Differences in locomotor behavior revealed in mice deficient for the calcium-binding proteins parvalbumin, calbindin D-28k or both

Maria A. Farré-Castanya, Beat Schwallera, Patrick Gregorya, Jaroslaw Barskic, Céline Mariethoz, Jan L. Erikssong, Igor V. Tetskog, David Wolferd, Marco R. Celioa, Isabelle Schmutze, Urs Albrechte, Alessandro E.P. Villab

a Unit of Anatomy, Department of Medicine, University of Fribourg, CH-1700 Fribourg, Switzerland
b Neuro-heuristic Laboratory, University of Lausanne, CH-1005 Lausanne, Switzerland
c Max-Planck-Institute of Neurobiology, AG Cellular Dynamics, Am Klopferspitz 18, 82152 Martinsried, Germany
d Institute of Anatomy, University of Zurich, Switzerland
e Unit of Biochemistry, Department of Medicine, University of Fribourg, CH-1700 Fribourg, Switzerland
f Laboratoire de Neurobiophysique, INSERM U 318, Université “Joseph Fourier” Grenoble 1, France
g Institute for Bioinformatics, GSF, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany

Received 26 June 2006; received in revised form 28 December 2006; accepted 2 January 2007
Available online 5 January 2007

Abstract
We investigated the role of the two calcium-binding proteins parvalbumin (PV) and calbindin D-28k (CB) in the locomotor activity and motor coordination using null-mutant mice for PV (PV−/−), CB (CB−/−) or both proteins (PV−/− CB−/−). These proteins are expressed in distinct, mainly non-overlapping populations of neurons of the central and peripheral nervous system and PV additionally in fast-twitch muscles. In a test measuring repeated locomotor activity during 18–20 days, the analysis revealed a slightly increased activity in mice lacking either protein, while the lack of both decreased the number of beams crossed during active periods. An increase in the characteristic speed during the first 8 days could be attributed to PV-deficiency, while the elimination of CB in CB−/− and double-KO mice decreased the percentage of fast movements at all time points. In the latter, additionally a reduction of the fastest speed was observed. The alterations in locomotor activity (fast movements, fastest speed) strongly correlate with the impairment in locomotor coordination in mice deficient for CB evidenced in the runway assay and the rotarod assay. The graded locomotor phenotype (CB > PV) is qualitatively correlated with alterations in Purkinje cell firing reported previously in these mice. The presence or absence of either protein did not affect the spontaneous locomotor activity when animals were placed in a novel environment and tested only once for 30 min. In summary, the lack of these calcium-binding proteins yields characteristic, yet distinct phenotypes with respect to locomotor activity and coordination.

Keywords: Parvalbumin; Calbindin-D28k; Knockout mice; Locomotor activity; Purkinje cells

1. Introduction
Parvalbumin (PV) and calbindin D-28k (CB) are high-affinity calcium-binding proteins (CaBPs) of the EF-hand family which are expressed in distinct classes of neurons [1], for a general review, see [2,3]. They are implicated to contribute to the regulation of Ca2+ homeostasis by acting as cytosolic Ca2+ buffers [4–6] and recent experiments on CB suggest that this protein might have additional functions as a Ca2+ sensor [7,8]. PV is almost exclusively expressed in inhibitory GABAergic interneurons in different regions including the neocortex, cerebellum, hippocampus and the reticular nucleus of the thalamus (RTN) of the mammalian brain and also in few projection neurons. CB-positive cells include both, interneurons and projection neurons including pyramidal cells. Co-expression of PV and CB is rare and the only clearly defined neuron population expressing high levels of both CaBPs is the Purkinje cells of the cerebellum [4,9]. Additionally, PV is expressed in fast-contracting muscles [10].

In recent years, the generation of knockout mice for PV [11], CB [12], as well as the CB antisense transgenic CB-deficient...
mice [13] have contributed to the understanding of the physiological role of these CaBPs. Previous work reported several physiological alterations at neuronal and muscular levels in these animals. PV−/− mice revealed electrophysiological alterations associated with PV-expressing cortical neurons [14], cerebellar stellate and basket cells [15,16] and hippocampal PV-immunoreactive interneurons [17]. CB−/− mice showed impairment in motor coordination associated with altered synaptic Ca2+ transients in Purkinje cell dendrites [12]. Furthermore, expression of PV and/or CB was reported to be altered in human patients and animal models of epilepsy and other neurological disorders. A loss of CB immunoreactivity in a majority of dentate granule cells was seen in mesial temporal lobe epilepsy (mTLE) patients [18]. Loss or decreased immunoreactivity to PV and CB from GABAergic neurons was observed in epileptic gerbils [19] and in experimental rats with induced cortical displasia [20].

Moreover, reduced immunoreactivity for PV and/or CB was reported in surviving cerebellar Purkinje cells [21] of patients with sporadic cerebellar ataxia-1 (SCA-1) and in an animal model using SCA-1 transgenic mice [22]. Decreases in PV expression were one of the most consistent findings in postmortem brains of patients with schizophrenia, bipolar disorder and major depression [23] and also downregulation of CB expression was reported before [24]. The results obtained in human patients and genetically modified mice clearly indicate that the presence or absence of PV or CB has significant effects on synaptic transmission and are likely to have profound effects at the systemic level including behavior, learning or memory. In this report, the influence of PV and CB on spontaneous locomotor activity and motor coordination was investigated using mice deficient for PV, CB or both. This former analysis is considered essential to reveal basic defects in sensory motor processing [25], which should be taken into account before performing more complex behavioral tests in view to detect putative links to neuropathologies. Mice were either tested in a novel environment for 30 min, in the second paradigm, animals were tested 18–20 times in 15-min trial sessions and finally the motor coordination was assessed on the runway assay previously described for the CB−/− mice [12] and on the rotarod. All three groups of mice did not exhibit any obvious behavioral alterations when observed under standard housing conditions [11,12,26]. Preliminary results related to this study have been presented in abstract format [27].

