Adaptive Motor Control: Neuronal Mechanisms Underlying (Targeted) Searching Movements

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Eva Berg

aus Freiburg i. Br.

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Berichterstatter:
Prof. Dr. Ansgar Büschges
Prof. Dr. Peter Kloppenburg

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Abstract

Animals move through a complex environment and therefore constantly need to adapt their behavior to the surroundings. For this purpose, they use sensory information of various kind. As one strategy to gain tactile cues, animals perform leg searching movements when loosing foothold. The kinematics of these searching movements have been well investigated in the stick insect. In this thesis, the modification of stick insect searching movements following a tactile cue are explored as an example of a sensory-motor system that adapts to environmental conditions. Furthermore, the premotor neuronal network underlying the generation of searching behavior is investigated.

Searching movements were studied in animals with a single intact leg that was free to move in the vertical plane. After several cycles of searching movements, a stick was introduced into the plane of movements such that animals would touch it with its distal leg. As is known from previous studies, in such a situation stick insects try to grasp the object that they touch. In my experiments, the stick was retracted as soon as a brief contact with the animals' leg had occurred. Therefore, animals could not grasp the stick.

I could show that following this short tactile cue, stick insects modify their searching movements to target the former position of the object (PO). Targeting occurs by a change in two parameters of searching movements: animals (i) shift the average leg position of their searching movements towards the PO and (ii) confine searching movements to the PO by a reduction in movement amplitude. These two parameters, position and amplitude, can be changed independently of each other. Searching movements are flexibly adjusted to different locations of the object which demonstrates the targeted response to be a situation-dependent adaptive behavior. The targeted response outlasts the tactile stimulus by several seconds suggesting a simple form of short term memory of the PO as proposed for targeted movements of other insects.

Vision is not necessary for a targeted response. Instead, tactile cues from leg sensory organs are important. Two proprioceptive organs, the trochanteral hairplate (trHP) and the femoral chordotonal organ (fCO), are crucial for targeting. Other sensory organs like tactile hairs and campaniform sensilla are dispensable. The brain is not necessary for a targeted response, therefore the adaptation of searching movements is likely to be mediated on the thoracic level.

The premotor neuronal network underlying searching movement generation was investigated using the same single-leg preparation as described above. Nonspiking interneurons (NSIs) of the premotor network were recorded intracellularly during searching movements. Additionally, EMG recordings of the four main leg muscles that generate searching movements in the vertical plane were recorded.

The membrane potential of previously described, as well as newly identified NSIs providing synaptic drive to leg motoneurons is shown to be phasically modulated during searching. Therefore, NSIs are part of the premotor network for the generation of searching movements. NSIs that were previously described to contribute to the generation of walking behavior are shown to contribute to the generation of searching behavior. When artificially de- or hyperpolarized by current injection, several NSIs are able to induce changes in searching movement parameters like position, amplitude, velocity of movements, or inter-joint coordination. One NSI is able to drive or stop searching movements. Each NSI acts on a specific set of parameters.

The same NSIs that were recorded during searching also were recorded during walking behavior. In comparison, NSI membrane potential modulations during searching are smaller in amplitude and more undulated than during walking. In contrast, fast transitions in NSI membrane potential are closely coupled to step phase transitions during walking. The most prominent difference in NSI membrane potential occurs during step phase (when walking) as compared to flexion phase (during searching). This difference might be attributed to load signals from campaniform sensilla. Analogous to results of previous studies in the stick insect, this highlights the importance of sensory feedback in shaping the motor output.

Finally, NSIs were recorded intracellularly while animals with their searching leg made contact with the stick that was introduced into the plane of movement. First results indicate that the response of a given NSI to this contact is characteristic and depends on the direction of touch.

Zusammenfassung

Tiere bewegen sich in einer komplexen Umwelt und müssen daher fortwährend ihr Verhalten an die Gegebenheiten anpassen. Zu diesem Zweck greifen sie auf sensorische Informationen vielfältiger Art zurück. Um taktile Informationen zu erhalten, führen viele Tiere Suchbewegungen mit ihren Beinen aus, wenn sie Bodenkontakt verlieren. Diese Suchbewegungen sind für die Stabheuschrecke auf Verhaltensebene bereits gut beschrieben. In der hier vorliegenden Arbeit untersuche ich, wie Stabheuschrecken ihre Suchbewegungen auf einen taktilen Stimulus hin ändern. Dabei betrachte ich Suchbewegungen und ihre Veränderungen beispielhaft für ein sensomotorisches System, das an einen Stimulus aus seiner Umwelt adaptiert. Des Weiteren untersuche ich das prämotorische neuronale Netzwerk, das zur Erzeugung der Suchbewegung beiträgt.

Um Suchbewegungen zu untersuchen, wurden Tiere mit nur einem intakten verbleibenden Bein verwendet. Die Tiere konnten das Bein in der vertikalen Ebene frei bewegen. Nach einigen ungestörten Suchzyklen wurde ein Stab in die Bewegungsebene geführt, den die Tiere im folgenden Suchzyklus mit der distalen Tibia berührten. Wie aus Ergebnissen vorheriger Untersuchungen bekannt ist, versuchen Tiere in einer solchen Situation, den Stab zu greifen. In den hier vorgestellten Experimenten wurde der Stab aus der Bewegungsebene entfernt, sobald die Tiere ihn mit dem Bein berührt hatten. Die Stabheuschrecken konnten den Stab daher nicht greifen.

Ich konnte zeigen, dass Stabheuschrecken ihre Suchbewegungen in Folge dieser kurzen Berührung verändern und an die Stelle zielen, an der sie den Stab berührten. Diese Anpassung erfolgt durch Veränderung zweier Parameter der Suchbewegung: zum Einen (i) verschieben die Tiere die mittlere Position ihrer Suchbewegungen zu der Position der vorangegangenen Stabberührung (PO) hin, zum Anderen (ii) verringern sie die Amplitude ihrer Suchbewegungen und beschränken somit ihre Bewegungen auf den Bereich der Stabberührung. Die beiden Parameter, Bewegungsposition und Bewegungsamplitude, können unabhängig voneinander verändert werden. Die Suchbewegungen werden an verschiedene Positionen der Stabberührung angepasst, was zeigt, dass die gezielte Antwort eine flexible, situationsabhängige Reaktion ist. Die gezielte Antwort überdauert den kurzen Stimulus um mehrere Sekunden und deutet somit die Existenz einer Art Kurzzeitgedächtnis für die Position der Stabberührung an. Eine solche Art von Gedächtnis wurde bereits für gezielte Beinbewegungen anderer Insekten vorgeschlagen.

Visuelle Informationen sind für die beschriebene gezielte Reaktion nicht nötig. Stattdessen spielen taktile Reize eine wichtige Rolle. Zwei propriozeptive sensorische Organe, das trochanterale Haarfeld (trHP) und das femorale Chordotonalorgan (fCO), sind für eine gezielte Reaktion von entscheidender Bedeutung. Andere sensorische Organe, wie taktile Haare oder campaniforme Sensillen, sind entbehrlich. Das supra-

ösophageale Ganglion ist für eine gezielte Reaktion nicht nötig, was darauf hinweist, dass die Anpassung der Suchbewegung auf thorakaler Ebene erzeugt wird.

Die gleiche Präparation wie vorab beschrieben, wurde auch für Versuche zur Untersuchung des prämotorischen neuronalen Netzwerkes zur Generierung von Suchbewegungen verwendet. Das Membranpotential nicht-spikender Interneurone (NSIs) des prämotorischen Netzwerkes wurde intrazellulär abgeleitet, während die Tiere Suchbewegungen ausführten. Zusätzlich wurde die elektrische Aktivität jener Muskeln aufgezeichnet, die das Bein in der vertikalen Ebene bewegen.

Das Membranpotential bereits bekannter sowie neu identifizierter NSIs, die Motoneurone erregen, wird phasisch moduliert während das Tier Suchbewegungen ausführt. Somit sind diese NSIs Teil des prämotorischen Netzwerkes zur Erzeugung von Suchbewegungen. NSIs, die, wie in vorherigen Studien beschrieben, an der Generierung von Laufbewegungen beteiligt sind, wurden hier als an der Generierung von Suchbewegungen beteiligt beschrieben. Durch Strominjektion herbeigeführte Manipulationen im Membranpotential einzelner NSIs können zu Veränderungen der Suchbewegungen führen. Veränderte Parameter umfassen die Position, Amplitude und Geschwindigkeit der Bewegung, sowie die Koordination der verschiedenen Beingelenke. Ein einziger, bestimmter NSI zeigt einen generellen Einfluss auf Suchbewegungen, indem er deren Generierung fördert oder unterdrückt. Es konnte gezeigt werden, dass einzelne NSIs spezifische Parameter beeinflussen.

Das Membranpotential der NSIs wurde sowohl während Suchbewegungen, als auch während Laufbewegungen aufgezeichnet. Im Vergleich zeigt sich, dass das Membranpotential während Suchbewegungen wesentlich schwächer und wellenförmig moduliert wird als während Laufbewegungen. Während des Laufens treten schnelle, starke Änderungen im Membranpotential auf, die eng an die Übergänge der verschiedenen Laufphasen gebunden sind. Der stärkste Unterschied im Membranpotential der NSIs tritt im Vergleich von Stemmphase (während des Laufens) und Flexionsphase (während des Suchens) auf. Dieser Unterschied könnte durch Belastungsinformationen der campaniformen Sensillen verursacht sein. Dies hebt, wie bereits die Ergebnisse früherer Studien, den wichtigen Beitrag sensorischer Information zur Erzeugung motorischer Aktivität hervor.

Im letzten Teil der Arbeit wurde das NSI Membranpotential aufgezeichnet, während die Tiere mit ihrem suchenden Bein den Stab berühren, der in die Bewegungsebene geschoben wurde. Erste Ergebnisse deuten an, dass während des Kontakts das Membranpotential einzelner NSIs charakteristisch moduliert wird und diese Modulation von der Richtung der Berührung abhängt.

Contents

Abbreviations

angle α	overall joint angle
angle β	coxa-trochanter-joint angle
angle γ	femur-tibia-joint angle
CPG	central-pattern-generator
CS	campaniform sensilla
CTr	coxa-trochanter-joint
EMG	electromyogram
fCO	femoral chordotonal organ
FTi	femur-tibia-joint
MN	motoneuron
NSI	nonspiking interneuron
PO	position of object
RMP	resting membrane potential
SEG	subesophageal ganglion
sRMP	resting membrane potential for searching behavior; leg resting midair
ThC	thorax-coxa-joint
trHP	trochanteral hair plate
TTa	tibia-tarsus-joint
wRMP	resting membrane potential for walking behavior; leg resting on treadwheel

1 Introduction[1]

Animals that move through the environment constantly need to adapt their movements to the surrounding conditions. This may be necessary in order to avoid dangers and find food, but also to negotiate obstacles or balance irregularities in the walking substrate, air or water flow. To name just a few examples, lampreys show escape swimming when tactile stimulation mimics a predator (Islam and Zelenin, 2008), hawk moths adapt their flight path in order to track odor plumes (Rutkowski et al., 2009), cockroaches walk over or tunnel under a shelf depending on tactile information and surrounding light conditions (Harley et al., 2009), and walking stick insects use different gaits depending on the ascending slope of the ground (Grabowska et al., 2012). For the purpose of adaptation, sensory information of various modality is used which can provide information in the long distance range (visual, auditory, olfactory cues) or short distance. As such, in vertebrates and invertebrates tactile cues play a prominent role in the exploration of the environment (Prescott et al., 2011). For example, humans use their hands for haptic exploration (Cote, 2014), harbor seals (Grant et al., 2014) and rodents (Diamond et al., 2008) use whiskers to palpate objects. Many insects constantly use antennal exploration to gain information about their environment during locomotion (cockroach: Camhi and Johnson, 1999; Harley et al., 2009; Baba et al., 2010; cricket: Honegger et al., 1990; Horseman et al., 1997; stick insect: Dürr, 2001; Schütz and Dürr, 2011). As another form of tactile exploration, insects also perform leg searching movements when losing ground contact, e.g. when approaching a gap. Such leg searching movements have been reported for stick insects (Bläsing and Cruse, 2004a,b; Dürr, 2001), locusts (Pearson and Franklin, 1984), cockroaches (Delcomyn, 1987) and fruit flies (Pick and Strauss, 2005).

[1]Parts of the introduction are already published in "E. Berg, A. Büschges, and J. Schmidt (2013) *Single pertubations cause sustained changes in searching behavior in stick insects*" J. Exp. Biol. 216, 1064-1074. The authors contributions are: EB, AB, and JS designed research; EB performed experiments, analyzed data and prepared figures; EB, AB, and JS wrote manuscript. Parts of the introduction are taken from the publication literally or with minor modifications. Additional sections were added where necessary.

In the present thesis, I investigated leg searching movements of the stick insect *Cuniculina impigra*, first, on a kinematic level in order to test whether searching movements are adapted in response to tactile stimulation. Second, I electrophysiologically investigated the premotor neuronal network underlying the generation of searching movements. This will be introduced in the following in more detail.

Leg searching movements In the stick insect, the behavioral context and kinematics of leg searching movements have already been described by Karg et al. (1991) and Dürr (2001). Searching movements were shown to be rhythmic stereotyped movements which consist of several cycles (Karg et al., 1991; Dürr, 2001). Movements in all three main leg joints contribute to searching (Dürr, 2001). These are the thorax-coxa (ThC) joint for pro- and retraction of the leg, the coxa-trochanter (CTr) joint for levation and depression of the femur, and the femur-tibia (FTi) joint for extension and flexion movements of the tibia. During searching, movements in the CTr and FTi joint rhythmically move the tarsus from a dorsolateral position to a ventromedial position (Dürr, 2001). Imposed on this movement is a retraction of the leg with low amplitude oscillations (Dürr, 2001).

The coordination of leg joints during searching has been described in detail for the CTr and FTi joint in a single-leg-preparation that was restricted to move in the vertical plane (Karg et al., 1991). A downward movement is initiated by a depression of the femur which is followed by a flexion movement of the tibia. During the subsequent upward movement, levation and extension occur simultaneously. Correspondingly, the antagonistic pools of motoneurons (MNs) supplying the CTr and FTi joint are each alternatingly active (Fischer et al., 2001; Schmidt et al., 2001). The *protractor* and *retractor coxae* muscles moving the ThC joint (Dürr, 2001) and correspondingly their innervating MNs (Fischer et al., 2001) are alternatingly active with a much slower frequency. Therefore, each of the two MN pools is active for several cycles of searching movements (Fischer et al., 2001).

Adaptation of searching movements – analysis on the kinematic level

As one form of behavioral adaptation limb movements of animals can be targeted. Examples shown in vertebrates include reaching movements of monkeys (Georgopoulos, 1996) and targeted scratching movements of dogs (Sherrington, 1906), cats (Sherrington, 1910), and turtles (Mortin et al., 1985).

Targeted movements of body appendages (limbs and antennae) are also found in invertebrates like insects. These movements may be directed towards non-transient stimuli, like cricket antennal movements that follow a visual cue (Honegger, 1981), but also

towards transient stimuli. For example, stick insects target their front legs towards the location of short preceding antennal contact (Schütz and Dürr, 2011).

In some cases, targeted movements outlast the stimulus. This may happen on different time scales. For example, in a motor learning paradigm bees were allowed to scan the position of an object for several minutes with their antennae. After removal of the object, the bees still targeted the position (Erber et al., 1997).

On a shorter time scale, locusts perform targeted hind leg scratching movements that outlast the tactile stimulus on their wing (Matheson, 1997, 1998). Furthermore, locusts walking on a ladder target their front legs to rungs under visual control (Niven et al., 2010). When a rung has been relocated during a front leg's swing phase, searching movements are performed at the original location of a rung. Because of this observation and the absence of immediate modification of the step, a memory for the rung position has been suggested (Niven et al., 2010).

Inspired by these experiments, I became interested in the mechanisms employed by a sensory-motor system to adapt to such an unexpected event. A stick insect searching for foothold in a bush may touch a leave or a twig that moves out of reach after the first contact, e.g. because of the elasticity of the material. I hypothesized that a change in searching strategy, i.e. searching movements confined to the former location of the object to regain contact, may result after such an event. Such a change in searching strategy would provide the opportunity to identify mechanisms underlying adaptation of movements in a largely intact sensory-motor system. As one aspect, such a change could employ a short-term memory in the range of seconds, as inferred from rung-searching locusts. Therefore, I aimed to study how searching movements of a stick insect's leg are changed in response to a transient contact of the leg with an object.

Generation of searching movements – analysis on the neuronal network level

As mentioned above, the kinematics of stick insect searching movements have been described (Karg et al., 1991; Dürr, 2001) and the motoneuronal activity during searching movements was investigated (Fischer et al., 2001; Schmidt et al., 2001). The contribution of the premotor network to searching movement generation has not been a subject of research yet. At the premotor level, searching movements are likely generated by an interplay between central rhythm generating networks (central-pattern-generators; CPGs) which produce a basic motor pattern and modulating influences that shape the pattern. Such an interplay has been shown to underly many rhythmic behaviors in both vertebrates and invertebrates e.g. swimming (lamprey: Grillner, 2003; tadpole: Roberts et al., 1998; mollusc: Getting et al., 1980), flight (locust: Robertson and Pearson, 1985; Ausborn et al., 2007), walking (cat: Grillner and Zangger, 1979; rodent:

Kiehn, 2006), crawling (leech: Eisenhart et al., 2000), scratching (turtle: Robertson et al., 1985), respiration (Smith et al., 1991; Feldman et al., 2013), heartbeat (leech: Kristan et al., 2005), and stomach movements (crustacean: Marder and Bucher, 2007).

Generating a central rhythm CPG networks are able to produce rhythmic activity in the absence of movement related sensory feedback that might pattern activity (e.g. Marder and Calabrese, 1996). Different mechanisms can underly rhythm generation (e.g. Marder and Bucher, 2001). In some networks, endogenously bursting pacemaker neurons produce rhythmicity (e.g. pyloric rhythm in the crustacean stomatogastric ganglion: Miller and Selverston, 1982; vertebrate respiration: Smith et al., 1991). In other networks rhythmic activity emerges from the interplay of synaptic interconnectivity and intrinsic properties of the constituent neurons (e.g. lamprey: Grillner, 2003, leech heartbeat: Calabrese et al., 1989).

The CPG networks are located close to the muscles they control (Büschges et al., 2011), e.g. CPGs generating limb movements are located in the spinal and ventral nerve chord, respectively (e.g. turtle: Robertson et al., 1985; locust: Berkowitz and Laurent, 1996a). CPG activity can be elicited by application of neuroactive substances like pilocarpine or N-methyl-D-aspartate (NMDA) on the isolated nervous system. This "fictive behavior" in some animals largely resembles the motor pattern observed during a specific behavior in the intact animal (lamprey: Grillner, 2003; crayfish: Mulloney and Smarandache-Wellmann, 2012). In other animals, e.g. the stick insect, only certain aspects of the behavior are reflected (Büschges, 2005).

In several studies, a modular organization of motor networks has been suggested (review: Tresch et al., 2002; Grillner, 1981). According to this conception, a network responsible for the generation of a behavior can be subdivided into several smaller networks which are responsible for the generation of a part of the behavior. For example, results from the walking mudpuppy (Cheng et al., 1998) or scratching turtle (Stein, 2008) suggest that pools of extensor and flexor MNs can be independently controlled to be rhythmically active. The complex rhythmic motor activity of a multi-segmented limb therefore is thought to arise from the concerted action of such modules.

A modular organization of CPGs is supported also by results of studies in the stick insect. Pharmacological activation of the networks that control limb movements resulted in alternating activity of antagonistic motoneuron pools with different frequencies in each leg joint (Büschges et al., 1995). Thus, in the stick insect each joint is thought to be controlled by a separate CPG producing alternating activity in antagonistic MN pools.

Shaping the central rhythm With respect to limb movements, three types of synaptic inputs are known to shape the centrally generated output: descending, intersegmental, and sensory intrasegmental information. Additionally, neuromodulators can strongly affect CPG activity (Marder, 2012).

Descending tonic input from higher neuronal centers is shown to provide drive to the central pattern generating networks located on the spinal and thoracic level, respectively. For example, in the lamprey tonic excitation from the mesencephalic locomotor region (MLR) elicits rhythmicity of swim MNs in the spinal cord (Sirota et al., 2000; Grillner, 2003). In insects, descending drive from head ganglia, i.e. supraesophageal and subesophageal ganglion, was shown to maintain ongoing motor behavior (Kien and Altman, 1984; Ridgel and Ritzmann, 2005; Gal and Libersat, 2006). Gal and Libersat (2006) showed that "walking-related behaviors" in the cockroach, i.e. walking, righting, swimming as opposed to "flight-related", are promoted by inputs from the subesophageal ganglion. On the thoracic level, a tonic depolarization that is found throughout rhythmic activity in MNs (Büschges et al., 1994; Ludwar et al., 2005) is thought to be the physiological correlate of the received descending drive (Ludwar et al., 2005). A tonic depolarization is seen also in several nonspiking interneurons (NSIs) (Ludwar et al., 2005; Rosenbaum, 2013).

Intrasegmental sensory signals have been shown to influence timing and magnitude of the centrally generated motor output in both vertebrates and invertebrates (reviews: e.g. Büschges, 2005; Pearson, 2000). For example, during stick insect walking, signals regarding load and position of the leg were shown to control step phase transitions (Akay et al., 2004; Cruse, 1985) and the strength of the muscle activation during stance (Akay et al., 2001). Load, movement, and position signals are shown to have access to individual joint CPGs of the own or adjacent leg joint (Bässler, 1986; Hess and Büschges, 1999; Bucher et al., 2003; Akay et al., 2004). For example, flexion movement signals of the FTi joint were shown to elicit levator MN activity and terminate depressor MN activity in the CTr joint (Hess and Büschges, 1997, 1999). Such inter-joint reflexes therefore contribute to movement coordination in the multi-segmented leg.

Sensory neurons provide input to spiking and nonspiking interneurons and rarely have direct connections with motoneurons (Burrows, 1987b; Burrows and Pflüger, 1988; Pearson et al., 1976). Sensory neurons always make excitatory synapses but can provide inhibitory effects indirectly via intercalated neurons (section 7.10 Burrows, 1996). Sensory signals are processed state dependent (Bässler, 1986; Hess and Büschges, 1997; Akay et al., 2007). This is, signal processing has been shown to depend on whether animals are in a resting or active state, e.g. walk (Bässler, 1986; Hess and Büschges, 1997) or on the walking direction (Akay et al., 2007). State dependent differences in signal processing can concern the strength of the effect elicited by the signal (Hess and

Büschges, 1997) or can even invert its sign: For example, flexion signals of the femoral chordotonal organ (fCO), a proprioceptor that measures movement and position of the FTi joint, excites the *extensor tibiae* muscle in the resting animal but inhibits extensor activity in the active state (Bässler, 1986). In the stick insect, intrasegmental sensory signals are shown to have a strong impact on the motor activity and overrule intersegmental signals (Borgmann et al., 2012).

Local premotor interneurons Local premotor interneurons are subdivided into two classes according to their membrane properties and thus usual mode of information transfer: spiking local interneurons and nonspiking local interneurons. Both classes receive and integrate sensory information from inter- and intrasegmental sensory neurons and are shown to control MN activity (stick insect: e.g. Büschges, 1989, 1990; locust review: Burrows, 1996). Local spiking interneurons have received little attention in the stick insect but were well investigated in the locust (Burrows, 1996).

In insects, nonspiking interneurons (NSIs) were first described in the walking system of the cockroach (Pearson and Fourtner, 1975) and intensively studied in locusts but also in stick insects (Siegler, 1985; Burrows, 1996; Büschges, 1990; Büschges et al., 1995; Driesang and Büschges, 1996; Hess and Büschges, 1997; von Uckermann and Büschges, 2009). The defining property of NSIs is their graded transmitter release according to gradual changes in their membrane potential (Burrows and Siegler, 1978). Therefore, NSIs affect their postsynaptic neurons in a finely tuned fashion. Wilson and Phillips (1982) showed that NSIs are able to tonically release transmitter thereby permanently influencing their postsynaptic neurons. Not much is known about the transmitters that are released; however, GABA has been shown to be important (Wildmann et al., 2002).

Especially from studies in the locust, NSIs are known to receive excitatory signals from local sensory neurons (Burrows et al., 1988; Laurent and Burrows, 1988), input from spiking local interneurons (Burrows, 1987a), intersegmental projections (Laurent and Burrows, 1989a) and other NSIs (Burrows, 1979). NSIs process signals in distributed antagonistic pathways (Büschges, 1990; Bässler, 1993a). For example, as shown in the stick insect, NSIs that –when depolarized– provide excitatory synaptic drive to extensor MNs may be depolarized or hyperpolarized by the same sensory signal (Büschges, 1990). Therefore, in a given behavior some pathways support, others oppose the ongoing movement (Büschges, 1990; Bässler, 1993a; von Uckermann and Büschges, 2009; Rosenbaum, 2013).

In the stick insect, NSIs contribute to leg movement and posture control. NSIs that are able to affect MN activity were shown to receive load, movement, and tactile sensory signals from sensory organs of the own or other leg joints (Akay, 2002; Büschges,

1990; Hess and Büschges, 1997; Kittmann et al., 1996). Therefore, NSIs contribute to coordinating movements of the multi-jointed leg. Two NSIs are known which provide drive to MNs of all three leg joints. These two interneurons, NSI E4 and I4, were shown to be part of the central rhythm generating network for joint control (Büschges, 1995). Single NSIs, when artifically depolarized, could affect aspects of the pilocarpine induced centrally generated rhythm like cycle frequency, MN burst strength, or occurrence of "spontaneous recurrent patterns" (SRPs) which are thouhgt to represent fictive step phase transitions (Büschges, 1995).

In more recent studies NSI activity has been described during walking behavior, when animals were stepping on a treadwheel (Schmitz et al., 1991; von Uckermann and Büschges, 2009; Rosenbaum, 2013). In certain NSIs, the strength of the membrane potential modulations was shown to correlate with stepping velocity (von Uckermann and Büschges, 2009; Rosenbaum, 2013). In a few studies, NSIs were shown to influence behavioral output; mostly they could disrupt stepping of the leg (Schmitz et al., 1991; Kittmann et al., 1996; von Uckermann and Büschges, 2009).

This thesis In the first part of this thesis, I tested whether stick insects adapt their searching movements to a transient tactile stimulus on the leg. I hypothesized that indeed, searching movements are confined to the former position of an object animals briefly touched with their leg. If the hypothesis proves true, this paradigm might allow to investigate in a largely intact sensory-motor system how e.g. sensory organs, changes in muscle activity, or moto- and premotor neurons contribute to movement adaptations.

In the second part, I aimed to extend the present knowledge regarding neuronal activity underlying searching movements by investigating premotor nonspiking interneuron activity. This is particularly interesting for several reasons:

First, NSIs are described to be part of the centrally activated leg joint control networks (Büschges, 1995) and have been shown to participate in the generation of walking behavior (von Uckermann and Büschges, 2009; Rosenbaum, 2013). However, whether NSIs contribute to the generation of searching movements is as yet unknown. Assuming that the premotor network for joint control is involved in the generation of multiple leg movements, I postulate that NSIs which contribute to the generation of walking behavior also contribute to the generation of searching behavior.

Second, NSIs were shown to exert drive on postsynaptic MNs (Büschges 1990; Hess and Büschges 1997; Akay 2002; Rosenbaum, 2013) and to modify certain aspects of centrally generated "fictive" motor rhythms (Büschges, 1995). Furthermore, the membrane potential modulations of certain NSIs during walking behavior correlated with stepping velocity (von Uckermann and Büschges, 2009; Rosenbaum, 2013). However,

this does not imply causality. As yet, whether single NSIs can actually change the behavioral output was investigated only in few cases (Schmitz et al., 1991; Kittmann et al., 1996; von Uckermann and Büschges, 2009). Therefore, I aimed to test whether single identified NSIs can modify searching movements. The results are interesting also with respect to the kinematic analysis of targeted movements described in the first part of the thesis.

Third, if NSIs are involved in the generation of walking and searching behavior, their membrane potential modulations are likely to differ because of different sensory signals they receive during either behavior. A comparison of NSI membrane potential modulations during both behaviors might indicate which are the characteristic differences. This might suggest sensory signals that shape the motor pattern towards either walking or searching activity.

Experiments All experiments were performed with a single-leg-preparation as previously described by Karg et al. (1991). This preparation is advantageous because it prevents intersegmental sensory information. A restriction of leg movements to the vertical plane facilitates movement analysis. For experiments regarding the modification of searching movements upon object contact, a stick was introduced into the plane of leg movements. When animals touched the stick during searching movements they initiated stereotyped grasping movements in order to hook the stick with the claw (Bässler et al., 1991). However, during my experiments the stick had immediately been retracted, therefore animals could not hook the object. Because the preparation is stationary, intracellular recordings could be performed during ongoing searching behavior. For recordings during walking behavior, a treadwheel was added to the setup such that animals could perform stepping movements. Additionally, leg movements were continuously videotaped and leg muscle activity electromyographically recorded.

2 Materials and Methods

Animals

Experiments were carried out on adult female stick insects *Cuniculina impigra* Brunner von Wattenwyl 1907 from the colony maintained at the Zoological Institute, Biocenter Cologne. Animals were kept at constant temperature (22–24°C), 60% humidity and under a 12 h:12 h light:dark cycle. Experiments were performed at room temperature (20–24°C) and under dimmed light conditions.

2.1 Kinematic and electromyographic analysis of targeted searching movements [1]

Preparation

For experiments, all legs except the left front leg were cut off mid-coxa. Animals were mounted dorsal side up with insect pins or dental cement (Protemp II, 3M ESPE, Seefeld, Germany) on a foam platform, the coxa of the remaining leg being located on the edge of the platform. The leg was fixed at an angle of 90° with respect to the body axis by applying dental cement to the thorax–coxa (ThC) joint. Movements of all other leg joints (coxa–trochanter (CTr), femur–tibia (FTi), tibia–tarsus (TTa) and tarsal joints) were not restricted, thus the animal could freely move its leg in the vertical plane. Animals were not able to touch the ground. Accordingly, when analyzing searching movements of the middle leg, all legs except the middle leg were cut and the middle leg was fixed as described previously.

[1] Major parts of the methods section concerning behavioral and electromyographic analysis of targeted leg searching movements are already published: E. Berg, A. Büschges, and J. Schmidt (2013) *Single perturbations cause sustained changes in searching behavior in stick insects* Journal of Experimental Biology 216, 1064-1074. The authors contributions to the paper are as follows: EB, AB, and JS designed research; EB performed experiments, analyzed data and prepared figures; EB, AB, and JS wrote manuscript. Except for minor modifications, the methods section is taken literally from the paper. Methods of experiments not included in the paper were added in the appropriate parts -i.e. methods concerning ablation of femoral chordotonal organ or supraesophageal ganglion, as well as movement analysis of the mesothoracic leg.

Ablation experiments For experiments regarding the influence of vision, the animals' view was either blocked by a black paperboard between head and leg or their eyes were covered with black ink.

In several experiments, the trochanteral hairplate (trHP, termed BF1 by Wendler, 1964) was shaved off with a razor blade (Akay et al., 2001). Success of the ablation was verified by means of a scanning electron microscope (Quanta FEG 250 ESEM, FEI) after the experiments.

The influence of tibial and tarsal sensory information (tactile hairs, campaniform sensilla) was eliminated by cutting the leg distal to the FTi joint. The tibial stump was hollowed out with an insect pin to prevent signals from campaniform sensilla (CS) groups 6A and 6B (Zill et al., 2011), which are located just distal to the FTi joint. The tibia was replaced by a wooden stick of appropriate length and mass to serve as prosthesis. Values for length and mass had been obtained beforehand from mean values of several animals.

In some experiments additionally all trochanteral and femoral CS were ablated by indentation of the cuticle with a pin. Success of ablation was verified by means of a scanning electron microscope (Quanta FEG 250 ESEM, FEI) after the experiments.

In order to prevent the femoral chordotonal organ's (fCO) influence, a small opening was cut into the dorsal femoral cuticle approximately mid-femur, such that the cuticle could be opened like a door. Then, the fCO receptor apodeme was cut. Lost hemolymph was replaced by saline (pH 7.2, Weidler and Diecke, 1969) and the opening closed again. For "sham"-experiments, the same procedure was applied except for actually cutting the apodeme. After "sham"-experiments had been recorded, searching movements of these animals were also recorded with cut fCO-apodeme.

To ablate the supraesophageal ganglion (henceforth "brain"), a window was cut into the frontal head cuticle between the eyes. The circumesophageal connectives between brain and subesophageal ganglion were cut to remove the brain from the head (Fig. 3.10).

Electromyograms For recordings of *levator* and *depressor trochanteris* muscle activity, two copper wires (57 µm, insulated except for the tip) were inserted into each muscle through small holes in the dorsal and ventral posterior coxa of the front leg before fixation of the coxa (Rosenbaum et al., 2010).

Experimental setting

Searching movements were elicited by slightly touching the animal at the abdomen with a paintbrush ('tickling') or by a puff of air directed at the antennae or abdomen.

A metal stick with a tip made of fiberglass was used as an obstacle to be introduced into the plane of leg movement. The stick was mounted to a micromanipulator, aligned in parallel to the animal and could be quickly moved forward by approximately 1 cm. The stick was always touched by the most distal third of the tibia. The stick was located caudal to the intact front leg, and thus was moved into the plane of movement from behind. A coil spring enabled a fast retraction of the stick as soon as the animal had touched it. The stick was black with a small red marker (fluorescent pigment, Dr. Kremer Farbmühle, Aichstetten, Germany) at the very tip to make it detectable during video analysis of the leg's movements. In random order, the stick was introduced into the plane of leg movements in one of four different positions (henceforth 'position of object' (PO); see Fig. 3.1 C). The experimental setting for the analysis of middle leg searching movements was the same; the position of the stick was adapted to the position of the middle leg accordingly.

Data acquisition and analysis

Video Leg movements were recorded from a frontal view at a frame rate of 50 Hz (AVT Marlin, Allied Vision Technologies, Stadtroda, Germany) and stored on a computer using firmware (AVT ActiveCam). If electromyogram (EMG) signals were obtained simultaneously, then both film and EMGs were recorded using Spike2 software (Version 5.20, CED, Cambridge, UK). Yellow fluorescent markers (fluorescent pigment, Dr. Kremer Farbmühle, Aichstetten, Germany) were applied to the leg (Fig. 3.1 C) to facilitate the analysis of leg movements. Pigment fluorescence was evoked by LED illumination (λ=395 nm) and filtered by a high-pass filter (λ>575 nm) mounted on the camera lens.

Movements were tracked using WinAnalyze (Version 2.2 2D, Mikromak, Berlin, Germany) or MATLAB (Version 7.11, MathWorks, Natick, MA, USA) using a custom-written program (StickAnalyze, written by Dr. Till Bockemühl, Zoological Institute, University of Cologne, Cologne, Germany). As the size of animals varied, positions of the leg were not expressed in coordinates but as an angle, α, which was formed by a straight line from the coxa to the distal end of the tibia and a horizontal that was set by the coxa and a reference marker to the right side of the animal (Fig. 3.1 C). Positions above the horizontal were defined as positive values of α, positions below the horizontal as negative values. When considering positions of the CTr and FTi joints, I used angles β and γ, respectively. The coordination of the CTr and FTi joints during searching movements is very stereotyped; movements in both joints are generally coupled (Fig. 3.1 B; see Results). Because of this coupling, the same α is generally defined by very similar β and γ values in each animal and the distal tibia repeatedly moves on very similar trajectories during consecutive searching cycles (Fig. 3.1 A). Therefore, α

sufficiently describes the position of the most distal third of the tibia, which was the location of contact with the stick.

Calculation of average leg position before and after contact with the object For each experiment, average leg position as the average α was calculated for four consecutive searching cycles directly before and after leg contact with the object. α values were obtained for each video frame in four cycles. For example, four searching cycles in 2.4 s give 120 α data points. The average of these gives the average leg position. For each experiment, the pooled data points obtained from four cycles before and after leg contact with the object were used to test for statistical significance of differences of the means (Mann–Whitney U-test, α=0.05). Amplitudes were calculated for half-cycles of searching movements. Thus, eight values obtained from four cycles before touching the object were pooled and compared with eight values after touching (Mann–Whitney U-test, α=0.05). No pause occurred within each of the four searching cycles and animals were not 'tickled' along the way. Animals were only included in analyses if at least one experiment was performed with the PO above the horizontal (0, +15 and +25 deg) and one with PO=–40 deg. If the distance from the PO to average leg position before touching was <10°, the experiment was excluded from the statistical analysis of the change of average leg position, but included in all other analyses. In experiments regarding the influence of the fCO on a change in searching movements after contact, experiments with less than four cycles before leg contact were included in the analysis in order to increase the number of experiments. Experiments had a minimum of 2.5 searching cycles in experiments with cut fCO-apodeme, "sham"-operated apodeme and corresponding intact leg.

Statistical analyses were conducted in SPSS (IBM, Armonk, NY, USA) and MATLAB. Curves were fitted to the amplitude and average leg position data sets, respectively, in MATLAB. Data points directly after touch were left out of the fit if the maximum deviation of values from control occurred in the second cycle instead of the first after touch (amplitudes: one data point in 7 of 23 cases; average leg position: three data points in 3 of 21 cases). The mean angular speed was calculated by averaging the absolute values of the first derivation of α from one minimum of the leg position to the next minimum.

Electromyograms EMG signals were amplified (custom-built amplifier, model 102 Electronics workshop, Zoological Institute, University of Cologne, Cologne, Germany) 3000–10,000 times according to signal amplitude and filtered (low-cut 100 Hz, high-cut 2 kHz). The data were digitized with a rate of 12.5 kHz (Micro 1401k II, CED) and stored using Spike 2 (Version 5.20, CED, Cambridge, UK).

EMG recordings were analyzed in Spike 2 using the 'bursts' script for evaluation of burst duration. Cycles were measured from the onset of levator muscle activity to the next onset of levator muscle activity. A custom-written script was used for calculation of integrals of electrical muscle activity. To obtain the ratio of levator to depressor trochanteris muscle activity, the sum of levator integrals of four searching cycles was divided by the sum of depressor integrals of four searching cycles both before and after touching the object. The resulting ratios were normalized to the ratio before touching the object. Figures were created in Origin (Version 6.0, Microcal Software, Northampton, MA, USA) or MATLAB and modified in Corel Draw (Version 13, Corel, Ottawa, CA).

A single experiment consisted of four searching cycles before touching the object and four cycles after touching the object.

N = number of animals, n = number of experiments (throughout the section concerned with behavioral and electromyographic analysis).

2.2 Electrophysiological analysis of the premotor network for leg searching movements

In order to investigate the activity of the premotor network during searching movements, intracellular recordings of nonspiking interneurons (NSIs) were performed simultaneously with video recordings as described previously. Again, muscle activity was recorded from *levator* and *depressor trochanteris* muscles and in addition from *extensor* and *flexor tibiae* muscles. Due to the better accessibility of the mesothoracic ganglion as compared to the prothoracic ganglion, these experiments were performed with the single middle leg preparation.

