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DIPLOMARBEIT

Titel der Diplomarbeit

Studies on the role of the calcium binding mitochondrial carrier Aralar in development/differentiation/survival of mesencephalic dopaminergic neurons in mice

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To my father

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Summary

Aralar, a mitochondrial aspartate/glutamate carrier (AGC), belongs to the family of Calcium mitochondrial carriers (CaMC) and is a component of the malate-aspartate NADH shuttle (MAS). It has been shown essential for proper mitochondrial function [1].

Having in mind that differentiating neurons have increasing energetic demands, that differentiating neurons produce elevated levels of reactive oxygen species (ROS), which could expose mitochondria to an increased stress[2], and that the MAS shuttle function could be implied in differentiation and survival abilities of cells[3], the matter arises whether Aralar could be relevant for cell differentiation and/or survival capacity.

In this thesis Aralar´s eventual role in dopaminergic neurogenesis was investigated.

Expression studies, using immunohistochemistry of ventral mesencephalon (VM) cryostat slices, immunocytochemistry of ventral mesencephalic (VM) primary culture and mES cells and western blot methods, carried out in CD1 wildtype mice showed abundant Aralar expression during critical phases of dopaminergic neurogenesis.

Comparative in vivo immunohistochemical and in vitro immunocytochemical expression studies of VM at embryonic ages E11.5, E13.5, e14.5 and E15.5 of WT(+/+) and KO (-/-) aralar mice revealed differences in dopaminergic neuron harvest (evaluated by tyrosine hydroxylase expression) and expression differences of Foxa2, a dopaminergic transcription factor, as well as the cell cycle marker Ki67 and the apoptotic marker casp3. Therefore an impact of Aralar on cell survival capacity due to missing mitochondrial integrity is suggested. Furthermore MAS shuttle activity deprivation could explain metabolic changes which could influence transcriptional control of dopaminergic neurogenesis.

Moreover mES cell lines from aralar (+/-) mates have been established and characterised. Pluripotency of established lines has been approved by immunocytochemistry and cytochemistry. Still genotyping, antibiotic selection and western blot analysis demonstrated that no ara (-/-) mES cell line could be derived. This lead to the conclusion that Aralar might already be implicated at a very early point of development maybe because of restricted energy supply. Aralar deficiency at blastocyst stage might difficultate their detection.

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Abbreviations

a/p	anteriour/posterior
A9/A10	dopaminergic neurons area in VM mouse brain
AA	amino acids
AADC	aromatic L-amino acid decarboxylase
AGC	aspartate/glutamate carriers
a-KG	a-ketoglutarate
a-KGDH	a-ketoglutarate dehydrogenase
A	Amper
AMPD	2-amino-methyl-1,3-propanediol
AP	alkaline phosphatase
APS	ammonium persulfate
Ara	Aralar
asp	aspartate
AST	mitochondrial and cytosolic aspartate aminotransferases
B27	supplement growth factor
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
β-IIItub	β-III Tubulin
β-ME	β-mercaptoethanol
Ca^{2+}	Calcium
$CaCl_2$	calcium chloride
CaMC	calcium mitochondrial carrier
Casp3	Caspase3
CaU	Ca2+ uniporter
cer	cerebellum
Cit	Citrine
C-terminal	carboxy-terminal
d	dorsal
DAB	3,3'-diaminobenzidine
DABCO	1,4 diazobicyclo-(2,2,2)-octane

DAns	dopaminergic neurons
dATP	deoxy adenosine triphosphate
dCTP	deoxy cysteine triphosphate
ddH_2O	double distilled water
dGTP	deoxy guanine triphosphate
dm	Dorsomedial
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DPX	distrene-80 plasticizer xylene (synthetic resin)
DTT	Dithiothreitol
dTTP	deoxy thymidine triphosphate
E	embryonic age
E14	mES cell line
ECL	electro chemic luminescence
EDTA	ethylenediaminetetraacetic acid
EF-hands	EF-hand-motif.
En1/2	Engrailed ½
ESC	embryonic stem cells
EtBr	ethidium bromide
FBS	fetal bovine serum
FCS	fetal calf serum
Fgf8	fibroblast growth factor 8
Foxa2	Forkhead box protein A2
g	Gram
GABA	gamma-aminobutyric acid
Gbx2	gastrulation brain homeobox 2
GDNF	glial cell line-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
glut	Glutamate
h	Hour
Ham´s F12	Medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP	horse radish peroxidase
hESCs	human embryonic stem cells
HET	heterozygotus
Hind III	endonuclease
HSC	hematopoietic stem cell
HSC2002	human stem cell medium
IDH	isocitrate dehydrogenase
I-r1	isthmic region
IsoCit	Isocitrate
kDA	kilodalton
KH2PO4	monopotassium phosphate
Ki67	Kiel67 proliferation marker
KO	knock out
KO SR	knock out serum replacement
L6	mES cell line
L7	mES cell line
lge	lateral ganglionic emincence
L-Glu	L-glutamin
LIF	leucemia inhibitory factor
Lmx1a	LIM homeobox transcription factor 1, alpha
Lmx1b	LIM homeobox transcription factor 1, beta
m	mamillary body
mAB	monoclonal antibody
mal	malate
MAS	malate-apartate NADH shuttle
Mash1	achaete-scute complex-like 1
Mb	midbrain
MC	mitochondrial carrier
mDAn	mouse dopaminergic neuron
MDH	Malate dehydrogenases
MEF	mouse embryonic fibroblast
MEM	mimimum essential medium
MetOH	Methanol
$MgCl_2$	magnesium chlorid

mge	medial ganglionic eminence
mhb	midbrian-hinbrain border
min	minute(s)
µl	mycro liter
Msx1/2	msh homeobox ½
N2	supplement growth factor
Na_2HPO_4	disodium phosphate
$Na_4P_2O_7$	sodium diphosphate
NaCl	sodim choloride
NAD^+	oxidated nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NaF	sodium fluoride
NaH2PO4	sodium phosphate
Nest	Nestin
ng	nano gram
Ngn2	neurogenin 2
Nkx6.1	NK hemeo box, family 6, member A transcription factor
Np-40	nonyl phenoxylpolyethoxylethanol
N-terminus	amino-terminus
Nurr1	nuclear receptor related 1
o/n	over night
OAA	oxalacetic acid
ob	olfactory bulb
Oct4	octamer binding transcription factor 4
OGC	A-ketoglutarate–malate carrier
or	optic recess
Otx2	Orthodendicle homeobox2 transkription factor
p	Prosomere
PA6	mouse stromal cell line
pAB	plyclonal antibody
PAGE	polyacryleamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS+Triton
PCR	polymerase chain reaction

PD	Parkinson disease
PDH	pyruvate dehydrogenase
pen/strp	penicillin/streptomycin
PFA	paraformaldehyde
PHEM	buffer see immunocytochemistry recipies
poly-lys	poly lysine
Ptx3	Bicoid-related homeodomain-containing transcription factor
Pyr	pyruvate
raldh1	retinal dehydrogenase 1
RN	red nucleus
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecylsulfate
Shh	sonic hedgehog
SNpc	sustancia nigra pars compacta
Sox 2	sex determining region Y-box 2
sP	secondary prosencephalon
SR	serum replacement
SSEA1	stage-specific embryonic antigen-1
Succ-CoA	succinyl-CoA
T20	tween 20
TAE	Tris base, acetic acid, EDTA
TBS	Tris-buffered saline
TE	Trophoectoderm
TEMED	N,N,N',N'-tetramethylethylenediamine
TH	Tyrosine hydroxylase
TM1-6	six tilled transmembrane helixes
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
TRp Hyd	Trypthophane hydroxylase
VTA	ventral tegmental area
Wnt	wingless int1

1. Introduction

1.1. Parkinson

The illness of Parkinson (PD) was first described by James Parkinson (Parkinson, 1817). Affected individuals show an accumulation of the following symptoms of complex motor activities:

1. rhythmic tremor
2. loss of control of voluntary movements (Akinesia) or deficiencies in the start of those (slow movements, bradykinesia)
3. muscular rigidity
4. instable posture
5. later symptoms like dementia [4, 5]

These symptoms are caused by the selective cerebral loss of dopaminergic neurons (DAns) localized in the pars compacta of the substantia nigra (SNpc). As a consequence the relative level of the neurotransmitter dopamine is reduced in its target regions which are the striatum (nucleus caudatus/putamen), the cortex and the nucleus accumbens.

Various treatment options for PD do exist including pharmalogical treatment, deep brain stimulation, cell replacement strategies etc.. Pharmalogical treatment aliviates the symptoms to a certain extend but prolonged administration can result in severe side effects. Deep brain stimulation targeting the subthalamic nucleus can bring ease but is only appropriate and efficient for a reduced number of patients

Cell therapy for PD tries to diminish the illnesses symptoms, by subjecting cells in vitro a re-/de-/differentiation program, for obtaining DAns, which are able to integrate at the place of axonal degeneration and to establish contact with the target cells thereby re-establishing neurotransmission.

Human foetal tissue has been used demonstrating some clinical efficacy [6] still it confronts us with problems like being not expandable or being difficult to standarize regarding tissue composition, quality and viability [7] aside from ethical restrictions.

These severe limitations demand for alternative cellular sources. In this regard stem cells in general and mainly embryonic stem cells provide an unlimited source of cells.

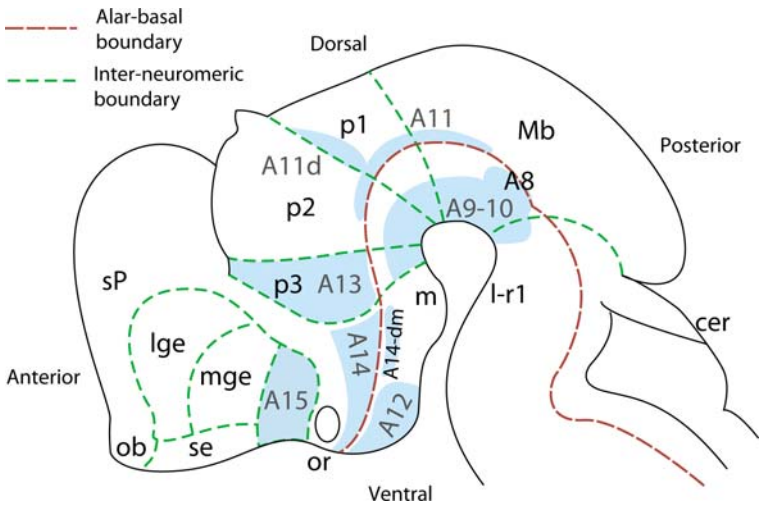
Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocyst and are characterized by their extensive selfrenewal capacity and their potential to differentiate into any cell type of the body, including neuroepithelial tissue and specific neuronal cells [8-10].

It has already been possible to generate cells with mDAn phenotype from mES cells of which the developmental program in vitro recapitulates the temporal course of normal mDAn maturation [11] [12] [13-15].

However there are three major challanges that still have to be overcome:
Firstly sufficient quantities of differentiated cells of the desired type have to be achieved.
Secondly so far only poor post graft survival has been achieved in animal models transplanting ESCs [11, 15-19].
When it comes to human embryonic stem cells (hESCs) the cell harvest of graft survivers is even worse even under immunosuppression treatment in the murine model of PD [17, 20].
Thirdly phenotype heterogenity leading to teratoma formation has to be diminished [21, 22].
Moreover, as grafts, also to the formerly immunologically previleged thought system, the brain, are allogenic, at the moment accompanying immunosupression treatment is indispensable for graft survival. hESCs banking or nuclear transfer as well as induced pluripotent stem cells (IPs) could be used as an alternative to reduce disparities between donor and recipient in a future.

1.2. Midbrain dopaminergic (mDA) neuron development in mice [23]

Midbrain dopaminergic neurons (mDAn) are present in two areas of the adult mouse brain: 1. the substantia nigra pars compacta (SNc or A9 group) and 2. the ventral tegmental area (VTA or A10 group). These two neuron populations have different projection sides wich also correspond to different functions. SNc projects to the dorsolateral striatum and regulates motor functions and the VTA projects to the ventromedial striatum and cortical areas is thereby contributing to the mesocortical limbic system and regulating emotional behaviour and mechanisms of natural motivation and reward.



1) Fig. 1: Dopaminergic neurons in the CNS [24].

Schematic illustrating the location of Th$^+$ neurons, corresponding to A8-A15 groups, in mouse brains ranging from E12.5-E14.5. The DA groups (light blue areas) are depicted in relation to the neuromeric map [subdivision into transeverse segments[25] and the alar (dorsal) and basal (ventral) plate subdivision.

Looking chronologically at SNc mDAn development one can figure out three major transitions which are influenced by diffusable factors secreted by signaling centers as well as cell intrinsic factors within the progenitors[24, 25]

(1) Regionalisation of neural plate before ~E9 (progenitors)

(2) Midbrain cell fate determination ~E9 to E11 (immature mDAns)

(3) Terminal differentiation ~E11 onwards (mature mDAns)

During regionalisation two axis of the neural plate can be made out: the anterior posterior (a/p) and the dorsal ventral (d/v) axis. During neurulation a tube is formed in which the neural plate midline occupies the ventral-most side of the evolving nervous system, receiving extrinsic signals from the underlying mesoderm and signals from within the neural plate itself. During early regionalisation the midbrain-hinbrain border (mhb) also called Isthmic Organizer is established, defining a spatial seperation along the a/p axis. Fgf8 expression and a corepressive interplay of Otx2 and Gbx2 maintain the position of this signaling center. Within the mhb signaling factors Wnt and En1/2 are induced for proliferation and survival of the VM cells. Sonic hedgehog (Shh) is responsible for patterning along the d/v axis being induced by extrinsic Shh signaling from the underlying notochord. It is expressed only in the most ventral population, giving rise to the mDAn. Endogenous lateral Otx2 expression seems to restrict Shh expression to the ventral domain thereby preventing the Shh inhibition of lateral Nkx6.1 expression zone, which generates red nucleus (RN) progenitors. Within the later Nkx 6.1 expression zone emerges a narrow band of Nkx2.2, which later gives rise to GABAergic neurons [26].

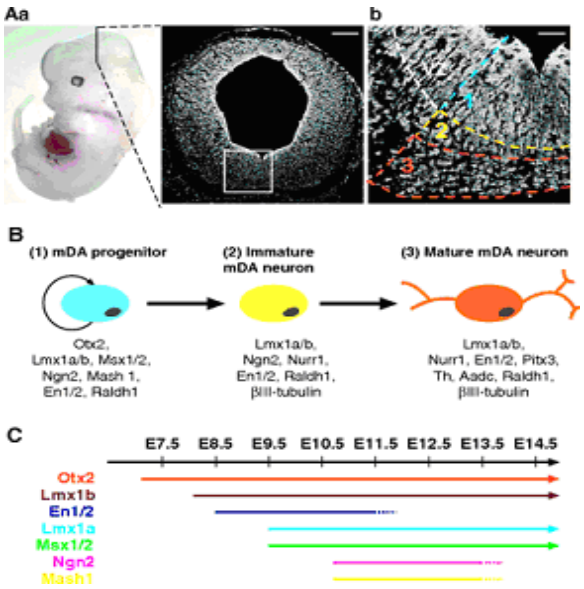
mDA progenitors are derived from radial glial like floor plate cells, which means that at a certain point they have to accomplish a switch to neuronal fate maybe by expression of proneural genes like Ngn2 or Mash1[27] [28].

The developing neuroepithelium of the midbrain (mb) proliferates, thereby getting thicker and is getting layered. The ventral ventricular zone cells maintain their proliferative neural stem cell character while other cells differentiate further, start to migrate laterally and ventrally and exit the cell cycle. The laters start to express Lmx1a, Foxa2, lmx1b, Msx1/2, Ngn2 and at early stages keep on expressing Shh. Shh induces Lmx 1a expression (E9). Cells that downregulate Shh at a later stage still seem to be capable of giving rise to mDAn progenitors if they continue to express Lmx1a. Msx1 acts synergistically with Lmx1a inhibiting Nkx6.1 lateral expression allowing thereby Ngn2 proneural gene expression. Ngn2 mediates transition from the proliferative zone to the intermediate zone, where cells start to express Nurr1 (E10.5).

Foxa2 influences Ngn2 and later on proper Nurr1 and En1 expression in a dosage dependent manner. [29]

Mash1 which coexpresses with Ngn2 is not required for mDA neuron development, but it is able to compensate partially Ngn2 function in mDA progenitors [27].

For obtaining the final characteristics of mDAn the progenitors start to express TH, the rate limiting enzyme for dopamine (DA) production, and aromatic L-aminoacidic decarboxylase (AADC). These cells also specifically express En1/2, which seems to be crucial for the maintenance of neurons of the SNc but not of the VTA. It is believed that ventrolateral cells go on to form the SNc and more medial ones give rise to the VTA. Pitx3 is required for SNc cell maintenance but not for VTA. Lmx1b expression becomes restricted to mDAns of the mb.



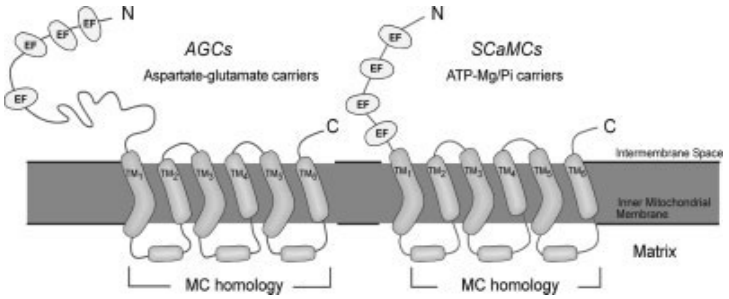
2) Fig. 2: Molecularly distinct DA cell populations in the mouse ventral midbrain.[24]

(A, part a) An E12.5 mouse embryo. The black line marks the plane of section shown in the image to the right. (A, part b) An enlarged view of the ventral midbrain (square box in A, part a), illustrating the position occupied by mitotic progenitors, immature and mature postmitotic neurons of the mDA lineage. (B) The sequential timing of transcription factor activation and defined marker expression in mDA progenitors. The curved arrow indicates cycling cells. (C) The timing of expression of transcription factors in mDA progenitors (not in subsequent steps of the lineage) are shown. Dotted lines indicate that the precise timing of expression shutdown has not been determined.

Functional maturation of mDAns takes place by extension of axonal and dendritic processes. This process starts at E11 when mDAn axons project rostrally. By E14.5 many of them have reached their targets (mainly the striatal cells) [30]. Guidance molecules such as the homeodomain proteins En1 and En2 are proposed to be implicated in this process as also in the induction of guidance molecule receptors expression like Ephrin-A2 and Ephrin A5. Slits and Netrins may also be involved in target recognition by nigrostriatal mDAns [31]. Upon target innervation, mDAn axons likely compete for establishing synapses and for survival. Growth factors, notably GDNF and BDNF, are implicated in this process [32].

1.3. Aralar

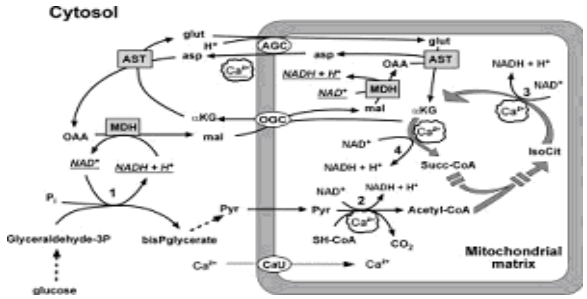
Aralar as well as its isoform Citrine are mitochondrial carriers located in the inner membrane. They belong to the calcium mitochondrial carriers (CaMCs) family, which have N-terminal extensions harbouring a number of EF-hands the archetypical $Ca2^{+}$-binding motifs. Within this group they belong to the subgroup of apartate/glutamate carriers (AGC) being members of the malate-aspartate NADH shuttle (MAS). Aralar is encoded by a gene on chromosome 2q31 [33] and Citrin on chromosome 7q21 [34].



3) Fig. 3: Secondary structure of calcium-binding mitochondrial carriers[36].