2. Materials and methods

2.1. Subjects

The subjects for the analysis of repeated locomotor activity were 97 female mice, on average 5 months old and weighing 26–29 g. The design of the study aimed to minimize the number of experimental animals and all experiments were performed with permission of the local animal care committee and according to the present Swiss law and the European Communities Council Directive of 24 November 1996 (86/609/EEC). Mice were housed in groups of three to four individuals on either a 12 h/12 h light/dark cycle with light onset at 7 a.m. with free access to food and water. In this study we analyzed PV−/− mice produced from targeted E14 cells [11] with either a mixed C57BL/6J × 129OlaHsd (B6 × 129P2) or a B6 background (backcrossed for eight generations; B6PV−/−). The mixed background PV−/− mice were maintained as a homozygous stock with at least 10 intercrosses generations. The breeding scheme among mixed PV−/− was aimed to avoid homozygous fixation of allelic combinations not related to the PV gene locus by systematic randomizing of breeder pairs from the original PV−/− homozygotes derived from heterozygous (PV+/−) couples. As the control groups for the mixed and B6 background, an F1 generation of B6 and 129P2 or B6PV−/+ littermates from heterozygous B6PV+/− breedings was used, respectively. Also for CB−/− mice produced from R1 stem cells [12], two different genetic backgrounds were tested: mice with a mixed background B6 and 129R1 (129Sv × 129SvJ) [28], or mice that had been backcrossed to B6 for seven generations (B6CB−/− and B6CB+/+, both derived from heterozygous B6CB+/− matings). Control mice for the B6 × 129R1 CB−/− background were either B6 × 129R1 CB+/+ littersmates produced from heterozygous B6 × 129R1 CB+/− breedings or a F1 generation of B6 × 129SvS, which was assumed to be similar to B6 × 129R1 [28]. PV−/− CB−/− mice, which were bred from the mixed CB−/− and PV−/− mice [26] have a genetic background of B6 × 129P2 × 129R1 and were compared to a pool of 20 mice consisting of 10 each with a B6 × 129P2 or B6 × 129SvS background. Wildtype (WT) animals used in this study included B6, 129SvS, 129P2 and F1 generations of B6 × 129SvS and B6 × 129P2. All animals, including WT ones, were genotyped by PCR as described before [26].

2.2. Analysis of repeated locomotor activity

The animals were analyzed in a black rectangular box (380 mm wide × 420 mm long × 400 mm high) located in a soundproof room. The box was open at the top and illuminated by a dim light. Three parallel infrared beams (labeled F1, F2, F3) were used to detect the movements of the animals (Fig. 1). The distance between F1 and F2 was 140 mm and between F2 and F3 was 80 mm. The investigator left the soundproof room before the beginning of the recording and monitored the mouse’s behavior from the outside by means of a video camera placed on the ceiling over the box. Digital pulses were generated when the infrared beams were interrupted and the time stamp of these occurrences was recorded by a Macintosh IIfx (Apple Computers, Cupertino, CA) personal computer equipped with digital acquisition boards (National Instruments, Austin, TX). All signals were recorded at a time resolution of 1 ms and stored digitally for further off-line analysis.

All experiments were performed between 1 p.m. and 7 p.m. Mice of either genotype or genetic background were subdivided into groups and tested in different series. Each series consisted of three to four different groups, e.g. different genotypes (wildtype, PV−/−, CB−/−, and PV−/− CB−/−) or different genetic backgrounds (B6, 129P2, F1 B6 × 129P2) of three to four animals of each group. Thus, a series consisted on the average of 12 mice from at least three different groups. For each series the passage order was randomized at the first session and varied from day to day following the initial randomization scheme. The mice were recognized by color codes and numbers. In all experiments, the breeder kept the correspondence of code and genotype secret to the investigators until the end of all experiments. During one session a mouse was placed in the apparatus close to the F1 infrared beam (Fig. 1) and allowed to move unhindered during 15 min. The sessions occurred at a rate of one per day. Mice were analyzed
2.3. Spontaneous activity exploring an unfamiliar environment

by means of the multi-parameter activity monitor Tru Scan and its associated

duration of the tests. This corresponds to a number of events per unit of time.

of various genetic backgrounds: B6, 129S5, 129P2 and F1 gen-

groups. Grouped values were called

were crossing two adjacent beams (F2–F3) within a delay of 40 ms. These events

F1 beam crossings. The arrow indicates the mode of the distribution used to calculate the characteristic speed. The y-axis is scaled in rate units (beams crossed/second) and smoothed after convolution with a moving Gaussian-shaped bin of 25 ms width. The dashed line corresponds to the confidence intervals assumed assuming that a Poisson distribution underlay the time series. This figure illustrates the distribution of one particular mouse.

During 18–20 sessions, yielding an overall time of observation of at least 4.5 h per animal. Sessions for the entire experiments were divided into four blocks: block 1 (days 1–3), block 2 (days 4–8), block 3 (days 9–13) and block 4 (days 14–18 or 20). Block 1 of each mouse was analyzed separately and yielded information about the spontaneous locomotor activity in a novel environment. The data from all 18–20 sessions was pooled and analyzed as “repeated locomotor activity”.

For each mouse we defined a set of parameters that were used to describe individuals as well as locomotor activity grouped by genotype. Grouped values are expressed as the mean ± standard error of the mean (S.E.M.). Statistical evaluation of genotype factor was assessed by non-parametric Kruskal–Wallis and Mann–Whitney U-tests. The average activity (AA) was expressed in arbitrary units corresponding to the total number of beam crossings divided by the total duration of the tests. This corresponds to a number of events per unit of time. Whenever the mouse remained without crossing any beam during 10 consecutive seconds this was counted as one inactive period with regard to locomotion. We defined the parameter locomotor inactivity (LIA) as the ratio of the time spent in inactive periods divided by the total duration of the tests. Based on this, we defined a locomotor index LI = AA/(1 – LIA) × 100. This index referred to the number of beam crossings per active time times 100, thus providing a more objective parameter of the subject’s mobility.

