RESEARCH PAPER

Acta Neurobiol Exp 2023, 83: 404–413
DOI: 10.55782/ane-2023-2472

Early development of the *Tsc1* Purkinje cell specific mouse knockouts

Anna Sługocka[1,2], Marta Anna Przybyła[1,3], Jarosław Jerzy Barski[1,2]*

[1] Department for Experimental Medicine, Medical University of Silesia, Katowice, Poland,
[2] Department of Physiology, School of Medicine in Katowice, Medical University of Silesia, Katowice, Poland,
[3] Laboratory of Molecular Biology, Institute of Physiotherapy and Health Science, Academy of Physical Education, Katowice, Poland,
** Email: jbarski@sum.edu.pl*

Tsc1 is a gene which expression results in hamartin, a protein involved in regulation of the mTOR1 pathway. Inactivation of *Tsc1* gives rise to hyperactivation of the mTOR1 machinery, increased proliferation and growth of cells with subsequent cell degeneration and cell death. In humans, mutations of *Tsc1* result in an inherited disorder - tuberous sclerosis complex (TSC) with the concomitant multiorgan non-malignant tumors (tubers), epileptic seizures and autistic-like manifestations. General mouse knock-outs, homozygous for the inactivated *Tsc1* alleles do not survive and die at early embryonal stages. To circumvent this problem, we utilized the Cre/loxP system and removed *Tsc1* specifically in Purkinje cells using the pcp2/L7Cre mouse strain and the *Tsc1*tmDjk/J strains. Because of the published results showing the autistic-like symptoms after the same crossbred, we have decided to look closer at the early postnatal period of these mutants. Surprisingly no evidence of any behavioral alterations were found, including the ultrasonic vocalizations of newborns. We decided to focus more attention on the interpretation of data, including a more detailed statistical evaluation of our results.

Key words: Purkinje cells, pcp2/L7Cre, *Tsc1*, early development, behavior

INTRODUCTION

Tuberous sclerosis complex (TSC) is an inherited multi-organ disease resulting from hyperactivation of the mTOR signaling pathway, which in turn is caused by mutations in one or both genes, *Tsc1* and *Tsc2*. Expression of these two genes results in two protein inhibitors of mTOR: hamartin and tuberin. Hyperactivation of the mTOR pathway results in formation of non-malignant tumors – hamartomas, which may develop in many organs including brain, where subependymal nodules (SENs) or subependymal giant cell astrocytomas (SEGA) can be also observed (Napolioni et al., 2004; Boer et al., 2008; Orlova and Crino, 2010; Lipton and Sahin, 2014). These malformations are responsible for various neurological and psychiatric manifestations including autistic-like traits present in about 20-60% of patients suffering from TSC (Curatolo, 2004; Jeste et al., 2008). Study results published recently indicate that one of the brain parts responsible for autistic-like behaviors is the cerebellum (Fatemi et al., 2012; Rogers et al., 2013; Sudarov, 2013). This evolutionary old part of the central nervous system (CNS) seems to be involved not only in pure motor functions, but also in cognition and control of emotions (Wang et al., 2014; Dickson et al., 2017). Many studies showed that in patients with malfunctions in the cerebellum additional cognitive deficits were stated (Rogers et al., 2013). Our own preliminary data (unpublished results) and other data coming from Purkinje cells (PC) specific *Tsc1* knock-out mice (Tsai et al., 2012) suggested autistic-like neurodevelopmental disorders in these animals. To verify these indications and to broaden the analysis on other developmental stages, we have decided to analyze the same mouse strain in the first three weeks of life.

Received 24 January 2022, accepted 25 October 2023

METHODS

Animals

In order to stop the expression of *Tsc*1 in PC, female mice carrying the floxed *Tsc1* gene (*Tsc1*tmDjk/J, Jackson Laboratory, Bar Harbor, USA) (Kwiatkowski et al., 2002) were crossed with males expressing Cre recombinase exclusively in PC (B6.129-Tg(Pcp2-cre)2Mpin/J, Center for Experimental Medicine, Katowice, Poland) (Barski et al., 2000). It has to be stressed that for the ultrasonic vocalizations (USV) analysis (isolation test) the maternal genotype has to be carefully selected. If the dam had even only one not-functional *Tsc1* allele, observed USV alterations could potentially depend on the dam's behavioral insufficiency (Tsai et al., 2012). For this reason, only females with both functional *Tsc1* alleles were chosen to deliver newborns for the isolation test. As the effect of that breeding, all genotypes according to the Mendelian rules were obtained. For further steps, animals of both sexes were assigned to following groups: full *Tsc1* knockouts (KO), single *Tsc1* allele knockouts (HET), and animals with both fully functional *Tsc1* alleles which served as control (WT). In the first publication describing the B6.129-Tg(Pcp2-cre)2Mpin/J strain we were able to confirm first signs of Cre recombinase activity at PND 6 using the ß-galactosidase reporter strain (Barski et al., 2000). To assess more precisely the starting point of the pcp2/L7Cre transgene expression, we utilized animals from the B6.Cg-Gt(ROSA)26Sortm9 (CAG-tdTomato)Hze/J strain and crossed with abovementioned Cre expressing mice (Madisen et al., 2010). Standard PCR reaction was performed to assign animals to proper groups.

Animals were housed in the animal facility in the Department for Experimental Medicine in a 12 h dark-light cycle environment, temperature 22°C, humidity 50% with access to standard food and water *ad libitum*. Whole experiment was planned and carried out in accordance with guidelines of the Local Committee for Animal Welfare, license number 24/2013.

