INTRINSIC PROPERTIES OF MOUSE LUMBAR MOTONEURONS

REVEALED BY INTRACELLULAR RECORDING IN VIVO.

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ABSTRACT
We have developed an in vivo model for intracellular recording in the adult anaesthetised mouse using sharp microelectrode electrodes as a basis for investigations of motoneuron properties in transgenic mouse strains. We demonstrate that it is possible to record post-synaptic potentials underlying identified circuits in the spinal cord. Forty-one motoneurons with antidromic spike potentials (> 50 mV) from the sciatic nerve were investigated. We recorded the intrinsic properties of the neurones including input resistance (mean 2.4±1.2 MΩ), rheobase (mean 7.1±5.9 nA) and the duration of the afterhyperpolarization (mean 55.3±14 ms). We also measured the minimum firing frequencies (F_{min}, mean 23.5 ±5.7 SD Hz), the maximum firing frequencies (F_{max}; >300 Hz) and the slope of the current/frequency relationship (f-I-slope) with increasing amounts of current injected (mean 13±5.7 Hz/nA.). Signs of activation of persistent inward currents (PICs) were seen, such as accelerations of firing frequency or jumps in the membrane potential with increasing amounts of injected current. It is likely that the particular anaesthetic regime with a mixture of Hypnorm and Midazolam is essential for the possibility to evoke PICs. The data demonstrate that mouse spinal motoneurons share many of the same properties that have been demonstrated previously for cat, rat and human motoneurons. The shorter AHP duration, steeper f-I slopes and higher F_{min} and F_{max} than in rats, cats and humans are likely to be tailored to the characteristics of the mouse muscle contraction properties.
INTRODUCTION

Most of what is known today concerning the intrinsic properties of adult mammalian spinal motoneurons has been obtained from electrophysiological studies in which the adult cat has traditionally been the model of choice. This has been due to the mechanical stability and longevity normally required for such investigations (Eccles 1957; Kernell 2006). Subsequently these techniques were extended to investigations using rat in vivo preparations (Granit et al. 1963; Gardiner and Kernell 1990; Bakels and Kernell 1993 ab; Button et al. 2006, 2007, 2008). Changes in the intrinsic properties of motoneurons are a feature of a number of disorders for which transgenic mouse models are now available (for example amyotrophic lateral sclerosis (ALS); Pieri et al. 2003; Kuo et al. 2004; 2005, Zona et al. 2006; Bories et al. 2007). This, together with the development of transgenic mice with mutations affecting the normal development of spinal cord circuitry now make it crucial to be able to perform such experiments in the adult mouse in vivo.

Despite early pioneering work using intracellular recording of neurons in-vivo in the adult mouse spinal cord (Huizar et al. 1975; Biscoe et al. 1975, 1977; Martin et al. 1978) and some recent initiatives (Alstermark and Ogawa 2004; Graham et al. 2004, 2007; Manuel et al 2009), the electrophysiological techniques necessary for these kind of investigations have not yet been developed for routine use in the mouse. This is due to the technical demands associated with the small size and the much greater sensitivity to the stress of invasive surgery of the mouse. Consequently a number of laboratories have been developing in vitro models. However, such preparations are limited with respect to the age of the mice and thus do not allow for studies of developmental, compensatory and degenerative changes.

The primary aim in this study was therefore to develop an adult in vivo mouse model in which the intrinsic properties of neurons and neuronal circuitry can be investigated. We demonstrate that it is possible to record the intrinsic properties of motoneurons intracellularly in the anaesthetized adult mouse as has been performed in the cat and that these properties are consistent with the smaller size of mouse motoneurones and appear to be tailored to meet the characteristics of mouse muscle contraction properties. This data from the normal adult mouse will form a control for future comparisons with relevant transgenic strains. We also demonstrate that using an
anaesthetic regime of hypnorm and medazolam it is possible to see evidence of the plateaux potentials that are mediated by persistent inward currents and that these appear to be a common feature of mouse motoneurones. Furthermore, in this model we demonstrate that it is also feasible to record postsynaptic potentials underlying identified circuits in the spinal cord such as monosynaptic EPSPs and disynaptic Ia reciprocal IPSPs.

METHODS

The results presented in this study originate from 15 adult (≥ 20 weeks, 25 grams) mice of the C57BL/6J strain (10 female, 5 male). We selected this particular strain of mouse as a number of transgenic mice that we wish to investigate (future experiments) were created from this strain. The experimental procedure was approved by the Danish Animal Experiments Inspectorate. Anaesthesia was briefly induced with isoflurane for a duration sufficient to allow for a stress-free injection of a longer lasting anaesthesia. A longer lasting anaesthesia was induced using a cocktail of 1 part fentanyl (Hypnorm 0.315mg/ml), 1 part Midazolam (5mg/1ml) and 2 parts sterile water (0.15ml of cocktail/25g) injected subcutaneously. Three intra-peritoneal cannulas were inserted for the drug delivery and anaesthesia was maintained by 0.2 ml doses of this cocktail at 20 minute intervals through one of the cannula. A tracheal cannula was inserted and atropine (0.02mg) was administered subcutaneously to reduce mucous secretions.