Preparation and experimental setting

The animal was placed on a platform, all legs except the left middle leg were cut, and the coxa of the left middle leg was fixed as described previously (section sec. 2.1). Therefore, the animal was free to move its middle leg in the vertical plane. Searching movements were elicited by 'tickling' or a puff of air directed at the antennae or abdomen, as described above (sec. 2.1).

To additionally allow walking movements, a custom made treadwheel could be added to the setup during the experiment. It was composed of two styrofoam drums of 40 mm diameter, 28 mm width and 50 mm center distance that were connected by a belt of crepe tape. The two drums were each mounted on a micro-DC motor (DC1516,

Faulhaber, Schönaich, Germany) of which one supported the treadwheel's movements to reduce friction. The second motor can serve as tachometer but its output was not recorded during these experiments (for details see Gabriel et al., 2003). The treadwheel was placed rectangular to the platform, i.e. the body axis of the animal, beneath the middle leg. Therefore, the animal could perform steps with fixed ThC joint. The treadwheel height was adjusted such that the femur position was approximately horizontal when the FTi position was 90°.

The treadwheel was also used to test the influence of FTi joint position and movement, i.e. information from the femoral chordotonal organ, on the membrane potential of NSIs. For this purpose, the treadwheel was manually moved back and forth when the leg was resting on it, thereby flexing and extending the FTi joint (i.e. elongating and relaxing the fCO-apodeme) while the CTr joint was not moved.

Data acquisition

Video recordings Leg movements were recorded in the same way as described in sec. 2.1, except for a different software (Spike2 Video Recorder, implemented in Spike2, Version 7.09, CED, Cambridge, UK) that was used to specify (the same) recording parameters. A custom written Spike2 sequencer script was used to trigger the recording with 50 Hz. The trigger signal was recorded in Spike2 in order to detect if any video frames were lost during the recording.

Electromyographic recordings The activity of *levator* and *depressor trochanteris* muscles was recorded as described in the previous section sec. 2.1. Additionally, the activity of *extensor* and *flexor tibiae* muscles was either recorded simultaneously with one pair of copper wires inserted trough holes into the proximal posterior femur at medium height or with separate pairs of wires inserted dorsally and ventrally, respectively. The wires were attached to the cuticle by dental cement (Protemp II, 3M ESPE, Seefeld, Germany). EMG signals were recorded and digitized as described above (sec. 2.1).

Intracellular recordings The animal was opened by a dorsal midline incision from prothorax to metathorax. The cuticle was fixated to remain open with minuten pins. The gut was left intact but pulled and placed beside the animal. The cavity was filled with saline (pH 7.2, Weidler and Diecke, 1969). Fat tissue and tracheae were removed to expose the mesothoracic ganglion. The nerves nl2 *(nervus lateralis* 2; containing *protractor coxae* MNs), nl5 *(retractor coxae* MNs), *nervus anterior* and *posterior* were cut on both sides. The *nervus cruris* contralateral to the intact leg was crushed several times with a forceps to prevent afferent and efferent signals. The

ganglion was placed on a custom made platform (steel, covered with wax) introduced into the thoracic cavity from the front, passing through the gap between the pro-mesothoracic connectives, in order to ensure a stable position throughout recordings. The ganglion was fixated on the platform by pinning the surrounding fat tissue down with cactus spines (*Nopalea dejecta*). To facilitate electrode penetration, the ganglion sheath was treated with a proteolytic enzyme (Pronase E, Merck, Germany) for 40 s. Sharp microelectrodes with a resistance of 15-30 MΩ were made out of borosilicate glass (GB 100TF-8P, Science Products, Hofheim, Germany) using a Sutter Micropuller (P-1000, Sutter Instruments, Novato, CA, USA) and filled with 3M KAc/ 0.1M KCl electrolyte. Signals were recorded in bridge mode (intracellular amplifier SEC-10L, npi electronics, Tamm, Germany) using Spike2 software (Version 7.09, CED) on a personal computer.

Intracellular stainings For experiments in which neurons were stained in addition to electrophysiological characterization, 5% neurobiotin (Vector Laboratories Inc, Burlingame, CA, USA) was added to the electrode solution. Neurons were filled with this solution by applying depolarizing current pulses (+2 nA, 400 ms pulse duration, 1 Hz) for up to 15 minutes. The ganglion was removed from the animal while 45-60 minutes were allowed for dye diffusion. Afterwards the ganglion was fixated for 20 minutes with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS) and 5% Triton X (Fluka, Buchs, Switzerland) and then fixated for 2-16 hours in 4% PFA. The ganglion was washed (3x15 minutes with PBS) and then treated with Streptavidin-Cy3 (1:500 in PBS, Sigma-Aldrich, St. Louis, MO, USA) in 0.5% Triton X and 2% normal goat serum (Vector Laboraties Inc, Burlingame, CA, USA) over night on a shaker at 4°C. After washing (3x15 minutes with PBS) and dehydration with an ascending ethanol series (50%, 70%, 90%, 2x100%; 10 minutes each) the ganglion was mounted on a concavity object slide in methylsalicylate and scanned with a confocal laser scanning microscope (LSM 510 meta, Carl Zeiss, Germany). Throughout the thesis, images of the stainings are shown from a dorsal view and the anterior side of the ganglion is oriented towards the upper margin of the image.

Identification of nonspiking interneurons The neuron's membrane potential modulations were recorded from their neuropilar aborizations. The output connections of NSIs were determined by observing the effect of depolarizing and hyperpolarizing current puls injections onto EMG recordings and leg movements. The membrane potential modulations during walking, as well as the response to stimulation of the fCO (via treadwheel), was used to match the neurons to identified neurons that had already been described previously (e.g. Büschges, 1990; Sauer, 1996; Hess and Büschges, 1997;

Stein and Sauer, 1998; Akay, 2002; von Uckermann and Büschges, 2009; Rosenbaum, 2013).

Neurons were considered nonspiking if they fulfilled the following criteria: 1) No spike occurred throughout the whole recording, i.e. a) no spikes could be evoked by unspecific stimulation of the abdomen or antennae when activating the animal to perform searching or walking movements; b) no spike was induced by stimulation of sensory organs; c) there was no rebound spike after hyperpolarizations; d) there was no spike when changing the membrane potential by injections of depolarizing current. 2) Nevertheless, when de- or hyperpolarized by current injections the neuron affected the leg motoneurons by eliciting activity in EMG recordings or leg movements (see also Burrows and Siegler, 1976; Hengstenberg, 1977; Burrows, 1981; Wilson, 1981; Siegler, 1985; Büschges, 1990). Morphologically, neurons were classified according to their soma location and their main processes, i.e. primary and secondary neurites.

All NSIs shown in this thesis had a clear and reproducible influence on leg motoneurons shown either by eliciting activity in one or more EMG recordings and/or leg movements when de- or hyperpolarized by current injections. The analyses of recordings of 53 NSIs were included in this work, of which 41 NSIs were stained.

Data analysis

Video recordings were processed as described in the previous section (sec. 2.1) and the position signals of both CTr and FTi leg joints, as well as the overall angle α, were imported into the corresponding Spike2 file containing EMG and intracellular recordings. Electromyographic and intracellular recordings together with position information were analyzed with Spike2 and Matlab (Version 7.11, MathWorks, Natick, MA, USA) using custom written scripts, and Microsoft Office Excel 2007. Figures were prepared with MATLAB (Version 7.11, MathWorks, Natick, MA, USA) and Corel Draw (Version 13, Corel, Ottawa, CA).

For NSIs that were recorded at least three times, average membrane potential, peak-to-peak potentials, and standard deviations were calculated as weighted means. When quantifying the effect of tonic de- or hyperpolarizing current injection during ongoing searching behavior, current pulses were included in the analysis if 1) the pulse was given during ongoing searching behavior; 2) the pulse had a duration of at least two searching cycles. Changes in searching movements were considered to be an effect of current injection if they started within two cycles of searching movements from start of current injection. Changes in CTr and FTi joint coordination were evaluated regarding the center of the trajectories (when FTi was plotted against CTr joint position). If this center moved horizontally or vertically along the x- or y-axis, this was not considered

a change in coordination but rather a restriction to a fragment of the previous working range (for examples see Fig. 3.7). If the center moved diagonally towards higher x- and lower y-values, this was considered a change in coordination.

N = number of animals, n = number of searching cycles or steps (throughout the electrophysiological analysis of the premotor network).

3 Results

3.1 Kinematic analysis of targeted searching movements [1]

When slightly touched on the abdomen or activated by a puff of air, stick insects performed stereotypical searching movements in the vertical plane with their foreleg (Fig. 3.1 A,B) (Karg et al., 1991). Searching movements covered a wide range of 75±17 deg (measured as α; see Fig. 3.1 C; N=14, n=76, ~300 cycles) and were centered on a rather stable average leg position. As described by Karg et al. (1991), downward movements started with depression of the CTr joint whereas the FTi joint remained fully extended or was finishing its extension movement from the previous searching cycle. Approximately halfway down the searching range, CTr movements slowed down whereas the FTi joint started to flex (Fig. 3.1 A, black lines). During upward movements, the FTi joint was extended together with a simultaneous or immediately following elevation of the CTr joint (Fig. 3.1 A, magenta lines). The coordination of both joints was highly consistent throughout consecutive cycles of searching movements, as can be seen when the FTi angle (γ) is plotted against the CTr angle (β), which results in closely matching trajectories (Fig. 3.1 B).

[1] Major parts of the section concerning behavioral and electromyographic analysis of targeted leg searching movements are already published: Berg, Büschges, and Schmidt *Single perturbations cause sustained changes in searching behavior in stick insects* Journal of Experimental Biology 216, 1064-1074, 2013. The authors contributions to the paper are as follows: EB, AB, and JS designed research; EB performed experiments, analyzed data and prepared figures; EB, AB, and JS wrote manuscript. Except for minor modifications, this results section is taken literally from the paper. Results of experiments not included in the paper were added in the appropriate parts -i.e. results concerning ablation of femoral chordotonal organ, campaniform sensilla, or supraesophageal ganglion, as well as movement analysis of the mesothoracic leg.

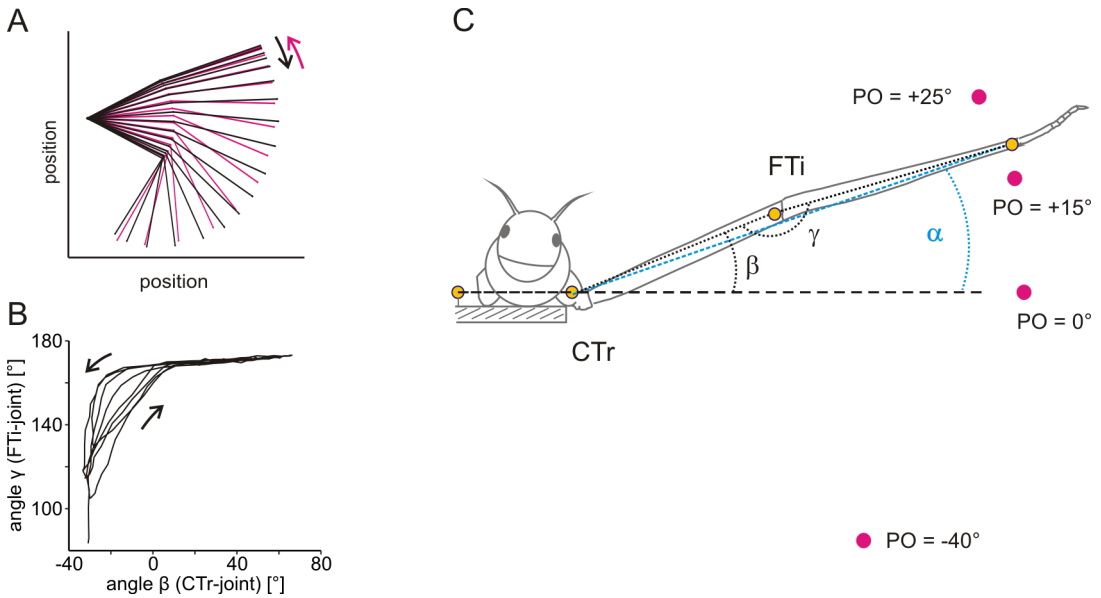

Figure 3.1 – (A) Schematic of leg kinematics during downward (black) and upward (magenta) movement of an undisturbed searching cycle. (B) Movements of the femur–tibia (FTi) joint plotted against movements of the coxa–trochanter (CTr) joint for several cycles of searching movements. Arrows indicate upward and downward movements of the leg. Coordination of both joints movements are very stereotyped. (C) Schematic drawing of the experimental setup (modified from von Uckermann and Büschges, 2009). The leg is shown from the camera's perspective. Magenta circles indicate the four different positions of the object (POs); yellow circles denote markers. Angle α gives the position of the distal tibia relative to the body, angle β gives the position of the coxa–trochanter joint and angle γ gives the position of the femur–tibia joint.

3.1.1 Qualitative analysis of searching movements

To determine whether searching movements are modified after touching an object with the leg, after four cycles of undisturbed searching movements, a stick was moved into the plane of movement from behind such that the animal would eventually touch it with its distal tibia. Immediately after contact, the stick was retracted and searching movements were observed. The stick was alternately placed in one of four positions (Fig. 3.1 C). Upon touching the object, generally two parameters of the searching movements changed. Fig. 3.2 shows these changes in searching movements of a single animal for all four POs. Searching trajectories are plotted as angles (α) over time. First, upon touching the object, the average leg position was shifted towards the PO. It was shifted upward if the PO was located above the average leg position before touching (Fig. 3.2 A–C) and downward if the PO was located below (Fig. 3.2 D). Subsequently, searching movements were gradually shifted back towards the initial average leg position. Second, upon touching the object, the amplitude of searching movements was instantly decreased and gradually increased again. The combined occurrence of these changes may be interpreted as a targeted searching movement that wanes over time. Henceforth, movements that shifted towards POs and that were reduced in amplitude

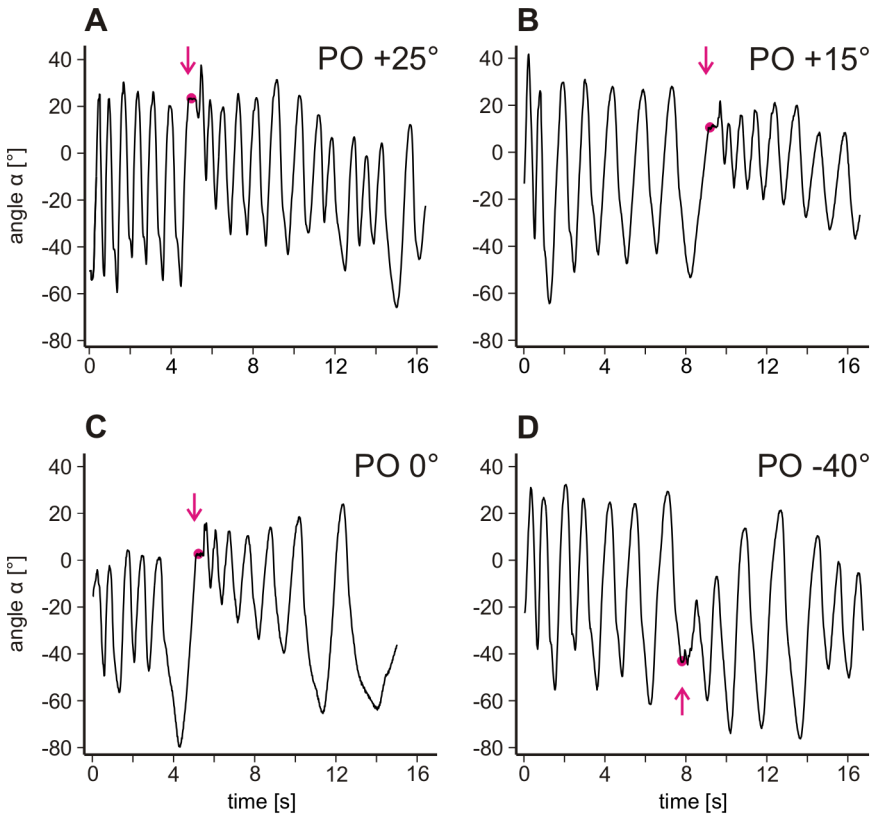

Figure 3.2 – Searching movements plotted as angle α over time. All four experiments are recorded from the same animal and examples are shown for each of the four different POs used: +25° (A), +15° (B), 0° (C) and −40° (D). Magenta arrows indicate the point in time when the object was touched, and magenta circles indicate the PO. The large amplitude before contact in C is an outlier with no relation to the subsequent contact.

will be named targeted searching movements. Movements that showed changes in only one or none of the two parameters were considered non-targeted.

The mean angular speed of leg searching cycles did not change after touching the object (Fig. 3.3 A). Single experiments (colored lines) could show a decrease or increase in speed; however, these changes were not systematic, as average data (black line) were not different from control values (Fig. 3.3 A). As a consequence of the unchanged angular speed and the decrease in amplitude, the cycle duration of searching movements instantly decreased upon touching an object and subsequently increased again (Fig. 3.3 B). Fig. 3.3 shows the results for targeted searching movements at a PO of +15° (N=9, n=15). Curve progression was similar for non-targeted searching movements as long as the amplitude of movements decreased (N=4, n=5; not shown). If the amplitude was not decreased, both angular speed and cycle duration tended to increase (N=2, n=2). Despite their overall stereotypy, searching movements in different experiments displayed a considerable amount of variability regarding initial average leg position and size of amplitude (compare cycles of undisturbed searching movements in Fig. 3.4). Also, average leg position and amplitude of searching movements could

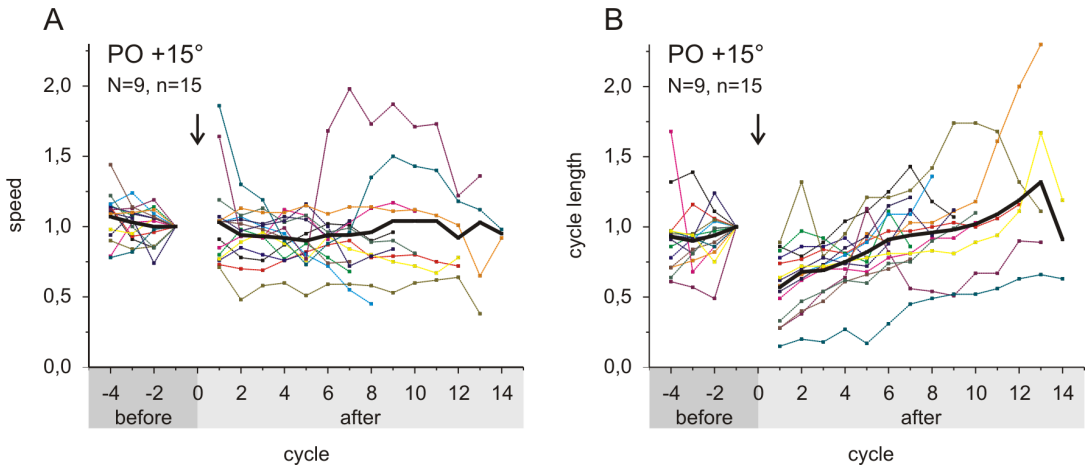

Figure 3.3 – Mean angular speed (A) and cycle duration (B) of searching movements plotted over cycle number. Values are normalized to the last cycle before touching the object. Cycles before touch (−4 to −1) are highlighted in dark gray on the x-axis; cycles after touch (cycle 1 to end) are highlighted in light gray. Colored curves depict single experiments; black curves show mean values. Arrows indicate the point in time when the object was touched. N, number of animals; n number of experiments.

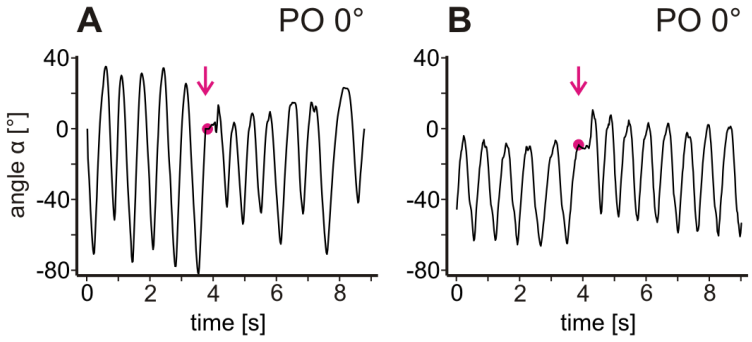

Figure 3.4 – Average leg position and movement amplitude may change independently after contact with the object. Searching movements plotted as angle α over time for two experiments with a PO of 0°. Magenta arrows indicate the point in time when the object was touched, and magenta circles indicate the PO. (A) Amplitudes change but average leg position remains constant. (B) Average leg position changes but amplitudes remain constant.

be altered independently of each other. Fig. 3.4 A shows an experiment in which the amplitude changed but average leg position remained the same, whereas in Fig. 3.4 B only the average leg position was changed.

3.1.2 Quantitative analysis of searching movements

For a quantitative measure of the observed changes, I tested for differences in average leg position and amplitude between four cycles directly before and after touching the object. The results show that the average leg position was significantly shifted towards the PO in most cases for all four POs (73–91% of experiments, Mann–Whitney U-test,

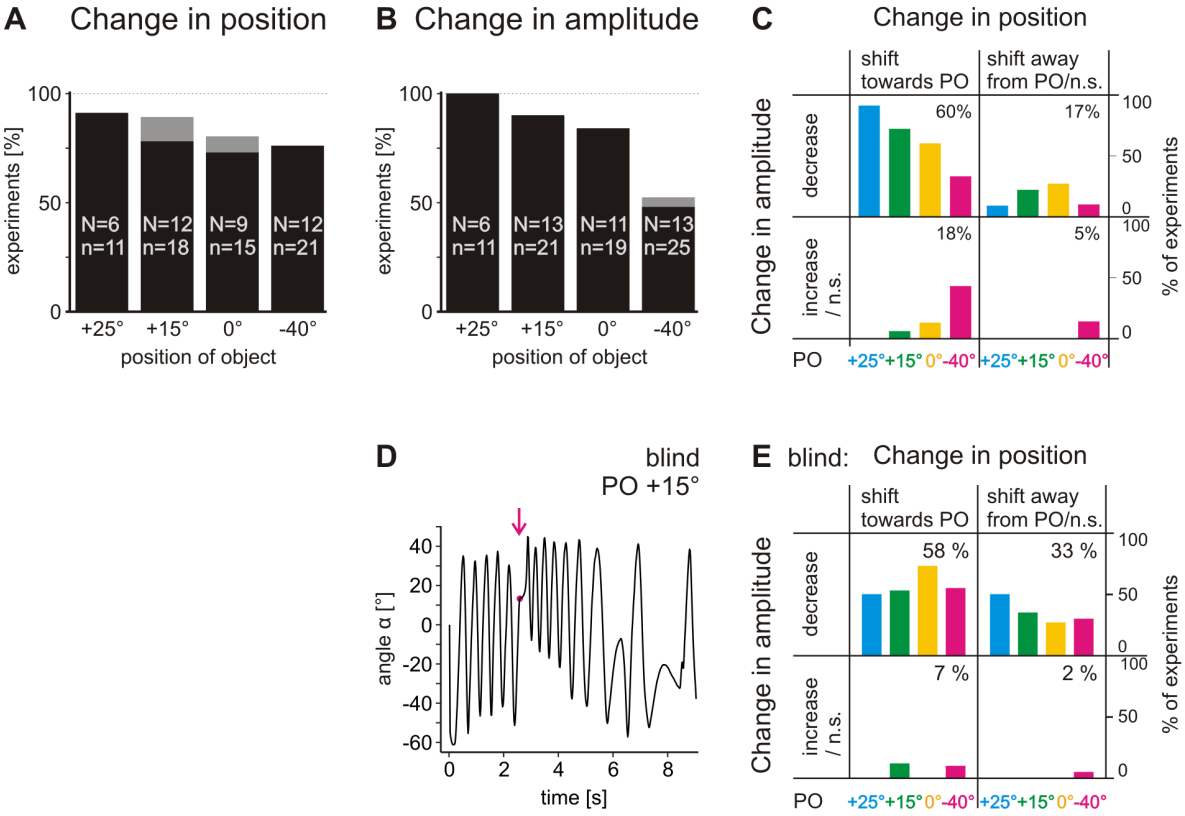

Figure 3.5 – Changes in average leg position and movement amplitude upon touching the object. (A) Change in position. Black bars, percentage of experiments in which average leg position shifted significantly towards the PO; gray bars, percentage of experiments in which average leg position shifted significantly away from the PO. Experiments are separated according to the respective POs of +25°, +15°, 0° or −40°. Non-significant changes are indicated by gaps between bars and the 100% line. (B) Change in amplitude. Black bars, percentage of experiments in which amplitudes significantly decreased; gray bars, percentage of experiments in which amplitudes significantly increased. (C) Frequency of appearance of different combinations of changes in average position and changes in amplitude for the different POs. POs are indicated by color. Left quadrants: average leg position was significantly shifted towards PO. Right quadrants: average leg position was significantly shifted away from PO or not significantly (n.s.) shifted. Upper quadrants: amplitudes decreased significantly. Lower quadrants: amplitudes increased significantly or did not change significantly. Numbers in quadrants give the mean relative frequency of the combined occurrence of a change in position and in amplitude. PO +25°: N=6 animals, n=11 experiments; PO +15°: N=12, n=18; PO 0°: N=9, n=15; PO −40°: N=12, n=21. (D) Searching movements of a blindfolded animal plotted as angle α over time for an experiment with a PO of +15°. (E) Experiments with blindfolded animals grouped according to the combination of changes in leg position and amplitude after contact with the object. Other details as in C. PO 25°: N=3, n=8; PO 15°: N=7, N=17; PO 0°: N=6, n=15; PO −40°: N=7, n=20.

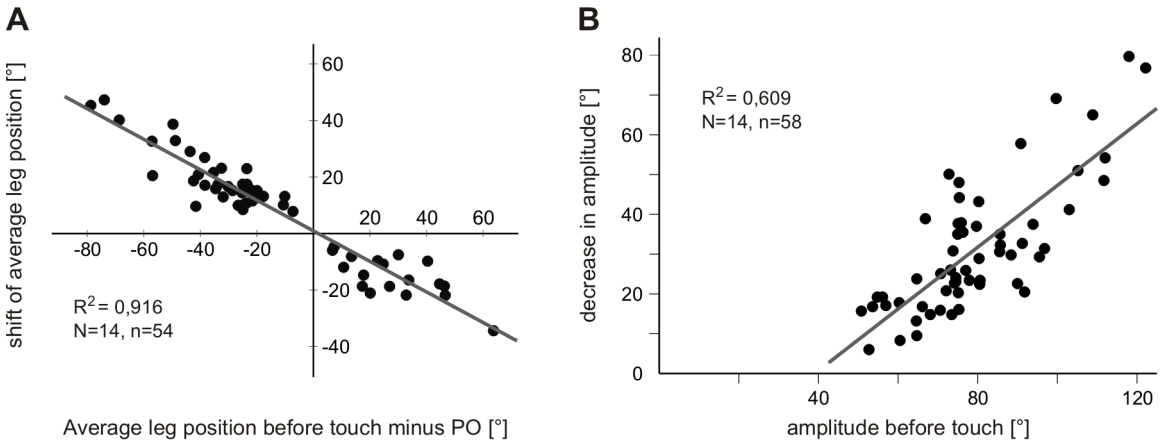

Figure 3.6 – Extent of changes in average leg position and movement amplitude. (A) The magnitude of shifts in average leg position depends on the difference from the PO to the average leg position before touch. Each data point denotes the extent of shift of leg position for one experiment. Data points in the upper left (lower right) quadrant come from experiments in which the PO was located above (below) the average leg position before touch. (B) Magnitude of decrease in movement amplitude depends on amplitude before touch. The regression line is drawn in gray. N, number of animals; n, number of experiments.

$P<0.05$; Fig. 3.5 A). Only rarely was the average leg position significantly shifted away from the PO (gray bars) or not significantly shifted at all. Also, the amplitude of leg movements was decreased significantly in the majority of experiments for POs of +25°, +15° and 0° (84–100% of experiments, $P<0.05$; Fig. 3.5 B, black bars). When disturbed at PO=–40°, the amplitude decreased significantly in 48% of experiments ($P<0.05$). A more comprehensive view on leg searching movements is provided in Fig. 3.5 C, which displays the frequency of appearance of different combinations of changes in average position and changes in amplitude for the different POs. Targeted searching movements, that is a shift of average leg position towards the PO and at the same time a reduction of amplitude, occurred in the majority of experiments (60–91%) for POs of +25°, +15° and 0° (upper left quadrant of Fig. 3.5 C). Only for a PO of −40° was a targeted response not the predominant behavior, as the amplitudes of movements were often not decreased significantly (magenta columns in upper and lower left quadrants in Fig. 3.5 C). Searching movements that, upon touching the object, were not shifted towards the PO and did not show decreased amplitudes only occurred in 5% of experiments (lower right quadrant). Visual information was not necessary for the generation of targeted searching. In experiments performed with blindfolded animals (N=8, n=76), both average leg position and amplitude changed in the same way as in sighted animals (Fig. 3.5 D). Evaluation of the frequencies of different combinations of changes in both parameters resulted in an average percentage of targeted responses

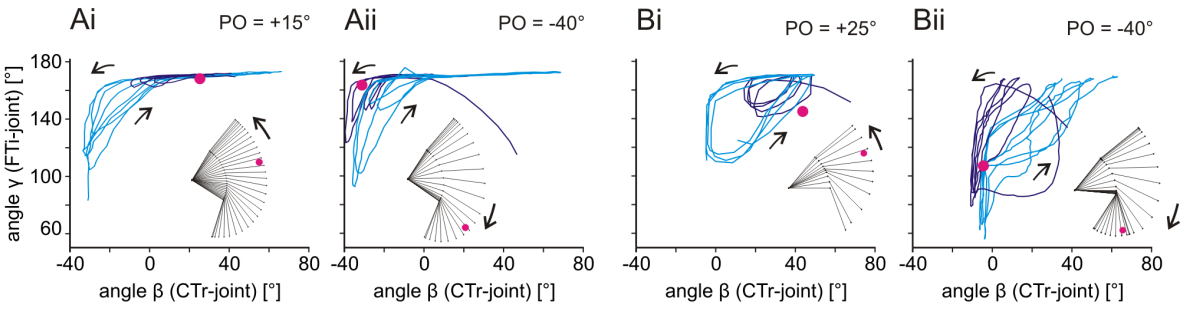

Figure 3.7 – Searching movements plotted as γ (FTi angle) against β (CTr angle). Cyan trajectories depict four cycles of searching movements before contact with the object; dark blue trajectories depict four cycles after contact. Magenta circles indicate leg joint angles when objects were touched (and thus the PO). Black lines show leg kinematics of half a cycle of undisturbed searching movements of the respective experiment. (A) Experiments from the same animal, with POs of +15° (Ai) and –40° (Aii). (B) Experiments from another animal, with POs of +25° (Bi) and –40° (Bii).

(58%; Fig. 3.5 D) very similar to that in sighted animals (60%; Fig. 3.5 C). In animals that responded with targeted searching movements to touching the object, the magnitudes of shifts were highly correlated with the distance between the PO and the average leg position before touching the object (R^2=0.916, P<0.001; Fig. 3.6 A). This high correlation indicates the shift towards PO as a flexible situation dependent adaptive behavior. On average, leg positions were shifted by 62±23%. For amplitudes, the magnitudes of changes were moderately correlated with the size of the amplitude before touch (R^2=0.609, P<0.001; Fig. 3.6 B). On average, amplitudes changed by 38±14%.

3.1.3 Contribution of CTr and FTi joints to targeted searching movements

So far, leg positions have been described by a single angle, α. The coordination of the CTr and FTi joints during searching movements is very stereotyped, movements in both joints are generally coupled (Fig. 3.1B). Thus, α is a sufficiently precise single parametric measure of the overall leg movements to demonstrate the targeted response that animals perform upon touching an object. However, α does not describe how each of the two involved joints, CTr and FTi, contribute to the leg trajectories during searching movements and especially to the changes in searching movements upon touching an object. Thus, I plotted angle γ (FTi joint) against angle β (CTr joint); four examples from two animals are shown in Fig. 3.7. As can be seen in Fig. 3.7, upon contact with the object, the movements of both joints were changed regarding their center and amplitude. The extent to which they contributed to the leg's new trajectory mainly depended on the PO: upon contact in an upper position, changes in α were mainly due to a decrease in FTi joint movements (Fig. 3.7 Ai,Bi). Maximal

extension of the FTi joint was almost unchanged. Changes of CTr joint movements were not as pronounced, but contributed to the change in α. Upon contact in a lower position (Fig. 3.7 Aii,Bii), it was mainly the amplitudes of CTr joint movements that were decreased and centered around a new position, whereas movements of the FTi joint remained relatively unchanged compared with contact in upper positions. However, the relative contribution of each joint to a change in searching movements varied from animal to animal (compare Fig. 3.7 A and 7B). Fig. 3.7 also shows that even upon contact with an object, the stereotyped coordination pattern of both joints basically did not change. Rather, leg movements were confined to a section of the previous working range of searching movements while retaining the inter-joint coordination of the respective section. I never observed that amplitudes (α) were decreased by increased movements at the CTr joint and compensating increases in movements at the FTi joint. While the point of contact with the object did not change from animal to animal and a single animal's joint coordination was rather stereotypical, joint angles β and γ could vary among animals for a given α (compare Fig. 3.7 Aii and 7Bii).

3.1.4 Trochanteral muscle activity underlying targeted searching movements

In order to analyze the muscle activity underlying the observed changes in searching movements, EMGs of *levator* and *depressor trochanteris* were recorded. The antagonists showed alternating bursting activity correlated with femoral levation and depression (Fig. 3.8, top two traces). Rectification and low-pass filtering (smoothing with a time constant of 0.05 s) of EMG recordings (RS-EMG) (Gabriel et al., 2003) give an approximation of the overall excitation of the respective muscles (Fig. 3.8, middle two traces). Changes in the integrals of RS-EMGs (gray areas below the curve) of the levator and depressor muscle were accompanied by shifts of average leg position (cyan line) upon touching the object. Upon contact, mainly depressor activity decreased, thereby causing an increase in the ratio (from 1 to 6) of levator to depressor activity (Fig. 3.8 A). This relative increase in levator activity was accompanied by an upward shift of the average position of the femur (β, cyan line) and the entire leg (α, cyan line) (Fig. 3.8 A, bottom two traces). A decrease in the ratio (from 1 to 0.003) of levator to depressor activity was accompanied by a downward shift of the average position of the femur and the entire leg (Fig. 3.8 B). Such changes in activity were observed in 17 out of 18 experiments. The change in ratio was due to either altered activity in both the levator and depressor trochanteris or changes in activity in only one of the muscles. In some cases femoral levation and depression was accompanied by activity in only one of the antagonists (Fig. 3.8 B, second, fourth and sixth traces from the top).

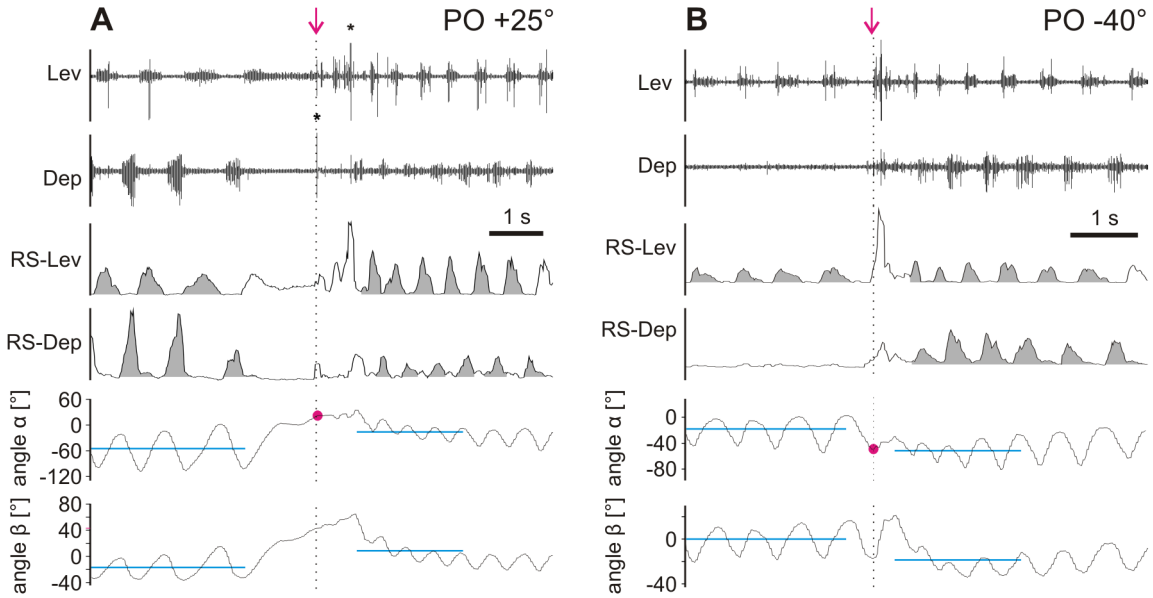

Figure 3.8 – Activity in levator and depressor trochanteris muscles in experiments with a PO above (+25°; A) and below (−40°; B) the average leg position, shown as original EMGs (two top traces) and after rectification and smoothing (RS-EMG; two middle traces). Asterisks mark two muscle potentials that have been clipped. Gray areas indicate integrals of RS-EMGs. Lower two traces show the position of the entire leg as angle α, and the position of the femur as angle β (CTr joint). Cyan lines indicate average leg position and femur position before and after touching the object. Please note that four cycles before touch were included in analysis but only three cycles are shown. Magenta arrows indicate the time point of touch, and POs are marked by magenta circles.

