Characterization of Neonatal Vocal and Motor Repertoire of Reelin Mutant Mice

Emilia Romano1,3*, Caterina Michetti2*, Angela Caruso3, Giovanni Laviola1, Maria Luisa Scattoni2*

1 Behavioural Neuroscience Section, Department of Cell Biology & Neuroscience, Istituto Superiore di Sanità, Rome, Italy, 2 Neurotoxicology and Neuroendocrinology Section, Department of Cell Biology & Neuroscience, Istituto Superiore di Sanità, Rome, Italy, 3 Bambino Gesù Children’s Hospital Istituto Di Ricoero e Cura a Carattere Scientifico, Rome, Italy

Abstract

Reelin is a large secreted extracellular matrix glycoprotein playing an important role in early neurodevelopment. Several genetic studies found an association between RELN gene and increased risk of autism suggesting that reelin deficiency may be a vulnerability factor in its etiology. Moreover, a reduced reelin expression has been observed in several brain regions of subjects with Autism Spectrum Disorders. Since a number of reports have documented presence of vocal and neuromotor abnormalities in patients with autism and suggested that these dysfunctions predate the onset of the syndrome, we performed a fine-grain characterization of the neonatal vocal and motor repertoire in reelin mutant mice to explore the developmental precursors of the disorder. Our findings evidence a general delay in motor and vocal development in heterozygous (50% reduced reelin) and reeler (lacking reelin gene) mutant mice. As a whole, an increased number of calls characterized heterozygous pup’s emission. Furthermore, the typical ontogenetic peak in the number of calls characterizing wild-type pups on postnatal day 4 appeared slightly delayed in heterozygous pups (to day 6) and was quite absent in reeler littermates, which exhibited a flat profile during development. We also detected a preferential use of a specific call category (two-components) by heterozygous and reeler mice at postnatal days 6 and 8 as compared to their wild-type littermates. With regard to the analysis of spontaneous movements, a differential profile emerged early in development among the three genotypes. While only slight coordination difficulties are exhibited by heterozygous pups, all developmental precursors of the disorder. Our findings evidence a general delay in motor and vocal development in reelin mutant pups.

Introduction

A number of studies implicate the glycoprotein reelin in the etiology of several neurodevelopmental disorders such as schizophrenia [1,2], lissencephaly [3] and autism [4–6]. Reelin is a protein of the extracellular matrix involved in regulation of embryonic brain development [7,8]. In particular, this protein plays an important role in regulating neuronal migration and brain lamination and promoting dendrite maturation, axonal growth, and the establishment of synaptic contacts [5,9–12]. In addition, reelin seems to act after embryonic development in synaptogenesis and synaptic plasticity [13].

Spontaneous reeler mutant mouse (Rl, lacking Reelin gene) presents lamination defects in cerebral cortex, hippocampus and cerebellum. The cerebellum shows a decreased number of Purkinje cells [14] leading to a severe cerebellar hypoplasia and an ataxic phenotype characterized by uncoordinated and unsteady gait, imbalance, tremors and usually early death around the time of weaning [15–19]. Due to its serious physical impairments, reeler gait, imbalance, tremors and usually early death around the time of weaning [14] leading to a severe cerebellar hypoplasia and an ataxic phenotype characterized by uncoordinated and unsteady gait, imbalance, tremors and usually early death around the time of weaning [15–19]. Due to its serious physical impairments, reeler mice are not considered as a reliable animal model for basic behavioral research and its use has been so far limited to the study of neuronal migration and of the etiology of human lissencephaly [3,20].

Unlike Rl mice, levels of reelin in heterozygous mice (Het) are reduced by 50% compared to wildtype (Wt) and the lamination defects in the CNS are absent. This molecular depletion produces a range of subtle neurobehavioral consequences including increased impulsivity and disinhibited behavior [6,21,22], impaired executive function [23,24], associative learning deficit [25], reduced social motivation [26,27] and reduced pre-pulse inhibition [28].

Reduced reelin expression has been observed in several brain regions of subjects with autism [4,29,30]. Post-mortem studies [29], showed that reelin is reduced in cerebellum (~40%), superior frontal (~70%) and parietal (~70%) cortices. These brain regions have been implicated in the mediation of the three core behaviors that are impaired in autism: social behavior, language and communication, and repetitive and stereotyped behaviors. In particular, the frontal and parietal cortices influence the planning and organization of behaviour and are closely related to the recognition of language and memory for words. The cerebellum has been proposed to serve as an integrative area providing...
‘correct predictions about the relationship between sensory stimuli’ and its dysfunctions may directly relate to cognitive impairments. Moreover, several genetic studies found an association between RELN gene and increased risk of autism [31–36]. Altogether these genetic and molecular studies suggest that the reelin deficiency may be a vulnerability factor in the pathology of autism. Since several reports have documented the presence of vocal and neuromotor abnormalities in patients with autism and suggested that these dysfunctions predate the onset of the syndrome, we performed a fine-grain characterization of the neonatal vocal and motor repertoire in reelin mutant mice to explore the developmental precursors of this disorder [37]. The elucidation of the developmental onset of autism will be crucial in designing early intervention strategies to reduce the incidence and impact of autism-related abnormalities.

To address the hypothesis that Het and RI mice display motor and vocal alterations [30] and that autism-like phenotypes [39] in these mutant mice can be detected at an early developmental stage, we analyzed development of ultrasonic vocalization (USV) patterns and spontaneous motor behavior throughout the first two postnatal weeks. Ultrasonic vocalizations, emitted by mouse pups in response to separation from the lactating mother, are considered a reliable index of social motivation and provide a very sensitive insight into the early emotional development [40–43] thus representing a suitable and reliable tool for the identification of the early communication deficits in autism animal models [21,37,38,44–50]. The primary goal of the present study was to detect any unusual component of vocalizations in RI and Het mice at infant stages, relevant to the absence of crying, and the unusual guttural grunts and squeals, reported for some babies that were later diagnosed with autism [51,52]. Our results evidence a genotype-dependent deviation in ultrasonic vocal repertoire and a general delay in motor development in reelin mutant pups.