The time stamps of each beam crossing were used to construct time series. We computed the crosscorrelogram of the time series associated with the two most distant infrared beams crossings, thus corresponding to the distribution of F3–F1 crossings as a function of the time interval between F3 after F1 crossings. The characteristic speed (expressed in mm/s) was defined as the distance between F3 and F1 (equal to 220 mm) divided by the time lag corresponding to the mode of this distribution measured on the crosscorrelogram of F1 beam crossings as a function of the lag after F3 beam crossing (Fig. 2). The ordinate of the crosscorrelograms is related to time in order to standardize for different total assay length. From the same crosscorrelogram also the fastest speed (expressed in mm/s) was calculated. In addition, we counted the number of times a subject was crossing two adjacent beams (F2–F3) within a delay of 40 ms. These events were called fast movements and we analyzed the proportion of these events with respect to the total number of beam crossings. At the beginning of each session, the subjects were placed in the apparatus close to the F1 beam.

2.4. Runway assay

Tests were performed by the same experimenter on age- (4–6 months old), sex-, and weight-matched animals (group sizes were between 8 and 20 mice). Each mouse was tested in five consecutive trials per day for 5 days on a rod (100 cm × 2 cm × 2 cm) with small obstacles [29], as used before for the CB–/− mice [12,30]. Slips of the forelimb and hindlimb were counted on the side visible for the experimenter. Values are expressed as numbers of slips per trial for a given day. Data was analyzed using a two-way repeated ANOVA model with between factor genotype (wildtype strains of various genetic backgrounds (see above and PV−/−; CB−/−; PV−/−CB−/− mice)) and within factor day (1–5). Differences were considered significant at P<0.05. Results are expressed as mean ± 1 standard error.

2.5. Rotared assay

Mice were tested on a Rotarod device (TSE Systems GmbH, Bad Homburg, Germany) using three different protocols: constant speed 20 rpm, accelerating 5–20 rpm and accelerating 10–40 rpm. PV−/− (five females/four males) and control WT (13/5) mice were on the B6 background and the PV−/−CB−/− (12/10) on the mixed background were compared to one of the mixed background groups (B6 × 129P2; 6/13). The age of the mice on the starting day (constant speed) ranged from postnatal days P28–P44. The first accelerating protocol started 2 days later and the second one approximately 3 weeks after the start of the rotared experiments (P49–P57). Each mouse performed up to 12 trials over 2 days on each protocol, with 6 trials per day separated into 2 batches of 3 trials. The mice were allowed to rest for at least 10 min between trials and the 2 daily batches of trials were separated by at least 2 h. Data was analyzed using the Prism® software (GraphPad, San Diego, USA) and expressed as average latency to fall ± S.E.M. Statistical analysis was performed using Student’s t-test, P<0.05 was considered statistically significant.

2.5.1. Locomotor activity monitoring

Mouse housing and handling was performed as described [31]. Activity records are double-plotted so that each day’s cycle activity is plotted both to the right and below that of the previous cycle. We used the Clocklab software (Actimetrics) for wheel running data acquisition and analysis. Locomotor activity counts were assessed from days 5 to 25 with six animals per genotype.

3. Results

The genetic background of transgenic or knockout mice is an important factor in behavioral tests [25,32]. Hence, the locomotor tests reported here were first validated with wildtype strains of various genetic backgrounds: B6, 129S5, 129P2 and F1 geno-
The characteristic speed (mm/s) was 166 ± 0.6 for B6 mice, 20 ± 0.6 for B6 × 129P2, 52 ± 0.9 for the 129P2, and 30 ± 1.7 for the 129S5. The fastest speed (mm/s) was 563 ± 11.3 for B6 mice, 49 ± 4.7 for B6 × 129P2, 267 ± 10.4 for the 129P2, and 231 ± 9.0 for the 129S5. The two parameters related to the speed of movement, the characteristic speed and the fastest speed for each genetic background or genotype was calculated. The two parameters related to the speed of movement were more pronounced in the 129 groups. Interestingly, the locomotor index (LI) was not dependent on the presence or absence of PV in both genetic backgrounds. For a more detailed analysis, the 18–20 days trial session was separated in four blocks (1–4) of 3–6 days (for details, see Section 2). The average activity in the PV−/− group was increased in all four blocks when compared to the WT group, but differences were smaller during block 2 (Fig. 3b). The hallmark of both PV−/− mouse groups with respect to the speed parameters was an increased characteristic speed, whereas all other values were not statistically different between the two groups (PV−/− and PV+/+) in both genetic backgrounds (Table 2A). The higher characteristic speed was observed only in blocks 1 and 2, i.e. during the first 8 days of the trial (Fig. 3a; two-tailed Mann–Whitney U-test, P<0.05); while at later times the characteristic speed of all four groups was indistinguishable.

For the mixed CB mice, littermates (CB−/− and CB+/+) in both genetic backgrounds (Table 2A) were used, in the case of mixed PV animals, a line of mixed PV−/− mice was generated by systematic randomization mating for more than 10 generations. Additionally, for both null mutations (CB and PV), lines that were backcrossed for 7 (CB) or 8 (PV) generations to B6 mice resulting in B6CB−/− or B6PV−/− lines were tested.