3.1.5 Persistency of changed searching movements

I was interested in the time it takes for altered searching movements to be restored to initial values. Therefore, initial values were calculated for both parameters (average leg position and amplitude) from the four cycles (eight half-cycles resulting in eight data points) before touching the object (solid cyan lines in Fig. 3.9). For cycles after touch, average leg position and amplitudes of movements were calculated per half-cycle and plotted over time. A curve (see above) was fitted to each resulting data set (solid dark blue lines in Fig. 3.9). Prediction bounds (dotted lines) were adjusted such that they contained 90% of the data points. I evaluated 23 experiments from five animals. Amplitudes were evaluated for all 23 experiments; the average leg position was evaluated for only 21 experiments as a result of exceptionally large variations in leg position before touching the object in two experiments. In 91% of experiments evaluated regarding the amplitude and 81% regarding average leg positions, exponential fits yielded R^2-values larger than 0.8 or resulted in the best R^2-values (as compared with linear, power or logarithmic fits). However, in several cases the data could be well fitted ($R^2 \geq 0.8$) by more than one function type. In 36% of cases the best fit was yielded by

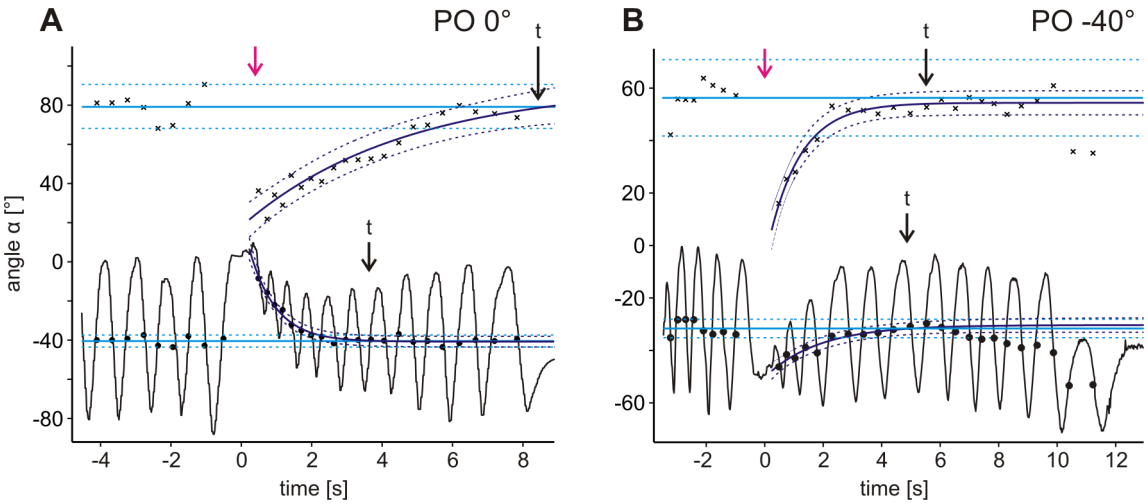

Figure 3.9 – Restoration of changes in searching movements upon touching the object at POs of 0° (A) and –40° (B). Black curves depict searching cycles throughout an experiment, plotted as angle α over time. Magenta arrows indicate the point in time when the object was removed after touch and searching movements were resumed. Black circles give average leg positions for half-cycles of searching movements; black crosses give mean amplitudes of half-cycles of searching movements. Initial mean values of average leg position and amplitudes before touch are displayed as cyan lines. Dark blue lines indicate best fits to average leg position and amplitude data after touch, showing the restoration of average leg position and amplitude; dotted cyan and dark blue lines give corresponding prediction bounds. Distances between magenta arrow and the two arrows marked 't' indicate recovery time for amplitude (upper) and average leg position (lower).

either power, linear or logarithmic curves. Nevertheless, in all 44 evaluated cases I used exponential fits to determine the duration of the changes. The time from removal of the object (magenta arrow, Fig. 3.9 A) to the intersection of the initial value fit and the exponential fit (arrows 't', Fig. 3.9 A) was interpreted as the time required to restore initial values. In approximately half of the experiments, the exponential fit closely approached the initial value but did not intersect. In those cases, a different approach was used: the duration of change was defined as terminated when the exponential fit had achieved 99% of its final value, thus the slope was close to zero (arrow 't' in upper trace, Fig. 3.9 B). Both approaches resulted in similar durations. In seven experiments, initial amplitudes or average leg positions were not regained, that is, even prediction bounds did not intersect. These cases were excluded from data evaluation. For amplitudes, it took 6±2.9 s to be restored to initial values, and for average leg positions it took 6±4.0 s. However, as is obvious from Fig. 3.9 A, the duration required to regain the initial average leg position or amplitude could differ even in the same experiment.

3.1.6 Necessity of the brain for targeted searching movements

It has been shown in the literature that animals with lesioned higher neuronal areas (brain and/or subesophageal ganglion (SEG)) are still able to perform coordinated leg movements. For example, cockroaches show flying or walking behavior after ablation of the SEG or brain, respectively (Gal and Libersat, 2006). Graham (1979) showed that headless stick insects walk with almost unchanged coordination. Such headless/ head lesioned animals have been shown to have difficulties with obstacle negotiation, block climbing or avoidance turning (Harley and Ritzmann, 2010). On the other hand, in various preparations headless animals have been shown to modify their movements due to sensory information. For example, headless locusts can be trained to maintain a certain leg position with repeated electric stimuli (Horridge, 1962); locusts, with isolated metathoracic ganglion, target their hind leg to a tactile stimulus on their wing (Matheson, 1997); and *Drosophila* larvae, without input from the brain, modify crawling due to a light stimulus (Berni et al., 2012). Thus, it seemed favorable but not sure whether stick insects without brain can target their leg searching movements to the position of an object as described previously.

Therefore, in several animals after a number of control experiments (Fig. 3.10 I), the circumesophageal connectives were cut (scheme in Fig. 3.10 A) and the brain removed from the head capsule. After ablation, animals were still able to perform a targeted response (Fig. 3.10 B). If upon object contact searching movements were shifted significantly towards the PO, the magnitude of the shift depended on the distance from PO to average leg position before contacting the object ($R^2 = 0.87$; Fig. 3.10 C).

The correlation was almost as strong as with intact brain (Fig. 3.10 Iiii; $R^2 = 0.91$). This demonstrates the targeted behavior to be a flexible situation-dependent response even without brain. The decrease in movement amplitude was only weakly correlated to the size of the amplitude before touch ($R^2 = 0.23$; Fig. 3.10 D) and was weaker than during control experiments ($R^2 = 0.48$; Fig. 3.10 Iiv). For experiments in which upon contact the average leg position was significantly shifted towards the PO, its distance (after contact) to the PO was not significantly different from the distances obtained in control experiments ($p = 0.23$; ranksum test;Fig. 3.10 E). Thus the average leg position was adapted equally accurate by animals with and without brain. Similarly, the absolute sizes of movement amplitudes after contact were statistically the same ($p = 0.07$). However, when amplitudes after object contact were measured relative to the size of amplitudes before contact, they were significantly larger for experiments of brain ablated animals ($p = 0.01$;Fig. 3.10 F). On average (arithmetic mean, magenta cross), the amplitudes were only decreased to 67% of amplitudes before contact in animals without brain but to 56% in animals with intact brain.

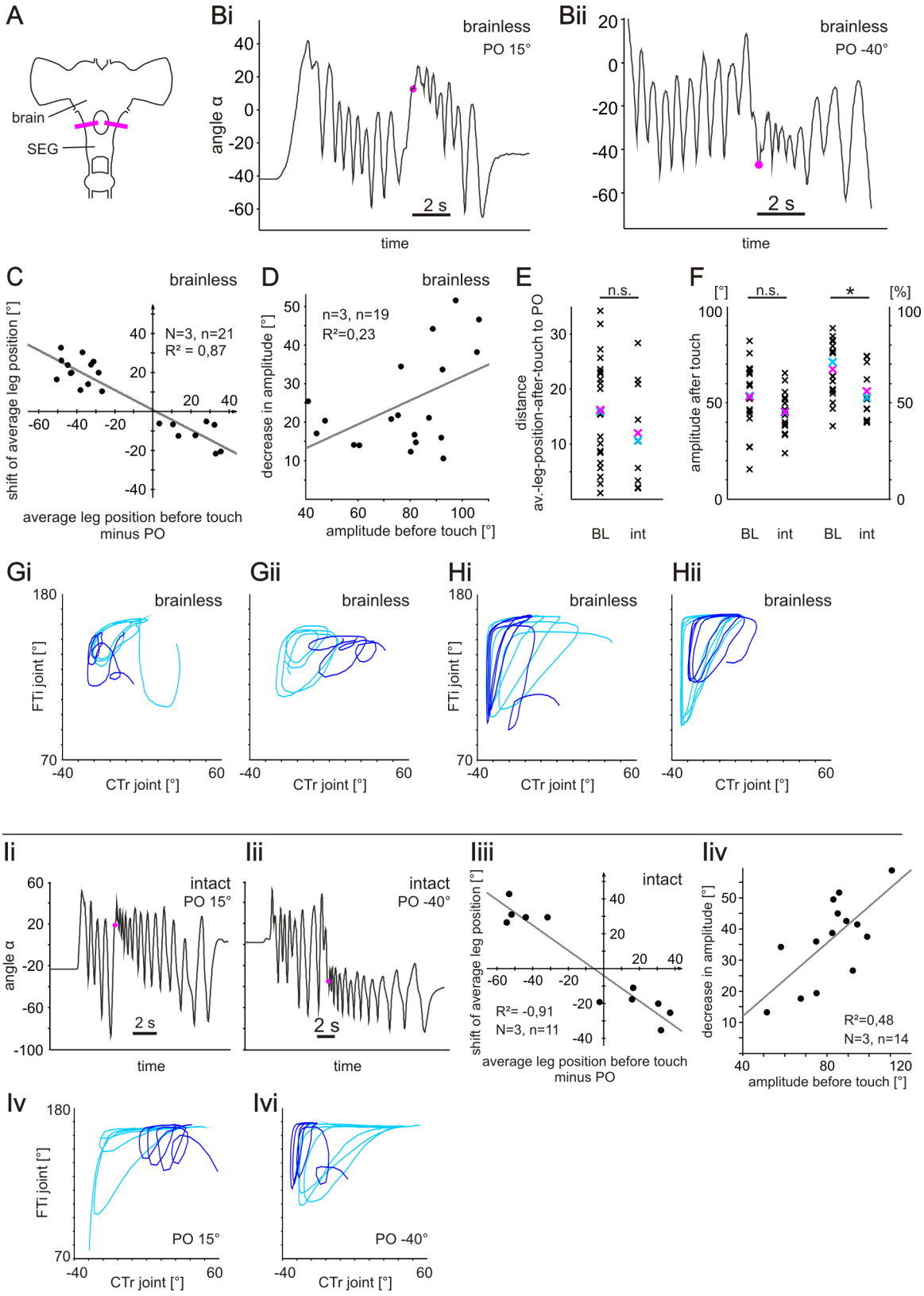

Figure 3.10 – Targeted searching movements without brain (A-H) and control experiments with intact brain (I). For detailed figure legend see next page.

Fig. 3.10: Targeted searching movements without brain. (A) Scheme of head ganglia with cutting sites (magenta bars) of circumesophageal connectives. (B) Searching movements of two experiments plotted as angle α over time for PO=+15 °C (Bi) and PO=−40 °C (Bii). Magenta dot indicates PO. (C) The magnitude of shifts in average leg position plotted against the difference from the PO to the average leg position before touch. Each dot denotes the extent of shift of leg position for one experiment. The regression line is drawn in gray. (D) Decrease in movement amplitude plotted against amplitude before touch. (E) Distance of average leg position after contact to PO for searching movements without brain (BL) and with intact brain (int). All experiments in which the average leg position was significantly shifted towards PO were included. Each black cross denotes a single experiment. (F) Movement amplitudes after contact. All experiments with a significantly reduced amplitude were included. Scale and data on left: absolute values of amplitude; on right: values relative to amplitude before contact. n.s.=non-significant; *:$p<0.05$. (G) Searching movements of experiments shown in B plotted as FTi joint angle against CTr joint angle, indicating joint coordination. Cyan trajectories depict searching movement cycles before contact with the object; dark blue trajectories depict cycles after contact. (H) Searching movements of two further experiments of the same animal. (I) Searching movements of the same animal with intact brain. Figure details as described in A-H.

The coordination of leg joints seemed to become slightly more variable after ablation of the brain in some cases. This is visible when CTr and FTi joint positions of the two experiments shown in Fig. 3.10 A are plotted against each other (Fig. 3.10 G, size and regularity of cyan trajectory and dark blue trajectory). However in other experiments, the same animal after brain ablation showed the same coordination as during control experiments with intact brain (compare exemplary experiments in Fig. 3.10 H and I). Thus, CTr and FTi joint coordination remained robust also in searching movements performed without brain.

In summary, animals do not need a brain for a targeted response. Average leg position is adapted equally well to the position of the object. Movement amplitudes are reduced to a slightly lesser extend after brain ablation when compared to control amplitudes. The intra-leg coordination largely remains the same as during control experiments.

3.1.7 Role of leg sensory signals in targeted searching movements

As shown in Fig. 3.5 and Fig. 3.10, neither visual information nor the brain is necessary to perform a targeted response. Therefore, the execution of this response is very likely to be based on information from leg sensory organs. In this case, the animal may use different sensory organs for two purposes: firstly, to sense contact with the object and secondly, to determine the actual position of the leg at the same time and thus gain information on the position of the object. Therefore, in four sets of experiments I selectively prevented input from leg sensory organs providing either of the two types of information.

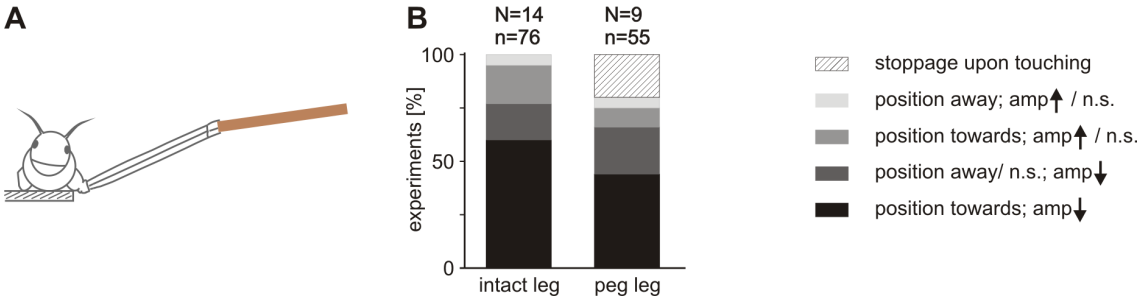

Figure 3.11 – Impact of tibial and tarsal sensory organs on the change of searching movements upon touch. (A) Schematic of preparation for 'peg leg' experiments. The tibia was substituted by a wooden stick. Figure modified from von Uckermann and Büschges (2009). (B) Quantitative comparison of data from experiments with intact legs and 'peg legs'. Black bars depict the percentage of experiments in which a targeted reaction (position shift towards PO, amplitude decrease) was shown upon touch. Dark gray bars show the percentage of experiments in which only amplitudes were decreased. Medium gray bars show the percentage of experiments in which only the average leg position was shifted towards the PO. Light gray bars indicate the percentage of experiments in which average leg position was not shifted towards the PO nor were amplitudes decreased. Hatched bars depict experiments in which animals stop searching movements. n.s., not significant; amp↓, significant decrease in amplitude; amp↑, significant increase in amplitude.

Strong candidates for coding contact with the object are sensory organs located on the tibia and tarsus, e.g. tactile hairs and campaniform sensilla (Zill et al., 2011), as well as proximal campaniform sensilla that were described to be active due to force resulting from resisted movements (Zill et al., 2012). The position of the femur is measured by the trochanteral hairplate (trHP; BF1 by Wendler, 1964), which is known as an essential sensory organ in the coxa–trochanter joint control loop (Schmitz, 1986a,b; see Fig. 3.13 A). The hairplate sensilla are deflected when the femur is lifted. Position and movement of the FTi joint is measured by the femoral chordotonal organ (fCO), a group of mechanosensory neurons located in the proximal femur with attached apodeme. The apodeme spans the length of the femur and is attached with its second end to the proximal tibia (Füller and Ernst, 1973; Hofmann et al., 1985). Therefore the apodeme and fCO-neurons are stretched when the FTi joint is flexed or relaxed when the FTi joint is extended.

In a first set of experiments, to prevent sensory information from the tibia and the tarsus, the tibia was cut distal to the FTi joint (Bässler et al., 1991), which abolished the tibial and tarsal tactile hairs and the tarsal campaniform sensilla, amongst others. The tibial stump was hollowed out to exclude influences of campaniform sensilla groups 6A and 6B (Zill et al., 2011), which are located on the proximal tibia. The missing tibia was replaced by a wooden stick (Fig. 3.11 A). With this 'peg leg', animals were still able to show a targeted response upon touching the object in the same manner as with the intact leg. The percentage of targeted responses was 55% (Fig. 3.11 B)

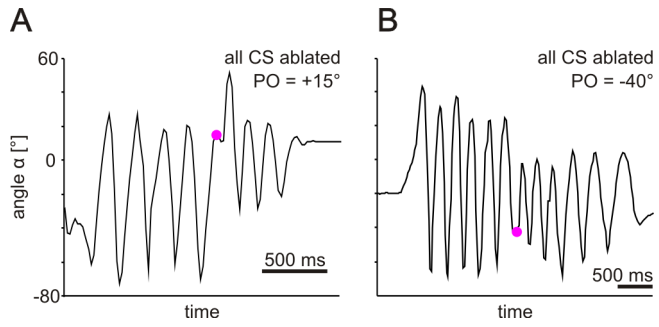

Figure 3.12 – Exemplary searching movements of one animal plotted as angle α over time with all leg campaniform sensilla ablated for PO = 15° (A) and PO = -40° (B). The time point and position of the PO is indicated by a magenta dot.

and thus only slightly less than in intact animals (60%; Fig. 3.5 C). The magnitude of shifts of average leg position was correlated with the distance between the PO and the average leg position before touching the object (R^2=0.793, P<0.001); the magnitude of changes of amplitude was correlated, but again more weakly, with the amplitude before touching the object (R^2=0.587, P<0.001). The coordination of CTr and FTi joint movements remained the same as in animals with an intact leg (not shown). However, some animals (20% of experiments) did not respond to touching the object at all but instead quit searching movements (Fig. 3.11 B, shaded bar). Such a behavior was not observed in animals with an intact leg.

In a second set of experiments, all femoral and trochanteral campaniform sensilla were ablated in addition to replacement of the tibia by a peg leg (N=4, n=49). Still, three of four animals were able to perform a targeted response as can exemplarily be seen in Fig. 3.12. It was not analyzed, if targeting occurs as frequent and precise as with intact leg.

In a third set of experiments following ablation of the trHP, animals performed searching movements with increased amplitude (mean amplitude 105±22° instead of 85±17° with intact leg; N=4, n=16 (intact), n=31 (trHP ablated)) but coordination of CTr and FTi joint movements remained the same as with the intact leg. The percentage of experiments in which a targeted response was exhibited decreased considerably from 58 to 21% after ablation of the trHP (Fig. 3.13 B, black bars). This decrease was mainly due to animals failing to reduce the amplitude of movements (66% of experiments, Fig. 3.13 B, light and medium gray bars; Fig. 3.13 Cii), whereas the average leg position was not shifted towards the PO in 46% of experiments (Fig. 3.13 B, light and dark gray bars). Noticeably, in some trials (17%), animals neither decreased amplitude nor shifted average leg position (Fig. 3.13 Ci). In animals that showed a decrease in amplitude, the range and mean of the reduction (16° to 60°, mean=33°) was similar to that of the controls (12 to 76°, mean=34°). However, in contrast to the intact animals, the size of reduction was not correlated with the size of the amplitude before touching the

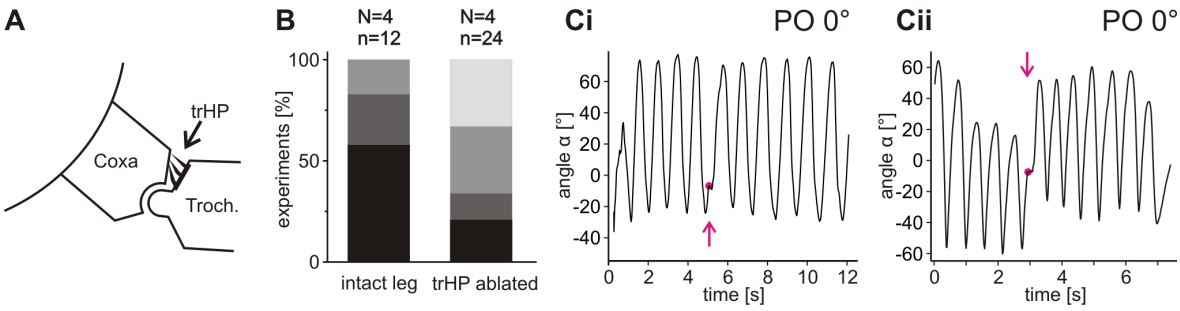

Figure 3.13 – Impact of trochanteral hairplate (trHP) ablation on the change of searching movements upon touch. (A) Schematic of the trHP. Ablation of the trHP largely impairs amplitude control and shifting of average leg position towards the PO. Modified from Wendler (1964). (B) Quantitative comparison of data from experiments with intact trHP and data from experiments with ablated trHP. Figure legend as in Fig. 3.11 B. (C) Searching movements plotted as angle α over time for experiments with ablated trHP.

object (R^2=0.058, versus R^2=0.657 with intact trHP). The extent of shifts of average leg position still correlated with the distance between PO and the average leg position before touching the object (R^2=0.644, P<0.001), albeit the correlation was weaker.

Finally, when the fCO-apodeme was cut in another set of experiments, animals were still able to target high POs (PO, +25°, +15°, 0°) (Fig. 3.14 A); they succeeded in 65% of experiments as compared to 56% in experiments of the same animals with intact leg (see Fig. 3.14 D,E, gray bars). However, animals could not target low POs any more (PO = -40°; Fig. 3.14 B); their success rate with ablated fCO-apodeme dropped to 5% as compared to 63% in experiments of the same animals with intact leg (Fig. 3.14 D,E; black bars; Fig. 3.14 C). This drop was due to a failure to reduce the amplitude of searching movements (59% of experiments, Fig. 3.14 D; black bars). As was assured in sham experiments with a subset of animals (N=3), the decrease in targeting performance was not due to damage to cuticle or muscle that might have occurred during the ablation of the fCO-apodeme. During sham experiments, animals were able to perform a targeted response both for high POs (56% of experiments, Fig. 3.14 E, gray bars) and for low POs (71% of experiments, Fig. 3.14 E, black bars; Fig. 3.14 C). Some animals with cut fCO-apodeme did not respond to touching the object at all but instead stopped searching movements (Fig. 3.14 D). Similar to results by Karg et al. 1991, when the fCO-apodeme was cut, also the coordination of CTr and FTi joints changed: animals tended to either not flex the FTi joint (Fig. 3.14 Fi) or, if flexed, the FTi joint remained flexed for a longer time than with intact leg also during upward movements (Fig. 3.14 Fii). For comparison, the coordination of CTr and FTi joint during searching movements with intact leg (Fig. 3.14 Fiii) and after sham operation (Fig. 3.14 Fiv) are shown. In contrast to results by Karg et al. (1991), no searching movements with sole FTi joint movements occurred; on the other hand, searching movements with the same coordination as seen in animals with intact leg occurred in 39% of experiments (not

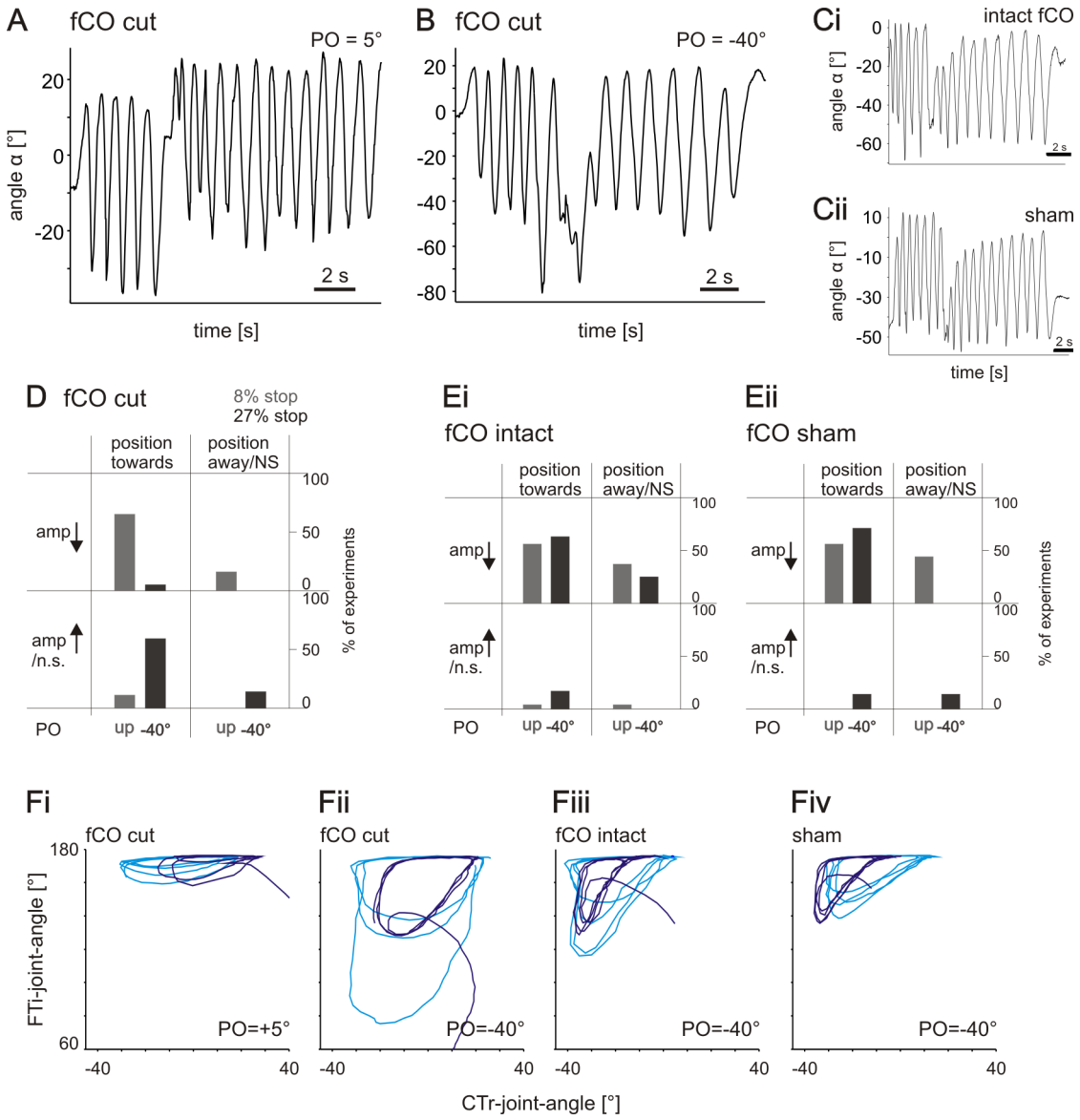

Figure 3.14 – Impact of fCO-apodeme ablation on the change of searching movements upon touch. Searching movements plotted as angle α over time for experiments with cut fCO-apodeme (A,B), intact fCO (Ci) or sham operated animals (Cii). (D) Quantification of the response to contacting the object at high POs (gray bars; PO=0°,15°,25°) or low PO (black bars; PO=-40°) for experiments with cut fCO-apodeme. Left quadrants: average leg position was significantly shifted towards PO. Right quadrants: average leg position was significantly shifted away from PO or not significantly (n.s.) shifted. Upper quadrants: amplitudes decreased significantly. Lower quadrants: amplitudes increased significantly or did not change significantly. Thus, the left upper quadrant indicates a targeted response. High POs (=0°, 15°, 25°): N=8 animals, n=37 experiments; low PO (=-40°): N=8, n=22. (Ei) Quantification for experiments with intact fCO. High POs: N=8, n=27; low PO=-40°: N=8, n=24. (Eii) Quantification for experiments with sham operated fCO. High POs: N=3, n=9; low PO=-40°: N=3, n=7. (F) Searching movements of one animal plotted as FTi joint angle against CTr joint angle, indicating joint coordination. Cyan trajectories indicate searching cycles before contact with the object; dark blue trajectories depict four cycles after contact. (Fi,Fii) Experiments with cut fCO-apodeme; (Fiii) with intact fCO-apodeme; (Fiv) with sham opareated fCO-apodeme.

shown).

Thus, ablation of tibial and tarsal sensory organs decreased the number of experiments in which animals continued their searching movements upon touching the object. If searching movements continued, animals still performed a targeted response. Additional ablation of femoral and trochanteral CS did not prevent targeting. On the other hand, ablation of trHP or fCO-apodeme heavily impaired the accuracy of the targeted response.

3.1.8 Targeted searching movements of the middle leg

The legs of either segment –pro-, meso-, or metathorax– can perform cyclic stereotyped searching movements as can be observed when stick insects are lifted up in the air and which has been described by Dürr (2001) for animals that step into a gap with either one of their legs. Still, a targeted response as described in the previous sections might be a peculiarity of animals performing front leg searching movements. This might be assumed, firstly, because front legs are the legs that animals mostly search with when walking on even ground and probably while climbing in bushes as might be expected from results by Dürr (2001). Secondly, for leg coordination during walking, front legs have been shown to uncouple from the walking system (Grabowska et al., 2012) thereby, again, assuming a special role. As yet, it is not sure whether stick insects can perform targeted searching movements with middle or hind legs in the same way as shown for front legs. Using the same experimental setup as previously, I tested whether stick insects can target their middle leg searching movements, too.

Fig. 3.15 A shows two examples of middle leg searching movements while the animal makes contact with the object at PO = 15° (Fig. 3.15 Ai) and PO = -40° (Fig. 3.15 Aii), respectively. After several cycles of stereotyped undisturbed searching movements and contact with the object (magenta dot in Fig. 3.15 A), animals change their searching movements to target the position of the object. The coordination of CTr and FTi joints for these two examples is shown in Fig. 3.15 B. Qualitatively, the coordination is the same during middle leg searching and front leg searching (compare Fig. 3.7), both for undisturbed searching cycles (cyan trajectory) and the targeted response (dark blue trajectory).

When quantifying the middle leg responses to object contact, however, a targeted response on average occurred in 33% of experiments (Fig. 3.15 C, left upper quadrant, black bar) and thus less often than during front leg responses (front leg: 60%, Fig. 3.5 C). This is mainly due to an increased percentage of responses in which the movement amplitude is not significantly reduced (39% on average, Fig. 3.15 C, lower left quadrant, black bar) as compared to front leg responses (front leg: 18%, Fig. 3.5 C).

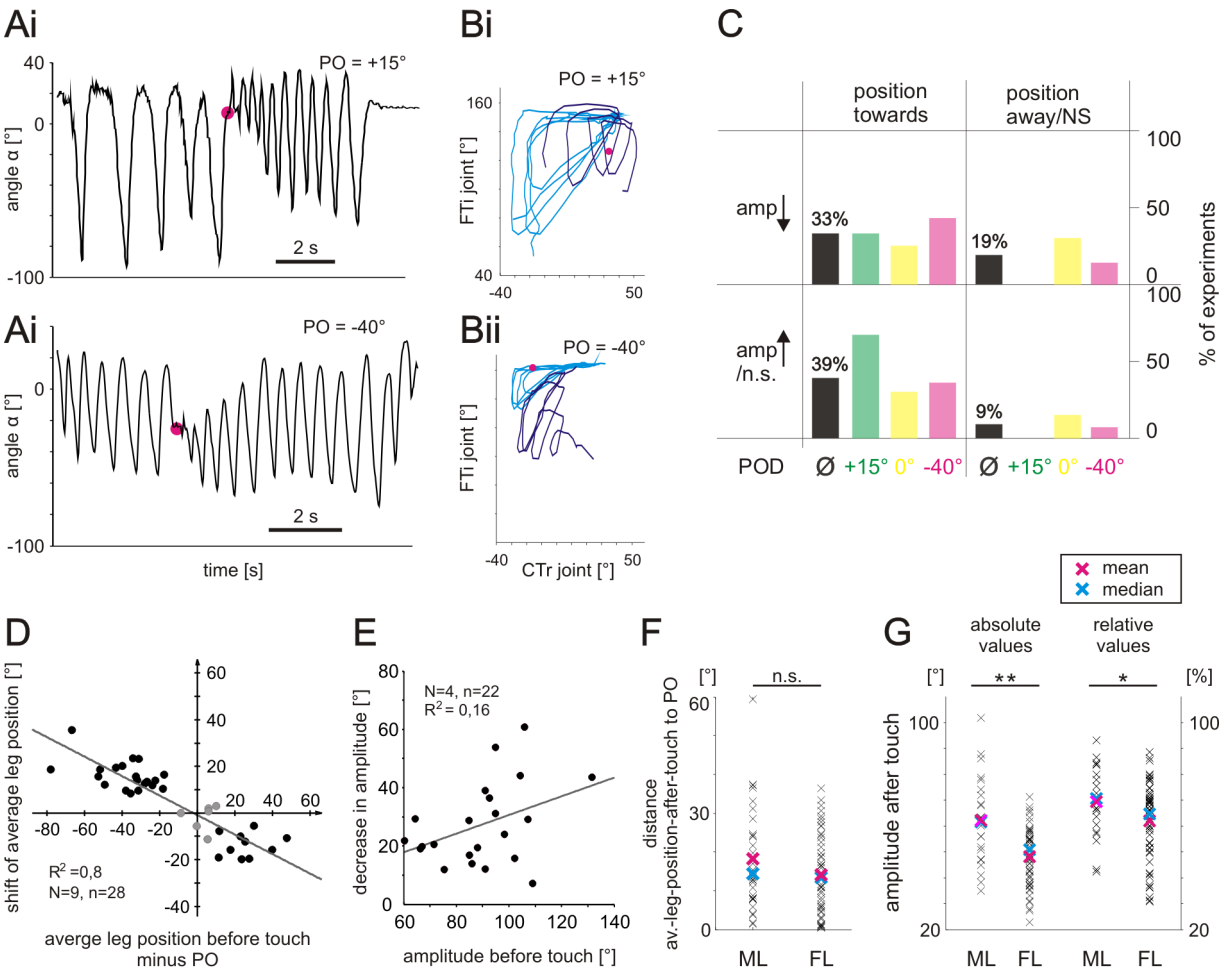

Figure 3.15 – Targeted searching movements with the mesothoracic leg. (A) Searching movements of two experiments plotted as angle α over time for PO = +15° (Ai) and PO = -40° (Aii). Magenta dot denotes PO. (B) Searching movements of the same experiments plotted as FTi joint angle against CTr joint angle, indicating joint coordination. Cyan trajectories depict searching movement cycles before contact with the object; dark blue trajectories depict cycles after contact. Magenta dot: joint coordination at time point of contact. (C) Quantification of responses to contacting the object. Responses to different POs (+15°, 0°, -40°) are color coded. Black bars and percentages denote the result of pooled data of all POs. Further legend: see Fig. 3.5 C PO +15°: N=6, n=9; PO 0°: N=9, n=20; PO -40°: N=8, n=14; pooled data: N=9, n=43. (D) The magnitude of shifts in average leg position plotted against the difference from the PO to the average leg position before touch. Each dot denotes the extent of shift of leg position for one experiment. The regression line is drawn in gray. (E) Decrease in movement amplitude plotted against amplitude before touch. (F) Distance of average leg position after contact to PO for searching movements of the middle leg (ML) and front leg (FL). All experiments in which the average leg position was significantly shifted towards PO were included. Black crosses denote single experiments. (G) Movement amplitudes after contact. All experiments with a significantly reduced amplitude were included. Scale and data on left: absolute values of amplitude; on right: values relative to amplitude before contact. n.s. = non-significant; * = $p<0.05$; ** = $p<0.01$. N = number of animals, n = number of experiments.

For experiments in which animals, upon object contact, significantly shifted their average leg position towards the PO, the magnitude of the shift was correlated with the distance between PO and average leg position before contacting the object (R^2=0.8; Fig. 3.15 D; each black dot denotes one experiment; pooled data from several animals). This correlation is *not* only due to the position of the two data groups that result from 1) a PO above average leg position before contact (black dots in upper left quadrant) versus 2) PO below average leg position before contact (black dots in lower right quadrant) relative to each other. This is shown when the parameter space is extended by not excluding those experiments whose distance between the PO and the average leg position before contacting the object was <10° (compare sec. 2.1; gray dots in Fig. 3.15 D). These data points exactly bridge the gap between the two previous groups. In middle leg responses that showed a significant decrease in movement amplitude, the magnitude of this decrease was not correlated ($R^2 = 0.16$; Fig. 3.15 E) to the amplitude before contact. This is different from results in front leg responses (Fig. 3.6 B).

The accuracy of middle leg responses was further assessed by measuring the average leg position of the targeted response (i.e. leg position after contact) relative to PO (Fig. 3.15 F). This distance on average was the same in middle and front legs (p= 0.2376; Mann-Whitney-U-test). In contrast, the amplitudes of targeted searching movements remained larger for the middle leg than for the front leg, both for absolute values (p=0,0011, Mann-Whitney-U-test) and relative to the amplitude before contact (p=0,0433).

In summary, animals are able to target the position of an object also with their middle leg, albeit not as often and not as accurate as with front legs. Especially movement amplitudes are reduced less in middle leg responses compared to front leg responses.

Summary

So far, it was shown that stick insects can adapt their leg searching movements to target the position of an object they had touched before with their leg. They did so by flexibly shifting the average leg position towards the position of the object and decreasing movement amplitudes. The targeted response lasted several seconds while it declined. The overall electrical muscle activity changed in accordance with the shift of average leg position during searching movements. During targeted searching movements, the coordination of CTr and FTi joint remained the same and searching movements were restricted to a fraction of the previous working range. Vision was not necessary for a targeted response and neither was the brain as a whole; but the trochanteral hairplate and the femoral chordotonal organ seemed to be important sensory organs. Contrarily, replacing the tibia with a wooden stick and ablation of all campaniform sensilla on the

femur and trochanter did not severely impair targeting. Finally, animals performed targeted responses also with their middle leg, albeit to a lesser extent.

3.2 Activity of premotor nonspiking interneurons during searching movements

The description and analysis of targeted searching movements in the first part of this thesis raises the question how this targeted response is generated on the neuronal level. As we know from the presented behavioral experiments with brain ablated animals, the targeted response is likely to be mediated on a local thoracic level. Therefore, further investigations were concentrated on the thoracic neuronal network.

A first necessary step in the analysis of the neuronal basis of targeted searching is to investigate how undisturbed searching movements are generated by the neuronal network. This has been analyzed in part by Fischer et al. (2001) and Schmidt et al. (2001) who recorded the motoneuronal activity during searching movements. The premotor interneuronal activity has not been investigated yet.

Therefore, nonspiking interneurons (NSIs), a class of local premotor interneurons, were investigated. NSIs transmit information gradually (Burrows and Siegler, 1978), as transmitter is released according to gradual shifts in their membrane potential. With respect to the previously described graduated targeted response, this makes them interesting candidates for further investigations.

In the stick insect, NSIs are known to process sensory signals and to contribute to intra- and inter-joint reflexes (Büschges, 1990; Hess and Büschges, 1997, 1999; Akay et al., 2004). In more recent studies (Schmitz et al., 1991; von Uckermann and Büschges, 2009; Rosenbaum, 2013), NSI activity has been described during walking behavior. Whether NSIs are involved in the generation of leg searching movements, has not been investigated yet. Furthermore, membrane potential changes in certain identified NSIs correlate with stepping velocity during walking (von Uckermann and Büschges, 2009; Rosenbaum, 2013). Whether single NSIs can actually cause changes in behavioral output so far has not systematically been investigated. While in several cases, de- or hyperpolarization of NSIs has been shown to change MN activity (e.g. Büschges, 1990; Ludwar et al., 2005; Schmitz et al., 1991), only in few cases the impact on ongoing behavior was investigated (Schmitz et al., 1991; Kittmann et al., 1996; von Uckermann and Büschges, 2009).

During the experiments described in the following, the intracellular NSI activity was recorded from the neuropil of the mesothoracic ipsilateral hemiganglion, while animals performed searching movements with their mesothoracic leg. As a monitor of MN activity, electromyograms (EMGs) of the four main leg muscles moving coxa-trochanter and femur-tibia joint were recorded and leg movements videotaped for reference. Recorded NSIs were identified and classified according to their morphology, the effect of de- and hyperpolarizing current injections during rest on EMG signals and leg movement, and

membrane potential modulations during searching movements. In order to compare recorded NSIs with recordings of previous studies, their membrane potential modulations during walking on a treadwheel were recorded, as well as the response to femoral chordotonal organ stimulation. The responses of newly identified NSIs are shown in this chapter, the ones of previously identified NSIs are included in the appendix for comparison.

