Studies on the Renshaw Cell without its inhibitory effect on alpha motoneurons

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Summary
Renshaw cells mediate recurrent inhibition on α-motor neuron (α-MN) in the spinal cord. The Renshaw cells are stimulated by side branching axons (collaterals) originating from the α-MN. The Renshaw cell then fires back to a α-MN. The Renshaw cell is stimulated by acetylcholine released from the α-MN, and when activated it releases the inhibitory amino acids GABA and glycine on to the α-MN. These inhibitory amino acids are packed into vesicles by a transporter called VIAAT, short for vesicle inhibitory amino acid transporter. For a very long time it has been very hard to study the function of Renshaw cells in locomotion. Deleting the VIAAT gene via the Cre/lox system enables us to study the behavior of living, unobstructed moving animals. The focus of this work has been to establish the content of the VIAAT knockout and to investigate to what extent the Renshaw cells affects locomotion in mice.

By performing in situ hybridization using a riboprobe anti-sense for VIAAT and double-staining this with antibodies binding to the Renshaw cell marker cabining I found the knockout mouse to be successful. Further I confirmed that VIAAT-mediated Renshaw cell signaling is involved in shaping synaptic input to motor neurons, but that the number of synapses on the Renshaw cell is unchanged.

Introduction

Neuronal Circuits
A human brain consists of approximately 100 billion neurons. A very rough estimate is that a neuron has about 7000 connections in average (a Purkinje cell has about 50 000 connections). This leads to the conclusion that a brain consists of about 100-billion connections (if you suppose that every neuron has a unique set of connections).

Scientist has used more simple systems such as central pattern generators in their studies regarding behavior and the circuits involving, the neural basis of movement (Grillner, 1975, Kiehn, 2006, Grillner, 2007, Goulding, 2009, Ramirez et al., 2004). These central pattern generators are much simpler than actual networks in the brain when speaking of the number of neurons involved in the circuit. A neuron never works on its own but instead works in circuits (Frank and Wenner, 1993). A circuit can be distinguished from a network in the way that a network is a group of interconnected neurons and a circuit is a whole functional unit and these units somehow influence each other (Kiehn, 2010). These neuronal circuits are organized into collections and process different kinds of information and control essential bodily functions as summarized in Jessell (2000). A lot of circuits exist and they vary depending on what kind of function they are intended to control (Grillner and Jessel, 2009).

A neuronal circuit consists in general of three different types of neurons. Afferent neurons carry information toward the central nervous system. Efferent neurons carry information away from the central nervous system and spinal cord. Interneurons are the third type of nerve cells and take part in circuits at a local aspect, meaning that they are connecting the afferent neurons with the efferent.

There are four types of neural circuits that underlie locomotion (Grossman et al., 2010). The first one being the local circuitry, consisting of lower motor neurons that are in charge of performing movements, independently of if they are voluntary or not, and the local circuit neurons.
The second circuitry consists of neurons where the cell bodies can be found in the brainstem or cerebral cortex. These neurons are called upper motor neurons and they send their axons to the local circuit neurons. Upper motor neurons are neurons that originate in the motor cortex within the cerebral cortex and redirect motor information from the cerebral cortex to the motor neurons responsible for carrying out actual work on the muscles. For a schematic picture of this see Fig. 1. One should keep in mind that it is quite rare that these lower neurons directly speak with the lower motor neurons, but instead talk through the local circuit neurons innervated in a motor neuron pool. The best way to describe a motor neuron pool is a set of motor neurons that innervate a single muscle. One can further describe the motor neuron pool as a pool that encloses all the neurons that are responsible for contracting a muscle (Sherrington, 1947).

Figure 1. The lower motor neurons are in charge of performing movements. This is mediated through the α-MN. The information from the motor cortex via the cerebral cortex and the spinal cord is redirected downwards through the Upper motor neurons. The upper and the lower motor neurons talk to each other through local circuit neurons. Adapted from Purves, Neurobiology, 4th edition.
These motor neuron pools are not randomly oriented in the spinal cord but are grouped into segments. Different segments in the spinal cord coordinate and control different types of muscles. Some parts control postural control and balance while some other controls something else. The motor neuron pools consist of alpha, beta and gamma motor neurons (see Fig. 2 for a schematic picture of a α-MN).

The remaining two are not neurons but structures, the cerebellum and the basal ganglia, respectively.