Materials and Methods

Animals

B6C3Fe heterozygous female and male mice, originally purchased from Jackson Laboratories (USA) were bred in our laboratory. Two females were housed with one male in (33 cm×13 cm×14 cm) Plexiglas boxes, with sawdust bedding and a metal top. After two weeks of mating, male mice were removed, dams were housed individually in Plexiglass cages (33×13×14 cm), and daily checked for delivery. Mice were maintained on a reversed 12:12 h light: dark cycle (lights on at 18:30 h). The temperature was maintained at 21±1°C, relative humidity (60±10%). Mice genotype was determined at weaning (pnd 25) by RT-PCR on tail samples. Mice are weaned into cages with sawdust bedding. Animals provided drinking water and a complete pellet diet (wheat, corn, barley, soybean meal, soybean protein, maltodextrin, com gluten, sunflower meal, maize flour, minerals, vitamins). Mice were weaned into cages of same sex pairs. After weaning mice were housed in pairs within Plexiglass cages (27×21×14 cm), with sawdust bedding. Animals were provided drinking water and a complete pellet diet (Macrolina, Settimo Milanese, Italy) ad libitum. Pups were tattooed on the paw with animal tattoo ink (Ketchum permanent Tattoo Inks green paste, Ketchum Manufacturing Inc., Brockville ON Canada) by loading the ink into a 30G hypodermic needle and inserting the ink subcutaneously through the needle tip into the center of the paw. The procedure was performed at two days of age, immediately after behavioral testing. The procedure causes only minor brief pain and distress and does not require the use of anesthesia. All procedures were in accordance with the European Communities Council Directive (86/609/EEC) and formally approved by Italian Ministry of Health.

Ultrasonic vocalizations in separated pups

Litters chosen for testing contained more than six pups. Body weights and body temperatures of pups were measured after the ultrasonic vocalization test on pnd 2, 4, 6, 8 and 12. On each day of testing, each pup was placed into an empty glass container (diameter, 5 cm; height 10 cm), located inside a sound-attenuating styrofoam box, and assessed for ultrasonic vocalizations during a three minute test. At the end of the three minute recording session, each pup was weighed and its axillary temperature measured by gentle insertion of the thermal probe in the skin pocket between upper foreleg and chest of the animal for about 30 seconds (Microprobe digital thermometer with mouse probe, Stoelting Co., Illinois, USA). No differences in patterns of calling were detected in a comparison of male and female pups, therefore data were collapsed across sex.

An Ultrasound Microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 10–180 kHz, recorded the pup vocalizations in the sound-attenuating chamber. The microphone was placed through a hole in the middle of the cover of the styrofoam sound-attenuating box, about 20 cm above the pup in its glass container. The temperature of the room was maintained at 22°C. Vocalizations were recorded using Avisoft Recorder software (Version 3.2). Settings included sampling rate at 250 kHz; format 16 bit. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 1024 points and a time window overlap of 75% (100% Frame, Hamming window). The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency of 15 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Call detection was provided by an automatic threshold-based algorithm and a hold-time mechanism (hold time: 0.01 s). An experienced user checked the accuracy of call detection, and obtained a 100% concordance between automated and observational detection. Parameters analyzed for each test day included number of calls, duration of calls, total calling time, qualitative and quantitative analyses of sound frequencies measured in terms of frequency and amplitude at the maximum of the spectrum.

Qualitative analyses

We classified every USVs emitted at pnd 2, 4, 6, 8, 12, in nine distinct categories, based on internal pitch changes, lengths and shapes, using our previously published categorization [37]. Classification of USVs included nine waveform patterns described below: 1) Complex calls displayed one component containing two or more directional changes in pitch, each ≥6.25 kHz; 2) Two-component calls consisted of two components: a main call (flat or downward) with an additional punctuated component towards the terminal dominant frequency at 12.5 kHz; 3) Upward-modulated calls exhibited a continuous increase in pitch that was ≥12.5 kHz, with a terminal dominant frequency at ≥6.25 kHz more than the pitch at the beginning of the vocalization; 4) Downward-modulated calls exhibited a continuous decrease in pitch that was ≥12.5 kHz, with a terminal dominant frequency at ≤6.25 kHz less than the pitch at the beginning of the vocalization; 5) Chevron calls resembled an ‘inverted-U’, which was identified by a continuous increase in pitch ≥12.5 kHz followed by a decrease that was ≥6.25 kHz; 6) Short calls were punctuated and shorter than 5 ms; 7) Composite calls were formed by two harmonically independent components, emitted simultaneously; 8) Frequency steps were instantaneous frequency changes appearing as a vertically discontinuous “step” on a spectrogram,
but with no interruption in time; 9) Flat calls displayed a constant
beginning and the ending of the pitch frequency remained
constant (±3 kHz of each other).

Call category data were subjected to two different analyses: a)
genotype dependent effects on the frequency of vocalizations
emitted by each subject at pnd 2, 4, 6, 8, 12; b) genotype-
dependent effects on the probability of producing calls from each
of the nine categories of USV, as described below under Statistical
analysis.

Righting reflex

After each USV recording session, each pup was placed on its
back over a flat surface, and the time needed to return to the
natural position (all four paws on the floor) was measured. This
reflex requires complex coordination between head, trunk, and
paws. The reflex was tested once with a cut-off latency of 60 s.
Latencies were measured in seconds, using a stopwatch for
righting reflex.

