Behavioural Brain Research 374 (2019) 112113

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

FGF-2 isoforms influence the development of dopaminergic neurons in the murine substantia nigra, but not anxiety-like behavior, stress susceptibility, or locomotor behavior

Friederike Freiin von Hövel[a,b,1], Ina Leiter[a,b,1], Regina Rumpel[c], Alina Langenhagen[a,b], Dirk Wedekind[c], Christine Häger[c], André Bleich[c], Rupert Palme[d], Claudia Grothe[a,b,*]

[a] Institute of Neuroanatomy and Cell Biology, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hanover, Germany
[b] Center for Systems Neuroscience (ZSN), Hanover, Germany
[c] Institute for Laboratory Animal Science, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hanover, Germany
[d] Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

ARTICLE INFO

Keywords:
FGF-2
Dopaminergic system development
LMW
HMW
Behavior
Glucocorticoids

ABSTRACT

Background: Loss of fibroblast growth factor 2 (FGF-2) is responsible for the development of an increased number of dopaminergic (DA) neurons in the murine substantia nigra pars compacta (SNpc). Furthermore, dysregulation of its expression patterns within the central nervous system (CNS) is associated with behavioral abnormalities in mice. Until now, the contributions of the individual FGF-2 isoforms (one low (LMW) and two high molecular weight (HMW) isoforms) in the CNS are elusive.

Methods: To unravel the specific effects of FGF-2 isoforms, we compared three knockout mouse lines, one only deficient for LMW, one deficient for HMW and another lacking both isoforms, regarding DA neuronal development. With this regard, three time points of ontogenic development of the SNpc were stereologically investigated. Furthermore, behavioral aspects were analyzed in young adult mice, supplemented by corticosterone measurements.

Results: Juvenile mice lacking either LMW or HMW develop equal supernumerary DA neuron numbers in the SNpc. Compensatory increased LMW expression is observed in animals lacking HMW. Meanwhile, no knockout mouse line demonstrated changes in anxiety-like behavior, stress susceptibility, or locomotor behavior.

Conclusions: Both FGF-2 isoforms crucially influence DA neuronal development in the murine SNpc. However, absence of LMW or HMW alone alters neither anxiety-like nor locomotor behavior, or stress susceptibility. Therefore, FGF-2 is not a determinant and causative factor for behavioral alterations alone, but probably in combination with appropriate conditions, like environmental or genetic factors.

1. Introduction

Fibroblast growth factor 2 (FGF-2) is abundant in the central nervous system (CNS) and widely expressed in neurons of the ventral midbrain (VM) [1]. There, expression patterns play a major role in the development and maintenance of dopaminergic neurons (DA), especially within the substantia nigra pars compacta (SNpc) [2].

in vitro, exogenously applied FGF-2 stimulates survival and neurite outgrowth in DA neuronal cultures and also possesses neuroprotective effects [3,4]. Contradictory, *in vivo* analyses of mice overexpressing FGF-2 revealed a decreased number of DA neurons and a reduced cell density in the SNpc [5]. Meanwhile, mice lacking FGF-2 displayed 36% more tyrosine hydroxylase (TH) positive neurons in this area, while the number of DA neurons within the VTA was not altered [6]. Further investigations of FGF-2 deficient mice discovered the onset of the observed phenotype between embryonic (E) day 14.5, demonstrating an increased proliferation rate of DA progenitor cells in the subventricular zone (SVZ), and postnatal day (P) 0, characterized by a decreased apoptosis rate [6].

In rodents, FGF-2 is translated in three different isoforms originating

* Corresponding author at: Claudia Grothe, Hannover Medical School, Institute of Neuroanatomy and Cell Biology, Carl-Neuberg-Straße 1, 30625 Hanover, Germany.
E-mail address: grothe.claudia@mh-hannover.de (C. Grothe).
[1] contributed equally.

https://doi.org/10.1016/j.bbr.2019.112113
Received 20 June 2019; Received in revised form 24 July 2019; Accepted 24 July 2019
Available online 02 August 2019
0166-4328/

from the same mRNA. The low molecular weight FGF-2 (LMW) isoform (18 kD) is expressed starting at the AUG codon [7], whereas, two high molecular weight FGF-2 (HMW) isoforms (20.5 and 21 kD) are generated by translation from an alternative upstream CUG codon [8]. While HMW is restricted to the cell nucleus, LMW can be found in the nucleus, in cytoplasm, and is also secreted resulting in autocrine or paracrine effects [9,10]. Besides their different localization, both isoforms also differ in their spatiotemporal expression throughout development of the nigrostriatal system. Thereby, LMW is the dominant isoform in the embryonic phase, and HMW increases during development and is more pronounced in adult mice [11]. These findings suggest different regulatory impacts of FGF-2 isoforms on development of the DA pathway.

Disturbances in the DA and glutamatergic (GA) system as well as in the fibroblast growth factor signaling have been related to several neuropsychiatric and neurodegenerative disorders [12–16]. Recently, there is increasing evidence linking the level of FGF-2 expression with anxiety behavior in rodents [17–19]. Furthermore, FGF-2 deficiency was associated with hyperactivity in mice, caused by maladjustment between the GA and the DA system [20].

To identify the specific roles of FGF-2 isoforms in the development and function of the DA system, mouse strains lacking FGF-2 and mice lacking either LMW ($LMW^{-/-}$) or HMW ($HMW^{-/-}$) were closely evaluated with regard to DA neuron development as well as behavioral characterization with special focus on anxiety behavior, stress sensitivity, and locomotor activity.

2. Methods and materials

2.1. Animals and tissue preparation

All experimental protocols were permitted by the local authorities (Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) Hannover, Germany) and followed the German Animal protection Act (33.12-42502-04-13/1205). The experiments were performed using mice lacking FGF-2 ($FGF\text{-}2^{tm1Zllr}$) [21] or with animals only deficient for LMW ($FGF\text{-}2^{tm2Doe}$/J) [22] or HMW ($FGF\text{-}2^{tm3Doe}$/J) [8].

All strains were maintained on a C57BL/6 J background, retrospectively compared by single-nucleotide polymorphism (SNP) analysis. Therefore, B6 J.Cg-Fgf-2tm3Doe/*J* and B6 J.Cg-Fgf-2tm2Doe/*J* and B6;Cg-Fgf-2tm1Zllr have been compared to reference strains B6 J and B6 NCrl using a marker set of 39 SNPs. This marker set has been composed at the Institute for Laboratory Animal Science, Hannover Medical School to distinguish several B6 substrains. Informative SNPs were selected from published data [23,24] as well as from results of our own experiments (unpublished data). Positions of SNPs are in accordance to dbSNP release 150. DNA was isolated from ear biopsies by using a MasterPure™ complete DNA Purification Kit (Lucigen, Middleton, USA) in accordance with the manufacturer's instructions. SNP genotyping was carried out by LGC Genomics (Hoddesdon, UK). This marker set has been also successfully used earlier [25].

Both mouse strains deficient for isoforms of Fgf-2 show a SNP profile equal to reference strain B6 J. The genetic background of the strain deficient for both Fgf-2 isoforms ($B6;Cg\text{-}Fgf\text{-}2^{tm1Zll}$) is not standardized. This background is characterized by SNP genotypes homozygous and heterozygous for B6 J and B6 NCrl alleles (Table 1).

Genotypes were determined by PCR as previously described [26,27], and gene mutation was verified by sanger sequencing (GATC, Eurofin Genomics, Ebersberg, Germany).

All animals were kept in the same temperature- and humidity-controlled room on a 14-h light/10-h dark schedule and housed in open cages as groups or individuals with food and water available *ad libitum*. The hygienic status was routinely monitored according to the FELASA recommendations [28]. No evidence of infectious agents was revealed except for occasional positive tests for *Rodentibacter pneumotropica* and *Helicobacter spp*.

Embryos at the E14.5 time-point were generated by time-mated homozygous mice, with the day of vaginal plug defined as E0.5. All older homozygous $FGF\text{-}2^{-/-}$, $LMW^{-/-}$ and $HMW^{-/-}$ knock-out (ko) animals and their respective $FGF\text{-}2^{+/+}$, $LMW^{+/+}$ and $HMW^{+/+}$ wt littermates were obtained by crossbreeding of heterozygous (het) animals. Neonatal mice (P0) were defined by the day of birth, and P28 designated as juvenile mice, while older animals were indicated as young adult mice.

2.2. Western Blot analysis

Embryos and neonatal (P0) mice were decapitated, while all older mice were sacrificed by cervical dislocation after CO_2 inhalation for tissue preparation.

For protein expression analysis, striatum (ST, P28 and P0) or ganglionic eminence in the forebrain (FB, E14.5), and VM tissue samples from individual young adult male and female mice (n = 3 per genotype) or pooled from three animals (E14.5 and P0) were dissected and homogenized with radioimmune precipitation assay (RIPA) buffer, consisting of 800 µl RIPA base stock (20 mM tris − HCl, 137 mM NaCl, 25 mM β-Glycerophosphate, 2 mM EDTA, 1% Triton-X-100, 1% Sodiumdeoxychlorate, 1 mM Sodiumorthovanadate), 2.6 µl 5 M NaCl, 20 µl 50x Protease Inhibitor (Roche, 11873580001), and 177 µl Millipore water per milliliter [11]. For expression analyses, 100 µg (only for FGF-2) or 20 µg of total protein were denatured by boiling in Laemmli buffer (except for VGLUT1 samples), and applied to a 15% (only for FGF-2) or 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). After blotting on nitrocellulose membranes (Amersham Protran Premium 0.45 NC nitrocellulose Membrane, GE Healthcare), membranes were blocked with 5% milk powder in Tris-buffered saline containing 0.03% Tween 20 buffer (TBST) for one hour at room temperature.