3.1. Repeated analysis of locomotor activity and locomotor activity monitoring (wheel running)

Mice were tested in a box for 18–20 consecutive days for 15 min per trial applying a scheme as described in Section 2 in order to avoid any bias with respect to order of testing or day time. All parameters are based on the crossing of infrared beams (F1–F3) in the box. The measurement of the parameters average activity (AA) and the locomotor inactivity (LIA) revealed significant differences between B6 on one hand and 129S5 and 129P2 on the other hand (Table 1). B6 mice were significantly more active and correspondingly, periods of inactivity were more pronounced in the 129 groups. Interestingly, the locomotor index (LI) was not much different between groups. The effect of speed characteristic during locomotor activity, the analysis of individual crosscorrelograms (Fig. 2) allowed to determine the average speed for each genetic background or genotype (representing the most frequent time of that subject to cross F1 beam after F3 beam crossing). Additionally, by analysis of the crosscorrelograms, the fastest speed for each genetic background or genotype was calculated. The two parameters related to the speed of movements were significantly affected by the genetic backgrounds. Both the characteristic speed and the fastest speed were lower in the 129 groups compared to the B6 mice. In most parameters analyzed, the F1 groups were in-between the values measured for the B6 and the 129 groups and again the LI parameter was unaffected and the same as for the pure lines. The percentage of fast movements was somewhat higher in the F1 lines as compared to the parental lines, but the effect was not very pronounced; the 129P2 group had clearly the smallest percentage of fast movements.

Tests of PV−/− or CB−/− mice were carried out with animals with either a backcrossed B6 background or a mixed background as described above. Since most previously published data on these mice was carried out with animals of mixed genetic background, the number of mice tested in these groups was larger compared to the groups with the backcrossed B6 background. In both genetic backgrounds, PV−/− mice were more active (Table 2A), as evidenced by the increased average activity and the concomitant decreased LIA. Although differences in the B6 background were less evident, this might also be related to the small number of animals tested. The number of beam crossing during active periods (LI) was not dependent on the presence of PV in both genetic backgrounds.

To obtain a mixed background B6 × 129R1 and B6 × 129P2, respectively [28]. In both cases, targeted ES cells had been injected into B6 blastocysts and the chimeras mated to B6 [11,12]. The resulting heterozygous mice (CB+/− or PV+/−) have a mixed background B6 × 129R1 and B6 × 129P2, respectively, and were used to breed wildtype (+/+ or knockout (−/−) littermates with mixed genetic backgrounds. While for the mixed CB background only littermates from mixed heterozygous pairs were used, in the case of mixed PV animals, a line of mixed PV−/− mice was generated by systematic randomization matings for more than 10 generations. Additionally, for both null mutations (CB and PV), lines that were backcrossed for 7 (CB) or 8 (PV) generations to B6 mice resulting in B6CB−/− or B6PV−/− lines were tested.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>F1 B6 × 129S5</th>
<th>F1 B6 × 129P2</th>
<th>129S5</th>
<th>129P2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals (n)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Average activity</td>
<td>0.24 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Locomotor inactivity (%)</td>
<td>38.0 ± 2.9</td>
<td>60.2 ± 2.8</td>
<td>63.9 ± 4.9</td>
<td>90.4 ± 1.4</td>
<td>75.8 ± 4.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Locomotor index</td>
<td>37.8 ± 1.9</td>
<td>58.2 ± 2.6</td>
<td>34.4 ± 2.4</td>
<td>39.6 ± 4.7</td>
<td>24.8 ± 1.5</td>
<td>0.0011</td>
</tr>
<tr>
<td>Fast movements (%)</td>
<td>7.7 ± 0.6</td>
<td>11.3 ± 0.9</td>
<td>8.6 ± 0.8</td>
<td>9.1 ± 1.7</td>
<td>4.0 ± 0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Characteristic speed (mm/s)</td>
<td>166 ± 30</td>
<td>131 ± 20</td>
<td>106 ± 14</td>
<td>117 ± 16</td>
<td>84 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td>Fastest speed (mm/s)</td>
<td>563 ± 58</td>
<td>516 ± 52</td>
<td>420 ± 49</td>
<td>267 ± 30</td>
<td>231 ± 31</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*The various parameters are defined in Section 2. A group comparison was carried out by Kruskal–Wallis test.*
Table 2A

Comparison of locomotor parameters between PV−/− and wildtype mice on two genetic backgrounds: a B6 background and a mixed B6 × 129P2 background

<table>
<thead>
<tr>
<th></th>
<th>B6PV−/− (n = 4)</th>
<th>B6PV+/+ (n = 7)</th>
<th>PV−/− (n = 10)</th>
<th>F1 B6 and 129P2 (n = 10)</th>
<th>PV−/− (all)a</th>
<th>PV+/+ (all)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average activity</td>
<td>0.25 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>141 ± 10</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>Locomotor inactivity (%)</td>
<td>43.0 ± 1.4</td>
<td>53.7 ± 3.6</td>
<td>49.6 ± 4.2</td>
<td>63.9 ± 4.9</td>
<td>78 ± 5</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Locomotor index</td>
<td>44.0 ± 1.8</td>
<td>51.7 ± 3.4</td>
<td>37.6 ± 2.2</td>
<td>34.4 ± 2.4</td>
<td>102 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Fast movements (%)</td>
<td>10.3 ± 0.7</td>
<td>12.7 ± 1.2</td>
<td>10.7 ± 0.4</td>
<td>8.6 ± 0.8</td>
<td>112 ± 6</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Characteristic speed (mm/s)</td>
<td>81 ± 15</td>
<td>64 ± 2</td>
<td>143 ± 16</td>
<td>106 ± 14</td>
<td>132 ± 12</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Fastest speed (mm/s)</td>
<td>300 ± 41</td>
<td>382 ± 102</td>
<td>487 ± 21</td>
<td>420 ± 49</td>
<td>105 ± 6</td>
<td>100 ± 12</td>
</tr>
</tbody>
</table>

We tested whether the difference between the two “all” groups was equal to zero (chi-square test). P-values were classified as **P < 0.005, *P < 0.01 and NS: P > 0.05; not significant.

a Since the parameters in PV+/+ mice are significantly different in the mixed and the B6 background (Table 1), the wildtype values of both genetic backgrounds were set to 100% PV+/+ (all), and the corresponding PV−/− groups PV−/− (all) calculated according to the two wildtype groups.