Spontaneous movements

Concomitant with the vocalizations recording on pnd 2, 4, 6, 8
and 12, mouse pups were also videorecorded for analysis of
spontaneous movements. Frequency and duration of behavioral
items were analyzed by an observer blind to mouse genotype. We
used the NOLDUS OBSERVER software V 10XT (Noldus
Information Technology, Wageningen, NL, USA) to score the
videotapes. In accordance with previous studies focused on
neonatal rodent behavior [53–56], the following behavioral
dimensions were scored: locomotion (general translocation of the
body of at least 1 cm in the glass container), immobility (no visible
movement of the animal when placed with all the four paws on the
floor), side (no visible movement of the animal when laying on the
side), head raising (a single rising of the head up and forward), head
shaking (a single lateral displacement of the head), face washing
(forepaws moving back and forth from the ears to the nose and
mouth), wall climbing (alternating forelimb placing movements on
the wall of the container), nose probing (pushing the snout against
floor or walls of the apparatus), pivoting (locomotor activity
involving the front legs alone and resulting in laterally directed
movements), circling (circular locomotor activity involving the all
legs and resulting in laterally directed movements), and curling
(roll, vigorous side-to-side rolling movements while on the back;
curl, a convex arching of back while on side or back, bringing head
in a closer opposition to hump/hindlimb region).

Statistical analysis

A mixed-model Analysis of Variance (ANOVA) with Repeated
Measures was performed to analyze genotype-dependent effects on
neonatal USVs and spontaneous movement responses, with the
genotype (Wt vs Het vs Rl) as factor and pnd or call categories as
the repeated measures. Probability of vocalizations within strain
was calculated as number of calls in each category for each
subject/total number of calls analyzed in each subject and
standardized by angular transformation. Since no sex differences
were detected, data were collapsed across sex. Post-hoc compar-
isons were performed using Tukey HSD Test only when a
significant F-value was determined. For all comparisons, signifi-
cance was set at P = 0.05.

Results

Body weight gain

Body weight did not differ between genotypes [genotype:
F(2,73) = 1.00, ns; genotype x day: F(8,292) = 1.603, ns] and, as
expected, progressively increased with age [F(4,292) = 232.12;
p<0.0001] (data not shown).

Pup separation vocalizations

Changes of ultrasonic vocalizations (USVs) over time were
characterized by an inverted U-shape across the first 12 postnatal
days of age, as confirmed by the main effect of day [day: (F
(4,292) = 26.412, p<0.0001] (Figure 1A). Wildtype pups showed
a peak of emission at pnd 4, while it was slightly delayed to pnd 6 in
Het pups. By contrast, the profile of emission in RI mice appeared
quite flat, without a peak, across the five days of testing. Wildtype
and Het pups emitted same number of calls at pnd 4. Total
number of vocalizations varied across genotypes [F(2,73) = 5.601,
p<0.005]. In particular, Het emitted a significantly higher
number of calls than Wt and RI mice. No differences in genotype
were found for mean duration, peak frequency and peak
amplitude of USVs [duration: F(2,73) = 2.575, ns; peak frequency:
(F(2,73) = 0.459, ns; peak amplitude: (F(2,73) = 0.041, ns]. As
shown in Figure 1B, Het pups emitted USVs for longer time than
the other genotypes [F(2,73) = 5.042, p<0.05]. (Figure 1B).

No differences were detected in body temperatures of RI, Het
and Wt as measured after each separation test [genotype:
(F(2,73) = 0.269, ns; genotype x day: F(8,292) = 0.790, ns], (data
not shown).

Classification of ultrasonic vocalizations into distinct
categories

Figure 2A illustrates genotype-dependent variation on frequen-
cy of calls at pnd 2 [F (2,73) = 5.55, p<0.005]. Het and RI pups
emitted a significantly higher number of two-component calls than
Wt pups [p<0.05 after post hoc comparison performed on
frequency x calls subtype interaction: F (16,584) = 2.39, p<0.005].
As illustrated in Figure 2B, a genotype-dependent effect has been
found also at pnd 6 [genotype: (F (2,73) = 2.96, p = <0.05], with
Het emitting a significantly higher number of two-component calls
than the other genotypes [genotype x calls subtype interaction: F
(16,584) = 2.57, p<0.0001]. No differences among genotypes were
evidenced on other subtypes of calls later on in development (see
Figure S3).

Pattern of sonographic structure among genotypes

Proportions of calls within each category are shown in Figure 3.
Wt, Het and RI pups emitted a wide spectrum of call categories. At
pnd 2, Wt pups mainly emitted chevron, complex, downward, flat
and two-component calls, along with low prevalence in production of
upward, composite, frequency steps and short calls. On pnd 4, Wt pups
showed a decrease in the emission of the complex calls in favor of
the flat calls. On pnd 6, as well as on pnd 8, a reduction of the flat
and downward calls appeared in favor of two-component and short calls.
This profile appeared even more pronounced on pnd 12, with
general vocal repertoire reduced and mainly defined by the
concomitant presence of two types of calls: two-components and
shorts. Het and RI pups (see second and third column), already on
pnd 2, exhibited a differential vocal repertoire in comparison to
Wt controls. Indeed, mice of both mutant genotypes emitted a
high proportion of two-component calls and a low proportion of flat
calls, whereas other types of vocalizations remain unchanged. This
profile was still there on pnd 4. The data on pnd 6 indicate that the
vocal repertoire of Het and RI pups was represented by 50% of
two-component calls. This profile was still present on pnd 8, then
reducing later on. In fact, on pnd 12, Het pups still persisted in
emitting primarily the complex and two-component calls, while Wt
emission was characterized by a prevalence of two-component and
short calls. At this age, RI pups showed a vocal repertoire characterized by the two-component, short and complex calls, indicative of a phenotype halfway between Wt and Het pups (see exemplary sonograms in Figure 4).

Proportions of calls within genotype and sex are shown in Figures S1 and S2.