In both CaMCs, the carboxy-terminal half corresponds to the MC homology region, and the N-terminal extension harbors Ca^{2+}-binding EF-hand motifs. The MC homology region, about 300 amino acids long, is represented according to its homology with that of adenine nucleotide translocase in its carboxyatractylosidebound form[37]. TM1-6 corresponds to the six tilted transmembrane helices characteristic of all MCs, three of which are kinked (1, 3, and 5 because of the presence of conserved prolines. Three shorter helices in the loops between TM 1-2, TM 3-4, and TM 5-6 face the matrix and are parallel with the membrane surface. The MC region forms a basket opening to the intermembrane space, with a funnel-shaped cavity inside that ends on a narrow pit close to the matrix surface [37]. The N-terminal extensions of AGCs are longer than those of SCaMCs, and the overall distribution and sequence of EF-hand motifs is unrelated to that of SCaMCs, which are very similar to calmodulin.

The transport reaction catalysed by the AGCs is the only irreversible step in the malate-aspartate shuttle and a main point of regulation. Aralar exchanges matrix aspartate for external glutamate plus a proton. The major route of aspartate production in the central nervous system is thought to be mitochondrial and it depends on Aralar for aspartate efflux to the cytosol**.** In the cytosol NADH is produced during glycolysis and shuttled into mitochondria by MAS. Blocking of MAS gives a hold on glycolysis, which can lead to energy deprivation of the cell [35].



4) Fig. 4: Ca^{2+} activation of the aspartate-glutamate carrier (AGC)–malate–aspartate NADH shuttle pathway (aralar–MAS)[36]

and the calcium uniporter–mitochondrial dehydrogenases pathway (CaU–mitDH) by calcium. The MAS is made up of four enzymes, mitochondrial and cytosolic aspartate aminotransferases (AST) and malate dehydrogenases (MDH), and two mitochondrial carriers, the AGC and the a-ketoglutarate–malate carrier (OGC). MAS activity results in the transfer to the mitochondria of reducing equivalents generated with the reaction of glyceraldehyde-3-phosphate dehydrogenase (1). Extramitochondrial Ca^{2+} can activate the aralar–MAS pathway, resulting in increased mitochondrial NADH, underlined. Mitochondrial matrix Ca^{2+}, taken up through the $Ca2^{+}$ uniporter (CaU), activates pyruvate dehydrogenase, PDH (2), isocitrate dehydrogenase, IDH (3), and a-ketoglutarate dehydrogenase, a-KGDH (4), generating intramitochondrial NADH, in not underlined.

Aralar and Citrin have an overlapping distribution in embryogenesis. In fact, abundant expresssion of both isoforms can be observed in brain (including midbrain) of E11 mouse embryos [36].

In the adult brain only Aralar is present and Citrin gets restricted to the rest of the body beeing implicated also in the urea cycle. Aralar is mainly expressed in neurons in comparison to glial cells and this Aralar expression augments with neuronal maturation.

Deficiency of Aralar (in KO mice) induces a dose dependent decrease in mitochondrial activity (or dysfunction of mitochondria) in brain, mainly in neurons and in skeletal muscle leading to death 20 days postnatal [1].

These knock out mice have also allowed the discovery of new functions of aralar: 1) it is required for synthesis of brain aspartate and N-acetylaspartate, a neuron-born metabolite that supplies acetate for myelin lipid synthesis in the brain and 2) it is essential for the transmission of small Ca2+ signals to mitochondria via an increase in mitochondrial NADH [37].

1.4. Evidence for possible Aralar implication in mDA neuron developement and pluripotency

Mitochondria not only have a key role in electron transport and oxidative phosphorylation, they are also the main cellular source of free radicals and are involved in calcium homeostasis and in the regulation of cell-death pathways [38].

Multiple lines of evidence suggest a pathogenic role of oxidative damage and mitochondrial dysfuncion in PD [39].

Cellular ability to regulate Reactive Oxygen Species (ROS) levels and respond to oxidative stress levels will in part be determined by the volume and efficacy of mitochondrial mass. [40]

Pluripotency seems to be associated with anaerobic metabolism and a poorly developed mitochondrial network. Differentiation leads to activation of mitochondrial biogenesis according to the metabolic requirements of the specific cell type[41].

Mitochondrial dysfunction and the thereby caused lack of sufficient energy supply at such an early state of development could deprive cells drastically in their capacity of differentiation and therefore in their ability to give rise to dopaminergic neurons.

Hematopoietic stem cells (HSC) and neural stem cells appear to show low ROS levels compared to more mature cells [2]. Therefore one could conclude that pluripotent cells produce little ROS species, which changes upon differentiation.

It is likely that dopamine and its metabolism, which generates reactive oxygen species, place an additional burden on dysfunctional mitochondria, thus accelerating neuronal loss. Alternatively, functionally compromised mitochondria may result in early defects in neurotransmission, resulting in an imbalance of cytosolic dopamine in the longterm, which could be neurotoxic (more than other neurotransmitters) [3] and dedifferentiation [42, 43].

In the case of Aralar deficiency the more vulnerable mitochondria might render the cell less competent to deal with higher ROS levels.

All the above data demanded for an analysis of the role of these carriers played during pluripotent state and midbrain dopaminergic neuron differentiation, development and survival.

2. Hypothesis of this diploma thesis

a) Given that differentiating neurons have increasing **energetic demands** Aralar could influence the process of dopaminergic neural and neuronal differentiation, due to its importancy for mitochondrial integrity.

b) Pluripotent cells seem to produce relatively low levels of **ROS**. Once they start differentiating ROS production rises. More fragile mitochondria of Aralar deficient mice might be limited in their capacity to deal with higher ROS levels.

c) Considering the function of Aralar as the major aspartate supplier for the cytosol in brain neurons, increased Aralar expression with neuronal maturation supposes that its deficiency carries out its **effects at a later point of embryonic development as to say from when neurons reach maturity onwards**.

3. Objectives of this diploma thesis:

a) Technical set up of cryostat cutting, immunohistochemical and immunocytochemical techniques, optimizing the derivation of primary culture, their culture conditions and optimizing MEF feeder cell derivation as well as blastocyte derivation.
b) Comparative in vivo immunohistochemical expression studies of different characteristic dopaminergic markers as well as cell cycle markers in coronal brain sections at 3 stages of dopaminergic neuron evolution (E11.5,E13.5,E15.5) of WT(+/+) and KO (-/-) Aralar embryos.
c) Comparative in vitro immunocytochemical expression studies of characteristic dopaminergic markers as well as cell cycle markers in primary culture of E12.5,E13.5 and E14.5 neural dopaminergic precursors of the ventral mesencephalon of WT(+/+) and KO (-/-) Aralar mice
d) Derivation mES cell lines from blastocytes of E3.5 HET(+/-) mated Aralar mice. Comparative in vitro immunocytochemical, cytochemical and Westernblot expression studies of these lines regarding their pluripotency and their differentiation capacity towards a dopaminergic fate.

4. Materials and Methods:

4.1. Immunochemistry Buffers and solutions:

PB 1L 0.2M pH 7,4

Solution A: 0.2M

NaH_2POH_2O Mw=137.99 g/mol

27.598 g/1000 ml 6.9 g/259 ml

Solution B: 0.2M base

$Na_2HPO_412H_2O$ Mw= 358.14 g/mol

71.628g/1000 ml 57.36 g/800ml

pH was adjusted by adding solution B to solution A

PBS: 1 L 1x pH 7.4

PB 0.2M	50 ml
NaCl	8.76 mg
KCl	200 mg
H_2O	950 ml

Blocking solution:

10% serum (horse, goat or chicken)

0.25% Triton

PBS 1x

Master mix (working solution)

1% serum (horse, goat or chicken)

0.25% Triton

PBS 1x

Moviol/Dabco:

6g of glycerol were mixed with 2.4g of moviol (Calbiochem Ref.475904, Polysciences Ref. 17951) in a 50ml centrifuge tube containing a rotation magnet. During mixing 6 ml of MiliQ H_2O were added and the solution was incubated for 2 hours at room temperature. Then 12ml of 0.2M Tris pH8.5 and 230 µl of Thimerosal 1% (weight/volume in H_2O) were added.

Then another 10 minutes of incubation at 50°C followed moving the tube continuously until all the moviol was dissolved. At the end the solution was centrifuged at 5000xg for 15

minutes in order to remove solid lumps. Then 2.5% DABCO (1.4-diazobicyclo-(2,2,2)-octane) were added. Aliquotes of 1ml were stored at -20°C until usage.

4.2. Immunohistochemistry:

4.2.1. Mounting:

Not dehydrated slides were mounted with vectashield (commercial fluorescent mounting solutionfor; Distrene-80- plasticizer- xylene) and stored at 4°C. Glass cover was removed by leaving the slide in PBS for some minutes for doing an additional immunological stain.
For mounting with moviol or DPX dehydration was performed using a rising alcohol series ending with xylol.

4.2.2. Fluorescent labelling

Slides were edged with an ImmunoPen (Calbiochem cat. no: 402176) for preventing the applicated liquid to float away. Then they were washed twice with PBS for 10 minutes. A blockage of 10% serum according to the secondary antibody, 0,25% Triton in PBS is applied for one hour. The incubation in the primary antibody diluted in 1% serum and 0.25% Triton in working solution was left over night (o/n) at +4°C.
This was followed by 3 washing steps using working solution. Afterwards the sections were incubated in the secondary antibody (see table 1 for references) at RT (room temperature) for one and a half hour. This was followed by two washes with working solution each 10 minutes. Finally the sections were washed once with distilled water for getting rid of salts.

4.2.3. 3,3'-Diaminobenzidine (DAB) labelling

Slides were washed twice with PBS for 10 minutes. Then the endogenous peroxidase was inhibited for 30 minutes in H_2O_2 3%, MetOH 10% in PBS. The sections were washed 3 times for 10 minutes again. Then the sections were blocked for one hour in 10% serum, 0.25% triton in PBS for one hour. The primary antibody was incubated in working solution o/n. The slides were washed again 3 times in working solution before incubating them in the secondary antibody, which is coupled to biotin, diluted in PBST for one and a half hour.

During this incubation time the Vectastain ABC solution (cat. no PK6100), which contains hydrogen peroxide coupled to avidine, was prepared mixing 1 drop of solution A with 1 drop of solution B in 5ml of 0.25% triton and storing this mix for 30 min before usage.
The slides were washed again 3 times in working solution and were then incubated in the ABC mix for one hour, allowing avidine to stick to biotine. Afterwards the sections had to be washed once in working solution and twice in PBS. For detection 10ml of PBS were mixed with 5 mg DAB and 4µl of H_2O_2 (30%) (beneath laminar flow). Diaminobenzidine is oxidized by hydrogen peroxide to give a dark brown color.
To stop the reaction the mix was washed off by PBS. The slides have been dehydrated by exposing them to an accelerating alcohol chain (60% - 80% - 90% - 100%) finally exposing them to xylol and mounting them immediately with DPX.

4.2.4. Nissl Stain (cresil violet)

The slides were exposed to the following solutions:
Alcohol 100% for five minutes, alcohol 95% for 5 minutes, distilled water for two minutes, cresil violet for 10 minutes, distilled water for five minutes, alcohol 70% twice two minutes, differentiation solution for one minute or less, dip into alcohol 95% three to five times, alcohol 100% twice for two minutes and finally xylol . The slides were mounted with DPX immediately without drying out.
Cresil violet solution preperation:
2.5g of cresil violet acetate were disolved in 100ml of distilled water and afterwards filtered.

4.3. Immunocytochemistry

Cells were grown on coated dishes or on coated coverslips, depending on the cell type.
Fixed cells were washed three times with PBS and could be stored in PBS at 4°C. Unspecific binding sites were blocked with blocking solution for 1 h at room temperature.
Cells were incubated o/n with primary antibodies, diluted in working solution. Prior to incubation with the secondary antibody cells had been washed
3 times with working solution for 10 min. Cells were incubated with fluorescence-labelled secondary antibody, diluted in working solution, for 2 h at room temperature in darkness.
Cells have been washed three times with PBS for 10 min and rinsed immediately
with ddH_2O before they were mounted with moviol.

In case of antibodies directed against membrane proteins neither blocking solution nor working solution contained any detergent.

4.3.1. Hoechst staining:

1µl of Hoechst stock (1mg/ml) was diluted in 5000µl PBS 1 and cells were incubated in this solution for 10 min. After washing once with PBS the crystal slides were mounted with moviol. In case of using no crystal glasses the wells were stored with PBS at 4°C.

4.4. Cytochemistry:

4.4.1. Alkaline Phosphatase (AP) Detection Kit (Millipore, cat no. SCR004):

Material: Fast Red Violet solution (o.8g/L stock), Naphthol AS-BI phosphate solution (4 mg/ml) in 2-amino-methyl-1,3-propanediol (AMPD) buffer (2mol/L), pH 9.5

Staining Protocol:
ES cells have been cultured for one day prior to analyzing AP activity, at low to medium density.
On day one, media was aspirated and ES cells were fixed with a fixative (e.g. 4% paraformaldehyde in PBS) for 1-2 minutes (Note: overfixation longer than 2 minutes results in the inactivation of AP).
Fixative was aspirated and rinsed with 1x rinse Buffer. Wells must not dry.
Reagents for AP staining are prepared (FVR:naphtol:water 2:1:1 ratio)
0.5 ml of stain solution was added to each 24-well plate and incubated in dark at RT for 15 minutes.
Staining solution is aspirated and wells were rinsed with 1x rinse buffer. Cells were covered with PBS to prevent drying and photos of colonies expressing AP (red stem cell colonies) and differentiated colonies (colorless) were taken.

I. Table 1: Applied Antibody concentrations and commercial references

primary antibodies	Reference	single IHH	double IHH	DAB	ICC	Host
Aralar	Housemade	1/100	1/100		1/100	Rabbit
β-tubulin III	Sigma T2200	1/250				Rabbit
β-tubulin III	Sigma T28660	1/300	1/500		1/2000	Mouse
Caspase 3					1/200	Rabbit
En1	Santa Cruz SC-8110	1/50	1/25			Goat
Foxa 2	SC-7554	1/100	1/100	1/200		Goat
GFAP	Sigma Clone GA5 G3893				1/300	Mouse
Ki67	RS RM-9106-S1	1/300	1/300		1/300	Mouse
Nestin	Abcam ab5968					Rabbit
Nestin	BD Transduction laboratories N17220-150	1/500				Mouse
Neurogenin 2	Santa Cruz SC-1923	1/100	1/50	1/100		Goat
Nurr1	Santa cruz SC-5568	1/50		1/200		Rabbit
Oct4	Santa Cruz Biotechnolgy A1509				1/300	Rabbit
Sox 2	Millipore AB5603	1/300			1/300	Rabbit
SSEA1	Development Hibridome bank				1/50	Mouse
TH mAb	Millipore mAB 318	1/100	1/100		1/100	Mouse
TH pAb	Chemicon AB152	1/100	1/100		1/500	Rabbit
TH pAb	Pel-Freeze P40101-0	1/100	1/100		1/500	Rabbit
TRp Hyd (serotonin)	Sigma T8575				1/500	Goat
secondary antibodies						
Alexa 555 α mouse	Molecular Probes	1/300	1/300	1/300	1/300	Donkey
Alexa 488 α rabbit	Molecular Probes	1/300	1/300	1/300	1/300	chicken
Alexa 488 α rabbit	Molecular Probes	1/300	1/300	1/300	1/300	Goat
Alexa 555 α goat	Molecular Probes	1/300	1/300	1/300	1/300	Donkey
BA 1000 α rabbit	Vector			1/200		Goat

4.5. Cell culture methods:

4.5.1. Preperation of culture dishes

Using Polylysine:

The culture dishes were covered with polylysine (10μg/ml) and incubated for at least one hour (or o/n) at 37° C. Then they are washed three times with PBS.

If crystal slides were used the polylysine was applied at a higher concentration 25-30μg/ml.

Using polylysine and laminin:

With polylysine pretreated culture dishes were covered with laminin (1µg/ml of 1000x) and incubated o/n.

Using gelatin:

Gelatin 0.1% in PBS was used to treat dishes for cultivation of embryonic stem cells or feeder cells and 0.2% gelatin for cultivation of cells on crystal slides.

4.5.2. Passaging of cells:

All media and used components (Trypsin /EDTA, PBS/ EDTA, gelatin, media) were assimilated to room temperature. The old medium was drawn off the culture dishes. The cells were washed with PBS/EDTA for 3-4 min. For trypsinizing a certain amount of trypsin was added depending on the size of culture dish and cultured for 5 minutes until all cells have deattached from the culture dish.

	P6	P12	P24	P35	P60	P100	Flask 600
Ml	200	100	50	250	400	1000	2000

For stopping the reaction 1 ml medium was added. The mixture was transferred to a falcon tube and centrifuged for 5 min at 1000 rpm. The medium was carefully drawn off and cells were resuspended in 1ml of medium.

For counting 10µl of trypan blue and 1µl of cells were mixed. 10µl were then pipetted into a Neubauer counting chamber and an area consisting of 16 smaller quadratic panels was counted. The desired solution was calculated so that the right amount of medium could be added to the cells. In the end the medium containing the resuspended cells was distributed to the new previously gelatinized dishes.

4.5.3. Mitotical inactivation

Once feeder cells (MEFs or stromal PA6 cells) of a dish had almost reached total confluency (~90%) they were treated with mitomycin C. This procedure keeps the cells in a non proliferative state.

The medium was drawn off the culture dish. 10µl mitomycin C (1mg/ml)/10ml of cell according medium (endconcentration: 10µg/ml)were applied for 2 up to 4 hours. Alternatively 1µl mitomycin C (1mg/ml)/ 1ml of medium was applied o/n (10ml/ p100).

4.5.4. Freezing of Cells

The medium was taken off. Cells were washed with PBS EDTA. One ml of trypsin was added to a p100 dish and incubated for 5 minutes at 37°C. The reaction was stopped by adding 1ml of medium. Then cells were easily removable from the dish for centrifugation in 20ml of medium for 5 minutes at 1000 rpm. The supernatant was taken off and the pellet was resuspended in freezing medium (10% DMSO in normal medium for MEF or PA6 or mES). Cells were kept at -80°C after at least 24 h stored in liquid nitrogen.

4.5.5. Thawing of Cells

Frozen cells were smoothly rotated in the water bath before resuspending in 1ml of medium. Then the resuspended cells were mixed with another 9ml of medium for centrifugation at 1000 rpm for 5 minutes. The supernatant was taken off and the cell pellet was resuspended in 1ml of medium.
According to the desired concentration a certain amount of resuspended cells was distributed equally on a pretreated culture dish. The dishes were moved smoothly from east to west and from north to south, thereby preventing cells from accumulation in the center and growing radially outwards, which would have lead to cell death in the center, poor cell harvest at the edges.

4.5.6. Derivation of dopaminergic neuronal precursors of the ventral mesencephalon

Culture dishes (24 or 48 wells) coated with only poly-lysine and dishes coated with poly-lys + laminine (1µg/ml) have been prepared in advance.
One box with ice was prepared. The embryonic age 13.5 (E13.5) embryos were extracted from the uterus of the female and put directly into cool PBS. The horns were put into a 100-mm-diameter culture dish with 20ml of PBS. A culture hood equipped with a dissection scope and dissection forceps and scissors that have been autoclaved (or cleaned and submerged in 70% ethanol) were used. The embryos and placenta from the uterine horns were released by cutting the walls of the horn and then each embryo was separated from the placenta and surrounding membranes. The heads were cut off. The ventral mesencephalon was dissected and cut into small pieces, which were put in PBS with glucose (5% autoclaved). Once all embryos have been processed the pieces were all choped by pipetting them in pasteur pipets of different diameters.