Newly identified NSIs were named according to the nomenclature used in previous studies in the stick insect (Büschges, 1990; Hess and Büschges, 1997; Akay, 2002; von Uckermann and Büschges, 2009; Rosenbaum, 2013). The first letter of the name indicates which muscle is driven by the NSI. If the neuron is described morphologically and electrophysiologically, this letter is a capital; if the neuron is described only electrophysiologically, it is a small letter. A second letter indicates whether the influence is excitatory ("e") or inhibitory ("i"). If a nonspiking interneuron provides drive to several muscles, all influences are listed, separated by a dot. Finally, a number is asigned in ascending order.

For example, NSI Li.De3, when depolarized in the resting animal, inhibited levator muscle activity and excited depressor muscle activity (and elicited corresponding leg movements). Because in previous studies an interneuron of the type Li.De was already identified and named Li.De2 (Rosenbaum 2013; NSI De2 renamed to NSI Li.De2; see also appendix), a "three" was assigned. It should be noted that neurons from previous studies were only considered if they had been stained. This was necessary because for many neurons the description of their input and output properties was too fragmentary to re-identify them in new recordings. Accordingly, the list of names of known interneurons was adjusted and some interneurons renamed. For a list of corresponding old and new names see Tab. 4.1 in appendix.

3.2.1 Nonspiking interneurons influencing the coxa-trochanter joint

Several NSIs that provide synaptic drive to motoneuron pools of the coxa-trochanter joint have been described previously. Hess and Büschges (1997) and Hess (1998) analyzed the contribution of those interneurons to interjoint reflexes elicited by sensory signals from the femoral chordotonal organ. Rosenbaum (2013) described the interneurons' activity during forward and backward walking. None of my recordings matched one of these previously known interneurons; instead several newly identified interneurons are described in this section. According to the criteria explained above, the newly identified interneurons were named Li.De3, De8, de9 and Le3, Le4, di3.

Nonspiking interneurons supporting depression of the leg

NSI Li.De3 is a newly identified interneuron that was recorded and stained three times. Intracellular stainings (neurobiotin, streptavidin-cy3) revealed the distinct morphology of NSI Li.De3 (Fig. 3.16 A). The soma is located posteriorly with a large c-shaped main neurite that bifurcates at its posteromedial end. Two characteristic secondary neurites project laterally, another secondary neurite originates between these two neurites and projects medially. When depolarized by current injection during rest, NSI Li.De3 caused leg depression and in some cases subsequent extension (Fig. 3.16 B). It excited *depressor trochanteris* motoneurons (MNs) (Fig. 3.16 Bi) or inhibited *levator trochanteris* MNs as shown in a second animal (animal 2; Fig. 3.16 Bii; morphology and further physiological description is provided in appendix for comparison). In a third animal, no muscle activity was detectable in EMG recordings upon current pulse injection into NSI Li.De3, although during searching movements a clear signal was measured. Stimulation of the femoral chordotonal organ (fCO), by FTi joint flexion and extension via treadwheel movements, phasically depolarized (following flexion) and hyperpolarized (following extension) the membrane potential of NSI Li.De3 (Fig. 3.16 C).

During episodes of searching movements, the membrane potential of NSI Li.De3 was more hyperpolarized than the resting membrane potential (48.3 mV $\pm$ 1.5) (Fig. 3.16Di, N = 3/3). This hyperpolarization decreased but did not invert into a tonic depolarization when the membrane potential level was artificially hyperpolarized by current injection to values as negative as -75 mV (not shown; N = 2/2). Phasic membrane modulations occurred, i.e. the membrane potential was gradually repolarized during downward movements of the leg and hyperpolarized during upward movements (Fig. 3.16 Dii; gray bars denote downward movements) thereby supporting leg movements. This phasic modulation had an average peak to peak value of 7.6 mV ($\pm$1.7) and can be seen best in overdraws of membrane potential modulations during searching movements (Fig. 3.16 E). Overdraws were triggered by the extreme positions of angle α (peak and trough of total leg movement; Fig. 3.16 Ei) and the extreme positions of the CTr and FTi joint movements (Fig. 3.16 Eii-Eiii). At peak movement positions, the membrane potential was maximally hyperpolarized (Fig. 3.16 Ei-Eiii, top row); at trough movement positions, the membrane potential was maximally repolarized (Fig. 3.16 Ei-Eiii, bottom row). Because the resulting average membrane potential modulations (black traces) were very similar for the corresponding trigger points of overall-, CTr-, and FTi joint movements, such overdraws will subsequently be presented only for overall movements (angle α) during the rest of the thesis.

When tonically depolarized or hyperpolarized during searching behavior, no effect of NSI Li.De3 on leg movements could be seen (Fig. 3.16 F, N=3/3, 20 pulses). This may seem somewhat surprising in the light of the previously presented results, where phasic

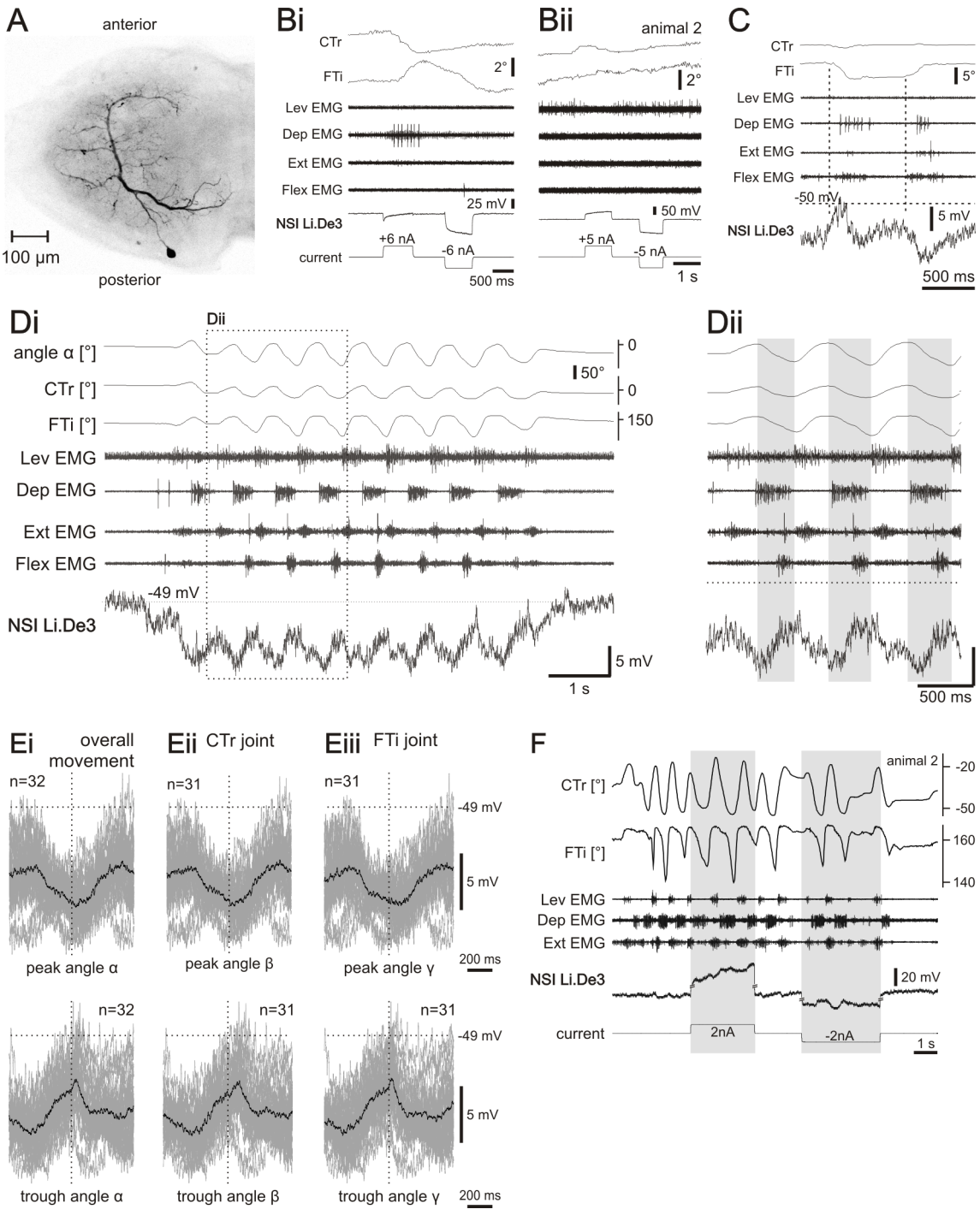

Figure 3.16 – Characterization of NSI Li.De3 and membrane potential modulation during searching behavior. For detailed figure legend see next page.

Fig. 3.16: Membrane potential modulation of NSI LiDe3 during leg searching movements. (A) Morphology of NSI Li.De3 from dorsal view. (B) Current injection into Li.De3 caused leg depression and subsequent extension. Depressor muscle activity increased (animal1) or levator muscle activity decreased (animal2; for further characterization see appendix). (C) Response of NSI Li.De3 to fCO stimulation. Dotted lines indicate start of passive leg movements. (Di) Membrane potential modulation of NSI Li.De3 during a sequence of searching movements. Traces from top to bottom depict: overall leg movement ("α"), coxa-trochanter-joint movement ("CTr"), femur-tibia-joint-movement ("FTi"); electric muscle activity of levator ("Lev EMG"), depressor ("Dep EMG"), extensor ("Ext EMG"), flexor ("Flex EMG"); intracellular recording of NSI Li.De3. (Dii) Section of Di (as indicated by dotted box) with extended time scale. Gray bars denote downward movement of the leg (i.e. decrease in angle α). Dotted line indicates resting membrane potential. (E) Overdraws of membrane potential modulation during searching movements; triggered by peak values of angle α, CTr or FTi joint movements (Ei,Eii,Eiii; top row) or trough values (bottom row). Gray traces depict single sweeps, black trace depicts average potential. n=number of sweeps (F) Tonic de- and hyperpolarization of NSI Li.De3 during ongoing searching behavior did not affect leg movements.

membrane changes were clearly correlated to leg movements and the NSI was able to elicit leg depression when depolarized during rest. Possible reasons will be addressed in the discussion.

Another newly identified interneuron, NSI De8, caused excitation of depressor MNs when depolarized by current injection during rest. This was shown by a clear depression of the CTr joint and a signal, although weak (arrow), in the depressor EMG recording (Fig. 3.17 B). The soma of NSI De8 is located at the anterolateral margin of the ganglion (Fig. 3.17 C). From there, the main neurite projects posteriorly in an inverted c-shape. One secondary neurite originates from the anterior region and projects medially; numerous secondary neurites originate from the posterior region end and project further posterior. NSI De8 was depolarized following both passive flexion and extension of the leg (Fig. 3.17 D).

During searching movements, the membrane potential was maximally depolarized during depressor MN activity, i.e. during downward movements. Towards the end of downward movements, i.e. at the transition to upward movements, De8 membrane potential abruptly hyperpolarized just below resting membrane potential. During the following upward movement of the leg, the membrane potential slowly depolarized again (Fig. 3.17 A, E). An eye-catching feature of De8 are the large IPSPs the NSI received simultaneously with large units occurring in the levator EMG recording (Fig. 3.17 Fi, enlargement from Fig. 3.17 A as indicated). This co-occurrence is shown nicely by an overdraw of membrane potential modulations triggered by large levator EMG signals (overdraw Fig. 3.17 Fii, exemplary large levator signals see Fig. 3.17 Fi, bottom traces). This effect is seen even clearer in the recording and corresponding membrane potential overdraw of another NSI De8 that is shown in Fig. 3.17 G. If all EMG levator muscle

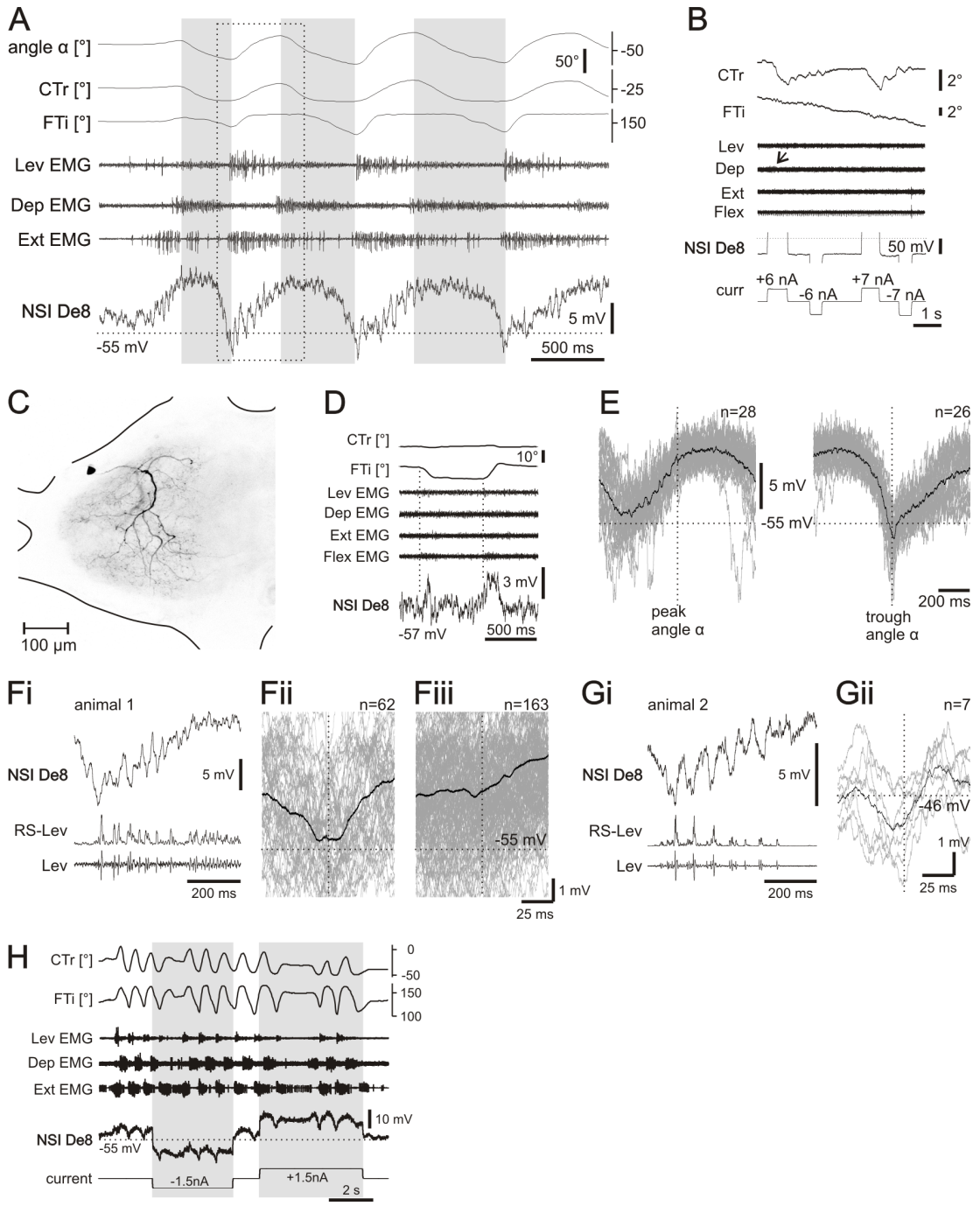

Figure 3.17 – Membrane potential modulation of NSI De8 during leg searching movements. (A) Membrane potential modulation of NSI De8 during searching movements. Gray bars denote downward movement of the leg (i.e. decrease in angle α). Dotted line indicates resting membrane potential. (B) Depolarizing current pulses into De8 cause leg depression. (C) Morphology of NSI De8; dorsal view; anterior-posterior as in Fig. 3.16. (D) Response of NSI De8 to fCO stimulation. n = number of sweeps (E) Overdraws of membrane potential modulation during searching movements; Gray traces depict single sweeps, black trace depict average potential. (F) De8 receives characteristic IPSPs simultaneous to levator muscle activity. (Fi) Enlargement from A as indicated shows exemplary NSI membrane potential modulations during levator EMG activity. For details see text. (Fii,Fiii) Membrane potential overdraws triggered to large levator EMG signals (Fii) or all levator EMG signals (Fiii). (G) Same as in Fi,Fii; shown for a second animal. (H) Tonic de- and hyperpolarization of NSI De8 did not influence searching movements. Gray bars denote times of current injection.

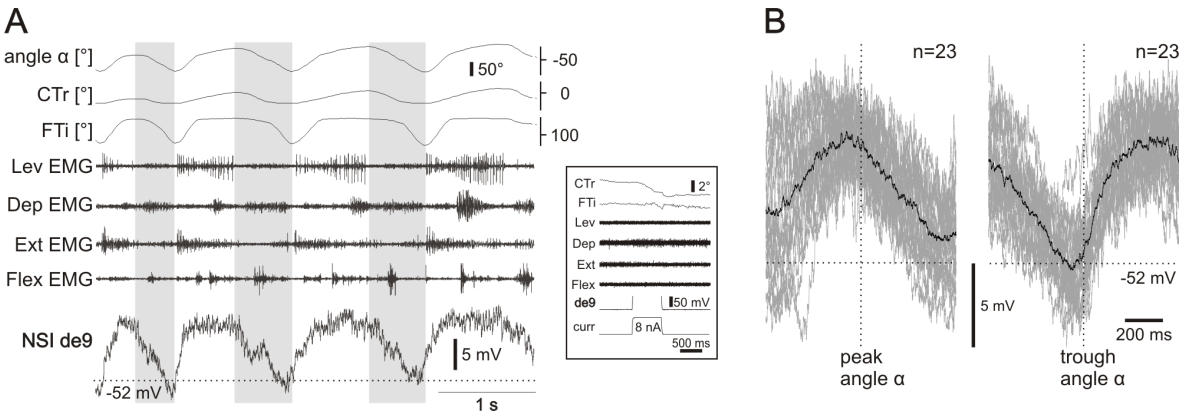

Figure 3.18 – Membrane potential modulation of NSI de9 during leg searching movements. (A) Membrane potential modulation of NSI de9 during searching movements. Gray bars denote downward movement of the leg (i.e. decrease in angle α). Inset: Depolarizing current pulses into de9 cause depressor muscle activity and leg depression. (B) Overdraws of membrane potential modulation during searching movements; Gray traces depict single sweeps, black trace depicts average potential. n = number of sweeps.

signals, irrespective of size, were included as trigger, no such co-occurrence can be detected (Fig. 3.17 Fiii). While generally the recorded membrane potential modulation could as well have been produced by EPSPs in antiphase to levator signals, the notion that the occurring PSPs were inhibitory and in phase with levator signals was supported by the slight but reliable inhibition below resting membrane potential at the transition from down to upward movement. At this point, too, the inhibition was accompanied by a large signal in the levator EMG.

When tonically injecting depolarizing or hyperpolarizing current into NSI De8 during ongoing searching behavior, no effect on searching movements could be detected (Fig. 3.17 G; N=1, 15 pulses). NSI De8 was recorded twice and stained once.

In contrast to the previously described NSIs whose membrane potential modulations supported leg movements during searching behavior, another newly identified NSI, de9, opposed leg movements. Depolarization of NSI de9 during rest lead to excitation of the depressor muscle and to a clear depression of the femur (Fig. 3.18 A inset). However during searching movements, NSI de9 was depolarized above resting membrane potential during upward movement of the leg and repolarized during downward movements, i.e. during depressor activity (Fig. 3.18 A,B). NSI de9 was recorded two times.

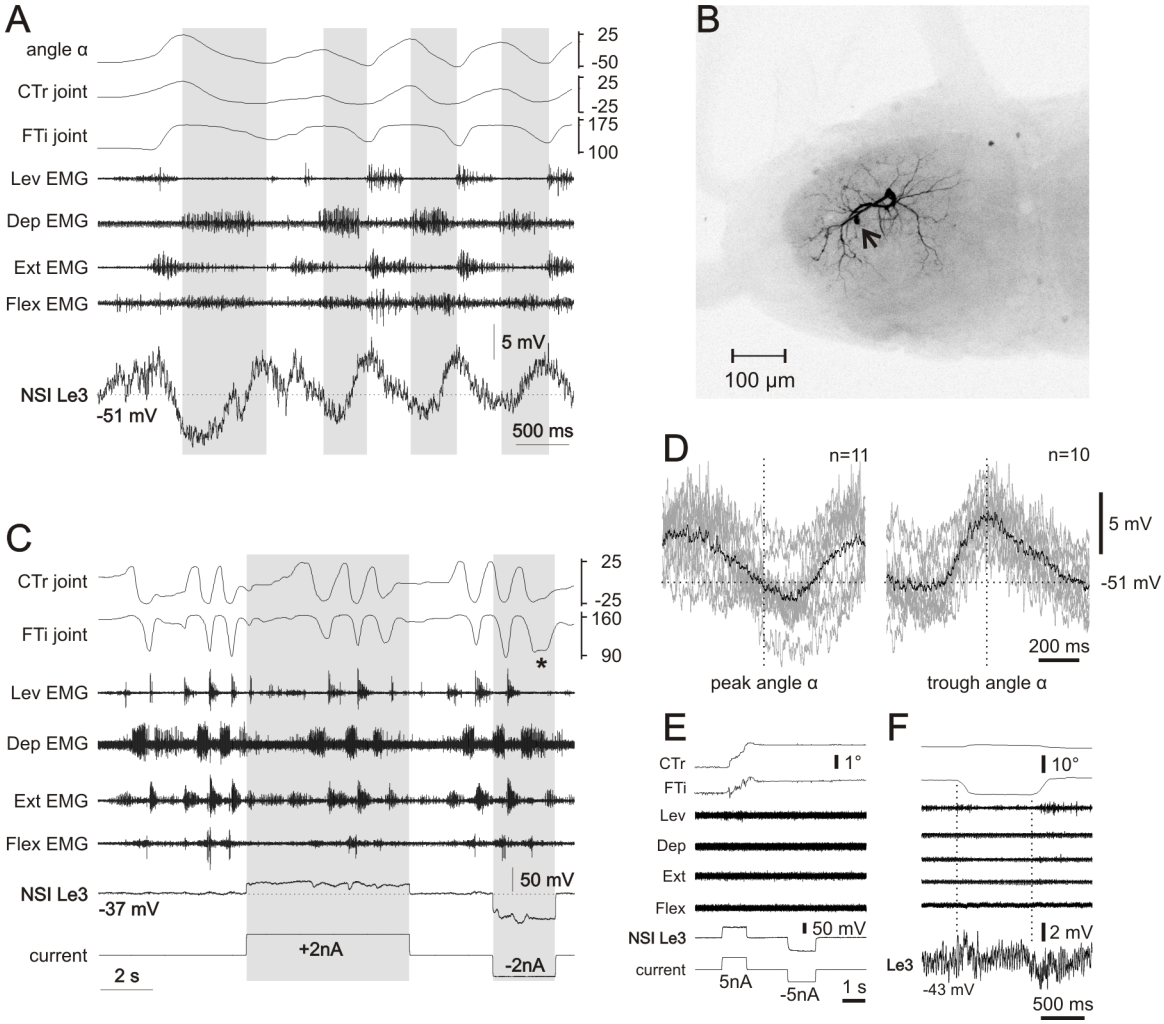

Figure 3.19 – Membrane potential modulation of NSI Le3 during leg searching movements. (A) Membrane potential modulation of NSI Le3 during searching movements. Gray bars denote downward movement of the leg (i.e. decrease in angle α). (B) Morphology of NSI Le3 from dorsal view. (C) Tonic current injection into Le3 changes searching movements. (D) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (E) Depolarizing current injection excites levator muscle activity and leg levation and extension. (F) NSI Le3 response to fCO stimulation. Dotted lines indicate start of passive leg movements.

Nonspiking interneurons supporting levation of the leg

Current pulse injection into nonspiking interneuron Le3 lead to excitation of the *levator trochanteris* muscle and to a clear levation of the femur, accompanied by a smaller extension of the FTi joint (Fig. 3.19 E). Stimulation of the fCO led to phasic depolarization (following flexion) and phasic hyperpolarization (following extension;Fig. 3.19 F). The soma of NSI Le3 was located in the middle of the hemiganglion (halfway anterior-posterior, halfway medial-lateral; see arrow in Fig. 3.19 B). The main neurite projected anteromedially, then formed a hairpin coil and projected posterolaterally. During searching behavior, the membrane potential was hyperpolarized below resting mem-

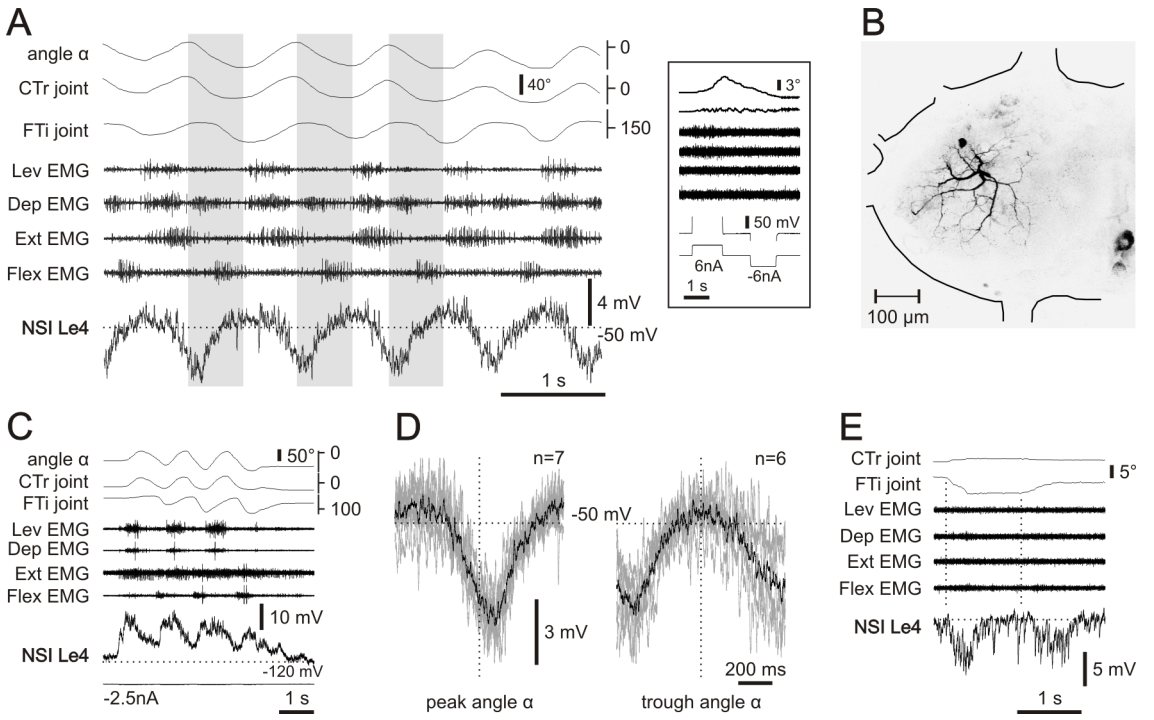

Figure 3.20 – Membrane potential modulation of NSI Le4 during leg searching movements. (A) Membrane potential modulation of NSI Le4 during searching movements. Gray bars denote downward movement of the leg. Inset: Depolarizing current injection causes levator muscle activity and leg levation. (B) Morphology of NSI Le4. (C) Membrane potential modulations at hyperpolarized resting membrane potential. A tonic excitation is visible. (D) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (E) NSI Le4 response to fCO stimulation. Dotted lines indicate start of passive leg movements.

brane potential (RMP = -51 mV) during the first half of downward movements and quickly depolarized above resting membrane potential during the second half. The membrane potential was maximally depolarized at the minimum of leg searching movements (Fig. 3.19 A). During upward movements of the leg, the membrane potential slowly hyperpolarized (Fig. 3.19 A,D). Thus, whether NSI Le3 supported or opposed leg movements during searching behavior was not clear. Tonic injection of depolarizing or hyperpolarizing current during ongoing searching behavior weakly affected FTi joint movement amplitude (Fig. 3.19 C). In the majority of cases, flexion was reduced during depolarization (4 of 5 pulses); during hyperpolarization, flexion was strengthened (2/3 pulses). Also, if animals rested between single cycles of searching movements, the leg remained flexed more often if Le3 was hyperpolarized (e.g. Fig. 3.19 C, see asterisk in FTi joint trace). NSI Le3 could be recorded and labeled one time.

As a second NSI that elicited femur levation when depolarized with current pulses during rest (Fig. 3.20 A, inset), Le4 could be recorded. Stimulation of the fCO resulted in a phasic hyperpolarization following both flexion and extension of the leg

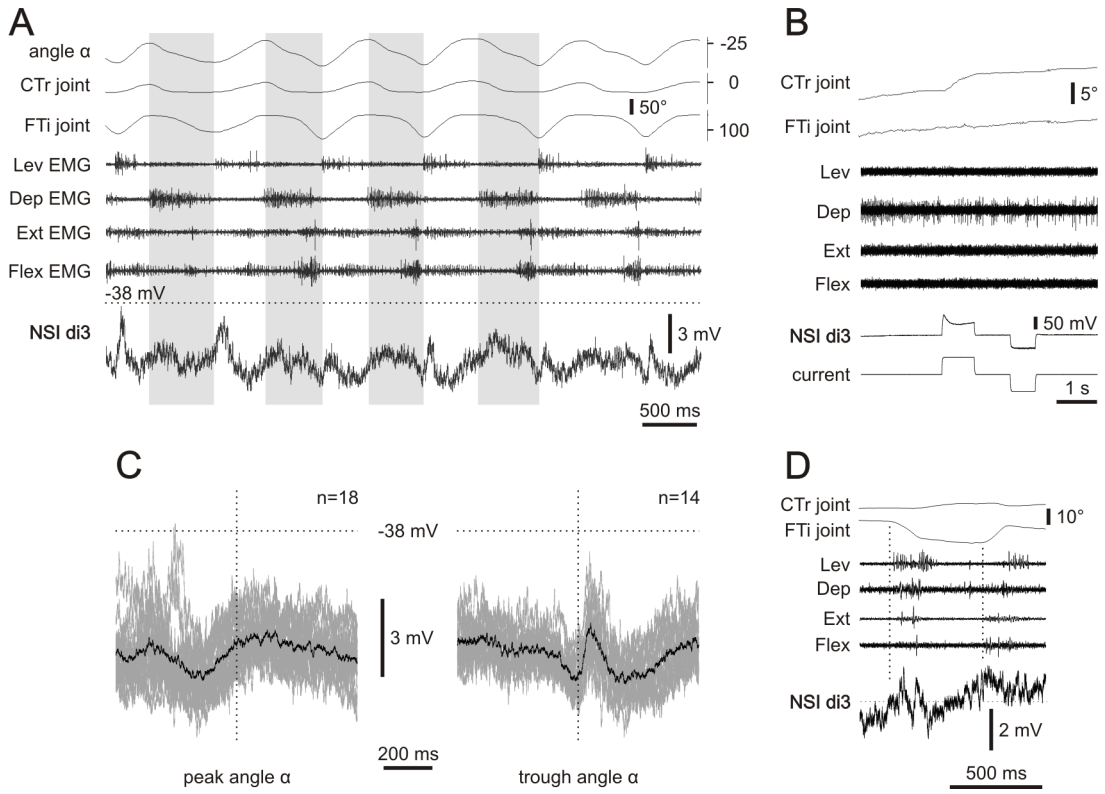

Figure 3.21 – Membrane potential modulation of NSI di3 during leg searching movements. (A) Membrane potential modulation of NSI di3 during searching movements. Gray bars denote downward movement of the leg. (B) Depolarizing current injection causes inhibition of depressor muscle activity and leg levation. (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI di3 response to fCO stimulation. Dotted lines indicate start of passive leg movements.

(Fig. 3.20 E). NSI Le4 had a anteriorly located soma with posteriorly directed broad neurites (Fig. 3.20 B). The resting membrane potential of NSI Le4 was -50 mV. During searching movements, the membrane potential showed a strong phasic hyperpolarization during the first part of downward movements and repolarized during the second part (Fig. 3.20 A, D). The membrane potential remained repolarized or slightly depolarized during the first part of upward movements and then was hyperpolarized shortly prior to the maximum position of the leg. Therefore the activity of NSI Le4 neither clearly supported nor opposed leg movements during ongoing searching behavior. During searching behavior, NSI Le4 received synaptic tonic excitation, as was visible when the resting membrane potential artificially was held hyperpolarized by current injection (Fig. 3.20 C). Phasic excitation and inhibition occurred on top of the tonic excitation (excitation Fig. 3.20 C, inhibition Fig. 3.20 A). NSI Le4 was recorded and labeled once.

NSI di3 was recorded as a third NSI that elicits femur levation when depolarized with current pulses during rest; it caused inhibition of depressor muscle activity (Fig. 3.21 B). NSI di3 could be recorded once. During this recording NSI di3 had a resting membrane

potential of -38 mV. The membrane potential was hyperpolarized to around -42 mV during searching behavior. The tonic hyperpolarization was still visible when the membrane potential level was hyperpolarized to -70 mV by current injection (-1.5 nA; not shown). Phasically, the membrane potential was weakly repolarized during the second half of upward leg movements. The membrane potential remained at the same level or slowly hyperpolarized during the first half of downward movements (i.e. depressor muscle activity). During the second half of downward movements (i.e. mainly flexor muscle activity), the membrane potential was hyperpolarized faster, with a subsequent sharp membrane potential peak after the end of flexor muscle activity (Fig. 3.21 A, C). Following flexion of the FTi joint (fCO elongation) the membrane potential of NSI di3 was phasically depolarized (Fig. 3.21 D). No reliable change in membrane potential could be measured after extension of the FTi joint.

3.2.2 Nonspiking interneurons influencing the femur-tibia joint

Nonspiking interneurons providing synaptic drive to motoneurons of the femur-tibia joint have been described in detail. Büschges (1990) identified many of those NSIs according to their morphology, response to current injection during rest, and response to stimulation of the femoral chordotonal organ (fCO). Further NSIs were described in consecutive work of Sauer (1996) and Akay (2002); also the influence of fCS stimulation on several NSIs was investigated Akay (2002). von Uckermann and Büschges (2009) described NSI membrane potential modulations during walking for a single leg preparation stepping on a treadwheel. Finally, using the same single leg preparation, NSI activity during forward and backward walking was analyzed by a recent work of Rosenbaum (2013).

Nonspiking interneurons supporting flexion of the leg

One previously described interneuron that supports FTi joint flexion, NSI I1, could be recorded. Additionally two new interneurons, NSI I5 and fe1, were identified.

Because NSI I1 had been described previously (Büschges, 1990; von Uckermann and Büschges, 2009), recorded interneurons could be unambiguously identified as I1 due to their morphology (Fig. 3.22 B) and electrophysiological properties. (This is, response to fCO stimulation via treadwheel (appendix), response to current injection during rest (Fig. 3.22 D), membrane potential modulation during walking behavior (Fig. 3.43).) As described previously (Büschges, 1990), when NSI I1 was depolarized by current injection during rest, extensor muscle activity was inhibited and therefore a flexion of the FTi joint elicited (Fig. 3.22 D). As visible in Fig. 3.22 D, also a small levator muscle signal was elicited. This signal probably was due to interjoint reflexes caused by flexion of the FTi joint (Hess and Büschges, 1997), especially as the levator signal was delayed with respect to stimulus and flexion onset. It should be noted, however, that according to the literature (Büschges, 1995), the effect of NSI I1 on levator motoneuron activity has not been analyzed yet.

During searching behavior, the membrane potential of NSI I1 was rhythmically modulated. NSI I1 was strongly hyperpolarized below resting membrane potential (Fig. 3.22 A, C) during the first part of upward leg movements; during the second part NSI I1 started to slowly repolarize, which continued during the first part of downward movements of the leg. Towards the end of downward movements, I1 membrane potential was steeply depolarized just above resting potential. This depolarization often coincided with flexion of the FTi joint during searching movements. Therefore, NSI I1 activity supported leg movements during searching behavior. Besides phasic synaptic

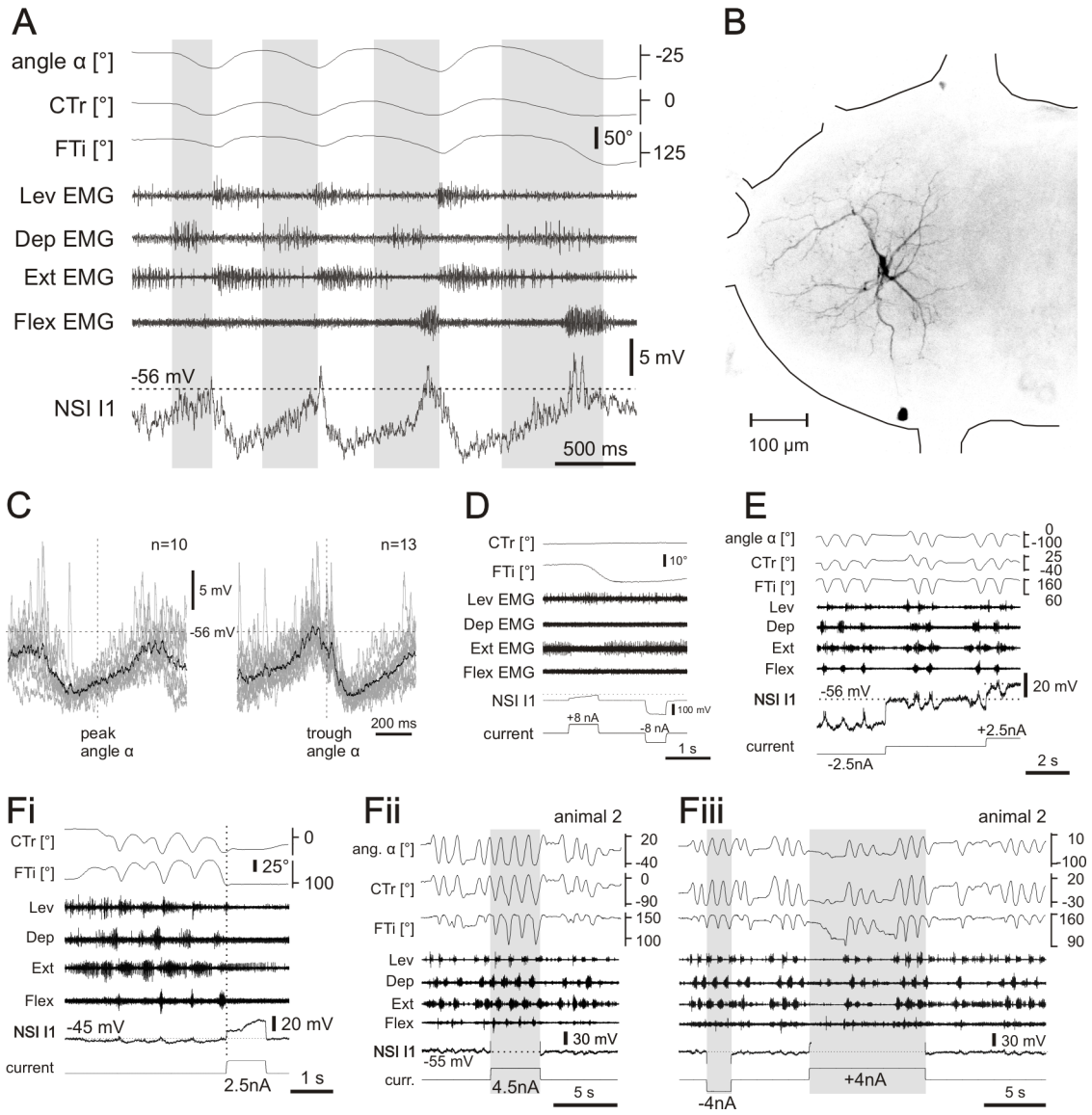

Figure 3.22 – Membrane potential modulation of NSI I1 during leg searching movements. (A) Membrane potential modulation of NSI I1 during searching movements. Gray bars denote downward movement of the leg, i.e. a decrease in angle α. Traces from top to bottom depict: overall leg movement ("angle α"), coxa-trochanter-joint movement ("CTr"), femur-tibia-joint-movement ("FTi"); electric muscle activity of levator ("Lev EMG"), depressor ("Dep EMG"), extensor ("Ext EMG"), flexor ("Flex EMG"); intracellular recording of NSI I1. (B) NSI I1 morphology from dorsal view. (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) Depolarizing current injection causes inhibition of extensor muscle activity and leg flexion. Also levator muscle activity is elicited. (E) Synaptic inputs to NSI I1 become visible at hyper- and depolarized resting membrane potential. (F) Current injection during searching behavior affected leg movements. Leg movements either stopped (Fi) or amplitudes of continuing searching movements changed (Fii, Fiii). Also leg resting position between sequences of searching was influenced (Fiii).