When analyzing each USV category separately on different postnatal days (see Figure 5, Panels A, B, C, D, E), a main effect of genotype was found for the probability of producing different categories of calls (this index is obtained by calculating the number of calls in each category for each subject/total number of calls analyzed for each subject): [two-components], pnd 6: F(2,70) = 3.692, p < 0.05; short, pnd 6: F(2,70) = 3.328, p < 0.05; flat, pnd 6: F(2,70) = 3.573, p < 0.05; upward, pnd 12: F(2,35) = 3.367, p < 0.05; complex, pnd 12: F(2,35) = 7.845, p < 0.001]. Specifically, on pnd 6, Wt pups emitted less two-component calls than RI pups, more short calls than Het and RI pups, and more flat calls than RI pups (post hoc, p < 0.05). At pnd 12, Het pups emitted more complex and upward calls than Wt subjects (post hoc, p < 0.05).

Righting reflex

The righting reflex, measured as latency to turn back onto all four paws when placed on the back [57], was tested at pnd 2 to 12 after the USV and spontaneous movements recording session. As expected, righting reflex latencies differed significantly across pnd [F(4,204) = 24.290, p < 0.0001], with all genotypes reaching the full development of the reflex on pnd 12. Post hoc comparisons performed on the two way interaction genotype x day [F(8,204) = 2.241, p < 0.05] indicated that Het spent more time to turn their body than Wt and RI pups on pnd 2 (p < 0.01) (see Figure 6A).

Analysis of spontaneous movements

Curling behavior is measured by time spent from pups rolling on the back. This behavior is associated to the development of the righting reflex. Curling duration rapidly decreased across the five days of observation and disappeared on pnd 12 [main effect of age: F(3,219) = 26.238, p < 0.0001]. A main effect of genotype was found [F(2,73) = 11.151; p < 0.0001]. Post hoc comparisons performed on the interaction genotype x days of testing [F(6,219) = 8.149; p < 0.0001] showed that curling durations in Het and RI mice were longer than Wt on pnd 2 (p < 0.05), and persisted longer in RI mice on pnd 4 (p < 0.05), thus suggesting a motor coordination deficit in Het and RI pups in getting an upright position (see Figure 6B).

Face washing requires coordination and equilibrium since the pup is standing up on its hindlimb and washing its face with forelimbs. For this reason, it was exhibited and analyzed only at day 12 of observation. A main effect of genotype was found for frequency and duration of face washing [frequency: F(2,73) = 4.726; p < 0.05; duration: F(2,73) = 5.822; p < 0.005], with RI pups being significantly much less involved than Wt and Het subjects, (see Figure 6C).

A similar profile was observed for frequency and duration of wall climbing, as confirmed by a significant genotype x day interaction [F(6,219) = 2.899; p < 0.05; F(6,219) = 2.692; p < 0.05, respectively], (see Figure 6D). Post hoc comparisons showed that, on pnd 12, RI pups spent consistently less time climbing the walls of the glass container than Wt and Het pups (P < 0.05).

Time spent in locomotion rapidly increased across the five days of observation [F(4,292) = 102.346; p < 0.0001]. Post hoc comparisons performed on the interaction genotype x time intervals [F(8,292) = 2.767; p < 0.05] revealed that RI mice were more active than Wt and Het mice on pnd 12 (P < 0.05), (see Figure 7A).

Analysis of frequency of circling behavior yielded a significant main effect of genotype [F(2,73) = 3.972; p < 0.05], with RI mice exhibiting a lower number of circling episodes than the other genotypes at all days of testing (see Figure 7B). A similar profile was evident for duration of circling but the genotype effect just missed the statistical significance [F(2,73) = 2.393; P = 0.098].

ANOVA performed on frequency of nose probing detected a main effect of genotype [F(2,73) = 4.017; p < 0.05], with RI mice performing less nose probing than Wt and Het pups (see Figure 7C). In general, the profile of frequency of nose probing exhibited by Wt and Het pups was characterized by an inverted U-shape profile across the five days of observation, with a peak on pnd 6 [time intervals: F(4,292) = 7.526, p < 0.0001]. Instead, the nose probing profile of RI pups appeared delayed in time and showed its peak on pnd 8 (see Figure 7C).

A main effect of age was found for head shaking [frequency: F(4,292) = 14.551; p < 0.0001; duration: F(4,292) = 7.388; p < 0.0001]. Post hoc comparisons performed on the genotype x time intervals interaction [frequency: F(8,292) = 2.767; p < 0.05; duration: F(2,73) = 2.941; p < 0.05] revealed that RI pups spent
significantly less time in shaking their heads than Wt on pnd 2 \((p<0.05)\) and less time than Wt and Het littermates on pnd 4 \((p<0.05)\), (see Figure 7D).

No differences between genotypes were observed for head raising, side, pivoting, and immobility behaviors (data not shown).

**Discussion**

Reelin is a glycoprotein playing a role in regulating neuronal migration and brain lamination during development. The reduced or complete lack of reelin signaling impairs neuronal connectivity...
Figure 3. Pie graphs show the percentages of the different call categories for the three genotypes, Wt, Het, RI during five days of testing (pnd 2, 4, 6, 8, 12). Percentages were calculated in each genotype as number of calls in each category for each subject/total number of calls analyzed for each subject. Number of total calls analyzed at pnd 2: Wt = 1823; Het = 8301; RI = 2776. Pnd 4: Wt = 4064; Het = 10015; RI = 2708. Pnd 6: Wt = 3522; Het = 11642; RI = 3017. Pnd 8: Wt = 2511; Het = 6613; RI = 1603. Pnd 12: Wt = 705; Het = 3692; RI = 614.

doi:10.1371/journal.pone.0064407.g003
and synaptic plasticity leading ultimately to the cognitive deficits present in autism and schizophrenia. So far, limited studies have investigated the contribution of reelin deficiency to the establishment of the early motor and social/communicative deficits present in these neurodevelopmental disorders [6,80]. To this aim, the present study provides, for the first time, a fine-grain characterization of neonatal vocal and motor repertoires in reelin mutant mice, a genetic line widely used as animal model of autism and schizophrenia. In rodent models of neurodevelopmental disorders, it is critical to conduct behavioral phenotyping during the early developmental period in order to document the precise onset of symptoms, identify transient signs, and provide a basis for the timing of early intervention [58,59]. Moreover, performing experiments during the first two postnatal weeks allowed us to include the study also the homozygous reeler mouse, presenting an impaired phenotype generally leading to death shortly after weaning. The inclusion of RI mice is crucial for the assessment of dose-dependent effects in genetic vulnerability [6].

