Lurcher mice exhibit potentiation of GABA$_A$-receptor mediated conductance in cerebellar nuclei neurons in close temporal relationship to Purkinje cell death

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Running head:
GABAergic transmission in Lurcher cerebellum

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Abstract

In heterozygous Lurcher mice (Lc/+), the Purkinje cells (PCs) degenerate almost totally during postnatal development. On the other hand, their projection target, the deep cerebellar nuclei (DCN), shows little signs of degeneration and seems to play an important role in maintaining a residual cerebellar function in Lc/+.

We asked if the DCN in Lc/+ develop cellular adaptations allowing them to cope with the loss of GABAergic PC input. Using whole-cell patch-clamp recordings, we measured IPSCs from DCN of Lc/+ and wild-type mice (WT). In experiments on phenotypically striking Lc/+ studied well after the onset of the PC degeneration, we found enlarged average synaptic conductances (g_{syn}) compared to WT. We next investigated postnatal mice before and after the onset of PC death. In younger animals ≤ postnatal day (p) 13 no difference was found in g_{syn} between the two groups. At p14, g_{syn} in Lc/+ showed an increase, while those in WT stayed on the level found in younger animals. A peak-scaled non-stationary fluctuation analysis suggests that an increase in the average number of channels open at peak is the basis for the change in g_{syn}. The changes in g_{syn}, suitable to increase the efficacy of GABAergic transmission, occur in close temporal relationship to PC death and, thus, may reflect a functional adaptation to the loss of the DCN’s main GABAergic afferents.
Introduction

The GABAergic PC projection onto the DCN undergoes a degeneration in the spontaneous, semidominant mouse mutation *Lurcher* (Phillips 1960). Homozygous mutants (*Lc/Lc*) die shortly after birth. Heterozygous mice (*Lc/+*) become ataxic as a result of an apoptotic death of cerebellar PCs during postnatal development (Caddy and Biscoe 1979; Wets and Herrup 1982). In *Lc/+*, the first signs of PC degeneration are observed at p8 (Dumesnil-Bousez and Sotelo 1992) and by p26, about 90% of PCs have disappeared (Caddy and Biscoe 1979). On the other hand, the majority of DCN neurons survive, although deprived of their inhibitory Purkinje cell input (Heckroth 1994; Sultan et al. 2002). In view of the almost total disruption of cerebellocortical signal processing, the ataxia in *Lc/+* is surprisingly mild and even some degree of sensorimotor learning is retained (Lalonde et al. 1996). The ataxia, however, is substantially aggravated by lesioning the DCN (Caston et al. 1995). Hence, the DCN seem to play a beneficial role for the motor performance of *Lc/+*. We asked if there are any cellular adaptations in the DCN of *Lc/+* allowing them to compensate for the functional consequences of the massive denervation. To this end, we investigated the properties of IPSCs recorded in DCN of mutants in comparison to WT during postnatal development before and after the onset of the PC degeneration.


Methods

Animals and molecular genotyping

Heterozygous B6CBACa-Aw-J/A-Lc (Jackson Laboratories, Bar Harbor, ME, USA) were mated with B6CBA (Charles River, Sulzfeld, Germany) mice. B6CBA mice were also used as wild-type controls. Animals were kept and used in experiments according to the institutional and national animal care guidelines (also conforming with NIH guidelines on animal care). Animals from the Lurcher progeny not showing the characteristic Lc/+ phenotype (ataxic gait and tendency to fall) were genotyped. The genomic DNA was isolated (DNeasy Tissue Kit, Qiagen, Hilden, Germany). Primers (MWG-Biotech, Ebersberg, Germany) 5'-TAAAAGCATATTGATGTTGTTG-3' or 5'-GCACTGAATGTGTATGACTTCAG-3' and 5'-CAGCATTTGTCAGGTTTGGTGAC-3' (Zuo et al. 1997) were used in PCR to amplify the region of interest including a guanine to adenine transition at nucleotide position 1960 in exon B of the mouse δ2 glutamate receptor gene. The PCR product was purified (CONCERT Rapid PCR Purification System, Gibco BRL, Rockville, MD, USA) and sequenced employing the big dye terminator chemistry (PE Biosystems, Weiterstadt, Germany). The sequences were analyzed with an automated 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the fluorescent dideoxynucleotide technology and evaluated using Lasergene software (Dnastar, Madison, WI, USA).