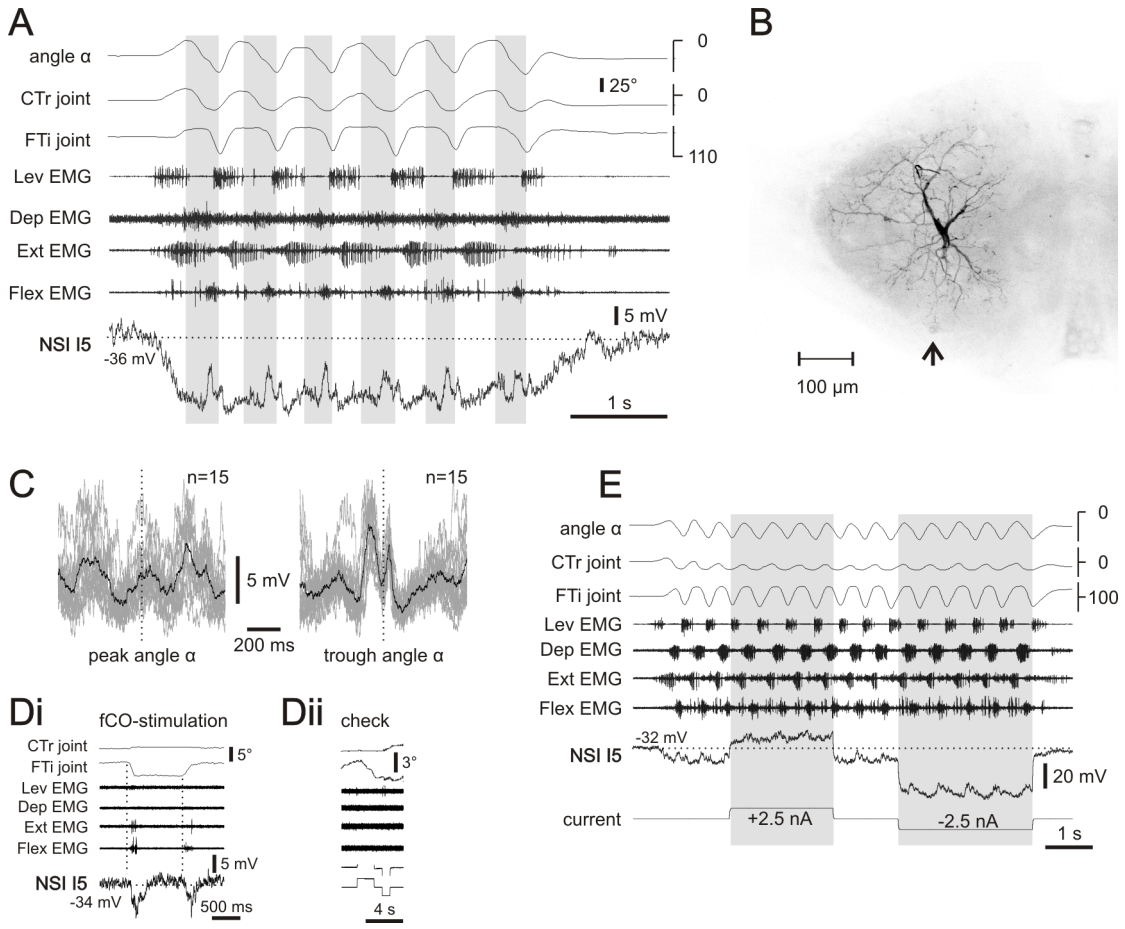

Figure 3.23 – Membrane potential modulation of NSI I5 during leg searching movements. (A) NSI I5 membrane potential modulations during searching movements. Gray bars denote downward movements of the leg. (B) Morphology of NSI I5. (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) Characterization of NSI I5 by fCO-stimulation and current pulse injection. Dotted lines indicate start of passive leg movements. (E) Tonic current injections during searching behavior do not affect leg movements.

inhibition, NSI I1 also received phasic excitation, as is visible by increased amplitudes of phasic depolarization when I1 is hyperpolarized (Fig. 3.22 E).

Injection of depolarizing or hyperpolarizing current into I1 during searching behavior often caused a stop of searching movements (Fig. 3.22 Fi; up to 42% of experiments; N=3/3). If searching movements continued, the amplitude of FTi joint movements and the resting position between bouts of searching movements were altered. More specifically, during tonic injection of depolarizing current the amplitude of FTi joint movements increased due to larger flexion movements (Fig. 3.22 Fii, Fiii; up to 87% injections, N=2/3) and the animal's leg rested in more flexed positions in between searching movements (Fig. 3.22 Fiii; up to 62% of injections, N=2/3). NSI I1 was recorded three times and labeled twice.

Another nonspiking interneuron supporting leg flexion, NSI I5, could be recorded and

stained twice. The soma of NSI I5 was located posteriorly, with a thin primary neurite that ramified into thicker neurites approximately in the middle of the hemiganglion (halfway anterior-posterior, halfway medial-lateral) (Fig. 3.23 B). Its membrane potential was hyperpolarized by passive leg flexion and extension (Fig. 3.23 Di). Thus, morphology and physiology in response to fCO-stimulation were similar to NSI I8 (Akay, 2002). However, the NSI's activity during walking behavior differed from the activity described by von Uckermann (2008).

During searching behavior, the membrane potential of NSI I5 was more negative than the resting membrane potential and was phasically modulated (Fig. 3.23 A). The tonic hyperpolarization might exclusively be contributed to the relatively positive resting membrane potential of -36 mV; however, also in a second recorded NSI I5 with a resting membrane potential of -50 mV such a hyperpolarization was observed (as negative as -65 mV). Phasic membrane potential modulations reached a maximum during leg flexion just prior to trough leg position (Fig. 3.23 A,C); a second local maximum was located directly after trough leg position. During upward movements of the leg, the membrane potential remained more negative. Therefore, the activity of NSI I5 supported leg movements during searching behavior. When NSI I5 was de- or hyperpolarized by tonic current injections during ongoing searching behavior, no alteration of leg searching movements could be observed (Fig. 3.23 D, N=1, 9 pulses).

As a third interneuron eliciting FTi joint flexion, NSI fe1 was newly identified. When depolarized by current pulse injection during rest, the FTi joint was flexed strongly; when hyperpolarized, the FTi joint was extended (Fig. 3.24 B). Muscle activity of *extensor* and *flexor tibiae* were recorded in the same EMG channel, nevertheless it can be assumed that EMG signals during flexion are due to flexor muscle activity. This is also consistent with the later on recorded EMG signals during searching movements. The small effect of current injection on the levator muscle that can be seen in Fig. 3.24 B, probably is caused by interjoint reflexes due to flexion of the FTi joint (Hess and Büschges, 1997); especially, this can be assumed as the latency of levator activity is more than 450 ms with respect to the signal.

During searching movements, the membrane potential of NSI fe1 was depolarized during downward movement of the leg (Fig. 3.24 A,C). The maximum membrane potential coincided with flexor muscle activity just prior to trough leg position. Directly afterwards, at the transition to upward movements of the leg, NSI fe1 was sharply hyperpolarized just below resting membrane potential and repolarized during the following upward movement.

When NSI fe1 was artificially depolarized by tonic current injection during ongoing searching behavior, a clear impact on leg searching movements in CTr- and FTi joint was visible. First, the position of FTi joint movements was shifted to more flexed posi-

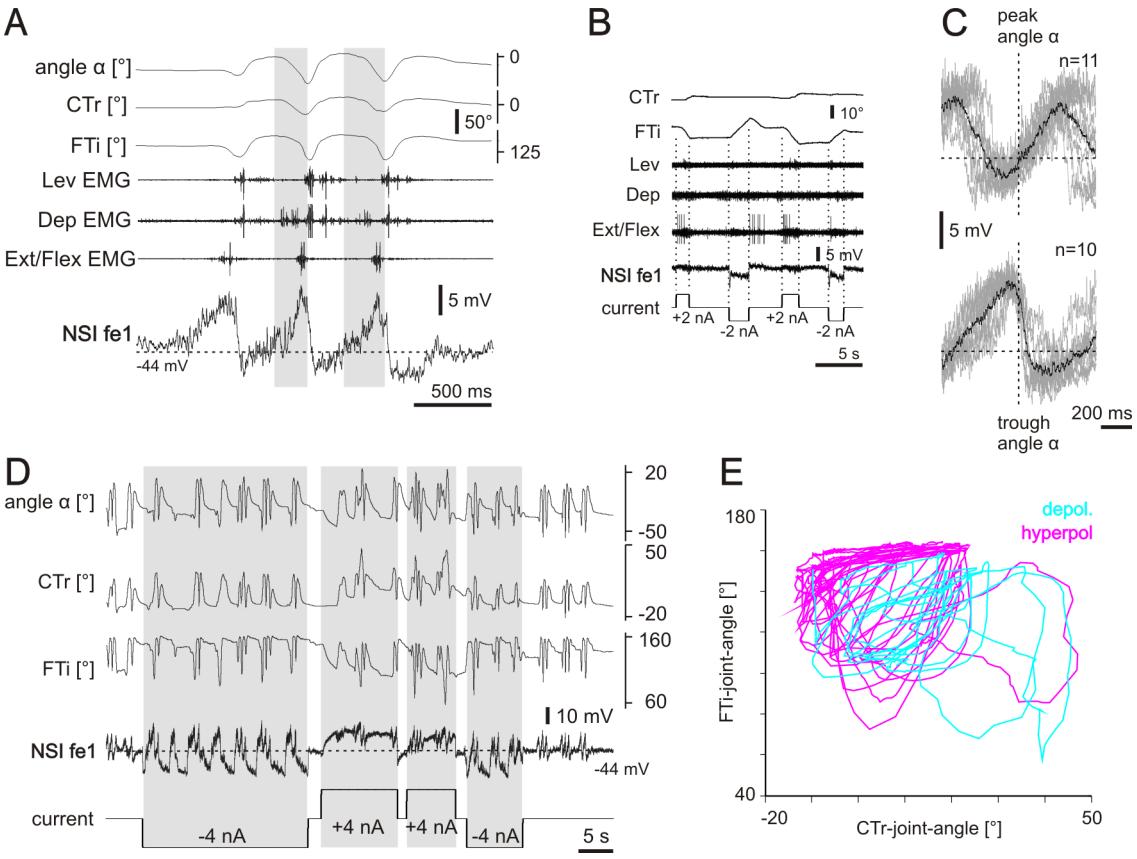

Figure 3.24 – Membrane potential modulation of NSI fe1 during leg searching movements. (A) Membrane potential modulation of NSI fe1 during searching movements. Gray bars denote downward movement of the leg. (B) NSI fe1 excites flexor muscle activity and leg flexion. (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) Tonic current injection affects searching movements. (E) FTi joint angles plotted against CTr joint angles for several searching movement cycles; this indicates joint coordination during tonic depolarization (cyan trajectories) or hyperpolarization (magenta trajectories).

tions (Fig. 3.24, 2/2 pulses). Notably, this did not go along with a change in amplitude of FTi joint movements -which was not affected- but was achieved by a shift of both trough and peak FTi joint position to more flexed positions by approximately 10°. Second, and corresponding to changes in position during movement, also the joint's position when resting in between bouts of searching movements was more flexed (approximately 50°). Furthermore, also the position of CTr joint movements shifted to more elevated positions (approximately 10°, 2/2 pulses); again, by shifting the trough CTr position to more elevated values, not by changing movement amplitudes. The joint's position when resting was more elevated, too (approximately 20°). Corresponding to such changes in both CTr and FTi joint, the coordination of the two joints during searching movements was changed when NSI fe1 was depolarized. This is visible, when FTi joint position is plotted against CTr joint position for several cycles of searching movements while NSI fe1 is depolarized by current injection (Fig. 3.24 E, cyan trajec-

tory). When fe1 was depolarized, a combination of elevated CTr joint angles (indicated by high values on x-axis) and simultaneously flexed FTi joint angles (medium/low values on y-axis) occurred (compare cyan trajectory in right/bottom part of Fig. 3.24 E). This combination did not occur during searching movements with uninfluenced membrane potential. When NSI fe1 was hyperpolarized by current injection, no change in searching movements was apparent (Fig. 3.24 D; E magenta trajectory). NSI fe1 was recorded once.

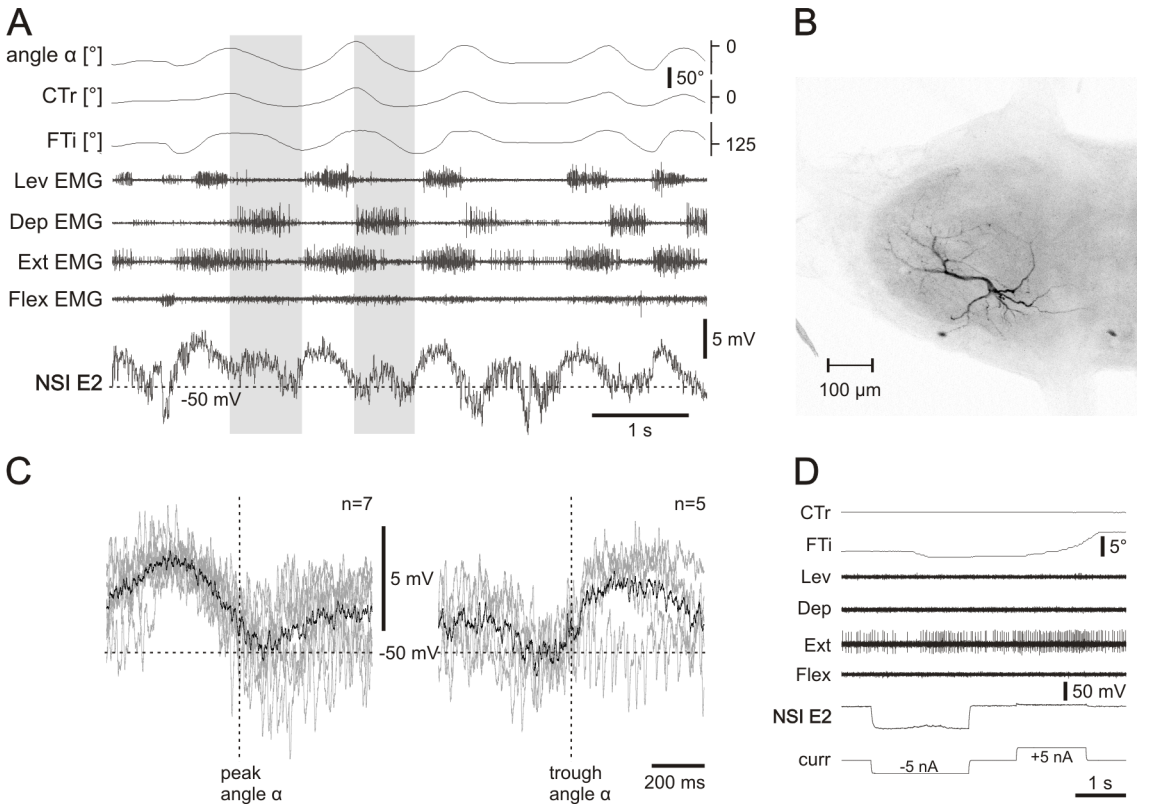

Figure 3.25 – Membrane potential modulation of NSI E2 during leg searching movements. (A) E2 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. (B) NSI E2 morphology (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI E2 excites extensor muscle activity and extension.

Nonspiking interneurons supporting extension of the leg

Several nonspiking interneurons that support extensor tibiae muscle activity could be recorded. They had either been identified previously (NSIs E2, E5; Büschges, 1990; von Uckermann and Büschges, 2009) or were newly characterized (NSIs E10, E11, E12, ext1).

Previously described NSI E2 was identified by its morphology (Fig. 3.25 B), its response to current pulse injection (Fig. 3.25 D) and its activity during walking (see Fig. 3.44). During searching behavior, the membrane potential of NSI E2 was depolarized during upward movements of the leg, repolarized at peak leg position, again mildly depolarized during downward movements, and repolarized at trough leg position (Fig. 3.25 A,C). The peak-to-peak potential of these modulations was 7.2 mV. NSI E2 was recorded once.

A second nonspiking interneuron that could be recorded was identified as the previously described NSI E5 (Büschges 1990). E5 had been described to provide excitatory drive to the slow extensor motoneuron; correspondingly, when depolarized by current injections

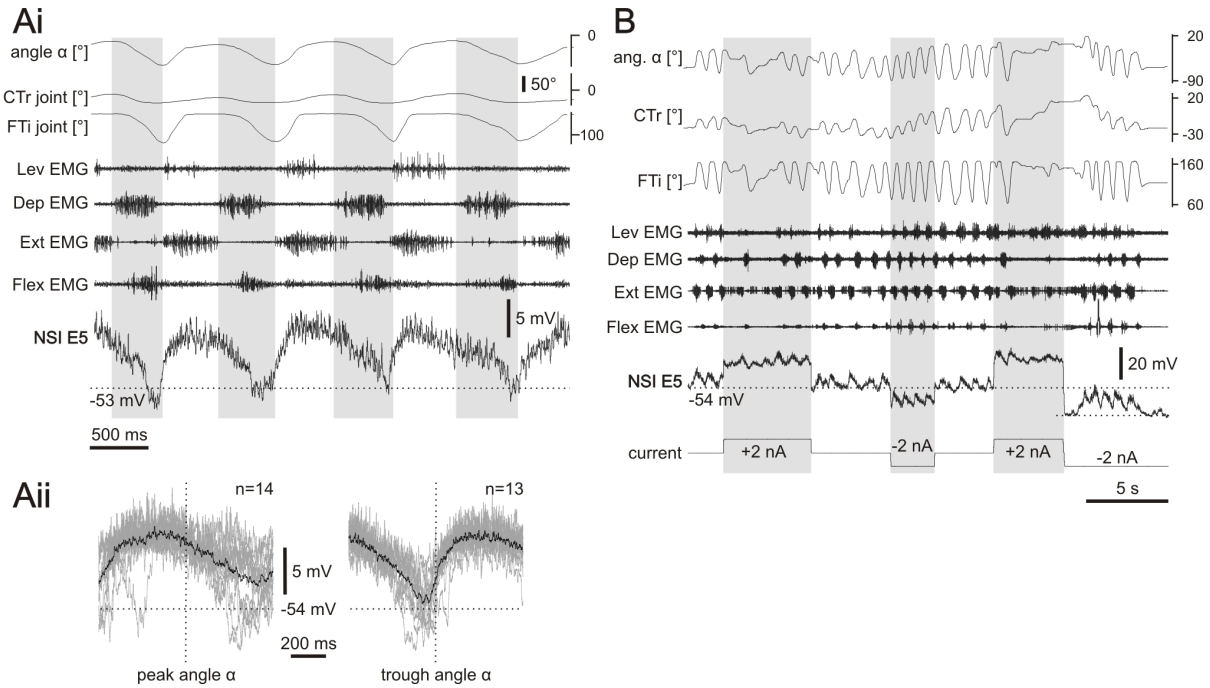

Figure 3.26 – Membrane potential modulation of NSI E5 during leg searching movements. (Ai) E5 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. (Aii) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (B) Tonic depolarizing current injections into E5 impeded searching movements and lead to decreased movement amplitudes.

during rest, the recorded NSI increased the frequency of a spontaneously active unit in the extensor EMG and elicited extension movements (see appendix). In addition, also the recorded response to fCO stimulation (see appendix) and membrane modulations during walking (see Fig. 3.44) were consistent with the known electrophysiology of E5 (Büschges 1990, von Uckermann and Büschges 2009).

During searching behavior, NSI E5 was quickly depolarized during the first part of upward movements and remained depolarized during further upward movements (Fig. 3.26 Ai,Aii). Just prior to peak leg position, NSI E5 started to slowly repolarize; it was strongly repolarized to resting membrane potential (or slightly below) during the second part of downward movements. Therefore the activity of NSI E5 supported leg movements during searching behavior. Membrane modulations were caused by phasic inhibitory and excitatory synaptic inputs as became visible during de- and hyperpolarizing current injections (Fig. 3.26 B). A tonic excitation underlying these phasic modulations was unmasked when E5 was hyperpolarized (Fig. 3.26 B; right side).

Also, depolarization of NSI E5 by current injection during ongoing searching movements impeded leg movements: While leg movements almost stopped, also movement amplitudes decreased, due to decreased flexion of the leg, for those searching cycles

that still occurred (Fig. 3.26 B; 4/5 pulses). Although in some parts of the recording it seemed that injecting hyperpolarizing current accelerated ongoing searching movements, this was not the case in other parts of the recording. Contrarily, impediment of searching movements alongside decreased amplitudes caused by depolarizing current injection was visible in other parts of the recording, too. NSI E5 was recorded once.

Nonspiking interneuron E10 is the first of several newly identified interneurons that elicited leg extension. It was recorded and stained three times. When depolarized by current injection during rest, it caused FTi joint extension (Fig. 3.27 D) by extensor muscle excitation (signal not visible in all EMG recordings; Fig. 3.27 Di,Dii). Upon fCO stimulation via treadwheel, the membrane potential of NSI E10 was phasically depolarized (following flexion) or hyperpolarized (following extension; Fig. 3.27 E). The soma was located anteriorly, with a primary neurite that projected posteriorly in a small inverted c-shape. Multiple neurites originated from the primary neurite and projected mainly medial and further posterior (Fig. 3.27 B).

During searching movements, E10 membrane potential was depolarized during downward movements of the leg, reaching its maximum potential at trough leg position. During upward movements of the leg, the membrane potential was repolarized and reached its minimum at peak leg position (Fig. 3.27 A,C). Therefore, NSI E10 activity neither clearly supported nor opposed leg movements during searching behavior. The average peak-to-peak value of membrane modulations was 14,2 mV ($\pm$5.4) and thus exceptionally large for modulations during searching movements. The membrane modulations were caused by phasic excitatory and inhibitory synaptic inputs as was revealed by de- and hyperpolarizing current injections (Fig. 3.27 F). During hyperpolarizing current injections, also a tonic excitation underlying phasic inputs became visible (Fig. 3.27 F).

Tonic current injection into NSI E10 during ongoing searching movements clearly influenced the motor output. Movement amplitudes of the FTi joint strongly decreased during injection of depolarizing current in a majority of pulses (Fig. 3.27 G; N = 3/3, in on average 80% of pulses). This was caused by a decrease in FTi flexion. In some cases, FTi joint movements were completely stopped and the FTi joint remained in an extended position (Fig. 3.27 Gii). On the other hand, hyperpolarizing current injection caused increased FTi joint movement amplitudes due to increased flexion (Fig. 3.27 G; N = 3/3; 72% of pulses in two animals, 25% in a third animal). Despite the clear result, how strictly movement amplitudes followed current injections differed between animals (compare Fig. 3.27 Gi and Fig. 3.27 Gii, the later displaying stricter influences).

As a second newly identified interneuron, NSI E11 could be recorded and stained four times. When depolarized by current injection during rest, the FTi joint was extended (Fig. 3.28 D). A corresponding signal in the extensor EMG was visible in

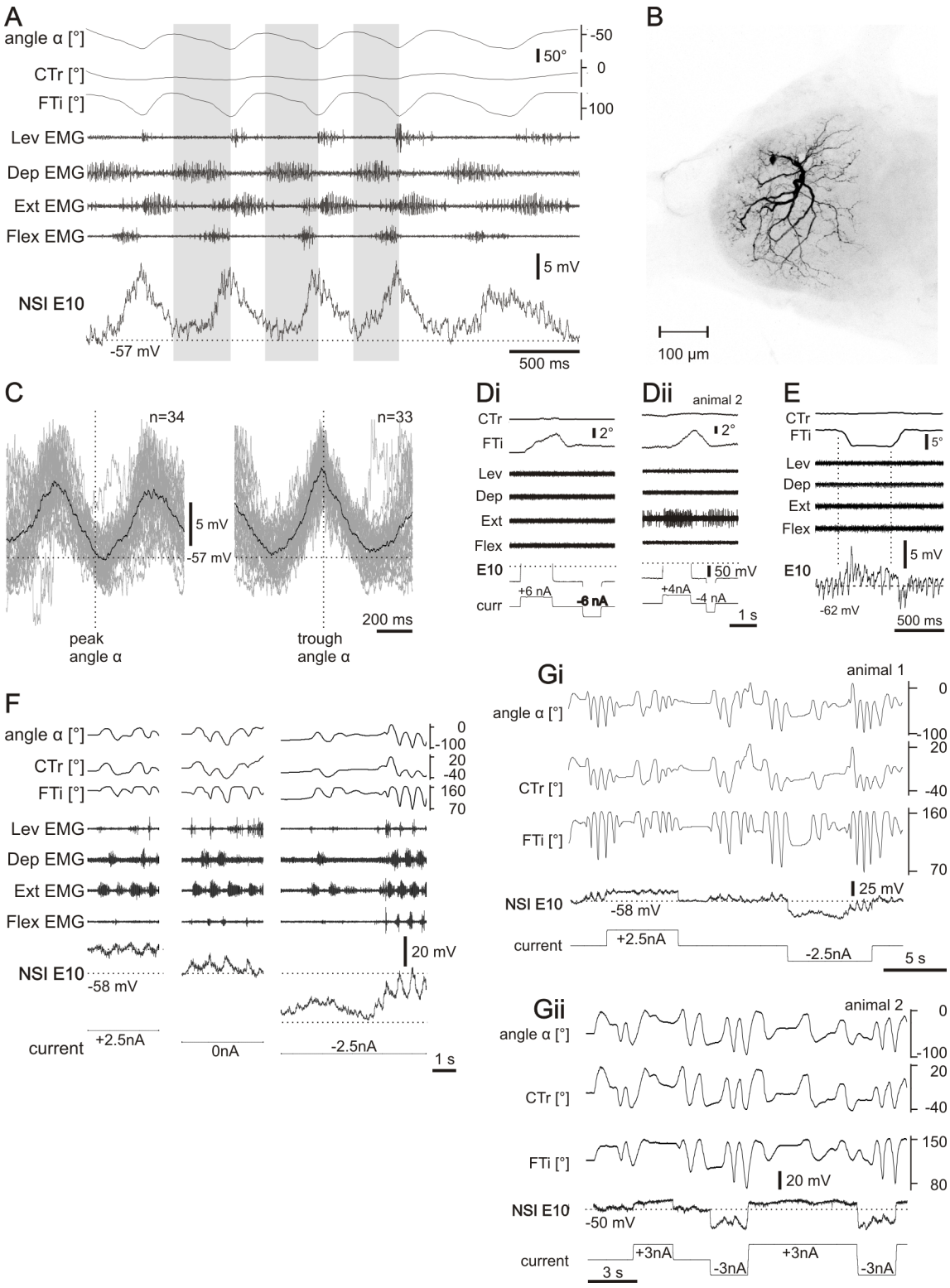

Figure 3.27 – Membrane potential modulation of NSI E10 during leg searching movements. (A) E10 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. (B) NSI E10 morphology (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI E10 excites extensor muscle activity and extension movements. (E) Response to fCO stimulation. (F) Membrane potential modulations during tonic current injections reveal phasic inhibitory inputs (left side, depolarizing current injection) and tonic, as well as phasic depolarizations (right side, hyperpolarizing current injection). (G) Influence of tonic current injections on searching movements; shown for the characterized animal (Gi,"animal 1") and another animal (Gii,"animal2").

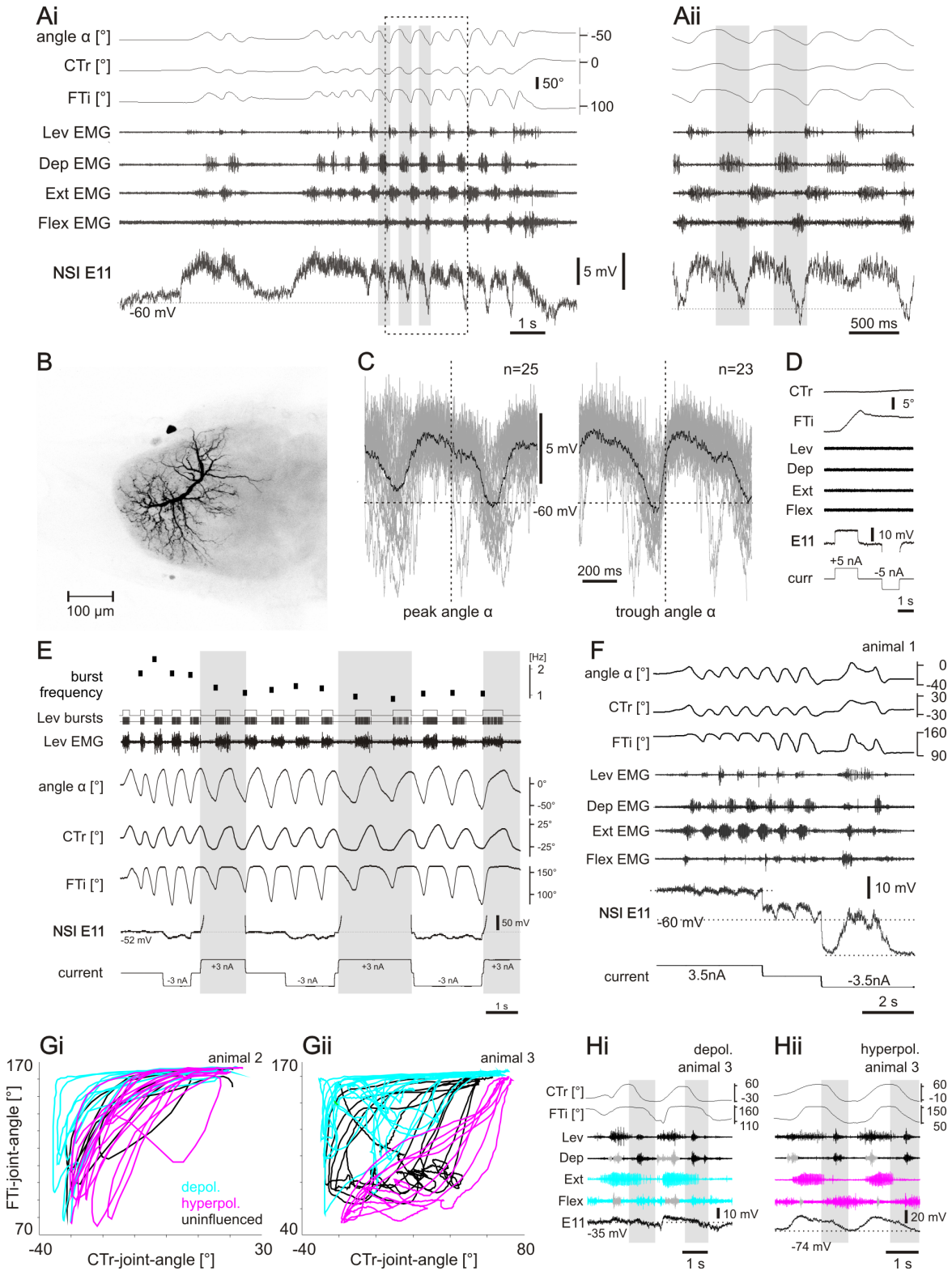

Figure 3.28 – Membrane potential modulation of NSI E11 during leg searching movements. (A) E11 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. Dotted box in Ai indicates details shown enlarged in Aii. (B) NSI E11 morphology (C) Overdraws of membrane potential modulations triggered by angle α peak (left) and trough (right). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI E11 causes extension movements when depolarized. (E) Tonic current injections into E11 affect searching movement velocity, frequency and FTi joint movements. (F) Membrane potential modulations during tonic current injections reveal phasic inhibitory inputs (left side; depolarizing current injection) and a tonic depolarization (right side; hyperpolarizing current injection). (G) FTi joint angles plotted against CTr joint angles for several searching movement cycles, thereby indicating joint coordination. Coordination shown during no current injection (black trajectories), depolarizing (cyan) and hyperpolarizing (magenta) current injections for two different animals. (H) Timing and strength of extensor and flexor muscle activation differ when E11 is depolarized (Hi) or hyperpolarized (Hii).

three of four animals; in one of them the frequency of a spontaneously active unit, thus SETi activity, was increased with depolarization and reduced with hyperpolarization (see appendix; "animal 3"). In response to fCO stimulation, E11 was hyperpolarized (by leg flexion) or depolarized (by extension) in three of four animals (see appendix). One animal showed a depolarization to both stimuli but showed no difference in all other aspects including morphology and therefore was grouped as NSI E11. Its staining is included in the appendix ("animal 2"). The soma of NSI E11 is located anteriorly (Fig. 3.28 B). The large main neurite projects posteriorly and bends laterally. Numerous much smaller neurites originate from the main neurite over its whole length and project in all directions.

During searching movements, E11 membrane potential was rhythmically modulated. In two animals a tonic excitation underlying these modulations was visible but sometimes masked by strong hyperpolarizations that occurred simultaneous to flexor EMG signals (Fig. 3.28 Ai). The membrane potential was phasically depolarized during upward movements of the leg, remained depolarized throughout the first part of downward movements and was sharply hyperpolarized during the second part of downward movements, i.e. simultaneous to flexion (Fig. 3.28 Aii,C). In three of four animals an indentation of the membrane potential at peak leg position as seen in Fig. 3.28 C (left panel) could be found. Membrane potential depolarization in part was caused by phasic excitation. This was shown by increased phasic depolarizations when the membrane potential was hyperpolarized by current injection (not shown).

Artificially de- or hyperpolarizing NSI E11 by tonic current injection during ongoing searching movements changed the motor output in manifold ways. When E11 was depolarized, the amplitude of FTi joint movements decreased (Fig. 3.28 E, gray bars; $N = 4/4$; 82-100% of pulses); when E11 was hyperpolarized, FTi joint movement amplitude increased, albeit less reliably (Fig. 3.28 E; $N = 3/4$; 40-71% of pulses). As a second effect when E11 was depolarized, movement velocity decreased. This effect was observed in two animals that displayed very regular and long sequences of searching movements. The decreased velocity can be seen in a reduced slope of movement trajectories, not only of the FTi joint, but also of the CTr joint and overall movement (angle α) (Fig. 3.28 E). Corresponding to this effect, the inter-burst frequency -shown for levator EMG bursts- decreased during continuous depolarizing current injection (Fig. 3.28 E; three top traces). In other words, the cycle duration of searching movements increased despite a reduction in FTi joint amplitudes emphasizing the effect of NSI E11 depolarization on movement velocity. As a third effect, in three of the animals the coordination of CTr and FTi joint movements changed with current injections into NSI E11 (Fig. 3.28 Gi,Gii). When E11 was depolarized (cyan trajectories), the FTi joint remained maximally extended during leg downward movements for a longer time

than with uninfluenced NSI E11 (black trajectories). This was caused in two of the three animals by an increased and prolonged extensor muscle activity as shown in Fig. 3.28 Gii. The opposite effect was caused by injection of hyperpolarizing current into E11: The FTi joint was flexed earlier during downward movements and was extended later during upward movements (magenta trajectories). This was caused by a shortened extensor muscle activity and a stronger and earlier flexor muscle activity (Fig. 3.28 Giii). In the fourth animal, such an effect of current injection on extensor and flexor muscle activity was detected also during changes in amplitude and velocity.

NSI E12 is another newly identified interneuron which could be recorded once. When depolarized by current injection during rest, it excited the *extensor tibiae* muscle and caused an extension of the FTi joint (Fig. 3.29 D). Stimulation of the fCO induced a phasic depolarization (following flexion) or hyperpolarization (following extension) and a tonic position dependent shift in E12 membrane potential (Fig. 3.29 E).

As opposed to the NSIs exciting the extensor muscle that were described in this work so far, NSI E12 activity during searching behavior was opposed to leg movements. During searching movements, the membrane potential was phasically modulated and more negative than the resting membrane potential of -57 mV. During upward movements of the leg, the membrane potential was hyperpolarized. Just prior to peak leg position the membrane potential started to slowly repolarize until it reached its maximum value at the trough leg position (Fig. 3.29 A,C). The repolarization was caused by excitation as was visible when NSI E12 was hyperpolarized by current injection. In this case the repolarizations (depolarizations) during downward movements of the leg increased in magnitude (Fig. 3.29 Fi). When NSI E12, by current injection, was held at a membrane potential of approximately -30 mV, the depolarization inversed. Now E12 was hyperpolarized during leg downward movements. Additionally, membrane resistance was drastically reduced during the depolarizing phase (Fig. 3.29 Fii). During other phases of searching movement cycles, the membrane resistance was reduced only weakly as compared to the resting state (Fig. 3.29 Fii; right panel). Current injections into E12 did not systematically affect leg searching movements (Fig. 3.29 G; 12 pulses).

Finally, another recorded interneuron induced an extension of the FTi joint when depolarized by current injection but no signal in the extensor/flexor EMG was visible (Fig. 3.30 B). Therefore, this interneuron was named NSI ext1 according to the elicited leg movement but unknown underlying change in muscle activity. During searching movements, its membrane potential was quickly depolarized during upward movements of the leg, i.e. simultaneous to extensor muscle activity. It remained at a depolarized membrane potential, and was repolarized to or hyperpolarized below resting membrane potential during late downward movement, i.e. simultaneous to flexor muscle activity (Fig. 3.30 A,C).