Heterozygous reeler pups separated from their mothers and siblings at pnd 2, 4, 6, 8 and 12 emitted significantly more calls and for longer time than their wildtype and homozygous littersmates. Moreover, a different profile of emission was detected in Het and RI mice in comparison to wildtype pups. In fact, while wildtype pups showed a peak of emission at pnd 4, Het’s peak of emission was slightly postponed to pnd 6, and profile of emission in RI mice appeared quite flat, without a peak, indicative of a potential delay in the emotional/communicative development in this mutant line. No differences were detected in body temperature upon USVs recording excluding the possibility of unusual thermoregulation in reeler mice. An increased number of USVs has been detected also in other animal models of autism such as Btbr T<sup>+<i>+</i></sup>tf/J, Shank2<sup>−<i>−</i></sup> and MeCP2 null mice, Tsc1<sup>+/−</sup> and Tsc1<sup>−<i>−</i></sup> conditional knockouts and mice with a chromosome 15q11–13 maternal deletion or a paternal duplication [37,46,60–64].

Our USV data are partially in contrast with previous data collected in this model [38] showing a reduced number of USV in Het and RI pups as compared to wildtype pups at pnd 7. Important differences in the experimental procedures, such as room temperature, may be implicated. In fact, the temperature of our experimental room was maintained at 22°C, while in Laviola’s study [38] the temperature was set at 26°C. Body temperature strongly affects ultrasonic vocalization emission, with higher temperature reducing the number of vocalizations [65]. Moreover, Laviola and colleagues measured ultrasonic vocalizations by a bat-detector tuned between 40 kHz and 60 kHz and were able to detect only calls within this range. Indeed, in our technologically improved and updated experimental setting, we have been able to detect any vocalization emitted in the frequency range of 10–180 kHz resulting in an increasing number of vocalizations recorded in each genotype.

We further investigated the specific types of calls emitted by the three genotypes at all days of testing. Specifically, according to our previous paper on pup vocalizations [37], we classified waveform patterns into nine categories of calls designated as complex, composite, downward, flat, frequency steps, short, two-components, upward and chevron. To our knowledge, this is the first report about a detailed analysis of pup vocal repertoire throughout the first two postnatal weeks of age. Aim of this deeper investigation was to detect subtle alterations in normative developmental aspects that could be potentially transferred to human studies for early detection of ASD [66]. Analysis of the vocal repertoire revealed a significant difference between genotypes in the use of specific categories. Wildtype pups, at pnd 2, emitted mainly 5 out of 9 categories (chevron, complex, downward, flat and two-components) while at pnd 12 their vocal repertoire appeared more limited (64%) and narrowed to two categories of vocalizations, such as two-component and short calls, and to a low number (29%) of chevron, complex and downward calls. This analysis allowed us to evaluate the development of the vocal profile of wildtype mice from a neonatal to a more adult-like repertoire. In fact, the call distributions showed by wildtype pups at pnd 12 are similar to what B6 males and females emitted in adult social contexts [67]. Het and RI pups emitted a restricted repertoire of calls at pnd 6 and 8 in comparison to Wt pups, limiting their vocal repertoire to the two-component (about 50%), chevron (about 20%) and complex calls (13%). At pnd 12, Het pups still persisted in emitting primarily the complex and two-component calls, while RI pups showed a vocal repertoire characterized by the two-component, short and complex calls, indicative of a phenotype halfway between Wt and Het pups. As a whole, from a detailed analysis of vocalizations in Het and RI pups, it was possible to detect a delay in reaching the peak-day of emission (6th vs 4th day), associated to a delay in reaching a more adult-like vocal profile. Altogether, these findings confirm and extend the presence in reelin mutant mice of quantitative and qualitative alterations in USVs already in a neonatal phase [38], indicative of the delay on the emotional and communicative development comparable to what has been found in children with ASD [68,69].

A number of deviations from normative motor development has been highlighted in Het and RI mice. When tested during the first 12 days of life, mutant mice exhibited longer curling and longer latency to right themselves when placed on their backs than Wt littermates. These two responses are strongly associated with each other, curling is a vigorous side to side rolling movement while on...
Neonatal Characterization of Reelin Mutant Mice

A  Two-components, pnd 6

B  Short, pnd 6

C  Flat, pnd 6

D  Complex, pnd 12

E  Upward, pnd 12
the back and it is aimed at righting. Moreover, RI pups showed a
deficit in face washing and wall climbing at pnd 12 as compared to
their Wt and Het littermates. Both these behaviors require
coordination and equilibrium since pup is standing up on its
hindlimb and washing its face or climbing the walls of the
container with forelimbs. Altogether these data suggested the
presence of a motor coordination deficit in mutant mice supported
by the fact that, in the first weeks of life, mutant mice show
progressive loss of Purkinje cells of the cerebellum [70,71], a brain
area that plays an important role in fine motor coordination. A
genotype-induced delay in the behavioral development of RI mice
is also suggested by a decrease in circling, nose probing and head
shaking behaviors and by the hyperactivity profile displayed by
mutant pups on pnd 12. As a whole, persistence of immature
motor behavioral responses characterizes the RI pups. Such motor
profile seems in line with the transient delays in sensory–motor
development shown by other animal models of ASD such as
Mecp2-null, Foxp2, and BTBR mice [37,46,47,56,72]. Early
neurological abnormalities found in RI and Het mice are
particularly intriguing, in view of subtle alterations in infantile
reflexes (such as body righting or head tilting) recently described in
infants later diagnosed for autism [73]. Studies on family videos
provided by parents of children later diagnosed as ASD have
highlighted the presence of both subtle deficits and pre-regression
developmental delays during the first months of life [74–76].
Infants with ASD had more often poor repertoire writhing general
movements as well as abnormal or absent fidgety movements than
control infants. Prospective studies performed on infant siblings of

