

How Is The Spinal Cord Organized To Produce Movement At Different Speeds?

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At this point in time, the lecture will focus on data from zebrafish that explore how patterns of activity within classes of spinal motoneurons and interneurons change in conjunction with changes in the speed of movement. Electrophysiology and imaging reveal orderly patterns of recruitment in spinal cord that relate where a neuron is located to the frequency or speed of the movement at which it is activated. They also show that there are switches in the classes of interneurons used to move at different speeds, with some interneurons that are active at slow speeds being inhibited at faster speeds. The evidence for what seems to be a simple pattern of recruitment will be presented, interspersed with outrageous statements designed to provoke people working on mammals and hopefully lead to discussion about principles of motor organization and whether anyone really cares about how a zebrafish spinal cord works.

Spontaneous Depolarization Waves of Multiple Origins in the Embryonic Rat CNS

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During development, correlated neuronal activity plays an important role in the establishment of the central nervous system (CNS). We recently reported that in the embryonic rat CNS, a widely-propagating correlated neuronal activity, termed the depolarization wave, is evoked by various sensory nerve inputs. A remarkable feature of the depolarization wave is how it spread broadly through the brain and spinal cord. In the present study, we examined whether the depolarization wave occurs spontaneously in the embryonic rat CNS, and if so, where it originates. In E15-E16 rat embryos, spontaneous optical signals appeared in association with the rhythmic discharges of cranial motoneurons, and propagated through the brain and spinal cord with similar characteristics to the evoked depolarization wave. The spontaneous wave originated in multiple locations including two restricted regions located in the dorsomedial pons and rostral ventrolateral medulla as well as the medial/caudal medulla and spinal cord. When the spinal cord was intact, the wave at E16 mainly originated in the lumbosacral region, although a wave associated with the oscillatory event was also generated in the cervical cord/caudal medulla. In preparations in which the spinal cord was transected, the contribution of the pons and medulla was more significant. These results show that the depolarization wave is triggered by the spontaneous activity of multiple neuronal populations which distribute widely from the pons to the lumbosacral cord. This network possibly behaves as a self-distributing system that maintains the incidence and complicated patterns of the correlated activity in the developing CNS.

Development of a Flexible Locomotor Pattern in the Spinal Cord of the *Xenopus* Tadpole

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The spinal networks that generate rhythmic locomotor movements are assembled very early in development, but generally their output lacks the flexibility and adaptability of more mature networks – features which must therefore be acquired as development proceeds. I will review aspects of the maturation of the *Xenopus* frog tadpole swimming network, which we have recently revisited using whole cell patch clamp recordings.

At the time of hatching, the swimming CPG in stage 37/8 embryos generates a motor rhythm in which rhythmically active spinal neurons, including motoneurons, discharge synchronously, once in each cycle of activity. However, the situation changes during the first day of larval life; by stage 42, neurons can fire multiply and, in association, the ventral root burst durations increase and become more variable allowing, for example, left-right variations that accompany fictive turning. Spinal neurons switch firing patterns; they can now fire multiply in each cycle and display a range of recruitment patterns not previously evident at stage 37/8.

The brief ventral root bursts in each cycle at stage 37/8 comprise synchronous firing in homonymous motoneurons coupled by electrical synapses. Presumably a loss of this electrical coupling is important for desynchronization of firing during stage 42 swimming. We tested this by applying gap junction blockers: 18 β -GA (30-90 μ M) produces a significant increase in ventral root burst durations, consistent with a desynchronization of neuronal firing, rendering the embryonic rhythm more reminiscent of later stage 42. Patch clamp recordings reveal that spinal neurons continue to fire only once per cycle but their spike timing becomes more variable relative to the ventral root rhythm. These data support the proposal that a decrease in electrical coupling is important for desynchronization of motoneuron firing during swimming but that this change alone cannot account for the developmental transition to multiple firing during swimming.

It has been shown previously that changes in the properties of NMDA receptors accompany the transition to the more flexible larval rhythm. We have assessed the role of the regulatory NMDA receptor glycine site and present evidence that: i) the site is important for swimming but it is not fully saturated throughout the swimming network; ii) enhanced activation of the site lengthens swim episodes and produced slow modulations of swimming consistent with the induction of intrinsic membrane potential oscillations; and iii) GlyT1b transport proteins are at least in part responsible for maintaining glycine concentrations below saturation in the vicinity of NMDA receptors.

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Sodium channels regulate dorsal root ganglion development in the developing zebrafish embryo

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Our research seeks to identify the essential roles of specific sodium channels in directing nervous system development. Our studies exploit the experimental advantages of zebrafish for genetic and molecular manipulations, embryological analyses, in vivo live imaging and physiological study. We focus on the neuronal *scn8aa* zebrafish sodium channel gene because it is expressed early in development and its elimination leads to significant defects in differentiation of specific sensory and motor neuron populations.

The results to be presented concern the role of the *scn8aa* in dorsal root ganglion development. Our preliminary data demonstrate that knock-down of the channel encoded by *scn8aa* leads to ectopic cell body position and aberrant axonal growth of dorsal root ganglion neurons.

Molecular events controlling early spinal cord development

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Tlx3 and Lbx1 determine the excitatory versus inhibitory neuron cell fate in the dorsal spinal cord

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Glutamatergic and GABAergic neurons mediate much of the excitatory and inhibitory neurotransmission, respectively, in the vertebrate nervous system. Others and we have recently shown that specification of these two basic neuronal cell types is controlled by a set of region-specific selector genes. In the dorsal spinal cord, the homeobox genes Tlx3 and Lbx1 determine the excitatory and inhibitory cell fates, respectively. In Tlx3 mutants, prospective glutamatergic neurons are transformed into GABAergic inhibitory neurons, whereas Lbx1 mutation causes an exactly opposite cell fate switch, from GABAergic to glutamatergic. Subsequent epistasis analyses showed that Tlx3 acts to antagonize Lbx1 in promoting glutamatergic differentiation. Most recently, we found that Tlx3 controls the expression of a set of neuropeptides that are critical in modulating the transmission of nociceptive sensory information. The studies suggest a coordinate regulation of both fast glutamatergic and slow modulatory peptide neurotransmitters in the dorsal horn relay station.

Excitatory Mammalian CPG Neurons

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The vertebrate spinal cord contains a central pattern generator or CPG that can produce the rhythmic movements of locomotion in the absence of peripheral and descending inputs. While the organization of the constituent interneurons in the CPG controlling swimming in non-limbed animals is known in great detail, less is known about the cellular organization of CPGs controlling walking in limbed mammals. Use of genetically modified mice including selective labeling, knockout and/or acute silencing of spinal neurons we have identified groups of ipsilaterally projecting excitatory neurons that might be directly involved in rhythm generation and/or coordinating left and right side activity.

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Genetic dissection of the locomotor CPG

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Specification of Spinal Motor Neurons from Human Embryonic Stem Cells

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Embryonic stem cells (ESCs), initiated and expanded from the inner cell mass of a blastocyst embryo, possess the potential to produce all cell/tissue types of an organism, including motor neurons. Human ESCs, under a chemically defined adherent colony culture for 8-10 days, robustly (>95%) differentiate to columnar neuroepithelial cells that uniformly express anterior transcription factors such as Pax6, Lhx2, Otx2, and Bfl1 but not posterior markers such as hox proteins. These early neuroepithelial cells, referred to as primitive anterior neuroepithelia, progress to definitive neuroepithelia that form neural tube-like rosettes and express Sox1/2 in addition to anterior transcription factors in the absence of morphogens. In the presence of RA, expression of anterior markers is eliminated and nearly all the cells express Hox proteins. The majority of the RA-induced cells express HoxC5 and C8 but not HoxC9/10 (lumbar cord) and few are positive for phox2b (hindbrain), suggesting that the neural progenitors are restricted to the cervical spinal cord fate. Treatment of the spinal progenitors with sonic hedgehog or purmorphamine, a small molecule that activates the SHH pathway, results in the specification of ventral progenitors, the majority of which express Olig2 or Nkx2.2 with few expressing Irx3 but not Pax7. Thus, hESCs are restricted to ventral spinal progenitors nearly completely.