These structures control movement by directing the upper motor neurons (see Fig. 1)

**Motor neurons**

There are three types of motor neurons in the body: alpha, beta and gamma. α-MN are innervating the extrafusal muscle fibers and thereby controlling the movement of the muscles. β-MN is involved in the motor spindle and intrafusal muscle fibers and is not nearly as good described as alpha and gamma.

One common hallmark among all motor neurons is the release of acetylcholine (Jessell, 2000). Acetylcholine can act as a marker for a general motor neuron population.

By staining for vesicular acetylcholine transporter (VACHT), which is a general cholinergic marker (Schafer et al., 1994; Arvidsson et. al., 1997) it is possible to visualize motor neurons. ChAT (cholinergic acetyltransferase) can be used to image cholinergic neurons in the brain and spinal cord. ChAT is an enzyme that synthesizes acetylcholine
from CoA and choline at cholinergic synapses. ChAT is synthesized within the body of the neuron and is therefore an excellent marker for motoneurons.

How do you distinguish a $\alpha$-MN from a gamma and beta besides its differences in function? The $\alpha$-MNs can be divided into the subpopulations fast and slow motor neurons. (Burke, 1981; Kernell, 1986; Banks, 1994). They not only differ in activation but the amount of force produced during contraction of muscles fibers (Burke, 2004).

Another distinct difference between motor neurons is the morphology and the size of the soma, where the $\alpha$-MNs are much larger than the gamma motor neurons (Eccles et al., 1960; Burke et al, 1977). Identifying these different types of neurons is rather easy due to these different morphological features (Fleshman et al., 1988; Gustafsson and Pinter, 1984; Kernell and Zwaagstra, 1981; Zengel et al., 1985).

The connections that influence the motor neurons are also different. $\alpha$-MNs obtain contact with Ia inhibitory interneurons that keeps it up to date with proprioceptive information (Eccles, et al., 1960). In general one can say that there are five senses (vision, smell, taste, hearing and touch. A six sense could be proprioception, the sensation of having a body.

When looking at the distribution between alphas and gammas one can see that gammas make up approximately one third of the total population of motor neurons.

Synaptophysin is a presynaptic vesicle protein and is present in more or less all neurons in the spinal cord and brain. By using a synaptophysin antibody it is possible to detect endogenous levels of total synaptophysin protein. This enables one to count and observe the number of positive presynaptic axon terminals (Arthur and Stowell, 2007).

**Renshaw cells - a negative feedback loop**

Studies have recognized a few classes of neurons in the ventral neural tube: motor neurons and four different kinds of central interneurons.

Among all the classes of interneuron in the ventral spinal cord some affect motor neuron excitability. The Renshaw cells (RCs) exhibit recurrent inhibition on motor neurons. The term recurrent inhibition was stated for more than 70 years ago by Birdsey Renshaw (Renshaw, 1946). He found a special type of inhibitory interneurons located in the ventro medial region of the ventral horn that were later found to be able to fire high-frequency bursts upon activation by $\alpha$-MNs (Eccles et al. 1954; Van Keulen, 1981; Thomas and Wilson, 1965).

