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Quantitative analysis of locomotor defects in neonatal mice lacking proprioceptive feedback

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Abstract

Proprioceptive feedback derived from specialized receptors in skeletal muscle is critical in forming an accurate map of limb position in space, and is used by the central nervous system to plan future movements and to determine accuracy of executed movements. Knockout mouse strains for genes expressed by proprioceptive sensory neurons have been generated that result in generalized motor deficits, but these deficits have not been quantitatively characterized. Here we characterize a conditional knockout mouse model where proprioceptive sensory neuron synaptic transmission has been blocked by selective ablation of munc18-1, a synaptic vesicle associated protein required for fusion of synaptic vesicles with the plasma membrane. Proprioceptive munc18-1 conditional mutants are impaired in surface righting—a dynamic postural adjustment task—and display several specific deficits in pivoting, an early locomotor behavior. Before the emergence of forward locomotion during postnatal development, animals explore their surroundings through pivoting, or rotating the upper torso around the relatively immobile base of the hind limbs. 3-D kinematic analysis was used to quantitatively describe this pivoting behavior at postnatal days 5 and 8 in control and munc18-1 conditional mutants. Mutant animals also pivot, but demonstrate alterations in movement strategy and in postural placement of the forelimbs during pivoting when compared to controls. In addition, brief forelimb stepping movements associated with pivoting are altered in mutant animals. Step duration and step height is increased in mutant animals. These results underscore the importance of proprioceptive feedback even at early stages in postnatal development.

Keywords

proprioception; locomotion; development; behavior

1. Introduction

The sense of proprioception, or the internal representation of an animal's limb position in space, is critical in motor planning and in determining if motor commands have been executed properly. Peripheral sensory neurons responsible for encoding muscle length, stretch, and tone provide critical feedback from muscle to the central nervous system and are the neural basis for the perception of proprioception [1]. Rapid changes in muscle length are

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encoded by group Ia sensory afferents associated with muscle spindles in the periphery [2], while muscle force is transduced by Ib afferents that terminate in specialized structures known as Golgi tendon organs (GTOs) [3]. On a molecular level, these proprioceptive sensory neurons can be differentiated from other sensory neuron types by expression of the calcium binding protein parvalbumin (PV), which is expressed only by proprioceptive sensory neurons [4–6]. Signals from these sensors influence motor neuron activity either via direct excitatory connections with MNs (Ia afferents) or indirectly through spinal interneuron pathways (both Ia and Ib afferents) [1].

Ablation of transcription factors expressed in proprioceptive sensory neurons or in muscle spindles produces striking phenotypes that severely limit purposeful movement. For example, the zinc-finger transcription factor *Egr3* is expressed in the intrafusal fibers of the muscle spindle and muscle spindles degenerate rapidly after birth in *Egr3* knockout mice [7]. This results in loss of synaptic transmission between Ia afferents and motor neuron targets in the spinal cord [8], and mutant animals present “marked ataxia characterized by a waddling, uncoordinated gait and abnormal limb positioning when stationary” [7]. Mice lacking the ETS-family transcription factor *Er81*, which is expressed by Ia and Ib afferents as well as intrafusal fibers, display consistent “limb ataxia and abnormal flexor-extensor posturing of their limbs” [4]. Similar phenotypes are also reported in knockout animals for other proprioceptive neuron restricted transcription factors [9].

While general descriptions of ataxia in these knockout mice have been reported, locomotor deficits of these animals have not been quantified or characterized in detail. Here we analyze performance of a conditional knockout mouse strain affecting proprioceptive neurons on an early dynamic postural adjustment task, surface righting, as well as quantify limb mechanics during early postnatal movement sequences. We chose to analyze a Cre-lox mouse genetic model where synaptic transmission in proprioceptive afferents is selectively blocked throughout postnatal development. *Munc18-1* is a synaptic vesicle associated protein expressed in all neurons and some other secretory cells such as pancreatic islet β -cells [10]. Ablation of *munc18-1* eliminates both spontaneous and action potential evoked neurotransmitter release in neurons [11–13]. Using a conditional allele for *munc18-1* allows cell-type specific knockout of *munc18-1* function in subsets of neurons [12, 14]. Expression of parvalbumin (PV) is restricted to proprioceptive sensory neurons during neonatal development [4, 5]. PV is first expressed beginning at E14.5 in large diameter sensory afferents in the DRG and is not expressed by neurons in the spinal cord until after the first postnatal week [4, 15]. We used a strain of mice where Cre-recombinase is knocked in to the *PV* locus (*PV-Cre*), restricting Cre expression (and *munc18-1* ablation) to proprioceptive neurons during early postnatal stages [16]. Thus in proprioceptive specific, *munc18-1* conditional mutant mice, proprioceptive neurons are rendered silent and unable to communicate proprioceptive sensory signals to spinal circuits. We found neonatal *PV-Cre; munc18-1 flox/flox* mutant mice are impaired in the surface righting task, a basic motor behavior. 3-D Kinematic analysis of an early voluntary motor behavior referred to as “pivoting” revealed forelimb coordination strategies unlike those in control animals as well as exaggerated forelimb movements and specific differences in forelimb placement affecting postural stability.