In the continual presence of RA/SHH (or purmorphamine) and neurotrophic factor cocktail, Olig2-expressing ventral spinal progenitors differentiate to HB9-expressing motor neurons. The Olig2-expressing progenitors and HB9-positive motor neurons account for 90% of the total differentiated progenies. Along further differentiation, the post-mitotic motor neurons become electrophysiologically active cholinergic (ChAT+) neurons and form functional synapses with surrounding neurons. The ChAT+ human neurons also induce clustering of acetylcholine receptors on myocytes, revealed by bungrotoxin staining, and formed neuro-muscular junctions, as evidenced by EM. Thus, the in vitro produced human neurons possess hallmarks of spinal cord motor neurons. They therefore provide a tool for studying the genesis, functional maturation, degeneration, and regeneration of human motor neurons.

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Characterization of the Spinal Neurons that Provide Locomotor Feedback to the Brainstem in Lamprey

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Descending systems controlling the spinal locomotor networks are kept informed of the state of locomotion by feedback from ascending spinal neurons. In lamprey, these neurons (spinobulbar neurons) project ventrolaterally to the brainstem as far as the caudal mesencephalon. Both ipsilaterally- and contralaterally-projecting spinobulbar neurons exist throughout the spinal cord but are densest in the rostral ten percent of the cord. Compared to mammals, this ascending system in lampreys is more direct, consisting of excitatory and inhibitory monosynaptic inputs from spinobulbar neurons to reticulospinal neurons, the main descending system in lamprey. With regard to inputs to spinobulbar neurons, they are rhythmically active during fictive swimming, and the population exhibits a wide range of timing relationships with nearby ventral root bursts. Spinobulbar neurons not only receive locomotor network inputs, but the same cells also receive direct and indirect inputs from primary mechanosensory afferents. With regard to their outputs, it appears that there is specificity in their connections to reticulospinal neurons as an individual spinobulbar neuron contacts only a subset of reticulospinal cells. In addition, uniquely identifiable reticulospinal neurons each exhibit different and consistent locomotor patterns from animal to animal due to the inputs they receive from the spinal cord. The spinobulbar neurons likely function as more than simple relay cells because some have mutual synaptic interactions with individual reticulospinal neurons (both mutual excitation and feedback inhibition have been found). Morphologically, the spinobulbar neurons have small- to medium-sized somata with dendritic arbors that are significantly less extensive than those of motoneurons and with dendrites often extending across the midline. Their axons exhibit multiple branches, with both ascending and descending axons in the cord. As to the overall organization of ascending and descending interactions, one hypothesis being pursued is that reticulospinal neurons control body movements by differential activation of dorsal and ventral myotomal quadrants and that spinobulbar neurons provide specific feedback regarding these quadrants of the spinal networks. Due to the relative simplicity of the lamprey nervous system and its motor control system, the spinobulbar neurons and their interactions with reticulospinal neurons may be advantageous for investigating the general organization of ascending systems in the vertebrate.

Interneurons Involved in Sensory-Induced Activation of Locomotor Pattern Generators in the Spinal Cord

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Stimulation of sacrocaudal afferents (SCAs) is a potent drug-free method to induce a locomotor-like rhythm in the isolated spinal cord of the neonatal rat and mouse. In this presentation we describe a group of intermediate-gray sacral interneurons that is involved in activation of the locomotor pattern generating circuitry by SCA stimulation. These neurons are contacted by terminals of non-segmental sacrocaudal afferents; their axons project to the contralateral lumbar cord via the ventral funiculi (VF), and they exhibit tonic or rhythmic discharge during SCA stimulation. The activity of the VF interneurons and the SCA induced rhythm is blocked by the mu-opioid receptor agonist DAMGO and restored in the presence of naloxone. We suggest that this opioid block involves suppression of synaptic transmission through the VF interneurons. The expression of mu opioid receptors in these interneurons provides an anatomical basis for this latter suggestion. Further studies are required to determine the synaptic connectivity of the VF neurons with lumbar networks in the rat and mouse spinal cord and assess the physiological significance of this crossed-sacral pathway.

Ionic Currents Shaping Activity of Neurons in the Mouse Spinal Locomotor Network

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The motor pattern generated by a CPG depends on the pattern of synaptic connections and the intrinsic firing properties of the network neurons. These are determined by the palette of ionic currents expressed in the neurons. We are exploring the roles of ionic currents and their modulation in the mouse spinal locomotor CPG. The riluzole-sensitive persistent sodium current, $I_{Na(P)}$, is present in motoneurons (MNs), and commissural interneurons (CINs). Riluzole (2-5 μ M) abolishes drug-induced or cauda equina-evoked fictive locomotion. The amplitude of ventral root bursts gets progressively weaker during riluzole application, but the frequency of the motor pattern is only marginally affected until the motor pattern is not detectable. Riluzole acts both on MNs and CINs in similar ways. The rhythmic synaptic drive to these neurons is progressively reduced in parallel with the reduction in VR output. Riluzole slightly depolarizes the spike threshold, and converts the tonic firing response to a current step to a single spike response. However, the amplitudes of evoked EPSPs in MNs and CINs is not reduced. These results suggest that $I_{Na(P)}$ plays a critical role in CPG neuron integration of synaptic inputs, and the strength of the spiking response to those inputs: when $I_{Na(P)}$ is reduced, the neurons within the CPG fire fewer spikes per cycle, resulting in a gradual weakening and eventual failure of the motor pattern. Since the cycle frequency is unaffected by riluzole, $I_{Na(P)}$ may not play a major role in the mechanisms for rhythm generation. We are also studying the mechanisms by which serotonin excites CINs during fictive locomotion. During 5-HT, synaptically isolated aCINs and dCINs depolarize and spike tonically. Their action potentials show several changes, including a lower threshold, smaller afterhyperpolarization, and wider spike. Using 2-photon calcium imaging, we found that serotonin reduces voltage-activated calcium accumulation at a subset of sites within CIN dendrites, with no effect at the remaining sites. Voltage clamp studies confirmed that the whole cell I_{Ca} is reduced by 5-HT. To understand how this could excite the neurons, we are starting to study 5-HT's effects on $I_{K(Ca)}$. The SK-channel blocker apamin mimics and occludes 5-HT's increase in CIN excitability (as seen by a shift in the F-I plot) and also occludes 5-HT's reduction of the AHP. Further experiments are planned to measure 5-HT's effects on $I_{K(Ca)}$ by voltage clamp.

Synaptic and Intrinsic Mechanisms regulating Locomotor-like Rhythms in Hb9 Interneurons

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Our recent studies have demonstrated that: 1) similar to motoneurons, medial lamina VIII interneurons express the homeobox gene Hb9 (Hb9 IN) in the mouse spinal cord, 2) Hb9 INs can generate membrane oscillations that are in phase with locomotor-like motor outputs, 3) neurochemically induced rhythms in Hb9 INs persist in the presence of CNQX, a non-NMDA receptor antagonist that blocks rhythmic motor outputs and 4) clustered Hb9 INs remain electrically coupled in juvenile mice that can walk. These findings raise the intriguing possibility that Hb9 INs are functional components of the locomotor central pattern generator (CPG). Locomotor patterns are determined by intrinsic properties and synaptic connections of neurons in the CPG circuitry. The primary objective of this study was to elucidate the synaptic and ionic mechanisms that underlie locomotor-like membrane oscillations in Hb9 INs. To determine whether glycine- and GABA-mediated synaptic transmission plays a role in rhythms generated by the mixture of NMA, 5-HT and dopamine, experiments were conducted in the presence of CNQX, picrotoxin and strychnine. Blocking fast inhibitory transmission did not alter the rhythms in Hb9 INs, but triggered slow bursts of motor outputs that were not correlated with the faster rhythms in Hb9 INs. Muscarinic and nicotinic receptor antagonists did not suppress the rhythms, suggesting that cholinergic synaptic inputs did not contribute to rhythm generation. These data indicate that rhythms in Hb9 INs can be produced independently of fast excitatory and inhibitory synaptic transmission. To identify the ionic currents underlying these rhythms, experiments were carried out in the presence of blockers of voltage-gated ion channels. Either riluzole (10 μ M) or nickel (200 μ M) abolished the rhythms, suggesting that both persistent sodium current and low-threshold calcium current contribute to locomotor-like rhythms in Hb9 INs.