A little bit of medium was added. The tissue was centrifuged 5 minutes at 400 rpm.
The pellet was resuspended in 1-2ml of medium and cells were counted. The cells were seeded at approx. 75-100.000 cells per well (if p24 were used). In case of polylysine and laminine coated culture dishes a drop of cell suspension was applied and left 5 minutes for adhesion. Then more medium was added (for p48 a drop of 10µl was applied containing 40000 cells and later on 150µl of medium is added, for p4 a drop of 20µl was applied containing 80-10000 cells and 200-300µl of medium was added).
In case of single embryo cultures cells of each embryo were counted, therefore cells had to be resuspended in very little medium (at the most 100µl) after centrifugation. Usually one embryo midbrain contained around 200000 cells. In case of polylysine all the cells were first resuspended in their final volume and then equally distributed to the wells. The cells were cultured at low oxygen tension for obtaining better results.

4.5.7. Derivation of mouse embryonic stem cells: [44]

Derivation of mouse embryonic fibroblasts (MEFs)
100mm tissue culture plates were prepared and labelled. Usually the MEFs from two embryos provide a sufficient number of cells for one plate.
A pregnant female mouse between 12.5 and 13.5 days post coitum was sacrifyed. The uterine horns with the embryos were dissected out and put into a 100-mm-diameter culture dish with 20ml of cold PBS. A culture hood equipped with a dissection scope and dissection forceps and scissors that had been autoclaved (or cleaned and submerged in 70% ethanol) were used. The embryos and placenta from the uterine horns were released by cutting the walls of the horn and then each embryo was separated from the placenta and surrounding membranes.
The heads were cut out and all the internal abdominal and thoracic organs (lungs, heart, liver, intestine, stomach, etc.) were removed from all embryos in a litter. The embryonal tissue was trypsinized by transferring all embryos to 35-mm-diameter dishes with 1ml of 0.25% trypsin/EDTA (two embryos per plate). The tissue was trypsinized 15 min in a 37°C incubator or water bath until tissues started to disintegrate. The tissue was pipetted up and down quickly with a 1ml pipette to achieve efficient dissociation. In case when more than ten embryos were obtained, the extra embryos were kept in ice-cold PBS for up to 15 min without affecting MEF viability. To short incubation may result in insufficient trypsinization and low yield, whereas too prolonged incubation may decrease the viability of MEFs. Needles and syringes have to be avoided for dissociation.

The cell suspension in trypsin was inactivated by pipetting it into the MEF culture medium (1ml of suspension per 10ml of medium). The embryos from one female were pooled in one 50ml tube (or in two 50-tubes when more than ten embryos are obtained). The large pieces of tissue sedimented. The supernatant was collected and seeded out as a homogenous suspension, keeping a ratio of two embryos per 100-mm plate.

The culture medium had to be changed 4-5 h after seeding to remove unattached cells. The MEFs were grown until 90% confluence (it usually takes 2-3 days). MEFs at this stage ready to be frozen were considered to be passage 1. The MEFs can be frozen for several weeks at -80°C or for unlimited time in liquid nitrogen.

If MEFs were going to be frozen down, cells from one 100-mm plate were frozen per single vial using standard cell culture techniques. When using frozen MEFs, the cells were usually ready for mytomycin treatment one day after thawing (if thawed, 1 vial per 100-mm dish). Alternatively, when thawed MEFs were confluent, they were split 1:3 to generate three plates of passage 2 MEFs.

Preparation of MEF feeder layer :

12-well, 24-well or 6cm plates were gelatinized by covering the bottom of wells with 0.1% gelatin for a minimum of 5 min at 37°C. The gelatin solution was removed just before next step. After mitomycin C inactivation, MEFs were washed once with

PBS, trypsinized, counted and seeded at a density of 20,000–25,000 cells per cm^2 onto gelatinized wells. The feeder layer covered the entire well. Wells in which the MEF feeder layer was not confluent have not been used.

Blastocyst extraction and ICM expansion

MEF medium was replaced with SR-ES medium 1–3 h before blastocysts extraction. Only freshly prepared MEF feeder layers were used (prepared the same or previous day) to avoid the retraction/detachment of feeder and in order to preserve feeder properties, which could negatively influence ESC derivation. Time-mated females at E3.5 were killed and immediately dissected putting their uteri into 10ml of HEPES/DMEM in 100-mm plate preheated to 37°C in the incubator. The uteri were transferred into 2ml of fresh preheated HEPES/DMEM in 35-mm dish or as an alternative into an organ tissue culture dish (more expensive but more convenient). The blastocysts were flushed out of the uterine horn under a dissection scope using a 1-ml syringe with 23-G needle. Then they were collected under an inverted microscope using 10ml pipette tips and a 20-ml pipette and transferred individually to wells of a 12-well plate containing a feeder layer of MEFs mitotically inactivated 1 d earlier. Blastocysts are not readily visible in dissection microscope. Using an inverted microscope with phase contrast made identification much easier. Blastocysts attachment to the MEF

feeder layer usually took 2 days. Any interference during this period has been avoided. After attachment, the blastocysts hatched. SR-ES medium has then been changed, and was subsequently replaced every second day while the inner cell mass expanded, until adopting a different morphology (usually on day 5 or 6). The plate has not been shaked or moved during attachment.

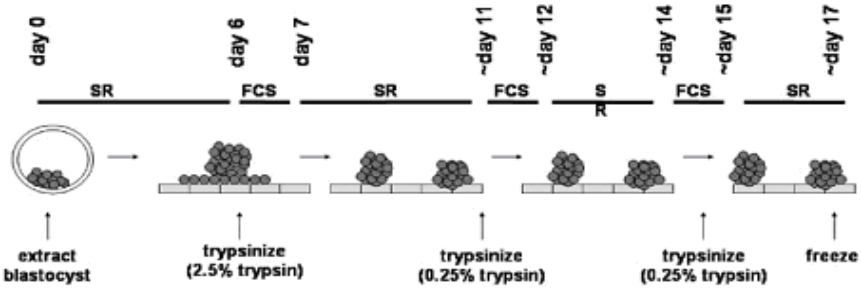
First trypsinization and ESC expansion

Appropriate numbers of 12-well plates with confluent layers of mitotically inactivated MEFs were prepared and labelled 1 day in advance. Two hours before trypsinization, the MEF medium was replaced with 1 ml per well of FBS-ES medium. 20-µl drops of 2.5% trypsin were made on a 60-mm-diameter culture dish. SR-ES medium was aspirated from the wells containing expanded blastocysts on MEFs and 200 ml of 0.25% trypsin-EDTA was added to the well. After 15 s, the trypsin-EDTA was aspirated, leaving only a wet surface. This procedure reduced stickiness of the cells and allows easier detachment. To mechanically detach an expanded blastocyst and surrounding MEFs, a 20-ml pipette and 10-ml pipette tip were used. Under a dissection microscope, 10µl of 2.5% trypsin from the 20-µl drops were pipetted onto each blastocyst (one at the time). Several circles were made with the pipette tip around the blastocyst to remove surrounding MEFs and separate the blastocyst (on the top of some MEFs) from the rest of the feeder layer. The blastocyst was aspirated back with the pipette and transferred into the remaining 10-µl drop of 2.5% trypsin.

MEFs cultured in SR-ES medium for 5–6 days produce large amounts of extracellular matrix resistant to trypsin, which increases their stickiness. To make sure that the blastocyst was successfully transferred it was checked for its presence under the dissection microscope. The cells were trypsinized in a drop for 5 min in the 37°C incubator and then dissociated into individual cells and small cell clumps, by pipetting up and down approximately ten times with a 10-ml pipette tip. Dissociation was monitored under the dissection microscope until the blastocyst was disintegrated into several smaller cell clumps. The cell suspension was pipetted into a well of a 12-well plate containing MEFs and FBS-ES medium. The next day, FBS-ES medium was replaced with SR-ES medium and the cells were let continue growing, changing the medium every second day. Several days after trypsinization, compact cell colonies with typical ESC colony morphology were detected in some of the wells (the number of colonies usually varied from 1 to 20). The colonies were monitored daily, focusing especially on colony morphology and growth.

ESCs were passaged according to their needs. All cells were trypsinized in a well with 0.25% trypsin-EDTA when most colonies in the well reached a certain morphology. The resulting single-cell suspension was mixed with FBS-ES medium and the cells were seeded in a dish (well) of suitable size containing fresh feeder cells. The next day, the medium was replaced

with SR-ES medium. Usually an additional trypsinization step was needed before the cells were grown on 60-mm-diameter dishes to sub-confluency before being frozen.



5) Figure 5. Scheme of extraction protocol [47]

4.5.8. Feeder layers for mES cells:

P 6cm diameter 800000 or at least 600 000 cells
P6 6 wells/plate 400000 or at least 300 000 cells
P4 4 wells/plate 80000 or at least 60 000 cells
P24 24 wells/plate 80000 or at least 60000 cells
P12 12 wells/plate 200000
PA6 were used for mES cell differentiation
MEFs were used for mES cell proliferation, blastocyte derivation (confluency and freshness, low passage ≤3 very important).

4.5.9. mES cell peculiarities:

Changing the medium, cells have been washed once with SRM. When trypsinized the 0,5% trypsin (+EDTA 0,2%) was applied. Cells had to be disintegrated pipetting them quite a few times with p1000 or p200 pipetts up and down. Too much disintegration before freezing cells down resulted in poorer cell survival. The centrifugation was performed at a maximum speed of 1000 rpm for 5 minutes.

4.5.10. Neomycine (G418) selection

Cells were seeded in SR-ES containing LIF and 500µg/ml G418 or 250 µg/ml G418 respectively. Growing cells have been monitored after 4 and 8 days and examined on cell mortality. Phase contrast images have been taken.

4.5.11. Differentiation of mouse embryonic stem cells towards dopaminergic fate (adjusted from Barberi et al. 2003 [11])

mES cells were trypsinized and replated at single-cell densities (depending on how many MEF cells they contained) on gelatine coated dishes containing a confluent PA6 cell layer prepared the previous day in SRM medium without LIF. Factors have been preresuspended in HSC 2000 medium. After 5 days of culture, 200ng/ml SHH and 25ng/ml FGF8 were added to the SRM for patterning, and then the medium was changed to N2 supplemented with SHH and FGF8 in the presence of 10ng/ml bFGF (days 8−11). At day 11, terminal differentiation was induced by withdrawal of SHH, FGF8 and bFGF and the addition of 200µM ascorbic acid, 20ng/ml GDNF and 10ng/ml BDNF.

If mES had already grown on gelatin for several passages 150 cells/well of p4 or p24 were seeded, if mES had so far been grown on a confluent MEF layer 200 cells/well of p4 were seeded, as cell countings included MEF cells.

4.5.12. Collection of cells for DNA extraction

Cells were washed twice with PBS before beeing trypsinized for 5 minutes. Afterwards 200µl of medium were added, the 15 ml tube was filled up with PBS for stopping trypsinization and cells (also mES) were centrifuged at 1000 rpm for 5 min. Supernatant was taken of. Pellet can be stored at - 20°C or resuspended in PBS.

4.5.13. Cell lysis for Western blot analysis

Cells were washed twice with cold PBS and put on ice immediately. 100µl of lysis buffer were added to P6 wells. Cells were scraped of the wells using cell scrubber and transferred into a 1.5ml eppendorf on ice. Then they were homogenized by sonication. Proteins either were used immediately or were stored at -20°C until their usage.

Lysis buffer: pH 7.5

Hepes	50mM
NaCl	150 mM
$MgCl_2$	1mM
$CaCl_2$	1 mM
$Na_4P_2O_7$	10mM
NaF	10mM

EDTA 2mM

Glycerol 10%

Np-40 1%

added just before usage:

1 inhibitor mix tablet Roche (ref.: 11836170001) in 10 ml lysis buffer.

4.5.14. Determination of protein concentration using Bradford assay

Reference curve: 1-50 µg/ml BSA

BSA (µl)	H_2O (µl)	Bradford
0	800	200µl
1	799	200µl
5	795	200µl
10	790	200µl
20	780	200µl
50	750	200µl

Stock BSA: 1mg/ml

After adding Bradford reagent samples were incubated between 5 minutes and one hour. Measurements were done at OD 595 nm. Linear zone of graph was chosen as a reference.

4.5.15. Media, solutions and equipment for cell culture

- Mouse stromal cell medium (PA6 cell line)
    - αMEM (Gibco, ref: 32571-028)
    - 10% FBS (bibco, ref: 10270-106)
    - 1% L-Glutamine
- PA6 freezing medium
    - 80% PA6 medium
    - 10% FBS
    - 10% DMSO
- Mitomycin C (Roche)
    - 10µg/ml (stock 1mg/ml), medium (PA6 or MEF)
- sterile and cool PBS with glucose (stock: 10%)
- N2 medium + non essential amino acids with and without B27 (1%)
- sterile Petri dishes for dissection

- 12 (Falcon, multiwell 12-well, cat. no. 353043), 24, 48 well, 35-, 60- and 100-mm tissue culture plates
- 2.5% (Gibco, cat. no. 15090-046), 0.25% trypsin (Gibco, cat. no. 25300-54)
- MEF culture medium: D-MEM supplemented with 10% FBS (Gibco, cat. no. 10270-106), penicillin (100 U ml–1)/streptomycin (100 mg ml–1) (Gibco, cat. no. 15140-122) and 2 mM L-glutamine (Gibco, cat. no. 25030-024)

If DMEM had been opened several weeks before use 1ml /100ml of glutamine was added.

- MEF freezing medium (MEF culture medium + 10% FBS (increases survival rate) + 10% DMSO)
- Dissection scope
- 10ml pipette tips (Gilson, Diamond D10, cat. no. F161632; Sarstedt, cat. no. 70.1130; or similar)
- 20ml pipette
- Dissection forceps and scissors
- PBS, without calcium or magnesium
- 70% (v/v) ethanol
- E12.5 or E13.5 mouse embryos
- 0.1% gelatin: 1 g of gelatin was disolved (Sigma, cat. no. G1890) in PBS 1x and sterilized by autoclaving 30 min at 120 °C
- HEPES/DMEM
    - 7.5ml Hepes 1M (100x, Gibco, cat. no. 15630-056)
    - 500ml Knock out (KO) DMEM (Gibco, cat. no. 10829-018)
- FBS-ES medium, a medium similar to SR-ES medium, in which SR was replaced by 20% ES cell-pretested FBS (PAA, cat. no. A15-080)
- 23-G needles (0.6 x 25 mm)
- 1-ml syringes
- Inverted microscope with phase contrast
- SR-ES medium (500ml)
    - 390ml KO DMEM (Gibco, cat. no. 10829-018) (light sensitive) in case of derivation, usually: 415ml.
    - 100ml (20%) KO serum replacement (SR) (Gibco, cat. no. 10828-028) in case of derivation, usually: 75ml (10%) KO SR
    - 5ml 200 mM L-Glutamine (Gibco, cat. no. 25030-024)
    - 500µl 1:1000 non-essential aminoacids
    - 5ml 10000 U/ml pen/strep (1:1000)
    - 500µl 1000 U/ml LIF (ESGRO, Chemicon) (1 µl/ml)
    - 1ml 0,1mM β-ME (stock: 88 µl β-ME in 25 ml PBS) (2 µl/ml)

For passaging or handling a LIF-free version of the medium was used.

- mES freezing medium
  - 90 % FBS (Gibco, cat. no. 10270-106)
  - 10% DMSO (Sigma cat. no, D2650)
- HSC 2002
  - DMEM/F12 (1:1) with Glutamax I, Liquid, 1x, Gibco cat. no: 31331-028. stored at 4°C)
  - glucose 30% (Sigma cat. no. G7021) DMEM/F12 was dissolved at 30% (w:v), filtered, aliquoted and frozen)
  - 1M HEPEs (Gibco cat. No. 15630-056) aliquoted and stored at 4°C
  - AlbuMAX-I (Gibco cat. no. 17502-048) 3mg/ml
- N2 medium
  - HSC 2002
  - N2 supplements 100x (Gibco cat. no. 17502-048) aliquoted and frozen
  - Non essential AA 100x (home made) Aliquoted and frozen
  - Pen/strep 1000x mix (home made) aliquoted and frozen
- PBS EDTA (0.02%): 50ml EDTA (0.2%, stock)
  450ml PBS

4.5.16. Fixatives and other solutions:

- PSP 0.1M
  - 25ml PFA 16%
  - 50ml PHEM 2x
  - 20ml saccharose 0.6M
  - 2.5ml H_2O
  - 2.5ml PB 0.2M
- PFA 16%: Dissolved in 50% H_2O and 50% PB 0.2M while heated to 50°C.

pH was adjusted to 7.4 using NaOH.

Filter through filter paper. Storage at -20°C is possible otherwise it is stored at 4°C.

- PHEM 2x
  - 120mM PIPES
  - 50mM HEPES
  - 20mM EGTA
  - 4mM $MgCl_2$

  dissolved in 50% H_2O and 50% PB 0.2M – pH was adjusted to 6.9
- Saccharose 0.6 M in 50% H_2O and 50% PB 0.2M

4.6. Animal treatment:

Aralar deficient animals were a kind gift of the laboratory of Prof. Jorgina Satrústegui:
These animals have been developed and published first by Jalil et al. [1].
slc25a12+/- (*Aralar*+/-) mice were housed with a 12-h light cycle and fed *ad libitum* on standard chow. Animals were sacrificed by cervical dislocation. All animal procedures were approved by European guidelines and guidelines of the Universidad Autonoma de Madrid (UAM).

4.6.1. Animal mating:

As aralar knock out mice are not viable enough time for being fertile aralar (+/-) females were mated with aralar (+/-) males for obtaining aralar (-/-) embryos and also (+/+) littermates of exactly the same age. The genotype of each embryo and also of the embryo derived primary cultures and ESC cultures was determined individually. Detection of vaginal plug was considered as embrionic day 0.5 (E0.5) postconception. At least three different embryos were used for each immunohistochemical staining.

Embryos or fetuses were removed from the uterus and their crown-rump length (CRL) was recorded. Specimens of the following average CRLs were selected: E11.5: 7-8mm, E13.5: 9 mm, E15.5: 16,13 mm.

4.6.2. Fixation and cutting of mouse brains and mouse embryos for immunohistochemistry:

After dissection of the embryo it can be stored for some minutes in cold PBS.
Then the embryos were transferred to paraformaldehyde 4% for two, four or 24 hours. Afterwards they were cryo-protected in firstly 20% and then 30% sucrose/PBS until the embryos were saturated and sank to the bottom of the tubes.
The tissue was embedded in O.C.T™ Compound (Tissue-Tek cat. no: 4583) using self made peel away molds and stored at -70°C until sectioning. Serial sections, in the sagittal planes, were cut at 14 µm on a cryostat. From animals older than E13 only heads were embedded younger embryos were embedded, as a whole. Prior to sectioning the olfactory bulbs, coronal sections were first oriented according to the nasal passages. Left-right and dorsal-ventral orientation were adjusted as needed. Final adjustments to the orientation were made in the region of the striatum. Then the orientation was maintained for the remaining sections.

The serial sections were thaw-mounted onto Menzel Superfrost Ultra Plus Microscope slides in sets of 8 upto 12 slides. Once the sections had dried onto the slides they were immediately stored in a slide box at -70°C until they were stained.

4.7. Genotyping:

4.7.1. Isolation of Nucleic acids from cultured cells (High pure PCR Template Preperation Kit, Roche, cat. no: 11796828001)

Adjustment of sample volume:
Medium with 10^4 - 10^6 cultured mammalian cells were centrifuged and the pellet was resuspended in 200µl PBS.

Sample lysis and DNA Binding:
To a nuclease free 1,5ml microcentrifuge tube 200µl of sample material, 20 µl of binding buffer, 40µl of proteinase K (reconstituted) were added, mixed immediately and incubated at 70°C for 10 min. Then 100µl of isopropanol were added and the well was mixed.
After inserting one High Filter Tube in one Collection Tube the sample was pipetted into the upper buffer reservoir of the filter Tube. The High Pure Filter Tube assembly was inserted into a standard table top centrifuge and centrifuged for 1 minute at 8,000 x g.
Afterwards sample was proceeded as described in Washing and Elution below.