Figure 5. Production of calls within genotype. Probability of producing calls from each of the nine categories of USV. * Data were expressed by
angular transformation. A) Two-component calls at pnd 6, B) Short calls at pnd 6, C) Flat calls at pnd 6, D) Complex calls at pnd 12, and E) Upward calls
at pnd 12. *p<0.05.
doi:10.1371/journal.pone.0064407.g005

Figure 6. Latency and/or duration of behavioral patterns shown by Wt, Het and RI pups on pnd 2, 4, 6, 8 and 12 during a 3-min
session. See methods for full description of neonatal behaviors. A) Righting reflex latencies. Pups acquired the righting reflex response at different
rates, with Wt and RI showing shorter latencies than Het at pnd 2. B) Curling, C) Face washing and D) Wall climbing. Data are expressed as mean ±
SEM. ** p<0.01 and *p<0.05; N = 17 Wt, 43 Het, 16 RI pups.
doi:10.1371/journal.pone.0064407.g006
children with ASD evidenced early motor delay such as poor motor coordination, abnormal postural control and atypical movements [77–80].

From our results, it appears that Het and Rl mice showed delay in the vocal and motor development in line with the alterations in these two systems seen in children with ASD and considered early warning signs of ASD. It is worth to notice that in terms of gene-dosing the deficiency of reelin in Het pups led to an alteration mainly in the vocal emission while the total lack of reelin in Rl pups primarily induce alterations at motor coordination level. Motor deficits present in Rl pups at the end of the second postnatal week could be the first signs of the cerebellar alteration generally leading to death shortly after weaning.

Altogether, our results emphasize the importance of conducting behavioral phenotyping during the early developmental period in order to document the precise onset of symptoms, to identify transient signs and provide a basis for the timing of early intervention. In conclusion, our data confirm that reelin mutant mice represent an interesting animal model for behavioral abnormalities seen in neurodevelopmental disorders like autism and schizophrenia [6,81] and highlight the importance of reelin during development.

Supporting Information

Figure S1 Pie graphs show the percentages of the different call categories for the three genotypes, (Wt, Het, Rl) in male pups during five days of testing (pnd 2, 4, 6, 8, 12).

Figure S2 Pie graphs show the percentages of the different call categories for the three genotypes, (Wt, Het, Rl) in female pups during five days of testing (pnd 2, 4, 6, 8, 12).

Figure S3 Production of ultrasonic vocalizations by call category. A) Frequency of ultrasonic vocalizations at pnd 4, 8 and 12. B) pnd 8 and C) pnd 12.

Materials S1

Acknowledgments

We are grateful to Luigia Cancemi and Giovanni Dominici for animal care.
Author Contributions

Conceived and designed the experiments: MLS GL. Performed the experiments: ER CM. Analyzed the data: ER CM AC. Wrote the paper: ER MLS.

References

1. Guidotti A, Auta J, Davis JM, Di-Giorgi-Gerevini V, Dwivedi Y, et al. (2000) Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. Arch Gen Psychiatry 57: 1061–1069.

2. Impagnatiello F, Guidotti AR, Pesold C, Dwivedi Y, Caruncho H, et al. (1998) A decrease of reelin expression as a putative vulnerability factor in schizophrenia. Proc Natl Acad Sci U S A 95: 15718–15723.

3. Hong SE, Shah RT, Huang DT, Shahwan SA, Grant PE, et al. (2000) Autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human RELN mutations. Nat Genet 26: 93–96.

4. Fatemi SH, Story JM, Halt AR, Realmuto GR (2001) Dysregulation of Reelin in Bel-2 proteins in autistic cerebellum. J Autism Dev Disord 31: 529–535.

5. Keller F, Persico AM (2003) The neurobiological context of autism. Mol Neurobiol 28: 1–22.

6. Lavidya G, Ogubene E, Romano E, Adriani W, Keller F (2009) Gene-environment interaction during early development in the heterozygous reeler mouse analysis. J Comp Neurol 494: 498–505.

7. Folsom TD, Fatemi SH (2012) The involvement of Reelin in neurodevelopmental disorders. Neuropharmacology.

8. Miroslav I (2010) Reelin-mediated signaling in neuropsychiatric and neurodegenerative diseases. Prog Neurobiol 91: 257–274.

9. Costa E, Chen Y, Davis J, Dong E, Noh JS, et al. (2002) REELIN and GAD67 Gene Expression in Brain of Heterozygous Reeler Mice. J Comp Neurol 148: 141–151.

10. Costa E, Davis J, Grayson DR, Guidotti A, Guidotti F, et al. (2001) Dendritic spine hypoplasticity and downregulation of reelin and GABAAergic tone in schizophrenia vulnerability. Neurobiol Dis 8: 723–742.

11. Niu S, Renfoot A, Quaascharla CC, Sheldon M, D’Arcangelo G (2004) Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dabl pathway. Neuroreport 41: 71–84.