Cancer Pain: Causes, Consequences and Therapeutic Opportunities

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Pain can have an extremely disruptive influence on cancer patients' quality of life. While significant advances have been made in the diagnosis and treatment of cancer, the basic mechanisms that drive cancer pain have remained poorly understood. New models of cancer pain have recently been introduced and for the first time a mechanism based understanding of the factors that generate and maintain cancer pain is beginning to emerge. Findings from these studies are driving the development of new mechanistically-based therapies which have the potential of improving both the quality of life and survival of cancer patients.

Spinal Cord Injury and Neuropathic Pain

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Pain is a debilitating consequence of spinal cord injury (SCI) and prevents attainment of predicted level of optimal functioning. Recent studies suggest pain occurs in 64% to 80% of SCI population, 1/3 of these are with severe pain and 47% report pain onset within 1 year after SCI.

SCI pain has multifactorial etiology, with more than one type of pain seen in the same patient. There are no diagnostic procedures or strategies that permit categorizing types of pain or predicting likelihood of successful treatments with various strategies.

Establishing a precise pain diagnosis and distinguishing amongst different pain types is the first step in arriving at a more specific pain diagnosis. Treatment approaches for each type of pain differ, hence the need for precise diagnosis.

SCI pain can be broadly divided into nociceptive and neuropathic pain. Nociceptive pain is due to irritation of nociceptors in the musculoskeletal or visceral tissues and occurs in presence of normally functioning nervous system. Neuropathic pain on the other hand is caused by a primary lesion in the peripheral or central nervous system.

Neuropathic pain in SCI is especially problematic because of diagnostic and therapeutic limitations. It can occur in spinal segments above, at or below the level of injury and may be caused by compressive mononeuropathy, nerve root compression, or direct injury to spinal cord. Neuropathic pain following SCI can be constant, spontaneous or paroxysmal and evoked by touch or contact. Many patients report pain starting at time of injury or shortly afterwards and worsening over time, resulting in increase in the area of pain or pain amplification.

In most cases neuropathic pain is characterized by negative (deficits) and positive (gain) sensory abnormalities. Bedside sensory testing for these abnormalities is feasible and is a valuable supplement to neurological testing.

Quantitative sensory testing (QST) is a laboratory technique for assessing pain and sensory abnormalities in neuropathic pain. Limitations of this technique include paucity of QST data in SCI pain, need for specialized equipment and trained personnel for administering the test and interpreting test results.

It is suggested that quantitative bedside sensory pain examination can be used to characterize the specific somatosensory phenotype of neuropathic pain state, help with developing more rational pain treatments, developing and evaluating new treatments in SCI and other patients with neuropathic pain.

Molecular Mechanisms of Post Spinal Cord Injury Neuropathic Pain

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Introduction:

Neuropathic pain (NP) represents a significant problem for patients following spinal cord injury (SCI). The mechanisms responsible for the development of such pain are not well understood. Investigations employing specific receptor agonists and antagonists as well as non-specific modifiers of secondary messenger systems have provided unique insight into the pathophysiology of this pain syndrome.

Materials and Methods:

Using the MASCIS rat SCI model and standardized testing for thermal hyperalgesia, we have been able to isolate several specific receptors which play a role in the development of NP. Sequential evaluation of the effects of the B1 receptor and the TRPV-1 receptor agonists and antagonists, the phosphodiesterase inhibitor Rolipram, and cyclooxygenase 1 and 2 inhibitors have been performed.

Results:

Administration of antagonists to the pain receptors B1 and TRPV-1 resulted in differential amelioration of TH. Administration of Rolipram significantly decreased the manifestation of TH, an unanticipated result. Cyclooxygenase inhibitors decreased the manifestation of TH, however animal morbidity was a significant problem with these drugs.

Conclusion:

The development and manifestation of NP following SCI is a complex process that utilizes components of nociceptive and inflammatory pathways. Interactions between these pathways may lead to unexpected results. Ongoing research indicates that interference with early inflammation may alter the late manifestation of NP. The mechanism and importance of this finding will be discussed.

Plasticity of Chloride Homeostasis as a Means to Control Neuronal Excitability

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Beyond modifying neurotransmitter release, receptor expression or properties, one way to modulate GABA_A and glycine receptor-mediated signalling is to alter the transmembrane gradient for chloride ions. Because intracellular chloride concentration ($[Cl^-]_i$) is low in neurons, and the reversal potential for chloride currents close to the resting membrane potential, small changes in $[Cl^-]_i$ can dramatically affect the strength, and even polarity of GABA/glycine-mediated transmission. Recent results indicate that chloride homeostasis can be actively regulated in the adult brain and affected by endogenous neuromodulatory agents.

We have recently identified altered chloride homeostasis as a novel mechanism to explain the central hypersensitivity characteristic of neuropathic pain. Following peripheral nerve injury in rats, a collapse of the transmembrane anion gradient occurs in neurons of the superficial dorsal horn of the spinal cord, a region critical for the relay of nociceptive input to supraspinal structures. This collapse of the anion gradient diminished the efficiency of GABA- and glycinergic inhibition and, in a subgroup of cells, inverted the action of GABA or glycine from inhibition to net excitation. This mechanism of disinhibition involved a reduction in the expression of the potassium-chloride exporter KCC2. Local blockade or knock-down of spinal KCC2 in intact rats markedly reduced nociceptive threshold, confirming that the reported disruption of anion homeostasis in lamina I neurons was sufficient to cause neuropathic pain. We then identified the intercellular signalling pathway responsible for the reduction of the anion gradient and its maintenance at a reduced level. It involves activation of spinal microglia by ATP following nerve injury and the ensuing release of BDNF, which in turn acts on neurons to causes a depolarizing shift in anion reversal potential. These findings, together with the observation of altered chloride homeostasis in other neurological disorders point to new targets for therapeutics.

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NMDA Receptors and in vitro Pain

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NMDA receptors in the spinal cord dorsal horn are importantly involved in the development of some forms of chronic pain. These receptors are expressed on neurons throughout the dorsal horn and on some central terminals of primary afferent fibers. It is not known if pain-critical NMDA receptors are expressed throughout the dorsal horn pain circuitry or if receptors at particular synapses are the most critical. Furthermore, while there is evidence for expression of all four NR2 subunits of the NMDA receptors within the dorsal horn, there is little information available about NMDA receptor subunit composition on specific neuron populations, for example, at the synapses between primary afferent fibers and lamina I projection neurons.

Several different approaches have been used in combination to study NMDA receptor expression in the spinal cord dorsal horn. To study pain in vitro, we have developed a technique to identify lamina I projection neurons in the spinal cord slice preparation. We do this by pre-labeling the neurons with tetramethyl rhodamine substance P that binds to the NK1 receptors expressed by the lamina I projection neurons. To identify NMDA receptors on lamina I neurons, we have combined physiological and pharmacological approaches to receptor subtype identification. These approaches, used in combination, are allowing us to identify specific receptor subtypes important for synaptic transmission onto projection neurons.

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Postnatal Tuning and Long Term Plasticity of Dorsal Horn Cell Receptive Fields

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Dorsal horn receptive fields undergo activity dependent tuning in the postnatal period; both low and high threshold excitatory and inhibitory fields are larger in the newborn and gradually reduce in size with age. The changing balance of excitatory and inhibitory receptive fields is likely to be a major factor underlying the decreasing excitability and increasing focus of nociceptive reflex activity over the first weeks of life. In vivo and in vitro experiments have demonstrated the role of dorsal horn NMDA receptor activation and CAMKII autophosphorylation in this tuning process. In addition, a striking switch from tonic excitation to tonic inhibition of dorsal horn cells, arising in the rostroventral medulla, takes place at 3 weeks which plays a further role in shaping receptive fields and nociceptive reflex activity.