Usually only the sciatic nerve was dissected except in 4 experiments where it was further dissected into its common peroneal and main tibial branches. Laminectomies were performed at vertebral levels T13 and L1 and mice were placed in a stereotactic frame with vertebral clamps on the T12 and L2 vertebrae. The mice were paralysed using the neuromuscular blocking agent Pavulon (diluted 1:10 with saline then 0.1 ml dose initially followed by 0.05ml doses every hour) and artificially ventilated at 70 breaths per minute (and a tidal volume of approximately 0.2 ml). Expired carbon dioxide levels were measured using a Capstar CO₂ analyser (IITC Life Science). The temperature was monitored using a rectal probe and maintained at 37°C using a heat pad underneath and a heat lamp above the mouse controlled by the output from the temperature probe. The electrocardiogram (ECG) was monitored using clips placed on the ear and rear foot and small doses of ephedrine (dosage to effect) were given in
response to slight drops in heart frequency. Due to possible central effects of ephedrine this was used only when necessary and usually this was restricted to a single dose when the mouse was initially transferred into the frame following surgery. Records were kept regarding the drugs given to each animal. There appeared to be no difference between those mice receiving the ephedrine and those that did not in terms of mean frequency/current relationships. Thus we believe that this limited dosage did not significantly affect our results.

Once paralysed, the same dosages of anaesthesia as previously used throughout the surgical preparation were given at 20 minute intervals (as during the preparation). Additionally the heart rate was monitored and in the event of any increase supplemental dosages of anaesthesia were administered.

The dissected nerves were placed on bipolar stimulating electrodes and a silver ball recording electrode was placed on the dorsal part of the spinal cord to record the incoming volleys from stimulation of the peripheral nerves. The dura was gently torn away from a small region on the dorsal surface of the spinal cord using fine watchmaker forceps. A sharp glass microelectrode filled with 2M potassium acetate (with resistances of 10 – 24 MΩ) was used to record intracellularly from spinal motoneurons identified by antidromic action potentials elicited by stimulation of one of the peripheral nerves. Using an electronic microdrive the microelectrode was slowly lowered into the spinal cord through the dorsal columns towards the ventral horn, tracking the extracellular field potentials of the motoneurons evoked by stimulation of the peripheral nerves. Successful impalement of a motoneuron was confirmed by the presence of an all-or-nothing antidromic action potential from stimulation of the sciatic nerve (or its common peroneal or tibial branches). Changes in membrane potential were recorded and amplified (10x) using the Axoclamp 2A amplifier (Axon Instruments Inc., Union City, CA, USA) using either bridge (for AHP measurements) or discontinuous current-clamp (DCC) mode (~3kH). The output was further amplified (10x for a low gain view and 100x for a high gain view) and then filtered (high pass 5kH for low gain view and low pass 1H for high gain view) using custom made amplifiers and was digitised using the 1401 analogue to digital converter (Cambridge Electronic Design, UK) and recorded using the Spike 2 software (Cambridge Electronic Design, UK).
Only motoneurons with antidromic spike potentials of > 50 mV, membrane potentials of -55 mV or more hyperpolarized (confirmed on exit from the cell) and repetitive firing in response to long lasting depolarizing current pulses were accepted for analysis (repetitive firing was usually not possible in cells with more depolarized resting potentials). Deterioration in the quality of the impalement (usually due to cardiovascular and respiratory associated movements) was evident as a clear reduction in the membrane potential below this level and failure to evoke repetitive firing. This was normally the point for termination of data collection and this is the reason why not all parameters were measured in every cell. Movements of the spinal cord are the biggest problem when trying to obtain stable intracellular recordings in the mouse. These were greatly reduced by raising the vertebral clamps until the spinal cord was kept at the same level as the head (or even slightly higher) and then gently stretching the cord by moving the rostral and caudal vertebral clamps apart from each other (without compromising the blood supply to the cord). By making only a hemi-laminectomy over the side of the spinal cord from which recordings were made the contralateral part of the lamina acted as a type of pressure plate further reducing movements.

Measurement of the post-spike afterhyperpolarization. The action potential in response to a short depolarizing current pulse (~1 ms duration) was recorded and averaged using Spike-2 (at least 10 sweeps). The exact return of the afterhyperpolarization to baseline was not always easy to detect, thus we also measured the time for the membrane potential to return to 1/3 of the peak amplitude of the afterhyperpolarization (AHP2/3).

Measurement of input resistance (RIN). The input resistances (using a 1-4 nA hyperpolarizing pulse of ~20 ms duration) were recorded in DCC mode and averaged using Spike-2 software.

Frequency-current (f-I) relation. This relationship was determined by creating frequency-current curves in one of 2 ways:

i) 400 ms square depolarizing pulses were given through the microelectrode evoking repetitive firing. This current was gradually increased for each pulse and the resulting
Increases in firing frequency was calculated by measuring the inter-spike intervals between the first and the second spike, between the second and the 3rd and between 3 consecutive spikes (and an average taken for the 3) occurring at 100 ms and 300 ms after the onset of the pulse. These intervals were expressed as instantaneous firing frequencies (Hz) and the frequency-current relationship given as the increase in firing frequency per nA current (Hz/nA).

ii) Slow triangular ramps of current were given through the microelectrode and the response was measured. Changes in firing frequency (measured in spikes per second) were plotted as frequency-current curves and the f-I relationship could be determined.