Electrophysiology and data analysis

The mice were deeply anesthetized by application of ketamine (150mg/kg i.p.). In some cases, the mice were intracardially perfused with ice-cold modified artificial cerebrospi-
nal fluid (ACSF) for 2min before further preparation. The modified ACSF contained (in mM) 126 sucrose, 2.5 KCl, 1.3 NaH₂PO₄, 3 MgCl₂, 26 NaHCO₃, 0.1 CaCl₂ and 20 D-glucose and was bubbled with 95%O₂-5%CO₂. The brains were removed and put in the ice-cold modified ACSF. The cerebellum was isolated and cut into two parasagittal halves. Parasagittal slices of 250-275µm thickness were obtained using a vibratome (VT1000s, Leica, Nussloch, Germany) and transferred into modified ACSF at room temperature. The modified ACSF was replaced within the next 90min with ACSF containing (in mM) 125 NaCl, 2.5 KCl, 1.3 NaH₂PO₄, 2 MgCl₂, 26 NaHCO₃, 2 CaCl₂ and 20 D-glucose, oxygenated with 95%O₂-5%CO₂ at room temperature. Experiments were performed with this ACSF to which the selective non-NMDA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione [DNQX, 20-25µM, for recordings of spontaneous inhibitory postsynaptic currents (sIPSCs)], and in some recordings also tetrodotoxine [1µM, to detect miniature inhibitory postsynaptic currents (mIPSCs)] and/or the GABAₐ antagonist bicuculline (10µM, Tocris, Bristol, UK) were added.

Whole-cell voltage-clamp recordings of the DCN were conducted with patch pipettes that had a resistance of 2.0-6.0MΩ when filled with a solution containing (in mM) 124 CsCl, 10 K⁺-HEPES, 5 EGTA, 4.6 MgCl₂, 4 K⁺ATP, 0.4 Na⁺GTP, 0.1 CaCl₂, and 5 QX-314 (Tocris, Bristol, UK), adjusted to pH 7.3 with CsOH, thus obtaining chloride-symmetrical experimental conditions. The patch procedure was visualized using a motorized (Luigs&Neumann, Ratingen, Germany) microscope (Axioscope, Zeiss, Göttingen, Germany) with water immersion objective (x40, Zeiss numerical aperture 0.75), infrared illumination, Normaski optics and infrared sensitive CCD camera (Newvicon C2400-07-C Hamamatsu, Japan). The recordings were performed with an EPC-7 or EPC-8 amplifier (Heka, Lambrecht, Germany). The data was sampled at a rate of 20kHz, and subse-
sequently low-pass filtered (cutoff frequency 1kHz) (Spike2, CED, Cambridge, UK). Membrane resistances for the recordings used for quantitative analysis were $494.8\pm379.4\text{M}\Omega$ (Mean±SD, throughout the paper) and the mean ratio serial resistance/membrane resistance was 0.078. The membrane and serial resistances did not differ significantly between genotypes. Neurons were voltage-clamped at $-70.4\pm4.4\text{mV}$. Data was not quantitatively analyzed if under these conditions the serial resistance increased more than 25% of the initial value or if the membrane resistance of the recorded neuron was below 150MΩ.