2. Materials and Methods

2.1 Animals

Proprioceptive specific *munc18-1* conditional mutants were generated using previously published mouse lines utilizing the Cre-lox strategy. Parvalbumin (PV) expression distinguishes proprioceptive sensory neurons from other neurons in the dorsal root ganglia. In the *PV-Cre* mouse line, a Cre-recombinase cassette with an internal ribosomal entry site

was inserted in the 3'-UTR of the *PV* locus immediately following the final exon, allowing for Cre-recombinase production in *PV*-expressing cells [16]. The conditional allele of *munc18-1* was generated by flanking exon 2 with lox-P sites (hereafter referred to as a floxed allele) [12]. Elimination of *munc18-1* exon 2 occurs in all cells expressing *PV* resulting in a cell-type specific knockout of *munc18-1*. Both strains were maintained on a mixed C57BL/6J-129 background. Animals were genotyped by PCR using primer pairs specific for relevant alleles (*PV-Cre* forward 5' GTCCAATTTACTGACCGTACACC; *PV-Cre* reverse 5'-GTTATTCGGATCATCAGCTACACC; *munc18-1* forward (common to both wild-type and floxed allele): 5'-CCTGTATGGGTACTGTTCGTTCTACTAAAATA; *munc18-1* wild-type reverse: 5'-TTCTGAACTTGAGGCCAGTCTGAGACACAG; *munc18-1* floxed reverse: 5'-TTGGTGGTTCGAATGGGCAGGTAG). Conditional mutants were produced from matings of *PV-Cre*+/+; *munc18-1* flox/+ and *munc18-1* flox/flox parents. All animals (male and female) from four litters were used for analysis.

Neonatal control and *munc18-1* conditional mutants were assayed daily to measure body weight and performance on the surface righting task from postnatal day 4 (P4) to weaning (P21). The majority of *munc18-1* conditional mutants failed to survive to weaning, but animals were analyzed for as many days as possible. Two age groups (P5/P6 and P7/P8) were utilized for quantitative kinematic analysis. Animals were raised in the Laboratory Animal Resources facility at Wright State University where they were housed in barrier isolation cages on a 12 light : 12 dark cycle. All procedures were conducted in accordance with Wright State University Animal Care and Use Committee guidelines.

2.2 Testing Conditions

Before daily behavioral testing, each cage was placed in the laminar flow hood where testing took place and left undisturbed for 5 minutes to acclimate to avoid possible mother/neonate stress. Mother and pups remained in the cage for the duration of testing with the exception of the test subject. Testing time for each subject was kept to a maximum of 5 minutes. A square testing arena for both the surface righting task and for video recording of pivoting behaviors was made from medium-density fiberboard (MDF) (35cm × 35cm × 1.5cm).

2.3 Qualitative analysis of surface righting behavior

To measure surface righting latency animals were gently placed in the supine position with all limbs projected upward. Both forelimbs were held in place before rapidly releasing them at the start of a timer. The timer was stopped at the point in which the subject successfully turned over and all four paws were in contact with the surface. A maximum of 60 seconds was allotted to complete the surface righting task and animals unable to complete the task in this time were given a latency of 60 seconds. Righting latencies of less than 1 second were assigned the minimum latency of 1 second. Each animal was tested three times per day and the latencies were then averaged.

2.4 Quantitative analysis of pivoting locomotor behavior

2.4.1 Acquisition of video data with KinemaTracer software—Digital video segments were captured with two *Dragonfly2* (*DR2-HICOL*) digital video cameras (Point Grey Research Inc, Richmond, BC, Canada), placed on two adjacent corners of the testing arena and the *MotionRecorder* module of the *KinemaTracer* software. Cameras were positioned on the surface of the arena and the animal was placed in the focal plane of both cameras and allowed to move freely. Each camera recorded (30 frames per second) profile views of the animal from different angles allowing for reconstruction of limb positions in 3-D following analysis with the *3-DCalculator* module of the *KinemaTracer* software (Version 1.3, *Kissei Comtec Co., LTD.*, Tokyo, Japan). Video was recorded until adequate pivot sequences were captured in full view of both cameras. At least 3 clear steps with the leading

limb were required to be considered an adequate pivot sequence. No maximum time was allotted to this particular task to allow for spontaneous demonstration of pivoting by each animal. A photograph of a 3-D calibration box was taken with each camera at the beginning of each video acquisition session. Furthermore, a 3-D model of the animal was created in the software, which in collaboration with the calibration box mapped the location of the animal in time and space.