The newborn dorsal horn also displays a remarkable plasticity in response to alterations in nociceptive afferent input in the first weeks of life. Early peripheral tissue inflammation or nerve injury result in long term changes in dorsal horn circuitry that are not observed when the same stimuli are applied to the mature organism. These changes will be described and it will be argued that they arise as a result of lack of tuning in dorsal horn circuits and immaturity of control systems during critical neonatal period.

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Combination Therapies to Improve Repair of the Injured Spinal Cord

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Due to the varied and numerous changes in spinal cord tissue following injury, successful treatment for repair may involve strategies combining neuroprotection (pharmacological prevention of some of the damaging intracellular cascades), axonal regeneration promotion (cell transplantation, genetic engineering to increase growth factors, neutralization of inhibitory factors, reduction in scar formation), and rehabilitation. Our goal has been to find effective combination strategies to improve outcome after injury to the adult rat thoracic spinal cord. Combination interventions tested have been implantation of Schwann cells (SCs) plus neuroprotective agents, growth factors administered in various ways, olfactory ensheathing cells (OECs), chondroitinase or cyclic AMP. The most efficacious strategy in our hands for the acute complete transection/SC bridge model, including improvement in locomotion, is the combination of SCs, OECs and chondroitinase administration (BBB 2.1 vs. 6.6, 3X more myelinated axons in the bridge, increased serotonergic axons in the bridge and beyond, and significant correlation between number of bridge myelinated axons and functional improvement). The most successful combination strategy for a subacute spinal cord contusion injury (12.5 mm, 10g weight, MASCIS) we have found to be SCs and elevation of cyclic AMP (BBB 10.4 vs 15; significant increases in white matter sparing, in myelinated axons in the implant, and in responding reticular formation and red and raphae nuclei; and a significant correlation between number of serotonergic fibers and improvement in locomotion. Thus, in two injury paradigms, these combination strategies as well as others studied in our laboratory have been found to be more effective than one intervention alone and suggest ways in which clinical application may be developed.

**Gaining access to the spinal circuitry capable of
generating a functional level of locomotion**

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The balance between intrinsic rhythmicity and pertinent afferent input in locomotor function after spinal cord injury

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The overall goal of this study was to investigate the differential importance of intrinsically oscillating locomotor networks and proprioceptive feedback in producing coordinated hind limb stepping in animals with complete spinal cord injury (SCI).

It has long been established that the lumbosacral regions of the spinal cord contain intrinsically oscillating networks that are capable of producing locomotor-like patterns composed of alternating flexor and extensor activations in the hind limbs of animals. The oscillatory activity does not require descending or afferent input; however, the timing and amplitude of the oscillations are modulated by sensory input conveying hip position and limb loading. The question we asked was: what is the relative role of intrinsic oscillation and afferent feedback in producing locomotor stepping in the hind limbs of cats with complete SCI? More specifically, we were interested in investigating whether activating the locomotor spinal networks (e.g., by using monoaminergic agonists or epidural electrical stimulation) is adequate for restoring robust stepping after SCI.

Experiments were conducted in adult cats with complete low thoracic SCI who were trained to step on the moving belt of a treadmill. After attaining proficient stepping, dorsal root rhizotomies were conducted to remove afferent input related to hip position, limb loading, or other sensory information, and the locomotor capacity was re-evaluated.

After training, animals with complete SCI were able to step on the moving belt of a treadmill with proper placement of the paw, and sufficient loading of the limbs. Following transection of the L3-L4 dorsal roots, which convey information regarding the position of the hip, hind limb locomotion was lost. Similarly, locomotion was lost after transection of the L5-S1 roots which convey information regarding the loading in the hind limbs. However, equally extensive rhizotomies but ones that retained partial input regarding hip position or limb loading had little effect on the locomotor stepping capacity of the hind limbs.

These findings demonstrate that after SCI, the intrinsic oscillations of the spinal locomotor networks alone are inadequate for producing stepping in the paralyzed hind limbs. Instead, pertinent sensory input, especially that conveying hip position and limb loading, plays a significant role in generating the observed stepping capacity on the treadmill. The results also highlight the importance of considering the organization of the afferent input in the lumbosacral enlargement when investigating the location and distribution of the spinal locomotor networks.

GluR1-Dependent Motor System Development

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Glutamatergic synaptic transmission provides the major excitatory drive into motor neurons and so might be an important player in the translation of activity into lasting changes in the motor neuron dendritic tree. Studies on the development of AMPA receptor subunit expression show that motor neurons express particularly high levels of GluR1 in prenatal and early postnatal life, while mature motor neurons do not express the subunit. Forcing mature motor neurons to express GluR1 (using viral vectors) leads to large-scale remodeling of the dendritic tree, indicating that GluR1 expression has morphogenic activity.

Studies of mice lacking GluR1 (complete nulls) were analyzed on behavioral tasks and in terms of motor neuron dendrite morphology. From at least postnatal day 10 onward, the dendritic tree of motor neurons in GluR1 null mice is smaller and less branched in comparison with wild type animals. Study of inputs onto motor neurons (using pseudorabies virus engineered to express GFP) reveals that the afferent inputs into motor neurons is disturbed in the null mice in comparison with wild type mice. In comparison with wild type animals, the GluR1 null mice are weaker on forelimb and hindlimb grip testing, and have less endurance on the rota-rod and treadmill tests. These motor defects are not due to fewer motor neurons in the null mice, but study of muscles reveals more type I fibers (which are smaller in diameter) in comparison with the wild type animals. These results suggest that GluR1-dependent motor system development is subserved by appropriate elaboration of motor neuron dendrites and segmental spinal cord connectivity.

We used the LoxP-Cre system to study the role of GluR1 specifically in motor neurons in motor development. The conditional knockout animals had a reduction in the size and complexity of the motor neuron dendritic tree in comparison with wild type mice. They display some (reduced grip strength), but not all (endurance on Rota-rod and treadmill tests), of the locomotor defects seen in the complete null mice. These results suggest that normal elaboration of motor neuron dendrites is associated with, and we suggest necessary for, the development of peak grip strength (as assessed by the grip strength meter). GluR1-dependent neuronal function at other levels of the neuroaxis is required for appropriate acquisition of motor behaviors assessed by the rota-rod and treadmill, such as endurance and coordination.

Short Term GABAergic Plasticity in the Developing Dorsal Horn

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The firing properties of dorsal horn neurones in the neonatal rat suggest that inhibition is less efficient in the immature spinal cord. This is unlikely to be explained by an absence of functional GABAergic inhibition as antagonism of GABA_ARs augments neuronal firing in vivo from the first days of life. An alternative hypothesis is that age-dependent differences in the short term plasticity of GABAergic neurotransmission could influence the relative excitability of the neonatal spinal cord.

Whole-cell patch clamp recordings were made in spinal cord slices at a range of postnatal ages (P3, P10 and P21) to examine mono-synaptic GABAergic inputs onto superficial dorsal horn neurones. Repetitive stimuli were applied focally to the dorsal horn to investigate dynamic changes in presynaptic GABA release. Firstly, the variability of single GABAergic IPSC amplitudes within cells was measured and these fluctuations were found to decrease significantly between P3 and P21. Secondly, paired pulse ratios were measured at a range of interstimulus intervals (25 msec - 5 sec). Facilitation was observed throughout the early postnatal period but the dependence of the ratio on the interstimulus interval varied significantly across age groups with a significant trend towards greater facilitation at later postnatal ages.

In other experiments trains of 40 stimuli were applied at various frequencies (1 to 20 Hz). Normalising the amplitude of the final IPSCs against the first response revealed frequency-dependent short term depression (STD) at all ages, although the magnitude of the STD did not differ significantly between postnatal ages. This investigation was then extended by measuring the amplitude of IPSCs evoked at set intervals after the end of the train. Here it was found that after a 20 Hz train cells from younger animals recovered significantly more slowly from depression.

