

Chapter 9

The turtle as a model for spinal motor circuits

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Introduction

The red-eared terrapin (*Trachemys scripta elegans*) has been used as an experimental model for investigating the neural circuits underlying movements for several decades [1]. Compared with other model organisms, the turtle has both advantages and shortcomings. First, due to its semiaquatic habitat and the frequent diving behavior, it has evolved mechanisms to withstand low-oxygen (hypoxic) conditions for long periods of time by relying on anaerobic metabolism in the nervous system. The caveat of this anaerobic metabolism is the acidification by lactate and protons. Nevertheless, the turtle is able to buffer the blood pH by Ca^{2+} and Mg^{2+} released from the shell [2], and thus is able to hibernate at underwater during the winter. This high tolerance for hypoxic conditions means that the blood can be replaced by oxygenated Ringer's solution, and the spinal cord kept alive in vitro, maintaining the integrity of motor circuitry for the duration of experiments. This allows experimental preparations where the parts of the body have been removed, defined as *semiintact* or *integrated preparations*. Combining this high tolerance with a steady flow of oxygenated Ringer's solution makes it easier to keep the neurons alive and the motor circuitry performing during these conditions. The in vitro and semiintact experiments are accomplished without the small movements associated with respiration and cardiovascular functions, which provides improved mechanical stability, so that long-term intracellular recordings are not only possible, but of highest quality. Parts of the turtle spinal cord are located in postcervical and presacral vertebrae (dorsal vertebrae, *D1–D10*), which are fused to the dorsal part of the shell [3, 4]. This property also contributes to the mechanical stability during electrophysiology. Due to this remarkable resistance to anoxia, the in vitro experiments can be performed in adults, which is difficult in mammals. Furthermore, the semiintact preparation can include large parts of the sensory periphery and several kinds of motor behaviors can be elicited (Fig. 1). Besides behaviors such as terrestrial stepping, forward swimming, and back-paddling, there are three forms of rhythmic hind limb scratching [5, 7] and each can be evoked

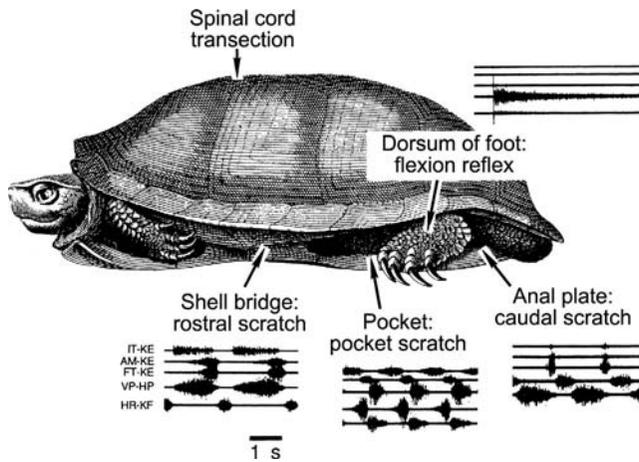


FIG. 1 Distinct motor behaviors of the spinalized turtle can be evoked by gentle somatic touch. After complete spinal transection at the rostral postcervical region (D3–D4, top arrow) the animal is still able to perform various motor reflex behaviors, depending on the sensory activation. Cutaneous touch of sutures in the shell bridge evokes triphasic rostral scratch with a distinct sequence of knee extensors, hip flexor, and knee flexor (*bottom left arrow* and electromyograms). Touching the region adjacent to the proximal portion of the hind limb induces pocket scratching (*middle bottom arrow*) where the knee and thigh then rub against the particular location within the receptive field [5, 6]. The receptive field of caudal scratching is the anal plate (*right bottom arrow*) where the heel of the hind limb rubs. The fourth behavior is the flexion reflex, where the limb is retracted following a gentle tap on the dorsum of the foot. (*Adapted with permission Stein PSG. Central pattern generators in the turtle spinal cord: selection among the forms of motor behaviors. J Neurophysiol 2018;119(2):422–40. <https://doi.org/10.1152/jn.00602.2017>.*)

