Activation of group I metabotropic glutamate receptors modulates locomotor-related motoneuron output in mice

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Running title: Group I mGluR-mediated modulation of mouse motoneurons

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Abstract

Fast glutamatergic transmission via ionotropic receptors is critical for the generation of locomotion by spinal motor networks. In addition, glutamate can act via metabotropic glutamate receptors (mGluRs) to modulate the timing of ongoing locomotor activity. In the present study, we investigated whether mGluRs also modulate the intensity of motor output generated by spinal motor networks. Application of the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG) reduced the amplitude and increased the frequency of locomotor-related motoneuron output recorded from the lumbar ventral roots of isolated mouse spinal cord preparations. Whole-cell patch-clamp recordings of spinal motoneurons revealed multiple mechanisms by which group I mGluRs modulate motoneuron output. Although DHPG depolarised the resting membrane potential, and reduced the voltage threshold for action potential generation, the activation of group I mGluRs had a net inhibitory effect on motoneuron output which appeared to reflect the modulation of fast, inactivating Na⁺ currents and action potential parameters. In addition, group I mGluR activation decreased the amplitude of locomotor-related excitatory input to motoneurons. Analyses of miniature excitatory postsynaptic currents indicated that mGluRs modulate synaptic drive to motoneurons via both pre and post-synaptic mechanisms. These data highlight group I mGluRs as a potentially important source of neuromodulation within the spinal cord which, in addition to modulating components of the central pattern generator for
locomotion, can modulate the intensity of motoneuron output during motor behaviour. Given
that group I mGluR activation reduces motoneuron excitability, mGluRs may provide
negative feedback control of motoneuron output, particular during high levels of
glutamatergic stimulation.

Key words: spinal cord, motor control, neuromodulation
Introduction

Neuromodulation is an important determinant of neuronal activity, endowing networks with the functional flexibility that is critical to the generation of complex and adaptable behaviours. Within motor systems neuromodulation is important for the control of rhythmic motor behaviours, such as locomotion, which must be adapted to suit the varied biomechanical and metabolic demands of different states, developmental stages or environments. During locomotion the basic timing and pattern of motoneuron activity is set by networks of local interneurons called central pattern generators (CPGs; Goulding 2009; Grillner 2006; Kiehn et al. 2010). Motor commands originating from locomotor CPGs are sent to motoneurons via pre-motor interneurons that utilise fast-acting ionotropic receptor-mediated neurotransmission (Cazalets et al. 1996; Hochman and Schmidt 1998; Orsal et al. 1986; Shefchyk and Jordan 1985). In contrast, neuromodulatory signalling affecting the output of the locomotor CPG typically involves the activation of slower-acting metabotropic receptors (Grillner 2006). Neuromodulatory inputs originate from well characterised ‘extrinsic’ supraspinal systems (e.g. brainstem raphe nuclei and locus coeruleus, reviewed by Heckman et al. 2009; Rekling et al. 2000; Schmidt and Jordan 2000) and less studied ‘intrinsic’ intraspinal neuromodulatory systems (Alaburda and Hounsgaard 2003; Dale and Gilday 1996; El Manira et al. 2008; Katz and Frost 1996; Zagoraiou et al. 2009).

Within spinal motor circuitry glutamate is best known for mediating fast, excitatory
synaptic transmission between CPG interneurons (Grillner 2006) and for transmitting locomotor drive from last order interneurons to motoneurons via the activation of ionotropic receptors (Cazalets et al. 1996; Fetcho et al. 2008; Hochman and Schmidt 1998; Orsal et al. 1986; Roberts et al. 2008; Shefchyk and Jordan 1985). However, glutamate may also act as an important intrinsic modulator of locomotor CPGs due to parallel activation of metabotropic receptors (Alaburda and Hounsgaard 2003; El Manira et al. 2002; Nistri et al. 2006). Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors which exist as eight different subtypes (mGluR1-8). These subtypes can be categorised into three groups (I - III) based on sequence homology, pharmacological profiles, and coupling to intracellular signalling pathways (Pin and Duvoisin 1995). mGluRs regulate neuronal activity throughout the CNS by modulating both the intrinsic properties of neurons and synaptic transmission. Group I mGluRs generally facilitate neuronal activity via postsynaptic mechanisms while group II and III mGluRs commonly inhibit activity by acting as pre-synaptic autoreceptors (Anwyl 2009; 1999; Pin and Duvoisin 1995).

In the spinal cord the activation of mGluRs, particularly group I mGluRs, has been shown to modulate locomotor networks that control swimming in lampreys (Krieger et al. 1998) and *Xenopus* tadpoles (Chapman and Sillar 2007) and walking in rats (Taccola et al. 2004). The role of mGluRs in locomotion is best described for the lamprey where group I mGluR activation leads to both short- and long-term increases in the frequency of locomotor activity.
(Krieger et al. 1998; Kyriakatos and El Manira 2007). These effects involve the modulation of synaptic transmission within the locomotor CPG (Kettunen et al. 2005; Kyriakatos and El Manira 2007), the depolarisation of spinal neurons via the blockade of a leak current (Kettunen et al. 2003) and the enhancement of NMDA-mediated currents (Krieger et al. 2000; Nanou et al. 2009). Similarly, activation of group I mGluRs increases the frequency of fictive swimming in *Xenopus* tadpoles via a reduction in inhibitory transmission (Chapman et al. 2008; Chapman and Sillar 2007).

Prior to the present study, analyses of the effects of mGluR activation during mammalian locomotion were limited to studies of isolated neonatal rat spinal cord preparations. These studies have revealed complex roles for group I mGluRs in mammals where the application of group I mGluR agonists either disrupts locomotor activity completely or slows it down (Taccola et al. 2004), while group I specific, but not general, mGluR antagonists also decrease the frequency of locomotor activity (Taccola et al. 2003; 2004). The cellular and synaptic effects of group I mGluR activation are also diverse in the rat with data supporting both inhibition (Marchetti et al. 2003) and enhancement (Marchetti et al. 2005) of synaptic transmission, and studies reporting motoneuron depolarisation associated with either no change (Marchetti et al. 2003) or an increase in input resistance (Marchetti et al. 2005). Given the complexities and unresolved roles of mGluR-mediated modulation in the mammalian spinal cord, further studies of the effects of group I mGluR activation on the function of
spinal networks and spinal neurons are needed. Furthermore, although it has been demonstrated that mGluRs can modulate locomotor rhythm generation, it remains to be determined whether mGluRs also modulate motoneuron firing and hence the intensity of locomotor-related output, as has recently been shown for other intrinsic modulatory systems (Miles et al. 2007; Zagoraiou et al. 2009). In the present study we have therefore investigated the effects of group I mGluR activation on the output of motoneurons in isolated mouse spinal cord preparations. We first demonstrate that the activation of group I mGluRs can modulate motoneuron output during locomotor activity. We then reveal cellular mechanisms by which activation of group I mGluRs can modulate both the synaptic excitation and intrinsic excitability of motoneurons and hence regulate the strength of motor outflow from the CNS.