The existence of both paired pulse facilitation and STD after repetitive stimulation at all postnatal ages suggests that the presynaptic terminals of GABAergic dorsal horn neurones from P3 rats have qualitatively similar release properties to those from the more mature nervous system. However, significant differences in the dependence of potentiation on presynaptic firing rate and the speed of recovery after repetitive stimulation could have implications for the integration of synaptic inputs within the developing superficial dorsal horn.

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Characterization of PC12 Cell Growth on Electrically Conductive Polypyrrole Coated Fabric

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Although the use of electricity in medicine has been largely shunned due to several misguided experiments early on, recent studies have demonstrated that low level electrical currents can have significant effects at the cellular level. Such effects in neurons include disruption of the cell cycle and increased axonal outgrowth. The following study explores the use of a synthetic polypyrrole fabric with conductive properties as a substrate for PC12 cells in culture. Preliminary results indicate that the fabric is conducive to neuron growth. The following phase of the project explores the characterization of the effects of low-level electric currents applied to the polypyrrole fabric. Cultures will be evaluated and compared on the basis of neurite outgrowth direction, density, length.

A Conformable Elastomer-Substrate Microelectrode Array (MEA) for Stimulation of Spinal White Matter Tracts

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We describe the evaluation of a novel microelectrode array (MEA) for spatially selective activation of spinal circuitry via surface stimulation of axonal tracts. The MEA wraps conformably around the circumference of the cord and is constructed of photopatterned gold traces within a very thin polydimethylsiloxane substrate (PDMS; Dow Corning Sylgard 184). This PDMS substrate confers elasticity and oxygen-permeability to the electrode array, which facilitates close proximity of multiple electrodes for electrical stimulus delivery.

Our experimental setup involves wrapping the MEA around the isolated in vitro hemisectioned spinal cord of the juvenile rat (P10-P15) and stimulating the thoracic (T4-T8) ventrolateral funiculus using a bipolar current configuration. We evaluate the degree of stimulus spread by recording surface compound action potentials (CAPs) at multiple circumferential sites distant from the site of activation. Briefly, a glass suction recording electrode (40-50 mm internal diameter) is placed 10-12 mm (5-7 segments) caudal to the stimulation site to record CAP responses in 50 mm lateral increments from the site of the peak response. Recorded CAP signals are then evaluated based on the rectified and integrated signal (mV*ms).

MEA stimulus values that reliably elicit a threshold CAP response require a charged-balanced single pulse of 700mA/500ms. This compares to a value of 300mA/100ms for bipolar tungsten electrodes. Selectivity of tract stimulation was demonstrated with a decremented then absent CAP on laterally adjacent axonal tracts. MEA selectivity compares favorably to that evoked with tungsten bipolar stimulating electrodes. Further, when the MEA is not placed conformably, but instead placed loosely in approximation to the cord, no recruitment of white matter tracts is discernable at any single-pulse value within the range of our stimulating device (800mA/200ms). Future MEA studies are directed at using white matter tract stimulation for selective recruitment of spinal cord functional systems.

Astrocyte dysfunction in neurological disease - a target for protective therapy

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Spinal Cord Dopamine and Restless Legs Syndrome

Shawn Hochman

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Restless Legs Syndrome (**RLS**) is a disorder involving abnormal limb sensations that peak at night, worsen at rest, and are reduced during movement. Dopaminergic (**DA**) systems are involved in RLS, as $D_{2\text{-like}}$ receptor agonists provide relief. The hypothalamic A11 DA neurons are implicated as they reside in a region that modulates autonomic function and exhibits circadian rhythmicity, both key features of the RLS phenotype. A11 DA projections are the only source of spinal DA, projecting diffusely, but richly innervating both spinal cord sympathetic preganglionic neurons (**SPNs**) and sensory input regions. As the A11 provides the only supply of spinal DA, studies on the actions of DA in spinal cord can be directly ascribed to A11 DA actions

This presentation will outline putative mechanisms involved in RLS, and propose that DA-induced dysfunction in spinal sensorimotor¹ and sympathetic² systems contribute to RLS. We have begun studies in mouse on spinal DA systems in these areas. First, using real-time PCR we show that all DA receptors are expressed in the cord with dominant D_2 expression. *In situ* hybridization revealed that expression for all DA receptors was in the gray matter, labeling motoneurons, and subpopulations of cells in most or all laminae including the superficial dorsal horn.

(1) *In vitro* studies show that $D_{2,3}$ receptors depress spinal reflexes, but DA-induced facilitatory actions are also observed including via a reduction in presynaptic inhibition. Two animal models of RLS are then studied: A11 lesioned rats and D_3 receptor knockout (**D_3 KO**) mice. A11 lesions reduce spinal DA and increase motor activity. D_3 KO mice similarly display increases in motor activity, as well as in reflex excitability.

(2) We show diurnal cycling of DA synthesis in spinal A11 terminals examined in the intermediolateral nucleus (**IML**), the location of SPNs. We also show that IML SPNs undergo diurnal cycling in their genetic expression profiles, including in D_2 receptor. Hence, DA transmitter synthesis and spinal DA receptors have a diurnal rhythm. Intriguingly, both DA synthesis and SPN expression profiles have altered diurnal expression patterns in the D_3 KO RLS mouse model.

Thus, A11 DA neurons modulate cord function, and likely with a strong diurnal dependence. Dysfunction in cyclical expression patterns may lead to the enhanced nighttime reflex excitability seen in RLS.

Spinal Respiratory Plasticity in a Rodent Model of ALS, the SOD1^{G93A} Mutant Rat

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ALS causes respiratory motoneuron degeneration and, ultimately, patient death by ventilatory failure. However, in a transgenic rat model of ALS that over expresses mutated superoxide dismutase-1 (SOD1^{G93A}), normal or even enhanced ability to increase breathing is observed until late in disease progression (20% decrease in body mass). Preliminary motor neuron counts suggest > 80% loss of phrenic motor neurons and > 60% loss of thoracic motor neurons, including inspiratory intercostal motor neurons. These observations pose a dilemma: how do SOD1^{G93A} rats preserve inspiratory capacity when faced with major losses of motor neurons innervating the respiratory pump muscles? We hypothesize that spinal and/or neuromuscular compensatory plasticity preserve breathing until such compensation is no longer possible; the rats will eventually die from ventilatory failure. Compensatory plasticity may arise from increased motor neuron activation, replacing lost contributions from dead or dying motor neurons, and/or motor neuron sprouting, increasing the size of motor units. To assess the former possibility, phrenic and hypoglossal nerve activity were assessed in end-stage SOD1^{G93A} or wild type rats at different levels of respiratory drive regulated by controlling arterial PCO₂. The apneic CO₂-threshold was not different between groups (SOD1^{G93A}: 41±1 mmHg; wild type: 42±2 mmHg). However, phrenic burst amplitude was reduced proportionately (~45%) between baseline conditions and severe hypercapnia. Thus, < 20% surviving phrenic motor neurons generate > 50% of maximal phrenic motor output, indicating that CNS plasticity amplifies their contributions to breathing. On the other hand, since phrenic motor output is reduced, other forms of compensatory plasticity are needed to fully restore normal inspiratory capacity (e.g. greater relative inspiratory intercostal activity and/or diaphragmatic motor end-plate plasticity); these possibilities have not yet been assessed. To begin investigations of compensatory plasticity in surviving phrenic motor neurons, we have been guided by our model of phrenic motor plasticity following intermittent hypoxia, phrenic long-term facilitation (pLTF). pLTF arises from serotonin-dependent increases in BDNF synthesis and release; subsequent TrkB receptor signaling strengthens synaptic inputs onto phrenic motor neurons, thereby amplifying phrenic motor output. Repetitive exposure to acute intermittent hypoxia enhances pLTF and up-regulates key molecules involved in the underlying mechanism. Preliminary investigations of SOD1^{G93A} rats provide evidence that similar mechanisms are operative during motor neuron disease. Progress to date will be discussed. Supported by ALS Association and NIH (HL89209, HL69024 and HL07654).