depending on where on the body is touched [8, 9]. The three forms of scratching each of which can be evoked by tactile stimulation within different receptive fields on the body surface with respect to the limb, i.e., rostral-, caudal- and pocket-scratching. Pocket scratching refers to the region of skin in the cavity where the limb is attached. The sequence of muscular activity is qualitatively different in these behaviors. The knee extensor muscle contracts in the late phase of the hip flexion (hip protraction) during rostral scratching. Pocket scratching produces simultaneous activity of knee flexor and extensor muscles, whereas in caudal scratching the knee extensor cycle occurs after the cessation of knee flexor [7]. This large behavioral repertoire allows investigation of the neural mechanisms behind multifunctionalism and decision making within the spinal circuitry. The elicited motor patterns also have a remarkable reproducibility where the intercycle variability within a bout of scratching is larger than the variability across trials [10]. Last, turtles are poikilotherms, with nervous systems that can function over a broad temperature range, contributing to their convenience as an experimental model.

Experimental model and historical overview

Various versions of the experimental model have been applied for different investigations over the years. After the initial observation that upon complete spinal

transection at the *D3–D4* spinal level the animal was able to perform reflex movements consisting of long-lasting hind limb scratching [11]. This motor behavior was induced by light mechanical touch applied to a suture of the shell bridge (Fig. 1). The first reported *in vitro* study on the turtle spinal cord [12] also included part of the shell and skin with the spinal cord left in the vertebral column (Fig. 2A). This preparation has sometimes been named the *integrated preparation* [13, 15–17] to emphasize the difference compared with experiments where the spinal cord is dissected out and isolated in a chamber (Chapter 11 in this book). The turtle preparation has also been used in conjunction with superfusion of drugs, either across a transverse cut surface [13, 15] (Fig. 2B) or along with the spinal cord in the immobilized turtle [18]. Other similar variants are the *ex vivo preparation* [14, 19], *brainstem-cervical spinal cord preparation* [20], and the *low spinal preparation* [5, 7–9, 18, 21–37], where the spinal column was transected at the dorsal postcervical segments *D2–D3*. Fictive swimming motor behavior is evoked by electrical stimulation of the dorsolateral funiculus (Fig. 2C) [14, 21–24, 36, 38]. Last, the turtle spinal tissue has been used in slice preparations for investigating a wide variety of membrane properties of spinal inter- and motoneurons. The membrane currents and channels were studied by intracellular recordings with the optional application of pharmacology, which can easily be applied to the Ringer medium while recording. Investigating cellular properties using spinal cord slices from turtles was introduced in early studies on the response properties of motoneurons [39–41]. The turtle has also been a model system to study spinal cord injury and neurogenesis and recovery. It was found that the deprivation of serotonergic fibers due to injury led to the emergence of local spinal interneurons that subsequently become serotonergic neurons [42].

A general advantage of the turtle preparation is the option of using hypothermia as anesthesia [21]. Hypothermia by cooling the animal for 2 h in a mixture of ice and water can be applied prior to euthanasia by decapitation and thus prevent potential long-term impact of chemical anesthetics on the integrity of the neuronal circuitry. Hypothermia as the sole means of anesthesia was recently reevaluated and approved for use in reptiles [43]. After euthanasia, the carapace is opened, the blood replaced via transcardial perfusion of Ringer's solution, and inner organs removed [44]. The preparation should be perfused continually with an oxygenated Ringer's solution to optimize the performance of the network [44] and minimize the pH gradient of the tissue [1]. This is most conveniently done through the spinal column, while keeping the neural tissue submerged or at least moist with Ringer's solution. For a detailed record on the procedure, see Ref. [44].

What has the turtle taught us about the circuits for locomotor control?

A number of important and diverse observations, relating to both cellular properties and the circuitry, have been collected about spinal motor control using the turtle.