Histology

For the histological analysis we used 28 weeks old animals from the reporter and from the knockout strains. Animals were anesthetized with ketamine – xylazine mixture (2 mg per kg of body weight) and perfused transcardially with PBS followed by 4% PFA in PBS. After dissection brains were fixed overnight in 4% PFA, and then immersed until saturation in 30% sucrose at 4°C. Embedded brain tissue (Tissue-Tec, Sakura, Japan) was sliced into 30 µm frozen sections by means of a cryostat microtome (Thermo Scientific, USA). Sagittal sections of the cerebellum were washed with PBS and placed on microscope slides with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, U.S.A). Immunostaining was performed according to the standard procedures using primary antibodies for calbindin D-28k (Swant, Bellinzona, Switzerland). Analysis of slices was performed in the Zeiss Axio Scope.A1 (Carl Zeiss Microscopy GmbH, Jena, Germany) microscope. Images were obtained in Zen 2 (blue edition) imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Isolation test and ultrasonic vocalizations (USV) recordings

All experimental procedures were designed according to the earlier set-up protocol (Hofer et al., 2002; Wöhr et al., 2008; Sungur et al., 2016).

USV recordings were carried out on every second postnatal day (PND) from PND 2 to PND 14. The dam was removed from the litter and placed in a separate room. To ensure a proper temperature for the newborns, the home cage was placed on a heating pad set at 34°C. After 10 min of rest animals were ready for the experiment. Recordings were performed in a separate room to ensure sensory isolation of a tested animal from the rest of the pups. Every pup was carefully placed on the test cage floor and the microphone was fixed 7 cm above the floor. Recordings were performed for 5 min and when finished, for each pup body temperature and weight were measured. In the end pups were marked with unscented ink to enable further identification and placed back into the home cage. Recordings were made with use of a condenser ultrasound microphone, Avisoft-Bioacoustics CM16/CMPA and the UltraSoundGate 116Hb recording interface (Avisoft Bioacoustics, Germany). Recorded vocalizations were analyzed by means of the integrated software Avisoft SASLab Pro (version 5.2) with the following setup of fast Fourier transformation: 512 FFT length, 100% frame, Hamming window, and 75% time window overlap. Automatic call detection provided by a build-in threshold-based algorithm (amplitude threshold: 240 dB; hold time: 10 ms; high-pass filter: 30 kHz) was verified by an experienced user.

To detect differences between experimental groups 6 parameters of recordings were evaluated: number of calls within the observation interval, total call duration, peak frequency, expressed as peak frequency at maximal amplitude, peak amplitude, expressed as maximal amplitude at maximal frequency, bandwidth,

expressed as difference between the minimal and maximal frequency in a single USV call, frequency modulation, latency – time elapsed to the first emitted call, mean call duration – mean duration of a single call which are the basic parameters used for USV evaluation (Bell et al., 1972; Roubertoux et al., 1996; Wöhr et al., 2008; 2011; Yang et al., 2012; Scattoni et al., 2013; Mosienko et al., 2015).

Fox's battery

From the first day on, when newborns were observed in the nest, a spectrum of reflexes and developmental milestones were studied. According to the well-established protocols known as Fox's battery (Fox, 1965; Wahlstein, 1974; Branchi and Ricceri, 2002; Castelhano-Carlos, 2010; Feather-Schussler and Ferguso, 2016; Crawley, 2019), sensory-motor development was observed in the first 21 days of life. Score 0 or 1 was attributed, respectively, to animals not presenting or presenting the reflex. The battery allows to specify the time of the first appearance of various reflexes and behavioral responses in early postnatal development. In this study following parameters were chosen:

- Eyes opening – expressed as the number of the postnatal day when first observed.
- Walking – the day when mouse pup was able to walk.
- Rooting reflex – expressed as a first day when animal is crawling forward and moving head in rooting fashion as a response to bilateral face stimulation.
- Cliff drop aversion – first day when the animal started to turn and crawl away when placed on the edge of the table.
- Auricular (auditory) startle response – presence of the whole-body startle-response after a short and loud sound stimulus.
- Postural reflex – the normal postural reflex is the extension of all four legs in order to maintain an upright, balanced position.
- Grasp reflex – in this study determined by observation of palmar reflex.
- Negative geotaxis – time needed to turn and crawl up after being placed upside down on the 45° angle slope. Measured from PND 2 to PND 10.
- Righting reflex – time needed to turn upright after being placed in supine position.

In the last two reflexes, if a task was not completed by the animal within 30 s measurement it was excluded from the analysis of the reflex.

At each day of testing animals were weighted and the temperature of their body was measured.

Statistics

Statistical analysis was performed in STATISTICA 12 Software (StatSoft Inc.). The distribution of each data set was checked for normality using the Shapiro-Wilk test. Homogeneity of variances was determined by the Levene test. If assumptions were fulfilled, differences among groups were planned to be compared using a one-way ANOVA or its modification for repeated measures, followed by the Bonferroni *post hoc* test. When assumptions for parametric analysis were not fulfilled, log transformation was performed as a try of normalization of data distribution. Most of these tries failed, therefore performing a coherent analysis employing parametric methods only was out of reach. It was decided to perform an alternative, nonparametric Kruskall-Wallis test with Dunn's multiple comparisons *post hoc* test or Friedman test with *post hoc* Wilcoxon test with Bonferroni correction for multiple comparisons (for repeated measures testing).

In all analyses the p value<0.05 was considered to be statistically significant. Results were presented as median +/- quartiles.

No formal analysis for intersexual differences was planned due to unequal sex groups.

RESULTS

Histology

To confirm the Cre protein activity in cerebellar Purkinje cells of adult animal slices were analyzed in a fluorescence microscope, revealing blue fluorescence of DAPI and red fluorescence of tdTomato protein (Fig. 1). Figs 1A and 1B present colocalization of these two signals in control animals, B6.129-Tg(Pcp2-cre)2Mpin/J and B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J animals respectively. In cerebellar slices, however from animals carrying both transgenes (Fig. 1C) the red fluorescence of the tdTomato protein can be observed. In course of the postnatal development first signs of Cre expression in the cerebellum, revealed as red fluorescence, were visible at PND 0 (Fig. 2A-C).