2.4.2 Video analysis and marker placement—After data acquisition, video recordings from each camera were reviewed manually on a frame-by-frame basis to place virtual markers in the software for a number of anatomical landmarks relevant to the pivoting behavior. Markers were placed at each of the four ankles of the animal (both forelimbs and both hind limbs). Markers were also placed on the vertebral border of each scapula (visible as bony protrusion under the skin on the back) to label the shoulders, each iliac crest to identify the hips and, finally at the tip of the nose to indicate the position of the head with respect to the shoulder. Two additional points were calculated by the software as the midpoint between the shoulders (center of shoulders (COS)) and the midpoint or center of the hips (COH).

To determine the reliability of manual marker placement, a test-retest approach was used on a subset of video segment frames. Markers were placed in these frames for each of the above mentioned anatomical landmarks and the x,y,z coordinates were noted. The same frames were then re-marked without the knowledge of the locations of the initial marker placements. The distance (mm) between the two sets of marks was measured to provide an estimate for the reliability of the manual marker placement. We found the average deviation between the test-retest locations for all markers to be 0.7mm (x-axis), 0.8mm (y-axis), and 0.3mm (z-axis). The (x,y,z) deviations of specific markers most relevant to the analysis of pivoting behavior were found to be: nose (0.3mm, 0.3mm, 0.3mm), shoulders (0.6mm, 0.8mm, 0.3mm), forelimb ankles (0.8mm, 0.7mm, 0.3mm).

Quantitative analysis of interlimb distance, forelimb location, and leading limb step parameters (duration, distance, amplitude) was performed using custom analysis routines developed in Matlab (The MathWorks, Inc, Natick, MA; version R2008a) utilizing 3-D coordinate output data provided from the *KineAnalyzer* module of the *KinemaTracer* software.

2.5 Statistical comparisons

Statistical comparisons between groups were performed using Student's t-test after first testing for normality of the distributions using the Kolmogorov-Smirnov test. Data are reported as mean $\pm$ standard error. Results were considered to be significant if $p < 0.05$.

3. Results

To study the neonatal consequences of a loss of proprioception, we utilized conditional mouse genetics to obtain mice that lack synaptic transmission in proprioceptive sensory neurons by crossing two existing transgenic mouse strains. Expression of *munc18-1* has been shown to be essential for fusion of synaptic vesicles with the plasma membrane and ablation of *munc18-1* blocks both spontaneous and evoked synaptic transmission from neurons [13]. A conditional allele of *munc18-1* has been generated and allows restricted ablation of *munc18-1* function based on Cre-recombinase expression in subsets of neurons [12, 14]. Expression of parvalbumin (PV), a calcium binding protein, is restricted to proprioceptive sensory neurons during neonatal development [17]. PV is first expressed beginning at E14.5 in large diameter sensory afferents in the DRG and is not expressed by neurons in the spinal cord until after the first postnatal week [15]. Using a transgenic animal

where Cre-recombinase is expressed in greater than 90% of PV expressing neurons allows us to restrict *munc18-1* ablation to the subset of sensory neurons providing proprioceptive information from muscles [16].

The frequency of *munc18-1* conditional mutants (*PV-Cre/+; munc18-1 flox/flox*) found in litters analyzed in these experiments was consistent with the expected Mendelian ratio, suggesting proprioceptive specific ablation of *munc18-1* did not result in embryonic lethality (11/38 animals or 28.9% of offspring in 4 litters). Genotypes of all animals were determined by PCR, but *munc18-1* conditional mutants could be readily distinguished from littermates from about P2 by general visual assessment of motor behavior. Animals were weighed daily from P4 to P21. Interestingly, *munc18-1* conditional mutants gained weight only until about P9 (Fig. 1A). From that time onward the body weight of mutants remained relatively constant, while the average weight of littermates continued to increase through the period analyzed.

We first analyzed the ability of *munc18-1* proprioceptive conditional mutants to perform a locomotor behavior typical of neonatal animals. One of the earliest movement sequences acquired allows for righting on a surface [18]. When the animal is placed on its back, it immediately begins to rock its torso back and forth in an effort to right itself. We measured the latency from the time an animal was released from the supine position until it was able to right itself and place all 4 paws on the surface of the testing arena. Even in normal animals, perfecting this skill takes several days, and individual differences in ability are particularly evident at early stages when animals are first attempting the task. For example, on the initial day of analysis (P4) some non-mutant littermates could perform the task in less than 3 seconds (5/27, 18.5%), while a similar number were unable to right themselves in the allotted 60 second test period (5/27, 18.5%).

Animals with the genotype *PV-Cre/+; munc18-1 flox/+* are functionally heterozygous for *munc18-1* in PV-expressing neurons and we wondered if differences in performance may be related to the genotype of non-mutant littermates. We compared the performance of animals of this genotype with both possible genotypes where Cre is not expressed, leaving both alleles of *munc18-1* unaffected (*PV-+/+; munc18-1 flox/+* and *PV-+/+; munc18-1 flox/flox*). We found no difference in surface righting latency at any age analyzed between functional heterozygous and normal *munc18-1* animals (data not shown, t-test p-value range 0.37 to 0.98). Consequently, littermates with either one or two functioning alleles of *munc18-1* were grouped together as control animals.