Fig. 3. Analysis of the characteristic speed (a) and the average activity (b). Various genotypes of mice, PV−/− (n = 10), CB−/− (n = 10), PV−/− CB−/− (n = 11) and WT controls (n = 10) were tested in a box for 18–20 days. The entire period was divided into four blocks of 3–5 days to observe changes in the parameters “characteristic speed” and “average activity” during the four time periods; blocks 1–4 correspond to data pooled from days 1 to 3, 4 to 8, 9 to 13 and 14–18, respectively; values are mean ± S.E.M. (a) The characteristic speed of PV−/− mice was significantly increased during blocks 1 and 2. : two-tailed Mann–Whitney U-test, P < 0.05, statistically significant vs. WT and PV−/− CB−/− in block 1 and vs. all other groups in block 2. (b) The average activity in block 1 was higher in all mutant genotypes when compared to the WT group (*P < 0.05).

the absence of CB−/− in both backgrounds, but the percentage of fast movements was slightly lower in both CB−/− groups (more prominent in mice with a B6 background) than in wildtype controls (Table 2B).

PV−/− CB−/− mice have a relatively complicated genetic background (B6 × 129R1 × 129P2) and since corresponding wildtype littermates were not available, we took the ensemble of the F1 groups (B6 × 129S5, B6 × 129P2; n = 10 for both groups) as the corresponding control group (for details, see Section 2). All activity parameters were essentially unchanged in the PV−/− CB−/− mice (AA, LIA), the only difference was a slightly lower locomotor index (Table 2C), indicating that mice deficient for both proteins tend to be less mobile during periods of locomotor activity (two-tailed Kruskal–Wallis test, P < 0.01). Analysis of the average activity during the four blocks revealed the activity to be higher in block 1 (similar

Table 2B

Comparison of locomotor parameters between CB−/− and wildtype mice on a mixed B6 × 129S5 background and on a B6 background

<table>
<thead>
<tr>
<th></th>
<th>CB−/− (n = 10)</th>
<th>F1 B6 and 129S5 (n = 10)</th>
<th>B6CB−/− (n = 3)</th>
<th>B6CB+/+ (n = 5)</th>
<th>CB−/− (all)a</th>
<th>CB+/+ (all)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average activity</td>
<td>0.18 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.18 ± 0.08</td>
<td>0.15 ± 0.05</td>
<td>119 ± 9</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Locomotor inactivity (%)</td>
<td>50.0 ± 3.4</td>
<td>60.2 ± 2.8</td>
<td>55.0 ± 13.9</td>
<td>63.0 ± 9.4</td>
<td>84 ± 5</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Locomotor index</td>
<td>35.8 ± 1.3</td>
<td>38.2 ± 2.6</td>
<td>38.0 ± 5.5</td>
<td>38.8 ± 3.5</td>
<td>95 ± 3</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Fast movements (%)</td>
<td>9.6 ± 0.4</td>
<td>11.3 ± 0.9</td>
<td>7.4 ± 1.3</td>
<td>11.5 ± 0.6</td>
<td>80 ± 5</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Characteristic speed (mm/s)</td>
<td>154 ± 13</td>
<td>131 ± 20</td>
<td>115 ± 54</td>
<td>93 ± 37</td>
<td>119 ± 10</td>
<td>100 ± 12</td>
</tr>
<tr>
<td>Fastest speed (mm/s)</td>
<td>551 ± 71</td>
<td>516 ± 52</td>
<td>426 ± 70</td>
<td>436 ± 117</td>
<td>105 ± 11</td>
<td>100 ± 8</td>
</tr>
</tbody>
</table>

P-values were classified as **P < 0.005, *P < 0.01 and NS: P > 0.05; not significant.

a For details, see text in Table 2A.
values as in the PV−/− and CB−/− groups), but in blocks 2–4 activity values were not different from the WT group (Fig. 3b). In the PV−/−CB−/− group, the most striking differences were observed in the significantly reduced percentage of fast movements (9.9 ± 0.7 versus 4.9 ± 0.9) and the decreased fastest speed (468 ± 37 mm/s versus 347 ± 20 mm/s; two-tailed Kruskal–Wallis test, \( P < 0.0001 \)). Fast movements were decreased during all four blocks and no significant differences existed between blocks; also in all other groups tested the percentage of fast movements was relatively constant during all four blocks (not shown). Interestingly, no significant changes were observed in the characteristic speed, although values were generally slightly lower than in the other groups (Table 2C and Fig. 3a).

In order to preclude that the observed changes related to locomotor activity reported above were the result of a generally altered diurnal behavior, mice were tested in a wheel-running assay. Animals were kept individually on a 12 h/12 h light/dark cycle and their wheel-running activity was monitored continuously for 25 days. We selected a wildtype control of endogenous circadian rhythms and entrainment to the environment. Two representative actograms from a WT and a PV−/−CB−/− mouse are shown in Fig. 4. The activity pattern is essentially the same, i.e. strong activity at the onset of the dark cycle subsiding towards the end and very little activity during the light cycle. The overall activity expressed as total wheel revolutions/min was calculated for the period between days 5 and 25, the first 4 days were considered as training period. The value for the WT group was 6.8 ± 2 rpm (mean ± S.E.M.; \( n = 6 \)) and for the PV−/−CB−/− group slightly lower, 5.4 ± 0.8 rpm (mean ± S.E.M.; \( n = 6 \); \( P > 0.5 \), \( t \)-test). Thus, diurnal activity is not significantly different between the null-mutant and the control group indicating that the differences presented above are genotypic differences not related to diurnal activity.

In summary, each genotype is characterized by a specific set of affected parameters when compared to their respective control group as summarized in Table 4. The sole absence of PV is manifested in an increased characteristic speed during the first 8 days of the experiment that drops in the second period to values recorded in the other groups. Deficiency in CB alone slightly decreases the percentage of fast movements, but otherwise these mice show the least differences when compared to the WT group. If both proteins are absent in PV−/−CB−/− mice, the percentage fast movements drops by approximately 50% compared to the corresponding WT group. Additionally the fastest speed is strongly reduced (Table 2C). This is in line with the previously reported impairment in motor coordination seen in CB−/− mice [12,30].