As mTOR hyperactivation results in degeneration and death of Purkinje cells, cerebellar cortex slices were incubated with calbindin D-28k antibodies (Fig. 4). Obvious signs of Purkinje cell death were visible in animals full knock-out for the *Tsc1* gene at week 28 (Fig. 3).

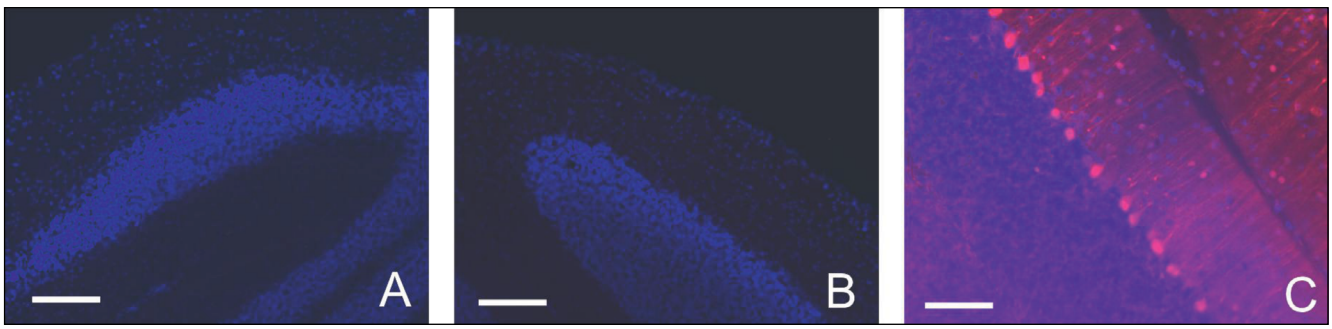

Fig. 1. Successful recombinase Cre action proved by the tdTomato protein expression (red) in the adult (28 weeks) mouse cerebellum. DAPI staining in blue (A) Control animal with *pcp2/L7Cre* transgene only, (B) Control animal from the B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J strain, (C) Animal from crossed strains: carrying *pcp2/L7Cre* and B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J transgenes. Scale bar = 50 μm.

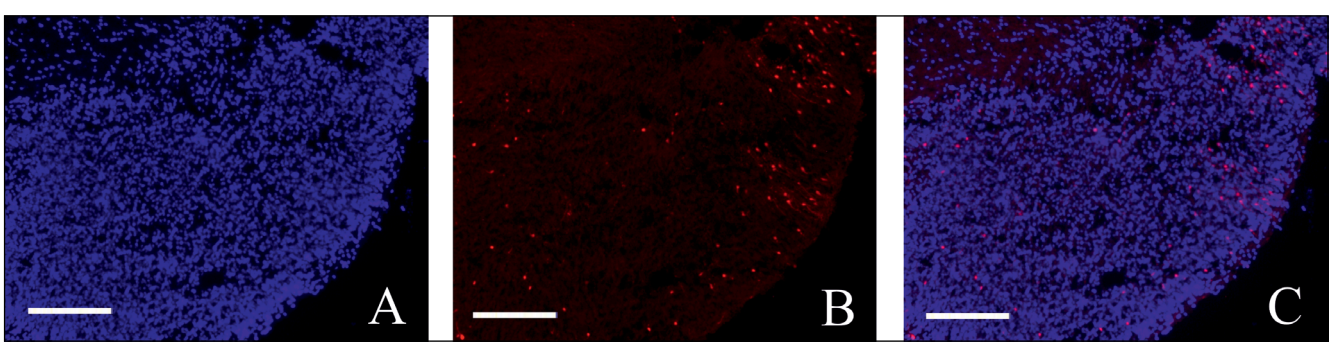

Fig. 2. tdTomato protein expression (red) in the cerebellum of a PND 0 animal as prove of Cre recombinase-based recombination. DAPI staining in blue. (A) Blue DAPI fluorescence, 461 nm, (B) Red tdTomato fluoresce, 581 nm, (C) Merge channels. Scale bar = 50 μm.

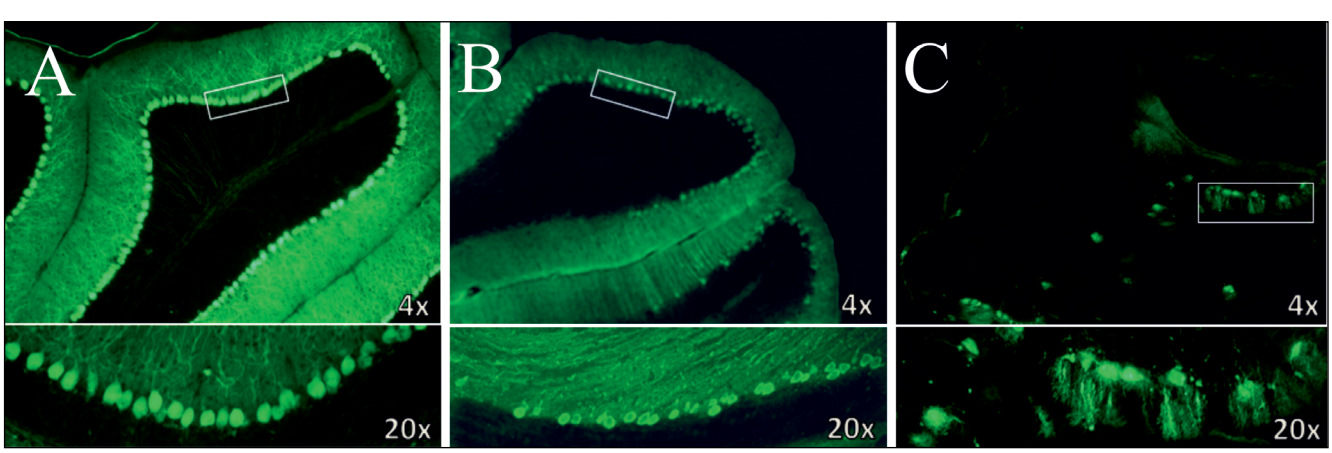

Fig. 3. Degeneration of Purkinje cells in the cerebellar cortex revealed by calbindin Cb-28k immunostaining. (A) Control group, both *Tsc1* alleles are active, (B) One *Tsc1* allele is inactivated, (C) Both *Tsc1* alleles are inactivated. Adult animals, 28 weeks old.