Righting latencies declined steadily on successive days of testing and control animals, as a group, mastered the behavior by P8/P9 (average latency at P8 = 3.5 ± 0.9 s, median latency = 1.3s, latency range = 1.0 – 16.0s, n = 25; Fig. 1B). In contrast, none of the *munc18-1* proprioceptive mutants analyzed at P4 were able to right themselves in the allotted 60 second time interval (0/11, Fig. 1B). In general, *munc18-1* mutants improved over time and maximal group performance was reached about P12 (average latency at P12 = 7.6 ± 1.2 s; median latency = 6.6s; latency range = 3.0 – 15.0s, n = 9; Fig. 1B). Nevertheless, the mutant group never reached the proficiency level of control animals and displayed significantly longer latencies after reaching maximal proficiency (average control latency at P12: 1.1 ± 0.1 s, median latency = 1.0s; latency range = 1.0 – 2.0s, n = 18; p < 0.001; Fig. 1B).

The number of mutants available for analysis declined with advancing postnatal age as animals failed to adequately gain weight. While we observed no obvious maternal neglect of mutant animals during feeding, their disturbed motor behavior may result in limited competitiveness with littermates during nursing. Any disadvantage of the mutants would become increasingly exaggerated as control animals acquire basic motor skills, for example

mastery of surface righting about P8/P9. This likely contributed to the high postnatal mortality rate. In fact, only two mutants survived for analysis between P18 and P21 and these animals were still unable to complete the surface righting task in a manner similar to controls (average mutant animal latency from P18-P21 = 7.2 ± 1.5 s). Dramatically reduced performance in surface righting illustrates the profound motor coordination deficits present in the absence of proprioception, but we wished to analyze more carefully movements of individual limbs during simple locomotor behaviors common in neonates.

Forelimb movements dominate early locomotor patterns in neonates. These movements resemble stepping and turning in more mature animals, but at early neonatal stages cause the animal to rotate the upper torso around the relatively immobile base of the hips and hind limbs. This “pivoting” behavior gradually disappears, presumably as descending inputs to the lumbar spinal cord mature allowing weight bearing at the hind limbs. Each forelimb plays a different role during pivoting. The leading limb (left forelimb for a left pivot) makes a series of small translations along the surface in the general direction of the movement and provides stability to the upper body. The opposite forelimb is referred to as the punting limb (right arm for a left pivot) and produces the force for pushing the torso laterally in the direction of the pivot [11]. This is achieved through extension of the arm at the elbow joint. Control and mutant animals routinely pivot in both directions and the forelimbs exchange leading and punting roles.

We sought to quantify aspects of this behavior to identify potential deficits resulting from a loss of proprioceptive feedback in *munc18-1* conditional mutants. 3-D video analysis was used to follow relevant body landmarks on a frame-by-frame basis from a series of video segments of animals making pivoting movements at both P5 and P8 (Fig. 2A; see Materials and Methods for detailed description of analysis and landmark identification). Control animals demonstrate pivoting behavior at P5, but the frequency of this behavior decreases after P8 as many animals are then able to execute rudimentary forward locomotion [18]. Surface righting performance in *munc18-1* conditional mutant showed improvement beginning at P8 (Fig. 1B), therefore analysis of movements at P5 and P8 would provide an opportunity to evaluate any differences in movement strategy in mutant animals at these two stages. Additionally, analysis at these time points would minimize possible confounds from the divergent trajectories of weight gain between control and *munc18-1* conditional mutants.

Animals would rotate to varying degrees during each episode of pivoting. The extent of each pivot was determined by measuring the angle formed by connecting the average location of the center of the hips during the pivot with the locations of the center of the shoulder at the beginning and end of the analyzed pivot (Fig. 2B). We found that at both P5 and P8 *munc18-1* conditional mutants were able to successfully pivot and that the average extent of each pivot was not significantly different than littermate controls (Fig. 2C; P5 control = $102.7 \pm 7.0^\circ$, n = 6; P5 mutant = $80.6 \pm 16.5^\circ$, n = 7; P8 control = $102.3 \pm 12.9^\circ$, n = 5; P8 mutant = $108.7 \pm 34.9^\circ$, n = 5). The duration of pivoting movements was variable in both control and mutant animals (1.0 to 2.5 seconds) and it was possible that *munc18-1* conditional mutants would pivot more slowly, but we found no significant differences in the rate of pivoting at both P5 and P8 (Fig. 2D; P5 control = $65.7 \pm 8.5^\circ/\text{sec}$, n = 6; P5 mutant = $63.1 \pm 11.5^\circ/\text{sec}$, n = 7; P8 control = $51.2 \pm 14.2^\circ/\text{sec}$, n = 5; P8 mutant = $74.3 \pm 23.6^\circ/\text{sec}$, n = 5).