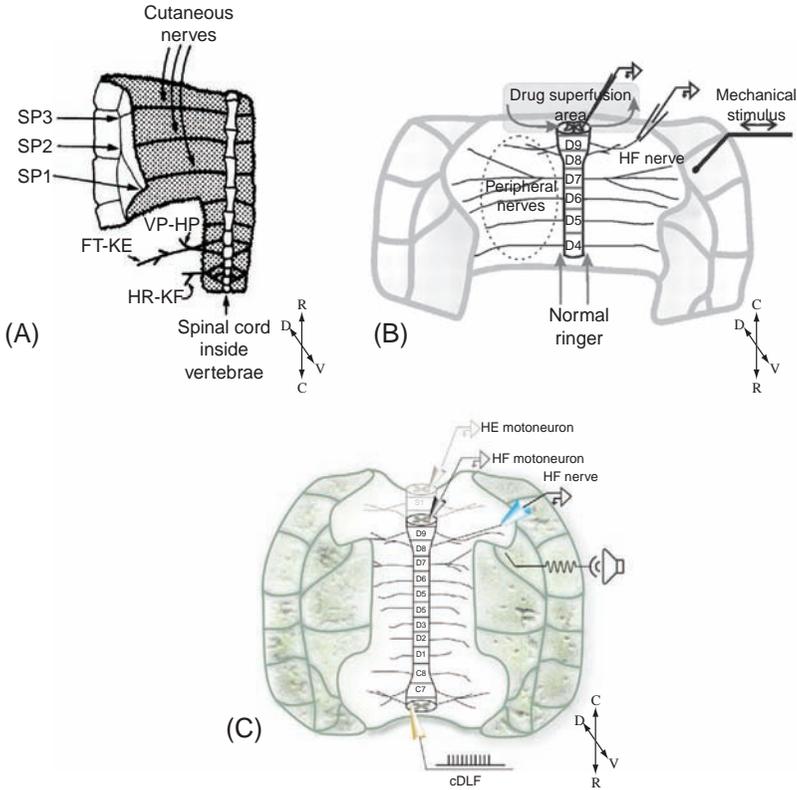


FIG. 2 Variants of the turtle preparation. (A) The original illustration of in vitro preparation first applied [12]. The dorsal parts of the carapace, which contained the cutaneous sensory nerves, were left intact while the ventral plastron was removed and the hind limb motor nerves (primarily the hip flexor and knee extensor nerves) were dissected out. (B) A variant with the upside-down configuration with access to the neurons on the cut surface where drug superfusion could also be applied [13]. The motor behavior (the scratch reflex) was induced by rhythmic mechanical touch (*upper right*). (C) A preparation with more vertebrae where the dorsolateral funiculus was stimulated to induce swimming behavior in conjunction with the evoked scratch behavior again by mechanical touch (*upper right*) [14]. D, dorsal; V, ventral; R, rostral; C, caudal. ((B) Copyright 2003 Society for Neuroscience. (C) Adapted with permission from R. Guzulaitis, J. Houmsgaard, A. Alaburda, *Irregular firing and high-conductance states in spinal motoneurons during scratching and swimming*, *J Neurosci* 2016;36(21):5799–807. <https://doi.org/10.1523/JNEUROSCI.0320-16.2016>. <http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.0320-16.2016>.)

Organization: Modular and distributed

The concept of neuronal modules is a major theme in spinal motor activity and work using the turtle has contributed to this idea [3]. A modular organization is defined as a “set of interneuronal populations that are each related to a single function at a single joint,” [28] which is similar to the “unit burst generator” hypothesis proposed by Grillner [45]. Modular organization

was also investigated with aid of the phenomenon termed "deletions," i.e., a variation of the motor pattern, where one of the motor phases is absent in one cycle [46]. The deletions occur for unknown reasons, but two important conclusions were extracted from their behavior. First, the spinal neurons that have correlated activity with the muscle, that fall out during a deletion, seem to all fall out and this suggests the concept of a module, i.e., a population of neurons with the same function. Second, the rhythm continues during a deletion, which suggests that the rhythmogenic network is independent of this module, and this is incompatible with the traditional half-center hypothesis [28, 46]. In attempts to pinpoint the location of the modules, the cord was sequentially transected while observing the motor rhythm and the rostral segments of the lumbosacral enlargement were found to be more rhythmogenic [29]. This is similar to the observations extracted from the spinal cord from newborn rodents, where the rhythmic activity was induced by 5-HT and NMDA [47, 48]. Nevertheless, the rhythmogenic circuitry seems to be distributed across several segments in the rostrocaudal direction, which has also been suggested by thoracic measurements [49] and retrograde axonal labeling [50]. Systematic lesioning indicates that the rhythm gradually decreases as well as the pattern as more segments are lesioned both for scratching and swimming [29, 30].