![Fig. 4. Representative actograms of a WT and PV−/−CB−/− mouse in the wheel-running assay. The wheel-running activity of individually housed mice was recorded for 25 days. It is plotted by aligning two consecutive days horizontally. Black vertical bars plotted side-by-side represent the activity (number of wheel revolutions). The height of each vertical bar indicates the accumulated number of wheel revolutions. The white and black bar at the top of the scheme depicts light (12 h) and darkness (12 h), respectively.](image-url)
3.2. Spontaneous activity exploring an unfamiliar environment

Mice were tested in this “classical” assay in a random order in a commercial activity cage for 30 min one time. Wildtype mice with different genetic backgrounds serving as controls for the knockout mice were tested. Since in the previous experiment (repeated analysis of locomotor activity), no significant differences between the F1 B6 × 129S5 and the F1 B6 × 129P2 groups (Table 1) were observed, the F1 B6 × 129P2 mice served as controls for both PV−/− and CB−/− mice and also only two pure strains (B6 and 129S5) were tested. The move number (Table 3) is the equivalent of the average activity (AA) parameter in the first experiment. Since the IR beams are more closely spaced in the commercial box, the parameters move time, rest time (similar to LIA) and total distance were recorded. Furthermore, the presence of beams in the X- and Y-axis additionally allowed to monitor the time spent and the distance covered in the center of the cage (center distance, center time) versus these parameters recorded along the walls of the cage (marginal distance, marginal time). Finally the beam in the Z-axis allowed the measurement of vertical breaks (rearing). All parameters (with the exception of move number) were significantly different between B6 and 129S5 mice (Table 3). The rest time was lower in B6 mice, they spent significantly more time in the center of the box and also the total distance covered during the 30-min trial was longer. Additionally, rearing in B6 mice occurred significantly more often than in the 129S5 group. In almost all of the parameters tested, the values for the F1 generation with a mixed background B6 × 129P2 were almost identical to the mean value between B6 and 129S5 (Table 3), that is, neither background is dominant with respect to these parameters.

When comparing mice lacking CB, PV or both with the corresponding control groups (CB+/+ and B6 × 129P2, respectively); no statistically significant differences were observed by ANOVA single factor analysis (P > 0.05 for all parameters). A direct comparison between the PV−/− and CB−/− groups revealed an increased center time for CB−/− mice compared to PV−/− mice (P = 0.0156). Additionally, the number of vertical breaks was somewhat higher in CB−/− than in PV−/− mice (P = 0.0472). The same effect was also seen when comparing CB−/− with PV−/− mice (P = 0.0466). If all mice analyzed were pooled in two groups only, i.e. mice having PV (F1 B6 × 129P2, CB+/+, CB−/−) or deficient for PV (PV−/−, PV−/− CB−/−) the rearing values were 4.10 ± 1.96 versus 2.64 ± 1.63, respectively. One-way ANOVA (P < 0.0071) revealed this parameter to be statistically different between the two groups and indicates that the lack of PV reduces the number of vertical breaks, which is considered as factor measuring exploratory activity.

3.3. Testing of motor coordination in the runway assay

CB−/− mice manifest impairment in motor coordination including deficits in the runway assay [12] that can be attributed to the absence of CB in Purkinje cells [30]. Since PV is also abundantly expressed in Purkinje cells and molecular layer interneurons, PV−/− and mice deficient for both CB and PV were tested in the same runway assay under the same experimental conditions. The assays with the different genetic backgrounds (B6, 129S5, F1 B6 × 129S5, congenic B6.129R1) revealed similar findings as previously reported for the wildtype littermates from the CB−/− mice [12]: differences between the various wildtype strains are minor with respect to both number of slips and the learning rate during the 5-day period when compared to the large differences in the CB−/− mice (Fig. 5A). Since the putative differences were much smaller in the PV−/− group, a detailed analysis of the WT strains was carried out beforehand (Fig. 5B). Differences in both the number of slips and the learning rate were observed in the four WT groups. The poorest performers were the B6 mice, while the number of slips was lowest in the F1 B6 × 129S5 group. The main effect of group is reproduced by one-way factorial ANOVA on average slip counts, with post hoc analysis revealing a significant difference between the groups B6 and F1 B6 × 129S5 (P = 0.0026). The main effect of group is reproduced by the non-parametric Kruskal–Wallis test (P = 0.0165). Also the learning rates were most different between B6 and F1 B6 × 129S5 factorial one-way ANOVA: P < 0.05; post hoc analysis B6 and F1 B6 × 129S5; P = 0.0128; Kruskal–Wallis test (P = 0.0443). In summary, the line plot indicates that B6 mice perform more poorly than the other WT strains, but compensate this by a steeper learning rate.