Closer examination revealed differing strategies in executing pivoting movements in controls and *munc18-1* conditional mutants. Figure 3A illustrates a representative pivoting episode from a P5 control animal. The degree of rotation from the original position steadily increases over time. This pattern was observed in 5/6 control animals at P5 and in 4/5 animals at P8. In contrast, the temporal profile of pivoting in *munc18-1* conditional mutants

was more episodic. While mutants on average achieved the same degree of rotation during an analyzed period of pivoting, the sub-second temporal profile suggested an alternating pattern between brief times of rotation and intervals where little rotation was produced (Fig. 3B). This pattern is evident in a stair-like profile in temporal plots of degrees of rotation in 6/7 mutants at P5 and 4/5 mutants analyzed at P8 (Fig. 3B for representative example).

We investigated whether coordination of left and right limb movement was associated with these differing strategies for torso rotation. Analysis of forelimb placement during pivot sequences revealed control animals begin the punting phase of a pivot movement from a position of relative stability where the right and left limbs are spaced some distance apart. During extension of the punting limb the upper torso is pushed laterally, crossing over the initial position of the leading limb which remains stationary and in contact with the surface. As a result of this movement the distance between the right and left limbs decreases (Fig. 3C, E, F). At the end of the extension, the leading limb is brought out from under the body again to provide stability for the next phase of punting and the distance between the two limbs is increased to the starting distance. This movement pattern was observed in 5/6 control animals at P5 and in 5/5 control animals at P8. *Munc18-1* conditional mutants, however, showed little evidence of a temporal pattern in the spatial relationship between the right and left limbs during pivoting (1/7 animals at P5; 1/5 animals at P8). Distances between the left and right limbs remained relatively constant during the pivoting motion (Fig. 3D, G, H).

In the absence of proprioceptive feedback, *munc18-1* conditional mutants may be unable to compensate for trunk instability brought about by crossing the trunk over the leading limb and therefore retain a wide forelimb stance during the pivot. Consequently, we analyzed forelimb placement throughout pivoting sequences at both P5 and P8. First, we measured the percent of time each forepaw was found lateral to its respective shoulder, as defined by a plane passing through the shoulder, but perpendicular to the line connecting the two shoulders (Fig. 4A, B). Consistent with our analysis of the dynamics of forelimb spacing during pivoting, leading limbs in control animals were often observed medial to the plane of the shoulder (Fig. 3F, Fig. 4C; P5 = $41.7 \pm 10.1\%$, $n = 6$; P8 = $39.4 \pm 11.9\%$, $n = 5$). While the leading limb was most often lateral to the shoulder (P5 = $58.3 \pm 10.1\%$, P8 = $60.6 \pm 11.9\%$), these results indicate an ability to coordinate lateral torso displacement without requiring constant lateral positioning of the leading limb. The position of the leading limb of *munc18-1* conditional mutants at P5, however, was more often lateral to the shoulder (Fig. 4C; $80.9 \pm 8.0\%$; $n = 7$; P5) with relatively little time ($19.1 \pm 8.0\%$) observed medial to the shoulder. Comparisons of leading limb placement did not reach statistical significance ($p = 0.112$, Student's t-test), but suggest general differences in trunk support strategies during pivoting in *munc18-1* conditional mutants at this earlier time point of analysis. By P8, however, lateral/medial placement of the leading limb in *munc18-1* conditional mutants had a distribution similar to that seen in control animals (Fig. 4C; lateral = $64.9 \pm 10.7\%$; medial = $35.1 \pm 10.7\%$). Analysis of punting limb positions showed predominantly lateral placement in control animals at both P5 and P8 (Fig. 4D; P5 = $96.7 \pm 1.5\%$, $n = 6$; P8 = $83.8 \pm 5.4\%$, $n = 5$). This is consistent with the role of the punting limb in providing force for torso movement in the direction of the pivot. Interestingly, *munc18-1* conditional mutants have different lateral / medial distributions at the two ages analyzed. At P5 the distribution was similar to control animals (lateral = $87.9 \pm 6.8\%$, $n = 7$), but at P8 the punting limb of mutant animals was often found medial to the shoulders (Fig. 4D; lateral = $64.7 \pm 9.4\%$; medial = $35.3 \pm 9.4\%$, $n = 5$).

Forelimb placement either rostral or caudal to the plane of the shoulders also has a significant impact on stability and the potential for force generation (Fig. 4E). As may be expected from the anatomy of the forelimb, we found placement of the leading limb to be

almost always rostral to the shoulders in both control and *munc18-1* conditional mutants at P5 and P8 (Fig. 4G; P5 control: $98.9 \pm 0.7\%$; P5 *munc18-1* mutants: $93.9 \pm 2.0\%$; P8 control: $97.4 \pm 2.1\%$; P8 *munc18-1* mutants: $91.3 \pm 3.6\%$). A similar situation was observed for the punting limb in control animals (Fig. 4H; P5: $87.9 \pm 3.9\%$; P8: $90.4 \pm 6.3\%$). In contrast, the punting limb in *munc18-1* conditional mutants was almost evenly distributed between rostral and caudal locations relative to the shoulders (Fig. 4H; P5 rostral = $47.1 \pm 9.7\%$, P5 caudal = $52.9 \pm 9.7\%$; P8 rostral = $43.5 \pm 14.4\%$, P8 caudal = $56.5 \pm 14.4\%$). The difference in distribution was highly significant (P5 control vs. *munc18-1* mutants, $p = 0.005$; P8 control vs. *munc18-1* mutants, $p = 0.027$; Student's t-test). This was consistent with our qualitative observations that the punting limb was often extended at the elbow in *munc18-1* conditional mutants (Fig 4F).