Development of Excitatory Inputs On Renshaw Cells

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The mechanisms by which adult synaptic circuits emerge from a complex set of immature synapses and embryonic interneurons are unknown. Central to this process is the development of distinct types of adult interneurons (INs). A major characteristic that defines adult INs is their synaptic input organization. How are distinct synaptic architectures developed on adult INs? Are synaptic inputs predetermined in early embryo by genetic programs or mature postnatally through use-dependent mechanisms? This talk will describe recent findings on Renshaw cell development that provides new information on these questions.

Renshaw cells develop from one subclass of embryonic INs, named V1, and constitute a distinct phenotype representing ~10% of all V1-derived INs. Thus, V1 INs diversify into several subtypes, but also share some common characteristics, including receiving projections from sensory axons. Not surprisingly Ia inhibitory interneurons (IaINs) are also V1-derived. However, Renshaw cells and IaINs are characterized by very different synaptic organization; IaINs preferentially respond to sensory inputs and Renshaw cells to motor axons. These observations beg the question of how neurons with such different synaptic organization arise from a common embryonic group.

Motor axon synapses are established first on Renshaw cells in early embryo. However at later embryonic stages proprioceptive axons invade the spinal cord and target V1-derived neurons including Renshaw cells. Contrary to expectation, sensory synapses, likely from Ia afferents, spread to all Renshaw cells in the neonatal spinal cord and proliferate in parallel to motor axon inputs during the first two postnatal weeks. Renshaw cells are thus competent of receiving a functional primary afferent input in neonates. However, sensory inputs on Renshaw cells become arrested in development (their density and synaptic apparatus are reduced) after the second postnatal week (just after weigh-bearing locomotion is initiated). Motor axon synapses, meanwhile, continue to proliferate and mature. As a consequence strong motor axon inputs and weak, if any, sensory inputs are matured on adult Renshaw cells. The maturation arrest of sensory synapses is prevented in animals in which central proprioceptive synapses are enhanced by excess muscle NT3. Similarly, Renshaw cells in animals with depleted levels of muscle-derived NT3 lose significantly more sensory synapses. Thus, activity-dependent mechanisms regulate sensory input deselection on Renshaw cells. Motor synapses react in a complementary manner, increasing in density when sensory synapses are reduced and decreasing when sensory synaptic density increases. The results suggest a competitive mechanism that regulates the balance of excitatory inputs from different sources on Renshaw cells and refines the final adult synaptic input that characterizes this interneuron.

Activation of the central pattern generator by stimulation of the ventral roots in the neonatal mouse spinal cord; possible mechanisms

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Spinal lumbar motoneurons have long been considered to be the solely output elements of the central pattern generator responsible for locomotor activity. We have shown recently that stimulation of ventral roots in the isolated neonatal mouse spinal cord preparation *in vitro*, results in locomotor-like activity. The mechanisms responsible for this type of activation are currently unknown. Ventral roots contain both somatic and autonomic (sympathetic) motoneurons, so that activation of either or both populations could mediate this effect. During early development (first postnatal week) motoneurons are connected to other motoneurons via electrical and chemical synapses. Although motoneuron axon collaterals activate inhibitory Renshaw cells, during the first postnatal week GABA and glycine may still be depolarizing because of the elevated intracellular chloride concentration of neonatal neurons. One observation that might help explain our original report that stimulation of ventral root axons resulted in locomotor-like activity in the presence of cholinergic blockers, is the release from motoneurons of a second fast excitatory neurotransmitter possibly glutamate or aspartate, in addition to acetylcholine. Antidromic stimulation of motor axons evoked depolarizing monosynaptic potentials in Renshaw cells that are depressed but not abolished by cholinergic receptor antagonists. This residual potential (~25% in amplitude) was abolished by the glutamate receptor antagonists APV and CNQX.

Tracing experiments with fluorescent dyes applied *in vitro* in the ventral roots resulted in labeling of motoneurons as well as sympathetic neurons in the lumbar regions of neonatal mouse spinal cords. Furthermore, calcium imaging experiments reveal that the sympathetic neurons are also activated antidromically following ventral root stimulation. If these neurons possess axon collaterals and make synaptic connections to other interneurons or even motoneurons, it may also implicate them in the activation of the central pattern generator.

Brainstem and Spinal Cord Rhythm Generating Mechanisms

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This session will focus on different rhythmic motor behaviors that are generated in brainstem (respiratory rhythm) and spinal cord (locomotion and scratching). These rhythmic behaviors have characteristics in common including a rhythm generating mechanism and a patterning or shaping mechanism. Also common to these systems is the concept of reconfiguration. Thus different rhythmic behaviors involving the same target muscle groups can emerge from reconfiguration of the same neural circuits. Also in common is the palette of ion channels that are involved in rhythm generation including, in some systems, persistent sodium (I_{NaP}) and Ca^{2+} -activated non-specific cation (I_{CAN}) currents. Commonality of synaptic connections is also apparent including the relative importance of synaptic inhibition.

Our work in the last few years has focused on the patterning of motor activity that is occurring within the rhythmic inspiratory burst (I-burst) of activity, and the role that synaptic inhibition has in generating this pattern. During the I-burst I-motoneurons fire clusters of action potentials, these arise from oscillatory synaptic inputs, and clusters occur at a particular frequency. Such clustering, which is also termed I-phase synchronous oscillations, is ubiquitous, being observed in all mammalian species studied. Also, they are found in the I-phase spike discharge in every inspiratory motor pool: phrenic, laryngeal, external intercostals and hypoglossal, as well as in medullary I-neurons. Functionally it has been found that oscillatory inputs to I-motoneurons increase their input-output efficiency, also the pattern of I-motoneuron spike timing is dependent on oscillatory inputs. Our recent work (Sebe et al., J. Neurophysiol. 96: 391, 2006), using the in vitro rhythmic neonatal mouse medullary slice preparation and recording hypoglossal motoneuron I-bursts, has shown that the average oscillation frequency increased with postnatal development, from 17 Hz during the first postnatal week to 38 Hz during the second week. Bath application of $GABA_A$ - and glycine-receptor antagonists significantly decreased these synchronous oscillations yet peak integrated I-burst activity was observed to increase. When the time course of $GABA_A$ mediated synaptic transmission was increased by bath application of zolpidem the median frequency of these synchronous oscillations was decreased. Thus inhibitory synaptic transmission is directly involved in the mechanism generating these I-burst synchronous oscillations and the time course of this transmission is a determinant of oscillation frequency.

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The Generation of Inspiration: Insights gained from a Brainstem Rhythm Generator

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Compared to walking, breathing is a relatively simple motor behavior. Proprioceptive feedback is less critical for generating the respiratory rhythm, and there are only three phases of rhythmic activity: Inspiration, Postinspiration and Expiration. Moreover, areas that are critical for the generation of the respiratory rhythm have been identified. One of the hallmark of respiratory rhythm generation is its plasticity and state-dependent reconfiguration. Breathing undergoes dramatic alterations in order to adapt to changes in metabolic demand and behavioral conditions. In mammals these changes are so dramatic that it was long believed that normal breathing (eupnea), gasps and sighs are generated by different neuronal networks. Here we show that the same neuronal network generates different forms of breathing based on network reconfiguration. This reconfiguration involves drastic changes in rhythm generating mechanisms. Under normal conditions heterogeneous types of pacemakers differentially control amplitude, frequency and stability of the respiratory rhythm. The relative contribution of these pacemakers and the number of active pacemakers is highly regulated by neuromodulators. By modulating pacemaker and network properties neuromodulators also regulate the number of sighs. During extreme conditions, such as in the absence of oxygen, many pacemakers and inhibitory neurons shut down and rhythm generation depends only on excitatory synapses and one subpopulation of pacemakers that drive the respiratory network.

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Synaptically Activated Intrinsic Conductances in Network-based Mechanisms for Respiratory Rhythm

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Breathing in mammals depends on respiratory neurons in the preBötzinger Complex (preBötC) that form excitatory networks. Glutamate receptors are critical for the synchronous bursts that form the basis for inspiration. The conventional explanation given by the *pacemaker hypothesis* is that excitatory synaptic interactions synchronize a rhythmogenic population of intrinsic bursting ‘pacemaker’ neurons in the preBötC.