Muscle twitch contraction times. In 2 mice we recorded the muscle twitch contraction time of the triceps surae muscles. In these mice the sciatic nerve was dissected, ligated and cut proximally. Distally the peroneal branch and the branches of the tibial nerve innervating muscles other than the triceps surae were sectioned; thus stimulation of the sciatic nerve resulted in a selective contraction of the combined triceps surae muscles. The knee region (femur and tibia) and the ankle were fixed by pins, and the Achilles tendon attached to a strain gauge.

Immunohistochemistry for Cav1.3 channels. Following the electrophysiological investigations, some of the mice were perfused and subsequently the spinal cord was processed for immunohistochemistry to verify the presence of Cav1.3 immunoreactivity in the spinal cord and large ventral horn neurons. The protocol was the same as recently described (Sukiasyan et al., 2009). Here only a short description will be given, focussing on the antibody against Cav1.3 and relevant controls. The rabbit anti- Cav1.3 antibody, purchased from Millipore-Chemicon is an affinity purified, polyclonal antibody raised against amino acid residues 859-875 of the Cav1.3 subunit of the rat brain voltage-gated calcium channel (accession No.: P27732). It recognizes all forms of Cav1.3 subunits from the voltage-gated calcium channel and does not cross react with any other calcium channel antigens tested so far (manufacturer’s data sheet). The specificity of the antibody was verified previously by Western blot on rat spinal tissue (Sukiasyan et al., 2009). Control staining was performed with Cav1.3 antibodies preadsorbed with a sufficient amount of the corresponding epitope peptides (supplied by manufacturer) or with the primary antibody being omitted; no positive reaction was then detected.
RESULTS

The results of this study are based on intracellular recordings from 41 motoneurons which were antidromically identified from the sciatic nerve (or one of its branches; the tibial nerve or the peroneal nerve, Fig. 1B). For each of these motoneurons the following parameters were recorded: 1) the post-spike afterhyperpolarization (AHP) following an action potential evoked by a short lasting (~1 ms) current pulse, 2) the input resistance, 3) the rheobase, and 4) the repetitive firing evoked by an intracellular rectangular current pulse of \( \geq 300 \) ms duration, and/or a triangular current pulse. As mentioned in the methods it was not always possible to obtain all of these parameters for every cell. However, the motoneurons included in the dataset all demonstrated repetitive firing following intracellular current injections. The resting membrane potentials for the cells included in the study ranged from -55mV to -83mV (mean -66mV, SD 7.7). Repetitive firing was usually not possible in cells with more depolarized membrane potentials. The resting membrane potential (at least within this range) was not correlated with either the AHP duration, AHP amplitude, the f-I relationship, rheobase or the input resistance.

While a full systematic description of synaptic activity is beyond the scope of this paper it should be noted that, in those experiments where the common peroneal and tibial nerve were dissected and stimulated separately, the monosynaptic Ia EPSP could be seen following stimulation of the homonymous nerve at stimulation intensities lower than that required to achieve antidromic activation (Fig 1A). Stimulation of the antagonist nerve produced disynaptic IPSPs (Fig. 1B, here polysynaptic IPSPs are seen in addition). The disynaptic Ia inhibition often required 2 shocks to appear (temporal facilitation) and was very small at membrane potentials more hyperpolarized than -50mv, however, they were greatly enhanced if the motoneuron was depolarized by injecting current. Polysynaptic EPSPs and/or IPSPs were almost always also observed following stimulation of either homonymous or heteronymous nerves. Under this anesthesia spontaneous synaptic activity could also be recorded (Fig 1C).

The AHP following a pulse-evoked spike is illustrated in Fig. 2 A-B. Even under the most stable recording conditions it can be difficult to judge with accuracy when the AHP returns to baseline. Furthermore, the precise return to baseline can sometimes be
obscured by the presence of a late afterdepolarization as has been described in cats (Kernell 1965). Since most of the recordings that we obtained were less stable than normally obtained in the cat and rat (Hultborn, personal observation), we decided to measure the duration from the start of the spike to the time at which the AHP amplitude had returned by 2/3 towards the baseline (AHP_{2/3}) as indicated by the arrows in Fig. 2B and where possible the estimated return of the AHP to baseline (AHP_d). Both measurements were performed from averaged records (n \geq 10 trials).

The distribution of AHP_{2/3} measurements for 38 motoneurons is shown in the frequency histogram in Fig. 2C. The relationship between the two different estimates of AHP-duration from 20 motoneurons for which the AHP_d could be measured (58.2 ± 17.2 (SD) ms) suggests that AHP_d = 1.8 \times (\text{AHP}_{2/3}) + 18 \text{ ms}. This allowed us to estimate the return to baseline for all 38 motoneurons, giving an average of 55.3±14 (SD) ms (see Table 1).

The input resistance was calculated from the voltage drop following a current pulse of 1-4 nA and \geq 20 ms as illustrated in Fig. 3A. The mean was 2.4 ± 1.17 (SD) MΩ with the distribution illustrated in Fig. 3B. Although not systematically investigated, we often noticed a sag following somewhat longer (and often larger) current pulses as illustrated in Fig. 3C (cf Ito and Oshima 1965).