Loss of proprioception affects movement trajectories as well as maintenance of posture [19]. We therefore analyzed movements of the forelimbs during pivoting at both P5 and P8 stages. In both control and *munc18-1* conditional mutants the leading limb makes a series of movements during pivoting to provide trunk stability as rotation progresses. These movements are a combination of “sliding” planar translations along the surface, and “steps” where the limb is briefly lifted off the surface as it moves laterally. Because the step portion had a definitive beginning and end point where the forelimb left and then returned to the surface, we were able to quantitatively analyze this segment of the movement. We first analyzed the distance of the step with regard to the plane of the surface. Both at P5 and P8 we found average step distance of *munc18-1* conditional mutants to be similar to those of control animals (Fig. 5A; P5 control: $9.6 \pm 1.3\text{mm}$; P5 *munc18-1* mutants: $6.9 \pm 1.1\text{mm}$; P8 control: $8.7 \pm 1.3\text{mm}$; P8 *munc18-1* mutants: $11.2 \pm 2.0\text{mm}$). This indicates the distance travelled across the surface during the time the forelimb is in the air is similar in control and mutant animals. Nevertheless, the average duration of each step was significantly longer in conditional mutants than control animals at both P5 and P8 (Fig. 5B; P5 control: $0.11 \pm 0.01\text{s}$; P5 *munc18-1* mutants: $0.15 \pm 0.01\text{s}$; P8 control: $0.10 \pm 0.01\text{s}$; P8 *munc18-1* mutants: $0.21 \pm 0.01\text{s}$). Given this finding we analyzed the path of each step in space and found that the height of each step was significantly exaggerated and was 2 to 3-fold greater in *munc18-1* conditional mutants at both P5 and P8 (Fig. 5C,D; P5 control: $1.9 \pm 0.4\text{mm}$; P5 *munc18-1* mutants: $4.9 \pm 0.8\text{mm}$; P8 control: $2.2 \pm 0.3\text{mm}$; P8 *munc18-1* mutants: $6.7 \pm 0.9\text{mm}$).

4. Discussion

In this study, we provide both qualitative and quantitative analysis of locomotor behaviors in neonatal mice lacking proprioceptive feedback to the central nervous system. The Cre-lox mouse genetic system was used to selectively block synaptic transmission in proprioceptive neurons during early postnatal development, effectively eliminating proprioceptive feedback during the time at which animals first begin to learn locomotor tasks. Throughout the first three weeks of postnatal development, *PV-Cre/+; munc18-1* conditional mutants perform poorly on the surface righting task, an early locomotor skill mastered by littermate control animals by P8/P9. While *munc18-1* conditional mutants do improve performance over time, they do not reach the level of proficiency of control animals in this task. Quantitative descriptions of limb movements during pivoting, another common early locomotor skill, using 3-D video kinematic analysis illustrates several differences in limb trajectory during movement and in postural placement of the forelimbs in *munc18-1* conditional mutants.

It is clear that *munc18-1* conditional mutants have a limited behavioral repertoire. Nevertheless, improvement is observed in surface righting behavior performance over time. This may be related to maturation of descending inputs to both the cervical and lumbar spinal cord during postnatal development [20]. Altman and Sudarshan describe different

strategies used during surface righting that suggest improving whole body coordination in neonatal rats throughout the same postnatal time course used in this study [18]. Initially (P0-P3) rats right themselves primarily via right and left rocking motions of the extended hind- and forelimbs. Righting with this strategy requires significant time and suggests an almost trial-and-error approach. Beginning at P3, head rotation in the direction of righting is observed followed by coordinated head, forelimb, and shoulder rotation at P7/P8. Finally, hips and hindlimbs move in coordination with the upper part of the body about P11/P12. In our experiments, *munc18-1* conditional mutants showed improvements in surface righting latencies at similar postnatal ages (P8 and again at P12, see Fig. 1B). These results suggest improvements observed in *munc18-1* conditional mutants may be related to developmental milestones whereby trunk and limb musculature become increasingly well coordinated by descending pathways. Optimal performance in the surface righting task, however, appears to require functional proprioceptive feedback, as evidenced by the impaired performance at any postnatal age of *munc18-1* proprioceptive conditional mutants.