Analysis of the postsynaptic currents was performed with the MiniAnalysis program (Synaptosoft, Decatur, GA, USA). For the analysis of the synaptic waveforms only synaptic events were evaluated that were not interrupted by other synaptic events. On average, $145\pm190$ events ($n=92$ recordings) were evaluated. $g_{\text{syn}}$ values were calculated as $g_{\text{syn}}=I_{\text{mean}}/(V_h - E)$, where $I_{\text{mean}}$ is the mean peak amplitude of the IPSCs, $V_h$ is the holding potential and $E$ is the measured equilibrium potential for the IPSCs of the respective recording. Decay time mentioned in this paper is the time it takes the current values to decay to $1/e$ of the peak values of the IPSC amplitudes. Peak-scaled non-stationary fluctuation analysis was performed using the respective module of MiniAnalysis, version 5.6.29 (adapted from the original algorithm by Traynelis et al. 1993). Those IPSCs that were interrupted by other events and did not return to baseline within the chosen time-window were discarded. The recording sections used for the non-stationary fluctuation analysis showed no statistically significant correlation between IPSC amplitude and time, decay time and time, IPSC amplitude and decay time, IPSC amplitude and half-width. The unitary current $(i)$ was estimated by fitting the equation $\sigma(t)^2=il(t)-$
\[ I(t)^2/N + \sigma^2_{\text{basal}}, \] where \( \sigma^2 \) represents the variance at given time \( t \) of the mIPSC, \( I \) the current at given time \( t \), and \( \sigma^2_{\text{basal}} \) the variance in baseline noise measured before the peak. The algorithm forces the probability of channel opening at the peak of the mIPSC to approach unity (1.0), consequently \( N \) represents the average number of channels open at peak (\( N_{\text{open,peak}} \)). Fitting was done after subtracting the baseline variance and without including the offset. The unitary conductance values (\( \gamma \)) were calculated as \( \gamma = i/(V_h - E) \).

Statistically significant differences and correlations were tested with the Student’s t-test and Pearson-Product-Moment-Correlation-Test for normally distributed data, otherwise with the Mann-Whitney-Test and Spearman-Rank-Order-Correlation-Test (italicized P values throughout the paper). Statistical significance was assumed for \( P < 0.05 \).
Results and discussion

Voltage-clamp recordings, performed from neurons in the DCN of mutant mice (p3-21, n=41) as well as from control animals (p4-28, n=51) consistently revealed dense patterns of IPSCs. These IPSCs could be blocked regularly in both, Lc/+ and WT (not shown, n=7 recordings) by application of 10µM bicuculline, indicating that they were GABAergic.

In those experiments in which mutants were studied well after the onset of the PC degeneration, i.e. ≥p14, the $g_{syn}$ values in Lc/+ were found enlarged compared to those in WT [for sIPSCs: $1.38±0.50nS$ (n=12, Lc/) versus $0.66±0.28nS$ (n=14, WT), $P≤0.001$; for mIPSCs: $1.23±0.51nS$ (n=10, Lc/) versus $0.57±0.21nS$ (n=19, WT), $P≤0.001$].

We next asked if the conductance changes appear in parallel to PC axonal degeneration or follow a different time course. Fig.1 shows recordings of sIPSCs and mIPSCs from the DCN of Lc/+ and WT at different postnatal days. An increase of $g_{syn}$ is obvious in the recordings obtained at p14 in the mutants, but not in the WT. $g_{syn}$ in animals ≤p13 did not show any statistically significant difference between Lc/+ in comparison to the healthy control group [for sIPSCs: $0.57±0.23nS$ (n=16, Lc/ ≤p13) versus $0.62±0.32nS$ (n=14, WT ≤p13), $P=0.95$]. The respective values for recordings of mIPSCs were $0.50±0.39nS$ (n=3, Lc/ ≤p13) versus $0.39±0.16nS$ (n=4, WT ≤p13), $P=0.62$. At p14, $g_{syn}$ in Lc/+ showed an increase while the ones in WT stayed on the level found in younger animals. Hence the change in conductance occurs only after the onset of PC degeneration. Fig.2A and 2B summarize the findings. The average synaptic conductances are plotted as a function of the postnatal age of the recorded neuron. An analysis of ampli-
tude distribution revealed that this increase in conductance was due to a decrease in the number of small events accompanied by an increase in the number of large events. There was no obvious indication for the appearance of a new population of IPSCs in $Lc/+\$ compared to WT $\geq p14$ (Fig.2C).