With depolarizing current injections (rectangular or triangular pulses) it was possible to evoke repetitive firing in all of the 41 motoneurons reported here. In some (other) motoneurons with acceptable amplitude (\geq 50 mV) of single spikes evoked by antidromic activation from the sciatic nerve or by a short (1 ms) intracellular current pulse it was not possible to evoke repetitive firing by longer lasting current pulses. In those cases there was often a short burst of action potentials of decreasing amplitude suggesting a rapid inactivation of Na^+-conductance; those motoneurons were not included in the present material. Several motoneurons initially responded with repetitive firing to long lasting current pulses and later developed an inactivation of repetitive firing, while still able to support single spikes. We thus believe that the lack of repetitive firing to long lasting current pulses reflects a pathological deterioration of the motoneuron. The frequency-current (f-I) relationship was determined either from rectangular current pulses of increasing amplitude (Fig. 4), or triangular current pulses (Fig. 5). With rectangular pulses it was possible to measure the f-I-relation for
both the initial inter-spike intervals (1st and 2nd inter-spike intervals in Fig. 4B,C) and
the more adapted frequency at 100 or 300 ms from the start of the current pulse (Fig.
4 A,C). Note the much steeper slope for the 1st interval than for the 2nd and later
intervals; it thus seems that much of the early adaptation occurs already following the
first inter-spike interval. The f-I slope for the “steady state” firing was 5.0 Hz/nA for
the illustrated motoneuron.

With triangular current pulses it was possible to determine the f-I relationship from a
single trial lasting only a few seconds (Fig. 5A). This current pulse injection could be
repeated a few times to check the reliability and determine a mean value for the
recorded motoneuron. It was important to determine how the f-I-relationship obtained
from a triangular pulse compares with the “adapted” (300 ms) f-I relationship
obtained from a large number of rectangular current pulses of different amplitudes.

The f-I slopes obtained by these different methods are illustrated for an individual
motoneuron in Fig. 5 B. The f-I-relationship with rectangular pulses was determined
as discussed in relation to Fig. 3 (open and filled circles in Fig. 5 B). When each inter-
spike interval during the ascending part of the triangular current pulse is plotted (filled
triangles) in relation to the injected current a very similar slope is seen. This was the
case for all 5 motoneurons for which both methods were used. We have therefore
preferentially used the triangular pulses for estimating the f-I-slopes. Since the f-I
slopes were very similar independent of which method was used, we have pooled the
results. Figures 5 C and D illustrate different types of responses to triangular current
pulses. Here we illustrate the most typical responses both during the ascending and
descending phases of the triangular pulses. In the motoneuron of Fig. 5 C there is a
linear increase in firing frequency during the ascending phase (17.7 Hz/nA; black
squares). Here the slope is very similar for the descending phase (16.5 Hz/nA; grey
triangles). Fig. 5 D illustrates the more common response with a significantly lower
firing frequency for the same current intensities during the descending phase, thus
reflecting an ongoing adaptation.

In some cells, in which higher currents were injected, a steeper slope (an acceleration)
developed at the end of the ascending phase (Fig 6 A). This “secondary range” is
assumed to reflect the recruitment of persistent inward currents (PICs; Powers and
Binder 2001; Hultborn et al 2004; Heckman et al. 2005). In addition to the clear
secondary range in the f-I slopes we also observed a number of other responses to the
current injections consistent with the activation of PICs. It was occasionally seen that
the de-recruitment occurred with lower current intensities than for the initial
recruitment (ΔI = 1.5 nA in Fig. 6 B). The difference in current at recruitment and de-
recruitment may reflect the size of the PIC (Hounsgaard et al. 1988a; Bennett et al.
2001; Button et al. 2006).

In some cells the firing suddenly ceased during the ascending part of the triangular
current injection. This usually occurred at high firing frequencies and appeared to be
accompanied by an apparent jump in membrane potential. The response pattern
illustrated in Fig. 6 C is likely to reflect the same recruitment of PICs. In this case
there was a repetitive firing during the beginning of the ascending phase, but suddenly
the firing was interrupted (spike inactivation) and the membrane potential “jumped”
around 14 mV. It thus seems likely that the activation of PIC contributed to a sudden
spike inactivation. As far as we are aware this pattern of response has not been
described for cat or rat motoneurons, but is likely to reflect a transition from the state
with full spikes without inactivation to the fully inactivated state. As in the present
example, the firing sometimes returned during the descending phase. Whilst the cell is
firing spikes it is hard to determine the exact contribution of PICs to this apparent
jump in membrane potential versus the return to baseline after the AHP. Illustrated in
Fig. 6 D is a “jump” in the membrane potential in a motoneuron in which the
repetitive firing had vanished following a long lasting intracellular recording. During
the ascending phase of a triangular current pulse (at 12.5 nA) there is a sudden “jump”
in the membrane potential (by 10.5 mV – from a membrane potential of -48 mV). A
somewhat similar jump (down) is then seen during the descending phase. When the
membrane potentials are superimposed for the ascending and descending phases - for
the same amount of injected current (Fig. 6 E) - the “counter-clockwise” hysteresis of
the membrane potential can be appreciated (i.e. the jump down occurs with a smaller
current than the jump up). A similar jump could also be seen following a rectangular
current pulse (Fig. 6 F; same neuron as in D-E) after a latency of around 140 ms.
These findings are also interpreted as a sign of an activation of PICs (plateau potential)
by the current pulse, and the latency in Fig. 6 F is likely due to the slow activation of
the PIC-conductance (cf Carlin et al. 2000).
Since the secondary range was usually only seen at high firing frequencies (above 150 Hz) and the jumps in membrane potential (together with spike inactivation) occurred at similarly high firing frequencies it was hard retrospectively to determine proportions of cells with PICs as not all motoneurons had been pushed to these extremes. However, in 13 motoneurones that were challenged with triangular current injections beyond the point at which cell fired at 150 Hz 10/13 cells showed secondary range firing. The transition from primary to secondary range almost always occurred between 140 and 185 Hz (mean 162 Hz, SD 11.4 Hz) and this was usually consistent across multiple trials, regardless of speed of current injection. If firing persisted beyond this point into the secondary range the firing frequencies reached between 223-433 Hz (mean 327 Hz, SD 59.9 Hz) before the spikes finally inactivated. All 3 cells that did not enter the secondary range of firing all 3 ceased firing with apparent jumps in membrane potential. The firing frequencies at which the spikes inactivated (150, 160 and 165 Hz) are consistent with the ranges at which the transition from primary to secondary range of firing occurred in the other cells. In the cells showing a secondary range of firing occasionally on some trials the spiking would fail with this apparent jump. This happened in 9 cells and this always occurred within the same range of firing frequencies where the transition from primary to secondary range occurred on previous trials in the same cell. Thus we believe that the this sudden cessation of firing (spike inactivation) accompanied by the apparent jump in membrane potential indeed represents the onset of a plateau potential mediated by persistent inward currents. Thus, in conclusion, all of these 13 motoneurons showed signs of persistent inward currents.