Methods

In vitro whole spinal cord preparation

All methods required to obtain tissue for in vitro experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Spinal cord preparations were obtained from post natal day (P)2 – 6 C57BL/6 mice using techniques similar to those described previously (Jiang et al. 1999). In brief, animals were killed via cervical dislocation,
decapitated and eviscerated before spinal cords were isolated from the mid-cervical to upper sacral segments in a chamber containing artificial cerebral spinal fluid (aCSF; equilibrated with 95% O₂ - 5% CO₂, ~ 4°C). For experiments in which whole-cell patch-clamp recordings were performed, following the removal of dura matter, a thin line of pia matter was scraped from the ventral surface of the spinal cord above the motor columns at the level of the 1st or 2nd lumbar (L1-L2) roots to allow access for patch pipettes (Miles et al. 2002).

**Ventral root recordings**

Glass suction electrodes were attached to L1 or L2 ventral roots of isolated spinal cord preparations. NMDA (5 μM), 5-HT (10 μM) and dopamine (50 μM) were added to the aCSF to induce rhythmic, left-right alternating bursts of locomotor-related ventral root activity (Jiang et al. 1999; Miles et al. 2007). Locomotor-related activity was left to stabilise (~1 hour) before subsequent drug applications. Signals were amplified, filtered (30 – 3,000 Hz), rectified and integrated (Qjin Design, ON, Canada) before being acquired at ≥1 kHz using a Digidata 1440A A/D board and AxoScope software (Molecular Devices, Sunnyvale, CA).

**Whole-cell patch-clamp recordings**

Whole-cell patch-clamp recordings were made from motoneurons visualised under infrared differential interference contrast (IR-DIC) microscopy. Most recorded cells were
confirmed to be motoneurons by the presence of antidromic action potentials in response to ventral root stimulation (<100 μA; ISO-Flex stimulator, A.M.P.I., Jerusalem, Israel). Patch electrodes (3–4 MΩ) were pulled on a horizontal puller (Sutter Instrument Company, Novato, CA) from filamented borosilicate glass (World Precision Instruments, Sarasota, FL).

Patch-clamp signals were amplified and filtered (4 kHz low-pass Bassel filter) with a MultiClamp 700B amplifier (Molecular Devices) and acquired at ≥10 kHz using a Digidata 1440A A/D board and pClamp software (Molecular Devices). Details of voltage and current-clamp protocols appear in the results section. Series resistance compensation (60%) was used during all voltage-clamp protocols.

**Data analysis**

Data from ventral root recordings were analysed offline using Dataview software (courtesy of W. J. Heitler, University of St Andrews). Whole-cell patch-clamp recordings were analysed using either Clampfit software (Molecular Devices) or, for mEPSC analyses, the Mini Analysis Program (Synaptosoft, Fort Lee, NJ). Na⁺ current activation and inactivation curves were fit with a Boltzmann function of the form: \( \frac{1}{1 + \exp \left( \frac{(V_{1/2} - V)}{k} \right)} \), where \( V_{1/2} \) is the half activation or half inactivation voltage, \( V \) is the test or conditioning voltage, and \( k \) is the slope of the fitted curve at \( V_{1/2} \). Boltzmann fits were performed using Microsoft Excel as described by Brown (2001). Data are reported as mean ± S.E. Differences
in means were compared using Student’s t-test. The Kolmogorov-Smirnov test was used to
test for differences in mEPSC amplitude or inter-event interval. Values of $p < 0.05$ were
considered significant.

**Solution and drugs**

The standard aCSF solution used for dissecting and recording contained 127 mM NaCl, 3
mM KCl, 1.3 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 26 mM NaHCO$_3$, and 10 mM
D-glucose (equilibrated with 95% O$_2$/5% CO$_2$). The standard patch-clamp pipette solution
contained 140 mM potassium methane sulfonate, 10 mM NaCl, 1 mM CaCl$_2$, 10 mM HEPES,
1 mM EGTA, 3 mM Mg-ATP, and 0.4 mM GTP-Na$_2$ (pH 7.2 – 7.3, adjusted with KOH).

For experiments investigating Na$^+$ currents in isolation, external and pipette solutions were
designed to eliminate Ca$^{2+}$ and K$^+$ currents and reduce Na$^+$ current amplitude to help
minimise voltage-clamp errors. The modified aCSF contained 10 mM NaCl, 105 mM choline
chloride, 3 mM KCl, 30 mM TEA-Cl, 10 mM HEPES, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM
D-glucose, 4 mM 4-AP, 1.5 mM kynurenic acid, 10 µM bicuculline, 5 µM strychnine, 0.5
mM CdCl$_2$ (gassed with 100% O$_2$, pH 7.3 - 7.4 adjusted with NaOH). The pipette solution
contained 100 mM caesium methane sulfonate, 30 mM TEA-Cl, 0.5 or 10 mM NaCl, 1 mM
CaCl$_2$, 10 mM HEPES, 1 mM EGTA, 3 mM ATP-Mg, 0.4 mM GTP-Na$_2$, (pH 7.2 – 7.3
adjusted with KOH, osmolarity adjusted to ~290 mosmol l$^{-1}$ with sucrose).
Pharmacological agents used included: NMDA (N-methyl-D-aspartic acid), 5-HT (5-hydroxytryptamine hydrochloride), dopamine (3,4-dihydroxyphenethylamine hydrochloride), strychnine ((-)-strychnine) and bicuculline (1(S),9(R)-(-)-Bicuculline methiodide), purchased from Sigma-Aldrich (St Louis, MO); DHPG ((S)-3,5-dihydroxyphenylglycine), LY367385 ((S)-(+)-(a-amino-4-carboxy-2-methylbenzeneacetic acid), MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride), and TTX, purchased from Tocris Bioscience (Bristol, UK).