USV recordings

As expected, the peak in the number of vocalizations was observed at PND 6 (Fig. 4). Number of calls per 1 minute is the parameter most commonly used for characterization of USV activity. Presented values reflect the number of single calls produced by pups during 5-minute recording and gives a simple view on the response intensity to isolation from the dam and littermates. This parameter, in most studies, is analyzed on a single day, mostly on PND 6 or PND 8, assumed as the point of the highest USV activity. However, when we look at the time course presented in Fig. 4, it becomes clear that the vocalization number varies strongly over time and analysis on one selected day, especially in developmental context, could lead to false interpretation of the data. The curves representing time course for all three groups look quite similar on particular days. In all groups the lowest number of calls was recorded at PND 2 and the highest at PND 6 - *post hoc* Wilcoxon test with Bonferroni correction for multiple comparisons revealed significant p-values levels in comparison of these days to all other days (Fig. 4).

Analysis did not reveal statistically significant effects of the genotype on: total number of calls, number of calls per minute, total call duration, peak frequency, peak amplitude, frequency modulation and bandwidth in any of analyzed time points (Table 1).

The latency to first call is a parameter reflecting the time of animal reaction to isolation from the dam and littermates. WT and HET had significantly different latency at PND 10 [H (2, N=44)=66.2121, p=0.0448 with *post hoc* p-value=0.0385] (Fig. 5).

Fox's battery

Statistical analysis did not reveal any significant differences among groups in respect to the time point of eye opening [ANOVA K-W result: H (2, N=33)=3.834232 p=0.1470]. On average heterozygous and knockout animals were opening their eyes at PND 13 while the control group at PND 14.

Newborns from all tested groups started to walk between PND 7 and PND 8. For the WT and HET groups, it was on average PND 8, while for the KO mice PND 7 [H (2, N=33)=2.858193 p=0.2395].

Rooting reflex was first observed between the 4th and 11th postnatal day. The median values for WT and HET mice was the same – PND 7, while for knockouts PND 9. KO group presented also the highest noted first appearance of that reflex – PND 11. Nonetheless ANOVA K-W test didn't reveal any statistically significant differences [H (2, N=33)=0.5316396 p=0.7666].

Similar results were obtained for cliff drop aversion. Although median values vary between 2 to 4 among genotypes, Kruskal-Wallis test results do not indicate statistically significant differences [H (2, N=33)=3.718416 p=0.1558]. Auricular startle response was present at PND 7 on average, in all three groups [H (2, N=33)=1.061732 p=0.5881]. No significant effect of the genotype was detected in postural reflex

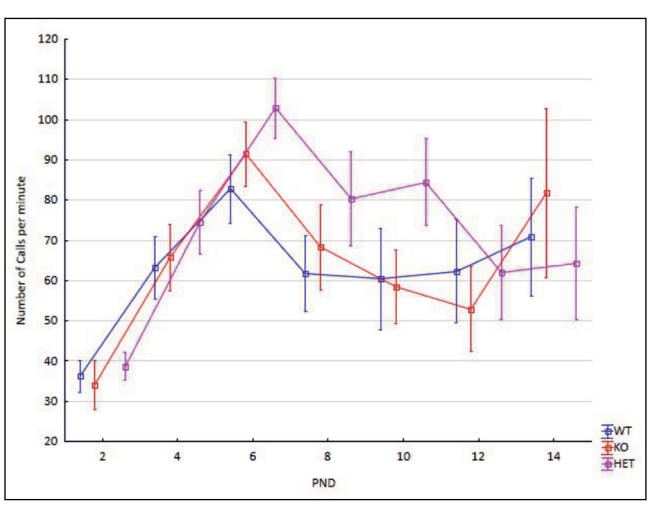

Fig. 4. Time course of changes in the number of calls per minute. KO – animals with both *Tsc1* alleles inactivated in PCs. WT – control group. HET – animals with single *Tsc1* allele inactivated in PCs. Significance of gradual increase in number of calls per minute during first 6 days was confirmed in *post hoc* tests. Median, +/- quartiles.

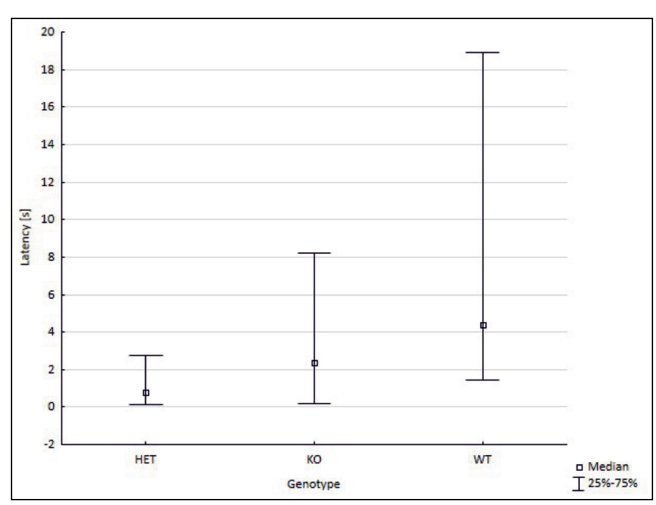

Fig. 5. Differences between groups in latency to first call at PND 10. Statistically significant was only the difference between the HET and WT groups (p<0,04). KO – animals with both *Tsc1* alleles inactivated in PCs. WT – control group. HET – animals with single *Tsc1* allele inactivated in PCs. PND – postnatal day. Median, +/- quartiles.