As plateau potentials in motoneurons have been partly attributed to the activation of Cav 1.3 channels we have also confirmed the presence of Cav 1.3 immunoreactivity in the spinal cord of some of the mice in this study (not illustrated). Immunohistochemistry using the antibody directed against the voltage-gated calcium channel Cav 1.3 produced widespread labelling in the grey matter of the spinal cord, including the dorsal horn, the intermediate zone and the ventral horn. In the ventral horn both somata of the large ventral horn neurons (presumably motoneurons) and their dendrites were clearly labelled. This labelling pattern is similar to that recently described for the rat spinal cord (Sukiasyan et al 2009) and also confirms the
motoneurone labelling seen in another mouse strain (Balb/C) with a different antibody (Jiang et al 1999).

It has been suggested that the duration of the AHP determines the minimal firing frequency during repetitive firing (see further in the Discussion). We have therefore determined the lowest firing frequencies from rectangular current pulses (with the lowest possible amplitude for repetitive firing). Fig. 7 illustrates the distribution of the “minimum firing frequencies” as a function of the AHPd. The variation in inter-spike intervals at the lower frequencies was indeed large, and it was often difficult to determine whether a long final interval represents a “true” interval during repetitive firing, or rather an interval after the end of repetitive firing and an accidental discharge due to a random synaptic input or a mechanical instability. We therefore omitted spike intervals >75 ms as they typically could not be confirmed at repeated trials.

As expected from previous results from the cat and the rat (see Discussion), there is a correlation ($P \leq 0.05$) between the estimated AHPd and the minimum firing frequency (Fig 7). Although the scatter was large, it is important to note that, as a whole, the range of “minimum firing frequencies” was clearly related to the range of AHP durations, although there was a tendency for the longest interspike intervals (lowest frequencies) to be somewhat shorter than would be expected from the estimated duration of the AHPs.

For the cat and the rat it has been described that the muscle twitch time corresponds to the AHP-duration (for references, see the Discussion). As a basis for a comparison of the relation between twitch time and AHP duration across species, the muscle twitches to single action potentials in the supplying nerve were recorded from the combined triceps surae muscle. The contraction time (CT) and twitch duration were 12 ms and 47 ms for the triceps surae. Figure 2 D illustrates the similarity between the twitch time course (combined triceps surae) and the AHP for the motoneuron illustrated in Fig. 2 A-B.
DISCUSSION

The present study demonstrates the feasibility of intracellular recording in the adult mouse in vivo preparation. Using the present experimental protocol we were able to obtain intracellular recordings for 1 – 6 hours after the surgical procedure (lasting 2-3 hours). Typically we could impale 2 – 8 motoneurons per animal with stable recordings (with membrane potentials better than -50 mv) for 5-10 minutes. The mechanical stability is certainly less than in the cat or rat, and this makes it more difficult to obtain recordings without artefacts originating from the pulse pressure and/or artificial respiration. The most stable recordings occur within the first 2 hours after the mouse was placed in the frame and artificially ventilated. Movement artefacts, usually cardiovascular, then gradually worsen over the course of the next few hours. The ability to record post-synaptic potentials from defined spinal circuits make this a good model to investigate the final compensation in the adult mice with mutations affecting the normal development of these circuits or specific neurotransmitter receptors.

