients with DS and treat other neurodegenerative diseases (Arbones et al., 2018).

Dyrk1a$^{\Delta/wt}$ heterozygous conditional mutant mice can be used also as an MRD7 model fo study the affectation of this syndrome in neurogenesis level. Future experiments carried in *Dyrk1a*$^{+/-}$ and *Dyrk1a*$^{\Delta/wt}$ mouse model could be used to discover therapeutic drugs like DYRK1A activators for future applications in diseases with decreased DYRK1A levels like MRD7.

Considering the importance of DYRK1A dosage imbalance in human diseases described in different mouse models, the proper objective of these chemicals is to ameliorate cognitive defects with a balanced level of DYRK1A.

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