12. Tissir F, Goiffnet AM (2003) Reelin and brain development. Nat Rev Neurosci 4: 98–115.

13. Wever EF, Bellet PV, Jones C, Christian J, Forster E, et al. (2002) Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. J Biol Chem 277: 39944–39952.

14. Mariani J, Crepel F, Mikoshiba K, Changeux JP, Sotelo C (1977) Anatomical, physiological and histochemical studies of the cerebellum from Reeler mutant mouse. Philos Trans R Soc Lond B Biol Sci 281: 1–28.

15. Caviness VS Jr., Siedman RL (1973) Time of origin or corresponding cell classes in the cerebral cortex of normal and reeler mutant mice: an autoradiographic study. J Comp Neurol 148: 141–151.

16. D’Arcangelo G, Curran T (1998) Reeler: new tales on an old mutant mouse. Bioessays 20: 233–244.

17. Falconer D (1951) Two new mutants, ‘trembler’ and ‘reeler’, with neurological actions in the house mouse (Mus musculus L.). Journal of Genetics 50: 192–205.

18. Geissler DB, Ehret G (2002) Time-critical integration of formants for perception of communication calls in mice. J Comp Neurol 148: 137–139.

19. Cohen-Salmon C, Carlier M, Roubertoux P, Jouhaneau J, Semal C, et al. (1985) Differences in patterns of pup care in mice. V–Pup ultrasonic emissions and pup care behavior. Physiol Behav 35: 167–174.

20. Geisler DB, Ehret G (2002) Time-critical integration of forms for perception of communication calls in mice. Proc Natl Acad Sci U S A 99: 9021–9025.

21. Noirot E (1972) Ultrasounds and maternal behavior in small rodents. Dev Psychobiol 5: 371–377.

22. Smotherman WP, Bell RW, Starzeck J, Elias J, Zachman TA (1974) Maternal care behavior. Physiol Behav 35: 167–174.

23. Geisler DB, Ehret G (2002) Time-critical integration of forms for perception of communication calls in mice. Proc Natl Acad Sci U S A 99: 9021–9025.

24. Noirot E (1972) Ultrasounds and maternal behavior in small rodents. Dev Psychobiol 5: 371–377.

25. Scattoni ML, Gandhy SU, Riceri L, Crawley JN (2008) Unusual repertoire of vocalizations in the ETBR Tm(1)J mouse model of autism. PLoS One 3: e3067.

26. Lavidya G, Adriani W, Gaudino C, Marino R, Keller F (2008) Paradoxical effect of prenatal acetylcholinesterase blockade on neuro-behavioral development and drug-induced stereotypes in reeler mutant mice. Psychopharmacology (Berl) 187: 331–344.

27. Silverman JL, Yang M, Lord C, Crawley JN (2010) Behavioural phenotyping assays for mouse models of autism. Nat Rev Neurosci 11: 490–502.

28. Cohen-Salmon C, Carlier M, Roubertoux P, Jouhaneau J, Semal C, et al. (1985) Differences in patterns of pup care in mice. V–Pup ultrasonic emissions and pup care behavior. Physiol Behav 35: 167–174.

29. Geisler DB, Ehret G (2002) Time-critical integration of forms for perception of communication calls in mice. Proc Natl Acad Sci U S A 99: 9021–9025.

30. Noirot E (1972) Ultrasounds and maternal behavior in small rodents. Dev Psychobiol 5: 371–377.

31. Smotherman WP, Bell RW, Starzeck J, Elias J, Zachman TA (1974) Maternal care behavior. Physiol Behav 35: 167–174.

32. Dutta S, Gangopadhyay PK, Sinha S, Chatterjee A, Ghosh S, et al. (2011) An association analysis of reelin (RELN) polymorphisms with childhood epilepsy in eastern Indian population from West Bengal. Cell Mol Neurobiol 31: 45–56.

33. Kelemenova S, Schmitthova E, Ficek A, Cecele P, Kubranksa A, et al. (2010) Polymorphisms of candidate genes in Slovak autistic patients. Psychiatr Genet 20: 137–139.

34. Persico AM, D’Agruma L, Maieronaro N, Tortaro A, Milertin R, et al. (2001) Reelin gene alleles and haplotypes as a factor predisposing to autistic disorder. Mol Psychiatry 6: 150–159.

35. Zhao D, Zhao X, Zang C, Mundo E, Macciardi F, et al. (2002) Reelin gene alleles and susceptibility to autism spectrum disorders. Mol Psychiatry 7: 1012–1017.

36. Scattoni ML, Gandhy SU, Riceri L, Crawley JN (2008) Unusual repertoire of vocalizations in the ETBR Tm(1)J mouse model of autism. PLoS One 3: e3067.

37. Lavidya G, Adriani W, Gaudino C, Marino R, Keller F (2008) Paradoxical effect of prenatal acetylcholinesterase blockade on neuro-behavioral development and drug-induced stereotypes in reeler mutant mice. Psychopharmacology (Berl) 187: 331–344.

38. Silverman JL, Yang M, Lord C, Crawley JN (2010) Behavioural phenotyping assays for mouse models of autism. Nat Rev Neurosci 11: 490–502.

39. Cohen-Salmon C, Carlier M, Roubertoux P, Jouhaneau J, Semal C, et al. (1985) Differences in patterns of pup care in mice. V–Pup ultrasonic emissions and pup care behavior. Physiol Behav 35: 167–174.

40. Geisler DB, Ehret G (2002) Time-critical integration of forms for perception of communication calls in mice. Proc Natl Acad Sci U S A 99: 9021–9025.

41. Noirot E (1972) Ultrasounds and maternal behavior in small rodents. Dev Psychobiol 5: 371–377.

42. Smotherman WP, Bell RW, Starzeck J, Elias J, Zachman TA (1974) Maternal care behavior. Physiol Behav 35: 167–174.

43. Geisler DB, Ehret G (2002) Time-critical integration of forms for perception of communication calls in mice. Proc Natl Acad Sci U S A 99: 9021–9025.