Following antibodies were used: rabbit anti-FGF-2 (1:750, SC-79, Santa Cruz Biotech., Heidelberg, Germany), rabbit anti vesicular glutamate transporter 1 (VGLUT1; 1:5.000, 135305, Synaptic Systems, Goettingen, Germany), rabbit anti membrane excitatory amino acid transporter 3 (EAAT3; 1:10.000, ab124802, Abcam, Berlin, Germany), rabbit anti TH (1:1000, ab152, Millipore, Darmstadt, Germany), and mouse anti fibroblast growth factor receptor 1 (FGFR1; 1:500; ab823, Abcam).

Glucocorticoid receptor (GR) expression was analyzed in hippocampus (HC) samples from young adult male animals (n = 4) with mouse anti-glucocorticoid receptor (1:500, SC-393232, Santa Cruz Biotech.).

Detection was performed on a Chemiluminescence Imager system (Intas, Goettingen, Germany). Equality of the protein amounts analyzed was ensured by Ponceau S (P-3504, Sigma-Aldrich, Munich, Germany) staining.

2.3. Tissue processing and immunohistochemistry

For bromodeoxyuridine (BrdU) incorporation assay time-mated female homozygous mice received a single intraperitoneal BrdU injection (100 µg/g body weight, Boehringer Ingelheim, 1,296,736) 20 hours before dissection of embryos at the E14.5 time-point [6]. For immunohistochemistry, embryos and neonatal (P0) mice were decapitated, while juvenile female mice (P28) were transcardially perfused with 4% paraformaldehyde (PFA) under deep ketamine/xylazine anesthesia.

Dissected brains of E14.5, P0, and P28 mice (n = 5–7 per time-point and group) were fixed overnight in 4% PFA at 4 °C and then transferred to 30% sucrose (Roth, Karlsruhe, Germany). Each brain was coronally sectioned on a cryostat. Adult brain sections (50 µm thickness, six series) were blocked with PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.3% TritonX-100 (9002-93-1, Sigma-Aldrich), before mouse anti-TH (1:5000, T1299, Sigma St Louis, MO,

Table 1
SNP profiles of Fgf-2 deficient strains on B6 background and reference strains B6J, B6NCrl.

SNP	MMU	Position	Locus	Reference		Tested background								
				C57BL/6 J	C57BL/6 N Crl	B6 J.Cg-Fgf-2^{tm3Doe}/J			B6 J.Cg-Fgf-2^{tm2Doe}/J			B6;Cg-Fgf-2^{tm1Zllr}		
rs13475814	1	36879483	*Tmem131*	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T
rs13476801	2	138480020		T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	C:C	C:C	T:C
rs13476956	3	5370727	*Zfhx4*	C:C	T:T	C:C	C:C	C:C	T:T	T:C	T:C	C:C	C:C	C:C
rs13477019	3	23824920	*Naaladl2*	T:T	A:A	T:T	T:T	T:T	T:T	T:T	T:T	A:A	A:A	A:A
rs13477132	3	58109942		G:G	C:C	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G
rs13477622	4	28322410		T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T
rs13477746	4	65944235	*Astn2*	T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	C:C	C:C	C:C
rs13477863	4	98297639		A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
rs13478736	6	46421423	*Cntnap2*	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T
rs13478783	6	60591379		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
rs13478995	6	117470880		C:C	G:G	C:C	C:C	C:C	C:C	C:C	C:C	C:C	C:C	C:C
rs13479233	7	47816324		T:T	G:G	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:G	T:G
rs13479522	7	129035694		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
08-015199792-M	8	15199792		T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	C:C	C:T	n.d.
rs13479733	8	43875316		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	G:G	G:G	G:G
rs13459107	9	28298786		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	G:G	G:G	G:G
rs13480122	9	31156626	*Aplp2*	T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T
rs13480619	10	57752462		T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T
rs13459122	10	80795365		T:T	A:A	T:T	T:T	T:T	T:T	T:T	T:T	C:C	C:C	C:C
rs13480759	10	109378627		C:C	T:T	C:C	C:C	C:C	C:C	C:C	C:C	C:C	C:C	C:C
rs13480829	10	129350405	*Olfr782*	G:G	A:A	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G
11-004367508-M	11	4367508		G:G	A:A	G:G	G:G	G:G	G:G	G:G	G:G	A:A	G:G	A:A
rs13481014	11	48117382		T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	C:C
rs13481117	11	79252230	*Wsb1*	G:G	T:T	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G
rs13481439	12	48965551		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
rs13481573	12	86909001		G:G	A:A	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G
rs13481634	12	106833655		A:A	C:C	A:A	A:A	A:A	A:A	A:A	A:A	C:C	C:C	C:C
rs13481676	13	6304055		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
rs13481734	13	27037150		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
13-041017317-M	13	41017317		T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T	T:T
rs31233932	14	124108797	*Fgf14*	C:C	T:T	C:C	C:C	C:C	C:C	C:C	C:C	C:C	C:C	C:C
rs13459145	15	7167980		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	G:A	A:A	G:A
15-057561875-M	15	57561875		A:A	G:G	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
rs4165065	16	17412079	*Snap29*	T:T	C:C	T:T	T:T	T:T	T:T	T:T	T:T	C:C	C:C	C:C
rs13483055	17	60319945		T:T	C:C	T:T	T:T	T:T	T:T	T:T	n.d.	C:C	C:C	C:C
rs13483296	18	35206506		A:A	T:T	A:A	A:A	A:A	A:A	A:A	?	A:A	A:A	A:A
rs13483369	18	54614841	*9330117O12RiK*	A:A	C:C	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A	A:A
19-049914266-M	19	49914266		G:G	T:T	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G	G:G
rs13483883	X	93699957		G:G	A:A	G:G	G:G	G:G	G:G	G:G	G:G	A:A	A:A	A:A

Genetic profile of B6J.Cg-Fgf-2^{tm3Doe}/J and B6 J.Cg-Fgf-2^{tm2Doe}/J is equal to B6J while the background of B6;Cg-Fgf-2^{tm1Zllr} contains SNP genotypes of B6 J and B6 NCrl in homozygous and heterozygous conditions. Positions of SNPs are in accordance to dbSNP release 150.

USA) was applied in blocking solution at 4 °C overnight. After washing the next day, biotinylated rabbit anti-mouse antibody (1:200, Dako, Glostrup, Denmark) was added in blocking solution for one hour at room temperature. This was followed by the avidin–biotin-complex ABC kit (Vector Laboratories, Peterborough, UK) and 3,3´-diaminobenzidin tetrahydrochloride (DAB; D5905, Sigma-Aldrich) with ammonium nickel sulfate intensification.

Neonatal brain sections (40 µm thickness, two series) were first postfixed in 4% PFA for 10 min at room temperature, followed by 10 min with 0.01 M citrate buffer (pH 6) at 95 °C. After blocking in PBS containing 5% normal goat serum (NGS, GibcoTM, Life technologies, Warrington, UK), 1% BSA, and 0.3% TritonX-100 at room temperature, sections were incubated with mouse anti-TH (1:1000, T1299, Sigma) and rabbit anti-cCasp3 (1:200, #9664, Cell Signaling, Frankfurt, Germany) in PBS containing 1% NGS, 1% BSA, and 0.3% TritonX-100 at 4 °C overnight. Secondary goat anti-mouse Alexa 488 (1:500, A-11029, Invitrogen) and goat anti-rabbit Alexa 555 (1:500, A-21429, Invitrogen) antibodies were applied for one hour at room temperature. Nuclei and apB were stained by 4,6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma-Aldrich).

For BrdU detection, E14.5 sections were treated as described previously [6]. Briefly, sections (40 µm thickness, three series) were denaturized with 2 M HCl for one hour at 37 °C before neutralizing in 0.1% borate buffer (pH 8.5) for five minutes. After blocking with PBS containing 5% NGS, 1% BSA, and 0.3% TritonX-100 at room temperature, sections were incubated with mouse anti-BrdU (1:100, 11 170 376 001, Roche) and rabbit anti-Lmx1a (1:6000, AB10533, Merck) in PBS containing 1% NGS, 1% BSA, and 0.3% TritonX-100 at 4 °C overnight. Secondary goat anti-mouse Alexa 555 (1:500, AB21422, Invitrogen) and goat anti-rabbit Alexa 488 (1:500, AB11034, Invitrogen) antibodies were used as described before.