Multifunctional and dedicated neurons coexist

Since the turtle preparation is capable of generating multiple distinct motor behaviors, in contrast to the neonatal mouse and rat preparations, it is an ideal test bed for the issues of multifunctionalism in spinal circuits [31]. The motor behaviors that have been studied in the turtle include three types of locomotion (forward stepping, forward swimming, and back paddling) [51], flexion withdrawal reflex, and three forms of hind limb scratching [5]. The scratching consists of a rhythmic circular movement of the hind limb rubs against the bodily region where a somatic touch or irritation has been imposed [32]. It is useful to study, because it is primarily an ipsilateral sensory-motor reflex; hence, the left versus right scratching represent two different behaviors. A group of interneurons that are strongly activated during limb withdrawal (flexion reflex, Fig. 1), but inhibited during other behaviors likely has more of a specialized function [33]. Although there are individual neurons dedicated to a particular behavior, the general observation from intracellular recordings from spinal neurons indicate that shared networks mediate various motor behaviors, e.g., swimming and scratching [34, 35]. When the transition zone between receptive fields is touched, e.g., rostral- and pocket scratching, the resulting behavior can be either of the two, or alternatively, a blend of the two. A blend can consist of consecutive alternation between two behaviors, or a hybrid, where the motor patterns has features of both behaviors simultaneously [3, 51, 52].

Synaptic excitation and inhibition: Balanced versus reciprocal

Due to the mechanical stability of the turtle preparation and the high reproducibility of the motor activity (Fig. 3), it has been possible to perform intracellular recordings to extract the mean synaptic currents and conductances as they change during the rhythmic motor activity [15, 19, 53, 54]. Hence, it has been possible to verify key concepts in spinal motor control and central pattern generators. A strong inhibition was observed together with excitation during rhythmic scratching [15], and the presence of a balanced network where there is concurrence of excitation and inhibition was proposed [55]. Although the possibility of concurrent excitation and inhibition had been reported previously [56] this was seen as a challenge to the consensus view of circuit organization and therefore caused controversy [57]. It was suggested that the experimental design of transecting the cord caused the synaptic input to be balanced [57]. A subsequent set of experiments, in which the cord was not cut, yielded similar results [58]. Another study [19] reported alternating excitation and inhibition and proposed that the previously observed concurrent inhibitory current was not synaptic, but rather a voltage-activated intrinsic K^+ -current. Nevertheless, the investigators left the remaining evidence unaddressed, e.g., the experiments of blocking the ligand-gated chloride conductance, and shortly thereafter they reported a contrasting observation that a "...substantial overlap between excitation and inhibition was detected in HF-like motoneurons" [53]. Here, HF-like motoneurons were defined as having a correlation between their membrane potential and the envelope of the hip flexor electroencephalogram, which was positive but less than 0.8. Hence, multiple observations suggest that at least a subset of motoneurons receive either direct balanced input or substantial overlap between excitation and inhibition, while other neurons receive an alternating excitation and inhibition. Not much significance has been associated with this observation by some investigators [3], while others suggest that balanced network activity is a manifestation of the negative feedback that is missing in the potentially unstable half-center and unit burst generator models [55]. The turtle preparation also allows for superfusion of drugs (Fig. 2B) where only the neurons close to the surface are affected by the drug, while not affecting the rest of the network. A series of investigations applied a glycinergic inhibitory antagonist (strychnine), which resulted in a strong depolarization of the on-cycle [10, 15, 54, 55]. When a substantial depolarization follows the blockage of synaptic inhibition, a parsimonious explanation is a simultaneous arrival of excitation and inhibition. For further discussion, see Refs. [3, 55, 59].

Cellular response properties

Slice experiments using the turtle spinal cord have not only been instrumental in initial descriptions of several key intrinsic properties of motoneurons and their modulation, but continue to provide valuable insight into other aspects of neural function. The investigation of cellular response properties of spinal neurons