Our results suggest that PICs are a common feature of mouse spinal motoneurons and are evoked at firing frequencies greater than 145 Hz, i.e. where the secondary range firing is initiated. This is much higher than seen for cat and rat and assists the motoneurones to fire at frequencies up to 430 Hz. PICs serve as a controlled “amplifier” of classical synaptic input to motoneurons, and appear to be the mechanism underlying the steeper f-I-relationship in the secondary range (Powers and Binder 2001; Hultborn et al. 2004; Heckman et al. 2005). These PICs are to a large extent mediated by nifedipine-sensitive voltage-gated L-type calcium channels (Hounsgaard and Kiehn 1989). As the PICs could be triggered at a relatively hyperpolarized levels, close to firing threshold (Hounsgaard et al. 1988a; Hounsgaard and Mintz 1988) they were attributed to a particular subgroup of L-type calcium channels, CaV1.3 (Alaburda et al. 2002). While it has been firmly established that non-inactivating components of Na⁺ channels also contribute to the PICs (Harvey et al 2006) it has not yet been possible to identify which subgroup of Na⁺-channels are responsible. The Cav 1.3 channel has been immunohistochemically labelled on mice spinal motoneurones (Balb/C strain, Jiang et al. 1999, Carlin et al. 2000) which was confirmed for the C57BC strain in this study.
Our current observations of PICs with Hypnorn/Midazolam anaesthesia demonstrates the feasibility for investigations of PICs in anaesthetized mouse preparations. This is important as other anaesthetics, in particular barbiturates, have been demonstrated to reduce/abolish the PIC in rats (Button et al 2006) cats (Hultborn and Kiehn 1992) and in vitro in turtle (Hounsgaard et al. et al. 1986; Guertin and Hounsgaard 1999). However in the rat Button et al. (2006) have described PICs under ketamine and xylazine anaesthesia being as strong as in unanaesthetized decerebrate preparation. Under ketamine/xylazine anaesthesia however, rat motoneurons had smaller AHPs than in decerebrate preparations and required more current to reach firing threshold but the f-I slopes, the presence of PICs and their amplitude was not affected by the ketamine/xylazine. (Button et al 2006). The exact effect that our protocol of Hypnorn and Midazolam has on the intrinsic properties of neurones is unknown. However it is unlikely that mice will survive the associated stress and blood loss following a decerebration rendering the decerebrate unanaesthetized preparation impractical for routine recording in vivo. Thus we believe this anaesthetic regime represents a good alternative for comparisons between experiments using the same anaesthetic in transgenic mice in which PICs may be affected such as the SOD-1 mouse mutants of ALS.

Our study provides data for comparisons not only with future data to be obtained in transgenic mice but also provides data for a cross-species correlation between motoneuron properties and their muscle unit contraction properties. In the present material of motoneurons supplying the sciatic nerve we found that the input resistance was around 2.4 MΩ (Table 1) which is far higher than in the cat and rat (see references listed with Table 2). This is as would be expected from the size of the mouse spinal motoneurons (McHanwell and Biscoe 1981; Hardesty 1902), which are smaller than in the rat and cat (see references listed with Table 2). The firing during steady state easily reaches > 300 Hz with an f-I relationship of around 13 Hz/nA. These values are far higher than seen for the cat and the rat (see references listed with Table 2). The instantaneous frequency for the first interval reached 800-1000 Hz (an interspike interval of 1 - 1.3 ms; see Fig. 4), and it is then seen that the second spike takes off from the early after-depolarisation following the initial spike. This short interval thus corresponds to the “doublets” described both in the cat (Kirkwood et al. 1996); the rat (Gorassini et al. 2000) and the human (as judged by the interspike
interval of motor unit potentials in EMG-recordings; see Kudina and Alexeeva 1992; Garland and Griffin 1999), although with much shorter intervals in the mouse. All in all, these basic features are qualitatively similar to that which has been described for the cat and the rat. However, there are significant quantitative differences which are further specified in Table 2, and in the following sections on the relation between the AHP duration, minimum firing frequency and the muscle contraction properties in the mouse and other species.

The AHP – firing rate relationship. Are the lowest firing frequencies in individual motoneurons matched with their AHP duration? The original observations of a strong correlation between AHP duration and minimum firing frequency stem from the work in the cat by Kernell (1965c; see also Wienecke et al. 2009). The literature regarding motoneurons in the cat, rat and turtle spinal cord all suggest that the firing rate is strongly influenced by the conductances contributing to the slow component of the AHP that follows the spike potential (see Powers and Binder 2001; Kernell 2006; Stauffer et al. 2007 for critical reviews with reference to all original work). In the present material (Fig. 7) there is a trend for an AHP /Fmin relationship with higher Fmin (shorter interspike intervals) with shorter AHP durations. Despite the large scatter, likely depending on the difficulty to determine the minimal firing frequency, this relationship reached statistical significance (p < 0.05). However, the ranges of AHP durations (41 – 76 ms) and the Fmin (14 – 41 Hz, corresponding to intervals of 24 – 73 ms) are certainly consistent.

The description of the relationship between the AHP and the f-I-slope also originate from the work by Granit and Kernell (Granit et al. 1963; Kernell 1965a,b,c). Experiments in which the AHP amplitude was reduced by various blockers (application of 5HT, or AHP K+ -conductance blockers) have indeed shown an associated increase of the f-I-slope (Zhang and Krnjevic 1987a,b; Hounsgaard et al. 1988b; Hultborn et al. 2004). The steeper f-I-slopes in the mouse (present material) than in other species (Table 2) may simply reflect that the total AHP current is smaller in the smaller sized mouse motoneurons.