Results

Effects of group I mGluR activation on locomotor-related motoneuron output

To investigate whether group I mGluRs modulate locomotor-related motoneuron output in mice, the group I mGluR agonist, DHPG (5 – 50 μM), was bath applied to in vitro spinal cord preparations in which rhythmic, left-right alternating bursts of locomotor-related activity were induced pharmacologically (5 μM NMDA, 10 μM 5-HT, 50 μM dopamine; Fig. 1A). Application of DHPG (5 μM; 15 mins) led to a significant decrease in the amplitude of bursts of locomotor-related activity recorded from lumbar ventral roots (Fig. 1A, 1Bi & 1Ci). The amplitude of locomotor bursts decreased gradually, reaching a minimum near the end of the 15 minute application of DHPG (18 ± 4.0 % reduction, n = 11; Fig. 1Ci), before returning to
control levels within ~10 minutes of drug washout (Fig. 1Bi). Analyses of ventral root
activity between locomotor bursts (inter-burst activity) revealed no change in baseline
activity when DHPG was applied (data not shown). These data suggest that the modulatory
effects of group I mGluRs on motoneuron output are specific to activity driven by locomotor
circuitry.

Application of DHPG also caused a significant increase in the frequency of
locomotor-related activity (Fig. 1A, 1Bii & 1Cii). The frequency of locomotor bursts
increased gradually, peaking near the end of the 15 minute application of DHPG (23 ± 8.8 %
increase, n = 11; Fig. 1Cii), before returning to control levels within ~10 minutes of drug
washout (Fig. 1Bii). Given the apparent excitatory effects of group I mGluRs on the
locomotor CPG network we also investigated whether DHPG (10 µM) alone or DHPG in
combination with NMDA (5 µM) could elicit locomotor activity. However, locomotor
activity was never observed under these conditions (n = 3).

Given that group I mGluR activation has long-term effects on locomotor activity recorded
from isolated lamprey spinal cord preparations (Kyriakatos and El Manira 2007), we assessed
whether higher doses of DHPG might also have long-term effects in isolated mouse spinal
cord preparations. Application of 10 µM DHPG (n = 9) again led to a significant decrease in
locomotor burst amplitude and a significant increase in locomotor frequency (data not shown),
with both effects of equivalent magnitude to those seen following the application of 5 µM
DHPG. Although the effects of 10 µM DHPG outlasted those of 5 µM applications, no long-term effects were observed. At higher doses (20 – 50 µM), DHPG also decreased the amplitude of locomotor-related bursts. However, this was accompanied by a rapid disruption in locomotor activity, with rhythmic activity ceasing several minutes after drug onset before returning after drug washout (data not shown).

DHPG activates both mGluR1 and mGluR5 subtypes of group I mGluRs. Thus, the activation of either receptor subtype could be responsible for the effects of DHPG on locomotor activity. To determine which receptor subtypes are involved, and to assess whether there is endogenous activation of mGluRs in our preparation, we utilised mGluR1 (LY367385) and mGluR5 (MPEP) specific antagonists. Antagonists were applied for 30 minutes prior to, and then during, the application of DHPG (5 µM, 15 mins; Fig. 2A & 2C).

Application of the mGluR1 antagonist, LY367385 (50 µM), alone had no significant effects on the amplitude or frequency of locomotor bursts (n = 7; Fig. 2A & 2B). However, LY367385 blocked the decrease in burst amplitude and increase in burst frequency normally induced by DHPG (Fig. 2A & 2B). Application of the mGluR5 antagonist, MPEP (50 µM), also had no significant effect on locomotor activity when applied alone (n = 6; Fig. 2C & 2D). MPEP did, however, block the DHPG-induced decrease in locomotor burst amplitude, but had no effect on DHPG-induced increases in locomotor frequency (Fig. 2Cii & 2Dii).

Given that LY367385 is a competitive mGluR1 antagonist, it is possible that we were
unable to uncover an endogenous role for these receptors due to weak competition of the
antagonist with endogenous glutamate. To address this we pre-incubated preparations in
LY367385 (50 µM) prior to the induction of locomotor activity and then subsequently
washed out LY367385 while locomotor activity was on-going. In these experiments
LY367385 again had no effect on locomotor frequency or burst amplitude (n = 5).
Together our data suggest that group I mGluRs are not activated during fictive locomotor
activity recorded in vitro, from the isolated mouse spinal cord. However, once stimulated,
group I mGluRs will modulate locomotor-related motoneuron output.

Effects of group I mGluR activation on motoneuron properties

To investigate the cellular mechanisms that underlie group I mGluR-mediated modulation
of locomotor-related motoneuron output, we analysed the effects of group I mGluR activation
on individual motoneurons. Whole-cell patch-clamp recordings were established from
motoneurons in isolated spinal cord preparations (Fig. 3A) under infrared differential
interference microscopy (IR-DIC). Most recorded cells were confirmed as motoneurons by
the presence of antidromic action potentials in response to stimulation of segmentally aligned
ipsilateral ventral roots (< 100 µA, duration 0.5 ms; Fig. 3B). The effects of group I mGluR
activation were assessed using bath applications of DHPG from doses of 10 µM up to 50 µM
to maximise the likelihood of revealing the full range of mGluR-mediated modulatory effects
We started by investigating the sub-threshold effects of group I mGluR activation on motoneurons. Application of DHPG significantly depolarised the resting membrane potential of motoneurons (10 µM, 11.4 ± 1.5 mV, n = 10; 50 µM, 16.0 ± 3.1 mV, n = 11; Fig. 3C & 3D). The membrane potential began to repolarise during the course of the drug application (15 – 20 min; data not shown) indicating desensitisation of receptors or other components of the group I mGluR signalling pathway, as seen in rat motoneurons (Marchetti et al. 2003). To investigate the currents responsible for the DHPG-induced depolarisation we measured the input resistance of motoneurons using small voltage steps (-70 to -55 mV, 2.5 mV increments, 10 ms duration) delivered in voltage-clamp mode (Fig. 3E). We were unable to reveal a significant change in input resistance with applications of 10 µM DHPG (n = 9, data not shown). However, at the higher dose of DHPG (50 µM) a significant decrease in input resistance was revealed (52.8 ± 10.1 to 37.3 ± 3.6 MΩ, n = 11; Fig. 3F). This effect was incompletely reversed following drug washout suggesting that mGluR activation is associated with long term effects on some conductances or that high doses of DHPG cannot be fully removed over the time course of our recordings. Current-voltage (IV) relationships were examined for DHPG-induced currents by subtracting IV relationships in control from those in the presence of DHPG (50 µM). IV relationships obtained from individual motoneurons were then pooled to reveal a linear IV relationship for DHPG-induced currents, with an
approximate reversal potential of -30 mV (n = 11, Fig. 3G). Finally, DHPG-induced currents remained in the presence of TTX (DHPG 50 µM; TTX 0.5 µM; n = 13; data not shown).