44. Noirot E (1972) Ultrasounds and maternal behavior in small rodents. Dev Psychobiol 5: 371–377.

45. Cohen-Salmon C, Carlier M, Roubertoux P, Jouhaneau J, Semal C, et al. (1985) Differences in patterns of pup care in mice. V–Pup ultrasonic emissions and pup care behavior. Physiol Behav 35: 167–174.
55. Spear LP, Penson J, Linville DG (1986) GABA and behavioral inhibition in the neonatal rat pup. Psychopharmacology (Berl) 90: 106–111.
56. De Filippis B, Ricceri L, Laviola G (2010) Early postnatal behavioral changes in the Mecp2-308 truncation mouse model of Rett syndrome. Genes Brain Behav 9: 213–223.
57. Fox VM (1965) Reflex-ontogeny and behavioural development of the mouse. Anim Behav 13: 234–241.
58. Branche I, Bichler Z, Berger-Sweeney J, Ricceri L (2003) Animal models of mental retardation: from gene to cognitive function. Neurorsci Biobehav Rev 27: 141–153.
59. Branche I, Ricceri L (2002) Transgenic and knock-out mouse pups: the growing need for behavioral analysis. Genes Brain Behav 1: 133–141.
60. Jiang YH, Pan Y, Zhu L, Landa L, Yoo J, et al. (2010) Altered ultrasonic vocalization and impaired learning and memory in Angelman syndrome mouse model with a large maternal deletion from Ube3a to Gabrb3. PLoS One 5: e12278.
61. Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, et al. (2009) Abnormal behavior in a chromosome-engineered mouse model for human 15q11–13 duplication seen in autism. Cell 137: 1235–1246.
62. Ricceri L, Cutuli D, Venerosi A, Scattoni ML, Calamandrei G (2007) Neonatal basal forebrain cholinergic hypofunction affects ultrasonic vocalizations and fear conditioning responses in preweaning rats. Behav Brain Res 183: 111–117.
63. Schmeisser MJ, Ey E, Wegener S, Bockmann J, Stempel AV, et al. (2012) Autistic-like behaviours and hyperactivity in mice lacking ProSAP1/Shank2. Nature 486: 256–260.
64. Won H, Lee HR, Gee HY, Mah W, Kim JL, et al. (2012) Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function. Nature 486: 261–265.
65. Shair RN, Brunelli SA, Masmela JR, Boone E, Hofer MA (2003) Social, thermal, and temporal influences on isolation-induced and maternally potentiated ultrasonic vocalizations of rat pups. Dev Psychobiol 42: 206–222.
66. Lahvis GP, Alleve E, Scattoni ML (2011) Translating mouse vocalizations: prosody and frequency modulation. Genes Brain Behav 10: 4–16.
67. Scattoni ML, Ricceri L, Crawley JN (2011) Unusual repertoire of vocalizations in adult BTBR T+tf/J mice during three types of social encounters. Genes Brain Behav 10: 44–56.
68. Silani G, Bird G, Brindley R, Singer T, Frith C, et al. (2008) Levels of emotional awareness and autism: an fMRI study. Soc Neurosci 3: 97–112.
69. Smith A (2009) The Empathy Imbalance Hypothesis of Autism: A Theoretical Approach to Cognitive and Emotional Empathy in Autistic Development. Psychol Record 59: 489–510.
70. Diamonte F, Assenza G, Marino R, D’Amelio M, Panteri R, et al. (2009) Interactions between neuroactive steroids and reelin haploinsufficiency in Purkinje cell survival. Neurobiol Dis 36: 103–113.
71. Hadj-Sahraoui N, Frederic F, Delhaye-Bouchaud N, Mariani J (1996) Gender effect on Purkinje cell loss in the cerebellum of the heterozygous reeler mouse. J Neurogenet 11: 45–50.
72. Santos M, Silva-Fernandes A, Oliveira P, Sousa N, Maciel P (2007) Evidence for abnormal early development in a mouse model of Rett syndrome. Genes Brain Behav 6: 277–286.
73. Teitelbaum O, Benton T, Shah PK, Prince A, Kelly JL, et al. (2004) Ehhkol-Wachman movement notation in diagnosis: the early detection of Asperger’s syndrome. Proc Natl Acad Sci U S A 101: 11909–11914.
74. Espostio G, Venuti P, Apicella F, Marutari F (2011) Analysis of unsupported gait in toddlers with autism. Brain Dev 33: 367–373.
75. Maestro S, Muratori F, Cesari A, Pecini A, Apicella F, et al. (2006) A view to regressve autism through home movies. Is early development really normal? Acta Psychiatr Scand 113: 68–72.
76. Phagava H, Muratori F, Einspieler C, Maestro S, Apicella F, et al. (2008) General movements in infants with autism spectrum disorders. Georgian Med News: 100–105.
77. Bhat AN, Galloway JC, Landa RJ (2012) Relation between early motor delay and later communication delay in infants at risk for autism. Infant Behav Dev 35: 838–846.
78. Bryson SE, Zwagenbaum L, Brian J, Roberts W, Szatmari P, et al. (2007) A prospective case series of high-risk infants who developed autism. J Autism Dev Disord 37: 12–24.
79. Flanagan J, Landa R, Bhat A, Bauman M (2007) Head Lag in Infants at Risk for Autism: A Preliminary Study. The American Journal of Occupational Therapy.
80. Zwagenbaum L, Bryson S, Rogers T, Roberts W, Brian J, et al. (2005) Behavioral manifestations of autism in the first year of life. Int J Dev Neurosci 23: 143–152.
81. Tordjman S, Drapier D, Bounot O, Graigner R, Fortes S, et al. (2007) Animal models relevant to schizophrenia and autism: validity and limitations. Behav Genet 37: 61–78.