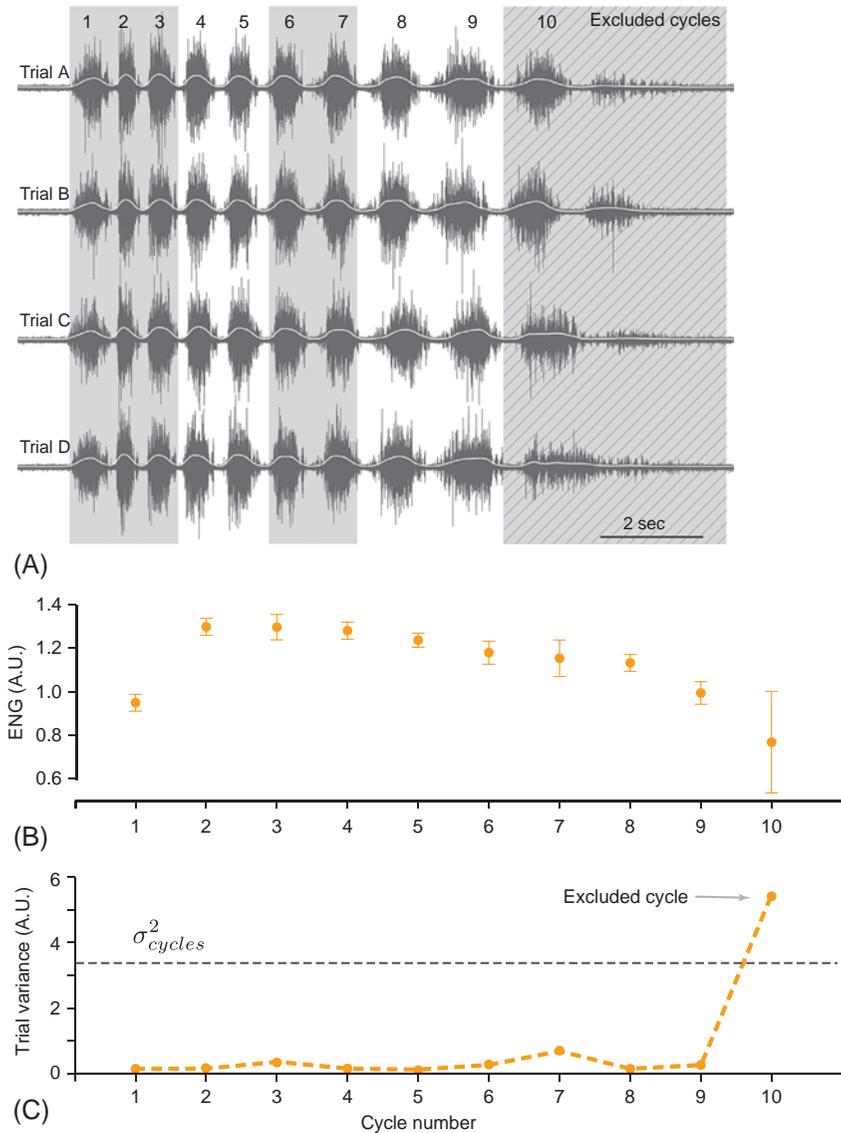


FIG. 3 High reproducibility in the generation of scratch motor patterns by somatic touch. (A) The hip flexor nerve activity during four consecutive trials has approximately 10 scratching cycles. (B) The nerve activity of each cycle is quantified by rectification (white lines in (A)) and integration (ENG). The mean and standard deviation across trials are plotted for each cycle. (C) The variance for each cycle across trials is small compared with the variance across cycles (broken gray line). The latter was used as an exclusion criterion of cycles, when investigating gain modulation and control of force. (Adapted with permission Vestergaard M, Berg RW. Divisive gain modulation of motoneurons by inhibition optimizes muscular control. *J Neurosci* 2015;35(8):3711–23. <https://doi.org/10.1523/JNEUROSCI.3899-14.2015>. URL: <http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.3899-14.2015>.)

can be conveniently performed using the adult turtle spinal cord because of the resistance to anoxia. Early studies on response properties were performed in the *in vitro* slices from the lumbar region and revealed plateau potentials in motor neurons [40, 41]. The slices were placed in a chamber with physiological Ringer's solution, in which drugs were applied. Serotonin was added to the saline bath, which transformed the response properties of motoneurons in a calcium-dependent manner [39]. The mechanisms underlying the M-current [60] and the impact of descending input via the dorsolateral funiculus on motoneuron response properties were studied in spinal circuits [61, 62]. Other issues, such as central fatigue, were investigated and suggested to be due to spillover effects of long-term release of serotonin during exhaustive exercise [17, 63]. Stimulation of the raphe nucleus in an integrated brainstem-spinal preparation promotes plateau potentials in spinal motoneurons [20]. The slice preparation studies showed that astrocytes responded to the neurotransmitter glutamate by releasing GABA in the dorsal horn [64].