4.7.2. Isolation of Nucleic Acids form mammalian tissue

Sample lysis and DNA binding:
25-50mg of tissue were used. Before starting the purification reaction, the Elution Buffer was warmed up to 70° C. To a nuclease-free 1,5ml micro centrifuge tube 25-50mg of sample material was added. Then 200µl Tissue Lysis buffer and 40µl proteinase K (reconstituted) were added and mixed immediately. This mixture was incubated for one hour at 55°C or until tissue was digested completely. The yield of nucleic acids was increased by cutting the sample with a scalpel in small pieces before incubation. 200µl binding buffer were added and mixed immediately. The mixture was incubated for ten minutes at 70°C. 100µl isopropanol (2-Propanol) was added and mixed well. Insoluble tissue segments were drawn out of the sample into a 1ml disposable pipette tipp. One High Filter Tube was inserted in one collection tube. The remaining liquid sample was pipeted into the upper buffer reservoir of

the Filter tube. The entire High Pure Filter Tube assembly was inserted into a standard table-top centrifuge and centrifuged for one minute at 92000 rpm (8,000 x g).

Washing and Elution:

The Filter Tube was removed from the collection tube and the flow through liquid in the Collection Tube was discarded. The Filter Tube was combined with a new Collection Tube. 500µl Inhibitor Removal buffer rare added to the upper reservoir of the Filter Tube and centrifuged for one minute at 92000 rpm. The Filter tube was then removed from the Collection Tube and combined with a new Collection Tube. 500µl washing Buffer was added to the upper reservoir of the Filter Tube and centrifuged for one minute at 92000 rpm. The Filter tube was then removed again from the Collection Tube and combined it with a new Collection Tube. 500µl washing Buffer was added to the upper reservoir of the Filter Tube and centrifuged for one minute at 92000 rpm. After discarding the flow through liquid the High Pure asambly was centrifuged for additional ten seconds at full speed before discarting the Collection Tube which ensured removal of residual Wash Buffer. To elute the DNA the Filter Tube was inserted into a clean, sterile 1,5ml micro centrifuge tube and 200µl prewarmed Elution buffer was added to the upper reservoir of the Filter Tube and centrifuged for five minutes at 92000 rpm.

By then the micro centrifuge tube contained the eluted DNA. The eluted DNA was either used immediately or stored at +2 to +8° C or at -25°C for later analysis.

4.7.3. Evaluation of quantity and quality of extracted DNA using Nanodrop-1000 UV/Vis-spectral photometer:

For making sure that the extracted samples contained enough DNA and were not contaminated by proteins spectralphotometry was used. After inserting a blank, in this case elution buffer of the extraction kit, 1µl of sample was inserted and measured for A 260 and A280. One D260 value corresponds 50µg of DNA, therefore the constant used by the program was 50. 260/280 ratios in the range of 1.8 to 2 were accepted regarding protein contamination. For PCR reactions quantities in the range of 100ng to 500ng were used:

4.7.4. PCR:

Used primers:
INT 13B: 5´-ACCACCACCAGCGTGTCAGC-3´
LTR: 5`-GTTCTCTAGAAACTGCTCAGG-3`
INT 13Fi: GATGTGAGAACTCACCAGTGT-3`
13Fi and 13B: produce wildtype (271 bp)
LTRF3 and 13B: produce knock out (406 bp)

dNTPs: dATP, dCTP, dGTP, dTTP (10mM)
100mM stocks are stored and diluted to 10mM.

Mixture of Oligos: LTRF3:13B:13Fi →1:1:0.5

PCR Program:

- initial denaturation	5 min 94°C	
- Cycles 30x:	1 min 94°C	
	30 sec 58 °C	
	30 sec 72°C	
- final extension:	5 min 72°C	

Mastermix:		Final concentration
Buffer 10x	2.5µl	
Mix Oligos	2µl	
$MgCl_2$	1µl	2mM
dNTPs	0.5µl (10mM)	2mM
dH_2O	12µl	
Total:	18µl	

Mix:		
	18µl Mastermix	
	5µl DNA	
	2µl Polymerase (Biotools 50mM 1:3 in dH_2O)	
Total:	25µl	

One negative control consisting of distilled water instead of DNA and positive controls for WT, KO and HET were always included.

4.7.5. Gel electrophoresis:

A 1,5% Agarose gel was prepared with TAE (150ml + 4µl Ethidium Bromide). Samples were mixed with loading dye (bromphenolblue). 20µl of sample were loaded. As a marker 2 µl of ϕ 29 DNA/Hind III (500ng/µl) (housemade) were used.
The electrophoresis was run at 100 V for approx. 50min.

Loading dye:
0.25% Bromophenol blue
40% saccharose
TAE 50x (1L):
242g TRIS
57ml cold acetic acid
100ml EDTA 0.5M
ad 1000ml H_2O

4.8. SDS Page and Western Blot

4.8.1. SDS PAGE

Gels were usually prepared one day before usage and stored at 4°C in a humide chamber for better polymerization. Before gel preparation gel boxes were cleaned and the according glass slides were inserted and fixed diagonally. Afterwards the boxes were confirmed to be leak proof and indications at the depth of the slot comb were drawn.
Gels were prepared according to the molecular weight of the proteins that were going to be detected. In case of aralar which has 74 kDA 10% gels were prepared.
The separation gels were prepared as follows in chronological order adding the radical starters for polymerisation in the end.

	6%		8%		10%		12%		15%	
	1 Gel	2 Gels	1 Gel	2 Gels	1 Gel	2 Gels	1 Gel	2 Gels	1 Gel	2 Gels
H_2O mQ	2.75	5.5	2.5	5	2.25	4.5	2	4	1.75	3.5
Acrylamide/Bisacrylamide mix mix 40%	0.75	1.5	1	2	1.25	2.5	1.5	3	1.87	3.75
Tris-HCl 1.5M pH8.8	1.3	2.6	1.3	2.6	1.3	2.6	1.3	2.6	1.3	2.6
SDS 10%	0.05	0.1	0.05	0.1	0.05	0.1	0.05	0.1	0.05	0.1
APS 10%	0.05	0.1	0.05	0.1	0.05	0.1	0.05	0.1	0.05	0.1
TEMED	0.005	0.01	0.01	0.01	0.005	0.01	0.01	0.01	0.01	0.01

After filling in the first gel until approximately 1cm below the indication (approx. 4ml) the gel was covered with isopropanol until it polymerized. After polymerization the isopropanol was taken off for filling in the stacking gel.

The stacking gels were prepared as follows :

Stacking gel:	4%	
	1 Gel	2 Gels
H20 mQ	2.3	4,6
Acrylamide/Bisacrylamide mix 40%	0.3	0.6
Tris-HCl 0.5M pH6.8	0.38	0.76
SDS 10%	0.03	0.06
APS 10%	0.03	0.06
TEMED	0.005	0.01

One liter of Tris-Glycin buffer was prepared.

Desired amounts of samples and unstained molecular weight marker were mixed with laemmli buffer and denatured for 3-5 minutes at 100°C. 10 – 20µl of samples and Kaleidoscope Prestained Standards marker from BIO-RAD (cat. no: 161-0324) were loaded. Unused slots were filled with H_2O+laemmli buffer. Buffer was added into the gel electrophoresis box. Electrophoresis was run at at 50 - 70 V until samples enter separation gel. Further on voltage was increased to 100 up to 200V. Electrophoresis was stopped when the dye front had started running off the bottom of the gel.

<u>Tris-HCl 1.5M (pH-8.8) 500 ml</u>

90.82g in 350ml dH_2O

Adjust pH 8.8 with HCl

ad 500ml

<u>Tris-HCl 0.5M (pH-6.8) 500ml</u>

30.27g Tris in 350ml H_20

Adjust pH 6.8 with HCl

ad 500ml

<u>SDS 10%</u>

25g SDS in 250ml H_20 cover with aluminium paper and leave it o/n.

<u>10% APS</u>

A pinch of APS was weighted and as many µl of H_2O as mg APS were added.

Laemmli buffer 2x

2.5ml Tris-HCl pH 6.8 0.5M

4ml SDS 10%

0.3ml EDTA 0.5M pH8

1ml β-mercaptoethanol 98%

2. 29ml glycerol

a little bit of bromophenol blue 20mg

Electrophoresis buffer5X 1L

15.12g Tris

72.06g glycine

5g SDS

Acrylamide mix

29.2 g acrylamide and 0.8 bisacrylamide were weighted on aluminium foil paper in a 250 ml flask using a spatula and a mouth cover. 50ml dH_2O were added and left wrapped in aluminium paper shaking o/n.

100ml dH_2O milliQ were added then the solution was filtered.

TBS 10x (1l)

NaCl 1.5 M 87,66g

Tris-HCL 0.1 M 12,15g

Ad 1000ml dH_2O adjust pH to 7.5

Ponceau red

0.2% Ponceau red in 3% acetic acid

1g ponceau red in 7.5ml acetic acid

4.8.2. Transfere using inmobilon-P filter

Transfer buffer

3.02g Tris

14.42g glicine

Disolve in 600ml dH_2O

ad 800ml dH_2O

at last 200ml pure metOH were added.

After finishing the electrophoresis, the gel was taken out, the stacking gel cut off and the separation gel rinsed in transfer buffer. One inmobilon-P filter was rinsed in 100% methanol for 15 seconds before incubating it in dH_2O for two minutes and passing it for at least 5 minutes to transfer buffer. The transfer cassette was mounted as follows from the positive pole side towards negative pole side (red):
White sponge, filter paper, inmobilon-P filter, gel, filter paper, white sponge rinsing everything in transfer buffer avoiding bubbles. The sandwich and a block of ice were inserted into a BioRad Mini Protean tetra cell blotting box containing already a rotary magnet and the same was filled with transfer buffer covering the upper electrode. The box was located on a magnetic stirrer. The transfer is usually run at 100V, Amperase indicating ~300A for one hour. If Ampere value was above 500 transfer buffer was changed. After transfer the filter was stained in ponceau red solution during 1-2 minutes for a reversible stain of proteins. For destaining the filter was washed with dH_20. The filter was activated in methanol for 10 seconds and dried for 15 minutes between two filters before immunostaining or storage at –20°C.

4.8.3. Antibody incubation using Inmobilon- P filter

Blocking solution:
BSA 1% (or 5% milk) Tween20 0.05% in PBS.
Filter was incubated with primary antibody (Aralar 1:1000) diluted at appropriate concentration in blocking solution for one hour at room temperature without prior blockage (5ml for one membrane). Primary antibody was reuptaken and could be stored for up to one week at -20°C. Membrane was washed twice in PBS before incubation with a horse radish peroxidase (hrp) coupled secondary antibody (GAR P/O goat α rabbit Nordic Immuno 1:5000) diluted in blocking solution for 30 minutes (5ml for one membrane). Membrane was washed again twice with PBS for 10 seconds and was then incubated in PBS Tween20 0.5% for permeabilisation until membrane looks transparent.
For detection membranes were incubated with HRP substrate solution (Western Lightening Chemiluminescence Reagent, Perkin Elmer Las, cat. no: NEL102) for 5 min and exposed to film (AGFA).

4.8.4. Stripping for Western blot

Sodium acid: Eliminates peroxidases of secondary antibodies.
TBS + sodium acid 0.2%
Membranes were washed in TBS for 10 minutes and incubated for 20 minutes at RT in stripping buffer. Afterwards they were washed twice in TBS for ten minutes at RT. For confirmation of signal elimination one revelation test with ECL reaction was done. Before incubation with another antibody membrane was dried or a blocking step was added.

4.9. Quantification and data analysis:

Images were obtained by an inverted Leica DM IRB microscope capturing images by a Leica DC 100 camara operated by Adobe PhotoShop 5.0 (Pantone Inc) and a vertical Axioskop2 plus microscope (Zeiss) coupled to a Coolsnap FX color camara navigated by Metavue 5.07 (Universal Imaging).
They have been processed in Adobe Photoshop CS3 extended and Image J.
For area quantification relative values of expression area against croped areas of the same size were calculated.
For cell quantification in stained tissue sections cell number per captured field was counted from various comparable sections of at least 3 different embryos. Mean value of different sections for each single embryo was used for statistical analysis.
For mES and primary culture cell quantification cell number per captured field or colony number per total number of colonies were counted as indicated in individual graphs.
Statistical analysis was performed using SPSS /Statistica doing independent sample T- Test an non paramaetric Mann Whitney test with a “n” of at least 3 and p-values ≤ 0.05.

5. Results

5.1. Set up of Immunohisto and Immunocytochemistry

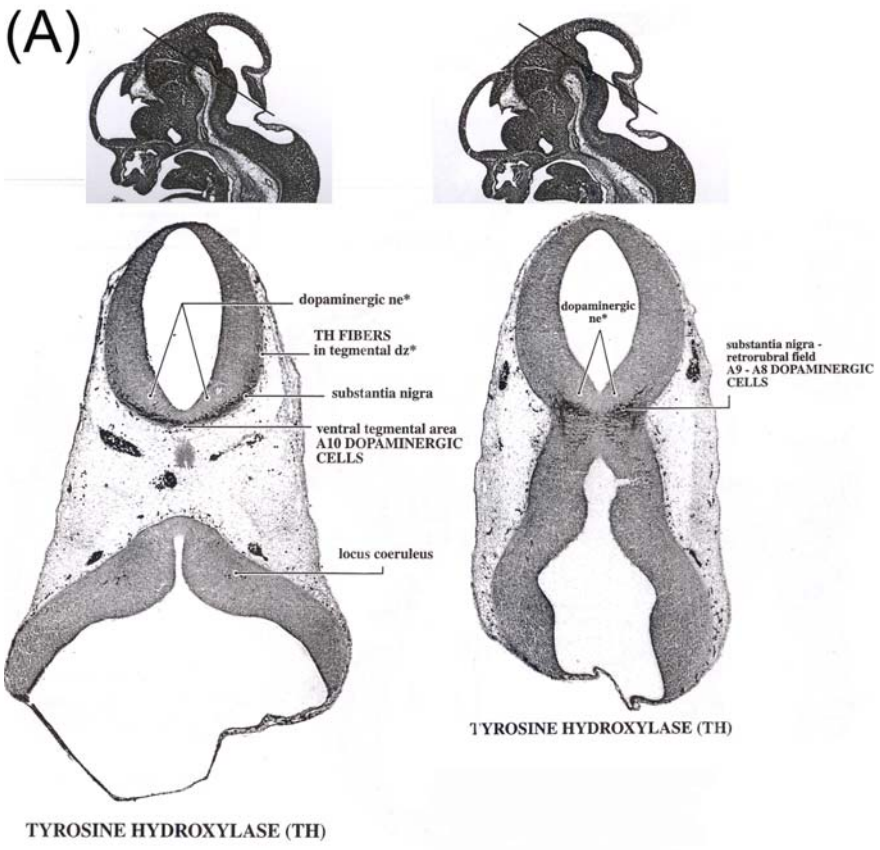
In a first attempt, appropriate PFA fixation time, embedding angle thickness of cryostat sections and antibody concentrations were determined in CD1 control brain slices of E13.5 and E14.5 embryos.

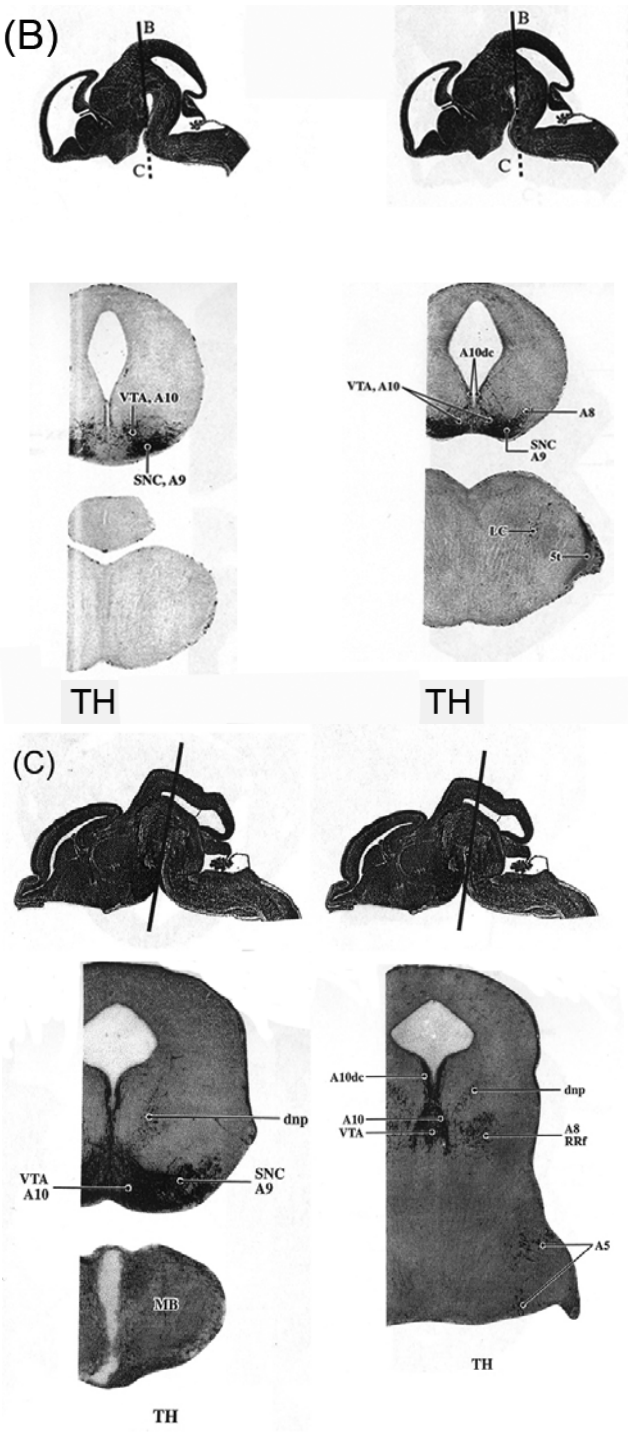
Different antibody concentrations were tested on brain slices cut in different angles, that were fixed for different time periods (either four hours or o/n).

No major differences in the outcome of immunohistochemistry could be observed between embryos fixed for o/n or four hours PFA. Therefore fixation time was fixed at four hours.

A table with determined antibody concentrations is attached in materials and methods.

Embedding has been optimised to obtain slices that show a similar tyrosine hydroxylase expression as seen in Fig. 6 on immunohistochemical stainings of cryostat mice embryo plates taken from Jacobowitz et al. 1998 Chemoarchitectonic Atlas of the Developing Mouse Brain [45]A9 and A10 areas of ventral mesencephalon.



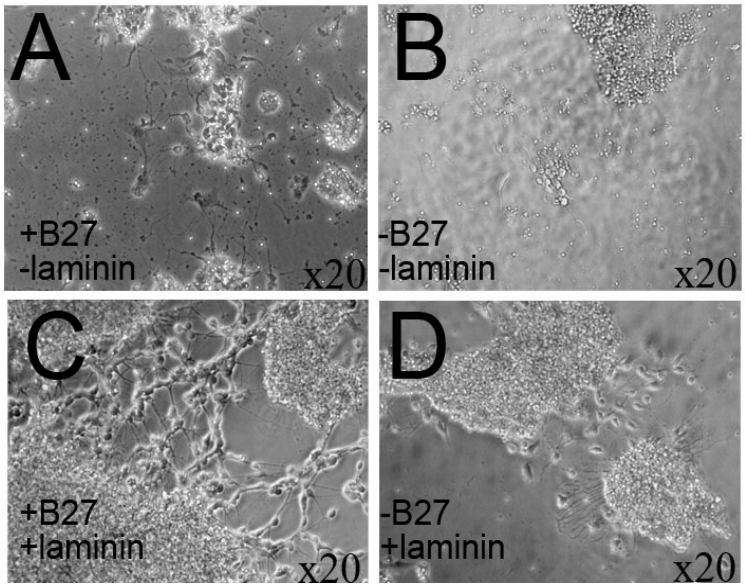


6) **Fig. 6: Ventral mesencephalic dopaminergic neurons of A9 and A10 area**

stained for Tyrosine Hydroxylase (TH) on cryostat sections of (A) E11.5, (B) E13.5 and (C) E15.5 mouse embryos. dnp dorsal noradrenergic pathway, MB mamilary body, RRf retrorubral field, SNC substancia nigra compacta, VTA ventral tegmental area.