We analyzed various parameters defining the properties of IPSCs known to undergo typical developmental changes in other inhibitory GABAergic systems. Fig.3 illustrates the developmental courses of the 10-90% rise time and the decay time for IPSCs in $Lc/+\$ and WT. The 10-90% rise time as well as the decay time of the sIPSCs showed a significant decrease with age in both, $Lc/+\$ and WT (10-90% rise time: for $Lc/+\$, $r=-0.64$, $P=0.004$, $n=18$; for WT $r=-0.75$, $P<0.001$, $n=20$; decay time: for $Lc/+\$, $r=-0.52$, $P=0.026$, $n=18$; for WT $r=-0.57$, $P=0.009$, $n=20$). These developmental changes reflect a speeding up of GABAergic IPSCs in both $Lc/+\$ and WT. The results can be very well brought into line with studies of GABAergic synapses in other parts of the mammalian CNS where developmental changes in the kinetics with unchanged IPSC amplitudes are reported and correlated with changes in the GABA$_A$ receptor subunit composition (Hollrigel and Soltesz 1997; Dunning et al. 1999, Vicini et al. 2001). Importantly, the maturation of the synaptic transmission in $Lc/+\$ seems to follow a normal pattern with the exception of a step-like increase in $g_{syn}$ in the mutant at p14. The data are consistent with a functional synapse with an enhanced inhibitory potency per afferent terminal at this point in time. One might speculate that intrinsic fibers/interneurons are involved in these compensatory mechanisms. Sultan et al. (2002) found indications for an increase in number and size of non-Purkinje cell GABAergic synapses in $Lc/+\$, most probably corresponding to interneurons.
An analysis of the frequencies of the IPSCs revealed no differences between mutants and WT, neither for animals <p14 [for sIPSCs: 2.0±1.8Hz (n=16, Lc/+)] versus 1.8±3.6Hz (n=14, WT), P=0.119; for mIPSCs: 3.2±2.3Hz (n=3, Lc/+)] versus 6.3±5.5Hz (n=4, WT), P=0.417] nor for animals ≥p14 [for sIPSCs: 14.2±11.6Hz (n=12, Lc/+)] versus 13.9±8.5Hz (n=14, WT), P=0.738; for mIPSCs: 10.1±8.2Hz (n=10, Lc/+)] versus 7.9±6.5Hz (n=19, WT), P=0.469].

In order to determine whether the potentiation of IPSC amplitudes in Lc/+ is due to a difference in the number of receptors or due to a modulation of the single receptor channel conductance, we applied a peak-scaled non-stationary fluctuation analysis as described by Traynelis et al. (1993). In addition to an estimation of the mean unitary conductance (γ) this method also estimates the average number of channels open at peak of the IPSC (N_{open,peak}). Using mIPSC recordings from Lc/+ (n=7) and WT (n=5) ≥p14 we found a statistically significant increase of N_{open,peak} from 22.65±7.59 in the WT to 45.45±16.11 in the Lc/+ (P=0.015). In contrast, γ showed no statistically significant difference between mutants and WT [42.60±21.96pS (Lc/+) versus 33.25±19.19pS (WT), P=0.462, Fig.4]. The most parsimonious explanation of the different N_{open,peak} is a change in the total number of postsynaptic receptors as has been shown by studies directly assessing the number of receptors (Nusser et al. 1998; Kilman et al. 2002). A more remote possibility, which, however, can not be excluded on the basis of the present data, is that neurotransmitter vesicle content increases in Lc/+.

The synaptic potentiation occurs in close temporal relationship to the PC degeneration which starts at p8 showing first signs of PC anomaly, progresses with first cell deaths at p12 (Dumesnil-Bousez and Sotelo 1992), and leads to the death of about 90% of PCs at
p26 (Caddy and Biscoe 1979). Therefore, the synaptic potentiation at p14 in the mutants may well be the consequence of the degeneration of the DCN’s only GABAergic afferents rather than a direct, primary element of Lc/+ pathology.

Electrophysiological in-vitro recordings from the DCN are possible in a limited postnatal time window only. The high myelinisation of the structure in older animals complicates the isolation of viable neurons and the formation of a stable patch configuration and - in our hands – renders such recordings virtually impossible in older animals. Due to this limited postnatal time window it is difficult or even impossible to issue a statement about the electrophysiological properties in older animals. At this moment it is difficult to decide if this is a transient or permanent mechanism of adaptation.