2.4. Stereological quantification of DA neurons

Cells were counted with the optical fractionator workflow of the StereoInvestigator® software (MBF Biosciences, VT, USA) under 40x magnification using an Olympus microscope (BX51, Olympus). Tissue thickness was determined at each section. The total number of TH positive (TH+) neurons within the SNpc was estimated in LMW$^{-/-}$ or HMW$^{-/-}$ as well as LMW$^{+/+}$ or HMW$^{+/+}$ female mice on P28 in every third section (120 x 120 µm grid size, 80 x 80 µm counting frame, 3 µm guard zone, accepted coefficient of error (CE, Gundersen m = 1) < 0.05). For quantification of BrdU/Lmx1a double labeled cells within the ventral mesencephalic SVZ of E14.5 LMW$^{-/-}$ or HMW$^{-/-}$ as well as LMW$^{+/+}$ or HMW$^{+/+}$ embryos, cells were counted on every third section (45 x 45 µm grid size, 30 x 30 µm counting frame, 2 µm guard zone, CE < 0.1, four rostral and four caudal sections). In P0 animals, the same counting parameters were used to estimate the

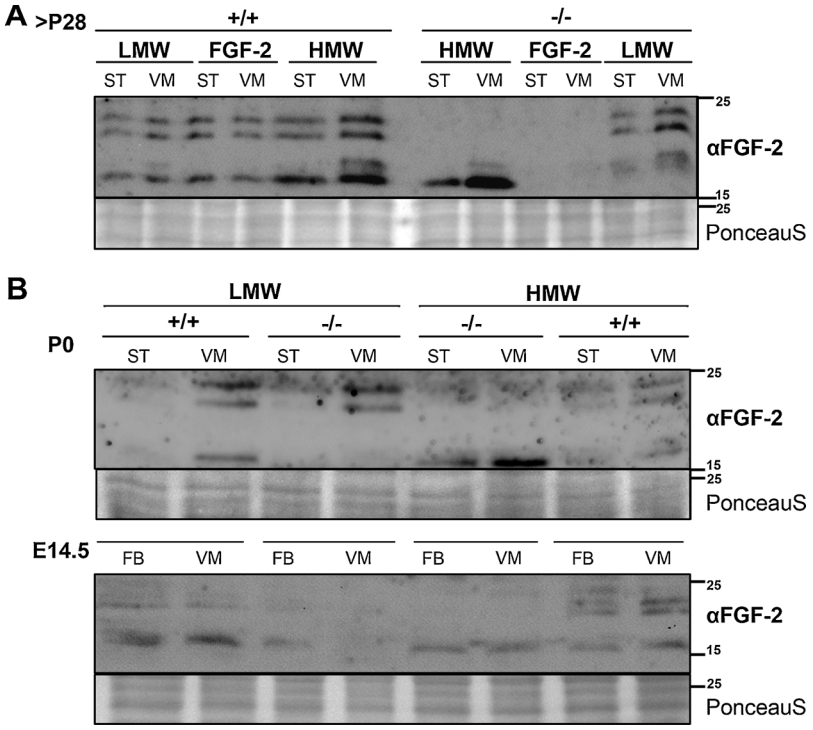

Fig. 1. FGF-2 expression pattern throughout development of the nigrostriatal system in isoform specific FGF-2 knockout mice. A) Western Blot analysis of striatum (ST) and ventral midbrain (VM) tissue samples from young adult mice lacking either FGF-2 (FGF-$2^{-/-}$), only LMW (LMW$^{-/-}$), or HMW (HMW$^{-/-}$) compared to their wt littermates. All FGF-2 isoforms (LMW with 18 kD, and HMW with 20.5 and 21 kD) were detectable in the respective wt (FGF-$2^{+/+}$, LMW$^{+/+}$, and HMW$^{+/+}$), while FGF-$2^{-/-}$ mice were lacking all isoforms. Meanwhile, LMW$^{-/-}$ only expressed both HMW isoforms, and HMW$^{-/-}$ only expressed LMW vice versa. B) FGF-2 expression patterns in isoform specific ko mice throughout development demonstrated that on P0, LMW$^{+/+}$ and HMW$^{+/+}$ expressed all FGF-2 isoforms in the VM, while minimal amounts of HMW were detectable in the ST. LMW$^{-/-}$ showed similar HMW expression in the VM, while no LMW was expressed. In comparison, newborn HMW$^{-/-}$ mice repeatedly demonstrated stronger signals of LMW in the VM as well as in the ST, without detectable HMW expression. On E14.5, LMW$^{+/+}$ and HMW$^{+/+}$ expressed all FGF-2 isoforms in the VM and the forebrain (FB), with LMW more pronounced. At this stage, in LMW$^{-/-}$ mice, no FGF-2 was detectable while HMW$^{-/-}$ mice expressed LMW in the VM as well as in the FB.

number of cells within the SNpc positive for cleaved caspase-3 (cCasp3+), for cCasp3+ and apoptotic bodies (apB/cCasp3+), apB alone or of TH + cells showing signs of preluded natural cell death on every other section (CE < 0.3). All results represent the estimated population using mean section thickness with counts.

2.5. Behavioral tests

All behavioral experiments were conducted in young adult (mean age 65.8 days per group) male FGF-$2^{-/-}$, LMW$^{-/-}$ and HMW$^{-/-}$ mice, and their respective wt littermates (n = 12 per group). The experimental room was kept at constant temperature, humidity, sound level, and brightness. Light intensity was measured by a luxmeter and dimmed to ≈ 70 lx. The experiments were carried out between 9:00 a.m. to 14:30 p.m. on one day for each test (open-field and subsequent modified light/dark test and elevated plus maze), respectively. After 30 min of acclimatization in their home cages in the experimental room, single animals were transferred from cages into the respective experimental setting in a beforehand defined randomized order. After every mouse the maze was cleaned with 70% ethanol to neutralize odour.

The open field arena was a force-plate actometer with a white quadrangular ground plate (42 cm x 42 cm) and transparent walls (31 cm height) [29,30]. Mice were released in the center of the arena for 10 min of exploration. Traveled distance, center-time (defined as the time spent in the central 6% of the open field area), low mobility bouts, and wall rears were analyzed as described previously [29]. Focal energy densities were scored automatically by the program analyzing the force data.

The modified version of light/dark test was set in a circular black plastic arena (105 cm diameter) with a white floor. A circular plastic anthracite animal house (12.5 cm diameter, 13 cm high) was located in the center, with a light source (> 1000 lx) arranged at about 40 cm distance to the entrance. Animals were placed in the house (≈ 4 lx) with their view on the opposite side of the entrance and time until the mouse left the house with all four paws at the same time was measured.

The white perspex elevated plus maze was standing 48 cm above the ground and had an arm length of 35 cm (5 cm arm width, 15 cm wall height). It was positioned equally in the room every time with one closed arm pointing to a corner of the room, the two open arms with similar distance to walls and the second closed arm pointing to the experimenter. Animals were placed with their heads in the center of the maze in view of the closed arm directed to the experimenter. Exploration behavior of the mice was filmed for five minutes and the time spend in open arms versus time spend in closed arms was measured by an investigator blinded to the experimental conditions. Results given as percentage of time spent in the open arm.

2.6. Analysis of fecal corticosterone metabolites and blood corticosterone

Fecal corticosterone metabolites (FCM) were measured with a 5α-pregnane-3β,11 β,21-triol-20-one enzyme immunoassay [31,32]. Therefore, feces were collected twice (one baseline measurement on a "day of rest" in the local animal facility and "stress" measurement of FCM six hours after stress, defined as transport to the experimental room) two hours after transferring animals to clean cages and stored at −20 °C.

Blood was taken from the facial vein of mice from each group (n = 5–7) directly after transport from the housing to the experimental room and stored as dried blood spots on Protein Saver™ 903™ Cards (10531018, GE Healthcare Bio-Sciences Corp.) at room temperature before analyzed [33].

2.7. Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was done using GraphPrism 6 (GraphPad Software, Inc., San Diego, USA) with Student's *t*-test, Kruskal-Wallis test or ANOVA followed by post hoc analysis as indicated. Statistical significance was set at $p < 0.05$.

3. Results

3.1. FGF-2 expression patterns in the developmental nigrostriatal system of isoform specific knockout mice

FGF-2 expression patterns were analyzed in the nigrostriatal system

of different ko strains throughout development. All FGF-2 isoforms were clearly detectable in the VM and ST of young adult FGF-$2^{+/+}$ as well as $LMW^{+/+}$ and $HMW^{+/+}$ mice, while no FGF-2 was expressed in FGF-$2^{-/-}$ mice (Fig. 1A). While FGF-2 HMW is physiologically expressed in two different isoforms, LMW is sometimes also detected as double signal. However, young adult $LMW^{-/-}$ only displayed one signal due to lack of the 18 kD isoform and showed intense expression of the HMW signals similar to the wt animals. Meanwhile, young adult $HMW^{-/-}$ mice lacking HMW seem to have increased expression of LMW in the VM and ST compared to all other animals, which was repeatedly observed.

At P0, HMW was similarly expressed in the VM of all groups except $HMW^{-/-}$. Additionally, no HMW was detectable in the ST of all groups. However, LMW expression was similar in the VM of $LMW^{+/+}$ and $HMW^{+/+}$ mice. Meanwhile, $HMW^{-/-}$ mice displayed a more pronounced LMW signal in the VM and ST on P0, while no 18 kD signal was detected in $LMW^{-/-}$ newborns (Fig. 1B).

During embryonal development, FGF-2 LMW was already expressed in the ST and VM of all groups except $LMW^{-/-}$ animals, where no 18 kD signal was observed. Both HMW signals were slightly expressed in VM and FB of $LMW^{+/+}$ and $HMW^{+/+}$ embryos, while none was detected in the ko animals at this early developmental stage (Fig. 1B).

3.2. Quantification of dopaminergic neurons throughout development of FGF-2 isoform specific knockout mice

Stereologically estimated DA neurons (TH+) within the SNpc of P28 juvenile $LMW^{+/+}$ and $HMW^{+/+}$ mice revealed values of 4037 ± 131.7 TH + cells per hemisphere (Fig. 2A, B). Compared to that $LMW^{-/-}$ mice displayed 16.1% more TH + cells reaching statistical significance (4719 ± 143.4 TH + cells per hemisphere). The difference compared to the respective wt group was even more pronounced in $HMW^{-/-}$ mice with 5103 ± 99.3 TH + cells per hemisphere and an average increase of 19.4%.