Pharmacology has been used to bolster support for the pacemaker hypothesis. In one type of experiment, drugs that modify burst frequency in synaptically isolated pacemaker neurons are shown to similarly regulate the frequency of the intact network. In the second type of experiment, drugs that block cellular conductances abolish or severely perturb network activity.

Yet both experiments fail to provide a causal link between pacemakers and network rhythmogenesis: (i) bath-applied drugs affect all the neurons and can regulate rhythmic frequency (or stop it altogether); and (ii) the use of nonselective agents, especially at high dosages, leads to the false conclusion that abolishing pacemaker properties stops (or perturbs) the rhythm. In both cases, these protocols induce network-wide effects on excitability and synaptic transmission, which are not specific to pacemaker neurons, but appear to be important for rhythmogenesis.

We performed ‘clean’ experiments in neonatal rodents (P0-5) in which Ca^{2+} -activated non-specific cationic current (I_{CAN})-mediated pacemaker neurons are developmentally precluded. Persistent Na^+ current (I_{Nap})-mediated pacemaker neurons can be blocked by bilaterally microinjecting the preBötC with 10- μM riluzole (RIL) or 20-nM tetrodotoxin (TTX), but rhythm remains at control frequency. This suggests that pacemaker neurons are not rhythmogenic.

Therefore, we re-examined the obligatory role of glutamate receptors from the perspective of the *group-pacemaker hypothesis*, which posits that recurrent synaptic excitation evokes postsynaptic conductances that then amplify synaptic input and thus promote inspiratory burst discharge in all preBötC neurons. We found that AMPA, NMDA, and group I metabotropic glutamate receptors (mGluRs) significantly contributed to inspiratory bursts: AMPA and NMDA receptors cause depolarization and mGluRs operate via an inositol 1,4,5-triphosphate (IP_3) receptor-dependent mechanism. Both factors converge to evoke I_{CAN} in all preBötC neurons (regardless of pacemaker properties), which generates 10-30 mV depolarizations that ‘drive’ inspiratory bursts. We conclude that I_{CAN} underlies robust inspiratory activity in preBötC neurons, and is only fully evoked by ionotropic and metabotropic glutamatergic synaptic inputs, i.e., by network activity. Our findings emphasize the importance of emergent network properties in respiratory rhythm generation.

Creating a Variety of Motoneuron Activity Patterns in the Two-level Mammalian Locomotor CPG

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With Ilya Rybak and colleagues at Drexel University, we have developed a computational model of the hindlimb locomotor CPG and its integration with spinal reflex circuits. Interacting populations of interneurons and motoneurons are modelled in the Hodgkin-Huxley style and the locomotor CPG is organized into separate interneuronal networks for the rhythm generator (RG) and for motoneuron activation (pattern formation, PF). Rhythm generation results from a combination of persistent sodium currents, mutual excitatory connections and reciprocal inhibition between the two half-centres in the RG network. The RG produces stable oscillations encompassing the full range of locomotor cycle periods and phase durations observed during cat locomotion and controls the timing of PF network activity. The separation of RG and PF networks allows the model to closely simulate a variety of sensory perturbations of locomotion as well as the ability to reproduce spontaneous deletions of motoneuron activity in which locomotor cycle timing and phase is maintained. In its simplest form, the PF network also consists of two half-centres coupled by reciprocal inhibition and creates two locomotor phases with non-overlapping, alternating activity in two antagonist (flexor and extensor) motoneuron populations. We now show how the model can be extended to reproduce the more complex and varied activity of motor pools innervating bifunctional muscles such as posterior biceps or semitendinosus (PBSt). Our analysis during fictive locomotion in decerebrate cats shows that PBSt activity falls into one of four patterns. Since these patterns occur without the influence of proprioceptive feedback or cortical control, they must be generated by the central locomotor circuitry. By making relatively simple additions to the PF network controlling PBSt motoneurons, their activity can be made to be biphasic (i.e. firing twice per cycle) or confined to the entire duration or a portion of either the flexion or extension phase. Importantly, this circuitry can also reproduce the behaviour of PBSt during deletions that affect hindlimb extensors or flexors. In summary our model shows how a simple two-level CPG organization with a separate rhythm generator and multiple, individualized pattern formation networks can provide a plausible explanation for a number of features of real CPG network operation including complex patterns of motoneuron activity. Supported by the NIH (R01 NS048844) and the Canadian Institutes for Health Research.

Neuronal Networks for Rhythmic Motor Behaviors of the Turtle Hindlimb

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Fundamental characteristics of central pattern generators (CPGs), neuronal networks responsible for producing motor behaviors, have been revealed in studies of the neuronal control of turtle hindlimb rhythmic movements (Stein, *J Comp Physiol A* 191: 213, 2005). The talk describes key features of this model system with results from past studies and directions for future studies. The turtle hindlimb participates in a variety of motor rhythms, e.g., scratching, swimming, and stepping. Each motor task exhibits several forms: rostral, pocket, or caudal scratch; forward or backward swim; terrestrial stepping or aquatic bottom walking. Some movement properties occur during all forms, e.g., alternation between hip flexion and extension. Other features are distinct for each form of a task: the specific phasing of knee extension within the cycle of hip rhythmic movements; the timing of high-force events, e.g., scratch rub, swim powerstroke. Scratch and swim have been studied in preparations with complete transection of the spinal cord just posterior to the forelimb enlargement. Fictive motor patterns recorded with ENG's in these spinal preparations are excellent replicas of the actual motor patterns recorded with EMG's in spinal preparations with hindlimb movement. Multiple lines of evidence support the concept of shared spinal circuitry among the CPGs that produce the motor rhythms for each form of each task. At the behavioral and motor-pattern levels, the existence of motor-pattern blends, e.g., switches and hybrids, supports the shared-circuitry concept. Analyses of motor-pattern after-excitabilities provide further insights into neuronal mechanisms of selection among behaviors. At the single-unit level, many spinal neurons are active during each of several motor behaviors: these neurons belong to shared circuits. Single-unit recordings during motor-pattern variations test a specific hypothesis of shared circuitry, the unit-burst-generator hypothesis of modular organization of spinal pattern generators (Grillner, *Neuron* 52: 751, 2006). Prior work supports the concept that hip-extensor interneurons are members of a hip-extensor module during rostral scratch. Work in progress provides evidence that knee-related interneurons belong to modules that are different from modules containing hip-related interneurons. Supported by NIH Grant NS30786.

An In Vitro Model for Investigating Spinal Control of Limb Movement

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Studies on mammalian locomotor rhythmogenesis have traditionally relied on the isolated rodent cord maintained *in vitro* with locomotor activity monitored at the ventral roots. However, the recorded motor activity cannot relate cellular events to behavioral biomechanics. Moreover, the role of sensory feedback from ongoing behavior is absent. While studies in the intact animal offer behavioral observability, they lack the cellular and neurochemical accessibility offered *in vitro*. To better relate spinal locomotor mechanisms to behavior, we developed a hybrid preparation that combines the neural accessibility of the isolated cord with unrestrained hindlimb locomotion.

We first investigated locomotion induced by bath application of NMDA ($\sim 2\mu\text{M}$) and 5HT ($\sim 35\mu\text{M}$) in a ventral-up neonatal rat cord with hindlimbs attached. We characterized the kinematics, joint coordination, and muscle activations of free air-stepping. Compared to *in vivo*, *in vitro* locomotion exhibited reduced frequency, range of motion, and stance-phase yielding, as well as altered ankle-knee coordination. We also observed a near-reversed phasing of vastus lateralis relative to tibialis anterior. Together these support an altered response due to reversed limb orientation and altered sensory feedback. To yield a more behaviorally-relevant model, we turned the isolated cord and hindlimb dorsal-up, allowing the limbs to locomote on a surface with more appropriate mechanoenvironmental interactions. Here the range of motion, stance-phase yielding, and ankle-knee coordination more closely resembled those observed *in vivo*, suggesting that sensory feedback contributes to locomotor patterning even in a reduced prep.