Table 1. Kruskall-Wallis H statistic with p-values for all USV isolation test parameters.

Parameter	Time point	K-W H statistic with p-values
Number of calls per minute [calls/min]	PND 2	H (2, N=68)=1.5074 p=0.4706
Number of calls per minute [calls/min]	PND 4	H (2, N=52)=1.2985 p=0.5224
Number of calls per minute [calls/min]	PND 6	H (2, N=50)=3.7565 p=0.1529
Number of calls per minute [calls/min]	PND 8	H (2, N=44)=1.1695 p=0.5573
Number of calls per minute [calls/min]	PND 10	H (2, N=44)=3,4541 p=0.1778
Number of calls per minute [calls/min]	PND 12	H (2, N=43)=0.2469 p=0.8839
Number of calls per minute [calls/min]	PND 14	H (2, N=41)=0.3915 p=0.8222
Total call duration [s]	PND 2	H (2, N=68)=0.6732 p=0.7142
Total call duration [s]	PND 4	H (2, N=52)=3.4920 p=0.1745
Total call duration [s]	PND 6	H (2, N=50)=4.8745 p=0.0874
Total call duration [s]	PND 8	H (2, N=44)=0.8497 p=0.6539
Total call duration [s]	PND 10	H (2, N=44)=3.0374 p=0.2190
Total call duration [s]	PND 12	H (2, N=43)=0.2780 p=0.8702
Total call duration [s]	PND 14	H (2, N=41)=0.2763 p=0.8710
Mean peak frequency [kHz]	PND 2	H (2, N=68)=2.8823 p=0.2366
Mean peak frequency [kHz]	PND 4	H (2, N=52)=3.4362 p=0.1794
Mean peak frequency [kHz]	PND 6	H (2, N=50)=2.4484 p=0.2940
Mean peak frequency [kHz]	PND 8	H (2, N=44)=1.9303 p=0.3809
Mean peak frequency [kHz]	PND 10	H (2, N=44)=1.0166 p=0.6015
Mean peak frequency [kHz]	PND 12	H (2, N=43)=1.8821 p=0.3902
Mean peak frequency [kHz]	PND 14	H (2, N=38)=5.1997 p=0.0743
Mean peak amplitude [dB]	PND 2	H (2, N=68)=0.5465 p=0.7609
Mean peak amplitude [dB]	PND 4	H (2, N=52)=1.3343 p=0.5132
Mean peak amplitude [dB]	PND 6	H (2, N=50)=3.6174 p=0.1639
Mean peak amplitude [dB]	PND 8	H (2, N=44)=0.4984 p=0.7794
Mean peak amplitude [dB]	PND 10	H (2, N=44)=0.7051 p=0.7029
Mean peak amplitude [dB]	PND 12	H (2, N=43)=0.3112 p=0.8559
Mean peak amplitude [dB]	PND 14	H (2, N=38)=0.9055 p=0.6359
Bandwidth [kHz]	PND 2	H (2, N=68)=0.0085 p=0.9958
Bandwidth [kHz]	PND 4	H (2, N=52)=4.3133 p=0.1157
Bandwidth [kHz]	PND 6	H (2, N=50)=0.4989 p=0.7792
Bandwidth [kHz]	PND 8	H (2, N=44)=0.9588 p=0.6192
Bandwidth [kHz]	PND 10	H (2, N=44)=4.4177 p=0.1098
Bandwidth [kHz]	PND 12	H (2, N=43)=1.2491 p=0.5355
Bandwidth [kHz]	PND 14	H (2, N=38)=0.1680 p=0.9194
Mean frequency modulation [kHz]	PND 2	H (2, N=68)=2.2205 p=0.3295
Mean frequency modulation [kHz]	PND 4	H (2, N=52)=4.4843 p=0.1062
Mean frequency modulation [kHz]	PND 6	H (2, N=50)=4.4014 p=0.1107
Mean frequency modulation [kHz]	PND 8	H (2, N=44)=1.6114 p=0.4468
Mean frequency modulation [kHz]	PND 10	H (2, N=44)=2.1205 p=0.3464
Mean frequency modulation [kHz]	PND 12	H (2, N=43)=0.7906 p=0.6735
Mean frequency modulation [kHz]	PND 14	H (2, N=38)=0.9520 p=0.6213

development [H (2, N=33)=0.6153411 p=0.7352]. Graph representing changes in the righting reflex during testing days clearly show decreasing time needed to complete the task, what is additionally supported by results of Friedman's ANOVA - χ^2(N=14, df=20)=252.62 p=0.0000 in the WT group, χ^2(N=14, df=20)=118.72 p=0.0000 in the HET group and χ^2(N=14, df=20)=164.74 p=0.0000 in the KO group).

The negative geotaxis was expressed as a number of seconds needed to complete the process of turning the body up on the 45° slope. The parameter was measured between PND 1 and PND 10. Median of the time needed to turn the body up on the slope was similar among groups and on consecutive days. Analysis revealed no statistically significant differences in this parameter (Table 2).

Among all genotypes mean body temperature was increasing from *ca.* 29°C at PND 1 to around 35°C at PND 21 (Fig. 6). Also, the weight of the animals was gradually increasing over time (Fig. 6).