On P0, the estimated number of TH + neurons was 2144 ± 286.1 TH + cells per hemisphere for $LMW^{+/+}$, while fewer cells were counted in $HMW^{+/+}$ mice (1826 ± 187.6 TH+). However, no significant differences were seen compared to the newborn $LMW^{-/-}$ (1620 ± 187 TH + cells) and $HMW^{-/-}$ mice (1588 ± 239.4 TH + neurons) (Fig. 2C). Postnatal apoptosis was stereologically analyzed by cCasp3 staining combined with DAPI to visualize chromatin clumps defined as apB (Fig. 2H). Estimated number of cells positive for both events in the SNpc did not differ between $LMW^{+/+}$ (123.4 ± 27.8 cCasp3+/apB + cells) and $HMW^{+/+}$ (126.2 ± 24.3 cCasp3+/apB + cells) newborns (Fig. 2D). Comparatively, $LMW^{-/-}$ (198.7 ± 47.4 cCasp3+/apB + cells) showed 37.9% increase on P0, even though this did not reach statistical significance. $HMW^{-/-}$ mice showed similar amounts of apoptotic events (155.1 ± 42.6 cCasp3+/apB + cells). This pattern was also seen when estimating the number of cells within the SNpc that were either cCasp3+ or apB+. $LMW^{+/+}$ (161.4 ± 33.6 cCasp3+ cells; 224.7 ± 39 apB + cells) and $HMW^{+/+}$ (165.3 ± 27.5 cCasp3+ cells; 220.8 ± 36.3 apB + cells) mice showed lesser apoptotic events compared to $LMW^{-/-}$ (247.3 ± 54.7 cCasp3+ cells; 325.8 ± 54.7 apB + cells) and $HMW^{-/-}$ (192 ± 51.5 cCasp3+ cells; 241.2 ± 37.8 apB + cells) mice (Fig. 2D–H). However, in very rare cases, cells positive for cCasp3 and / or apB were still recognizable as TH + neurons (zero to five counted cells per animal; data not shown).

In E14.5 embryos, quantification of co-localization of proliferating (BrdU+) and DA precursor cells (Lmx1a+) in the SVZ was performed using a stereological approach (Fig. 2K). The estimated cell populations of BrdU+/Lmx1a + cells in the SVZ of the VM were similar in all groups independent of FGF-2 expression ($LMW^{+/+}$: 3570 ± 456.1; $LMW^{-/-}$: 3629 ± 873.8; $HMW^{+/+}$ 3064 ± 375.3; $HMW^{-/-}$ 2974 ± 498.9 BrdU+/Lmx1a + cells). Segmenting the SVZ into three rostral and three caudal coronal areas according to earlier studies [6], revealed higher numbers of DA precursor cells within the rostral area of $LMW^{-/-}$ and $HMW^{-/-}$ embryos, respectively (Fig. 2I, J). $LMW^{-/-}$ mice (1324 ± 396.9 BrdU+/Lmx1a + cells) displayed 19% more DA precursors than their wt littermates (1111 ± 220.4 BrdU+/Lmx1a + cells), and $HMW^{-/-}$ mice (1174 ± 340.7 BrdU+/Lmx1a + cells) showed 9% more DA precursors than $HMW^{+/+}$ (1075 ± 288.0 BrdU +/Lmx1a + cells) (Fig. 2I).

3.3. Behavioral analysis

The force plate actometer revealed no differences among animal groups concerning the walked distance of about 100 m (Fig. 3A). The amount of time periods spent inactive (low mobility bouts; Fig. 3B) did also not differ between genotypes of each strain or among strains (FGF-$2^{+/+}$ 18.2 ± 4.1, FGF-$2^{-/-}$ 23.3 ± 3.9, $LMW^{-/-}$ 15 ± 2.3, $LMW^{+/+}$ 21.2 ± 2.6, $HMW^{+/+}$ 22.8 ± 4, $HMW^{-/-}$ 17.1 ± 3.5). Regarding rearing activity at the walls of the arena, $LMW^{-/-}$ mice showed less wall rears (30.2 ± 2.8) than their respective wt (44.7 ± 4), while $HMW^{-/-}$ mice (47.9 ± 5.4) showed more wall rears compared to their wt littermates (33 ± 5.5) (Fig. 3C). Both FGF-2 genotypes showed a comparable amount of wall rears (FGF-$2^{+/+}$ 26.9 ± 4.2; FGF-$2^{-/-}$ 22.8 ± 2.5). Looking at the energy density scores, the $LMW^{-/-}$ mice (152.7 ± 10.1) were significantly lower than $LMW^{+/+}$ mice (210.8 ± 15.9), whereas the $HMW^{-/-}$ group (211.7 ± 22.4) showed a tendency to a higher additive score compared to their respective wt (153.6 ± 20.3) (Fig. 3D). Again, both FGF-2 genotypes displayed similar results (FGF-$2^{+/+}$ 162.3 ± 15.8 and $FGF^{-/-}$ 144.1 ± 15.1).

FGF-$2^{+/+}$ and $LMW^{+/+}$ mice differed concerning the amount of wall rears (FGF-$2^{+/+}$ 26.9 ± 4.2; $LMW^{+/+}$ 44.7 ± 4) and concerning the time spent in the center within the first minute of each experiment, as the same result was obvious when comparing FGF-$2^{+/+}$ and $HMW^{+/+}$ mice (FGF-$2^{+/+}$ 6.5 s ± 2.3; $LMW^{+/+}$ 1.4 s ± 0.6; $HMW^{+/+}$ 1.4 s ± 0.6) (Fig. 3E). With regard to the time spent in the center during the whole experimental period of ten minutes, there was only a significant difference visible comparing FGF-$2^{+/+}$ and $HMW^{+/+}$ mice (FGF-$2^{+/+}$ 13.3 s ± 3.6; $HMW^{+/+}$ 5.8 s ± 1.7), with the results of the other genotypes in between (Fig. 3F).

In the modified dark-light test, no significant differences were observed within the analyzed mouse strains, but $LMW^{-/-}$ mice displayed the lowest mean latency time with 14.5 s ± 3.4 (Fig. 3G). The percentage of total time spent in the open arms of the elevated plus maze was between 10–25% for all animal groups analyzed (Fig. 3H).

3.4. Complementary analysis of behavior-related hormonal and protein expression status

Concentrations of fecal corticosterone metabolites were only significantly increased in $FGF^{+/+}$ (stress: 40.6 ng/ml ± 4; rest: 21.82 ng/ml ± 3.6) following stress induction (Fig. 4A). Meanwhile, similar blood corticosterone concentrations were measured in all groups following stress induction (Fig. 4B).

Additionally, glucocorticoid receptor (GR) expression patterns in hippocampal samples were not influenced by FGF-2 expression in young adult mice (Fig. 4C), which was also proved by densitometric analysis (Fig. S2). Furthermore, expression of TH, FGFR1, EAAT3, and VGLUT1 in ST or VM was also not influenced by genotype (Fig. 4D).

4. Discussion

In this study, we describe for the first time a similarly increased amount of DA neurons in the SNpc of juvenile mice either deficient for LMW or HMW. At this time-point, both isoforms were similarly expressed in wt mice. Until now, decreased FGF-2 expression in humans was correlated with major depression [34,35], and possible relations between FGF-2 gene polymorphisms and the response for