Altogether, the mouse motoneuron seems to be endowed with properties to sustain firing with higher frequencies than seen for other species investigated previously. As
discussed in the following section, the necessity for high firing rates may be related to
the very fast contraction times of the innervated muscles.

A comparison of motoneuron discharge properties and twitch contraction among
different species.
It is well known that muscles are comprised of many different types of motor units
and that the properties of the muscle units are matched with the motoneurons that
innervate them (see Burke 1981). Eccles et al. (1958) described (in the cat) that slow
and fast muscles were innervated by “tonic” and “phasic” motoneurons, respectively.
The tonic motoneurons were found to have longer lasting AHPs than the phasic
motoneurons, thus being matched to the contraction times of the fast and slow
muscles. Later Kernell (1965a) described a strict correlation between the duration of
the AHP and the $F_{\text{min}}$ across the spectrum of fast and slow motor units. Those studies
focused on differences between fast and slow muscles/motor units within the same
species. In this section we will discuss the information regarding the relationship
between AHP duration, minimum firing frequency and twitch duration across species
– from mouse to man.

A number of papers by A.V. Hill have focused on the relationship between the size of
an animal and the speed of muscle contraction (summarized in Hill 1950). One of the
characteristics of movements (such as locomotion) in smaller animals is the rapidity
as compared with those of larger animals that would require considerable differences
in muscle contraction speed/contraction time. In Table 2 we have summarized a
number of parameters for the motoneurons ($R_{\text{IN}}, AHP_{D}, F_{\text{min}}, F_{\text{max}}, f-I$-slope, and the
diameter of the largest motoneurons) as well as the muscle (units) (twitch contraction
time (CT) and twitch duration) for the mouse, rat, cat and human. The isometric
contraction time (time to peak) and twitch duration indeed differs markedly, with the
shortest times in the mouse and the longest in humans (mice < rats < cats < humans;
for references see Table 2). Consequently the firing rates needed for force gradation
by summation of muscle unit twitches are the lowest for humans and the highest for
mice. Our present findings (Table 1) are in accordance with this prediction. The
shorter duration of the AHP as well as the steeper f-I-slope in the mouse than in the
other species supports the view of the AHP as a major factor in the control of
motoneuronal discharge properties.
In Fig. 8 we illustrate the pivotal relationship between the duration of the AHP and the muscle twitch (contraction time as well as twitch duration) across the species. Using the median from several studies (specified in Table 2) there is indeed a striking relationship suggesting that the relationship between muscle twitch and AHP duration exists not only for slow and fast muscles within the same species, but also between species.

Even though the results quoted in Table 2 all relate to “mixed” (fast and slow) hind limb muscle (with one exception, the human first dorsal interosseus) there are several difficulties in finding comparable data. In some material the motor units are classified as fast, intermediate and slow. In other publications (as in our present material) there was no grouping in fast and slow units. In most publications the sampling method of motoneurons would favor “fast” motoneurons. For Table 2 we have focused on the mean values for mixed hind limb muscles (values for the slow soleus muscle have not been quoted; see further in the legend of Table 2). The AHP values for human subjects are estimated from the “interval death rate transform” of motor unit discharge ISI (inter-spike interval) histograms (Matthews 1996; Powers and Binder 2000; MacDonell et al. 2007). Since the data have originated from several studies with different techniques there is indeed a large variation, and therefore we have also indicated the range of values in Fig. 8 (quoted in Table 2).

To conclude;

This adult intracellular in vivo model is a feasible model in which to study the basic circuitry of the spinal cord as well as the intrinsic properties in spinal motoneurons of adult mice. This makes this model viable for investigating such features in transgenic mice in which the development of normal circuitry has been interrupted or in which the intrinsic properties of neurons may be altered. The present data demonstrate that mouse spinal motoneurons share many of the same properties that have been demonstrated previously for cat, rat and human motoneurons. The shorter AHP duration, steeper f-I slopes and higher $F_{\text{min}}$ and $F_{\text{max}}$ than in rats, cats and humans are likely to be tailored to the characteristics of the mouse muscle contraction properties.
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Figures and Legends:

Figure 1. A: Upper trace shows the membrane potential of a tibial motoneurone and the lower trace shows the incoming volley from stimulation of the tibial nerve recorded from electrodes placed on the dorsal surface of the spinal cord. Dotted lines indicate stimulation of the nerve at an intensity lower than that required to achieve an antidromic spike in the neuron. Here the monosynaptic Ia EPSP (dotted line, upper trace) can be seen at longer latency than the antidromic spike (solid line, upper trace). Traces are an average of at least 10 sweeps. B: Upper trace shows the membrane potential of a tibial motoneurone and the lower trace shows the incoming volley from stimulation of the peroneal nerve recorded from electrodes placed on the dorsal surface of the spinal cord. In the upper trace inhibition can be seen starting at latency consistent with being disynaptic. Traces are an average of 12 sweeps. C: Spontaneous synaptic activity recorded intracellularly from a motoneuron.