Parvalbumin (PV) is expressed by multiple neuron types in the adult central nervous system; hence Cre-recombinase would also be expressed in these neurons later in postnatal development [21, 22]. PV expression is prominent in a variety of interneuron populations. For example, a subset of glycinergic inhibitory interneurons in the ventral spinal cord directly involved in mediating reciprocal inhibition among antagonistic muscle groups have been shown to express PV [15]. This expression, however, is only detected after the first postnatal week [15]. In addition, subsets of GABAergic dorsal horn interneurons also express PV in adult animals [23]. The onset of PV expression in these interneurons is unknown, but as these interneurons are involved in processing of primary sensory information from receptors in the skin, ablation of *munc18-1* in these neurons is unlikely to produce the motor coordination defects observed in these experiments. There is also widespread expression of PV in GABAergic interneurons in the cortex and basal ganglia, but in mice relatively few neurons express PV during the first postnatal week compared with adult animals [17, 22, 24]. Generalized loss of GABAergic transmission following ablation of *munc18-1* in PV-expressing interneurons may lead to increased potential for seizures in *munc18-1* conditional mutants, but spontaneous seizures were not observed in our experiments.

Parvalbumin is also expressed in vestibular afferents beginning at E14 in rat [17, 25]. While we have not specifically tested vestibular function in *munc18-1* conditional mutants, two qualitative behavioral characteristics suggest proprioceptive defects, rather than possible vestibular deficits, are predominant in these animals. First, *munc18-1* conditional mutants in our experiments always attempted to right themselves in the surface righting task. While execution of the task was not as efficient as controls, mutant animals consistently exerted themselves to escape the supine position and return to the normal prone position. This behavior is in contrast to that reported in animals with vestibular specific defects produced either by toxin application or by genetic mutation. Using a modified righting task, termed “ground righting,” vestibular mutants appeared unable to determine up or down directionality and did not attempt to right themselves from a supine position [26, 27]. Second, mice and rats with vestibular dysfunction often display strong neck dorsiflexion and head bobbing [26, 28], but these behaviors were not observed in *munc18-1* conditional mutants (data not shown). Taken together, while we cannot exclude possible contributions from compromised vestibular function, these behavioral differences indicate motor behavioral deficits observed in *munc18-1* conditional mutants primarily result from deficits in proprioception.

Likewise, neurons in adult cerebellum express PV and could compromise the function of neural circuits related to locomotion if and when *munc18-1* is ablated following Cre-

recombinase expression [22, 29]. We interpret our kinematic results at P5 and P8 as being attributable to proprioceptive deficits for three reasons. First, PV in the cerebellum is developmentally regulated with significant expression detected after P10 [29]. Second, performance on the surface righting task improves over time in mutants, coincident with known milestones in trunk and limb coordination. Coordination of head, shoulder and hindlimb movements necessary to complete surface righting would likely be impaired if synaptic transmission in cerebellar circuits was blocked. Third, substantial loss of cerebellar function would likely impair *munc18-1* conditional mutants' ability to pivot, and while there are alterations in movement strategy, mutant animals are still able to pivot (Fig. 2C, D).

One of the most robust findings of our analysis was the exaggerated height of steps taken with the leading limb during pivoting. Both at P5 and P8, *munc18-1* conditional mutants had significantly greater amplitude when the forelimb lifts off the surface. This was coupled with longer step durations, but the average distance covered with respect to the surface was not different from control values. One possible explanation for this could be that dynamic tactile feedback may provide a sufficient signal to judge limb velocity, but only during the sliding phase of the leading limb's movement when the limb is in contact with the surface. In contrast to more widespread expression of PV over time in the central nervous system, PV expression in sensory neurons is stable and restricted to proprioceptive neurons [4, 17]. Thus, other sensory modalities, including the sense of touch are likely preserved in *munc18-1* conditional mutants. When the leading limb is lifted off the surface to complete the movement sequence of resetting the limb in a stable lateral position, tactile stimulation is lost and in the absence of functioning proprioceptive feedback the retraction from the surface is exaggerated. It is interesting to note *munc18-1* conditional mutants continue to use the same strategy as control animals in moving the leading limb where each movement sequence is comprised of both sliding and stepping components, as opposed to adopting a strategy favoring more sliding movements. This may suggest that even though steps are abnormal in mutants, it does not prevent the animal from completing the task.

Another consistent abnormality of *munc18-1* conditional mutant pivoting was the placement of the punting limb. In control animals a strong bias was observed for placement of the punting forelimb anterior to the shoulders during pivoting. In fact, the punting limb remains anterior to the shoulders approximately 90% of the time during a pivoting episode (Fig. 4H). In contrast, no such bias was observed in mutant animals. Punting limb position during a pivot sequence was almost equally divided between anterior and posterior compartments (40–50% anterior placement, 50–60% posterior placement; Fig. 4H). The elbow joint is in full extension when the limb is positioned posterior to the shoulders (Fig. 4F). This suggests dominant contraction of the triceps muscle group over the antagonist biceps group at the elbow. In contrast, when the limb is kept anterior to the shoulders, as in control animals, there is partial flexion at the elbow. It may be that stretch receptors in the biceps report joint angle during punting as the triceps contract to extend the elbow and generate force for lateral movement of the torso. A certain degree of extension may then trigger cessation of extension and a resetting of punting limb position for the next punting sequence. The absence of such a signal in mutant animals may result in unchecked contraction of the triceps leading to full extension of the elbow and posterior punting limb placement.