Population activity and motor behaviors

A novel use of the turtle preparation is the application of multielectrode silicon arrays to record from the lumbar cord network. Custom-designed multielectrode arrays (256 electrodes) were inserted into the turtle spinal cord, thus allowing recording of approximately 300 neurons during motor behaviors [65] (Fig. 4A). Population recordings of many neurons made it possible to investigate a number of issues. First, the distribution of the firing rates across the population was previously assumed to be a normal distribution. Nevertheless, it was found to be log-normally distributed, i.e., the logarithm of the firing rate was normally distributed (Fig. 4B). It implies a skewed distribution, where most neurons do not spike very much and a small number of neurons spike at very high rates [65]. This observation renders the widespread idea of a typical firing rate standard deviation an ill-suited description, and therefore these findings define a new arithmetic of motor networks [66]. Where does this log-normality come from? It can be explained by the following mechanism: The membrane potential is often normally distributed during intense synaptic input (Fig. 4C). This synaptic input distribution is converted into a skewed firing rate distribution by a non-linear transformation (Fig. 4D). Similarly, the mean membrane potential across the population is normally distributed, induce a skewed firing rate distribution across the population (Fig. 4E).

The lognormality is also an indicator of cellular diversity within the population. The distribution of firing rates across the population did not cluster into segregated groups. Rather there is a continuum, although not evenly distributed, of different activities covering the whole spectrum of firing rates. The irregularity of spiking is also an indicator of diversity within the population. Neurons that spike irregularly most likely receive both excitation and inhibition that puts the V_m below threshold where random fluctuations elicit action potentials.