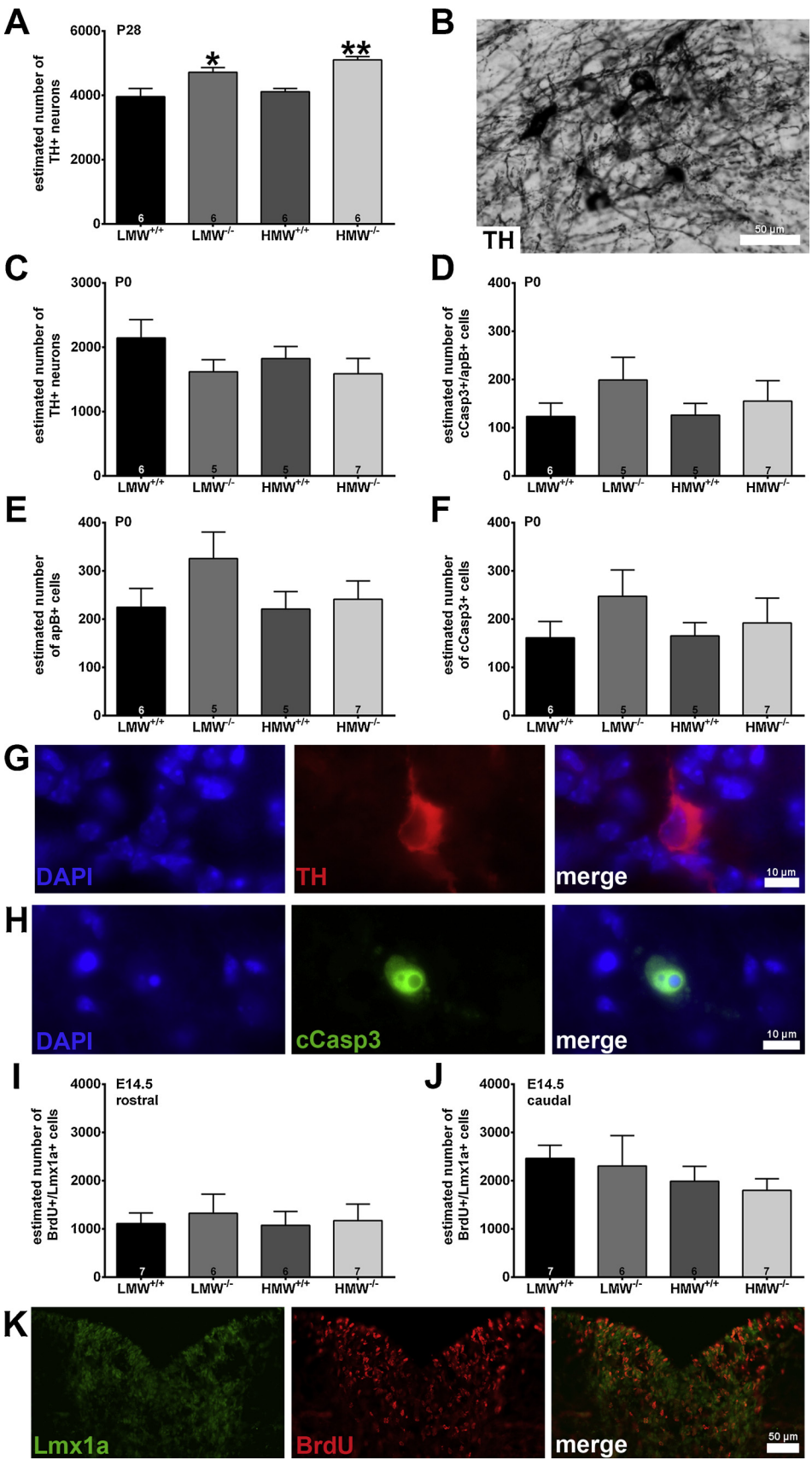

Fig. 2. Analyses of dopaminergic neurons in the ventral midbrain of LMW$^{-/-}$ and HMW$^{-/-}$ mice during ontogenetic development.
A) Estimated numbers of DA neurons (TH+) in the SNpc of juvenile LMW$^{-/-}$ and HMW$^{-/-}$ mice were significantly increased compared to their respective wt littermates (LMW$^{-/-}$ versus LMW$^{+/+}$ $p = 0.016$, HMW$^{-/-}$ versus HMW$^{+/+}$ $p = 0.002$). B) Demonstrates an example image of the TH DAB staining of the right SNpc at P28. C–H) Triple immunofluorescent staining of the SNpc of newborn mice was performed to stereologically estimate the number of DA neurons (TH+) (C) and simultaneously analyze the SNpc of LMW$^{-/-}$ and HMW$^{-/-}$ for the apoptosis marker cCasp3 and the appearance of apoptotic bodies (apB). Cells positive for both markers were slightly increased in LMW$^{-/-}$ and HMW$^{-/-}$ mice on P0 (D). Meanwhile, the cell population positive for cCasp3 (F, H) and the estimated number of cells with apB (E) were only enhanced in LMW$^{-/-}$ mice compared to all other groups. I–K) On E14.5, the number of proliferating DA precursor (BrdU+/Lmx1a+) cells was comparable in the rostral (I) and caudal (J) subventricular zone (SVZ) of all groups. G) Exemplary TH staining (red) including DAPI (blue) staining of neonatal brain tissue. H) Exemplary cCasp3+ cell (green) including apB stained with DAPI (blue). K) Distribution of BrdU (red) within Lmx1a+ cells (green) of the caudal SVZ 20 h after single BrdU injection. One-way ANOVA followed by Tukey´s post hoc test with $F(3, 20) = 10.85$, $p = 0.0002$, *$p < 0.05$ and **$p < 0.01$ compared to the respective wt (n as indicated in the graphs). No strain-specific significant differences were found between the analyzed wt animals.

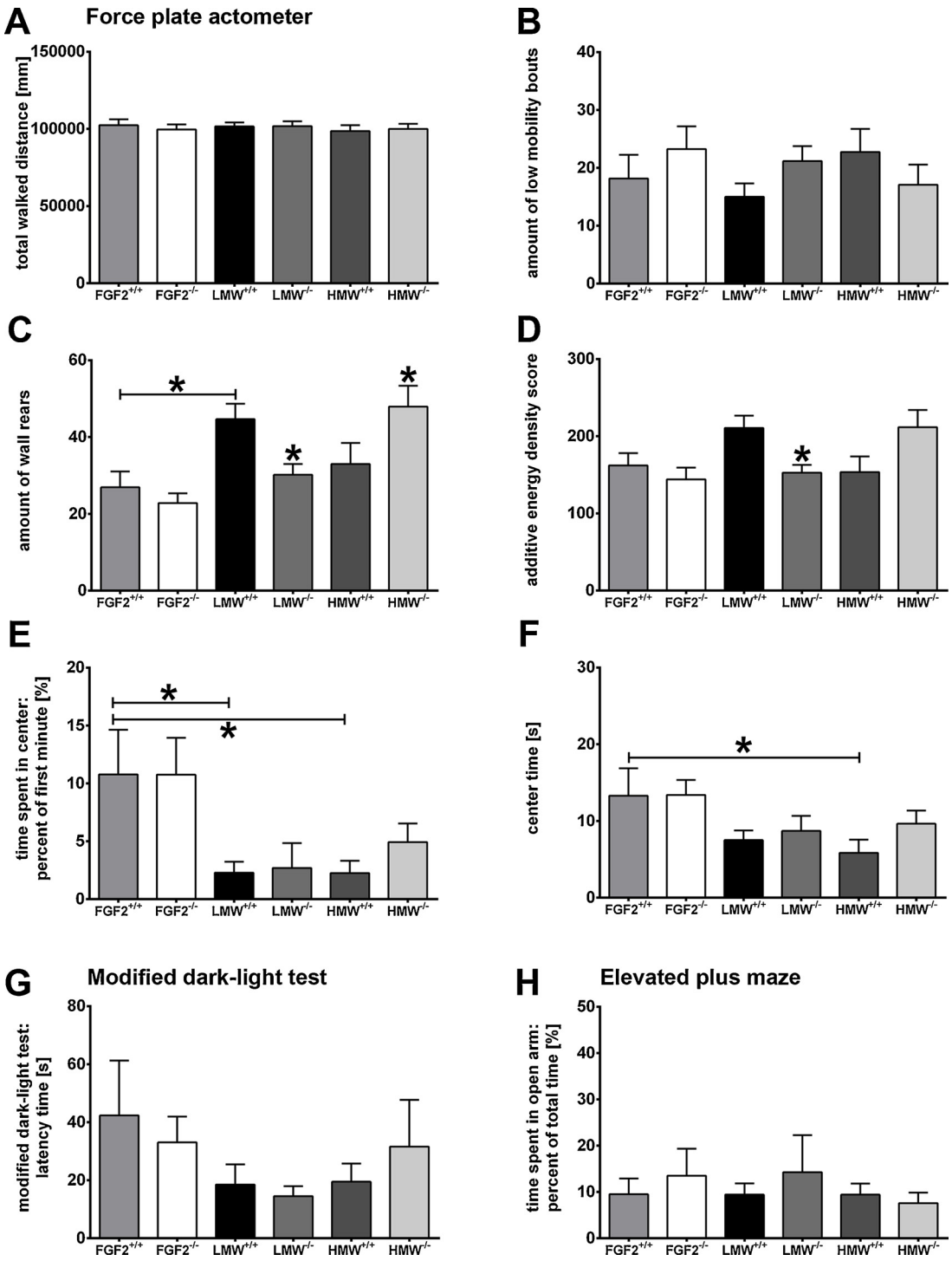

Fig. 3. Results of the behavioral analysis with no strain or genotype specific differences concerning locomotor activity or anxiety-like behavior.
A-F) Force plate actometer results are shown as total walked distance in mm (A), total number of low mobility bouts (B), total number of wall rears (C), total energy density score (D), and time spent in the center within the first minute of the experiment (E) and during the whole 10 minutes of the experiment (F) for all ko mice (FGF-$2^{-/-}$, $LMW^{-/-}$, and $HMW^{-/-}$) and their respective wt (FGF-$2^{+/+}$, $LMW^{+/+}$, and $HMW^{+/+}$) analyzed. No differences in the walked distance of about 100 m (A) or time periods spent inactive (B) were seen independent of mouse strain. In contrast, genotype specific differences within the $LMW^{-/-}$ and $HMW^{-/-}$ mice were observed concerning explorative behavior (C, D). Furthermore, hints for strain specific differences concerning explorative behavior and curiosity were given (C, E, F). G) In the modified dark-light test, no differences were observed within the groups with regard to time measured until entering the bright arena between respective littermates within one strain. However, $LMW^{-/-}$ mice had the shortest latency time. H) Timespan in the open arm of the elevated plus maze in percentage of the whole five minutes monitored revealed no differences between all strains analyzed. Comparisons among isoform-specific FGF-2 ko strains or wt of all three mouse strains: one-way ANOVA, Sidak's multiple comparisons test with C) $F(3, 44) = 3.577$, $p = 0.021$ for $HMW^{+/+}$ and $HMW^{-/-}$ ($p = 0.026$), $LMW^{+/+}$ and $LMW^{-/-}$ ($p = 0.031$) group comparisons and $F(2, 33) = 3.830$, $p = 0.032$ for wt group comparisons (FGF-$2^{+/+}$ versus $LMW^{+/+}$ $p = 0.010$), E) $F(2, 33) = 4.301$, $p = 0.022$ (FGF-$2^{+/+}$ versus $LMW^{+/+}$ $p = 0.016$, FGF-$2^{+/+}$ versus $HMW^{+/+}$ $p = 0.016$) and F) $F(2, 33) = 2.645$, $p = 0.086$ (FGF-$2^{+/+}$ versus $HMW^{+/+}$ $p = 0.036$) (except for D: Kruskal-Wallis test ($p = 0.033$), Dunn's multiple comparisons test ($LMW^{+/+}$ versus $LMW^{-/-}$ $p = 0.016$); comparisons within FGF-2 mouse strain: Student's t-test. *comparisons within same strain if not marked otherwise, with $*p < 0.05$. Twelve animals per group were analyzed.