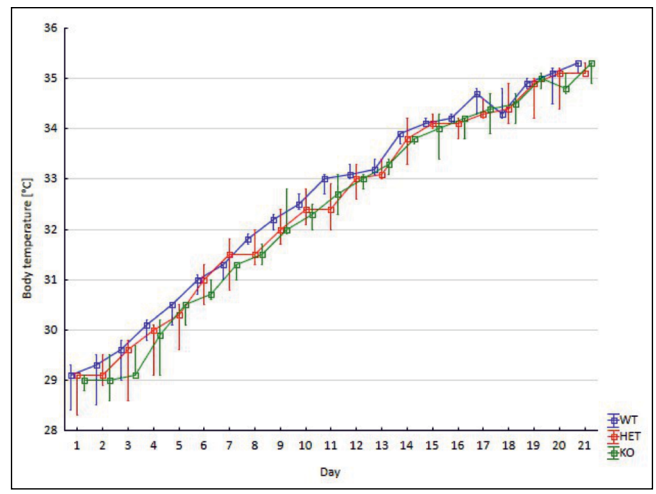

Fig. 6. Time course of changes in the body temperature. KO – animals with both *Tsc1* alleles inactivated in PCs. WT – control group. HET – animals with single *Tsc1* allele inactivated in PCs. PND – postnatal day. Median, +/- quartiles.

Table 2. Kruskall-Wallis H statistic with p-values for righting reflex and negative geotaxis test.

Time point	Negative geotaxis	Righting reflex
PND 1	H (2, N=16)=2.1787 p=0.3364	H (2, N=23)=0.5381 p=0.7641
PND 2	H (2, N=22)=1.8501 p=0.3965	H (2, N=20)=1.1934 p=0.5506
PND 3	H (2, N=24)=0.1606 p=0.9228	H (2, N=13)=1.4049 p=0.4954
PND 4	H (2, N=24)=2.8926 p=0.2354	H (2, N=17)=2.6781 p=0.2621
PND 5	H (2, N=21)=3.5548 p=0.1691	H (2, N=9)=10.1954 p=0.5501
PND 6	H (2, N=19)=3.4039 p=0.1823	H (2, N=13)=2.9807 p=0.2253
PND 7	H (2, N=21)=0.7291 p=0.6945	H (2, N= 15)=1.1420 p=0.5649
PND 8	H (2, N=23)=1.2034 p=0.5479	H (2, N=27)=0.0653 p=0.9678
PND 9	H (2, N=21)=2.0829 p=0.3529	H (2, N=29)=0.0817 p=0.9599
PND 10	H (2, N=14)=1.7809 p=0.4105	H (2, N=31)=0.8128 p=0.6660
PND 11		H (2, N=32)=4.1098 p=0.1281
PND 12		H (2, N=32)=1.5792 p=0.4540
PND 13		H (2, N=32)=1.0452 p=0.5930
PND 14		H (2, N=31)=2.5564 p=0.2785
PND 15		H (2, N=31)=0.1836 p=0.9122
PND 16		H (2, N=31)=5.9393 p=0.0513
PND 17		H (2, N=31)=2.1444 p=0.3423
PND 18		H (2, N=31)=0.3484 p=0.8401
PND 19		H (2, N=31)=1.2142 p=0.5449
PND 20		H (2, N=31)=1.0806 p=0.5826
PND 21		H (2, N=31)=0.0000 p=1.000

Slight differences in the body weight between genotypes were observed at PND 6 and PND 8 [H (2, N=50)=6.1442, p=0.0463 at PND 6 and H (2, N=50)=6.2428, p=0.0441 at PND 8]. *Post hoc* test revealed statistically significant differences between KO and HET groups at PND 6 and PND 8 (p=0.0453 and p=0.0389 respectively) (Fig. 7).

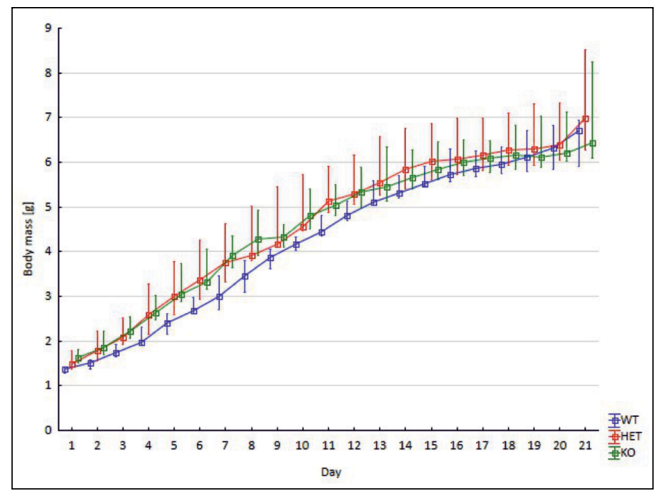

Fig. 7. Time course of changes in the weight. KO – animals with both *Tsc1* alleles inactivated in PCs. WT – control group. HET – animals with single *Tsc1* allele inactivated in PCs. PND – postnatal day. Median, +/- quartiles.

DISCUSSION

To compare our results with other data we decided to focus on studies utilizing the Purkinje cell specific mutants only and avoid in that way discussion with not strictly Purkinje cell dependent manifestations. Since development of the Purkinje cell specific transgenesis (Barski et al., 2000; Saito et al., 2005), many various mutations were introduced specifically into these neurons (Barski et al., 2002; Slugocka et al., 2017). In most cases however, less attention was paid on the early developmental stages of these mutant animals and traits analyzed in our study.