These data suggest that the activation of group I mGluRs leads to the activation or facilitation of a mixed-cation current in motoneurons.

The group I mGluR-mediated depolarisation of motoneurons is in apparent opposition to the decrease in locomotor-related motor output observed in ventral root recordings. Thus, we next investigated the effects of group I mGluR activation on motoneuron firing. Repetitive firing of action potentials was evoked in motoneurons by the injection of square wave depolarising current pulses (1s duration). To study the effects of DHPG on motoneuron firing in isolation from its depolarising effects, repolarising bias currents were applied to bring motoneuron resting potentials back to their control levels. Application of DHPG led to a reduction in the frequency of motoneuron firing in response to current injection (Fig. 4Ai).

This reduction in motoneuron excitability was evident as a rightward shift in the steady-state firing frequency versus injected current (f-I) relationships of 6 out of 8 motoneurons in response to 10 µM DHPG and 7 out of 11 motoneurons in response to 50 µM DHPG (Fig. 4Aii). In the remaining cells DHPG either had no clear effect on the f-I relationship or caused a leftward shift. Reduced excitability upon application of DHPG was also demonstrated by a significant reduction in the maximum firing frequency of motoneurons (10 µM, 26.3 ± 7.6 % reduction, n = 8; 50 µM, 16.1 ± 4.9 % reduction, n = 11; Fig. 4Aii). Furthermore, reduced
firing was observed in the presence of DHPG (50 μM) even when repolarising bias currents were not injected (n = 11).

To reveal mechanisms which may underlie the group I mGluR-mediated decrease in motoneuron excitability, we measured action potential parameters in control and in the presence of DHPG. Measurements of the height and maximum rate of rise of the first action potential evoked by depolarising pulses revealed a significant reduction in action potential height (10 μM, 5.5 ± 2.4 mV reduction, n = 9; 50 μM, 4.2 ± 1.8 mV reduction, n = 9; Fig. 4B) and a significant slowing of the maximum rate of rise (10 μM, 16.2 ± 5.0 % reduction, n = 9; 50 μM, 13.8 ± 5.9 % reduction, n = 9; Fig. 4C) upon the application of DHPG. Changes in these two parameters were evident whether the measurements were taken from the same current pulses in control and in the presence of DHPG or from current pulses which elicited similar firing frequencies in the two conditions. We also investigated whether group I mGluR activation affects the voltage threshold for the generation of action potentials in motoneurons. The voltage threshold was measured from the first action potential evoked by a ramp of depolarising current (100 – 500 pA/s; Fig. 4D). Surprisingly, the voltage threshold for action potential generation was significantly hyperpolarised during the application of DHPG (10 μM, 2.1 ± 0.7 mV hyperpolarisation, n = 8; 50 μM, 5.0 ± 1.6 mV hyperpolarisation, n = 9; Fig. 4D). Despite hyperpolarisation of the action potential threshold, a reduction in motoneuron firing was still evident in ramp protocols during DHPG application (Fig. 4D). Of note, there
also appears to be a reduction in the action potential after-hyperpolarisation in the recording depicted in Fig. 4Di. However, this was not a consistent observation across our recordings. Finally, hyperpolarisation of the action potential threshold was also apparent in recordings of motoneurons firing in response to square current pulses (Fig. 4Ai).

Given that group I mGluR activation leads to reductions in the height and maximum rate of rise of action potentials in motoneurons, we hypothesised that group I mGluR activation modulates Na⁺ channels that mediate the fast, inactivating Na⁺ current. This was tested by eliciting Na⁺ currents in motoneurons in voltage-clamp mode using a series of voltage steps (-70 to +20 mV, 2.5 mV increments, 10 ms duration) from a holding potential of -60 mV in control and in the presence of DHPG (Fig. 5A). Analyses performed on leak subtracted traces, recorded in standard external and pipette solutions, revealed that the density of fast, inactivating Na⁺ currents was significantly reduced during the application of DHPG (Fig. 5B & 5C). The peak current density, elicited by steps to -27.5 mV, was decreased by DHPG application (10 µM, 19.4 ± 8.4 % reduction, n = 9; 50 µM, 18.7 ± 5.3 % reduction, n = 11; Fig. 5B & 5C).

When recording large currents from large neurons there are is a high likelihood of voltage- and space-clamp errors. Although these errors may be equivalent in control conditions and in the presence of DHPG, it is also possible that changes in cell properties induced by DHPG may differentially affect the voltage control in the two conditions. We therefore also assessed
whether DHPG modulated Na\(^+\) currents when other channel types (Ca\(^{2+}\) and K\(^+\)) and synaptic transmission were blocked, and Na\(^+\) currents were reduced by using an external solution with lowered extracellular Na\(^+\) (10 mM NaCl; Carlier et al. 2006). In these modified solutions, Na\(^+\) currents elicited by depolarising steps (-70 to +20 mV, 2.5 mV increments, 10 ms duration) from a holding potential of -60 mV were more than 10-fold smaller than those recorded in standard solutions (Fig. 5D). With Na\(^+\) currents reduced and other channels and synaptic transmission blocked, the application of DHPG (50 µM) still led to a significant decrease in the peak density of Na\(^+\) currents (20.3 ± 7.8 % reduction, n = 6; Fig. 5E & 5F).