RCs make up about 10% of all inhibitory interneurons belonging to type V1 (Sapir et al., 2004; Alvarez et al., 2005). RCs have the ability to inhibit motor neurons when they receive synaptic inputs from excitatory collateral (side branching axons) from the alpha neuron's axon (Eccles et al., 1954). This activation of Renshaw cells elicits an inhibitory postsynaptic potential onto the MN and probably also on other Renshaw cells. They receive stimulation from side branching excitatory axons (also known as collaterals) from one or several $\alpha$-MNs (Renshaw, 1946; Eccles et al., 1954; Hultborn et al., 1979, Lamotte d’Incamps and Ascher 2008) (Fig 3).
This means that the α-MNs are inhibiting themselves by activating the Renshaw cells at the same time as they fire action potentials to muscle fibers and make them contract (Renshaw, 1946; Eccles et al., 1954; Hultborn et al., 1979). This relationship is however not entirely true. It is not yet clear if the α-MN is inhibiting itself, or if it is activating a Renshaw cell that is connected to another α-MN. There are some sources that indicate that the relationship between the number of RCs and MNs is 1:5 (Fyffe, 2007). Another possible scenario would be that a α-MN within the same motor neuron pool causes inhibition of other α-MN. This negative feedback keeps the Renshaw cell informed how strongly that particular motor neuron is firing. Surprisingly enough, the characteristic of the motor unit determines the level of the recurrent inhibition that the α-MN receives. The release of acetylcholine from α-MN activates the RC. When activated the RC releases inhibitory amino acids in the postsynaptic cleft facing the α-MN that once stimulated it. Figure 3 shows a schematic figure of a RC connected to a α-MN by collaterals. The inhibitory neurotransmitters of RCs are GABA and glycine (Curtis et al., 1976; Wilson et al., 1963). Once the Renshaw cell is stimulated an action potential travels through the cell and out into the synapse and causes vesicles filled with GABA and glycine to be released into the synaptic cleft. The amino acids are packed into vesicles by a transporter called Vesicular Inhibitory Amino Acid Transporter (VIAAT) (Fig 4). By blocking or deleting this VIAAT-gene it is possible to hold back the inhibiting effect of Renshaw cells.
There are some different theories regarding the likely function of the Renshaw cells in locomotion. They might be involved in recruitment/derecruitment of motor neurons (Bui et al., 2008; Hultborn et al., 2003; Kuo et al., 2003), produce synchronization among motor neurons (Maltenfort et al., 1998), and/or alter the activity in motor neuron pools (comprehensively reviewed in: Hultborn et al. 1979; Baldissera et al. 1981; Windhorst, 1990; Jankowska, 1992; Maltenfort et al. 1998; Mattei et al. 2003).

Combining well-known anatomical criteria such as size and shape, the morphology of the Renshaw cell with the expression of the protein calbindin (a calcium buffering protein which is found in high levels in Renshaw cells) when doing immunohistochemistry, Renshaw cells are easily identified. (Antal et al., 1990; Sanna et al., 1993; Carr et al., 1998)

**Cre/lox system**

The Cre/lox system is a tool to create knockout and conditional knockout animals. This is done by breeding two animals where one of them has the gene coding for the recombinase Cre and the other the gene of interest (gene X) flanked by two loxP sites.

Figure 4. Picture illustrating the role of VIAAT in vesicle packing of GABA and glycine. VIAAT (purple structures) is a transporter attached to vesicles in the postsynapse. Its function is to pack vesicles with GABA and glycine. The vesicles later release their content whenever the Renshaw cell receives stimulation from an α-MN. The picture indicates that GABA (dark green) and glycine (light green) is packed individually. This assumption is not yet known and should therefore not be taken as a fact.
Gene X carries a promoter that is specific for the cell type. Thereby you can modify gene expression by for example removing an unwanted piece of DNA. The Cre recombinase (short for causes recombination) is a DNA recombinase which is site specific. These sites are called loxP. When the excision of the DNA takes place the Cre protein first cuts the double stranded DNA at both loxP sites, which acts as some kind of “magnet” to the Cre protein; thereafter the two DNA pieces are reassembled by a DNA ligase. Due to the directionality of the loxP sites these can also be used to invert pieces of DNA (Fig 5).

![Diagram](image)

Figure 5. Schematic drawing illustrating the principle behind the Cre/lox system. Two different mice are breed where one have the target gene flanked with loxP sites and the other have the CRE gene that’s driven by a cell specific promoter. The outcome can either have CRE or not and if they do CRE recombinase will cut out the gene or a necessary part of it. Due to the presence of the cell specific promoter one can tailor the mouse in more or less anyway you want to.

A tool to knock out the Renshaw cells have been developed (the Cre/lox system; see Material and Methods section) which enables one to study the behavior of live, unobstructed moving animals.

Previous studies have shown that the gene Chrna2 is expressed, with the exception of a small population of large neurons within the area of motor neurons, in Renshaw cells in the spinal cord (Enjin, unpublished data). By using a cell specific promoter, in this case Chrna2, it is possible to get expression of Cre recombinase in Chrna2-expressing cells and thus perform a deletion of the VIAAT gene or an essential exon. Thereby we are able to create Renshaw cells lacking VIAAT. By having the VIAAT gene disabled the function of the Renshaw cells is removed.
Crossing mice carrying the VIAAT gene flanked with loxP-excision sites activated the Cre recombinase in the Renshaw cells. This system made it possible to delete the gene transcribing VIAAT transporter. With VIAAT gone the Renshaw cell is still there but the transporter responsible for packing the vesicles with inhibiting neurotransmitters is gone.