5.2. Set up of optimal primary culture conditions (B27/-B27)

For the determination of optimal primary culture conditions ventral mesencephalic precursors have were seeded on culture dishes precoated with only polylysine or culture dishes previously treated with polylysine and laminin and in medium with or without (w/o) B27. After four days cells have been monitored and phase contrast photos were taken.



7) **Fig. 7: Phase contrast images of primary culture of dopaminergic precursors from the ventral mesencephalon.**

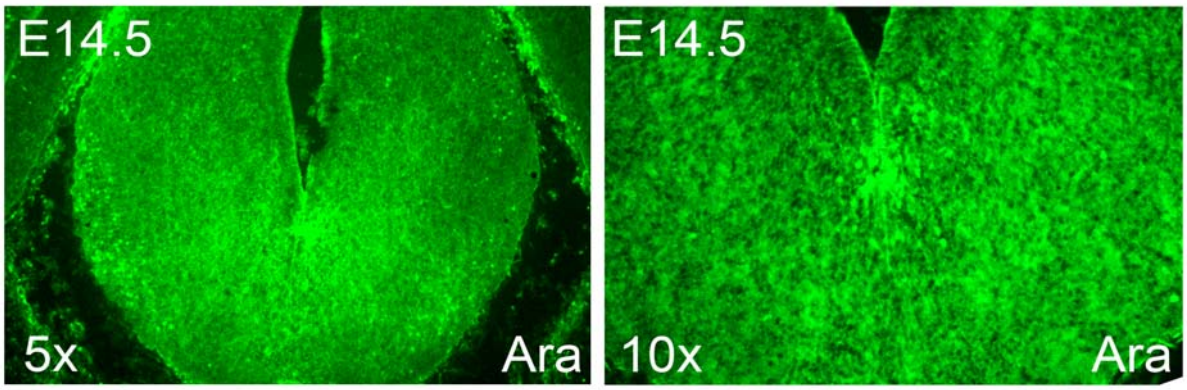
(A) N2 medium supplemented with B27 and wells treated only with polylysine; (B) N2 medium only and wells treated only with polylysine; (C) N2 medium supplemented with B27 and wells treated with polylysine and laminin; (D) N2 medium w/o B27 and wells treated with polylysine and laminin

As can be observed in Fig. 7 cell survival has been found best in culture dishes previously treated with polylysine and laminin and seeded in medium containing B27 (Fig. 7C) . Therefore these conditions were maintained for further primary cultures.

5.3. Analysis of Aralar expression in different experimental models in CD1 control mice

5.3.1. Expression in ventral mesencephalon of E14.5 mouse embryos in vivo

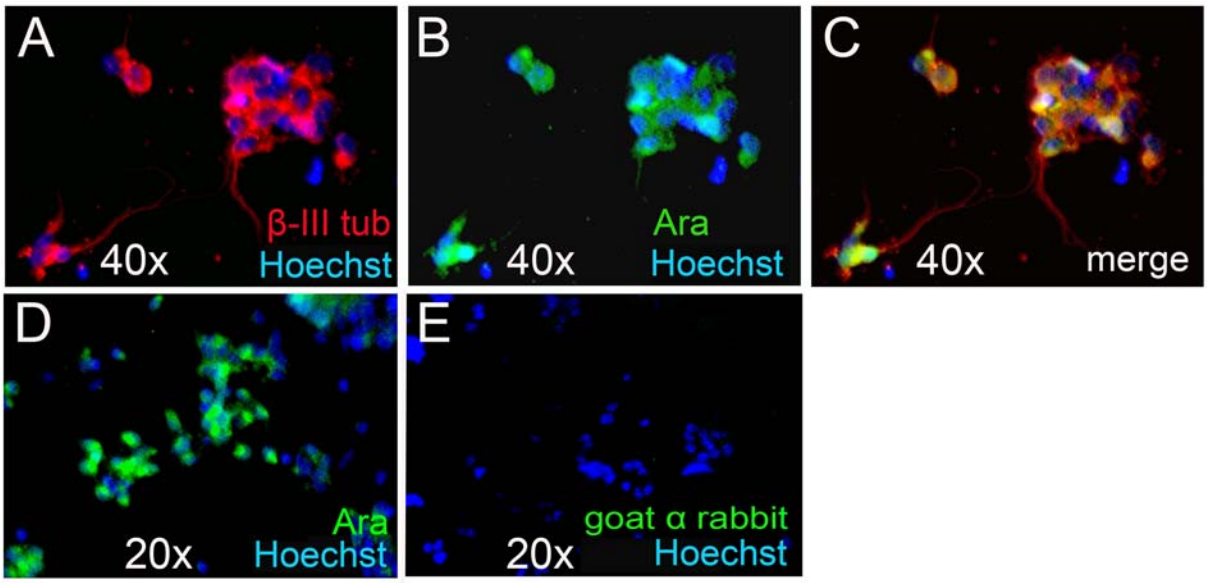
So far Aralar expression during embryonic development had not been studied in detail. Therefore wild type control embryos (CD1) were screened for Aralar expression in the time window of interest, as to say during dopaminergic neurogenesis. As expected Aralar was shown to be expressed abundantly in cryostat sections of ventral midbrain of E14.5 mouse CD1 control embryos (Fig. 8).



8) **Fig. 8: Immunohistochemical staining of CD1 ventral mesencephalic cryostat section at E14.5 for Aralar at 5x and 10x magnification.**

5.3.2. Expression in ventral mesencephalon of CD1 E13.5 mouse embryos in primary culture

Also primary culture of dopaminergic precursors derived from embryos at the age of E13.5 showed abundant Aralar expression in most of the cells including neurons (β-IIItub positive) (Fig. 9C). This expression was detected at cytoplasmatic level as could be expected from a mitochondrial protein (Fig 9B).The specifity of the antibody directed against Aralar was tested as well as can be seen in Fig. 9E.

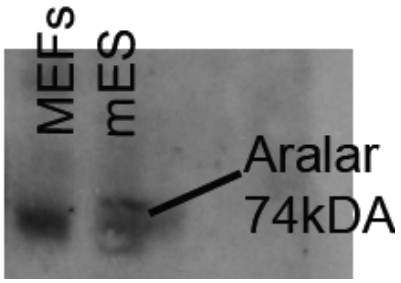


9) Fig. 9: Immunocytochemistry of primary culture derived from E13.5 CD1 mouse embryos.

(A) β-III tubulin, Hoechst 40x (B) Aralar, Hoechst 40x (C) Aralar, β-III tubulin, Hoechst merged 40x (D) Aralar, Hoechst 20x (E) negative control for secondary antibody Alexa 488 goat α rabbit, Hoechst 20x.

5.3.3. Protein expression in mouse embryonic stem cells (R1 line) in proliferation

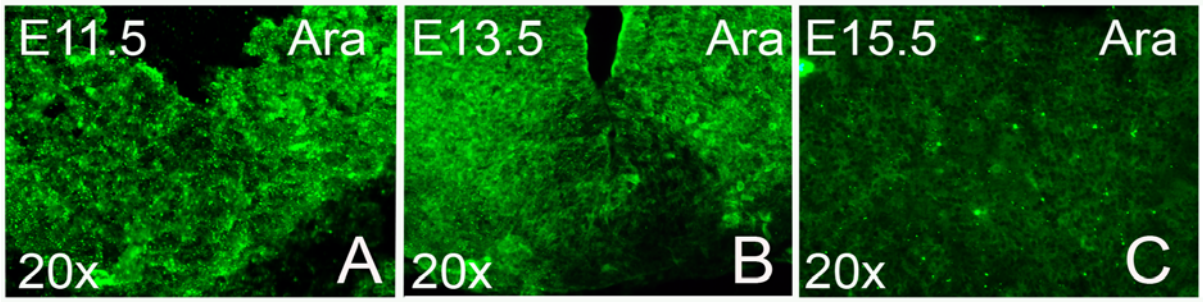
Furthermore the expression of Aralar during pluripotent state in proliferating mouse embryonic stem cells (R1 cell line) was analysed by western blot using mouse embryonic feeder cells (MEFs) of CD1 mice as a positive control. Also in this model abundant aralar expression could be detected (Fig10).



10) **Fig. 10: Western blot showing Aralar expression of mES *cells***

5.4. Analysis of ventral mesencephalic dopaminergic neurons during embryogenesis in vivo

In order to elucidate a possible implication of Aralar during dopaminergic neurogenesis, cryostat sections of aralar (+/+) and aralar (-/-) mice have been obtained and stained immunohistochemically. Before comparison of the two genotypes for expression of defined markers of dopaminergic neurogenesis, the presence of aralar at the embryonic ages and brain area of interest in the used mouse strain has been confirmed (Fig.11).

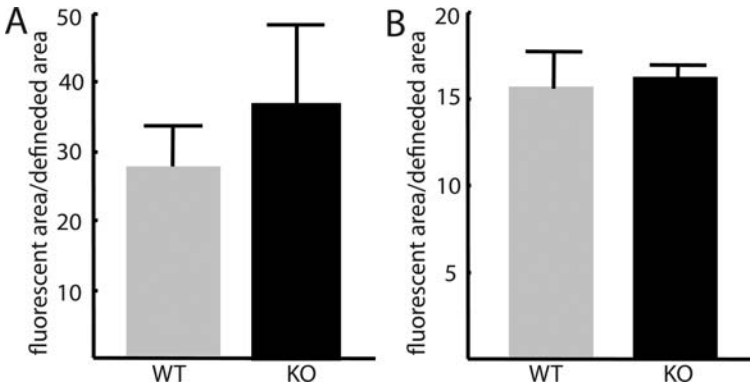


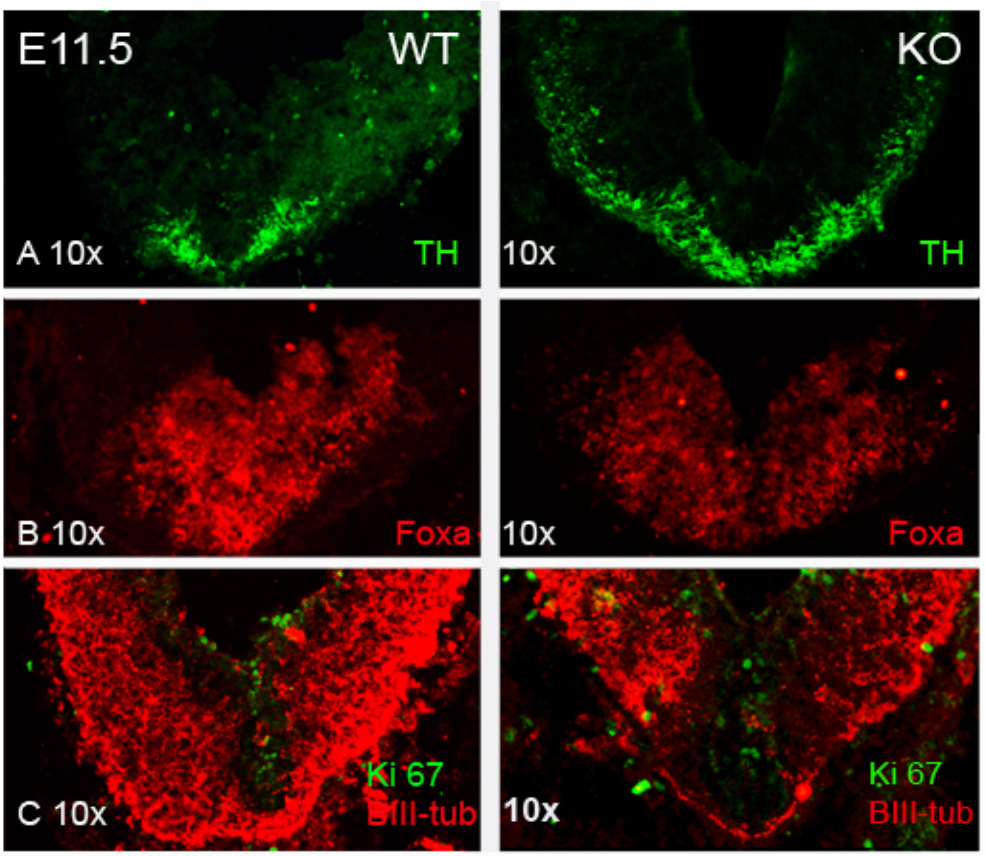
11) **Fig. 11: Immunohistochemistry for Aralar in cryostat sections of mouse embryonic midbrain of aralar WT mice at different ages**
(A) E11.5 20x; (B) E13.5 20x; (C) E15.5 20x.

For determination of changes in the quantitative outcome of dopaminergic neurogenesis Tyrosine hydroxylase expression was studied. Moreover Foxa 2 expression was looked at for investigating possible changes in the expression of pro dopaminergic transcription factors, that might lead to differences in cell fate programming.To gain insight in possible effects in cell cycle activity, expression of Ki67 versus β-III tubulin, a neuronal marker, were studied. Evaluation of Ki67 was done by cell counting in a predefined ventral mesencephalic area, whereas Foxa 2 and Tyrosine Hydroxilase have been analysed by relative measuremet of expression area against a predefined cropped ventral mesencephalic area.

At embryonic day E11.5 no statistical significant differences between knock out (KO) and wild type (WT) mice could be detected neither for TH nor for Foxa. Ki67 has not been counted at this age, as almost all cells are positive due to the required proliferative state of development (Fig. 12, Graph 1).

II. Graph 1: Quantification of comparative immunohistochemistry of E11.5 ara WT and KO mice.
(A) TH expression, 10x cropped area 1.3 x1, for graph: values /1000 (B) Foxa2: 10x cropped area 1x1, for graph: values /10000.



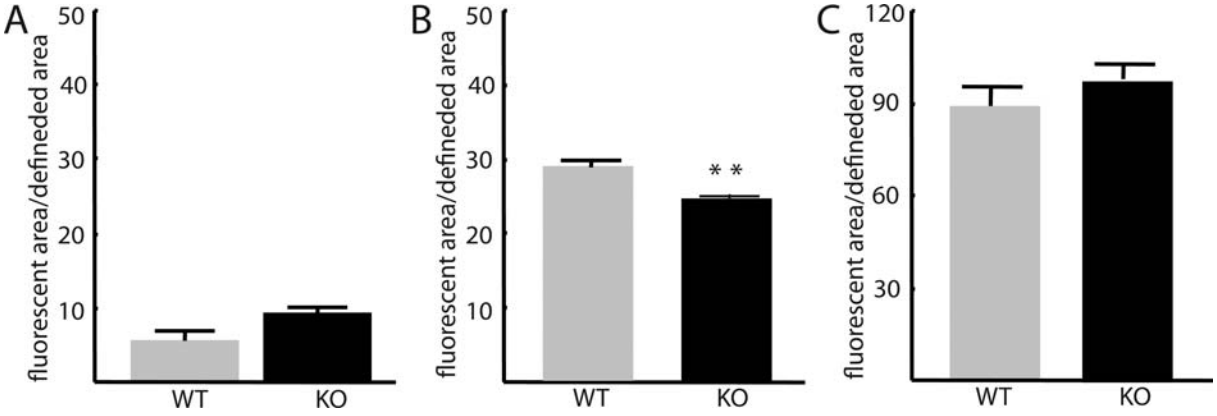


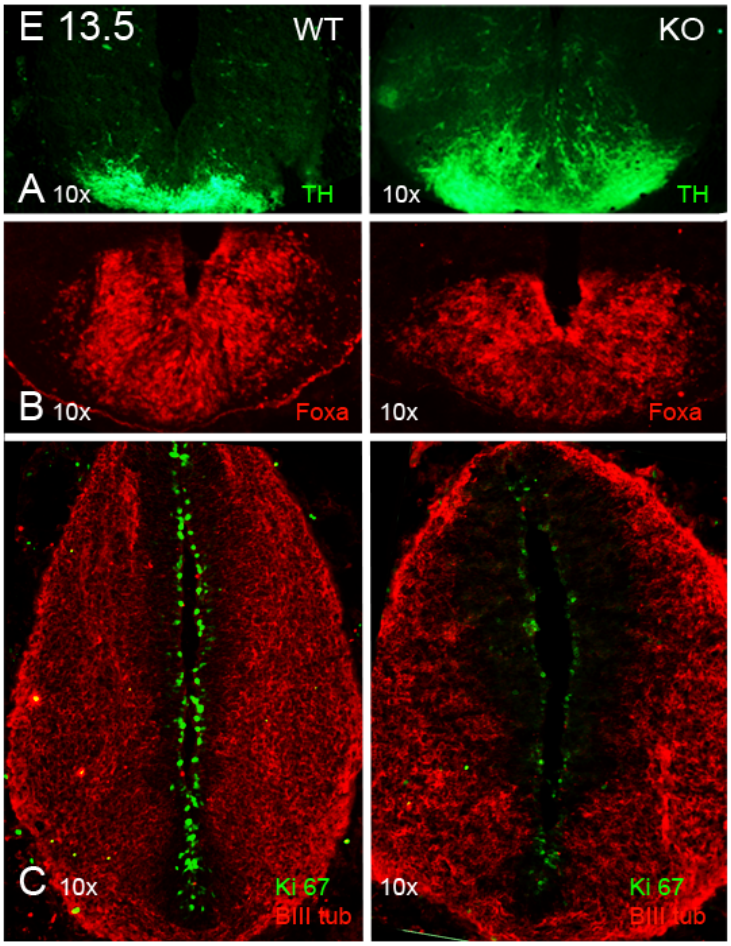
12) **Fig. 12: Immunohistochemical analysis of ventral mesencephalon at day E11.5.**

TH immunoreactivity for WT and KO can be observed in (A) at a magnification of 10x; Foxa2 immunoreactivity at 10x in (B); and Ki67/β-III tubuline immunoreactivity at 10x in (C).

At embryonic day E13.5 at first sight aralar knock out slices seemed to show increased TH expression, but decreased Foxa2 and Ki67 expression. Still further statistical analysis revealed that only decreased Foxa2 expression in aralar knock out mice was statistically significant (Fig. 13, Graph 2).