GABAergic synapses on DCN neurons have been shown to undergo long-term potentiation (LTP) in response to an electrical pulse train to their afferents in the white matter or after sole intracellular depolarization (Aizenman et al. 1998; Ouardouz and Sastry 2000). This form of LTP is accompanied by increases in mIPSC frequency without changes in their amplitude but stronger response to extracellularly applied GABA agonists, a finding that could be explained by the activation of previously ‘silent’ synapses (Ouardouz and Sastry 2000). While the potentiation of GABAergic IPSCs in Lc/+ as described here might be taken as a form of LTP, it must employ a mechanism different from the one described earlier (Ouardouz and Sastry 2000) because amplitudes of mIPSC are increased. Similar types of LTP, based on changes in N_{open,peak} without alterations in the \( \gamma \) values, have been observed in other GABAergic synapses, e.g. after kindling of hippocampal inhibitory synapses (Otis et al. 1994; Nusser et al. 1998) or after depriving activity at neocortical synapses (Kilman et al. 2002). An important implication of the present study is therefore that forms of LTP may not only be involved in learning and memory,
but may also be important for adaptive processes following brain damage or degeneration (Wilson et al. 1979; Mittmann and Eysel 2001) and that they might even have an adaptive value, compensating for the loss of the extrinsic inhibition.
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Figure legends

**Fig.1** Recordings of sIPSCs (A) and mIPSCs (B) in DCN neurons of Lc/+ and wild-type (+/+) mice at the indicated ages. The respective values for holding potentials (V_h), equilibrium potentials (E) and average synaptic conductances are also given. The recordings of neurons aged p11 and p14 are from the same neuron (before and after TTX application); note the effect of TTX application on the conductance values.

**Fig.2** Increase in the average synaptic conductances (g_{syn}) of GABAergic IPSCs at p14 in Lc/+ (A), but not in wild-type (B) mice. The g_{syn} values are plotted as a function of postnatal age of the recorded neuron. The insets give the mean values (with SD as error bars) for animals <p14 and ≥p14. * indicate statistically significant differences in g_{syn} in Lc/+ (for sIPSCs: P≤0.001, for mIPSCs: P=0.046). Cumulative IPSC amplitude histograms (C) for animals ≥p14. The IPSC amplitudes were grouped into bin widths of 20 pA. The number of IPSCs for each bin was normalized to the number of all IPSCs in the respective recording. The mean cumulative fractions are plotted versus the amplitude bins; for sIPSCs in Lc/+ (n=12 recordings; filled squares) and WT (n=14 recordings; unfilled squares), for mIPSCs in Lc/+ (n=11 recordings; filled triangles) and WT (n=19; unfilled triangles). The x-axis range is limited to 400pA. The increase in g_{syn} in Lc/+ compared to WT≥p14 is due to a decrease in the number of small sIPSCs and an increase in the number of large sIPSCs.

**Fig.3** Developmental decrease in the 10-90% rise and decay times of GABAergic IPSCs in Lc/+ (A and C) and wild-type (B and D) mice. The 10-90% rise and decay times were
calculated for each recording and the mean values (with SD as error bars) are plotted as a function of postnatal age of the recorded neuron.

**Fig.4** Peak-scaled non-stationary fluctuation analysis of mIPSCs (recordings from animals ≥p14). A: Variance-mean current-plot (for Lc/+ closed circles, for WT open circles) with the parabolic fit of the relationship (for Lc/+ dashed line, for WT solid line). Age of the animals: p21 (Lc+/+) and p18 (WT). 22 (Lc+)/+ and 41 events (WT) went into the average for the analysis. The respective mean unitary conductances (\(\gamma\)) and average numbers of channels (\(N_{\text{open,peak}}\)), as derived from the fitted curves, are also given. B: No statistically significant difference in the estimated mean unitary conductance (\(P=0.462\)) but a statistically significant increase in the average number of channels open at peak (*, \(P=0.015\)) in mIPSC recordings from Lc/+ aged p14 to 21 (p17.3±3.1) versus WT aged p15 to 23 (p17.8±3.3). The values are given as means (with SD as error bars). Only those events were included that fulfilled the strict criteria mentioned in the Method section. Only smooth average event traces were accepted for the non-stationary fluctuation analysis. 10 to 41 events (24±9) went into the average for the analysis.
A spontaneous IPSCs

B miniature IPSCs

Figure 1
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“Lurcher mice exhibit potentiation ...”
Figure 2
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Figure 3
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