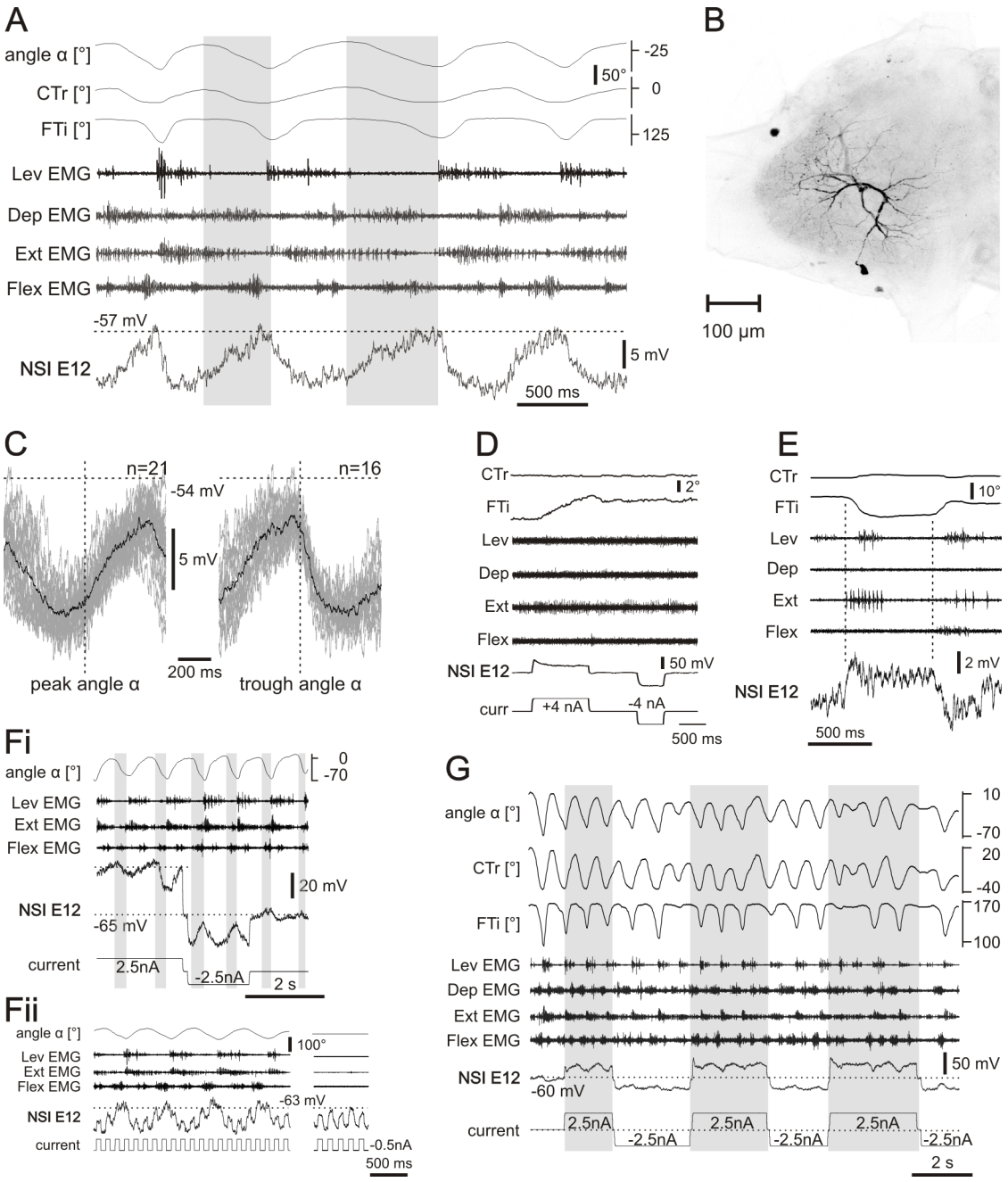

Figure 3.29 – Membrane potential modulation of NSI E12 during leg searching movements. (A) E12 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. (B) NSI E12 morphology (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI E12 excites extensor muscle activity and extension movements. (E) Response to fCO stimulation. (Fi) Tonic depolarizing current injection inverses phasic depolarization to phasic hyperplarization. (Fii) Membrane resistance is slightly decreased during searching movements as compared to the resting state and maximally decreased during phasic depolarizing inputs. (G) Tonic current injections into E12 did not affect searching movements.

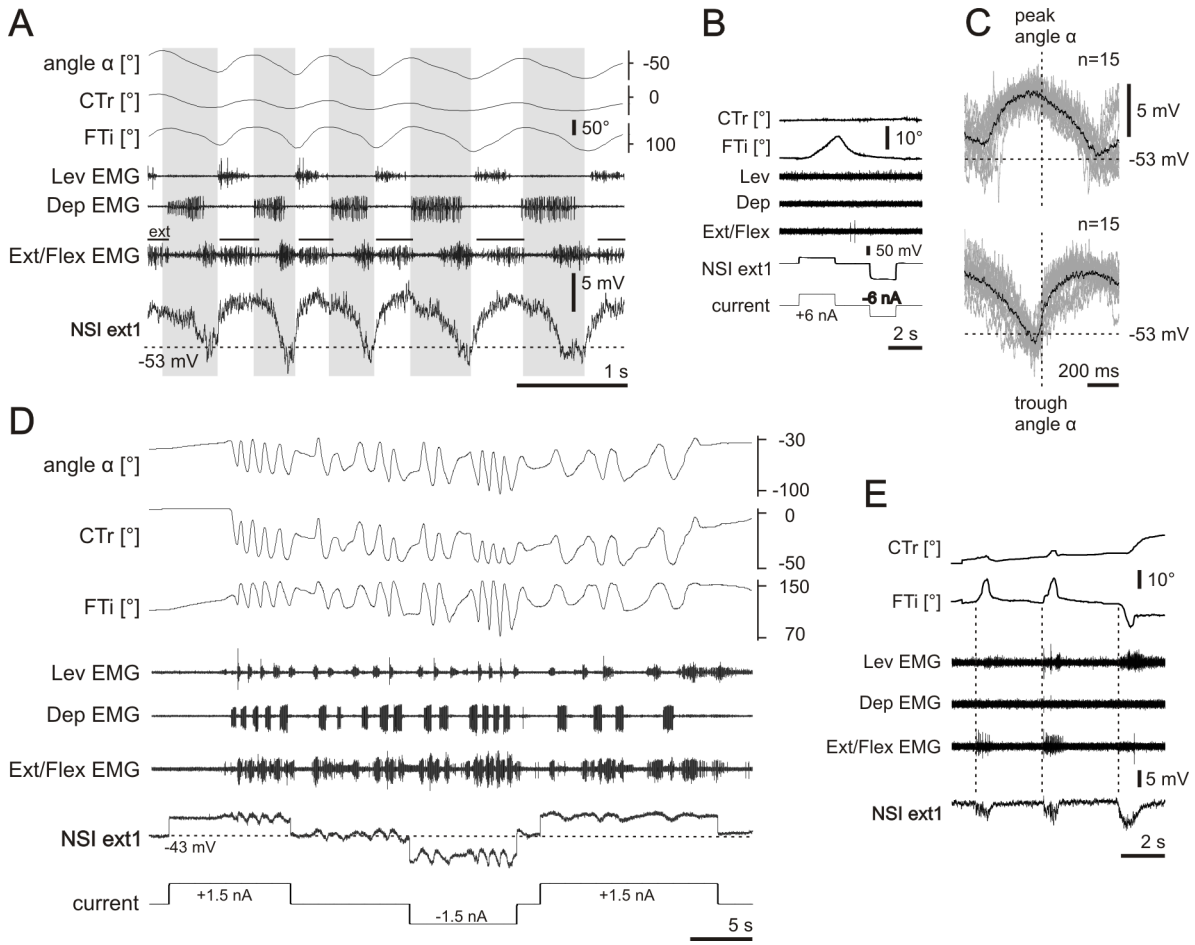

Figure 3.30 – Membrane potential modulation of NSI ext1 during leg searching movements. (A) NSI ext1 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. (B) NSI ext1 elicits leg extension (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) Tonic current injections into ext1 affect searching movements. (E) Response to fCO stimulation. Dotted lines indicate start of passive leg movements.

Leg searching movements could be changed by current injection into NSI ext1. When artificially depolarized, NSI ext1 caused the animal to search with decreased FTi joint movement amplitude (Fig. 3.30 D; two of two pulses); when hyperpolarized, the FTi movement amplitude was increased (two of two pulses). Fig. 3.30 D might also give the impression that the position of the CTr joint changes according to current injection. However, this was not true during another hyperpolarizing current injection that was applied. In this experiment fCO stimulation was not performed via a treadwheel but by bending and extending the leg manually with a paintbrush. Judged from this rather crude stimuli, NSI ext1 was hyperpolarized both to extension and flexion of the leg.

3.2.3 Nonspiking interneurons influencing multiple leg joints

So far, the NSIs described throughout this thesis influenced only the MN pools of one joint (CTr or FTi), when tested by current injections during rest. As opposed to this, the NSIs that will be described in this section affected both CTr and FTi joint. Some of them, NSIs I4, E4, Le.Ee1, have already been identified and described previously (Büschges, 1990, 1995; Büschges et al., 1994; Sauer, 1996; von Uckermann and Büschges, 2009; Rosenbaum, 2013), others were newly identified, i.e. NSIs Le.Ee2, Li.Ee1, Di.Ei1, Le.Di.EF1, Le.flex1, Le.flex2. Whether the effect on both joints is mediated centrally or sensory via interjoint reflexes was known or could be determined for some but not all of the described NSIs.

A first interneuron that affects multiple leg joints is NSI I4. It is known to provide excitatory synaptic drive to retractor, depressor, flexor MNs, and CI, as well as inhibitory synaptic drive to extensor MNs (Sauer et al., 1996; Büschges, 1995). Notably, NSI I4 has also been shown to be a part of the rhythm generating network for leg movements (Büschges 1995). NSI I4 could be identified here on the basis of its morphology (Fig. 3.31 B) and electrophysiological properties during walking (Fig. 3.46), following fCO stimulation, and current pulse injection (Fig. 4.4). I4 has a characteristic morphology, with its soma lying posteriorly and contralaterally to the investigated hemiganglion and leg. When the main neurite crosses the midline, several secondary neurites originate and project along the midline anteriorly and posteriorly. Ipsilateral to the innervated leg, the main neurite projects anteriorly and finally bends medially, with many arborizations. Therefore, I4 could be recognized even on weak stainings like the one shown in Fig. 3.31 B which belongs to the neuron exemplarily characterized here. A better NSI I4 staining is shown in Fig. 3.31 E.

During searching movements, NSI I4 membrane potential was tonically depolarized (4 to 10 mV) from resting potential, with weak phasic modulations (4.7 mV) on top. The phasic modulations were at least in part caused by phasic excitatory synaptic inputs: this became visible by decreased phasic modulations when NSI I4 membrane potential was held depolarized by current injection (Fig. 3.31 D). The membrane potential was phasically depolarized during upward movements of the leg and hyperpolarized during downward movements (Fig. 3.31 A,C).

Most interestingly, depolarization of NSI I4 by current injection strongly facilitated the generation of regular long sequences of searching movements. In two animals a depolarizing current injection into I4 –without any additional stimulus– led to the initiation of searching movements. In three other animals, when no current was injected in the NSI searching movements could not be elicited by tickling; however during depolarization of I4, regular searching movements were initiated easily with the first ticklings and stopped when I4 was no longer depolarized. Such an example is shown in Fig. 3.31 Fi,

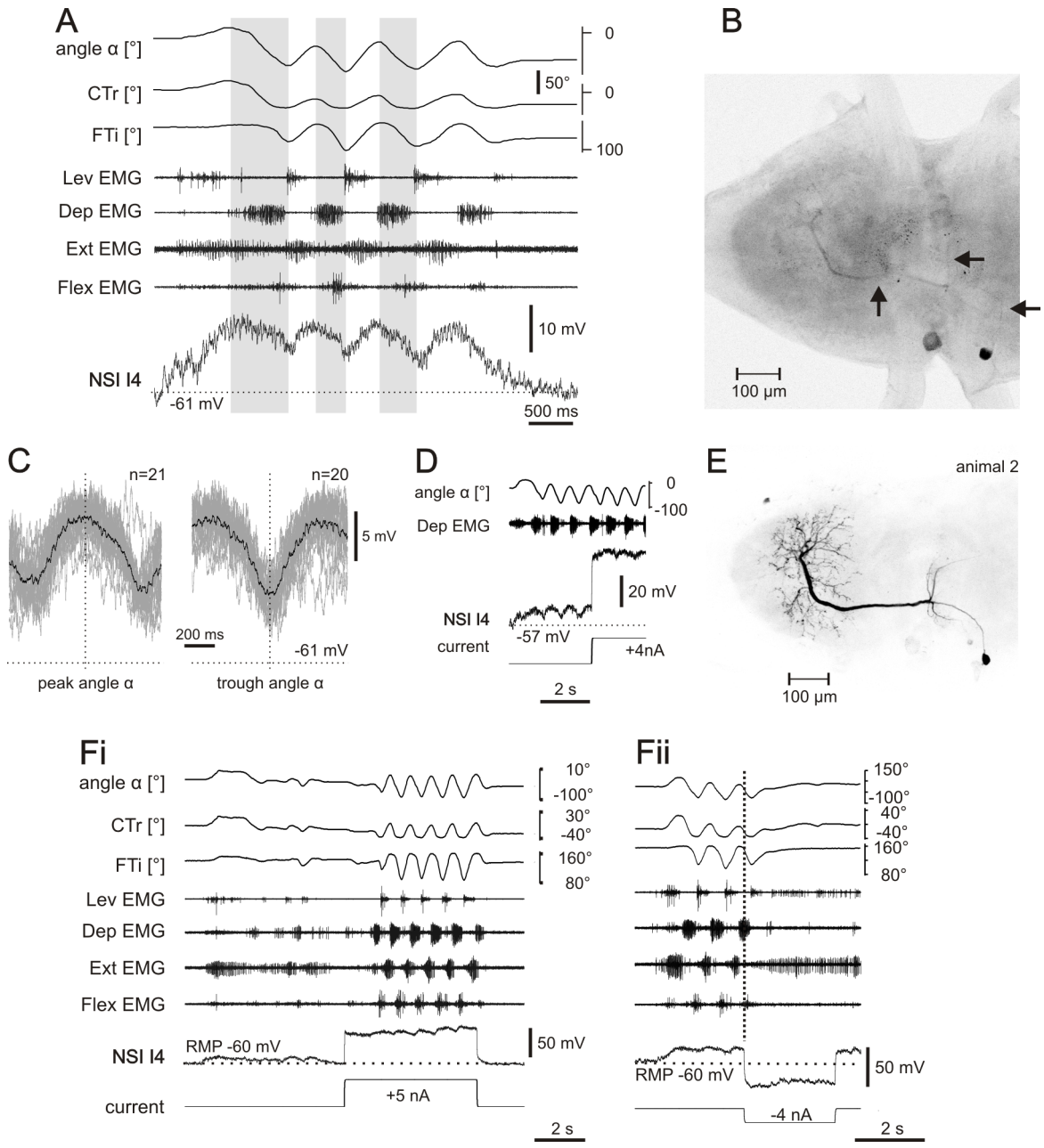

Figure 3.31 – Membrane potential modulation of NSI I4 during leg searching movements. (A) I4 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. Traces from top to bottom depict: overall leg movement ("angle α"), coxa-trochanter joint movement ("CTr"), femur-tibia joint movement ("FTi"); electric muscle activity of levator ("Lev EMG"), depressor ("Dep EMG"), extensor ("Ext EMG"), flexor ("Flex EMG"); intracellular recording of NSI I4. (B) NSI I4 morphology from dorsal view. Arrows denote the course of the primary neurite and arborizations at the midline. (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) At a depolarized resting membrane potential, phasic membrane modulations become smaller. (E) NSI I4 morphology of a different animal. (Fi) Tonic depolarizing current injection drives the generation of regular searching movements. (Fii) Hyperpolarizing current injection stops spontaneous searching movements.

where throughout the entire recording the animal was tickled. Another animal did not start searching movements immediately upon depolarization, but showed an increased rate of occurrence of searching cycles. On the other hand, when NSI I4 was hyperpolarized, ongoing spontaneous searching movements stopped, as is seen in Fig. 3.31 Fii. This was the case in five of six tested animals. I could not find evidence that the frequency of searching cycles or the velocity of movements depended on depolarization strength (N = 3). Neither could the searching rhythm be reset by depolarizing or hyperpolarizing current pulses that were applied during ongoing searching movements (N = 3). In two animals that performed searching movements also under "uninfluenced conditions", depolarization of I4 led to slightly increased movement amplitudes: The FTi joint was flexed and the CTr joint slightly more depressed. In one of these animals, when NSI I4 was hyperpolarized, searching movements didn't stop completely but continued with very little amplitude and high irregularity. Overall, NSI I4 had a very strong general effect on searching movements by being able to drive or stop searching behavior. NSI I4 was recorded nine and stained six times.

NSI E4 is a second interneuron influencing multiple leg joints that has been described previously (Büschges, 1990; Büschges et al., 1994; von Uckermann and Büschges, 2009). Accordingly, it could be identified here (N = 7) in comparison to known morphology and electrophysiological properties (see Fig. 3.32 B and Fig. 4.5). NSI E4 provides excitatory synaptic drive to protractor, levator and extensor MNs, as well as inhibitory synaptic drive to retractor and depressor MNs (Büschges, 1995). Morphologically, NSI E4 is similar to NSI I4, except that arborizations of secondary neurites at the midline are missing in NSI E4 (N = 6).

During searching movements, NSI E4 is depolarized during the second part of leg downward movements, remains at a depolarized level throughout upward movements, and is repolarized during the first part of downward movements (Fig. 3.32 A,C). These modulations are caused by true synaptic excitation and inhibition as becomes visible when NSI E4 is held at a hyperpolarized or depolarized membrane potential by current injection (Fig. 3.32 Dii). Also a tonic excitation underlying the phasic modulations becomes visible when interneuron E4 is artificially hyperpolarized (Fig. 3.32 Di).

In most experiments (N = 5/7), tonic de- or hyperpolarization of NSI E4 during ongoing searching behavior had no effect on leg movements (Fig. 3.32 E). Only in two experiments, an effect on FTi joint amplitude could be detected but its strength varied widely throughout the recording (compare Fig. 3.32 E and F). As opposed to NSI I4, NSI E4 did not generally drive searching movements. Interestingly, in one animal the searching rhythm could be reset by depolarizing current pulse injection (changing from hyperpolarizing to depolarizing current pulse injection, respectively; see Fig. 3.32 Gii) during ongoing searching movements. Whether the cycle duration of the disturbed

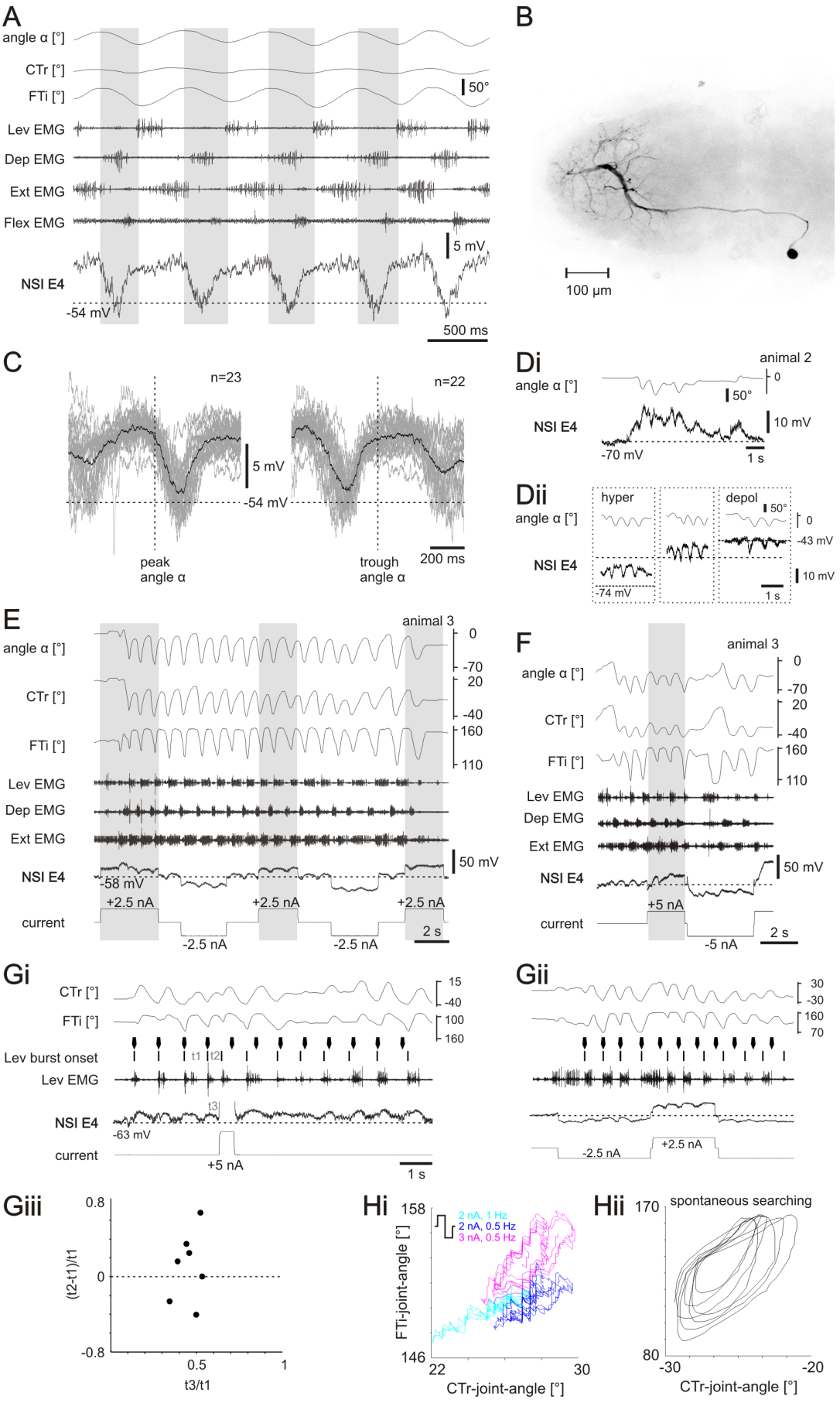

Figure 3.32 – Membrane potential modulation of NSI E4 during searching behavior. For figure legend see next page.

Fig. 3.32: Membrane potential modulation of NSI E4 during leg searching movements. (A) E4 membrane potential modulations during searching movements. Gray bars denote downward movement of the leg. (B) NSI E4 morphology (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n=number of sweeps. (Di) Sequences of searching movements during injection of hyperpolarizing current (-2nA) reveal a tonic depolarization underlying phasic modulations. (Dii) Membrane modulations are caused by phasic excitation and inhibition as revealed by hyper- and depolarizing current injections. (E) Continuous current injections during searching behavior do not influence leg movements. (F) In some cases, depolarizing current injection reduces searching movement amplitudes. (G) Depolarizing current pulses (or steps) reset the searching rhythm by shortening (Gi) or elongation (Gii) of searching cycles. (Giii) The effect is not phase dependent. t1=last undisturbed cycle; t2=disturbed cycle; t3=phase of pulse; (H)FTi joint position plotted against CTr joint position for leg movements that are induced by trains of alternating de- and hyperpolarizing current puls injections (Hi) and spontaneous searching movements (Hii).

searching cycle was shortened (Fig. 3.32 Gi) or prolonged (Fig. 3.32 Gii) was not phase dependent, however (Fig. 3.32 Giii). In one animal, trains of alternating de- and hyperpolarizing current pulses of different strength and frequency were injected into NSI E4. This elicited small leg movements (approximately 8°) in both CTr and FTi joint. Remarkably, movements in the two joints were coordinated similar to the coordination during spontaneous searching movements (see Fig. 3.32 Hi and Hii). Even the characteristic loop in coordination trajectories, i.e. differing joint coordination during upward and downward movements, could be seen (e.g. magenta trajectory in Fig. 3.32 Hi).

Another interneuron, NSI Ee.Le1, has previously been identified by Rosenbaum (2013). The morphology of an interneuron recorded here (Fig. 3.33 B) was the same as shown for NSI Ee.Le1 in Rosenbaum (2013). In the same way as described there, the recorded interneuron elicited levator and extensor muscle activity and correspondingly levation of the CTr joint (Fig. 3.33 Di). The FTi joint, however, was flexed instead of extended as would be expected from EMG activity. Nevertheless, the nonspiking interneuron recorded here, is assumed to be NSI Ee.Le1. For reasons of nomenclature consistency it is henceforth named Le.Ee1. Whether the simultaneous activation of levator and extensor MNs is mediated centrally or via sensory feedback had not been determined in Rosenbaum (2013). Here, in experiments with fixated FTi joint to prevent FTi joint movement, depolarizing current injection elicited activity in both levator and extensor muscle (Fig. 3.33 Dii). This points to a central connection exciting levator MNs, because the known sensory interjoint influences that might be responsible for the simultaneous activity rely on FTi joint flexion (Hess and Büschges, 1997). In response to fCO stimulation, NSI Le.Ee1 displayed a phasic depolarization (following flexion) or hyperpolarization (extension; Fig. 3.33 F).

During searching movements, Le.Ee1 was tonically depolarized (approx. 6 mV) and displayed small phasic modulations on top (6.3 mV) (Fig. 3.33 A,C). Le.Ee1 was de-

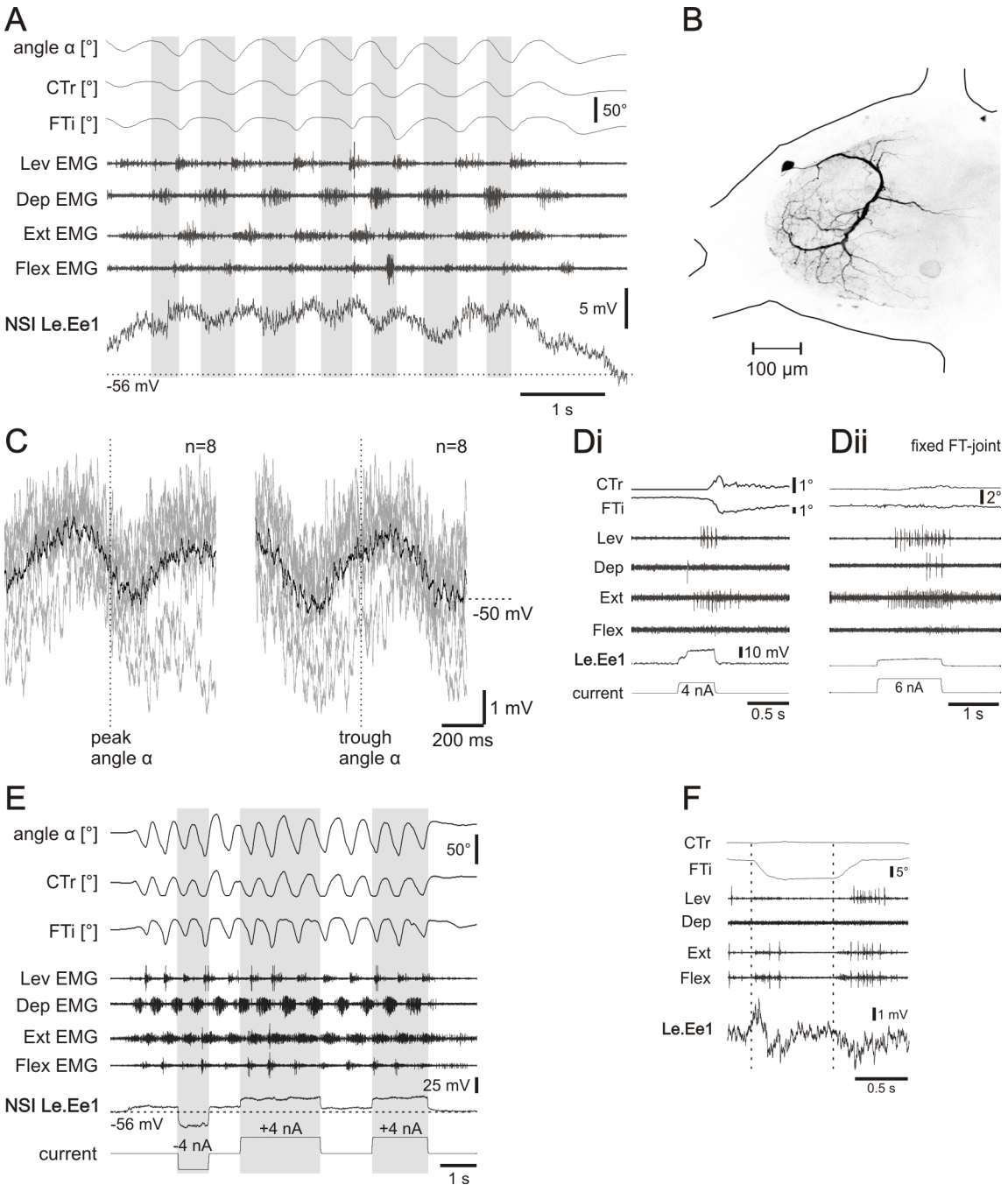

Figure 3.33 – Membrane potential modulation of NSI Le.Ee1 during leg searching movements. (A) Le.Ee1 membrane potential modulations during searching movements and underlying tonic depolarization. Gray bars denote downward movement of the leg. (B) Morphology of NSI Le.Ee1 (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI Le.Ee1 excites levator and extensor muscle activity, both with unrestrained (Di) and fixed FTi joint (Dii). (E) Continuous current injections during searching behavior do not influence leg movements. (F) Response of Le.Ee1 to fCO stimulation.

polarized during the second part of downward movements of the leg and during the first part of upward movements. Conversely, Le.Ee1 was hyperpolarized during the second part of upward and the very first part of downward movements (Fig. 3.33 A,C). Therefore, the membrane potential was maximally depolarized during upward movements and NSI Le.Ee1 supported leg movements during searching behavior. Tonic de- or hyperpolarization was not seen to influence leg searching movements (Fig. 3.33 E). NSI Le.Ee1 could be recorded once.

A second interneuron that excited levator and extensor MNs when artificially depolarized (Fig. 3.34 Dii), could be newly identified and was named Le.Ee2. It was recorded three times and could be stained twice (Fig. 3.34 B). Le.Ee2 was depolarized following passive flexion of the FTi joint and hyperpolarized following extension (N=3; Fig. 3.34 Di). In some cases, hyperpolarization was very weak, as e.g. in Fig. 3.34 Di.

During searching movements, NSI Le.Ee2 was phasically depolarized during downward movements of the leg and hyperpolarized during upward movements. This is shown for one animal in Fig. 3.34 Ai and C, and for a second animal -including better quality EMG recordings- in Fig. 3.34 Aii. Whether NSI Le.Ee2 supported or opposed leg movements during searching behavior was unclear from these recordings.

When NSI Le.Ee2 was manipulated by current injections during ongoing searching behavior, this affected several searching movement parameters. First, movement amplitudes of the FTi joint were clearly decreased during artificial depolarization of Le.Ee2 ($N = 3/3$; 91%-100% of pulses; Fig. 3.34 F). The decreased amplitudes were due to decreased flexion. Sometimes, FTi joint movements even seemed to stop. When NSI Le.Ee2 was hyperpolarized, FTi joint movement amplitudes increased ($N = 3/3$; 70%-100% of pulses; Fig. 3.34 F). Also the resting position of the FTi joint between bouts of searching movements was more flexed during hyperpolarization. Second, the position of the CTr joint movements shifted according to tonic current injection. During depolarization of Le.Ee2, the average position of femur movements was higher as compared to uninfluenced movements (Fig. 3.34 F). Correspondingly, femur movements shifted to lower positions during hyperpolarization. These positional shifts were not linked to a change in amplitude of CTr joint movements. Remarkably, interjoint coordination was not changed by de- or hyperpolarizing current injection, as is visible when FTi joint position is plotted against CTr joint position (Fig. 3.34 E). Rather, searching movements seem to be restricted to a smaller part of the working range of uninfluenced searching behavior. As a third parameter, the maximum speed of leg movements was reduced during depolarizing current injection into NSI Le.Ee2. Movement speed was measured as the speed of the distal femur and tibia (Fig. 3.34 G, top traces; $N = 1/1$). Simultaneously, changes in speed could also be detected in a changed steepness of movement traces. The steepness was significantly different ($p = 0.01$; Wilcoxon rank-sum test) in

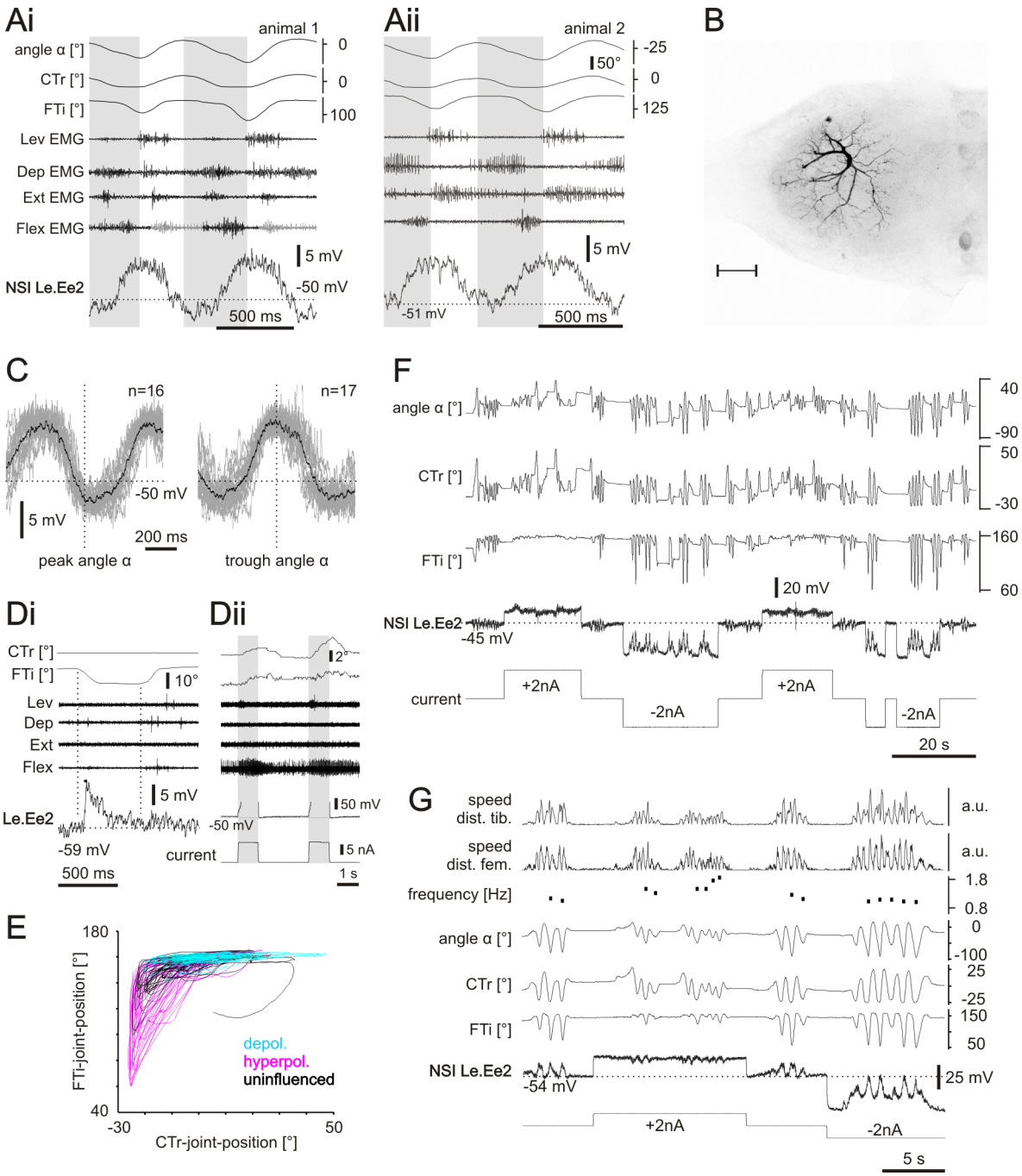

Figure 3.34 – Membrane potential modulation of NSI Le.Ee2 during searching behavior. (A) NSI Le.Ee2 membrane potential modulations during searching movements of two animals. (B) Morphology of NSI Le.Ee2. (C) Overdraws of membrane potential modulations triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (Di) Le.Ee2 response to fCO stimulation. (Dii) NSI Le.Ee2 excites levator and extensor muscle activity and leg movements. (E) Coordination of leg joints, depicted as FTi joint angle plotted against CTr joint angle. Coordination shown with no current injection (black trajectories), during depolarizing (cyan) and hyperpolarizing (magenta) current injection. (F,G) Tonic current injections during ongoing searching behavior affect leg movements. (F) Effects on FTi amplitude and CTr position and (G) movement velocity and cycle frequency are visible. Dotted horizontal lines denote RMP.

the depolarized condition compared to the uninfluenced or hyperpolarized condition. Finally, the frequency of searching cycles increased while NSI Le.Ee2 was depolarized and decreased during hyperpolarization. This might not be surprising because, as written above, also movement amplitudes decrease and increase, respectively. However, it indicates that the effect on FTi amplitude "overrules" the effect on movement velocity. Therefore, NSI Le.Ee2 is classified as an interneuron that mainly influences FTi joint movement amplitude and CTr joint movement position, but only weakly influences movement velocity.

NSI Li.Ee1 is another newly identified interneuron. When depolarized by current injection, it mainly caused extension of the FTi joint. Simultaneously, it excited extensor and inhibited levator muscle activity (Fig. 3.35 Di). This was the case even with fixed FTi joint (Fig. 3.35 Dii), therefore NSI Li.Ee1 seems to centrally inhibit levator muscle activity. For further characterization NSI Li.Ee1 morphology (Fig. 3.35 B) and response to fCO stimulation (Fig. 3.35 E) are shown.

During searching movements, NSI Li.Ee1 was tonically depolarized and phasically modulated (Fig. 3.35 A,C). Li.Ee1 was rapidly depolarized during initial upward movements of the leg, remained depolarized until peak leg position, and was slowly hyperpolarized during downward movements of the leg.