**Aim**

The Kullander lab has created a knockout mouse lacking the Vesicular inhibitory amino acid transporter (VIAAT). The gene Chrna2 drives the Cre expression. This setup enables us to delete VIAAT in Renshaw cells. My work has been concentrated around characterizing the expression of Cre in this particular knockout mouse. The specific question I am trying to answer is which cells are changed in the knockout and in what way. We know for sure that the Renshaw cells are affected but how?

**Results**

**VIAAT signal is gone in the ventral part of the lumbar spinal cord in Chrna2::Cre;VIAAT mice.**

Figure 6. VIAAT signal (red) is gone in calbindin positive cells (green). The picture was acquired by performing in situ hybridization on cryosectioned spinal cord from 4 weeks old mice. The probe is anti-sense for VIAAT (red). This in situ hybridization was co-stained with calbindin (green). Calbindin can be used as a selective marker for Renshaw cells. The picture is clearly showing that none of the calbindin positive Chrna2::Cre;VIAAT<sup>lox/lox</sup> cells are positive for VIAAT (right) while all of the calbindin positive cells in the wildtype are positive for VIAAT (left).

To find out if the Chrna2::Cre;VIAAT knockout was a complete successful knockout in situ hybridization was performed using a riboprobe for VIAAT. The tissue was co-stained with antibodies for calbindin (Fig 6). The Cre/lox system was used to create a possible knockout mouse lacking VIAAT in Chrna2 expressing cells (Chrna2::Cre;VIAAT). To control whether this gene knockout was successful or not we performed in situ hybridization and immunohistochemistry studies. Mice were sacrificed using perfusion with PBS and fixatives. Spinal cords from
four weeks old mouse were sectioned using a cryostat. VIAAT signal was visualized by performing in situ hybridization using a riboprobe specific for VIAAT mRNA. The same tissue was co-stained with antibodies specific for synaptophysin and calbindin (Fig 6). The primary antibody was visualized with Alexa 488 (calbindin, green) and Alexa 647 (VIAAT, red) secondary antibodies. The VIAAT signal is present in wildtype and abolished in Chrna2::Cre;VIAAT^tx/tx and thereby showing a complete knockout. This result confirmed that the knockout was successful.

**The number of calbindin synapses is greater on motor neurons in the knockout compared to the wildtype**

To examine if deletion of VIAAT from Renshaw cells affect synaptic input to motor neurons, antibody staining’s for ChAT, synaptophysin and calbindin. In average there is a difference in the number of synapses between the knockout mouse and the wildtype. The number of calbindin positive synapses and Chat positive cells are greater in the Chrna2::Cre;VIAAT mouse than in the wildtype. Two different sets of antibodies were used to detect the synapses in Renshaw cells and motor neurons. Immunohistochemistry was performed to visualize the synapses on motor neuron using antibodies positive for synaptophysin (blue), ChAT (red) and calbindin (green) (Fig 7). The numbers of calbindin synapses on motor neurons are in a greater number in the Chrna2-VIAAT^tx/tx than in the wildtype mouse (Fig 7B). Thus, VIAAT-mediated Renshaw cell signaling is involved in shaping synaptic input to motor neurons. However, when comparing the number of motor neuron synapses per Renshaw cell no difference is found. The picture shows calbindin cells (green), ChAT positive synapses (red), synaptophysin (blue) and VACht (red).

**Test of motor coordination skills revealed no differences in motor skill**

To understand the role of Renshaw cells in locomotion we analyzed the behavior of Chrna2::Cre;VIAAT in a set of behavioral test (steady rotorod, accelerating rotorod, beam walking and ladder walking). Because the Renshaw cells are thought to be part of the motor coordination we hypothesized that the Chrna2::Cre;VIAAT mice would perform as well as the wildtype in the basic testing. The mouse performed three trials with the ability to rest between each trial. No difference could be shown (Fig 8). The beam walking test challenged them in the way that we used different sizes of the beam, 25 mm and 12 mm. The graphs in Fig. 7 show the counting of the number of slips. A slip is defined as a partial or full slip with its hind leg. The time was measured during the trial and the number of slips counted afterwards using a video recorder. The beam walking test assesses motor coordination in mice but no difference could be found in the number of slips between Chrna2::Cre;VIAAT knockouts and the VIAAT^tx/tx (Fig 8C). Ladder walking was constructed in such a way that the mouse was allowed to walk on metal bars between two slides of acrylic glass. A number of bars had been removed in order to create a challenge. Each mouse performed three trials and was allowed to rest between each trial. Each trial was recorded and this footage was later used in the counting
of number of slips. A slip was defined as a partial or full slip with either the front or hind leg.