III. Graph 2: Quantification of comparative immunohistochemistry of E13.5 ara WT and KO mice.
*(A) TH expression: 10x croped area 0.9x 0.55, for graph: x100; (B) Foxa2: 10x cropped area 1x1, for graph: /10000 WT: 28,797 +/- 0,65731885, KO: 24,563 +/- 0,14113565, p≤0.003248** (C) Ki67: 20x not cropped.*

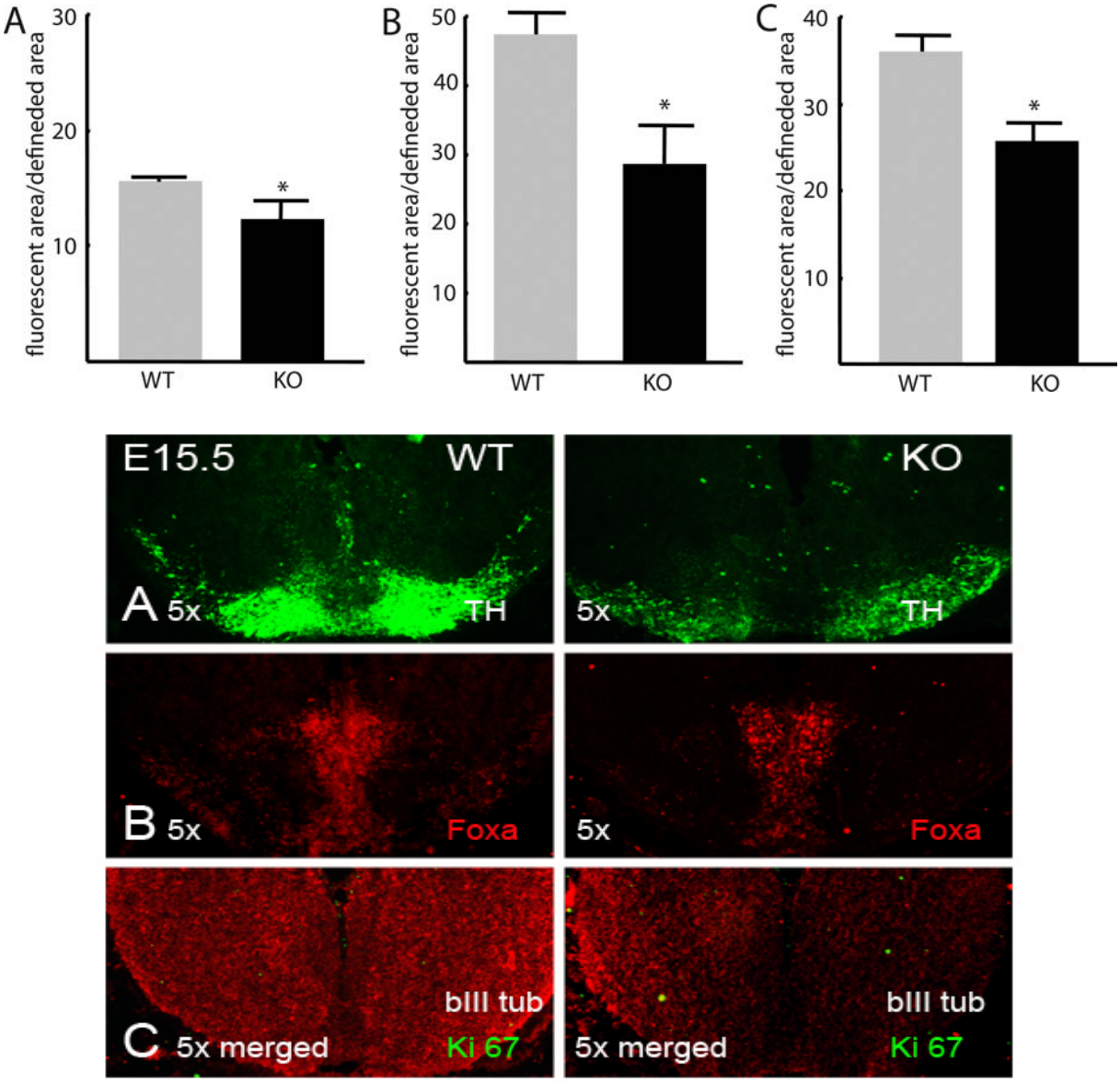




13) **Fig. 13: Immunohistochemical analysis of ventral mesencephalon at day *E13.5*.** *(A) TH in WT and KO mice 10x (B) Foxa2 in WT and KO mice 10x (C) Ki67/βIII tub in WT and KO mice 10x.*

At embryonic day E15.5 WT sections showed more TH expression in comparison to KO sections. This impression could be confirmed as significant by statistical analysis. Decreased Foxa 2 expression in aralar knock out sections is also maintained, but only reaches statistical significance doing non parametric Mann Whitney test. Moreover statistical analysis revealed significantly reduced Ki67 expression in aralar knock out sections at this age (Fig. 14, Graph 3).

IV. Graph 3: Quantification of comparative immunohistochemistry of E15.5 ara WT and KO mice. (A)TH: 5x cropped area 1.3x0.7, for graph: /10000, WT: 15,549 +/- 0,23126728 KO: 12,302 +/- 1,18884, p≤0,036472* (B) Foxa2: 10x cropped area 1.3 x 1, for graph: /10000, WT: 47,409 +/- 4,15435285 KO: 28,709 +/- 5,395658, p≤0,049535* (Man Whitney) (C) Ki67: 20x not cropped, WT: 36,03 +/- 1,78407773 KO: 25,61 +/- 2,07896609, p≤0,019045*.



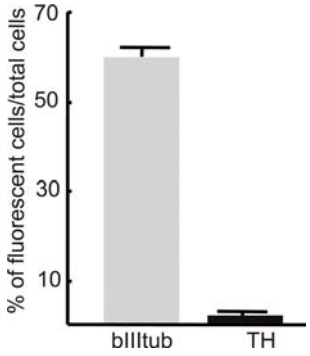
14) **Fig. 14: Immunohistochemical analysis of ventral mesencephalon at day *E15.5.*** *(A)TH 5x (B) Foxa2 5x (C) Ki67 5x.*

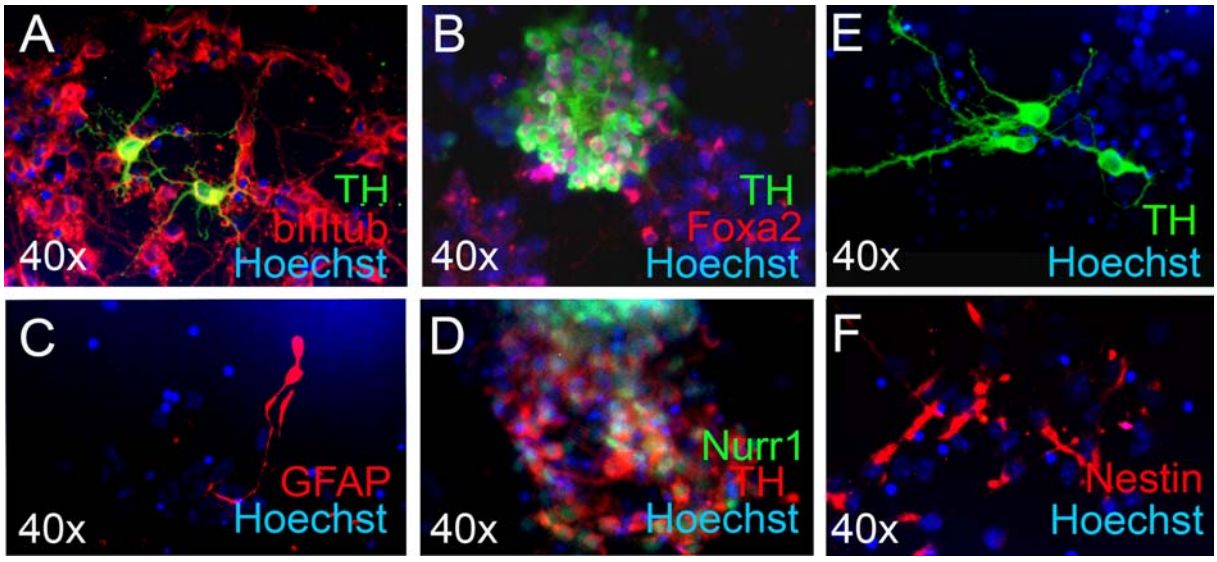
5.5. Analysis of ventral mesencaphalic dopaminergic neurons during embryogenesis in vitro

5.5.1. Phenotype characterization of WT Ara E13.5 primary culture of ventral mesencephalon

Ventral mesencephalon of wildtype aralar embryos was dissected as described in materials and methods and cultured for 3 to 4 days in medium containing B27. Then cells were fixed to subsequently stain them for proneuronal (β III tubulin), neural (Nestin), glial (GFAP) and dopaminergic (Tyrosine Hydroxylase, Foxa, Nurr1) immunocytochemical markers, thereby confirming their ventral mesencephalic characteristics (Fig. 15).

V. Graph 4: Quantification of phenotype characterization of primary culture VM E13.5 WT ara



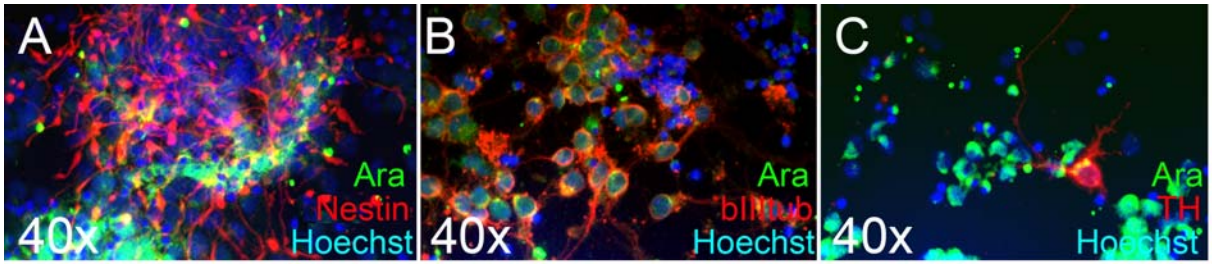


15) **Fig.15: Phenotype characterization of WT ara 13.5 primary culture of VM. Immunocytochemistry showing expression of** *(A) TH, βIIItub 40x; (B) TH, Foxa2 40x; (C) GFAP 40x; (D) TH, Nurr1 40x; (E) TH 40x; (F) Nestin 40x. Nuclei were counterstained by Hoechst.*

Almost no GFAP positive cells could be detected, which confirmed the neuronal character of the primary culture. Primary culture of day E13.5 was mainly positive for Nestin, β III tubulin and Tyrosine Hydroxylase. Most of the TH positive cells showed also immunoreactivity for Foxa2 and Nurr1, which indicates their dopaminergic fate decision (Fig. 15, Graph4).

5.5.2. Confirmation of Aralar expression in WT Ara E13.5 primary culture of ventral mesencephalon

Before analysing possible differences in primary cultures between Ara (+/+) and Ara (-/-) mice dissections the presence of Aralar in the obtained WT Ara E13.5 primary culture of ventral mesencephalon was confirmed doing double immunocytochemistry stainings in different cellular subtypes.



16) **Fig, 16: Immunocytochemistry of WT ara E13.5 ventral mesencephalic primary culture.**
(A) Ara, Nestin 40x; (B) Ara, β-III tub 40x; (C) Ara, TH 40x. Nuclei were counterstained by Hoechst (blue).

Aralar expression in neural cells (positive for Nestin), neuronal cells (positive for , β-III tub) and dopaminergic cells (positive for TH) could be confirmed (Fig. 16), suggesting a possible role of Aralar in all these cell subtypes.

5.5.3. Comparative immunocytochemical study of Ara (+/+) and Ara (-/-) ventral mesencephalic primary cultures (E13.5 and E14.5)

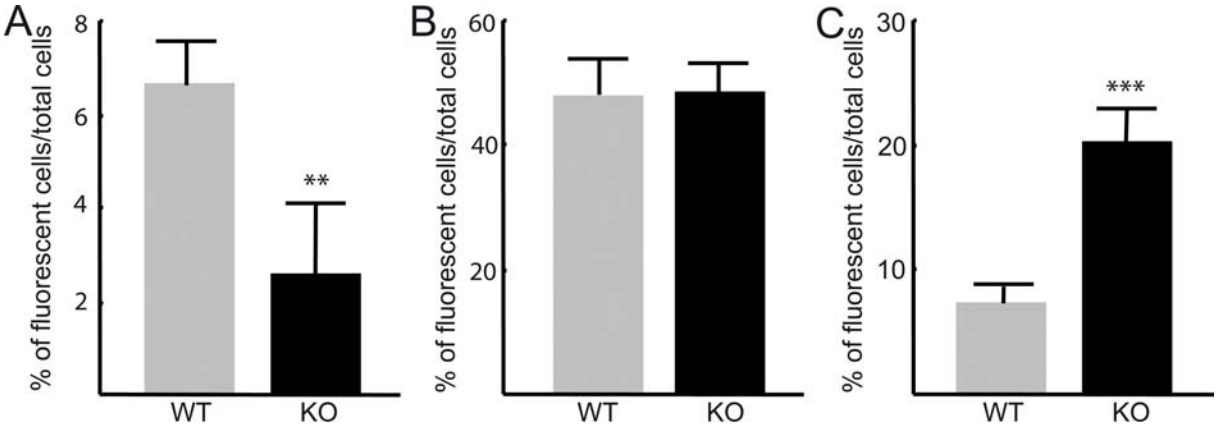
In order to do a comparative immunocytochemical study between Ara (+/+) and Ara (-/-) primary cultures ventral mesencephalic regions of respective E13.5 and E14.5 embryos have been dissected and cultured for 4 days. Afterwards cultures have been fixed for subsequent immunocytochemistry.

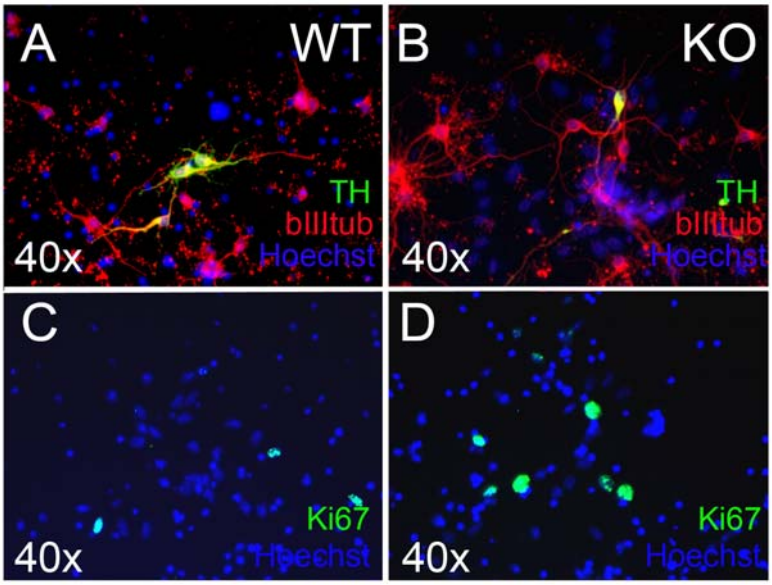
To gain insight in possible changes in cell death (apoptosis) and cellcycle activity expression of Ki67 and Caspase3 respectively were studied. Morevover β-III tubulin expression was looked at for investigating possible switches from neuronal to glial identity. Tryptophane hydroxylase was also applied, for excluding the possibility of differentiation into serotonergic

cells. TH expression was used to determine changes in the quantitative outcome of dopaminergic neurogenesis.

VI. Graph 5: Quantification of comparative immunocytochemistry of VM primary culture of WT and KO ara mice at E14.5

*(A) TH: WT:6,7+/-0,86922699 KO:3+/-1,41210751, p≤0,00484 ** (B) β III tub (C) Ki67: WT: 7,3+/-1,34989712 KO:20,3 +/- 2,56255081, p≤0,000053***.*





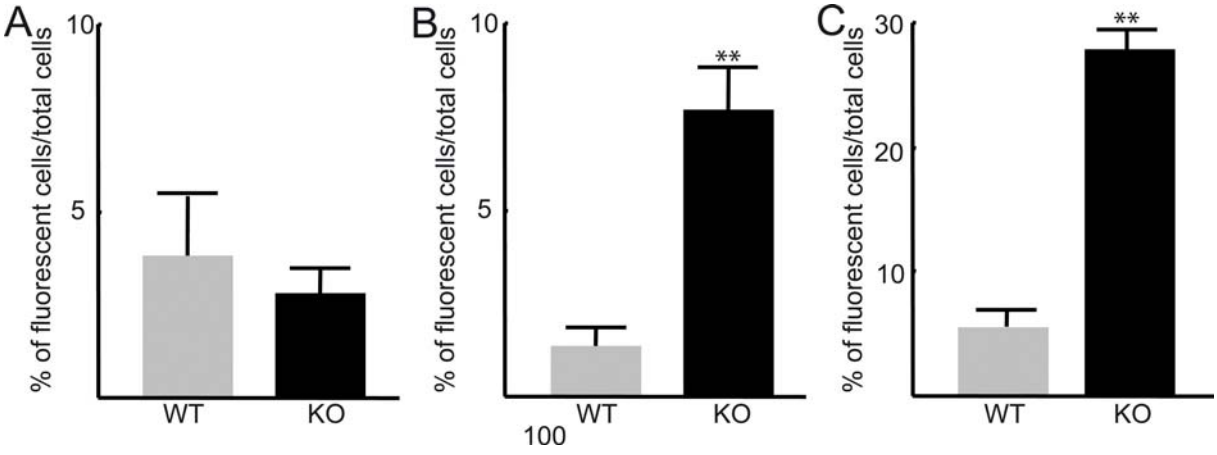
***17)* Fig. 17: Comparative immunocytochemistry of ventral mesencephalic primary culture WT and KO ara at E14.5**

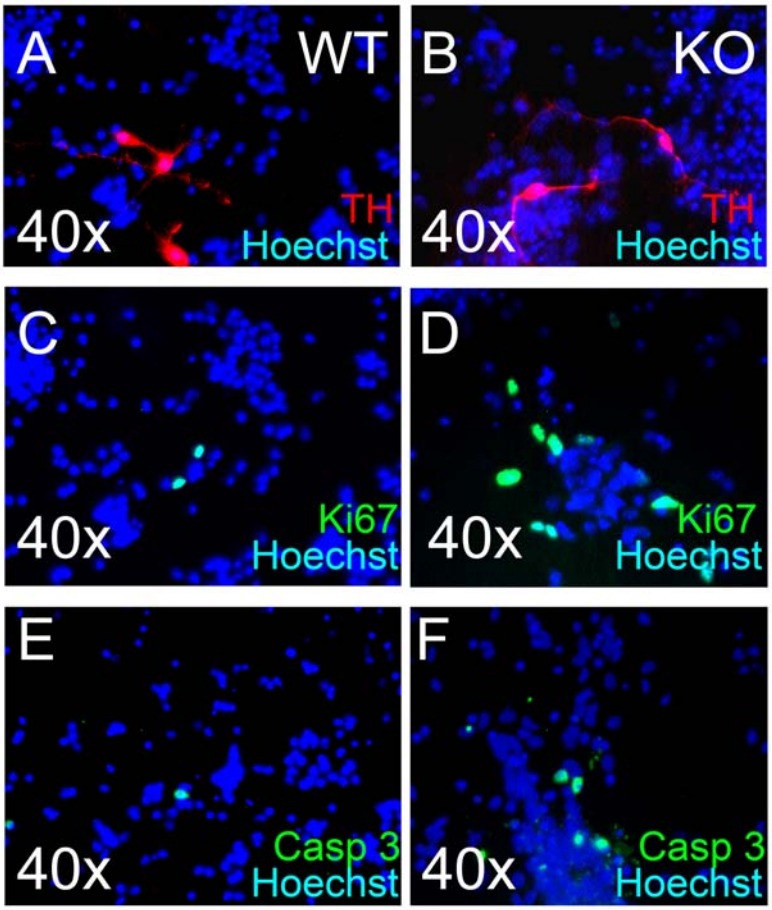
(A) WT TH, βIII tub 40x (B) KO TH, βIII tub 40x (C) WT Ki67 40x (D) KO Ki67 40x. Nuclei were counterstained by Hoechst (blue).

Ventral mesencephalic primary culture from embryonic day E14.5 revealed statistical significantly decreased TH expression in ara KO, β-III tub expression showed to be equal in ara WT compared to KO. Moreover cycling as shown by Ki67 stain was statistically significantly increased in ara KO cell cultures.

In a next step ventral mesencephlic primary culture at E13.5 was obtained, to obtain more insight into possible cellcycle differences that could lead to this outcome.

VII. Graph 6: Quantification of comparative immunocytochemistry of VM primary culture at E13.5
*(A) TH (B) Casp3: WT:1,3+/-0,44095855 KO: 7,7+/- 1,09290642, p≤ 0,000386**; (C) Ki67 WT: 5,5 +/ 1,44337567 KO: 27,83 +/- 1,42400062, p≤ 0,005792**.*





18) **Fig. 18: Comparative immunocytochemistry of ventral mesencephalic primary culture WT and KO ara at E13.5**

(A) WT TH 40x (B) KO TH 40x (C) WT Ki67 40x (D) KO Ki67 40x (E) WT Casp3 40x (F) KO Casp3 40x. Nuclei were counterstained by Hoechst (blue).

Also at embryonic day E13.5 statistically significantly elevated cell cycling as shown by Ki67 could be observed. Furthermore aralar knock out primary culture Casp3 activity significantly exceeded Casp 3 activity in wild type primary culture. For TH no statistically significant differences could be detected, still a trend to reduced TH expression in KO primary culture was observed (Graph 6). Almost no Tryptohphane positive cells could be observed neither in WT nor in ara KO primary culture (Fig. 18).