5. Conclusions

As a result of these studies we have identified specific alterations in movement patterns in neonatal *munc18-1* conditional mutants. Understanding these alterations in animals lacking all proprioceptive feedback provide an important framework against which other proprioceptive mutant strains can be compared. For example, Ia afferent function and/or connectivity is affected in both *Egr3* and *Er81* null mutants [4, 7]. Ib afferent function,

however, is not affected by these mutations [4]. Analysis of these mutant strains might provide new insight into specific roles of Ia versus Ib afferent signals in modulating movement programs.

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Research Highlights

- We analyzed locomotor behavior of neonatal mice lacking proprioceptive feedback.
- A Cre-lox strategy was used to ablate *munc18-1* in proprioceptive sensory neurons.
- 3-D kinematics revealed differences in limb trajectory during pivoting movements.
- Our findings delineate role of proprioception in execution of pivoting behavior.

Figure 1.

Munc18-1 conditional mutants demonstrate limited weight gain and impaired performance on surface righting task. (A) Body weight was measured daily from P4 to P21. (B) Time required to complete surface righting in mutants and control littermates was measured daily from postnatal days 4 to 21. Sample size (n) per age group is presented below the graph in B also applies to panel A. Sample size in mutants varied greatly as survival rate was highly unpredictable. Differences in control sample size can be attributed to interruptions in analysis. Data points represent mean values $\pm$ SEM.

Figure 2.

Quantitative kinematic analysis of pivoting behavior in neonatal mice. (A) Image of P5 control animal as example of marker placement for kinematic software analysis. The nose, right shoulder (RS), left shoulder (LS), right hand (RH), and left hand (LH) were manually placed points using KinemaTracer software to quantify movement. Locations of two markers (center of shoulders [COS] and center of hips [COH]) were calculated by software. With the exception of the COH, positions of hind limb markers (left iliac crest [LI], right iliac crest [RI], left foot [LF], and right foot [RF]) were not considered in data analysis. Actual camera placement for analysis recorded profile rather than aerial perspective views of each animal. (B) Schematic of a normal left pivot. Leading limb and punting limbs are

left and right limbs, respectively. represents the degree to which the mouse pivots. (C) Average number of degrees to which animals at P5 and P8 turn during an episode of pivoting. (D) Average rate of pivoting (degrees/sec) at P5 and P8.

Figure 3.

Representative examples illustrating different strategies for pivoting in control and *munc18-1* conditional mutants. (A) Pivot profile over time for a representative individual control animal at P5. Note strong linear trend in degrees of pivot over time. (B) Pivot profile over time for a representative individual *munc18-1* conditional mutant animal at P5. Note more stair-like profile of cumulative degrees of pivoting over time. Open arrowheads point to brief periods where turning is evident while filled arrowheads indicate periods where little change in degree of pivot is accomplished. (C) Interlimb distance in the same control animal as (A) shows a sinusoidal pattern over time. (D) No specific pattern is evident in the interlimb distance from same mutant animal as in (B). (E–H) Scaled plots of spatial relationships between relevant forelimb markers (Nose, RH, LH, RS, LS, COS) from individual frames at time points indicated in C and D. Dashed line between LH and RH shows interlimb distance at each time point. Solid line through LS, COS, and RS denotes plane of shoulder at each time point. Rotation of this plane is evident when comparing E with F or G with H, and illustrates animal rotation during pivot sequence.

Figure 4.

Analysis of forelimb placement during pivoting in control and *munc18-1* conditional mutant animals at P5 and P8. (A) Schematic indicating domains lateral and medial to each shoulder. (B) Image of a P5 control animal executing a left pivot and overlaid with kinematic analysis markers. (C) Graph indicating the percent of the total time during a pivoting episode where the leading limb is located either lateral or medial to the respective shoulder. (D) Analysis of punting limb position as in (C). (E) Schematic indicating domains rostral and caudal to the forelimbs with respect to the position of the shoulders. (F) Image of a P5 *munc18-1* conditional mutant animal overlaid with kinematic analysis markers executing a left pivot. (G) Graph indicating the percent of the total time during a pivoting episode where the

leading limb is located either lateral or medial to the respective shoulder. (H) Analysis of punting limb position as in (G). * = $P < 0.05$.

Figure 5.

Analysis of leading limb step parameters in control and *munc18-1* conditional mutant animals at P5 and P8. (A) Graph indicating average distance the forelimb travels with respect to the plane of the surface for an individual step. (B) Step duration (seconds) was significantly longer in mutants than in controls. (C) Average amplitude of each step during a pivot motion is significantly greater in mutants. (D) Plot of step amplitude against time required for each step from representative individual control and *munc18-1* mutant animals at P5. Step amplitude and duration is greater in the mutant animal. Scales for control animal plot also apply to *munc18-1* conditional animal plot. * = $P < 0.05$.