Figure 7. The number of synapses is increased in the Chrna2::Cre;VIAAT mouse while the number of motor neuron synapses is unchanged. A: Immunohistochemistry staining using antibodies for ChAT, calbindin and synaptophysin. Counting of the number of synapses revealed a greater number of synapses in the Chrna2::Cre;VIAAT than in the VIAATlox/lox. B: Antibody staining with ChAT, VACht and synaptophysin showed no differences in the number of synapses. The counting of the synapses is presented in the graphs to the right.

The analysis of these results showed no difference between the Chrna2::Cre;VIAAT knockouts and the VIAATlox/lox (Fig. 8D).

The rotorod (itch Life Sciences) evaluates skill in motor coordination and balance. Each mouse performed three trials and was allowed to rest between the trials. An automatic time measurement system was used in which the mouse itself stops the time by flipping a bridge when falling down. Each mouse performed two different kinds of trials: in one of them the rod was rotating steadily and in the other one it was accelerating (Fig 8 A and 8 B).
Figure 8. The Chrna2::Cre;VIAAT knock out shows no changes in its motor coordinating skills. A: Motor coordination and balance was measured in the rotorod test. The result revealed no deficiency. Two types of rotorod were tested in which one was at a steady speed and one accelerating. B: The beam-walking test assesses motor coordination in mice. The rotorod measures basic motor coordination whereas the beam walk measures fine motor coordination. No performance differences were found between Chrna2::Cre;VIAAT and a littermate control mice. Each mouse were given three trials in one day and allowed to rest for about 20 minutes in between each trial.

Discussion

The Renshaw cells are located in the ventral part of the spinal cord and act as an inhibitor of α-MNs. They are stimulated by contralaterals originating from the α-MN and then providing inhibitory input back onto the α-MNs. This is done through a process known as recurrent inhibition. GABA and glycine is packed into vesicles by the transporter VIAAT.

Previous studies have shown that the gene Chrna2 is expressed by Renshaw cells in the spinal cord. A transgenic mouse with Cre expression under the influence of Chrna promoter regions was constructed in order to get rid of the VIAAT signaling in Renshaw cells. Exactly what this means and if the Renshaw cell is still receiving stimulation from the α-MN or if the Renshaw cell is sending an inhibiting signal to MN has not been elucidated. Some theories have been presented about the role the Renshaw cells might have in locomotion. Previous studies have indicated that they take part in locomotor rhythm generation (Nishimaru et al., 2006). Some studies indicate that Renshaw cells do not participate in, or at least do not have a determining role in motor neuron burst termination. It has also been suggested that there is an interaction between commissural neurons and Renshaw cell during locomotion. Renshaw cells may participate in the recruitment or derecruitment of motor neurons, producing synchronization among these or alter the activity in motor neuron pools. It may also be the case that they differ in function in different developmental stages during circuit formation in the spinal. Our results show that VIAAT-mediated Renshaw cell signaling is involved in shaping synaptic input to motor neurons. The number of synapses is greater in the
Chrna2::Cre;VIAAT knock out than there is in the wildtype. But the number of synapses of motor neuron synapses on the Renshaw cell is not showing any difference. We hypothesized that these results somehow would affect the motor coordination. Most likely that the Chrna2::Cre;VIAAT would perform equally well in the basic motorical skill testing but it would fail in the finer. Surprisingly enough no difference could be seen.

Material and methods

Immunohistochemistry
The spinal cord was mounted using Sekura Tissue-Tek sectioned using a Microm HM 560 Cryostat (10µm) and let dry before further analysis. The sections were washed in PBST, 0.5% Triton-X (Sigma) to remove excess Tissue-Tek. Primary antibody was added and the slides were put in a humidified horizontal chamber over night at 4°C. The following day the primary antibody was washed away in PBS. Secondary antibodies and DAPI (Sigma) was added onto the sections and thereafter put in a humidified horizontal chamber for 1 hour at room temperature. The following hour the sections were washed in PBST. Slides were mounted using Mowiol (Sigma-Aldrich). Fluorescent staining was captured using a Zeiss LSM 510 meta microscope and LSM 510 software (Carl Zeiss). Images were adjusted and Z-stacked using ImageJ (v1.43u, http://rsb.info.nih.gov/ij/)