Histology

Expression start point of the Cre transgene is crucial for the experimental design of all Cre/loxP based recombination experiments. It determines the onset of activation or inactivation of the targeted allele. In the primary paper dealing with the generation of the first Purkinje cell specific Cre expressing mouse strain (Barski et al., 2000), developmental analysis of Cre expression revealed the start point at PND6. In this approach the GtROSA26 indicator mouse strain (B6,129S-Gtrosa26tm1Sor) was used, where recombination was traced as X-gal staining of ß-galactosidase. The ß-galactosidase approach was also used in the Zhang study (2004) with the same result supporting PND 6 as the Cre expression starting point. In 2005 Noboru Suzuki group published the knock-in pcp2-Cre mouse (Saito et al., 2005). Additionally, authors looked for the developmental time course of Cre expression by means of ß-galactosidase, claiming the expression starts for E17 and showing X-gal staining at PND 0. Keeping in mind the flaws of this method, in this paper we applied a much more sensitive reporter strain and namely the B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J mice (Madisen et al., 2010). In that case one can follow the recombination time course without additional staining procedures, because Cre-based recombination initiates expression of the red fluorescing tdTomato protein (Fig. 1). In that way we were able to define the expression onset at PND 0 already (Fig. 2), however we did not analyze embryos before delivery. Data presented above show clearly, that the developmental pattern of pcp2-Cre transgenes could be variable and depends on a transgenic approach used, but it is worth considering, that detection sensitivity is important factor.

USV recordings

Recorded data enabled us to establish the course of developmental variability of the selected USV parameters. All of the data obtained result not from the PC loss but only from the activation of the mTOR pathway, because even in the six weeks of life no significant PC loss was visible (own data, unpublished results). The most important message is that the general USV activity, expressed as vocalization rate, does not significantly differ, as result of mTOR activation, from the control animals. In all tested groups there is a clear and statistically significant peak around PND 6 (*post hoc* Wilcoxon test with Bonferroni correction for multiple comparisons revealed significant p-values levels in comparison of PND 6 to all other days) (Fig. 4). This result is different from the data presented in the paper of Tsai (2012), however these authors did not record USV on PND 8, so they showed a more stable time course resulted from their data. It's hard to compare our results with Tsai et al. (2012) not only because other time points were chosen to evaluate USVs, but also because of different statistical methodology. Even though we believe that in experimental conditions all animals in groups are homogenous, statistical conclusions should be cautious considering small sample size

($n \geq 8$). In the comparable experimental time course (PND 2 – PND 12) we have analyzed more animals, $n \geq 12$ for each time point and group. When we looked at other USV parameters, we noticed that most of the parameters are not significantly affected by the activation of the mTOR cascade.

The only one USV parameter where differences were observed is the latency to first call on PND 10, however only the difference between WT and HET groups reached the statistical significance level (Fig. 5). These alterations could suggest an increased sensitivity of pups to the separation from the dam. We already mentioned above that differences between our data and the data in Tsai paper could result from different statistical approaches applied for data analysis. However, this is not the only issue. The next methodological discrepancy is the recording of USV. It seems, according to Tsai paper, that pups for recordings were each time removed directly from the mother and the mother together with the remaining pups were in the same room where the recordings took place. In our experiments all pups were removed from the dam prior to experiment and placed on the heating pad in a separate room prepared for recordings, according to the protocol used by the Wöhr group (Wöhr et al., 2008). The next difference was the genotype of the mother. For recordings we did not use newborns from mothers with one deleted *Tsc1* allele. In that way we wanted to exclude the possible influence of the mother with impaired *Tsc1* expression level.

Fox's battery

Fox's battery is a set of simple tests used for evaluation of the early motor and behavioral development of the mouse according to developmental milestones. The milestones (selected simple reflexes – see the chapter "Methods") have to be achieved at defined time points during the first three weeks of the postnatal life (Wahlstein, 1974). Since Fox's paper published in 1965, the classic battery of tests has been modified many times to be better adapted to the researchers needs (Fox, 1965; Wahlstein, 1974; Branci and Ricceri, 2002; Yang et al., 2012; Feather-Schussler and Ferguson, 2016; Crawley, 2019). This experimental tool is of a big importance for the mouse life period, when more sophisticated motor or behavioral tests cannot be applied. Its advantage is also that the user obtains not only 0/1 results, but the level of impairment can also be evaluated. Our transgenic pups did not display any developmental impairments in respect to analyzed milestones and reflexes. We have to keep in mind however, that in the tested period of life no loss of Purkinje cells was visible even if clear signs of Cre activation were visible already at PND 0 (Fig. 2). There is only a very limited number of studies, which are reliable to our analysis. Autism related animal trials are focused mostly on older animals, where more specific test can be used. In the "classic" autism model, the Engrailed-2 knockout mouse, no abnormalities were found in respect to milestones however other tests revealed a strong impairment of social cues (Brielmaier et al., 2012). Developmental milestones are also (at least partially) preserved in an extremely motor affected mutant strain, namely the TrkB knockout mice (Bosman et al., 2006) (own observations, unpublished results).

CONCLUSIONS

We decided to publish these data, because of some contradictory results when compared with experiments carried out on the same animal model. Our results shown, that the comparable experimental approach may result in different conclusions and may depend on subtle modifications in methodology and statistical evaluation. Additionally, we have noticed only very sparse data coming from behavioral analysis of the early postnatal development, which is due to a very limited number of behavioral tools dedicated for young animals.

ACKNOWLEDGEMENTS

All experiments in that study were supported with grants provided by the Ministry of Science and Higher Education: KNW-2-009-D/5/N, KNW-1-098/N/7/I, KNW-1-110/K/5/0.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

REFERENCES

Barski JJ, Dethleffsen K, Meyer M (2000) Cre recombinase expression in cerebellar Purkinje cells. Genesis 28: 93–98.

Barski JJ, Lauth M, Meyer M (2002) Genetic targeting of cerebellar Purkinje cells: history, current status and novel strategies. Cerebellum 1: 111–118.

Bell RW, Nitschke W, Zachman TA (1972) Ultra-sounds in three inbred strains of young mice. Behav Biol 7: 805–814.