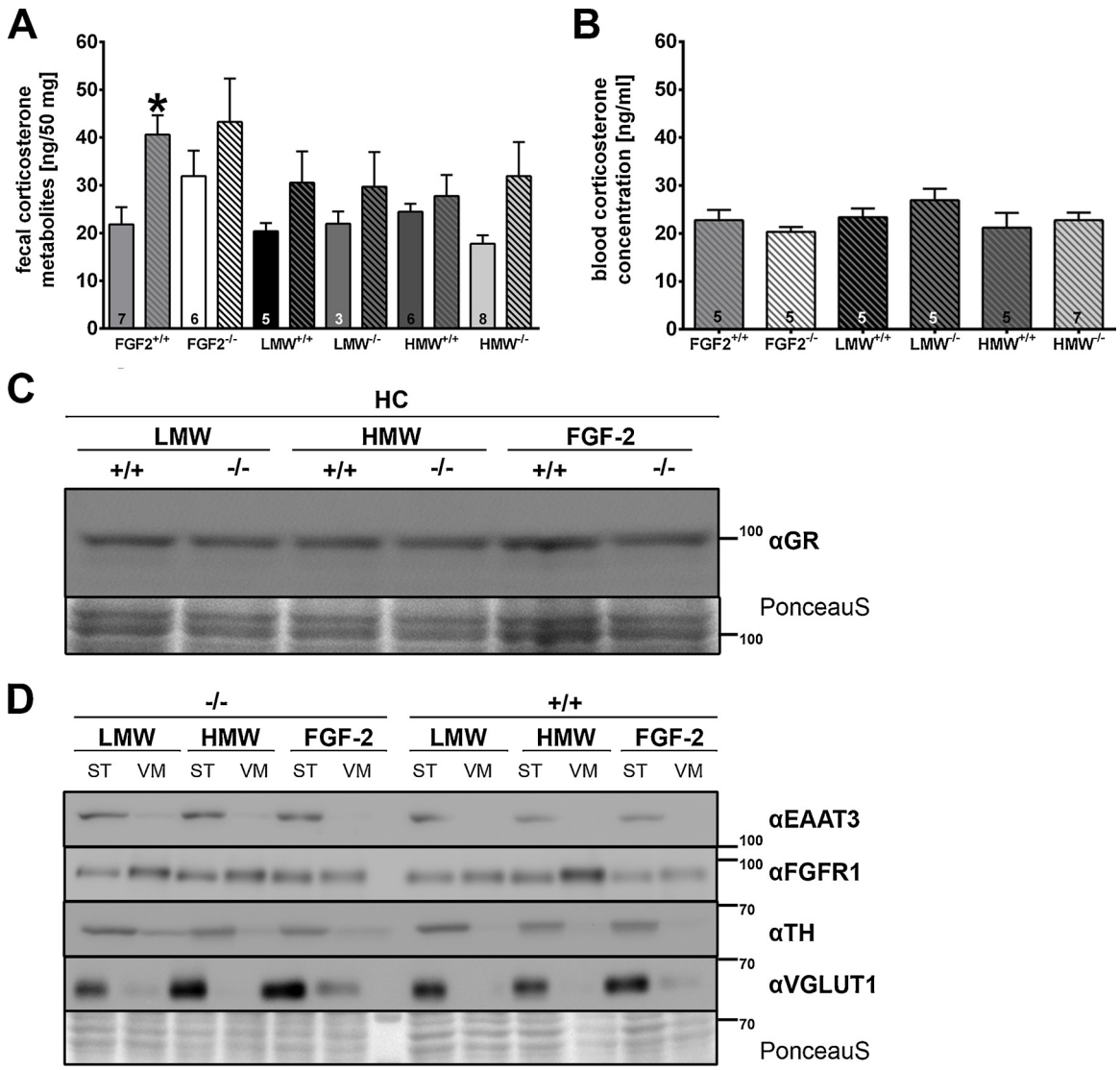

Fig. 4. Analyses concerning stress susceptibility in different FGF-2 knockout mouse strains.
A–B) Concentrations of corticosterone (metabolites) in fecal (A) and blood (B) samples of all ko mice (FGF-2$^{-/-}$, LMW$^{-/-}$, and HMW$^{-/-}$) and their respective littermates (FGF-2$^{+/+}$, LMW$^{+/+}$, and HMW$^{+/+}$). A) Comparable concentrations of fecal corticosterone metabolites were found among all groups before (blank bar) and after transport stress (striped bar), with only increased levels observed in FGF-2$^{+/+}$ animals after stress induction ($p = 0.021$). B) Blood corticosterone concentrations after transport with comparable levels for all groups analyzed. C) Glucocorticoid receptor (GR) expression measured by Western Blot in hippocampal (HC) samples was similar in all groups. The slightly darker band in the FGF-2$^{+/+}$ mice corresponds to the darker PonceauS staining and is therefore not conspicuous. D) Additional ST and VM tissue samples of young adult mice of all genotypes were analyzed for excitatory amino acid transporter 3 (EAAT), fibroblast growth factor receptor 1 (FGFR1), tyrosine hydroxylase (TH), and vesicular glutamate transporter 1 (VGLUT1) expression, with PonceauS verifying similar protein loads. Repeated analysis displayed no clear differences between ko and wt animals of the same strain. Statistics: A) Two-way ANOVA, Sidak's multiple comparisons test, with $F(1, 11)$ time factor (rest versus stress) = 11.25, $p = 0.006$, $F(1, 11)$column factor (genotype) = 0.925, $p = 0.357$, $F(1, 11)$interaction factor = 0.682, $p = 0.427$, *$p < 0.05$. B) Unpaired Student's t-test comparing FGF-2$^{+/+}$ and FGF-2$^{-/-}$ mice ($t(8) = 1.012$, $p = 0.341$), one-way ANOVA with $F(318) = 1.114$, $p = 0.369$, Tukey's multiple comparisons test comparing isoform-specific FGF-2 ko mouse strains.

antidepressant therapy (which seems to increase FGF-2 expression) have been described [36]. Furthermore, reduced FGF-2 immunoreactivity was demonstrated in Parkinson's disease patients [37]. FGF-2 promoted survival and differentiation of DA neurons *in vitro* and *in vivo* [2,5,38]. Interestingly, juvenile FGF-2$^{-/-}$ mice displayed 36% more TH + neurons in the SNpc, with the onset determined during the late stages of embryonic development (between E14.5 and P0) [6].

Because FGF-2 isoforms differ in their spatiotemporal expression pattern throughout the development of the nigrostriatal system, we hypothesized that different parameters could lead to supernumerary DA neurons in the adult SNpc. Therefore, we analyzed total FGF-2$^{-/-}$, LMW$^{-/-}$, and HMW$^{-/-}$ mice in comparison to their wt littermates at three developmental time-points (E14.5, P0, and P28 or young adult). LMW expression was pronounced in all embryos except LMW$^{-/-}$ embryos. On the day of birth, LMW and HMW were similarly expressed in the VM of LMW$^{+/+}$ and HMW$^{+/+}$ mice. Even though similar amounts of total protein were analyzed, no clear FGF-2 expression was measurable in the ST on P0, which is in contrast to earlier reports [11]. In young adult wt mice, all FGF-2 isoforms were equally detectable in the nigrostriatal system, which is in agreement with earlier studies [39]. Young adult LMW$^{-/-}$ mice displayed equal HMW expression. In contrast, HMW$^{-/-}$ mice displayed enhanced LMW signals. This phenotype in HMW$^{-/-}$ mice was already visible on P0 and might be a compensative upregulation.