In order to investigate the mechanisms by which group I mGluR activation modulates Na\(^+\) currents we also investigated the voltage dependence of Na\(^+\) current activation and inactivation in control conditions and in the presence of DHPG (50 µM; using modified solutions). Na\(^+\) current activation was measured using depolarising steps from a holding potential of -60 mV (-70 to +20 mV, 2.5 mV increments, 10 ms duration; Fig. 5G).

Steady-state inactivation of Na\(^+\) currents was investigated using steps to 0 mV (10 ms duration) from pre-pulses (50 ms duration) ranging from -90 to -5 mV (5 mV increments; Fig. 5H). The half-maximal activation voltage of Na\(^+\) currents was unchanged by DHPG (-31.4 ± 1.1 mV in control, -33.2 ± 1.7 mV in DHPG, n = 6; Fig. 5G). There were also no significant changes in the half maximal voltage of steady-state inactivation in the presence of DHPG (-51.0 ± 0.8 mV in control, -51.1 ± 0.9 mV in DHPG, n = 6; Fig. 5H). These data indicate that
group I mGluRs regulate Na\(^+\) current density by means other than modulating the voltage
dependence of activation or inactivation of Na\(^+\) currents.

Taken together, our results demonstrate that the activation of group I mGluRs has a range
of effects on the intrinsic properties of motoneurons. Although the effects include
depolarisation of the resting membrane potential and hyperpolarisation of the action potential
threshold, the net effect is a reduction in motoneuron output, most likely due to the
modulation of Na\(^+\) channels.

**Effects of group I mGluR activation on synaptic transmission to motoneurons**

Modulation of locomotor-related motoneuron output by the activation of group I mGluRs
could also involve regulation of the excitatory synaptic input received by motoneurons from
spinal locomotor networks. We therefore performed voltage-clamp experiments to analyse the
effects of group I mGluR activation on locomotor-related excitatory synaptic input to
motoneurons. In pharmacologically activated (5 µM NMDA; 10 µM 5-HT; 50 µM dopamine)
in vitro spinal cord preparations, rhythmic locomotor-related input was recorded from
motoneurons held at -60 mV (Fig. 6A). The rhythmic locomotor drive received by
motoneurons was in phase with bursts of activity recorded from segmentally aligned ventral
roots (data not shown). The amplitude of locomotor drive was significantly reduced by 10.4 ±
7.6 % when DHPG (10 µM) was bath applied (n = 10, Fig. 6A & 6B). Thus, group I mGluR
activation reduces the excitatory synaptic drive transmitted to motoneurons to stimulate bursts of locomotor-related output.

Next we investigated whether the activation of group I mGluRs modulates excitatory synaptic transmission to motoneurons via pre- or postsynaptic mechanisms. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in voltage-clamp mode while motoneurons were held at -60 mV (Fig. 6C). To isolate mEPSCs, TTX (0.5 µM), strychnine (5 µM) and bicuculline (10 µM) were added to the recording solution. Upon the application of DHPG (50 µM), both the amplitude and frequency of mEPSCs were decreased (Fig. 6C, D & E). DHPG-mediated decreases in mEPSC amplitude and frequency were evident as leftward shifts in cumulative frequency plots generated from data collected from individual motoneurons (Fig. 6D). DHPG application lead to a significant reduction in mEPSC amplitude in 7 out of 13 motoneurons and a decrease in mEPSC frequency in 8 out of 13 motoneurons. Average mEPSC amplitude across all motoneurons decreased significantly from 20.0 ± 0.8 to 19.2 ± 0.8 pA (n = 13, Fig. 6Ei), while average mEPSC frequency decreased significantly from 6.2 ± 0.8 to 4.9 ± 0.7 Hz (n = 13; Fig. 6Eii). Together these results indicate that the activation of group I mGluRs reduces excitatory synaptic transmission to motoneurons via both pre- and postsynaptic mechanisms.

Discussion
The activation of ionotropic glutamate receptors is essential for the functioning of the locomotor CPG (Beato et al. 1997; Talpalar and Kiehn 2010; Whelan et al. 2000) and for the transmission of locomotor drive from the CPG to motoneurons (Cazalets et al. 1996; Hochman and Schmidt 1998; Orsal et al. 1986; Shefchyk and Jordan 1985). However, glutamate can also control locomotor activity via the activation of metabotropic receptors which modulate the properties of spinal neurons and their connectivity (El Manira et al. 2002; Nistri et al. 2006). In the present study we demonstrate that group I mGluRs can modulate the intensity of locomotor-related motoneuron output. We also elucidate several mechanisms by which activation of group I mGluRs can alter motoneuron excitability and excitatory synaptic transmission to motoneurons and hence modulate motoneuron output. Interestingly, our data demonstrating a net reduction in motoneuron output upon activation of group I mGluRs represent a deviation from the typical facilitatory role reported for these receptors (Anwyl 2009; 1999; Delgado-Lezama et al. 1997; Dong and Feldman 1999; Pin and Duvoisin 1995; Svirskis and Hounsgaard 1998).

Although the focus of the present study was on the effects of group I mGluRs on motoneuron output, we also observed an increase in the frequency of locomotor-related activity which suggests that group I mGluRs modulate the activity of spinal interneurons involved in locomotor rhythm generation. These data are consistent with findings in lampreys (Krieger et al. 1998; Kyriakatos and El Manira 2007) and Xenopus tadpoles (Chapman et al.
The only previous data regarding the effects of group I mGluRs on mammalian locomotion, obtained from isolated rat spinal cord preparations, are less clear with both group I mGluR agonists and antagonists reducing the frequency of locomotor activity (Taccola et al. 2004). In order to fully understand the role of mGluRs in locomotor rhythm generation in mammals it will be important for future studies to investigate the effects of group I mGluRs on locomotor-related interneurons. Given that the interneuronal components of the mammalian locomotor CPG remain poorly understood in comparison to simpler vertebrates (Grillner 2006; Roberts 2000), the ongoing classification of distinct populations of locomotor-related interneurons in mammals will be critical toward future studies of mGluR-mediated modulation (Goulding 2009; Kiehn et al. 2010; Zagoraiou et al. 2009).

Our study represents the first report of modulation of the strength of locomotor-related motoneuron output following the activation of group I mGluRs. This modulation presents as a gradual, reversible decrease in the amplitude of bursts of locomotor-related motoneuron activity recorded from ventral roots. Our recordings of individual motoneurons highlight several mechanisms which may underlie group I mGluR-mediated modulation of motoneuron output including: sub-threshold effects on membrane potential; effects on fast, inactivating Na⁺ channels and motoneuron action potentials; and effects on synaptic input to...
motoneurons.