In situ hybridization
The mouse was perfused according to known procedures, briefly; Mice were anesthetized using 50:50 mixture of Dormitor (Orion) and Ketalar (Pfizer). This mixture was injected by an intraperitoneal injection. Mice were perfused in vitro by injection of phosphate-buffered saline (PBS) followed by 4% formaldehyde. The spinal cord was dissected out and fixed in 4% formaldehyde (Histolab) over night at 4°C. The fixed tissue was washed in PBS and mounted in 4% agarose and sectioned using a Leica VTs1000 Vibratome (Leica) (60um). The sections were put in PBS (136.9 µM NaCl (GTF, Sigma), 2.7 µM KCl (GTF, Sigma), 9.8 µM Na2 HPO4 (GTF, Sigma), 1.5 µM KH2 PO4 (GTF, Sigma)). If the sections are going to be stored in the freezer they first need to be dehydrated by successive washes in 25%, 50% and 75% methanol in PBT (PBS containing 0.1% Tween-20 (Sigma)) 10 minutes each. They are later on stored in 100% methanol at -20°C. Before use they need to be rehydrated by performing the dehydration washes reverted. The sections were bleached in 4% H2O2 in PBT for 30 minutes and ended by washing in PBT. Samples were treated with 0.5% Triton X (Sigma) for 5 min at RT. The samples were digested using proteinase K (20 µg/ml Merck) in PBT for 10 minutes at room temperature. The concentration depends on the thickness of the sections but also the age. Therefore it is set for each batch. The proteinase K treatment was stopped by washing in PBT several times in PBT. The sections were post-fixed for 30 minutes in 4% Formaldehyde (Histolab) followed by washing in PBT. The sections was prehybridized in hybridization buffer (50% formamide, 5X SSC, pH 4.5, 1% sodium dodecyl sulphate [SDS], 50 mg/ml tRNA (Sigma-Aldrich), 50 mg/ml heparin (Sigma-Aldrich) in PBT at 65°C. The probe (1 ng/ml) was denatured in hybridization buffer for 5 minutes at 80°C and later on added to the sections. To avoid evaporation the plate was sealed thoroughly. The sections were hybridized overnight (14-16 hours). The sections were washed in wash
buffer 1 (50% formamide (Sigma), 5xSSC (0.75 M NaCl, 75 mM sodium citrate), 1% SDS (GTF)) and 2 (50% formamide, 2xSSC (0.3 M NaCl, 30 mM sodium citrate, 0.1% Tween-20) three times 30 minutes each.

To block unspecific staining the sections were blocked using 1x blocking reagent (Roche) in TBST (0.5 M Tris-Cl pH 7.5, 1.5 M NaCl, 0.1% Tween-20 (MP Biomedicals Inc)). Anti-dig antibody Fab fragments 1:5000 (Roche Applied Science) was added to the sections and incubated at 4°C over night (14-16 hours). The Anti-dig antibodies bind to the RNA-probe.

The samples were washed in TBST (containing 2 mM Levamisol (Riedel de Haën) and developed by using Fast Red tablets (Roche, Germany) until staining became visible. Fast red tablets are used to visualize the Anti-dig antibody binding to the RNA probe. It produces an intense red staining. The reaction was stopped by successive washing in water.

Fluorescent staining was captured using a Zeiss LSM 510 Meta microscope and LSM 510 software (Carl Zeiss).

Images were adjusted and Z-stacked using ImageJ (v1.43u, http://rsb.info.nih.gov/ij/).

**Probe preparations**

A vector containing the desirable insert (i.e. the sequence you want your probe to have) was bought from Invitrogen. Before proceeding, the plasmid was sequenced in order to know which restriction enzyme to use for the linearization.

The plasmid was linearized using 20 µg plasmid, restriction enzyme 1% v/v, 1x restriction buffer (provided with the enzyme), and x µl H2O up to 200 µl. A 4% agarose (Sigma) gel was run to check that the linearization was completed. When confirmed one volume of phenol/chloroform/isoamyl alcohol (Sigma Aldrich) was added to the linearization mixture. The mixture was vortexed for some time until the solution becomes foggy, followed by centrifugation at 1700 g for 10 minutes at room temperature. The upper phase was transferred to a new tube and the DNA was precipitated using 1/25 vol of 5 M NaCl (Sigma) and 2 vol ice-cold 99.5% ethanol (Solveco) at -20°C over night. The tube was centrifuged for 10 minutes at 1700 g and washed with 70% ethanol. The remaining ethanol was removed and the pellet was resolved with DEPC-H2O (MP biomedicals Inc). Probe quality was controlled using the Nano-drop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