To verify whether the different expression patterns result in morphological changes and the hyperplasia phenotype seen in FGF-2$^{-/-}$,

we quantified DA neurons in the SNpc. Notably, both, LMW$^{-/-}$ and HMW$^{-/-}$ mice developed an increased number of DA neurons in the SNpc (16.1% in LMW$^{-/-}$, 19.4% in HMW$^{-/-}$), which correlates in total with the hyperplasia of TH + neurons in FGF-2$^{-/-}$ mice [6]. This result suggested that both isoforms have distinct developmental effects and that their interaction crucially influences the regulation of DA neuronal number in the SNpc. To determine the onset of this phenotype, E14.5 embryos were analyzed revealing that LMW$^{-/-}$ embryos developed in average 19% more DA precursor cells in the rostral SVZ compared to wt controls, while the difference in means was diminished to 9% in HMW$^{-/-}$ embryos. The number of DA neurons in the SNpc is not only predetermined by precursor cells, but possibly also influenced by natural ontogenetic cell death, with the highest peak of physiological apoptosis right after birth [40]. Furthermore, FGF-2$^{-/-}$ mice showed reduced apoptosis at this stage [6]. Interestingly, with our design-based stereological approach, we detected increased average estimations of morphological signs of cell death in LMW$^{-/-}$ and HMW$^{-/-}$ mice on P0 compared to their respective wt assuming similar partial, but minor influence leading to enhanced apoptosis at this developmental stage. Mean counts of apoptosis markers were increased in LMW$^{-/-}$ compared to HMW$^{-/-}$ mice, which is in accordance to *in vitro* studies, where HMW overexpression promoted apoptosis, while LMW had an enhanced anti-apoptotic effect [41]. In contrast, the apoptosis rate was reduced in wt littermates, leading to the hypothesis that interaction of the FGF-2 isoforms had compensating effects. Remarkably, the rarity of detected TH + cells showing signs of apoptosis suggested that DA neurons were less affected by apoptosis at this time-point. This observation is in agreement with other studies demonstrating that on P0, only 10–20% of apoptotic neurons were also TH+ [40]. The physiological regulation of DA neuronal development through natural cell death is still elusive, for example increasing DA neuron numbers were reported in the murine SNpc through maturation [40,42], supported by increasing TH mRNA expression in the VM one week after birth [43]. Thus, apoptosis would not seem to lead to a significant decrease in DA neuron number in the SNpc of mice after birth [42,44]. Therefore, it would be interesting to compare in the future total estimated neuron number with the number of TH + neurons to directly see potential changes in the relation. Importantly, rodent strains and age stages differ as well as qualitative and quantitative analysis methods.

Besides morphological impact, we aimed to evaluate behavioral phenotypic traits depending on FGF-2 expression, totally or only partially concerning one isoform. Previous studies related anxiety behavior and hypothalamic-pituitary-adrenal axis activity with FGF-2 expression [19]. In contrast, we did not observe an increased activity of the hypothalamic-pituitary-adrenal axis regarding corticosterone secretion during stress and hippocampal glucocorticoid receptor expression, or aggravated anxiety behavior in FGF-2$^{-/-}$ mice. Importantly, the cited study investigated a FGF-2 mouse strain with Black Swiss background, compared to wt littermates and wt of the same background [15]. With direct regard to anxiety-like behavior, there is evidence that Black Swiss mice are innately less anxious than C57BL/6 mice in the dark-light test [45]. Therefore, the strain might be more susceptible to loss of FGF-2. Additionally, mice were kept under different conditions (single housed in smaller cages) and increasing evidence exists relating caging systems, housing conditions as well as nutrition, and different mouse strains with deviations in anxiety and explorative behavior [46–48]. In addition, imbalances between GA and DA signaling leading to hyperlocomotion were reported [20]. Therefore, we further analyzed protein expressions related therewith (EAAT3, VGLUT1, TH). However, we did not find any differences among genotypes, which supported our behavioral results. Also, we observed no alterations in hippocampal GR expression, which was associated with increased FGF-2 expression and reduced anxiety-like behavior due to environmental enrichment by others [18,19]. Altogether, it seems that the C57BL/6 J background was more stress-resistant, be it due to housing conditions or due to genetics, highlighting that standardization of test protocols does not guarantee absence of systematic differences in results of different research institutes [49]. With the center time analyzed in the actometer in the first minute, we intended to evaluate possible freezing behavior due to the confrontation of the animal with a new environment. Interestingly, in this test, the FGF-2$^{+/+}$ mice stayed in place significantly longer than LMW$^{+/+}$ and HMW$^{+/+}$ mice. Of course, this way of interpretation depends on exactly identical conditions in all experimental runs. During the whole testing period of 10 min, conditions were always comparable. As results show at least a significant difference comparing FGF-2$^{+/+}$ and HMW$^{+/+}$ mice, we propose strain specific differences in behavior during exploration of new environments. This observation is in agreement with the results of the wall rear analysis demonstrating reduced explorative behavior of FGF-2$^{+/+}$ compared LMW$^{+/+}$, and also supported by the additive energy density score results. Additionally, comparing animals within one mouse strain demonstrated different behavioral activity for isoform specific ko mice. However, this observation could, of course be due to altered FGF-2 expression outside the nigrostriatal pathway.

Importantly, SNP analyses of mouse strain backgrounds verified discrepancies between FGF-2 and isoform specific ko strains further supporting our hypothesis that behavior is crucially influenced by the background of those mice. Finally, we would not interpret the varying center time in the open field experiments as a relevant difference in anxiety-like behavior or stress sensitivity. In fact, the results in the more sensitive elevated plus maze and modified dark-light test supported by corticosterone measurements revealed no differences among the experimental groups.

In conclusion, we show that FGF-2 isoforms influenced the development of the nigrostriatal system in mice, though without resulting in behavioral saliences. Nevertheless, we want to highlight that standardization of mouse background strains for better comparability among research centers is crucial.

Declaration of competing interest

All authors report no biomedical financial interests or potential conflicts of interest.

Acknowledgements

Friederike Freiin von Hövel was financially supported by the Konrad-Adenauer-Stiftung. This research did not receive any other specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

We thank Dietmar Schreiner (Biozentrum, University of Basel) for personal support, and Hella Brinkmann, Ulrike Dringenberg, Silke Fischer, and Natascha Heidrich (Institute of Neuroanatomy and Cell Biology, Hannover Medical School) for excellent technical assistance. We also acknowledge Edith Klobetz-Rassam (Department of Biomedical Sciences, Vienna) for analysis of the fecal samples and Erich Schneider (Institute of Pharmacology, Hannover Medical School) for providing the actometer, the elevated plus maze, and for converting the actometer raw data to text files. Concerning the actometer, we also thank Stephen C. Fowler (University of Cansas, USA) as principle inventor.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2019.112113.

References

[1] A. Cintra, Y.H. Cao, C. Oellig, B. Tinner, F. Bortolotti, M. Goldstein, R.F. Pettersson, K. Fuxe, Basic FGF is present in dopaminergic neurons of the ventral midbrain of the rat, Neuroreport 2 (1991) 597–600.

[2] C. Grothe, M. Timmer, The physiological and pharmacological role of basic fibroblast growth factor in the dopaminergic nigrostriatal system, Brain Res. Rev. 54 (2007) 80–91.

[3] G. Ferrari, M.C. Minozzi, G. Toffano, A. Leon, S.D. Skaper, Basic fibroblast growth factor promotes the survival and development of mesencephalic neurons in culture, Dev. Biol. 133 (1989) 140–147.

[4] C. Grothe, A. Schulze, I. Semkova, F. Muller-Ostermeyer, A. Rege, K. Wewetzer, The high molecular weight fibroblast growth factor-2 isoforms (21,000 mol. Wt and 23,000 mol. wt) mediate neurotrophic activity on rat embryonic mesencephalic dopaminergic neurons in vitro, Neuroscience 100 (2000) 73–86.

[5] M. Timmer, K. Cesnulevicius, C. Winkler, J. Kolb, E. Lipokatic-Takacs, J. Jungnickel, C. Grothe, Fibroblast growth factor (FGF)-2 and FGF receptor 3 are required for the development of the substantia nigra, and FGF-2 plays a crucial role for the rescue of dopaminergic neurons after 6-hydroxydopamine lesion, J. Neurosci. 27 (2007) 459–471.

[6] A. Ratzka, O. Baron, M.K. Stachowiak, C. Grothe, Fibroblast growth factor 2 regulates dopaminergic neuron development in vivo, J. Neurochem. 122 (2012) 94–105.

[7] B. Bugler, F. Amalric, H. Prats, Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor, Mol. Cell. Biol. 11 (1991) 573–577.

[8] M. Azhar, M. Yin, M. Zhou, H. Li, M. Mustafa, E. Nusayr, J.B. Keenan, H. Chen, S. Pawlosky, C. Gard, C. Grisham, L.P. Sanford, T. Doetschman, Gene targeted ablation of high molecular weight fibroblast growth factor-2, Dev. Dyn. 238 (2009) 351–357.

[9] A.D. Ebert, M. Laussmann, S. Wegehingel, L. Kaderali, H. Erfle, J. Reichert, J. Lechner, H.D. Beer, R. Pepperkok, W. Nickel, Tec-kinase-mediated phosphorylation of fibroblast growth factor 2 is essential for unconventional secretion, Traffic 11 (2010) 813–826.

[10] R.Z. Florkiewicz, A. Baird, A.M. Gonzalez, Multiple forms of bFGF: differential nuclear and cell surface localization, Growth Factors 4 (1991) 265–275.

[11] R. Rumpel, O. Baron, A. Ratzka, M.L. Schroder, M. Hohmann, A. Effenberg, P. Claus, C. Grothe, Increased innervation of forebrain targets by midbrain dopaminergic neurons in the absence of FGF-2, Neuroscience 314 (2016) 134–144.

[12] H. Bernheimer, W. Birkmayer, O. Hornykiewicz, K. Jellinger, F. Seitelberger, Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations, J. Neurol. Sci. 20 (1973) 415–455.

[13] O. Even-Chen, S. Barak, The role of fibroblast growth factor 2 in drug addiction, Eur. J. Neurosci. (2018).

[14] M. Laruelle, L.S. Kegeles, A. Abi-Dargham, Glutamate, dopamine, and schizophrenia: from pathophysiology to treatment, Ann. N. Y. Acad. Sci. 1003 (2003) 138–158.