We then tested the effects of octopamine ($\sim 10\mu\text{M}$), a trace amine, on ongoing 5HT/NMDA locomotion. Octopamine differentially modulated the strength of two quadriceps, increasing the strength of vastus lateralis (knee ext) and decreasing the strength of rectus femoris (knee ext/hip flex) (n=2). Thus, neuromodulators may modulate neuromuscular synergies, an observation undetectable at the ventral roots.

In conclusion, this preparation allows us to directly identify the behavioral effects of specific manipulations to the isolated cord. In addition, the model contributes sensory modulation typically absent *in vitro*. Finally, the potential to simultaneously monitor cellular events and behavioral outcomes provides a platform for studying the cellular substrates of behavior and spinal control of movement.

Trace Amines Excite Spinal Motor Circuits in the Isolated Neonatal Rat Spinal Cord

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The trace amines (TAs), tryptamine, tyramine, octopamine, and β -phenylethylamine (PEA), are present in the mammalian CNS in low concentrations with uncertain physiological actions. Chemically related to the classic monoamine transmitters, they are synthesized from the same precursor amino acids and require many of the same synthesis enzymes, including aromatic-L-amino acid decarboxylase (AADC). In 2001, a large family of metabotropic TA receptors was cloned. One of them, TA₁, had reported expression in the ventral horn, the location of spinal motor systems. Hence, we studied the properties of the TAs on the spinal motor system in the neonatal rat spinal cord.

Subpopulations of spinal neurons were immunolabeled for tryptamine, tyramine, octopamine, TA₁, and AADC. Labeling was dominant in the ventral horn and included motoneurons, providing anatomical evidence of an intrinsic spinal TA system.

Neuromodulatory actions were tested with bath application of the TAs while recording ventral root activity in the isolated spinal cord maintained *in vitro*. Applied alone, the TAs had two different effects: **1)** Tryptamine (n=18/19), tyramine (25/30), and octopamine (3/9), but not PEA (0/7) increased spontaneous motor activity. **2)** Tryptamine (n=2/19), tyramine (3/30), and octopamine (1/9) also produced slow rhythmic motor patterns. Co-applied with NMDA, the TAs recruited locomotor-like activity (LLA) having two different phenotypes: **1)** Tryptamine (n=9/14), tyramine (17/26), octopamine (3/8), and PEA (2/8) produced LLA similar to 5-HT/NMDA. **2)** Tyramine (n=7/26), octopamine (6/8), and PEA (7/8) also produced a complex form of LLA, characterized by a slower frequency rhythm that separated LLA into bouts. These ‘metarhythms’ were never seen with tryptamine or 5-HT, indicating selectivity to catecholamines.

TAs are found in high concentrations in foods such as cheese and chocolate. To further investigate behavioral relevance of the TAs on spinal motor behavior, we injected TAs i.p. into previously transected rats. Preliminary results support robust increases in hindlimb motor activity both for PEA and octopamine.

Overall, the existence of TA signaling systems in spinal neurons and their ability to recruit LLA both *in vitro* and *in vivo* support the notion that TAs are neuromodulatory transmitters in the mammalian spinal cord.

Glial Response to Injury in the SOD1G93A Model of ALS

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ALS is typically characterized as a motor neuron disease, but is also known to involve other cell types in the central nervous system. While the appearance of reactive astrocytes precedes or coincides with loss of motor neurons, the role of astrocytes in disease pathology is not well established. In spinal cord injury, reactive astrocytes are critical to preventing excessive neural damage. Although reactive astrocytes are present in ALS, neurodegeneration continues to progress. In order to test the ability of reactive astrocytes to function properly in the ALS rat, a mild injury was induced to the lumbar spinal cord of 60 day old pre-symptomatic rats. This injury model does not affect motor neuron survival and the blood brain barrier is healed within 2 weeks in wildtype rats. The response of mutant SOD1 astrocytes to injury and their ability to protect motor neurons will be evaluated. Glial response will be measured by proliferation and markers of reactive astrogliosis. Motor neuron numbers and early signs of degeneration such as loss of neurotransmitter phenotype will be analyzed. By inducing a reactive glial environment, our experiments will assess the contribution of astrocytes to motor neuron vulnerability in ALS.

Use of SOD1 Mutations in Embryonic Stem Cell Derived Motoneurons and Astrocytes as a Model for ALS

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Amyotrophic Lateral Sclerosis (ALS) is a fatal disease of selective cell death of motor neurons. Genetic linkage analysis has determined that ~5-10% of cases are familial, with ~20% of these cases caused by mutations in Cu/Zn-binding superoxide dismutase (SOD1). The cause of neurodegeneration has been implicated in a wide range of pathways, including protein aggregation, oxidative stress, mitochondrial damage, excitotoxicity and astrocyte involvement, though an appropriate *in vitro* model system does not yet exist. Recently, functional spinal motoneurons and spinal astrocytes have been generated through differentiation of human embryonic stem cells (ESCs). To create a useful model of motoneuron degeneration, wild type and mutant SOD1 sequences, fused to a GFP reporter, have been delivered into mouse and human ESCs using lentiviral infection. Motoneurons from transgenic mouse ESCs have been successfully derived, and human neurons expressing the mutant protein show abnormal protein localization and mitochondria with an upregulation of oxidative stress. This system will enable analysis between various SOD1 mutations and their effect in different species. Ongoing experiments are using motoneuron and astrocyte co-cultures to examine cell type specific effects of SOD1 mutations. A better understanding of early cellular events involved in ALS will be important to focus on more efficient therapies.

Okadaic Acid-Sensitive Protein Phosphatases Constrain Phrenic Long-Term Facilitation Following Sustained Hypoxia

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Phrenic long-term facilitation (pLTF) is a serotonin-dependent form of respiratory plasticity that manifests as a progressive augmentation of phrenic motor output following intermittent (IH), but not sustained hypoxia (SH). The mechanism(s) underlying pattern-sensitivity in pLTF is (are) unknown. Since certain protein phosphatases restrain the activation level of protein kinases, which are critical for multiple forms of neuroplasticity, including LTF, we hypothesized that protein phosphatase activation during sustained, but not intermittent, hypoxia confers pattern-sensitivity to pLTF. To test the hypothesis that protein phosphatase activation constrains pLTF following SH, we intrathecally injected okadaic acid, a non-specific serine/threonine protein phosphatase inhibitor (OA, 25nM, n=10), or vehicle (artificial CSF, n=11) over the C4 spinal cord in anesthetized, vagotomized, paralyzed and pump-ventilated male Sprague-Dawley rats prior to 25 min SH ($11\pm 1\%O_2$). 60 min post-SH, integrated phrenic burst amplitude was significantly increased in OA-treated *versus* vehicle-treated rats ($52\pm 21\%$ and $1\pm 8\%$ baseline, respectively). pLTF in rats with OA and SH was indistinguishable from vehicle-treated rats exposed to intermittent hypoxia (3, 5-min $11\pm 1\%O_2$ episodes; $50\pm 9\%$ baseline, n=13). Intrathecal OA failed to enhance IH-induced pLTF ($25\pm 10\%$ baseline, n=7) and intravenous methysergide, a broad-spectrum serotonin receptor antagonist, blocked pLTF elicited by SH and concomitant spinal phosphatase inhibition ($-3\pm 13\%$ baseline, n=6), suggesting that similar cellular pathway(s) are employed to elicit pLTF by these two treatments. Patterned hypoxia may regulate pLTF expression by differential generation of reactive oxygen species (ROS), which directly alters protein phosphatase/kinase activity. Intravenous injection of a superoxide dismutase mimetic (MnTMPyP) prior to IH abolished pLTF ($16\pm 10\%$ baseline, n=8). Furthermore, intrathecal OA revealed pLTF in MnTMPyP-treated rats that received IH ($69\pm 18\%$ baseline, n=4), suggesting that IH may decrease protein phosphatase activity via ROS, thus allowing the expression of pLTF. Taken together, these data suggest that the pattern of hypoxia may regulate pLTF by altering protein phosphatase (and/or protein kinase) activation, perhaps by differential ROS generation. The relevant OA-sensitive protein phosphatase remains to be identified. Supported by NIH HL89209, HL65383 and HL07654.