5.6. Mouse embryonic stem cell lines from Ara (+/-) mice

5.6.1. Generation of embryonic stem cell lines from Ara (+/-)

For obtaining wild type aralar and knock out aralar mouse embryonic stem cell lines heterozygoteous animals had to be mated, as knock out animals are not viable longer then 20 days. Therefore a modified protocol of Bryja et al. 2006 [44], was followed. From 3 pregnant females which permitted the extraction of 18 blastocysts 12 stable cell lines could be established (70% success).

5.6.2. Determination of Genotype of established cell lines

PCR

These cell lines have been grown on feeder free culture dishes for various passages to get rid of mouse embryonic fibroblast cells and were then genotyped.

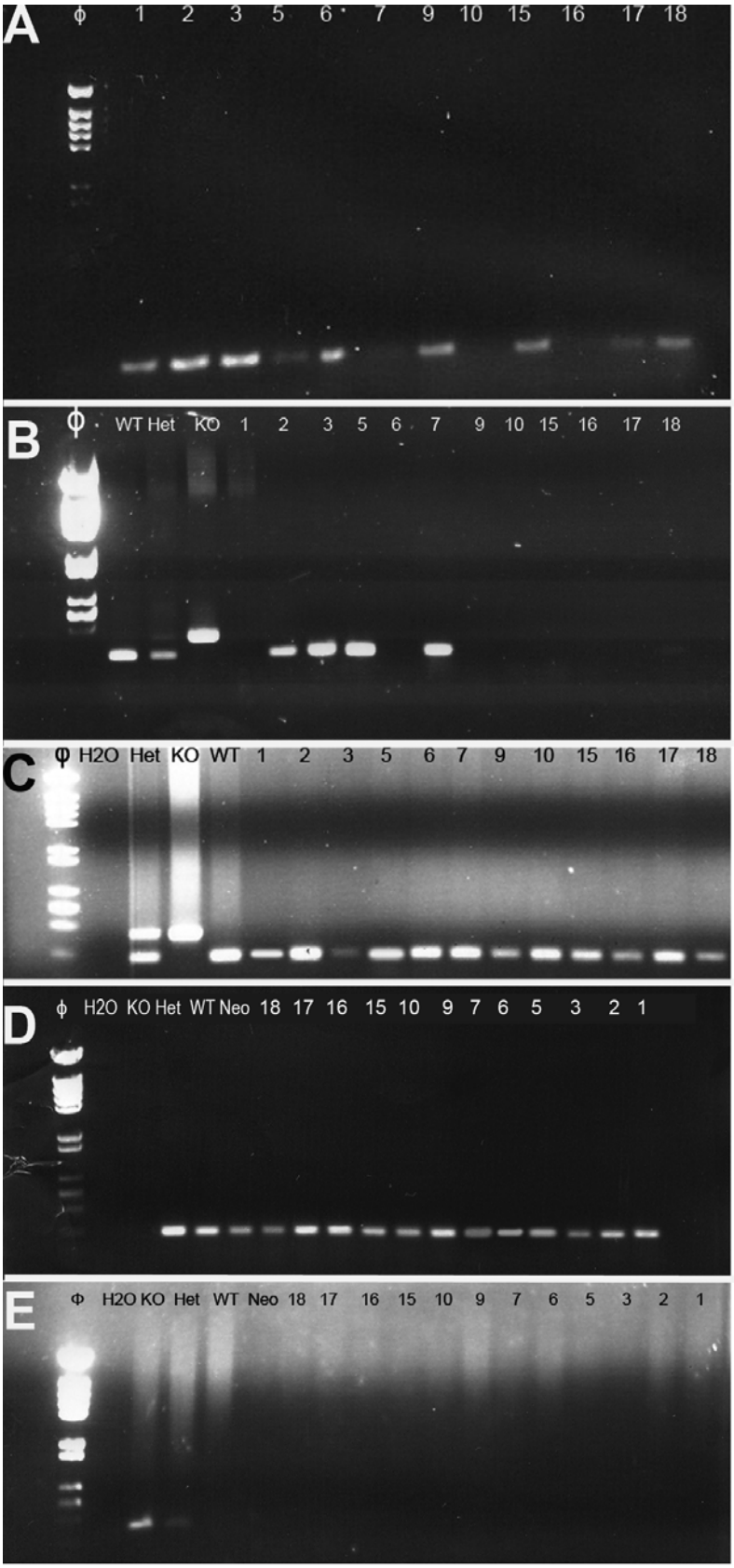
First DNA extraction followed by PCR for amplifying the knocked out and the wildtype sequence revealed only wild type bands in agarose gel electrophoresis (Fig. 19 A). This seemed very unprobable as usually a ratio of 5:5:10 for (-/-), (+/+) and (+/-) aralar respectively is reached. PCR was repeated, but lead to the same outcome (Fig. 19 B).

A possible resting contamination with MEF feeder layer cells which would have contained wild type aralar could have led to this result. As the wild type sequence is shorter and easier amplified by PCR the primers could have been used up for amplification the wildtype fragment. That is why after passaging cells again on gelatin another DNA extraction and genotyping was done. Also this second attempt failed to generate any knock out fragments (Fig. 19 C).

After passaging the lines one more time and doing another DNA extraction the PCR reaction has been split into two, one set of the 12 lines was amplified with only the primers for the knock out fragment and the other set of lines was amplified with only the primers for the wild type fragment. Like this the different fragments do not compete for their common primer.

Also these separated PCR reactions amplified only wild type fragments (Fig. 19 D,E).

The wild type genotype has been confirmed as well by Western blot analysis. All the 12 lines showed weak but persistent signals for the wild type proteins in Western blot. As at first sight one line (line 15, termed Neo) seemed to survive neomycine selection (explained later on), its cell lysate was included in the third DNA extraction, but it also turned out to produce only the wildtype protein upon PCR amplification, and failed to survive in a repetition of neomycine selection (Fig. 19 D,E).

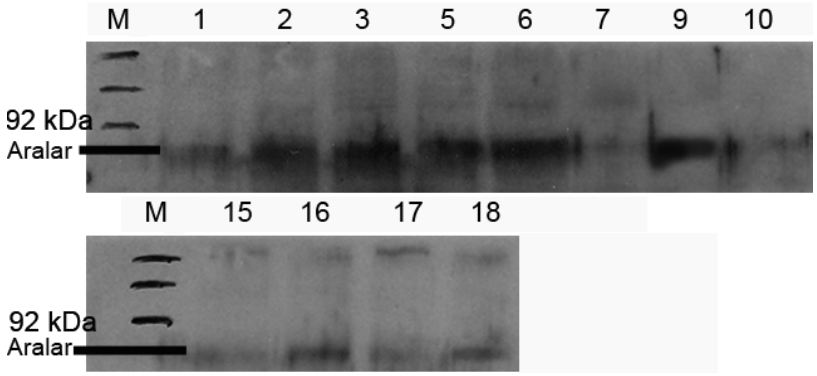


19) Fig. 19: Gelelectrophoresis of PCR Genotyping of established blastocyst lines

(A) First DNA extraction first PCR attempt (B) repetition of PCR (C) second DNA extraction after additional passage of cell lines on gelatine (D) third DNA extraction after another passage of cell lines on gelatine, PCR with ara WT primers only (E) third DNA extraction after another passage of cell lines on gelatine, PCR with ara KO primers only.

Western Blot

All twelve lines were analysed for the presence of wild type aralar by Western blot. Using this approach a possible contamination by MEF Ara protein would have been undetectable.

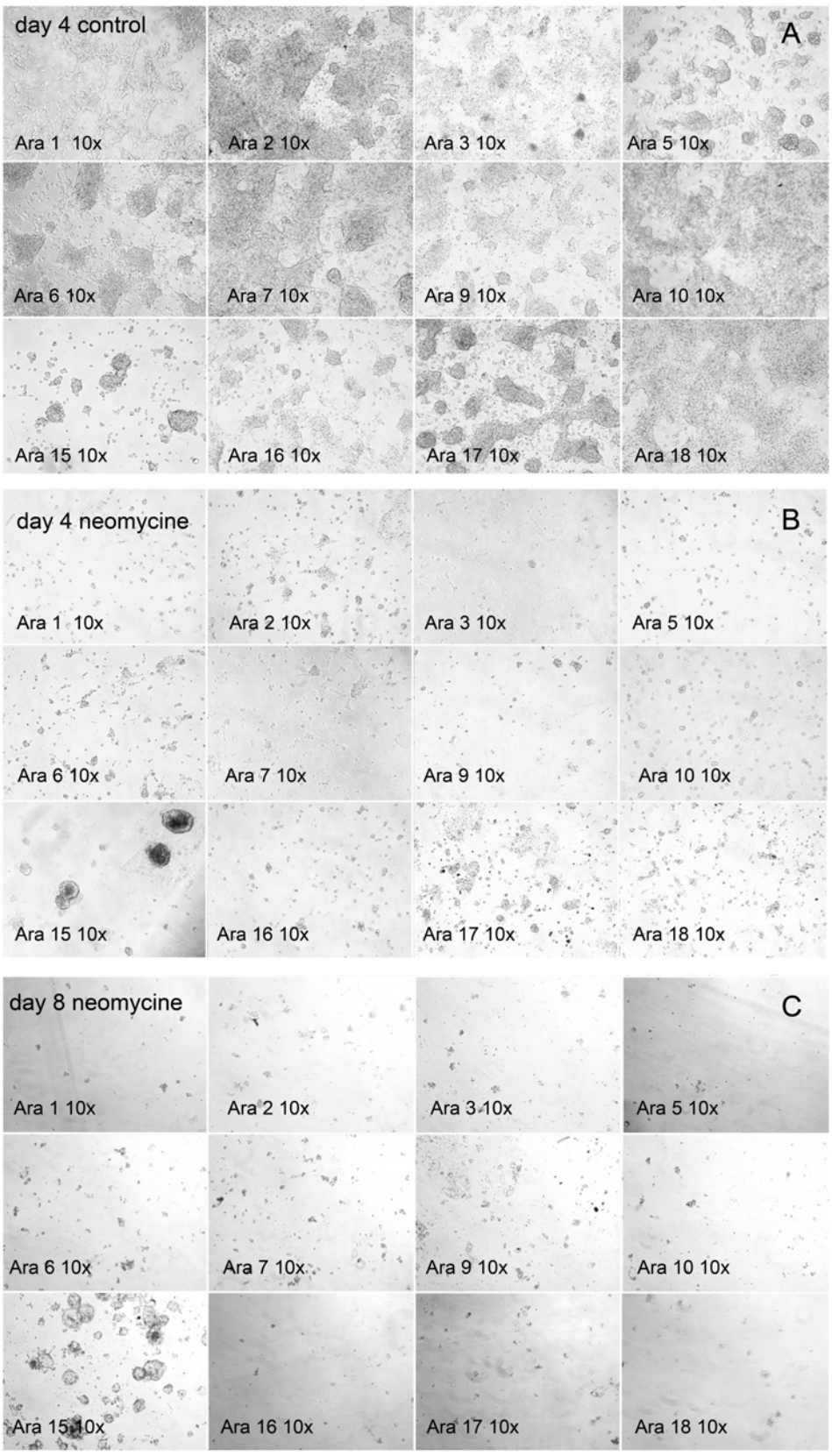


20) Fig. 20: Western blot of twelve derived blastocyst lines showing Aralar bands (74 kDa), marker (M)**.**

All the 12 lines showed weak but persistant expression signals for the wild type protein aralar in Western blot and thereby confirmed their wild type genotype (Fig. 20).

Neomycine selection

The original Aralar mouse transfection vector contained a G418 neomycine resistance. Therefore knock out cells as also heterozygotus cells should survive G418 selection.
So in a last attempt the twelve cell lines were seeded in the presence of 500 µg/ml G418 in SRM containing LIF, after four days concentration of the antibiotic was reduced to 250 µg/ml. Although at first sight line number 15 seemed to resist the selection after 8 days practically all cells were dead. A second selection (data not shown) starting with 250 µg/ml G418 right away did not lead to any surviving cell line either after 8 days of culture with the antibiotic.
None of the twelve lines survived neomycine selection which confirms furthermore that no heterozygoteous line could be established (Fig. 21).



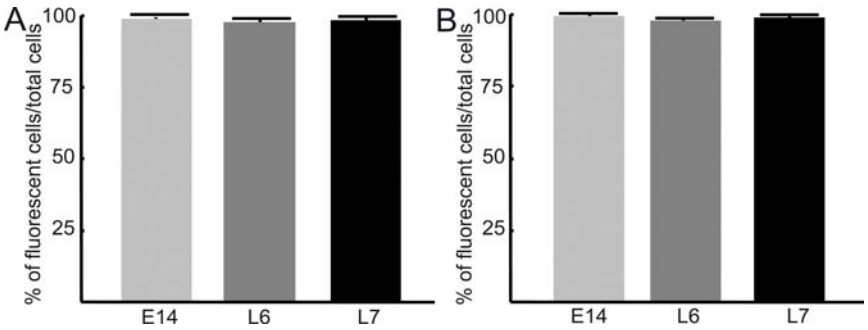
21) **Fig. 21: Phase contrast images of twelve derived blastocyst lines in neomycine selection**

(A) cell lines in control medium SRM + LIF after four days proliferation (B) cell lines in medium SRM + LIF containing 500 µg/ml G418 (C) cell lines in medium SRM +LIF containing four days 500 µg/ml G418 and four days 250 µg/ml G418.

5.6.3. Characterization of two Ara (+/+) versus one mES control cell line in proliferation

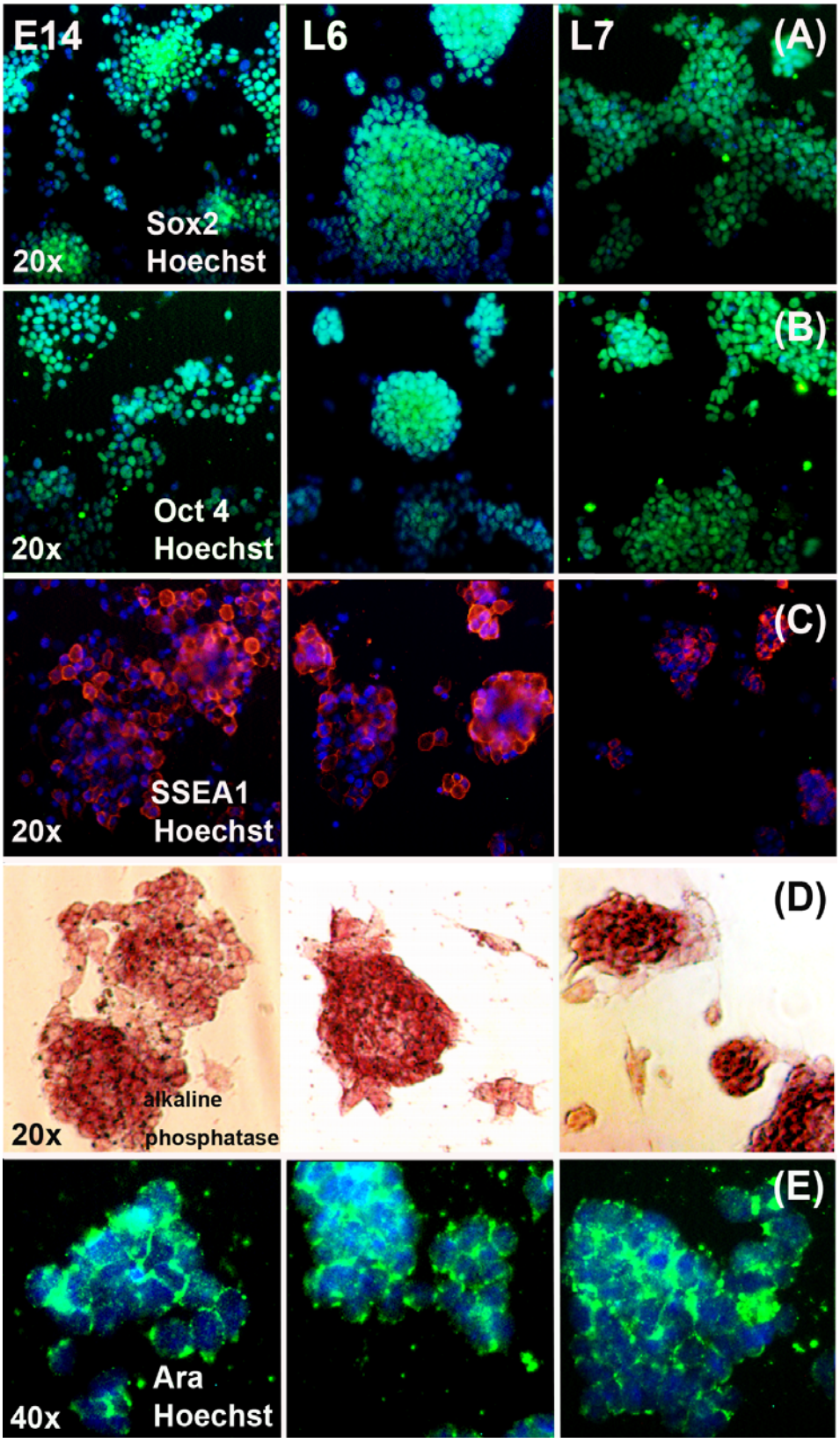
The pluripotent characteristics of two of the established lines, number six and number seven, have been explored in comparison to the comercial E14 mES line. Therefore cells have been fixed after culturing them in SRM medium containing leukemia inhibitory factor and checked for the expression of pluripotent markers Sox 2, Oct 4 and the SSEA1. Cytochemistry for alkaline phosphatase has also been done. All cell lines have been analysed for Aralar expression in proliferation.

VIII. **Graph 7: Quantitative comparison of pluripotency markers between E14, L6 an L7 cell lines in proliferation** *(A) Sox2 (B) Oct4.*



Pluripotency could be confirmed by cell counting of the markers Sox2 and Oct4. All three lines showed more than 95% of positive cells for pluripotent markers, Oct4 slightly excelling Sox2 expression percentage.

Aralar expression could be confirmed in all three cell lines by immunocytochemistry (Fig. 22).



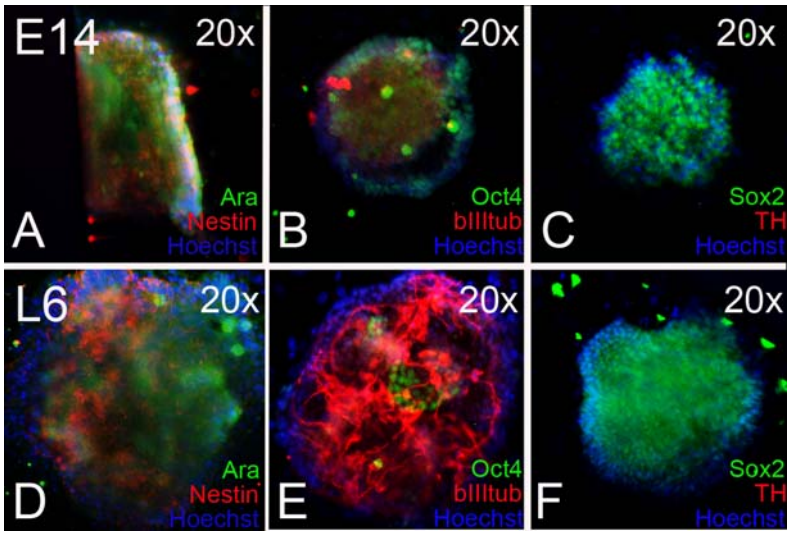
22) **Fig. 22: Immunocytochemistry and Cytochemistry of E14, L6 and L7 mES in proliferation**

(A)Sox2 (B) Oct4 (C) SSEA1 (D) Alkaline phosphatase (E) Aralar. Nuclei were counterstained by Hoechst (blue).