Firstly, we found that the activation of group I mGluRs transiently depolarises the resting membrane potential of mouse motoneurons. These data are consistent with reports of group I mGluR-mediated depolarisation of motoneurons in lamprey (Kettunen et al. 2003; Krieger et al. 1998), turtles (Svirskis and Hounsgaard 1998), and rats (Del Negro and Chandler 1998; Dong and Feldman 1999; Marchetti et al. 2005; 2003), but not *Xenopus* tadpoles (Chapman et al. 2008). mGluR-mediated depolarisations in motoneurons are typically associated with increases in input resistance, most often thought to reflect the blockade of leak K⁺ currents (Del Negro and Chandler 1998; Dong and Feldman 1999; Kettunen et al. 2003; Marchetti et al. 2005; Svirskis and Hounsgaard 1998). However, we found that in mouse motoneurons group I mGluR-mediated depolarisations were associated with a decrease in input resistance, with the underlying current reversing near -30 mV. These data are consistent with the opening of non-selective cationic channels which has been reported in response to activation of group I mGluRs in a range of neuronal types (Congar et al. 1997; Dong et al. 2009; Kolaj and Renaud 2010). In addition, other currents modulated by group I mGluRs which cannot be ruled out include those mediated by Na⁺-Ca²⁺ exchangers (Jian et al. 2010; Keele et al. 1997; Lee and Boden 1997) and Na⁺-K⁺-2Cl⁻ co-transporters (Schomberg et al. 2001).

The depolarisation of motoneurons suggests group I mGluRs should increase motoneuron excitability. However, we observed a reduction in locomotor-related motoneuron output upon
activation of group I mGluRs. Although membrane depolarisation could lead to a
depolarising block of action potentials this was unlikely based on the magnitude of the
depolarisation induced. Furthermore, the time course of the mGluR-mediated depolarisation
differed to that of the reduction in the amplitude of locomotor-related motoneuron output.
Instead, the net inhibitory effect of group I mGluRs on motoneuron output appeared to
involve the modulation of action potentials and repetitive firing of mouse motoneurons.
We found that the frequency of repetitive firing was reduced in mouse motoneurons upon
the activation of group I mGluRs. This is in contrast to the only other detailed studies of the
effects of group I mGluRs on the firing of individual vertebrate motoneurons, performed in
turtles, which demonstrated that activation of group I mGluRs enhances sustained firing or
plateau potentials via the facilitation of voltage-activated Ca\(^{2+}\) channels (Delgado-Lezama et
al. 1997; Svirskis and Hounsgaard 1998). In addition to demonstrating mGluR-mediated
reductions in repetitive firing in mouse motoneurons we have demonstrated mGluR-mediated
reductions in action potential height and maximum rate of rise which are consistent with
reduced Na\(^+\) channel availability (Miles et al. 2005). Consistent with this, voltage-clamp
analysis of fast, inactivating Na\(^+\) currents suggests a reduction in Na\(^+\) current density upon
activation of group I mGluRs. Although the conclusions from data concerning the effects of
group I mGluRs on Na\(^+\) current density may be complicated by voltage- and space-clamp
errors, we believe that we have significantly reduced these errors through the use of modified
recording solutions in which other channels types and synaptic transmission are blocked, and

Na\(^+\) currents are reduced (Cantrell et al. 1997; Carlier et al. 2006; Carr et al. 2003; Miles et al. 2005). Thus, our data indicate that the modulation of Na\(^+\) channel properties and subsequent inhibition of motoneuron firing is likely to contribute to group I mGluR-mediated reductions in locomotor-related motoneuron output.

We also found that the voltage threshold for action potential initiation was hyperpolarised following the activation of group I mGluRs in mouse motoneurons. Interestingly, it has recently been shown that an undefined intraspinal system hyperpolarises the voltage threshold for action potentials during fictive scratch in cats (Power et al. 2010). Given our findings in mice and previous work in rats showing modulation of the voltage threshold for spike initiation by protein kinase C (Dai et al. 2009), a downstream target of group I mGluRs (Pin and Duvoisin 1995), it seems plausible that group I mGluRs form part of the intraspinal system defined in the cat (Power et al. 2010) which controls action potential threshold in a state-dependent manner. Although the mechanisms underlying mGluR-mediated modulation of action potential threshold remain unclear, our data do not support a role for the modulation of the activation properties of fast, inactivating Na\(^+\) currents. Other potential mechanisms include the modulation of persistent Na\(^+\) currents (Carlier et al. 2006) and delayed rectifier K\(^+\) channels (Dai et al. 2002).

Although this is the first report of mGluR-mediated modulation of Na\(^+\) channels and
action potential shape in motoneurons, similar findings have been reported in cortical
pyramidal neurons (Carlier et al. 2006). In these neurons group I mGluR activation
hyperpolarises the inactivation of transient Na\(^+\) currents, which in turn decreases Na\(^+\) current
amplitude, reduces the height and maximum rate of rise of action potentials, and lowers the
frequency of repetitive firing (Carlier et al. 2006). In contrast to these results in pyramidal
neurons, in motoneurons we found that activation of group I mGluRs reduces Na\(^+\) current
density without changing the voltage dependence of steady-state inactivation. Our findings
are similar to previous reports in hippocampal neurons where phosphorylation of Na\(^+\)
channels, following the activation of either dopaminergic or muscarinic receptors, decreases
peak Na\(^+\) current without affecting the voltage dependence of activation or steady-state
inactivation (Cantrell et al. 1996; Cantrell et al. 1997). In such cases, reduced Na\(^+\) channel
availability may instead reflect modulation of slow inactivation (Carr et al. 2003), altered
rates of inactivation or a decrease in the unitary conductance of single channels.