Boer K, Troost D, Jansen F, et al. (2008) Clinicopathological and immunohistochemical findings in an autopsy case of tuberous sclerosis complex. Neuropathology 28: 577–590.

Bosman LW, Hartmann J, Barski JJ, Lepier A, Noll-Hussong M, Reichardt LF, et al. (2006) Requirement of TrkB for synapse elimination in developing cerebellar Purkinje cells. Brain Cell Biol 35: 87–101.

Branchi I, Ricceri L (2002) Transgenic and knock-out mouse pups: the growing need for behavioral analysis. Genes Brain Behav 1: 135–141.

Brielmaier J, Matteson PG, Silverman JL, Senerth JM, Kelly S, Genestine M, et al. (2012) Autism-relevant social abnormalities and cognitive deficits in engrailed-2 knockout mice. PLoS One 7: e-40914.

Castelhano-Carlos MJ, Sousa N, Ohl F, Baumans V (2010) Identification methods in newborn C57BL/6 mice: a developmental and behavioural evaluation. Lab Anim 44: 88–103.

Crawley J (2019) What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. John Wiley & Sons, Inc.

Curatolo P, Porfirio MC, Manzi B, Seri S (2004) Autism in tuberous sclerosis. Eur J Paediatr Neurol 8: 327–332.

Dickson PE, Cairns J, Goldowitz D, Mittleman G (2017) Cerebellar contribution to higher and lower order rule learning and cognitive flexibility in mice. Neuroscience 345: 99–109.

Fatemi SH, Aldinger KA, Ashwood P, et al. (2012) Consensus paper: pathological role of the cerebellum in autism. Cerebellum 11: 777–807.

Feather-Schussler DN, Ferguson TS (2016) A battery of motor tests in a neonatal mouse model of cerebral palsy. J Vis Exp (117).

Fox WM (1965) Reflex-ontogeny and behavioural development of the mouse. Animal Behaviour 13: 234–235.

Hofer MA, Shair HN, Brunelli SA (2002) Ultrasonic vocalizations in rat and mouse pups. Curr Protoc Neurosci Chapter 8: Unit 8.14.

Jeste SS, Sahin M, Bolton P, Ploubidis GB, Humphrey A (2008) Characterization of autism in young children with tuberous sclerosis complex. J Child Neurol 23: 520–525.

Kwiatkowski DJ, Zhang H, Bandura JL, et al. (2002) A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in *Tsc1* null cells. Hum Mol Genet 11: 525–534.

Lipton JO, Sahin M (2014) The neurology of mTOR. Neuron 84: 275–291.

Madisen L, Zwingman TA, Sunkin SM, et al. (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 13: 133–140.

Mosienko V, Beis D, Alenina N, Wöhr M (2015) Reduced isolation-induced pup ultrasonic communication in mouse pups lacking brain serotonin. Mol Autism 6: 13.

Napolioni V, Moavero R, Curatolo P (2004) Recent advances in neurobiology of tuberous sclerosis complex. Brain Dev 31: 104–113.

Orlova KA, Crino PB (2010) The tuberous sclerosis complex. Ann N Y Acad Sci 1184: 87–105.

Rogers TD, McKimm E, Dickson PE, Goldowitz D, Blaha CD, Mittleman G (2013) Is autism a disease of the cerebellum? An integration of clinical and pre-clinical research. Front Syst Neurosci 7: 15.

Roubertoux PL, Martin B, Le Roy I, et al. (1996) Vocalizations in newborn mice: genetic analysis. Behav Genet 26: 427–437.

Saito H, Tsumura H, Otake S, Nishida A, Furukawa T, Suzuki N (2005) L7/Pcp-2-specific expression of Cre recombinase using knock-in approach. Biochem Biophys Res Commun 331: 1216–1221.

Scattoni ML, Martire A, Cartocci G, Ferrante A, Ricceri L (2013) Reduced social interaction, behavioural flexibility and BDNF signalling in the BTBR T+ tf/J strain, a mouse model of autism. Behav Brain Res 251: 35–40.

Slugocka A, Wiaderkiewicz J, Barski JJ (2017) Genetic targeting in cerebellar Purkinje cells: an update. Cerebellum 16: 191–202.

Sudarov A (2013) Defining the role of cerebellar Purkinje cells in autism spectrum disorders. Cerebellum 12: 950–955.

Sungur AO, Schwarting RK, Wöhr M (2016) Early communication deficits in the Shank1 knockout mouse model for autism spectrum disorder: Developmental aspects and effects of social context. Autism Res 9: 696–709.

Tsai PT, Hull C, Chu Y, et al. (2012) Autistic-like behaviour and cerebellar dysfunction in Purkinje cell *Tsc1* mutant mice. Nature 488: 647–651.

Wahlsten D (1974) A developmental time scale for postnatal changes in brain and behavior of B6D2F2 mice. Brain Res 72: 251–264.

Wang SS, Kloth AD, Badura A (2014) The cerebellum, sensitive periods, and autism. Neuron 83: 518–532.

Wöhr M, Dahlhoff M, Wolf E, Holsboer F, Schwarting RK, Wotjak CT (2008) Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: an embryo-transfer study. Behav Genet 38: 579–595.

Wohr M, Roullet FI, Hung AY, Sheng M, Crawley JN (2011) Communication impairments in mice lacking Shank1: reduced levels of ultrasonic vocalizations and scent marking behavior. PLoS One 6: e20631.

Yang M, Bozdagi O, Scattoni ML, et al. (2012) Reduced excitatory neurotransmission and mild autism-relevant phenotypes in adolescent Shank3 null mutant mice. J Neurosci 32: 6525–6541.

Zhang XM, Ng AH, Tanner JA, Wu WT, Copeland NG, Jenkins NA, et al. (2004) Highly restricted expression of Cre recombinase in cerebellar Purkinje cells. Genesis 40: 45–51.