When NSI Li.Ee1 was de- or hyperpolarized by current injection during searching behavior, several searching movement parameters changed. First, FTi joint amplitudes decreased during a depolarization and increased during hyperpolarization (Fig. 3.35 Fi). Also FTi joint rest position between searching movements was more flexed during hyperpolarization. Second, the position of CTr joint movements was lowered during depolarization, but higher during hyperpolarization (Fig. 3.35 Fi). These changes in FTi joint movement amplitude and CTr joint movement position at the same time indicated a change in the coordination of the two joints (Fig. 3.35 Fii): While, during artificial depolarization of NSI Li.Ee1 (cyan trajectories), the FTi joint tended to be fully extended at very low positions of the CTr joint, it was flexed at much higher positions of the CTr joint during hyperpolarizing current injection (magenta trajectories). Especially the latter coordination differed clearly from coordination during uninfluenced searching movements. As a third parameter, in several parts of the recording the velocity of searching movements was affected by current injections. This is exemplarily shown in Fig. 3.35 G (left part of the recording). During artificial depolarization of NSI Li.Ee1 the steepness of movement trajectories was decreased, i.e. movement velocity was decreased; during hyperpolarization the steepness was increased, i.e. movement velocity increased. This was true for both the CTr and FTi joint. Finally, during depolarizing current injections, also the frequency of searching cycles decreased. This emphasizes the strong effect of depolarizing current injection into Li.Ee1 on movement

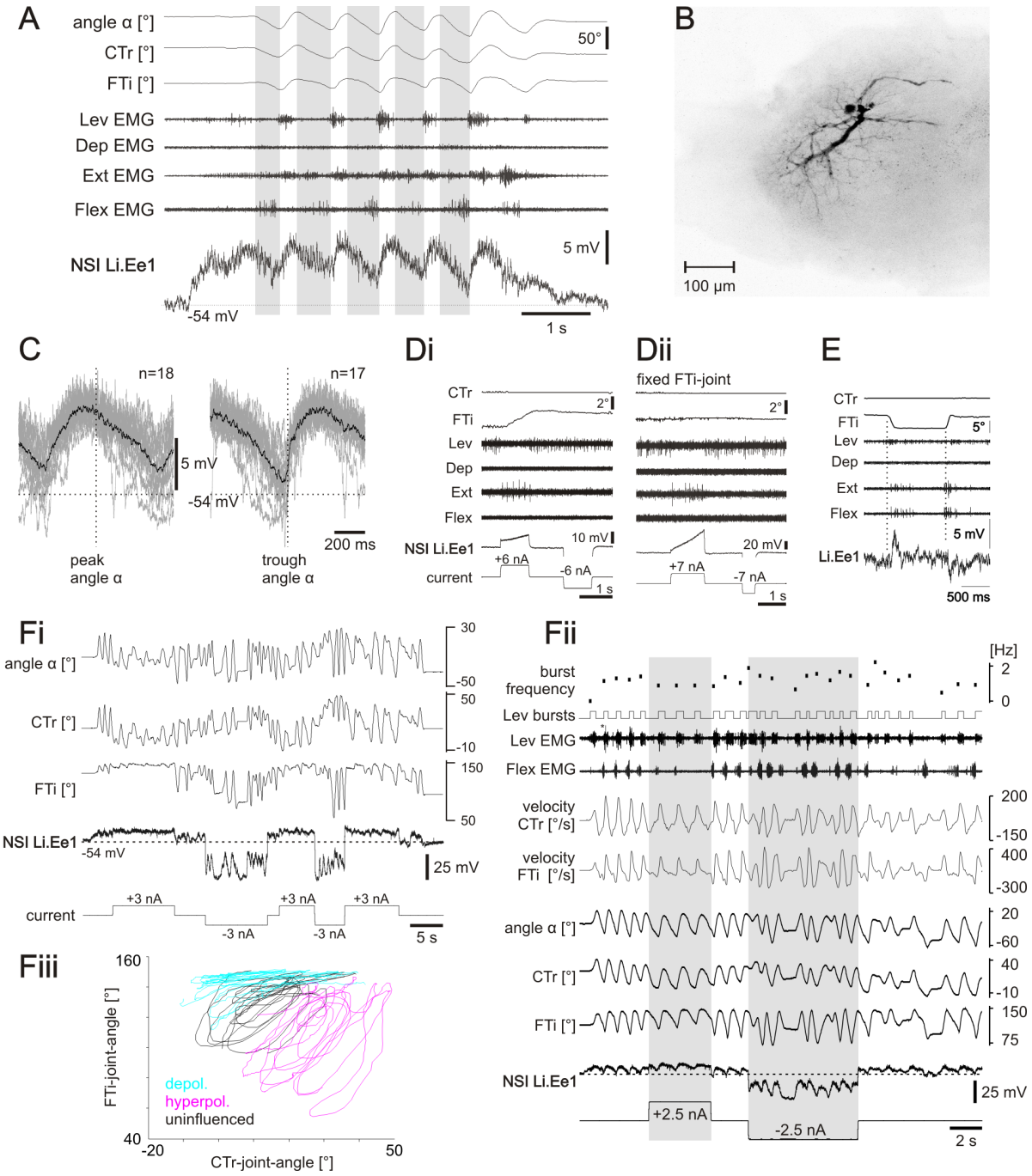

Figure 3.35 – Membrane potential modulation of NSI Li.Ee1 during searching behavior. (A) NSI Li.Ee1 membrane potential modulations and underlying tonic depolarization during searching movements. Gray bars denote downward movements. (B) Morphology of NSI Li.Ee1. (C) Overdraws of membrane potential triggered by angle α peak (left side) and trough (right side). Gray traces denote single sweeps, black traces denote average potential; n = number of sweeps. (D) NSI Li.Ee1 inhibits levator muscle activity and excites extensor muscle activity both with unrestrained (Di) and fixed (Dii) FTi joint. (E) Li.Ee1 response to fCO stimulation. (F) Tonic current injection during searching behavior influences leg movements. (Fi) Influence on FTi joint movement amplitude and CTr joint movement position. (Fii) Influence on movement velocity and frequency. (Fiii) FTi joint angle plotted against CTr joint angle, indicating joint coordination. Coordination shown without current injection (black trajectories), during depolarizing (cyan) and hyperpolarizing (magenta) current injection. Dotted horizontal lines denote RMP.

velocity; even compared to a clear reduction in FTi joint movement amplitude.

Fig. 3.36 shows the characterization of two (pairs of) neurons that were grouped together according to their morphology and certain aspects of their electrophysiology, despite differences in other electrophysiological aspects. Together, they were named NSI Di.Ei1. The (pair of) neuron(s) shown on the left side (subfigures 1), inhibits extensor tibiae muscle activity and elicits leg flexion, when depolarized by current injection during rest (Fig. 3.36 1D). It was recorded two times and stained once. The (pair of) neuron(s) shown on the right side (subfigures 2), also inhibits extensor tibiae muscle activity and elicits leg flexion but additionally inhibits depressor muscle activity and correspondingly elicits leg levation (Fig. 3.36 2D). It was recorded and stained once. The staining of either recording shows a pair of two neurons that were stained together (Fig. 3.36 1A,2A). Two further somata located nearby are stained and therefore might belong to this group, too. The morphology shown in the two stainings is very similar and characteristic. The somata are located closely together in an anterolateral location. From there the primary neurites shortly project posteriorly and split into three branches: one branch projects laterally and arborizes extensively, a second short branch projects anteromedially, and a third long branch projects medially to the midline of the ganglion. The staining shown in Fig. 3.36 2A, additionally shows another branch projecting to the midline (marked by an arrow). It should be noted that each branch consists of more than one neurite.

Both (pairs of) neurons were tonically depolarized during searching movements (Fig. 3.36 1B,2B). The membrane potential was phasically modulated. It was depolarized during upward movements of the leg and repolarized during downward movements (Fig. 3.36 1C,2C). For the group of neurons characterized in subfigures 2, the resting membrane potential was -44 mV when the activity during searching movements was recorded (Fig. 3.36 2C). However, the resting membrane potential later on was as negative as -52 mV (compare e.g. Fig. 3.36 2B). Both groups of neurons responded with depolarizations to a passive flexion or extension of the leg in order to stimulate the fCO (Fig. 3.36 1E,2E). Only one of the neurons was manipulated by current injection during ongoing searching behavior. This did not affect searching movements in an detectable way (Fig. 3.36 2F).

Another newly identified interneuron that influenced the MNs of several leg joints is NSI Le.Di.EF1. This interneuron was recorded and stained three times. When depolarized by current injection during rest, this neuron clearly excited levator and inhibited depressor muscle activity and therefore elicited a levation of the femur (Fig. 3.37 B). Additionally, in all recorded interneurons of this type, a simultaneous signal was visible in flexor and extensor EMGs after offset of depolarizing current stimuli. It was verified that this signal is not due to crosstalk between the EMGs. The signal did not cause

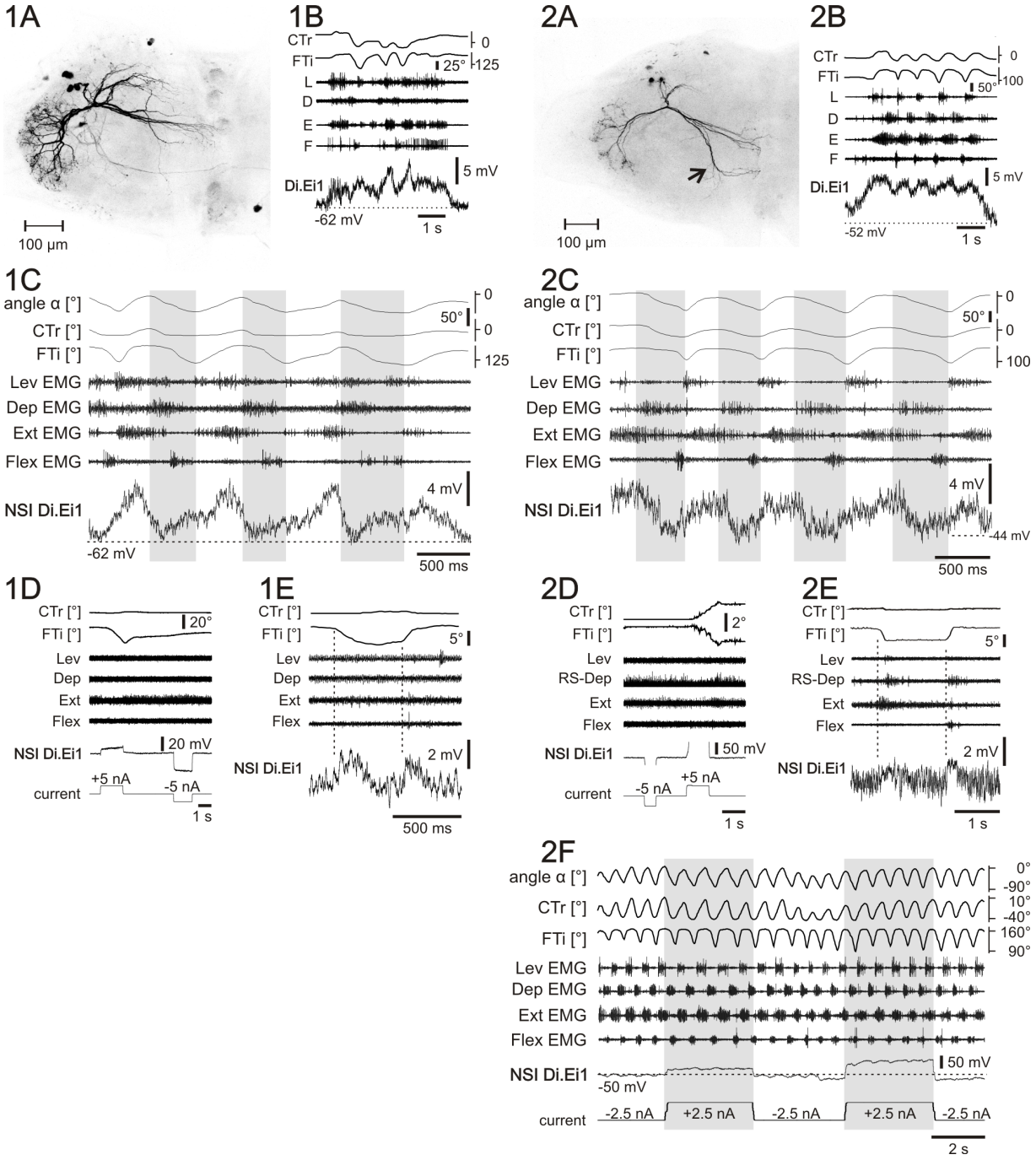

Figure 3.36 – Membrane potential modulation of NSI Di.Ei1 during searching behavior. Two neurons of type NSI Di.Ei1 are shown (subfigures 1 vs subfigures 2). The two neurons are similar in many but not all aspects and therefore are shown in comparison. (A) NSI Di.Ei1 morphology. Either staining shows a pair of neurons stained together. (B) Both neurons show a tonic depolarization underlying phasic membrane modulations. (C) Membrane modulations during searching movements. (D) One pair of neurons inhibits extensor muscle activity and elicits leg flexion (1D). The other pair of neurons inhibits extensor and depressor muscle activity and elicits leg flexion and levation (2D). RS-Dep = rectified and smoothed depressor muscle EMG. (E) Response to fCO stimulation. (F) Tonic current injection during searching behavior does not affect leg movements.

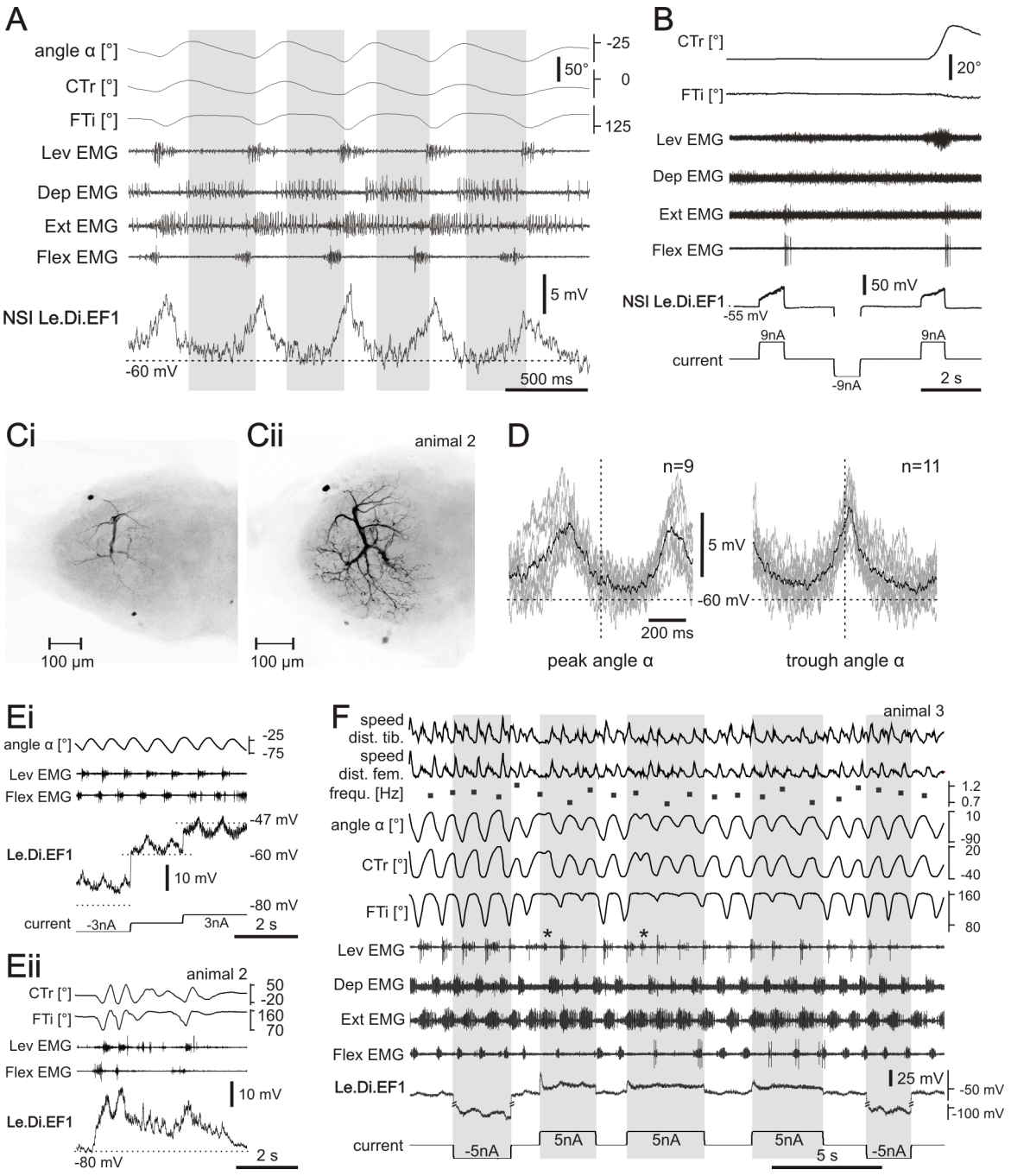

Figure 3.37 – Membrane potential modulation of NSI Le.Di.EF1 during searching movements. (A) Membrane potential modulations during searching movements. Gray bars denote leg downward movements. Dotted line indicates resting membrane potential. (B) NSI Le.Di.EF inhibits depressor muscle and excites levator muscle activity and leg levation. After offset of depolarizing current pulses, simultaneous signals in extensor and flexor EMG are detected. No reliable FTi joint movement is detected. (C) Morphology of the electrophysiologically characterized NSI Le.Di.EF (Ci) and a second specimen of another animal (Cii). (D) Membrane potential overdraws triggered at angle α peak (left) and trough (right). Gray traces denote single sweeps, black traces denote average potential. n is number of sweeps. (E) During searching, the membrane potential is phasically de- and hyperpolarization as revealed by hyper- and depolarizing current injection (Ei). An underlying tonic depolarization is visible during hyperpolarizing current injection (Eii). (F) Tonic current injection during ongoing searching behavior affects leg movements.

a movement in the FTi joint. Because of this unknown but reliably evoked signal, the interneuron was regarded as an NSI influencing multiple leg joints.

The morphology of the interneuron exemplarily characterized here is shown in Fig. 3.37 Ci. In a better staining, the morphology of a second interneuron is shown in Fig. 3.37 Cii. The interneurons response to fCO stimulation was very variable both within and between individuals. The responses ranged from phasic depolarizations following flexion and extension to depolarization/hyperpolarization or hyperpolarization/depolarization with a position component.

During searching movements, NSI Le.Di.EF1 membrane potential remained approximately at resting potential during the first part of leg downward movements and was depolarized during the second part (Fig. 3.37 A,C). The membrane potential did not remain depolarized but started to repolarize immediately after trough leg position during upward movements of the leg. Therefore, at least according to the shown effect on levator and depressor muscle, NSI Le.Di.EF1 supported searching movements. The phasic membrane modulations were caused by true phasic excitation and inhibition as can be seen in Fig. 3.37 Ei when NSI Le.Di.EF1 is depolarized to a new resting membrane potential (as indicated by the dotted line at -47 mV). A tonic excitation underlying phasic modulations became visible when NSI Le.Di.EF1 was tonically hyperpolarized by current injection (Fig. 3.37 Ei,Eii).

When tonically depolarized during ongoing searching movements, NSI Le.Di.EF1 led to reduced flexion of the FTi joint, thereby reduced movement amplitudes (Fig. 3.37 F; $N = 2/3$; 15/18 pulses). Hyperpolarizing pulses did not affect searching movements ($N = 3/3$; 17 pulses). The coordination of CTr and FTi joint movements remained the same ($N = 2/2$, not shown). Further, the maximum movement speed, which was measured in relative units as the speed of the distal tibia, decreased during depolarization (Fig. 3.37 F, top trace; $N = 2/3$; 8/15 pulses). This was reflected in the steepness of the overall movement trace (angle α, Fig. 3.37 F) which decreased significantly ($p = 0{,}0006$) during downward movements while Le.Di.EF was depolarized. Frequency did not systematically change, when NSI Le.Di.EF1 was depolarized ($N = 2/2$). In one animal, a short depolarizing current pulse (or the onset of a depolarizing current pulse as shown in Fig. 3.37 F) disturbed the searching rhythm by a prolongation of extensor and levator muscle activity (marked by asterisks in levator EMG trace; 4/13 pulses). Other depolarizing current pulses (or the onset of tonic depolarization) did not influence the rhythm but affected the amplitude of FTi joint movements as described above (3/13 pulses). An example is shown in Fig. 3.37 F (right side). The occurrence of one or the other possibility was not phase dependent (not shown).

NSI Le.flex1 is a further newly identified interneuron that affected several joints when manipulated by current injection. When artificially depolarized during rest, NSI Le.flex1

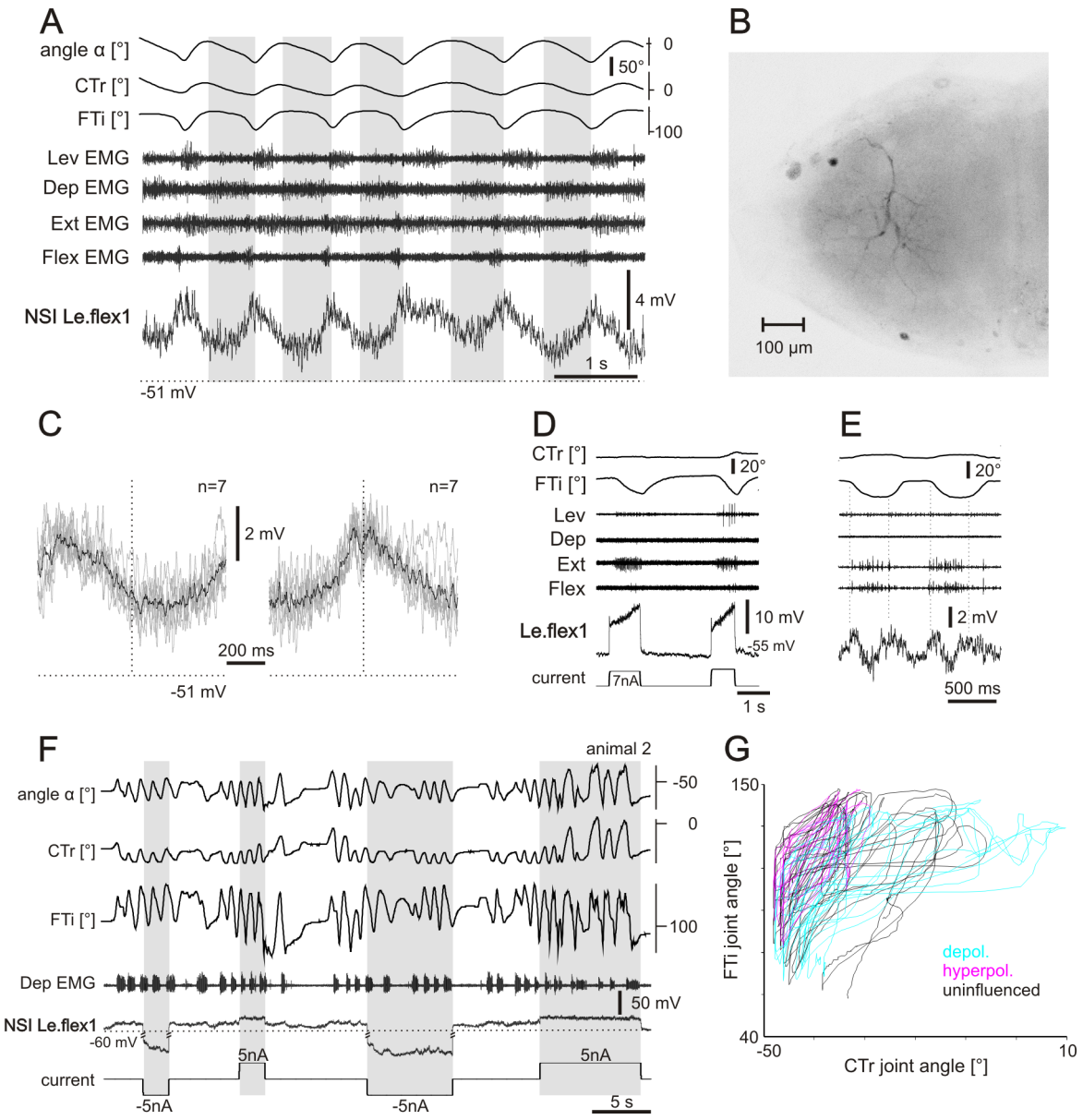

Figure 3.38 – Membrane potential modulation of NSI Le.flex1 during searching behavior. (A) Membrane potential modulations during searching. Gray bars indicate leg downward movements; dotted line indicates RMP. (B) Le.flex1 morphology. (C) Membrane potential overdraws triggered by angle α peak (left) and trough (right). Gray traces denote single sweeps, black traces denote average potential. n is number of sweeps. (D) NSI Le.flex1 excites levator and extensor muscle activity but leads to leg levation and flexion. (E) Response to fCO stimulation. (F) Tonic current injection into Le.flex1 affects searching movements. (G) FTi joint angle plotted against CTr joint angle for several searching cycles. This indicates joint coordination during uninfluenced searching movements (black trajectories), and during depolarizing (cyan) and hyperpolarizing (magenta) current injection.

elicited levation and flexion movements and corresponding levator muscle activity. However, no flexor muscle activity could be detected; instead a clear signal in the extensor EMG was measured (N = 2; Fig. 3.38 D). Therefore, according to the elicited movements, the interneuron was named Le.flex1. Its membrane potential was phasically depolarized during downward movements of the leg and repolarized during upward movements (Fig. 3.38 A,C) thereby supporting leg movements during searching behavior. The response to fCO stimulation was not very clear. The membrane potential seemed to be hyperpolarized (following flexion) and depolarized (following extension; Fig. 3.38 E) and displayed a position dependent tonic component (N = 2). The rough morphology is shown in Fig. 3.38 B.

When manipulated by current injections, NSI Le.flex1 affected the behavioral output. First, depolarization led to an increased flexion of the FTi joint, i.e. a larger FTi joint movement amplitude (Fig. 3.38 F; N = 1/2; 4/6 pulses). A hyperpolarization led to decreased flexion, i.e. a smaller movement amplitude (N = 1/2; 3/5 pulses). At the same time, also the position of FTi joint movements was shifted: during depolarization, the peak position of FTi joint movements decreased (approx. 10°), thus movement cycles were shifted to more flexed average leg positions (N = 2/2; 6/8 pulses). CTr joint movements were more elevated during 4 of 6 depolarizing current pulses. This was also reflected, when FTi joint movements were plotted against CTr joint movements (Fig. 3.38 G; cyan trajectory). NSI Le.flex1 was recorded twice and stained once.

Quite similar, another interneuron elicited levator muscle activity and flexion movements, but no activity was visible in either flexor or extensor EMG (N = 1). Therefore, this interneuron was named NSI Le.flex2. Its membrane potential modulations during searching movements were very similar to the modulations described for NSI Le.flex1. However, the phase was different: the membrane potential of NSI Le.flex2 was maximally depolarized just after trough leg position and therefore delayed by roughly 100 ms or 1/8 phase as compared to Le.flex1. In response to fCO stimuli, the membrane potential was hyperpolarized following both flexion and extension.

Depolarization of NSI Le.flex2 during searching behavior influenced the position of FTi and CTr joint movements. During depolarization, animals performed searching movements with a more flexed and less extended leg. Also, their leg was more elevated but less depressed. Whether the effect on CTr joint movement position was elicited directly or mediated via sensory signals by interjoint reflexes, could not be determined. At the same time, during depolarization the coordination of CTr and FTi joint changed, as is shown in Fig. 3.39 F. When NSI Le.flex2 was hyperpolarized, searching movements were largely unchanged (Fig. 3.39 E,F). As one slight difference in this situation, the FTi joint was even further extended than during uninfluenced searching movements (magenta trajectory in Fig. 3.39 F).

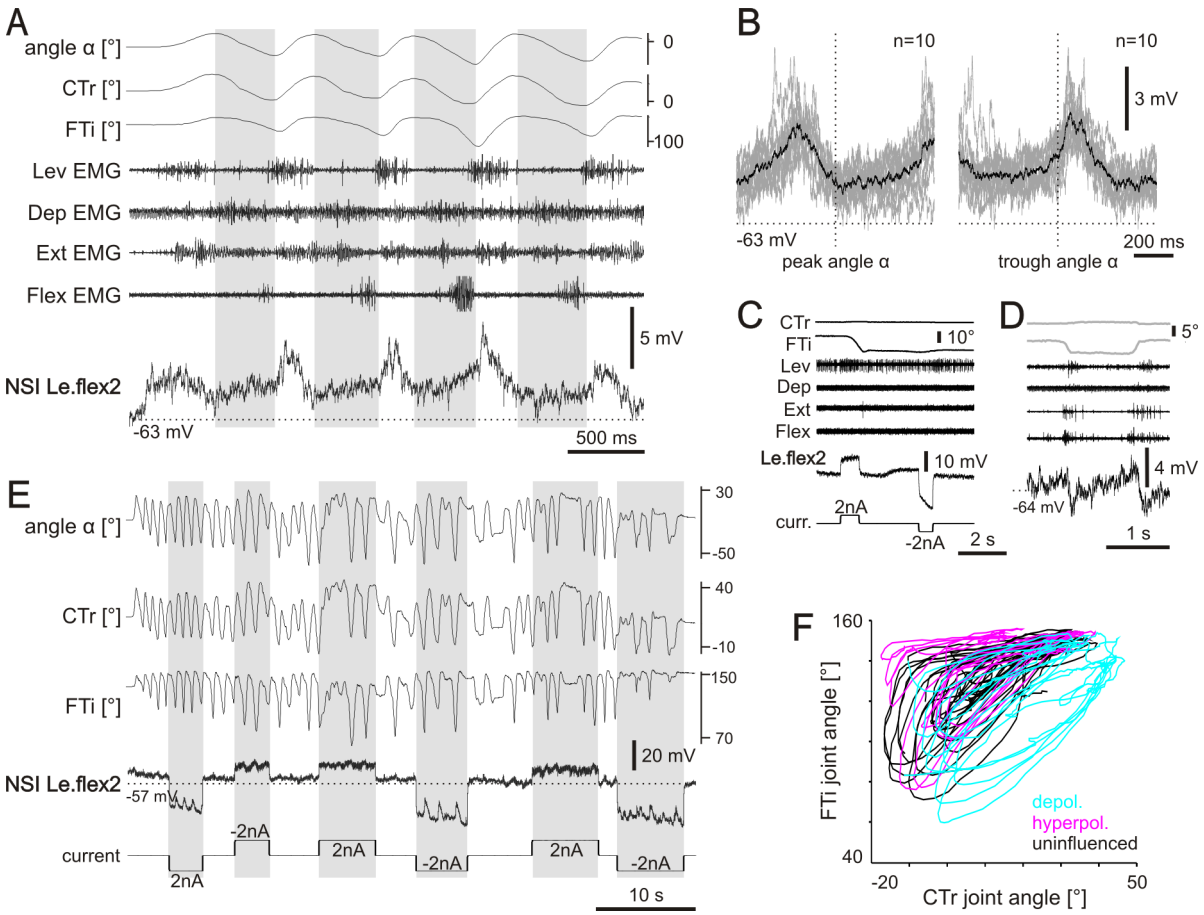

Figure 3.39 – Membrane potential modulation NSI Le.flex2 activity during searching behavior. (A) Membrane potential modulations during searching. (B) Membrane potential overdraws triggered by angle α peak (left) and trough (right). Gray traces denote single sweeps, black traces denote average potential. n is number of sweeps. (C) Le.flex2 excites levator muscle activity and small leg levation but strong leg flexion. (D) Response to fCO stimulation. (E) Tonic current injection during searching behavior affects leg movements. (F) Joint coordination of searching cycles during no, de- or hyperpolarizing tonic current injection.

SUMMARY

In the previous sections, intracellular recordings of 24 different types of nonspiking interneurons during leg searching movements were shown. All interneurons' membrane potential was phasically modulated with searching movements. Membrane potential modulations of most NSIs supported leg movements during searching behavior, only few opposed movements, and some neither clearly supported nor opposed leg movements. In some interneurons a tonic depolarization underlying phasic modulations was visible at the uninfluenced resting membrane potential level. Other NSIs revealed a tonic depolarization when their resting membrane potential was held at a hyperpolarized level by current injection. Three interneurons, NSI Li.De3, di3, I5, were tonically hyperpolarized at their uninfluenced resting membrane potential level during searching movements. No tonic depolarization could be seen even at hyperpolarized membrane

NSI	fCO elong/relax	sRMP	sp-p	N
Li.De3	d/h	-48.3 (±1.5)	7.6 (±1.7)	3
De8	h/d (1)	-55; -46	9.5; 11.5	2
de9	-	-50; -52	11.6; 10.3	2
Le3	d/h	-51	10.4	1
Le4	h/h	-50	7.7	1
di3	h/?	-38	3.3	1
I1	h/d	-53 (±3)	6.1 (±0.8)	3
I5	h/h	-36; -57	11.8; 11.8	2
fe1	-	-47	11.7	1
E2	d/h, pos	-50	7.2	1
E5	d/d	-53	11.5	1
E10	d/h, pos	-54.7 (±3.8)	14.2 (±5.4)	3
E11	h/d (3); d/d (1)	-53 (±5.3)	7.0 (±2.7)	4
E12	d/h, pos	-60	11.5	1
ext1	h/h	-53	10.6	1
I4	d/d	-56.4 (±4)	4.7 (±2.6)	9
E4	d/d	-58.3 (±3.9)	9.8 (±2.1)	7
Le.Ee1	d/h	-56	6.3	1
Le.Ee2	d/h	-52 (±2)	8.4 (±2.1)	3
Li.Ee1	d/h	-54	6.9	1
Di.Ei1	d/d	-54.7 (±6.4)	5.7 (±1.2)	3
Le.Di.EF	d/d; d/h; h/d, pos	-59.3(±1.2)	5.8 (±0.8)	3
Le.flex1	(h/d, pos)	-55; -56	4.6; 2.7	2
Le.flex2	h/h	-63	2.4 (±1.4)	1

Table 3.1 – The table summarizes results for all characterized NSIs: their response to fCO apodeme elongation ("fCO elong"; by passive leg flexion via treadwheel) and fCO apodeme relaxation ("fCO relax"; by passive leg extension via treadwheel); measured resting membrane potential ("sRMP"; measured with leg resting in air); the peak-to-peak value of membrane potential modulations during searching movements ("sp-p"), and the number of recordings in different animals ("N").

potential levels as negative as -75 mV.

When single NSIs were manipulated by continuous current injection, this often affected ongoing searching movements. The set of searching movement parameters that were affected was characteristic for individual NSIs. A summary is given in Fig. 3.40. Most often, NSIs affected movement amplitude (11 NSIs). But also other parameters like movement position (5), movement velocity (4), CTr and FTi joint coordination (5), or combinations of these were affected. One interneuron, NSI I4, provided general drive to searching behavior.

Most NSIs affected ongoing searching movements according to the synaptic drive they had been shown to provide, i.e. the leg movements they elicited when depolarized during rest. For example, NSI I1 lead to increased flexion of the leg when depolarized. Only four NSIs affected movements in another joint: Two neurons that provided synaptic drive to the FTi joint, NSIs E11 and fe1, extended their effect to influence also

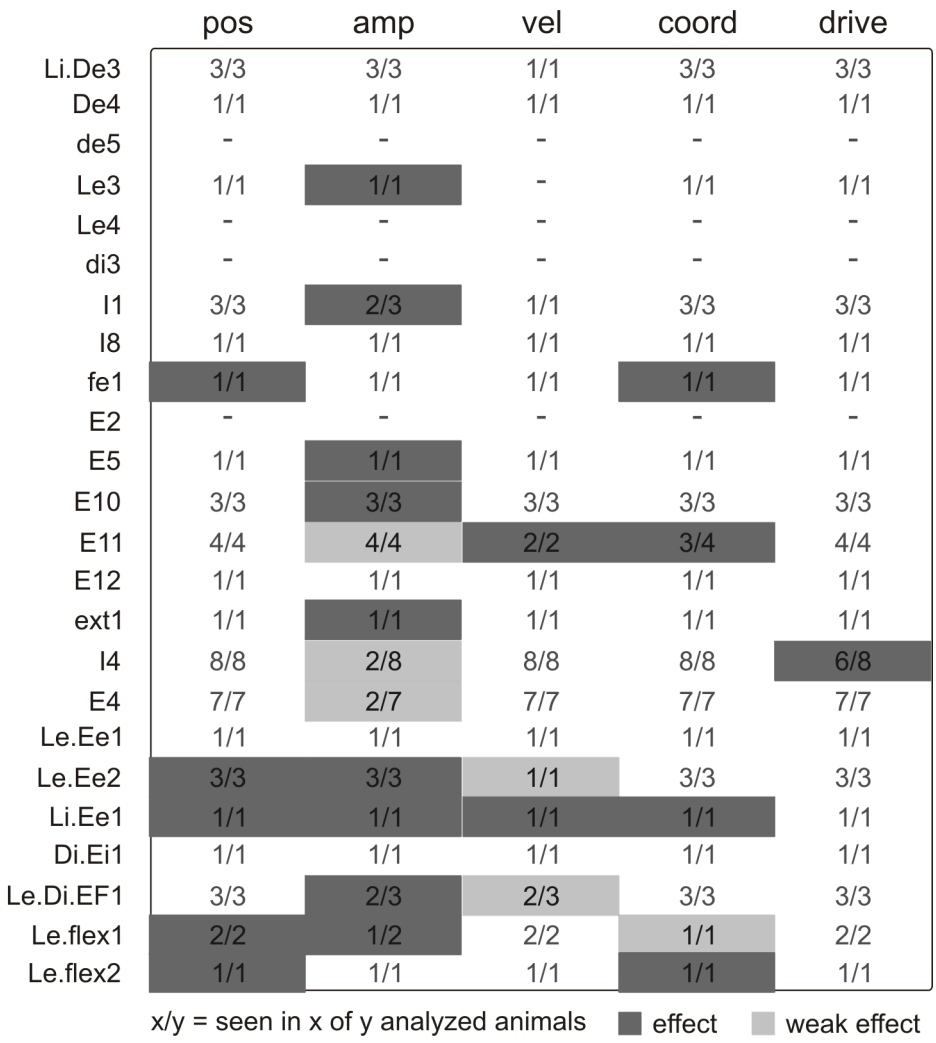

Figure 3.40 – Summary: Effect of tonic current injection into respective NSI on ongoing leg searching movements. Dark gray background: effect visible; light gray background: weak effect visible; white backgroung: no effect visible. Numbers indicate in how many animals the indicated effect was visible ("x") and tested ("y"). pos = position of searching movements (independent of effect on amplitude); amp = amplitude; vel = movement velocity; coord = coordination of CTr amd FTi leg joint; drive = general support or inhibitio of searching movements.

the CTr joint when manipulated during searching behavior. Another neuron, NSI Le3 affected FTi joint movement amplitudes. Finally, NSI Le.Di.EF1, did not elicit FTi joint movements when characterized, but strongly influenced FTi movement amplitude when manipulated during searching behavior.

3.3 Comparison of nonspiking interneuron activity during searching and walking

Most interneurons whose activity was described in the previous chapter during searching behavior, were also intracellularly recorded while animals were walking on a treadwheel. Besides their morphology and other electrophysiological criteria, the nonspiking interneurons' (NSIs) membrane potential modulations during walking were a characteristic feature in order to identify recorded interneurons and match them to previously described interneurons (von Uckermann and Büschges, 2009; Rosenbaum, 2013). Also, knowing their activity during walking will help to compare NSIs to interneurons that will be recorded in the future.

As a first result, all NSIs that were recorded showed phasic membrane potential modulations both during searching and walking behavior. This proves that interneurons are able to contribute to the generation of more than one behavior. But what is the difference in NSI activity whether the animal employs searching or walking behavior?

In order to compare membrane potential oscillations occurring during walking or searching, the stance phase during walking was assumed to correspond to the flexion phase during searching (i.e. the second part of the downward searching movement). This seems reasonable, first, because the same motoneuron (MN) pools are active, i.e. depressor and flexor MNs (retractor MNs were not considered in this study). Second, this approach resembles the method of previous studies analyzing walking behavior in semi-intact preparations, in which stance and swing phase were discriminated on the basis of extensor and flexor motoneuron or EMG activity (Bässler, 1993b; Fischer et al., 2001; von Uckermann and Büschges, 2009). There, extensor activity was interpreted to indicate swing phase, flexor activity to indicate stance. It should be noted, however, that of course sensory inputs are different during stance phase (walking) and flexion phase (searching), e.g. campaniform sensilla can be assumed to sense load during stance phase but not during searching behavior (Zill et al., 2012).