<p>| Table 3 |
| Locomotor parameters determined in the Tru scan box |</p>
<table>
<thead>
<tr>
<th>Move number</th>
<th>Rest time</th>
<th>Total distance</th>
<th>Marginal distance</th>
<th>Marginal time</th>
<th>Center distance</th>
<th>Center time</th>
<th>Vertical breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 (n=8)</td>
<td>7.78 ± 2.16</td>
<td>21.54 ± 11.0</td>
<td>187.47 ± 26.93</td>
<td>90.90 ± 24.42</td>
<td>42.97 ± 6.82</td>
<td>35.59 ± 13.5</td>
<td>17.03 ± 6.82</td>
</tr>
<tr>
<td>129S5 (n=14)</td>
<td>6.97 ± 2.17</td>
<td>36.90 ± 10.06</td>
<td>43.17 ± 28.07</td>
<td>35.17 ± 19.90</td>
<td>58.10 ± 3.70</td>
<td>2.46 ± 5.20</td>
<td>1.90 ± 3.70</td>
</tr>
<tr>
<td>F1 B6 and 129P2 (n=20)</td>
<td>9.13 ± 1.09</td>
<td>21.54 ± 4.51</td>
<td>104.79 ± 27.62</td>
<td>64.72 ± 12.45</td>
<td>51.88 ± 4.04</td>
<td>14.08 ± 7.12</td>
<td>8.13 ± 4.04</td>
</tr>
<tr>
<td>CB+/+ (n=8)</td>
<td>8.76 ± 1.32</td>
<td>18.55 ± 7.71</td>
<td>117.96 ± 43.31</td>
<td>71.14 ± 23.99</td>
<td>50.02 ± 5.11</td>
<td>15.06 ± 7.35</td>
<td>9.98 ± 5.11</td>
</tr>
<tr>
<td>PV−/− (n=12)</td>
<td>7.89 ± 1.22</td>
<td>21.04 ± 5.60</td>
<td>101.72 ± 46.82</td>
<td>65.91 ± 17.63</td>
<td>53.18 ± 7.12</td>
<td>15.00 ± 12.97</td>
<td>6.83 ± 7.12</td>
</tr>
<tr>
<td>CB−/− (n=8)</td>
<td>8.99 ± 1.09</td>
<td>15.66 ± 4.13</td>
<td>120.42 ± 43.45</td>
<td>65.52 ± 26.42</td>
<td>43.84 ± 6.38</td>
<td>22.33 ± 7.75</td>
<td>16.16 ± 6.38</td>
</tr>
<tr>
<td>PV−/− CB−/− (n=12)</td>
<td>9.19 ± 0.80</td>
<td>21.71 ± 6.83</td>
<td>89.79 ± 36.40</td>
<td>49.03 ± 12.63</td>
<td>48.93 ± 7.71</td>
<td>18.07 ± 15.30</td>
<td>11.07 ± 7.71</td>
</tr>
</tbody>
</table>

Each mouse from the different groups was tested in a single 30-min trial (bin size 0.5 min). Marginal time + center time = 60; rest time = 60 – active time, for other details, see Section 2.
On the other hand, the four groups PV–/–, CB–/–, double mutants and WT (the F1 B6 × 129S5 was selected as representative WT strain) differed clearly both with respect to overall performance and learning rate in the runway test (Fig. 5A; P < 0.0001 for the parameters genotype, day and interaction genotype-day). CB–/– were overall massively impaired compared to WT mice despite a slightly steeper learning rate (partial ANOVA CB–/– versus WT: P < 0.0001 for the parameters genotype, day and interaction genotype-day). PV–/– showed a significantly better performance than CB–/–, yet compared to WT their overall performance was still reduced (partial ANOVA PV–/– versus WT (days 2–5): genotype P < 0.005, day P < 0.0001, interaction P = 0.0466). For the comparison PV–/– versus WT, only days 2–5 were analyzed based on the results from the different WT strains that differences in performance in the WT groups are mainly manifested on the first day (Fig. 5B; interaction (days 2–5) corroborated by two-way repeated ANOVA P = 0.7828). Results for the PV–/–CB–/– mice were essentially the same as for the CB–/– animals and suggest that CB deficit is the major factor contributing to the observed motor coordination impairment in PV–/–CB–/–, since no synergistic effect of combined PV- and CB-deficiency could be detected.

### 3.4. Rotarod assay

The putative roles of CB and PV were assessed in a second Purkinje-cell-dependent task, the Rotarod assay. We tested two groups of knockout mice: the PV–/– mice that present a very minor effect in the runway assay and the PV–/–CB–/– mice that showed a pronounced one. Interestingly, the PV–/– mice performed better during the first task (constant 20 rpm) compared to the WT group, while for the more difficult tasks (accelerating rod 5–20 and 10–40 rpm) there were no differences between PV–/– and WT groups (Fig. 6). When analyzing days 1 and 2 at constant speed separately, the differences in performance were significant only at day 1, when PV–/– stayed on for longer periods (71.3 ± 7.1 s; mean ± S.E.M.) than WT mice (47.5 ± 4.0 s; P < 0.005). Qualitatively, WT mice apparently searched for a way to "escape" the test by turning around whilst on the rotarod and walking backwards, sometimes numerous times per trial, which often resulted in falling down, while PV–/– consistently walked in the direction (forward) as they were initially placed. Quantitatively, this effect was analyzed in the PV–/–CB–/– and the corresponding WT group (B6 × 129P2) during the 10–40 rpm protocol and the results are expressed as turns per mouse per minute on the rotarod device.
On average, WT mice turned around 1.3 ± 0.5 min⁻¹, while the double mutants never turned during the investigated period.

The PV−/−CB−/− mice had some difficulties learning the rotarod task (worse performance in the first test (20 rpm constant)), but at later times were equally good on the accelerating rod (5–20 and 10–40 rpm). As the difficulty of the task increased (10–40 rpm), the deficits in motor coordination were again manifested. Globally, the results of the rotarod and the runway give the same picture: minor changes in PV−/−, major changes when also CB is missing confirming the previous hypothesis that CB is the main determinant for the observed deficits in motor coordination.