In addition to decreased motoneuron excitability, we uncovered an mGluR-mediated
inhibition of excitatory locomotor drive to motoneurons which is likely to contribute to
reduced locomotor-related motoneuron output. The modulation of synaptic transmission
(both excitatory and inhibitory) by mGluRs is commonly reported in spinal neurons
(Chapman et al. 2008; Kettunen et al. 2005; Kyriakatos and El Manira 2007; Marchetti et al.
2005; 2003). We chose to concentrate on the effects of group I mGluRs on excitatory
transmission because modulation of rhythmic excitatory drive to motoneurons seemed most likely to underlie decreases in locomotor-related motoneuron output. It could be argued that modulation of inhibition, should it occur concurrent with rhythmic excitation (Berg et al. 2007) or contribute to recurrent inhibitory circuitry, could contribute to the reduced amplitude of locomotor-related motoneuron output observed upon activation of group I mGluRs.

Although we cannot rule out this possibility, it seems unlikely given that concurrent inhibition and excitation of motoneurons during locomotion appears not to be a feature of mammalian locomotion (Endo and Kiehn 2008) and previous data in the rat suggests that group I mGluRs actually depress recurrent inhibition to motoneurons (Marchetti et al. 2005).

Our data indicate that mGluR-mediated modulation of excitatory synaptic drive to mouse motoneurons involves both pre- and postsynaptic mechanisms. Although the exact processes involved remain to be investigated one possible pre-synaptic mechanism, based on our current findings, is that group I mGluR activation inhibits transient Na⁺ currents in pre-synaptic terminals. Other potential mechanisms include those revealed in the lamprey spinal cord where modulation of both excitatory and inhibitory transmission involves activation of postsynaptic group I mGluRs followed by the release and pre-synaptic actions of endocannabinoids and nitric oxide (Kettunen et al. 2005; Kyriakatos and El Manira 2007; Kyriakatos et al. 2009). In addition, data demonstrating modulation of both NMDA and AMPA receptor-mediated currents by group I mGluRs (Krieger et al. 2000; Nanou and El
Manira 2010; Nanou et al. 2009) provide potential mechanisms for postsynaptic modulation of transmission.

Taken together, data from our study and previous research demonstrate that the actions of group I mGluRs on motoneurons are complicated by the existence of multiple effects including the modulation of intrinsic neuronal properties and synaptic transmission. This may allow considerable flexibility in the actions of group I mGluRs if the multiple effects they mediate are controlled by pathways that are separable, for example, based on the group I mGluR subtype activated (mGluR1 versus mGluR5) or the second messenger signalling involved. Although it was not analysed in the present study, previous work supports the separation of some of the cellular effects of group I mGluRs. For example, in rat motoneurons mGluR1 activation is responsible for membrane potential depolarisation while mGluR5 activation triggers membrane oscillations (Marchetti et al. 2003). Meanwhile, in the lamprey spinal cord separate intracellular pathways downstream of mGluR1 receptor activation mediate blockade of leak channels versus the potentiation of NMDA currents (Nanou et al. 2009). Interestingly, in opposition to the independence of separate aspects of group I mGluR-mediated signalling, studies of striatal cholinergic neurons have demonstrated an interaction between group I mGluRs with mGluR5 modulating mGluR1 activity (Bonsi et al. 2005).

Although endogenous activation of group I mGluRs has been reported in lampreys
(Krieger et al. 1998) *Xenopus* tadpoles (Chapman and Sillar 2007) and rats (Taccola et al. 2004), under our experimental conditions we were unable to detect an endogenous role for group I mGluRs in the control of locomotor activity in mice. However, it is possible that our inability to detect an endogenous role for mGluRs reflects compensatory activation of serotonergic and dopaminergic receptors upon the antagonism of mGluRs. In addition, the reduced nature and simple state of our preparation does not preclude an involvement of mGluRs in the control of the locomotor CPG in the whole animal. Glutamatergic inputs to the CPG which are lost or inactive in our preparation, such as descending inputs implicated in the initiation of locomotion (Hagglund et al. 2010; Jordan et al. 2008), might activate both ionotrophic and metabotropic glutamate receptors (Delgado-Lezama et al. 1997).

In brainstem motoneurons, where mGluR activation also depresses excitatory inputs and mediates membrane depolarisation, mGluRs have been suggested to play a role in sculpting the final output of motoneurons by favouring strong synaptic inputs and amplifying their effects (Del Negro and Chandler 1998). Our data suggest that group I mGluR activation might mediate similar enhancement of the signal-to-noise ratio of synaptic inputs to spinal motoneurons by reducing excitatory inputs, but depolarising the resting membrane potential and hyperpolarising the action potential threshold. However, the inhibition of repetitive firing via the modulation of *Na*⁺ channels may be inconsistent with this. Alternatively, modulation of *Na*⁺ currents and repetitive firing may represent a separate role for group I mGluRs in
limiting the output of motoneurons, perhaps in the presence of intense glutamatergic input, to provide homeostatic control of motoneuron output (Carlier et al. 2006; Desai 2003). This might also serve to protect motoneurons from excitotoxic damage. Data indeed support a neuroprotective role for group I mGluRs in Amyotrophic Lateral Sclerosis (Anneser et al. 1999; Anneser et al. 2004; Aronica et al. 2001; Ma et al. 2006) where glutamate-mediated excitotoxicity is thought to contribute to motoneuron death (Boillee et al. 2006). Further analyses of the role of mGluRs in motoneurons may therefore be of importance, not only for understanding the control of motor outflow from the CNS, but also toward the design of new treatments for degenerative diseases which afflict spinal motor systems.
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References


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Figure Legends

Fig. 1. **Group I mGluR-mediated modulation of locomotor-related motoneuron output.**

(A) Raw (top) and rectified and integrated (bottom) traces showing the effects of the group I mGluR agonist DHPG (5 μM) on pharmacologically induced (NMDA 5 μM, 5-HT 10 μM, dopamine 50 μM) locomotor activity recorded from the lumbar ventral roots of an isolated mouse spinal cord preparation. (B) Time course plots showing a decrease in locomotor burst amplitude (i) and an increase in burst frequency (ii) in response to DHPG application (5 μM, 15 mins; n = 11). Each point represents 1 min worth of recording, normalised to control. (C) Pooled data, averaged from 5 min worth of recording in each condition, showing a significant decrease in locomotor burst amplitude (i) and increase in burst frequency (ii) following the application of DHPG (n = 11). *, significantly different to control.