[15] S. Simard, P. Shail, J. MacGregor, M. El Sayed, R.S. Duman, F.M. Vaccarino, N. Salmaso, Fibroblast growth factor 2 is necessary for the antidepressant effects of fluoxetine, PLoS One 13 (2018) e0204980.

[16] A.F. Terwisscha van Scheltinga, S.C. Bakker, R.S. Kahn, M.J.H. Kas, Fibroblast growth factors in neurodevelopment and psychopathology, Neuroscientist 19 (2013) 479–494.

[17] E. Eren-Kocak, C.A. Turner, S.J. Watson, H. Akil, Short-hairpin RNA silencing of endogenous fibroblast growth factor 2 in rat hippocampus increases anxiety behavior, Biol. Psychiatry 69 (2011) 534–540.

[18] J.A. Perez, S.M. Clinton, C.A. Turner, S.J. Watson, H. Akil, A new role for FGF2 as an endogenous inhibitor of anxiety, J. Neurosci. 29 (2009) 6379–6387.

[19] N. Salmaso, H.E. Stevens, J. McNeill, M. ElSayed, Q. Ren, M.E. Maragnoli, M.L. Schwartz, S. Tomasi, R.M. Sapolsky, R. Duman, F.M. Vaccarino, Fibroblast growth factor 2 modulates hypothalamic pituitary Axis activity and anxiety behavior through glucocorticoid receptors, Biol. Psychiatry 80 (2016) 479–489.

[20] P. Fadda, F. Bedogni, A. Fresu, M. Collu, G. Racagni, M.A. Riva, Reduction of corticostriatal glutamatergic fibers in basic fibroblast growth factor deficient mice is associated with hyperactivity and enhanced dopaminergic transmission, Biol. Psychiatry 62 (2007) 235–242.

[21] R. Dono, G. Texido, R. Dussel, H. Ehmke, R. Zeller, Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice, EMBO J. 17 (1998) 4213–4225.

[22] B. Garmy-Susini, E. Delmas, P. Gourdy, M. Zhou, C. Bossard, B. Bugler, F. Bayard, A. Krust, A.C. Prats, T. Doetschman, H. Prats, J.F. Arnal, Role of fibroblast growth factor-2 isoforms in the effect of estradiol on endothelial cell migration and proliferation, Circ. Res. 94 (2004) 1301–1309.

[23] K. Mekada, K. Abe, A. Murakami, S. Nakamura, H. Nakata, K. Moriwaki, Y. Obata, A. Yoshiki, Genetic differences among C57BL/6 substrains, Exp. Anim. 58 (2009) 141–149.

[24] E. Zurita, M. Chagoyen, M. Cantero, R. Alonso, A. Gonzalez-Neira, A. Lopez-Jimenez, J.A. Lopez-Moreno, C.P. Landel, J. Benitez, F. Pazos, L. Montoliu, Genetic polymorphisms among C57BL/6 mouse inbred strains, Transgenic Res. 20 (2011) 481–489.

[25] M. Smoczek, M. Vital, D. Wedekind, M. Basic, N.H. Zschemisch, D.H. Pieper, A. Siebert, A. Bleich, A Combination of Genetic and Microbiota Influences the Severity of the Obesity Phenotype in Diet-induced Obesity. Submitted, (2018).

[26] E. Kefalakes, S. Boselt, A. Sarikidi, M. Ettcheto, F. Bursch, M. Naujock, N. Stanslowsky, M. Schmuck, M. Barenys, F. Wegner, C. Grothe, S. Petri, Characterizing the multiple roles of FGF-2 in SOD1(G93A) ALS mice in vivo and in vitro, J. Cell. Physiol. (2018).

[27] A. Ratzka, O. Baron, C. Grothe, FGF-2 deficiency does not influence FGF ligand and receptor expression during development of the nigrostriatal system, PLoS One 6 (2011) e23564.

[28] M. Mahler Convenor, M. Berard, R. Feinstein, A. Gallagher, B. Illgen-Wilcke, K. Pritchett-Corning, M. Raspa, FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units, Lab Anim. 48 (2014) 178–192.

[29] S.C. Fowler, B.R. Birkestrand, R. Chen, S.J. Moss, E. Vorontsova, G. Wang, T.J. Zarcone, A force-plate actometer for quantitating rodent behaviors: illustrative data on locomotion, rotation, spatial patterning, stereotypies, and tremor, J. Neurosci. Methods 107 (2001) 107–124.

[30] E.H. Schneider, S.C. Fowler, M.S. Lionakis, M. Swamydas, G. Holmes, V. Diaz, J. Munasinghe, S.C. Peiper, J.L. Gao, P.M. Murphy, Regulation of motor function and behavior by atypical chemokine receptor 1, Behav. Genet. 44 (2014) 498–515.

[31] C. Touma, R. Palme, N. Sachser, Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones, Horm. Behav. 45 (2004) 10–22.

[32] C. Touma, N. Sachser, E. Mostl, R. Palme, Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice, Gen. Comp. Endocrinol. 130 (2003) 267–278.

[33] C. Häger, L.M. Keubler, S.R. Talbot, S. Biernot, N. Weegh, S. Buchheister, M. Buettner, S. Glage, A. Bleich, Running in the wheel: defining individual severity levels in mice, PLoS Biol. 16 (2018) e2006159.

[34] S.J. Evans, P.V. Choudary, C.R. Neal, J.Z. Li, M.P. Vawter, H. Tomita, J.F. Lopez, R.C. Thompson, F. Meng, J.D. Stead, D.M. Walsh, R.M. Myers, W.E. Bunney, S.J. Watson, E.G. Jones, H. Akil, Dysregulation of the fibroblast growth factor system in major depression, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 15506–15511.

[35] A. Mallei, B. Shi, I. Mocchetti, Antidepressant treatments induce the expression of basic fibroblast growth factor in cortical and hippocampal neurons, Mol. Pharmacol. 61 (2002) 1017–1024.

[36] M. Kato, G. Okugawa, M. Wakeno, Y. Takekita, S. Nonen, S. Tetsuo, K. Nishida, J. Azuma, T. Kinoshita, A. Serretti, Effect of basic fibroblast growth factor (FGF2) gene polymorphisms on SSRIs treatment response and side effects, Eur. Neuropsychopharmacol. 19 (2009) 718–725.

[37] I. Tooyama, T. Kawamata, D. Walker, T. Yamada, K. Hanai, H. Kimura, M. Iwane, K. Igarashi, E.G. McGeer, P.L. McGeer, Loss of basic fibroblast growth factor in substantia nigra neurons in Parkinson's disease, Neurology 43 (1993) 372–376.

[38] B. Reuss, K. Unsicker, Survival and differentiation of dopaminergic mesencephalic neurons are promoted by dopamine-mediated induction of FGF-2 in striatal astroglial cells, Mol. Cell. Neurosci. 16 (2000) 781–792.

[39] S. Giordano, L. Sherman, W. Lyman, R. Morrison, Multiple molecular weight forms of basic fibroblast growth factor are developmentally regulated in the central nervous system, Dev. Biol. 152 (1992) 293–303.

[40] V. Jackson-Lewis, M. Vila, R. Djaldetti, C. Guegan, G. Liberatore, J. Liu, K.L. O'Malley, R.E. Burke, S. Przedborski, Developmental cell death in dopaminergic neurons of the substantia nigra of mice, J. Comp. Neurol. 424 (2000) 476–488.

[41] X. Ma, X. Dang, P. Claus, C. Hirst, R.R. Fandrich, Y. Jin, C. Grothe, L.A. Kirshenbaum, P.A. Cattini, E. Kardami, Chromatin compaction and cell death by high molecular weight FGF-2 depend on its nuclear localization, intracrine ERK activation, and engagement of mitochondria, J. Cell. Physiol. 213 (2007) 690–698.

[42] K. Lieb, C. Andersen, N. Lazarov, R. Zienecker, I. Urban, I. Reisert, C. Pilgrim, Pre- and postnatal development of dopaminergic neuron numbers in the male and female mouse midbrain, Brain Res. Dev. Brain Res. 94 (1996) 37–43.

[43] T. Ivanova, C. Beyer, Estrogen regulates tyrosine hydroxylase expression in the neonate mouse midbrain, J. Neurobiol. 54 (2003) 638–647.

[44] J. Martí-Clúa, Natural apoptosis in developing mice dopamine midbrain neurons and vermal Purkinje cells, Folia Neuropathol. 54 (2016) 180–189.

[45] S. Flaisher-Grinberg, H. Einat, Strain-specific battery of tests for domains of mania: effects of valproate, lithium and imipramine, Front. Psychiatry (2010) 1.

[46] J. Ahlgren, V. Voikar, Housing mice in the individually ventilated or open cages - does it matter for behavioral phenotype? Genes Brain Behav. (2019) e12564.

[47] A.D. Kovacs, D.A. Pearce, Location- and sex-specific differences in weight and motor coordination in two commonly used mouse strains, Sci. Rep. 3 (2013) 2116.

[48] N. Pasquarelli, P. Voehringer, J. Henke, B. Ferger, Effect of a change in housing conditions on body weight, behavior and brain neurotransmitters in male C57BL/6J mice, Behav. Brain Res. 333 (2017) 35–42.

[49] J.C. Crabbe, D. Wahlsten, B.C. Dudek, Genetics of mouse behavior: interactions with laboratory environment, Science 284 (1999) 1670.