4. Discussion

Elimination of functional genes is assumed to produce a phenotype that might give clues about the specific physiological function(s) of the deleted protein. In case of proteins expressed in the CNS the phenotypic changes might be also manifested at the behavioral level. As a starting point, testing of spontaneous locomotor activity either in a novel environment or repeated recordings in a familiar environment were carried out with mice deficient for PV and CB. Since the available mice have a rather complex genetic background and since the test of “repeated locomotor activity” had not been previously validated with different wildtype strains, several pure (B6, 129S5, 129P2) or “mixed” (F1 of B6 × 129S5, B6 × 129P2) wildtype strains were analyzed beforehand. As reported before [25,32,34], 129 mice were significantly less active than B6 animals shown by the decreased average activity (AA) and the smaller total distance traveled. The exploratory activity of 129 mice was diminished: the center time and distance were decreased significantly and less rearing was observed as reported before [25]. The activity effects are very robust and seen in both assays, which were carried out in two different laboratories (Martinsried, Germany and Lausanne, Switzerland). While in some assays many different tests were performed with a number of “pure” genetic strains (e.g. [32]), much less information is available on F1 generations of pure strains [25,34]. In the latter report the authors point out that behavioral scores of F1 hybrid mice cannot be easily predicted from the knowledge of the parental strains and that for each hybrid strain the parameters for each behavioral test need to be determined. Since most of the common strains of knockout mice have been generated on a mixed B6 × 129 backgrounds, the characteristics of such mice were investigated in detail. Either congenic lines or F1 generations derived from pure B6 and 129 lines were investigated. In all tested parameters that were significantly different between B6 and 129 mice, the values of the F1 generations as well as the congenic line (CB+/+; B6 × 129R1) were almost identical to the average of the values for the pure strains. This was surprising, since in other F1 hybrids and behavioral tests (e.g. Y-maze rearing, tactile startle), such a simple correlation does not hold true [34]. As calculated for the parameters average activity, total distance, marginal distance, marginal time, fastest speed and vertical breaks (rearing) the arithmetic average was generally well within the S.E.M.-interval of these parameters (not shown). In addition, the S.E.M. values for the B6 × 129 backgrounds were not systematically larger
than these values for the pure strains. That is, even if differences between wildtype and genetically modified mice with a mixed genetic background lay within the boundaries set by the B6 and 129 backgrounds, statistically significant differences can be attributed to the genetic manipulations. Therefore, the comparison of data obtained with CaBP-deficient mice with that of genetically matched controls allowed us to assign distinct differences in these assays to the absence of a particular CaBP.

It has been shown previously that PV- and CB-expressing neurons are normal at the gross histological level in PV−/− and CB−/− mice with no apparent up-regulation of other CaBPs [11,12] supporting the original hypothesis that PV and CB are not essential for the basic properties of particular subtypes of cells [6]. Furthermore, these mice were normal and indistinguishable from the other littermates with respect to development, physical activity and behavior under standard housing conditions. Closer inspection of PV−/−, CB−/− and PV−/−CB−/− mice revealed significant functional alterations at cortical, hippocampal and cerebellar levels (for a review on the cerebellum, see [4]). Electrophysiological recordings of cortical, hippocampal and cerebellar sites in PV−/− mice showed important modifications in the function of the inhibitory systems [14,16,17,35]. Moreover, direct evidence that endogenous CaBPs play an important role in the cerebellar physiology was provided by behavioral analysis investigated in CB−/− mice and more recently in mice lacking the closely related protein calretinin. These mice revealed motor coordination impairment associated with alterations in Ca2+-transients and firing behavior of Purkinje cells [12,36].

It is interesting to note that deficiency in either one (PV, CB) or both of the CaBPs had no significant effect on the locomotor activity tested in a novel environment for only 30 min. On the other hand, detailed analysis of the activity during repetitive sessions in an environment without any external disturbances revealed distinct differences between all three genotypes (Table 4). It is important to stress that the differences between CaBP-deficient mice and control animals were robust and practical.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Summary of parameters related to locomotion and Purkinje cell electrophysiology in mice deficient for PV, CB or both</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV−/−</td>
<td>CB−/−</td>
</tr>
<tr>
<td>Average activity</td>
<td>↑↑</td>
</tr>
<tr>
<td>Locomotor inactivity</td>
<td>↓↓</td>
</tr>
<tr>
<td>Locomotor index</td>
<td>−</td>
</tr>
<tr>
<td>Characteristic speed</td>
<td>↑</td>
</tr>
<tr>
<td>Fastest speed</td>
<td>% of fast movements</td>
</tr>
<tr>
<td>Spontaneous activity</td>
<td>−</td>
</tr>
<tr>
<td>Vertical breaks</td>
<td>↓↓</td>
</tr>
<tr>
<td>Motor coord. impairment</td>
<td>↓♀</td>
</tr>
<tr>
<td>Simple spike firing ratea</td>
<td>↑</td>
</tr>
<tr>
<td>Complex spike (CS) durationb</td>
<td>↑</td>
</tr>
<tr>
<td>Spike pause after CSb</td>
<td>↓</td>
</tr>
</tbody>
</table>

(--) Unchanged; (+) increased; (-) decreased. Two symbols represent strong effects. P-values were classified as *P < 0.01; **P < 0.05; *0.05 > P > 0.1, tendency.

a Results from Ref. [35].
A sequence of events in the CaBP-KO tested here has been hypothesized to result in the observed phenotype: (I) altered Ca2+ signaling in the Purkinje cells of CB−/− [12], PV−/− and double-KO mice [39]; (II) changes in the firing properties of Purkinje cells resulting in an increased simple spike firing rate and the emergence of rhythmic activity of Purkinje cells that alters Purkinje cells neural code and facilitates the emergence of fast oscillation [35]; (III) disruption of functional cerebellar rostro-caudal functional organization.

Evidently CaBPs intervene at several levels of motor control including the peripheral nervous system (PNS), but currently no data is available on PNS alterations in the knockout strains tested here. The role of PV and CB in the PNS is likely to be revealed only in complex sensorimotor associative tasks rather than in simple locomotor tests. In conclusion, each of the genotype tested reveals subtle locomotor alterations, which are only observed when performing repeated tests. These small changes should not considerably affect the outcome of other behavioral tests related to the putative functions of these CaBPs in other brain regions.

Acknowledgements

We acknowledge the technical assistance of Simone Eichenberger, Line-Claude Chapuis and Aline Gioria from the Institute of Physiology, University of Lausanne. We thank Dr. Michael Meyer, Ludwig-Maximilian University, Munich, Germany for providing the CB−/− mice. This work was partially supported by the Swiss National Foundation (grant no. 3100-047291.96 to M.R.C. and 3100A0 100400/1 to B.S.).

References