One µg cleaved vector, DIG RNA labeling mix 1x (Roche Applied biosystems), 1x transcription factor (Roche), Ribolock Inhibitor 40 U/µl (Fermentas), RNA polymerase (T3, T7 or SP6, Roche) 20 U/µl was mixed in a tube and the volume was adjusted with DEPC-H2O up to 20 µl. It is important that you use the correct polymerase in order for the vector to be transcribed in an antisense way. By doing this you need to use the polymerase that has its promoter at the 3’end of the gene fragment. The solution was incubated for 3 hours at room temperature. After the incubation DNAseI (Fermentas) was added (2 U/µl). The RNA-transcript was precipitated with 0.5 M LiCl and 3 vol 99.5% ice-cold ethanol over night at -20°C. The tube was centrifuged at 1700 g for 15 minutes at 4°C. The obtained pellet was washed in 50 µl 70% ice-cold ethanol by centrifuging 1700 g for 5 minutes at 4°C. The pellet was resuspended in 100 µl DEPC-H2O.
Confocal laser scanning microscopy (CLSM)
A LSM 510 microscope was used when acquiring the pictures. When comparing traditional light microscopy with confocal LSM one finds one major difference. In a traditional microscopy all object points in your specimen are imaged at the same time, where a CLSM enables you to irradiate your specimen at in a point to point fashion. Using computing power the image is acquired point by point and later on put together to a 3D image.

It is the pinhole, or confocal aperture that makes it possible for the detector to point detect. A series of filters will selectively let through the fluorescent wavelengths emitted by the fluorescence and at the same time blocking the original excitation wavelength. The detector in the CLSM is only detecting light that has passed through the pinhole, which has a variable diameter. By focusing the laser beam to a limited spot one can point illuminate the object of interest. The pinhole permits light only from the focal plane and rule out all light coming from outside the focal plane and is therefore neither detected. This pinhole is variabled, due to this fact it is possible to vary the degree of confocality.

Behavioral testing
Rotorod
The rotorod (Iitch Life Sciences) test assesses motor coordination and balance (5). To make the mice custom to human contact they were handled for some time every day a week before, such as being weighed and tag-checked. The test consisted in walking on a
drum inside of the rotorod for a maximum of 120 s per trial, or until the mouse fell down. This was done for three trials, no matter if it were a consecutive one or not. The testing person was blinded to the mouse’s genotype.

To assess locomotor skills the mice was tested using a rotorod apparatus (IITC). They were placed on an elevated rod (3.2 cm in diameter) on both a steady rotorod and an accelerating one. The latency time for falling down was measured using timers that stopped whenever the mouse fell down on a tipping bridge.

**Beam walking**
The mouse has 30 s to walk the entire length of the beam, which measures 12 mm and 25 mm. The beam was 60 cm long and about 20 cm above the bench. The beam was held in place by boxes made of Styrofoam. If the mouse failed the first attempt the mouse was trained.

The test was designed such that there was an unpleasant environment (a bright light) and a familiar and “safe” one at the other end (in form of a shelter and some food) in order to motivate them to walk. A wooden beam was used because the mice found this one pleasant to walk on. The mice were placed in the unpleasant end of the beam and were thereafter allowed to walk alone to the other pleasant part. Each trial was recorded on video where the time and the number of slips (one or both hind limbs slipped from the beam) was measured.

**Ladder walking**
The mouse has 30 s to complete the trail. The trail consists of a construction made of two walls made from transparent acrylic glass. The space in between these walls is about 60 cm long and 8 cm wide. Placed along this are metal bars with a spacing of 1 cm. The mouse was placed in one end and allowed to walk by itself without assistance to the other side. By removing a random number of metal bars the degree of difficulty for the mouse to perform the task was increased. The removal of metal bars created spacing with either 1 or 2 metal bar gaps. The metal bar grid was elevated about 30 cm above the bench with cages filled with soft bedding material. Each mouse performed three consecutive trails.

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**References**


Lagerback PA, Kellerth JO (1985b) Light microscopic observations on cat Renshaw cells after intracellular staining with horseradish peroxidase. II. Journal of Comparative
Neurology 240:368-76