5.6.4. Differentiation of two Ara (+/+) versus one mES control cell line

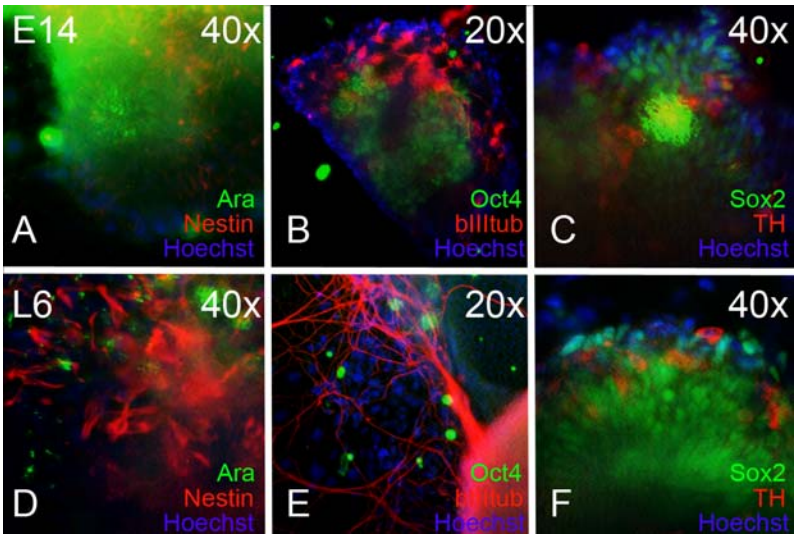
The same two lines have also been characterized for their differentiation capacity towards a dopaminergic fate in comparison to the commercial E14 mES cell line. Therefore the cells were seeded at single cell densities on PA6 feeder layers, LIF was withdrawn and differentiation factors have been added according to a slightly modified differentiation protocol from Barberi et al. 2006 [11] as described in materials and methods. Some wells have been fixed at each differentiation step as to say before each change of differentiation factors to follow the time course of differentiation. Then immunocytochemistry was performed for the following markers: Aralar, Nestin, β-III tubulin, Sox2, TH, and Oct4.

Both cell lines still showed pluripotent properties at day 5 of differentiation expressing Sox2 and Oct4, but started already expressing Nestin which means that they become neural precursors. At day 5 so far no TH positive could be detected. Yet at day eight of differentiation both lines started expressing TH at the borders of the colonies as a sign of obtaining dopamineric characteristics. Also βIII tubulin expression augments from day five to day 8 at the periphery of colonies, whereas remaining Sox2 and Oct4 expression gets more and more restricted to centers of colonies, where cells are supposed to maintain their pluripotent state. L6 seems to express slightly more Nestin and β-III tubulin (Fig. 23 A,B,D,E). Both cell lines show consistent Aralar expression throughout differentiation (Fig. 23 C,F, Fig. 24 C,F, Fig 25).



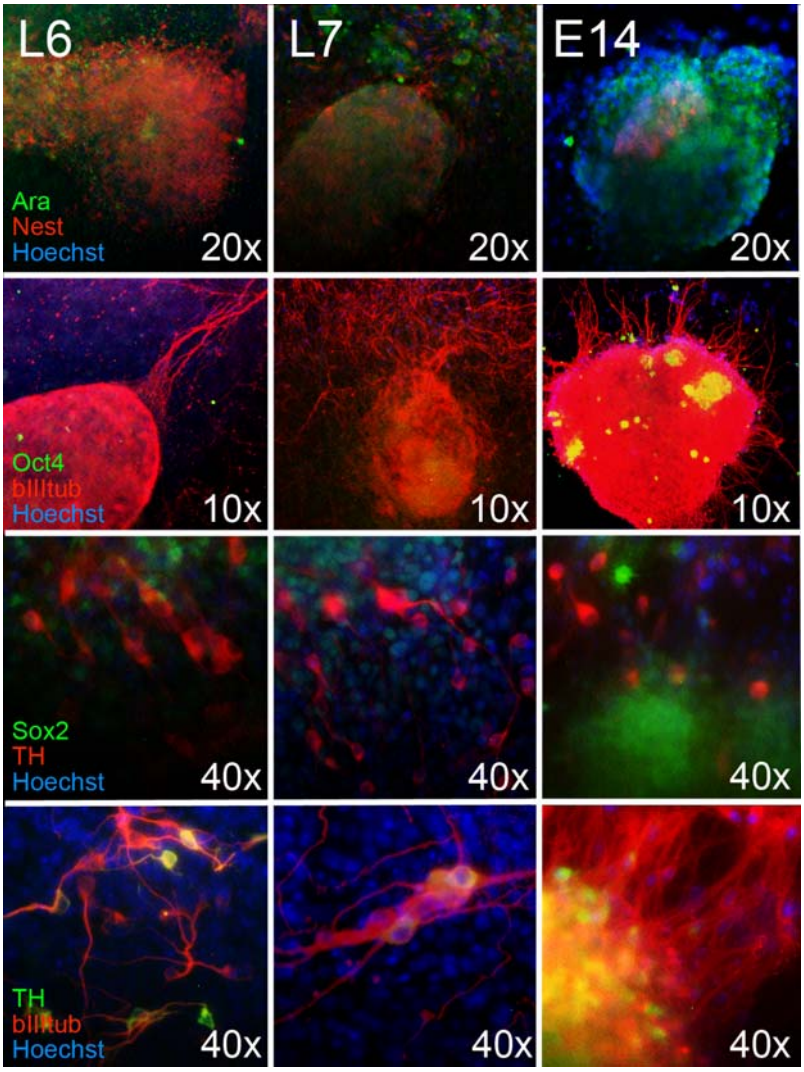
23) Fig. 23: Immunocytochemistry of time course of dopaminergic differentiation E14 and L6 mES cells at day 5 of differentiation

(A) E14 Nestin, Aralar 20x (B) E14 Oct4, βIII tubulin 20x (C) E14 Sox2, TH 20x (D) L6 Nestin, Aralar 20x (E) L6 Oct4, βIII tubulin 20x (F) L6 Sox2, TH 20x. Nuclei were counterstained by Hoechst (blue).



24) **Fig. 24: Immunocytochemistry of time course of dopaminergic differentiation E14 and L6 mES cells at day 8 of differentiation.**

(A) E14 Nestin, Aralar 40x (B) E14 Oct4, βIII tubulin 20x (C) E14 Sox2, TH 40x (D) L6 Nestin, Aralar 40x (E) L6 Oct4, βIII tubulin 20x (F) L6 Sox2, TH 40x. Nuclei were counterstained by Hoechst (blue).



25) Fig. 25: Comparative immunocytochemistry of finally differentiated mES cell lines E14, L6 and L7 (day 13)

6. Discussion

Parkinson`s disease (PD) is characterized by a progressive loss of of dopaminergic neurons (DAn) of SNpc. At the moment, no sufficiently effective treatment options for this disease are available. Still one possible therapy research has advanced quite significantly on, is cell therapy. As the usage of fetal tissue as a cell source is associated with ethical and low availability problems, research has shifted its interest on alternative cell sources like ES cells. Still one major problem that has to be overcome is poor dopaminergic differentiation cell harvest. Therefore it is necessary to study all possible influencing factors on dopaminergic neurogenesis.

Aralar, a mitochondrial glutamate/aspartate carrier, belongs to the family of CaMs and is member of the MAS NADH shuttle. Aralar has been shown essential for proper mitochondrial function[1].

Having in mind that differentiating neurons have increasing energetic demands, that differentiating neurons produce elevated levels of ROS, which could underlie mitochondria to an increased stress, and that the MAS shuttle function could be implied in differentiation abilities of cells the matter arises whether Aralar could be relevant for cell differentiation and for its survival capacity.

The aim of this thesis was to elucidate possible implications of Aralar in dopaminergic neurogenesis.

Aralar expression studies carried out in CD1 aralar wild type mice enforced the suspicion of its implications, as different experimental methods revealed that it is expressed in critical phases of dopaminergic neurogenesis. Immunohistochemical analysis in ventral mesencephalon of E14.5 mouse embryos (Fig. 8,9) , immunocytochemical analysis in E13.5 VM primary culture and Western blot of proliferating R1 mES cells (Fif. 10) confirmed abundant Aralar expression throughout embryonic development. Also immunohistochemistry of ara wildtype VM cryostat sections at ages E11.5, E13.5 and E15.5 was consistent with these findings (Fig. 11).

Comparative in vivo immunohistochemical expression studies of VM cryostat sections of WT(+/+) and KO (-/-) Aralar embryos revealed no statistical differences for neither TH nor Foxa2 at embryonic age E11.5 (Fig. 12) (Graph 1), probably because this stage of dopaminergic neurogenesis is too early for evaluation of any manifestation of differences in dopaminergic harvest by TH expression. At E13.5 first differences in Foxa2 expression can be observed, the decreased Foxa2 expression in ara knock out embryos at E13.5 (Fig. 13)

(Graph 2) is maintained in E15.5 (Fig. 14) (Graph 3) sections which already points to changes in dopaminergic neuron harvest. Experiments of Ferri et al. [29] have shown that decreased Foxa2 expression does not lead to changes in proliferation or cell death, but rather cause a change in fate decision. Indeed at E15.5 TH expression is statistically decreased in aralar kock out embryos. This could mean that a fate switch to gabaergic neurons is occurring, which will be interesting to explore in future experiments.
Moreover ara KO mice sections at E15.5 show less Ki67 expression (Fig.14) (Graph 3), this could be due to a generally enhanced cell mortality of precursors as an alternative to the proposed fate switch.

Comparative in vitro immunocytochemical expression studies of characteristic dopaminergic markers as well as cellular markers in primary culture of E13.5 dopaminergic precursors of the ventral mesencephalon of WT(+/+) and KO (-/-) Aralar embryos revealed that at embryonic day E13.5 (Fig. 17, Graph 6) cell cycling as shown by Ki67 expression levels was statistically significantly elevated in knock out aralar primary culture in comparison to wild type primary culture.
Furthermore percentage of Casp3 positive cells in aralar KO primary culture statistically significantly exceeded percentage of Casp3 positive cells in ara WT primary culture. Although the in vitro model can never be directly compared with the in vivo model an elevated cell death at E13.5 could explain why at E15.5 less Ki67 can be detected in tissue expression studies. For TH no differences could be detected (Fig. 17, Graph 6) which is consistent with in vivo findings.
Ventral mesencephalic primary culture from embryonic day E14.5 maintained statistical significant differences in Ki67 expression (Fig. 18, Graph 5), showing more expression in ara KO cells. Moreover TH expression was statistically significantly decreased in ara KO primary culture at E14.5.
One possible explanation could be higher ROS production as a consequence of impaired mitochondrial function during increasing energy demands for the differentiating precursors. Increased hematopoietic stem cell (HSC) ROS levels have been associated with lowered self-renewal, increased cell cycling and reduced HSC and progenitor viability [46].
When putative stem cells isolated from the retina were exposed to oxidative stress in vitro, the response of Muller cells was to increase proliferation and dedifferentiation [43].
So although first there are as much differentiating and differentiated cells in ara KO as in ara WT later elevated cell death occurs in ara KO. The dividing Ki67 positive cells might die, or might go back to a pluripotent state with lower ROS production [4] in which cells divide more and do not exit cell cycle is an attempt to compensate increased cell death.

Almost no Tryptohphane positive cells could be observed neither in wild type nor in ara knock out primary culture of E13.5, which confirms their ventral mesencephalic identity and shows that at least no fate switch towards a serotonergic fate occurs.
B-III tubulin expression showed no differences between ara WT compared to KO in E14.5 primary culture, which could be interpreted as no change towards a glial fate has taken place.

The derivation mES cell lines from blastocytes of E3.5 ara (+/-) mice mated mice led to the establishment of twelve mES cell lines. Two of these lines (line 6 and line 7) have been checked for their pluripotency by immunocytochemistry and cytochemistry comparing them to a commercially available mES cell line E14.
Sox2 and Oct4 positive cell counts exceeded in all three cases 95% of total cells, validated by Hoechst staining, which confirms their pluripotency. Slight differences between cell lines like lower Sox2 and Oct 4 percentages in L6 compared to line 7, could be explained by different seeding densities, which can influence their uninduced muted, light, starting differentiation at some cell colony boarders (Fig. 22) (Graph 7).

Furthermore when **differentiation was induced** applying a modified protocol from Barberi et al. [11] the lines were able to give rise to dopaminergic neurons to an expectable extend (Fig. 25). Following the time course of differentiation comparing L6 to L7 the expression of Sox2 and Oct4 slowly diminished from day 5 on whilst a lot of Nestin positive neuronal precursors could be seen (Fig. 23) whereas at day eight first TH positive cells could be observed at the boarders of colonies(Fig. 24). During this differentiation process Aralar showed consistent expression, which lead to the assumption that it is needed throughout the whole process. It is implausible that Aralar was indispensable for differentiation as immunohistochemical and immunocytochemical studies around the embryonic age of E13.5 showed that dopaminergic neurons were present, if also to lesser extend, in ara (-/-) animals. Still some impact seems very probable and will have to be investigated by comparative WT(+/+) and KO (-/-) mES cell studies.

Abundant Aralar expression could be observed (Fig. 22) in all 3 proliferating mES cell lines.
This observation is contradictory if one considers that in pluripotent mES the mitochondrial network is poorly developed, cells containing small numbers of inmature mitochondria. Therefore a smaller number of mitochondria would have to express Aralar even in a higher extend. The question remains which role Aralar could have at that point.

At the two-cell and eight-cell stages of blastocysts there is increased activity of the MAS in vivo and in vitro, which enables the transport of reducing equivalents across the mitochondrial membrane, thereby maintaining the redox balance between the cytoplasm and mitochondria and enabling the use of lactate and glucose as energy substrates [47].
ICM cells of the blastocyst have been shown previously to rely entirely on aerobic glycolysis for their energy requirements, converting 100% of the glucose taken up to lactate [48] which doesn´t need MAS shuttle activity.
On the other hand trophectoderm (TE) cells generate a significant proportion of their energy from oxidation, approximately 55% [48]. Mitchell et al. conclude that MAS dysfunction would mediate its effects on embryonic development in these very early stages via TE [49].
But maybe a difference in energy metabolism between ICM as part of a blastocyst and mES cells in proliferation in vitro, does exist, as to say, ICM might perform basically aerobic glycolysis and mES might use mainly lactate conversion to pyruvate by lactate dehydrogenase which needs elevated NADH recycling.

It was not possible to derive any knock out blastocyst line from heterozygotus aralar mice.

Among twelve embryonic stem cell lines that were established not even one turned out to be heterozygoteous or completely deficient for agc1, although mating of Het x Het mice usually results in a ratio of (+/-):(-/-):(+/+) 49:25:26 [1] The obtained genotypes have been confirmed by 3 different methods: westernblot (Fig. 20), genotyping via PCR (Fig. 19) and neomycin selection (Fig. 21).
This lead to the conclusion that Aralar might already be implicated at a very early point of development maybe because of restricted energy supply.
Studies using a AOA as a MAS inhibitor showed that eight –cell embryos developed to the blastocyst stage at high rates, but numbers of cells were significantly reduced in these conditions [49]. Hence one could conclude that at day 3.5 when blastocysts are collected it is only possible to collect WT blastocysts as KO and HET blastocysts are growth retarded and therefore hardly detectable.

The question remains whether Aralar is really indispensable for energy supply or it rather carries out its main function by its requirement for N-acetyl Aspertate production?

Recently Wibom et al. [50] have reported a case of a female child patient having a non functional mutated form of AGC1 who suffers from severe muscular hypotonia and psychomotor retardation affecting mainly motor skills. Magnetic resonance investigations showed global cerebral hypomyelination. However the patient had no substantial accumulation of lactate detected on magnetic resonance spectroscopy, which is considered

a marker for impaired oxidative phosphorylation. These findings do not support compromised neuronal energy production by the respiratory chain as a major pathogenetic mechanism in AGC1 deficiency, suggesting that the malate-aspartate shuttle may not be bioenergetically essential in the central nervous system. The glycerol-3-phosphate cycle, known to be active in the brain, is an alternative pathway of transfer for reducing equivalents into the mitochondria. The hypomyelination in the patient was confined to the cerebral hemispheres, with findings essentially normal in the cerebellum and brainstem. The ontogeny of tissue specific expression of AGC1 in the human central nervous system is unknown, and residual expression of AGC2 may explain the regional differences seen in the patient. [51].

Mitochondrial shuttle activity decrease in Aralar deficient mouse embryos can already be observed prenatally (around E15, unpublished data Beatriz Pardo), which means that Citrine is not fully able to compansate for Aralar during mouse embryonic development at this stage. Whether at that point Citrine expression gets already restricted to the periphery and therefore the mitochondrial dysfunction is due to a dose dependent decrease in Citrine or Citrine does not compensate at all but has another role in embryonic development still has to be determined.

Future studies will also have to expand the in vivo experiments to a postnatal stage exploring the time course of TH expression and other markers. Moreover it will be essential to derivate ara (-/-) mES cell lines or as an alternative knock down Aralar expression in the already established lines by RNAi, for observation of energetic and metablic changes that could lead to restrictions in differentiation capacity.

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Zusammenfassung

Aralar, ein mitochondrialler Aspartat /Glutamat Transporter, zählt zur Familie der Calcium mitochonriellen carrier und ist eine grundlegende Komponente des Malat-Aspartat NADH shuttle (MAS). Das Protein ist unerlässlich für eine einwantfreie Funktion der Mitochondrien[1].

Berücksichtigt man, dass differenzierende Neuronen einen erhöhten energetischen Aufwand haben, dass diese dadurch auch mehr ROS produzieren, welche die Mitochondrien einem größerem Stress aussetzten[2] and dass die MAS shuttle Funktion eventuell einen Einfluss hat auf die Fähigkeit der Neuronen sich zu differenzieren bzw. zu überleben[3], stellt sich die Frage ob Aralar möglicherweise eine Relevanz für Zelldifferenzierung beziehungsweise Zellüberleben hat.

In dieser Diplomarbeit wurde der etwaige Einfluss von Aralar auf dopaminerge Neurogenese untersucht.

Unter der Verwendung von Immunohistochemie in Cryostat Schnitten des ventralen Mesencephalons (VM), Immunocytochemie in Primärzellkultur des VM und mES Zellen und Western Blot Methoden, durchgeführt in CD1 Wildtyp Mäusen konnte eine abundante Aralar Expression während kritischer Phasen dopaminerger Neurogenese bestätigt werden.

Vergleichende immunohistologische and immunocytochemische Studien des VM im embryonalen Alters von E11.5, E13.5, E14.5 und E15.5 von WT(+/+) und KO (-/-) Aralar Mäusen deckten Differenzen der quantitativen Ernte an dopaminergen Neuronen (erhoben anhand TH expression) und Unterschiede der Expression des dopaminergen Transkriptionsfaktors Foxa2, wie auch des Zellcyklus Marker Ki67 und des apoptotischen Markers Casp3 auf. Eine mögliche Erklärung wäre ein Einfluss von Aralar auf die Zellüberlebenskapazität auf Grund von fehlender mitochondrieller Integrität. Darüber hinaus könnte das Abhandenkommen der MAS shuttle Aktivität metabolische Veränderungen hervorrufen und dadurch die Transkriptionskontrolle der dopaminergen Neurogenese beeinflussen.

Des Weiteren wurden mES Zelllinien durch aralar (+/-) Kreuzungen erzeugt und charakterisiert. Die Pluripotenz der Linien konnte durch Immunocytochemie und Cytochemie bestätigt werden. Genotypierung, antibiotische Selektion und Western Blot Analyse zeigten jedoch, dass keine Aralar defiziente Linie unter den hergeleiteten Linien war. Daraus wurde geschlossen, dass Aralar bereits zu einem sehr frühen Zeitpunkt der Entwicklung eine Rolle spielt, vielleicht auf Grund restringierter Energiebereitstellung durch defekte Mitochondrien. Aralardefizienz im Blastocystenstadium könnte deren Detektierung erschweren.

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- poster: Castillo C.G., Bueno C., Moreno B., Gapp K., Courtois E. T. C., Ramos M., Martinez-Serrano; Characterisation of human neural stem cell lines transfected with homologous recombination vectors: in vitro and in vivo studies; 622.26/B26, Sfn congress, 2008, Washington