Figure 2. Intracellular recording of the spike and post-spike AHP from an antidromically identified motoneuron evoked from intracellular current injection. A-B: An action potential (A) and the AHP (B) displayed at a different amplification. The downward arrows in B illustrate to where the AHP duration was measured (AHP_{2/3} and AHP_{d}, respectively). C: Frequency histogram for the AHP_{2/3} durations for 38 motoneurons. D: Comparison between the time course of an AHP (displayed downwards, from B) and the twitch contraction of the combined triceps surae muscle (displayed upwards); see further in the text. Time and voltage calibrations are shown separately for A and B. The time calibration is the same for the twitch and AHP in D; the twitch and AHP amplitudes are normalized to their peak values.

Figure 3. Input resistance and sag properties. A: The voltage response (upper trace) to a triangular current injection (lower trace). B: Frequency histogram of the input resistance from records as in A for 34 motoneurones. C: Example of a motoneuron with a pronounced sag seen with a longer rectangular hyperpolarizing current pulse. Voltage, current and time calibrations are shown separately for A and B.

Figure 4. Repetitive firing evoked by rectangular depolarizing current pulses. A: Upper traces show repetitive firing to two levels of depolarizing current pulses shown in the lower trace. B: Expanded record of the lower trace in A. Note the very short
first interval (black arrow) with a much longer 2nd interval (gray arrow). C: Diagram showing the relationship between the intensity of current and the instantaneous frequency for the first and second intervals and the mean for 3 inter-spike intervals at 100 and 300 ms after the start of the current pulse.

**Figure 5.** Repetitive firing evoked by triangular current pulses. A: Upper trace is the intracellular recording of the repetitive firing and the lower trace shows the injected current. B: The relationship between the instantaneous firing frequency of a motoneuron and the injected current obtained by rectangular current pulses (filled and open circles) and the ascending part of a triangular current pulse (triangles). C and D: f-I relationships in 2 different motoneurons obtained by triangular current pulses.

**Figure 6.** Evidence for plateaux potentials. A: The f-I relationship of a motoneurone showing a “secondary range” of firing (steeper slope) as the injected current was increased beyond 9 nA. B: Upper trace (AC-filtered in this example) is showing the membrane potential and the lower trace the injected current. Note the lower current at derecruitment than for recruitment of firing. C: Arranged as in B, but from another motoneuron (here with DC recording). The response to triangular current injection in a motoneurons in which the spike suddenly inactivates and the membrane potential shows a step depolarization (upper trace) D: The response to triangular current injection in a motoneuron in which the spike had spontaneously inactivated following a prolonged recording. The lower trace shows the current injection. E: Same records as in D, but with the ascending and descending phases of the triangular current injection superimposed (for upper trace the gray line is the ascending phase, and the black is the descending phase). The lower trace shows the current injection. F: Responses to a square pulse current injection in the same motoneuron as in D-E. The lower trace shows the current injection.

**Figure 7.** The relationship between AHPd duration and minimum firing frequency for 22 motoneurons.

**Figure 8.** The relationship between twitch contraction time/ twitch duration and AHP duration for human subjects and cats, rats and mice. The values are the medians of the studies presented in Table 2 (ranges given by the bars). The relationship with the
contraction time is shown with open circles, and the twitch duration time with filled
circles.

**Table 1.** Summary of the values obtained in the present study. Note that the f-I slope
was obtained for all recorded motoneuron (a criteria for the inclusion), while the
other parameters were not obtained for all motoneurons. The AHPd could be
measured in 20 motoneurons, while the AHP_{2/3} was measured in additional 18
motoneurons. From the relation between the AHPd and AHP_{2/3} in those 20
motoneurons (AHPd = 1.8( AHP_{2/3}) + 18 ms; see text), it was possible to estimate the
AHPd for the remaining 18 motoneurons (“AHPd calculated”).

**Table 2.** A comparison of motoneurone properties and twitch contraction among
different species. All values are obtained for mixed lower limb muscles (with slow
and fast units; values from the soleus muscle are not quoted), with the exception of
the first interosseous muscle in humans; Gossen et al 2003; Spiegel et al 1996).

Numbers in *italics* reflect that they are estimated from records or graphs in the cited
reference, rather than specifically given in the text or in tables. The numbers in
parenthesis refer to the references given below. The duration of the AHP in humans
were estimated from the “interval death rate transform” of motor unit discharge ISI
histograms (Matthews 1996; Powers and Binder 2000; MacDonell et al 2007). The
F_{Max} is usually not given with absolute numbers, but are extrapolated from the text or
illustrations in the cited reference. No references are given for F_{Max} in humans; the
range from a large number of publications is large, partly depending on the difficulties
in following single motor units during maximal contraction. Usually the cited
estimates range from 20 to 40 Hz. The size of the motoneurons has been
measured/estimated in many different ways. Here we have only given a single
reference, with the strength that all estimates (for all species summarized here and
many others as well) were obtained in the same way (Hardesty 1902). Cited
references: a) Gossen et al 2003 ; b) MacDonell et al 2008; c) Hardesty 1902; d) Van
Cutsem et al 1997; e) Spiegel et al 1996; f) Oda et al 2007a; g) Oda et al 2007b; h)
1965a; o) Kernell 1965c; p) Burke 1967; q) Emonet-Denand et al 1988; r) Denny-
Brown  1929; s) Bakels and Kernell 1993a; t) Button et al 2006; u) Gardiner and
A

Number of motoneurons

Duration of AHP 2/3 amplitude

C

D

Relative Amplitude %

Twitch

AHP

20ms

20mv

30ms

2/3

1/3

1mv

20ms
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