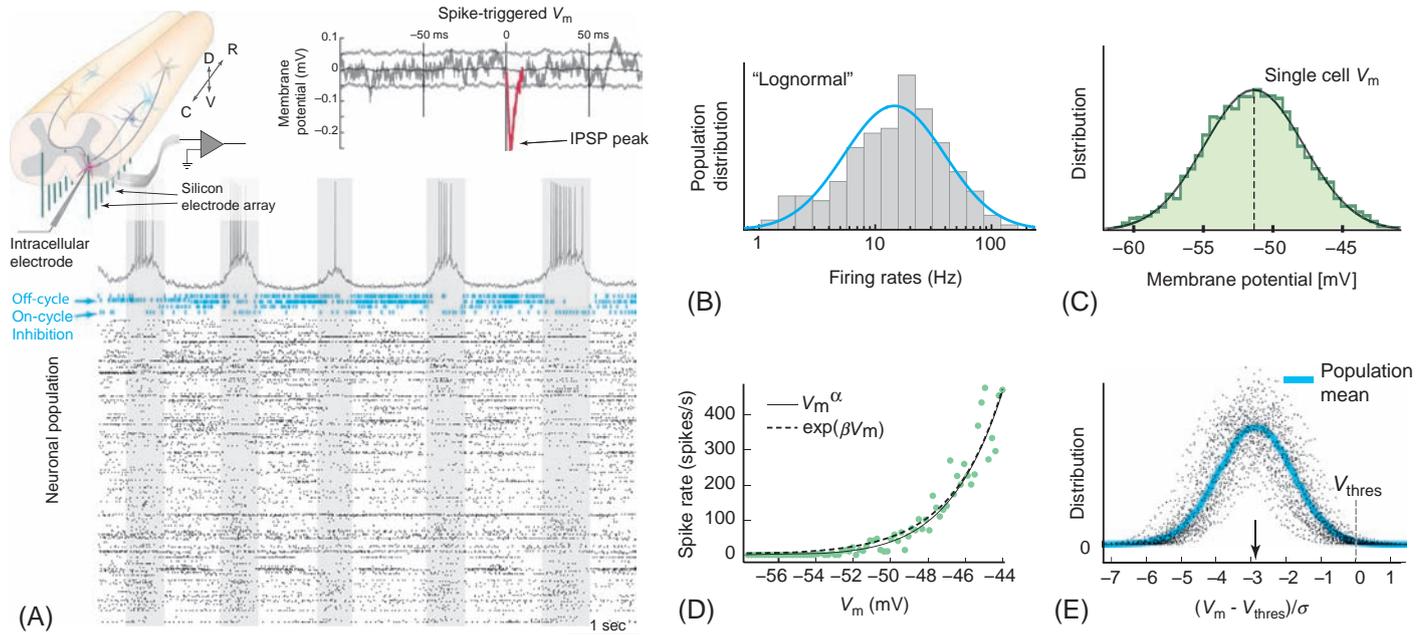


FIG. 4 Simultaneous extracellular population- and intracellular recordings. (A) Insertion of multielectrodes from ventral side of the lumbar cord together with an intracellular electrode allows simultaneous recording of the membrane potential (gray trace) of one neuron together with the spike activity of approximately 300 neurons (raster below). Four glycinergic inhibitory neurons (blue rasters) were identified via inhibitory postsynaptic potentials in the spike-triggered average membrane potential (inset, with negative red peak). (B) Firing rate distribution (spike count) across the population is approximately lognormal. Note the logarithmic x-axis. (C) Subthreshold membrane potential (on the burst peak in between spikes) has a normal distribution with strong fluctuations. (D) A nonlinear relationship between subthreshold membrane potential and firing rate can explain the lognormal firing rate distribution (B) if the input distribution (C) is normal, which it is. (E) The distribution of the mean membrane potential for all neurons minus the voltage-threshold and normalized to standard deviation $(V_m - V_{thres})/\sigma$ is also normal across population. The typical distance of the mean to the threshold is 3σ (arrow). ((A) Adapted with permission from M. Radosevic, A. Willumsen, P. C. Petersen, H. Lindéén, M. Vestergaard, R. W. Berg, *Decoupling of timescales reveals sparse convergent CPG network in the adult spinal cord*, *Nat Commun* 10 (1) (2019) 2937. <https://doi.org/10.1038/s41467-019-10822-9>. URL: <http://www.nature.com/articles/s41467-019-10822-9>. (B–E) Adapted with permission from Petersen PC, Berg RW. *Lognormal firing rate distribution reveals prominent fluctuation-driven regime in spinal motor networks*. *Elife* 2016;5:e18805. <https://doi.org/10.7554/eLife.18805>. URL: <https://elifesciences.org/content/5/e18805>; Berg RW. *Neuronal population activity in spinal motor circuits: greater than the sum of its parts*. *Front Neural Circuit* 2017;11:103. <https://doi.org/10.3389/fncir.2017.00103>.)

This is known as fluctuation-driven spiking [65, 66]. Interneurons in the turtle spinal cord often exhibit irregular spiking activity, both during scratching [15, 53, 65, 67] and swimming [14]. It is also interesting that although spinal neurons received intense synaptic input [15], the network connectivity was remarkably sparse [68]. The sparse connectivity was demonstrated by combining multielectrode extracellular recordings with intracellular monitoring in some experiments and pairwise intracellular recordings in other experiments [68]. Hence, it was possible to tease apart the correlation of synaptic input across pairs of spinal motor and interneurons that are active in-phase with each other. Together these results demonstrate that large distributed parts of the spinal population participate in the central pattern generation as well as the generation of rhythms and do so in a skewed manner.

Challenges using the turtle as a model

The challenge of using the turtle as experimental model is most notably the lack of transgenic strains and the access to the genetic toolbox that this would give. This prevents using the more common approaches of genetic identification of neuronal subtypes, selective optogenetics, and calcium imaging. This also makes it difficult to compare with other animal models, where the same neuronal subtypes can be genetically identified and compared. Part of the difficulty in the development of transgenic turtles is the slow growth and long breeding time of this animal. The age of the animals commonly used is approximately 5 years.

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About the author

Rune W. Berg completed undergraduate and master's degrees in biophysics/physics (1996 and 1998) at the University of Copenhagen. This was followed by a PhD (2003) in biophysics under the supervision of David Kleinfeld at University of California, San Diego, where he studied the cortical control of whisker movements in rats. After a short postdoc performing population recordings from spinal cord in awake rats under supervision of Henrich Cheng at the Taipei veteran's general hospital in Taiwan, Rune continued working on motor and inter neurons in the turtle spinal cord with Jorn Hounsgaard in Copenhagen. Berg then established his Lab in the Department of Neuroscience at the University of Copenhagen and received tenure as an Associate Professor in 2013. The purpose of this chapter is to review the experimental work performed using turtles in the context of neural control of locomotion.

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