Fig. 2. **Receptor subtype-specific effects of group I mGluR activation on locomotor-related motoneuron output.** (A) Time course plots showing that the mGluR1 antagonist, LY367385 (50 μM), blocked DHPG (5 μM)-mediated effects on locomotor burst amplitude (i) and frequency (ii) (n = 7). (B) Pooled data, averaged over 5 min worth of recording in each condition, again showing that DHPG had no effect on locomotor burst amplitude (i) or frequency (ii) in the presence of LY367385 (n = 7). (C) Time course plots showing that the mGluR5 antagonist, MPEP (50 μM), blocked DHPG-mediated effects on
locomotor burst amplitude (i). MPEP did not, however, block the effect of DHPG on locomotor burst frequency (ii) (n = 6). (D) Pooled data, averaged from 5 min worth of recording in each condition, also show no change in burst amplitude when DHPG is applied in the presence of MPEP (i) but that burst frequency (ii) was significantly increased by DHPG application in the presence of MPEP (n = 6). *, significantly different to control.

Fig. 3. Sub-threshold effects of group I mGluR activation on individual motoneurons.

(A) Schematic showing the experimental setup with a patch pipette targeting a spinal motoneuron and glass suction electrodes attached to the 2nd lumbar (L2) ventral roots. (B) Antidromic action potentials recorded from a motoneuron in response to stimulation of the ipsilateral ventral root. Arrow indicates stimulus artefact. (C, D) The resting membrane potential of motoneurons was depolarised in response to DHPG (50 μM) application (n = 11). (E) Current recorded in response to brief sub-threshold voltage steps (10 ms, -70 to -55 mV) in a motoneuron held at -60 mV in control conditions, in the presence of DHPG, and following drug washout. (F) Membrane resistance was significantly decreased by DHPG application. (G) The DHPG-induced current appeared to have a linear current-voltage relationship, with an extrapolated reversal potential of approximately -30 mV (n = 11). *, significantly different to control.
Fig. 4. **Group I mGluR-mediated effects on motoneuron firing.** (A) Repetitive firing in a motoneuron in response to brief (1 s) square current pulses in control conditions, in the presence of DHPG (50 µM) and after drug washout (i). The firing frequency versus injected current (f-I) relationship for this motoneuron was shifted to the right by DHPG application (ii). The maximum firing frequency of motoneurons was significantly reduced by DHPG application (iii) (n = 11). (B) The height of the first action potential evoked by a square current pulse was significantly decreased by DHPG application. (C) The maximum rate of rise of action potentials was also significantly reduced by DHPG. (D) The voltage threshold for action potential generation, measured by injecting a current ramp (i), was significantly hyperpolarised by DHPG application (ii). *, significantly different to control.

Fig. 5. **Group I mGluR activation modulates the fast, inactivating Na⁺ current.** (A) Fast, inactivating Na⁺ currents elicited in standard recording solutions by brief (10 ms) depolarising voltage steps (-70 to +20 mV) in a motoneuron held at -60 mV in control conditions, in the presence of DHPG (50 µM) and after drug washout. Plots of current-voltage relationships (B) and average peak current density (C) for fast, inactivating Na⁺ currents demonstrate a DHPG-mediated reduction in Na⁺ current density (n = 11). (D) Na⁺ currents recorded in control conditions and in the presence of DHPG (50 µM) in modified solutions designed to block Ca²⁺ channels, K⁺ channels and synaptic transmission
(TEA, 4-AP, Cs⁺, CdCl₂, bicuculline, strychnine and kynurenic acid), and to reduce Na⁺
current magnitude by lowering the concentration of extracellular Na⁺ (10 mM). Plots of
current-voltage relationships (E) and average peak current density (F) again demonstrate a
DHPG-mediated reduction in Na⁺ current density (n = 6). Normalised activation and
steady-state inactivation curves plotted in control conditions and in the presence of DHPG
demonstrate no change in the voltage dependence of activation (G) or steady-state
inactivation (H). *, significantly different to control.

Fig. 6. Effects of group I mGluR activation on synaptic input to motoneurons. (A)
Rhythmic locomotor-related drive currents recorded from a motoneuron held at -60 mV in
control conditions, in the presence of DHPG (10 µM) and after drug washout. (B) The
amplitude of locomotor-related drive was significantly decreased by DHPG (10 µM)
application (n = 10). (C) mEPSCs recorded from a motoneuron in the presence of TTX (0.5
µM), bicuculline (10 µM) and strychnine (5 µM). (D) Cumulative probability plots showing a
significant decrease in mEPSC amplitude (i) and frequency (ii) following DHPG (50 µM)
application. (E) Pooled data demonstrating that DHPG caused a significant decrease in both
mEPSC amplitude (i) and frequency (ii) (n = 13). *, significantly different to control.
<Fig. 1>

A

B

C

Bi 1.2

0.8

DHPG

Rel. Amp.

0 10 20 30 40 50 60 70 (min)

0 1 0 2 0 3 0 4 0 5 0 6 0 7 0

(min)

0 1 0 2 0 3 0 4 0 5 0 6 0 7 0

(min)

0 1 0 2 0 3 0 4 0 5 0 6 0 7 0

(min)

C

Ave. Freq.

DHPG

Rel. Freq.

0.6

0.8

1.0

1.2

Control DHPG Wash

Ave. Amp.

Control DHPG Wash

Control DHPG Wash

*
<Fig. 4>

Ai

Control DHPG Wash

Firing Freq. (Hz)

Max. Firing Freq. (Hz)

Firing Freq. (Hz)

Max. Firing Freq. (Hz)

Firing Freq. (Hz)

Max. Firing Freq. (Hz)

B

AP Height (mV)

AP max rate of rise (mV/ms)

AP Height (mV)

AP max rate of rise (mV/ms)

AP Height (mV)

AP max rate of rise (mV/ms)

C

AP Height (mV)

AP max rate of rise (mV/ms)

AP Height (mV)

AP max rate of rise (mV/ms)

AP Height (mV)

AP max rate of rise (mV/ms)

D

Control DHPG Wash

AP Height (mV)

AP max rate of rise (mV/ms)

AP Height (mV)

AP max rate of rise (mV/ms)

AP Height (mV)

AP max rate of rise (mV/ms)
A. Control, DHPG, Wash

B. I Na+ density (pA/pF)

C. Peak I density (pA/pF)

D. Control, DHPG

E. I Na+ density (pA/pF)

F. Peak I density (pA/pF)

G. GNa/GNa,max

H. hNa/hNa,max

<Fig. 5>