In the following, the physiology of those NSIs that were characterized and described for searching behavior in the previous chapter, will be described during walking behavior. Also their physiology during searching behavior will be shown again for direct comparison. Nonspiking interneurons were grouped according to the synaptic drive they were shown to provide in the previous chapter (sec. 3.2) and will be presented here in a corresponding order.

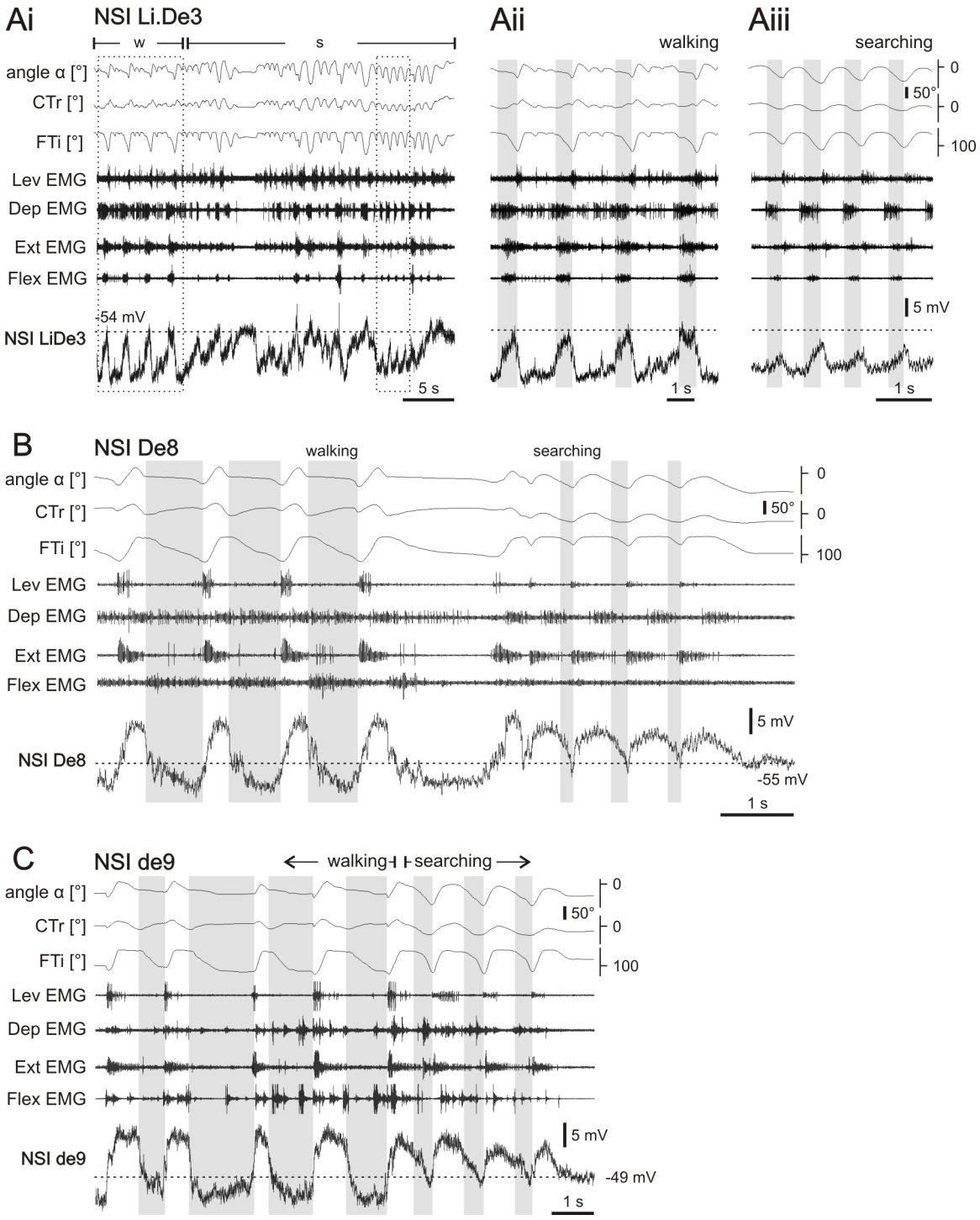

Figure 3.41 – Membrane potential modulation of NSI Li.De3 (A), De8 (B), and de9 (C) during walking and searching behavior. Physiologically, main differences between walking and searching occur during stance and flexion phase, respectively. Gray bars mark stance phase and flexion phase. "w" and "s" mark sequences of walking and searching behavior. Dotted boxes in Ai mark position of enlargements in Aii, Aiii. Traces from top to bottom depict: overall leg movement ("angle α"), coxa-trochanter-joint movement ("CTr"), femur-tibia-joint-movement ("FTi"); electric muscle activity of levator ("Lev EMG"), depressor ("Dep EMG"), extensor ("Ext EMG"), flexor ("Flex EMG"); intracellular recording of respective NSI.

3.3.1 Nonspiking interneurons influencing the coxa-trochanter joint

Fig. 3.41 shows interneurons that elicited depression of the CTr joint when depolarized by current injection during rest. In Fig. 3.41 Ai membrane potential oscillations of NSI Li.De3 during walking (left) and searching (right) are shown. Enlargements from this sequence are shown in Fig. 3.41 Aii and Fig. 3.41 Aiii. During both walking and searching behavior, the membrane potential was hyperpolarized below resting membrane potential. While walking, the membrane potential was completely repolarized during stance phase (gray bars). While searching, the membrane potential was repolarized more slowly and to a smaller extend during flexion of the leg (gray bars).

Fig. 3.41 B and C show the activity of NSI De8 and de9 during walking and searching. Both interneurons showed very similar changes in membrane potential modulations during walking and searching. Their membrane potential was depolarized during swing phase while walking and during upward movements while searching. The membrane potential remained depolarized during beginning depression. During flexion (while searching), the membrane potential slowly repolarized to resting membrane potential. However, during walking the membrane potential was quickly and strongly hyperpolarized below resting membrane potential. Thus, the peak-to-peak amplitude of membrane potential oscillations was much larger for walking behavior than for searching. Also, the transitions between de- and hyperpolarized membrane potential were much sharper and tightly coupled to the transition of swing to stance phase.

NSIs that were described to elicit a levation of the CTr joint, when depolarized by current injection during rest, are shown in Fig. 3.42. Their membrane potential modulations during swing (walking) and extension phase (searching) were qualitatively the same. Contrarily, during stance phase NSIs Le3 (Fig. 3.42 A) and Le4 (Fig. 3.42 B) received strong input that led to membrane hyperpolarization (left panels, gray bars), while during flexion phase of searching behavior they were depolarized (right panels, gray bars). Noticeably in NSI Le3, the peak to peak value of membrane potential modulations was larger during searching than during walking behavior.

In a third interneuron, NSI di3 (Fig. 3.42 C), the membrane potential was hyperpolarized below resting membrane potential during both walking and searching. While during walking behavior, NSI di3 was sharply and strongly repolarized during stance phase, during searching behavior this repolarization was not present (Fig. 3.42 C, right side, gray bars). Instead, the membrane potential was slowly hyperpolarized. Interestingly, at the end of flexion phase a short repolarizing peak was visible that coincided with the hyperpolarization at stance-swing transition during walking.

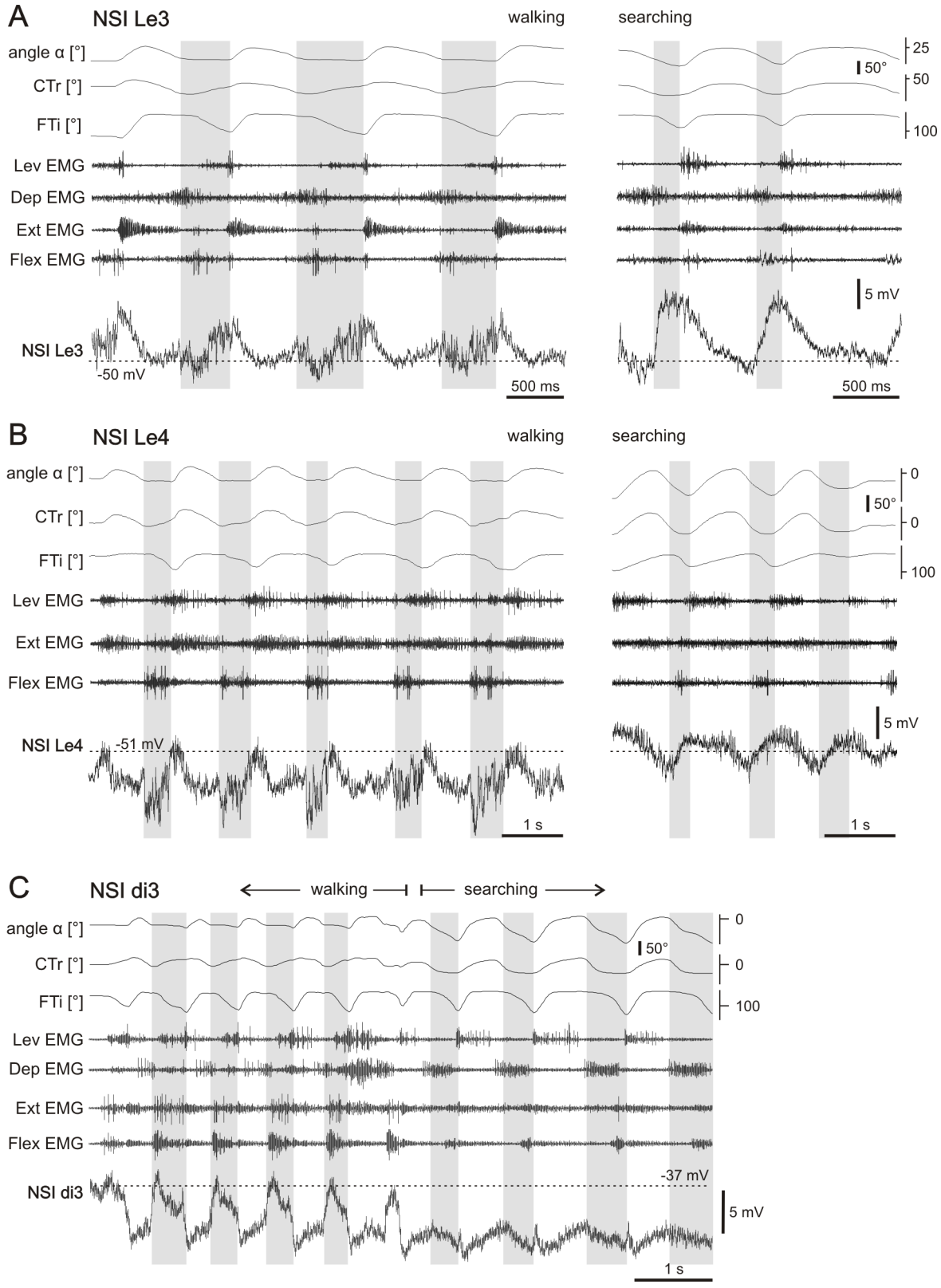

Figure 3.42 – Membrane potential modulation of NSI Le3 (A), Le4 (B), and di3 (C) during walking and searching behavior. Main differences in membrane potential between walking and searching occur during stance and flexion phase. Gray bars mark stance phase and flexion phase, respectively. Dotted lines indicate RMP.

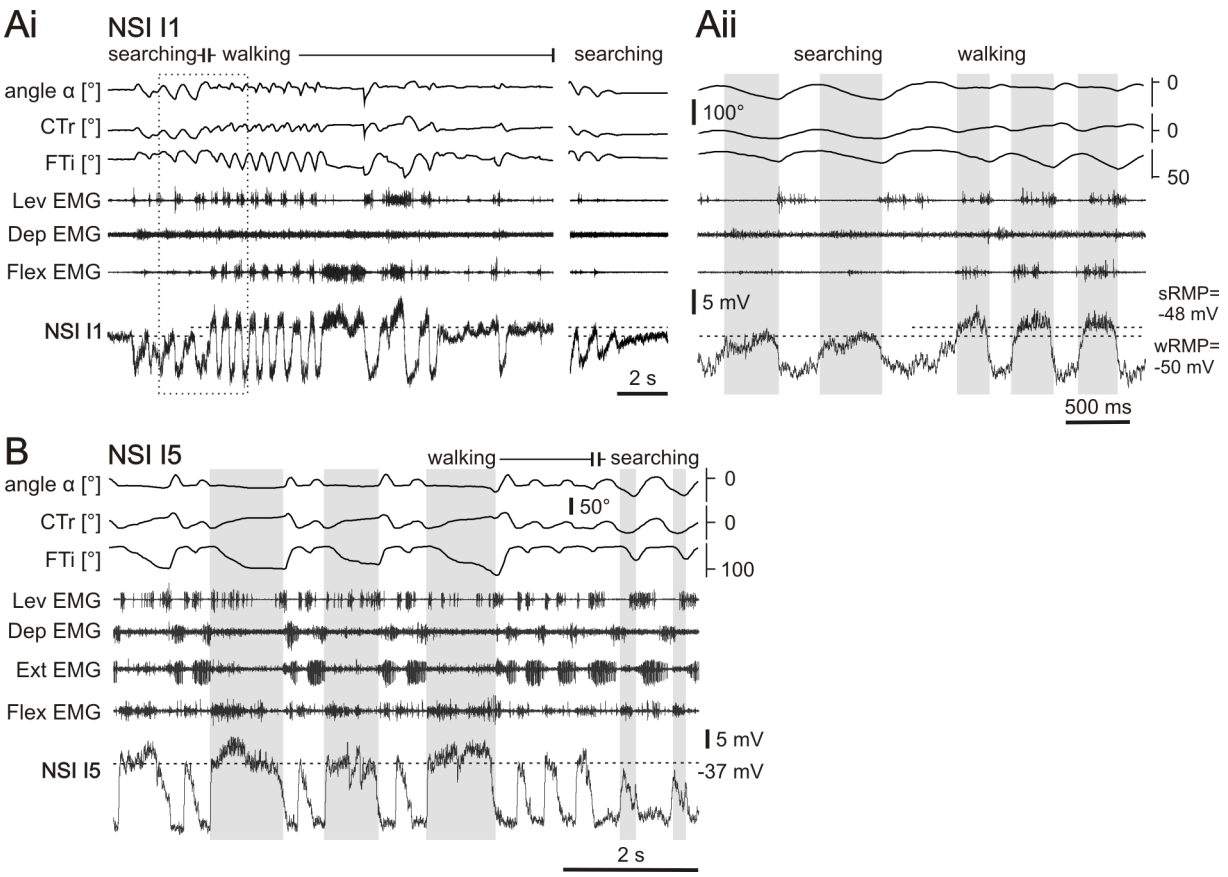

Figure 3.43 – Membrane potential modulation of NSI I1 (A) and I5 (B) during walking and searching behavior. Main differences in membrane potential between walking and searching occur during stance and flexion phase. Gray bars mark stance and flexion phase, respectively. Dotted horizontal lines mark resting membrane potential for searching ("sRMP") and walking behavior ("wRMP"). In Ai sRMP is shown before (left) and after (right) a sequence of walking behavior. Dotted box in Ai marks the position of the enlargement in Aii.

3.3.2 Nonspiking interneurons influencing the femur-tibia joint

Fig. 3.43 shows recordings of NSIs I1 (Fig. 3.43 A) and I5 (Fig. 3.43 B). As described in the previous chapter, NSI I1 membrane potential was hyperpolarized below resting membrane potential during upward movements of the leg during searching. In the same way, the membrane potential was hyperpolarized to approximately the same potential also during swing phase in walking behavior. The transition in membrane potential both during searching (flexion>extension) and walking (stance>swing) was fast. When searching, during flexion phase the membrane potential was repolarized to resting membrane potential (sRMP = -50 mV; Fig. 3.43 Ai,Aii; left sides). In contrast, during stance when walking the membrane potential was depolarized above resting membrane potential (Fig. 3.43 Ai,Aii; right side). The transition from hyperpolarization to depolarization was faster during walking than during searching. Interestingly, the resting membrane potential of NSI I1 was more negative during searching; i.e.

when the leg was rested in air (sRMP -50 mV), than during walking behavior when the animal rested its leg on the treadwheel (wRMP -48 mV). This was *not* due to a change in recording quality as is proven by the same sRMP before and after a walking sequence (Fig. 3.43 Ai). This effect was observed in all recorded interneurons if this type (N = 3; ΔRMP = 2.8 mV ($\pm$0.9)).

NSI I5 displayed similar membrane potential modulations as described for NSI I1 (Fig. 3.43 B). It was hyperpolarized during swing phase (walking; left side of figure) and extension phase (searching; right side of figure). During stance, the membrane potential was depolarized slightly above resting membrane potential (Fig. 3.43 B; gray bars, left side). In contrast, during flexion phase the membrane potential was depolarized sharply but did not reach resting membrane level (Fig. 3.43 B; gray bars, right side). Throughout the walking sequence, the animal sometimes touched the treadwheel but did not initiate a step and instead again lifted the leg. This lead to a sharp depolarization, albeit not as strong as during proper steps (Fig. 3.43 B, left side). NSI I5 resting membrane potential was the same during walking and searching (N = 1).

In Fig. 3.44 and Fig. 3.45, walking and searching sequences of five interneurons that elicit leg extension when artificially depolarized are shown. Again, the main difference in their membrane potential modulations occurred during stance phase and flexion phase, respectively. In three of these neurons –NSIs E2, E5, E11– this meant that in the respective phase the membrane potential was much more hyperpolarized during walking than during searching behavior (Fig. 3.44). The modulations' peak to peak value was larger during walking than during searching. Also, membrane potential transitions were sharper during walking than during searching. In two of these neurons, NSIs E5 and E11, the resting membrane potential during walking was clearly more negative than during searching behavior (Fig. 3.44 B,C; E5: ΔRMP = 6 mV, N = 1; E11:ΔRMP = 5.4 mV ($\pm$2.1), N = 4).

In one interneuron, NSI E10, the membrane potential during searching behavior was increasingly depolarized throughout leg flexion (Fig. 3.45 Ai (right side), Aiii). In contrast, when walking, no depolarization occurred during stance phase (Fig. 3.45 Aii). Instead, large postsynaptic potentials were visible throughout stance and the membrane potential remained approximately the same. Directly after stance, the membrane potential was strongly depolarized. Membrane modulations during swing movements (when walking) were very similar to those during leg extension (when searching). Also in this interneuron, the resting membrane potential of walking behavior (measured with leg resting on treadwheel) was more negative (wRMP = -61 mV) than the resting membrane potential of searching behavior (leg resting midair; sRMP = -56 mV; N = 3). The peak to peak values of membrane potential modulations were approximately the same during walking and searching behavior.

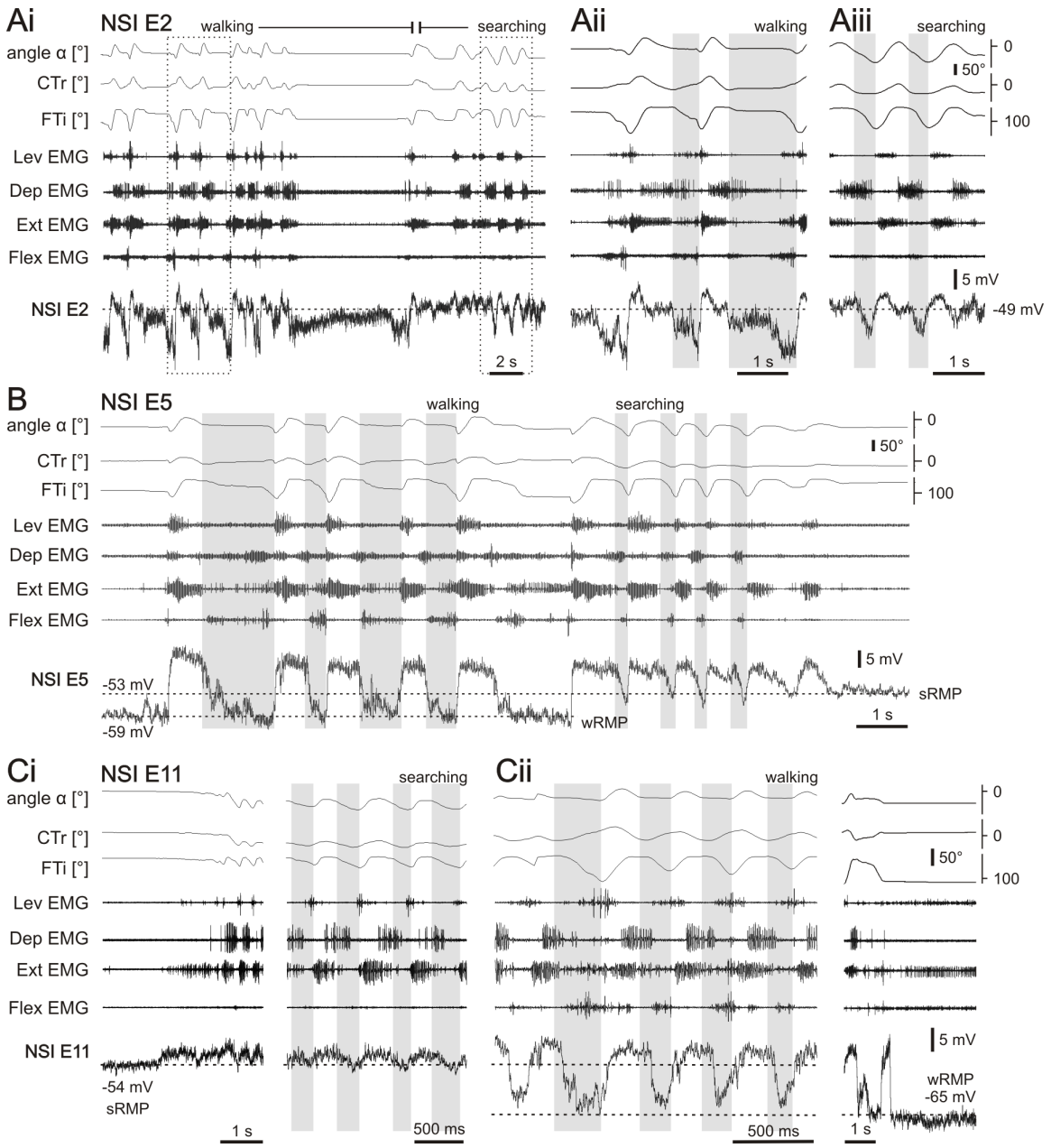

Figure 3.44 – Membrane potential modulation of three NSIs providing excitatory drive to the extensor muscle: NSIs E2 (A), E5 (B), E11 (C) shown during walking and searching behavior. Main differences in membrane potential between walking and searching occur during stance and flexion phase. Gray bars mark stance and flexion phase, respectively. Dotted boxes in Ai mark positions of the enlargements shown in Aii, Aiii. In B and C (far left, far right), the different resting membrane potentials for searching ("sRMP") and walking ("wRMP") behavior are indicated.

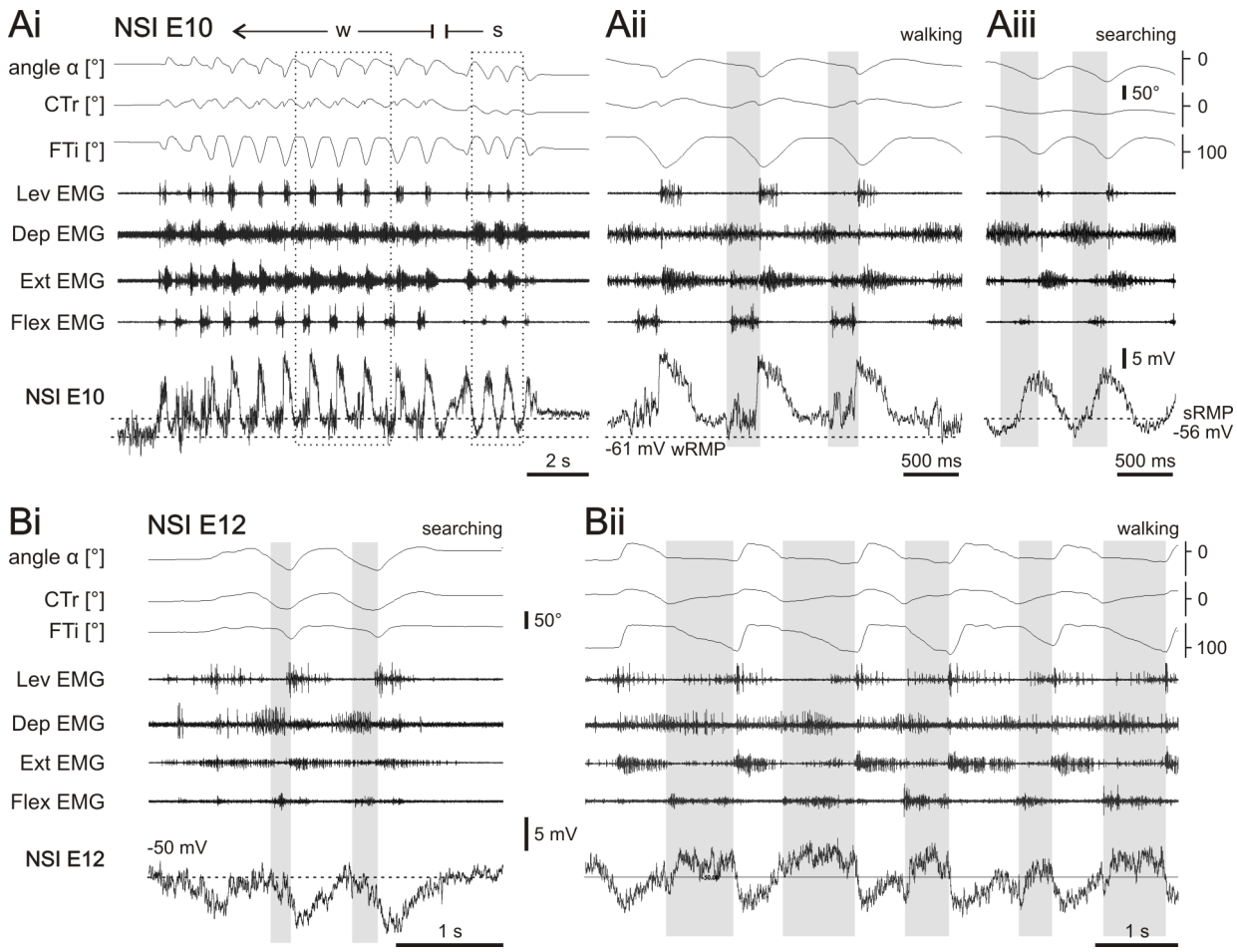

Figure 3.45 – Membrane potential modulation of two NSIs providing excitatory drive to the extensor muscle: NSIs E10 and E12 are shown during walking and searching behavior. Physiologically, main differences between walking and searching occur during stance and flexion phase. Gray bars mark stance and flexion phase, respectively. Dotted boxes in Ai mark positions of the enlargements shown in Aii, Aiii. Different resting membrane potentials for searching ("sRMP") and walking ("wRMP") behavior are indicated.

In another interneuron that provided drive to the extensor muscle, NSI E12 (Fig. 3.45 B), the membrane potential was more depolarized during stance phase (while walking) than during flexion phase (while searching). Also here, the transition from depolarized to hyperpolarized membrane potential was much sharper during walking than during searching. During swing phase and extension phase, respectively, the membrane potential was very similarly hyperpolarized below resting membrane potential. No change in resting membrane potential throughout walking and searching could be detected ($N = 1$).

3.3.3 Nonspiking interneurons influencing multiple leg joints

Finally, also interneurons that influenced more than one leg joint displayed clear differences in membrane potential modulations between stance (while walking) and flexion phase (while searching) (Fig. 3.46, Fig. 3.47, Fig. 3.48). For example, the membrane potential of NSIs Li.Ee1 (Fig. 3.46 A) and I4 (Fig. 3.46 B) during searching movement flexion phase was slowly repolarized to resting membrane potential. When walking, the membrane potential was strongly hyperpolarized below resting membrane potential. Interestingly, in one walking cycle, NSI I4 displayed a less strong hyperpolarization (Fig. 3.46 B, left panel, middle). There, the animal performed a proper step but with less flexion, thereby performing a shorter step (compare FTi position trace, Fig. 3.46 B). The membrane potentials of all other interneurons described in this section were slowly depolarized during searching movement flexion phase. Again, during stance phase when walking their membrane potential was hyperpolarized (Fig. 3.46 C, Fig. 3.47, Fig. 3.48) and remained hyperpolarized throughout stance.

Qualitatively, in all interneurons membrane potential modulations were the same during swing (walking) and extension phase (searching). In some interneurons, these modulations were remarkably preserved between behaviors also quantitatively (e.g. NSIs Li.Ee1, I4, Di.Ei1; Fig. 3.46). In other interneurons the maximum membrane depolarization was stronger when walking than when searching. For example, this is shown nicely in Fig. 3.47 A for NSI Le.Di.EF. In this interneuron, the membrane potential was slowly repolarized throughout both swing phase (walking) and extension phase (searching). However, the maximum membrane depolarization at stance>swing transition (walking; Fig. 3.47 Aii,Aiv second panel from right) was stronger than at trough leg position during searching (Fig. 3.47, Aiii, Aiv right panel). This can be seen also in NSI E4 (Fig. 3.47 Bii) and NSIs Le.Ee1, Le.Ee2, Le.flex1 (Fig. 3.48).

In several interneurons, the resting membrane potential that was observed with walking behavior (i.e. when the leg was resting on the treadwheel; "wRMP") was more negative than with searching behavior (leg resting in air; "sRMP"). This was the case for NSIs Li.Ee1 (ΔRMP = 8 mV; Fig. 3.46 A; N = 1), Le.Di.EF1 (ΔRMP = X mV; Fig. 3.47 Ai), E4 (ΔRMP = X mV; Fig. 3.47 Bi), Le.Ee2 (ΔRMP = X mV; Fig. 3.48 B). This effect was not observed in NSI I4 (N = 6).

Summary

All nonspiking interneurons that were recorded during both searching and walking showed phasic membrane potential modulations during both behaviors. Differences in membrane potential modulations mainly occurred during stance phase as compared to flexion phase during searching. Most NSIs were more strongly hyperpolarized during

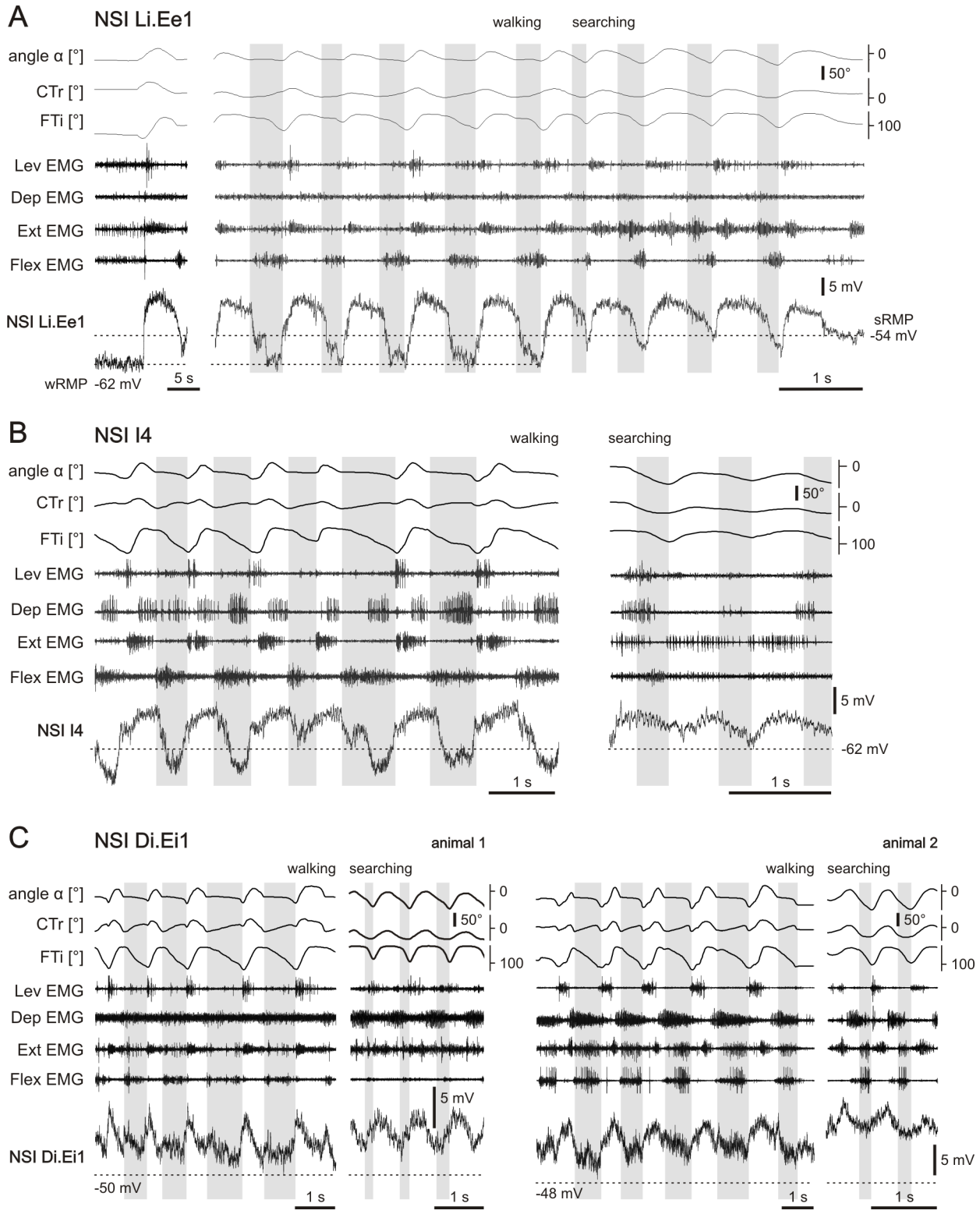

Figure 3.46 – Membrane potential modulation of three NSIs influencing multiple leg joints: NSIs Li.Ee1 (A), I4 (B), and Di.Ei1 (C) shown during walking and searching behavior. Analogous to Fig. 3.36, two neurons of type NSI Di.Ei1 are shown in comparison in (C). Main differences in membrane potential between walking and searching occur during stance and flexion phase. Gray bars mark stance and flexion phase, respectively. Dotted lines indicate resting membrane potential during searching behavior. Where different, resting membrane potential is indicated for searching (sRMP) and walking (wRMP).

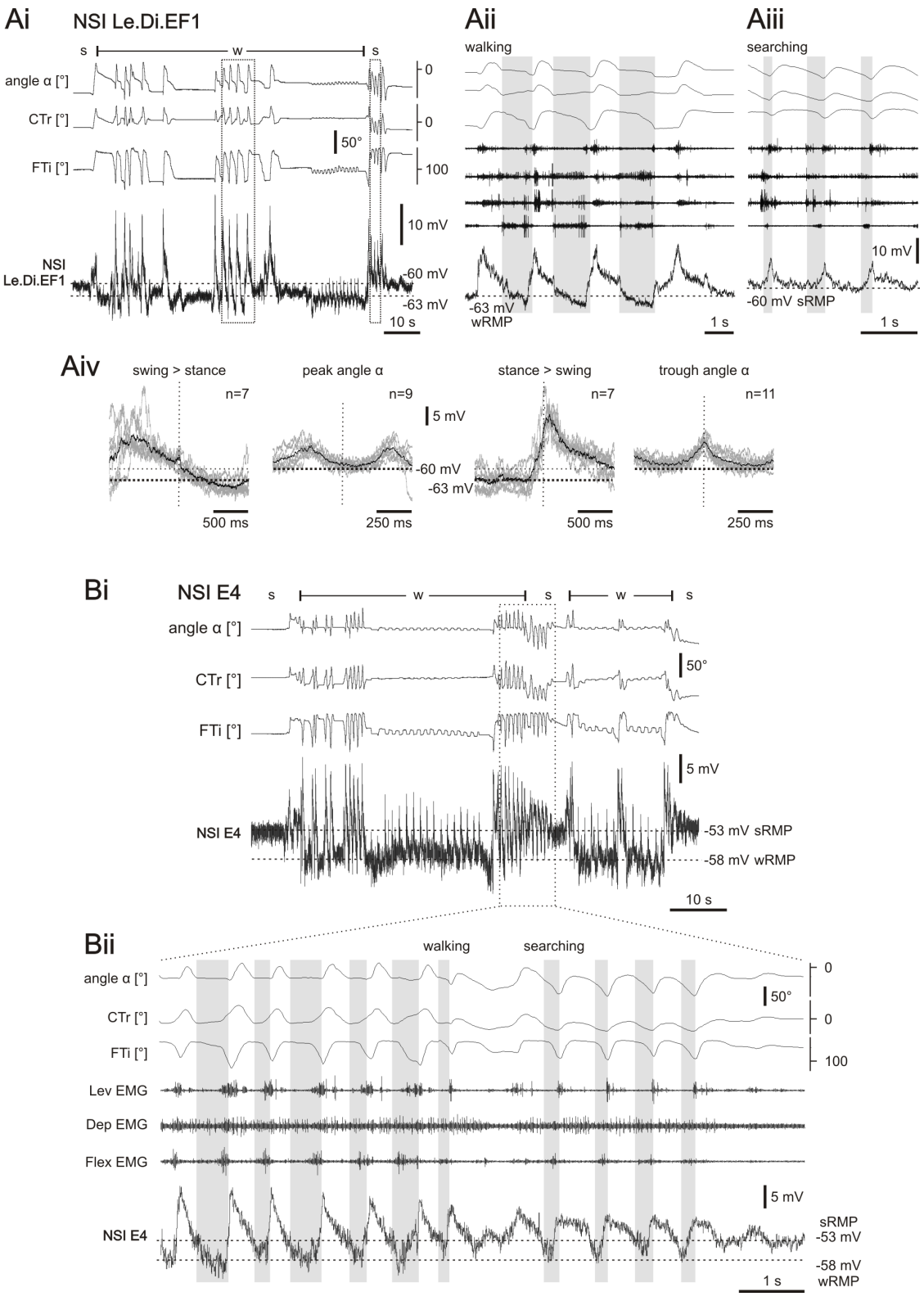

Figure 3.47 – Membrane potential modulation of NSIs Le.Di.EF1 (A) and E4 (B) during searching and walking behavior (A) NSI Le.Di.EF1: Overview of changes in resting membrane potential (RMP) during searching and walking (Ai). Physiology during walking (Aii) and searching (Aiii). (Aiv) Membrane potential overdraws triggered by swing>stance transition and angle α peak value (left) or stance>swing transition and angle α trough value (right). (B) NSI E4: Overview of changes in RMP during searching and walking (Bi); phasic membrane potential modulations (Bii). Dotted boxes in A,B indicate position of enlargements shown in Aii, Aiii and Bii. Gray bars denote stance or flexion phase, respectively. "s" searching behavior; "w" walking behavior.

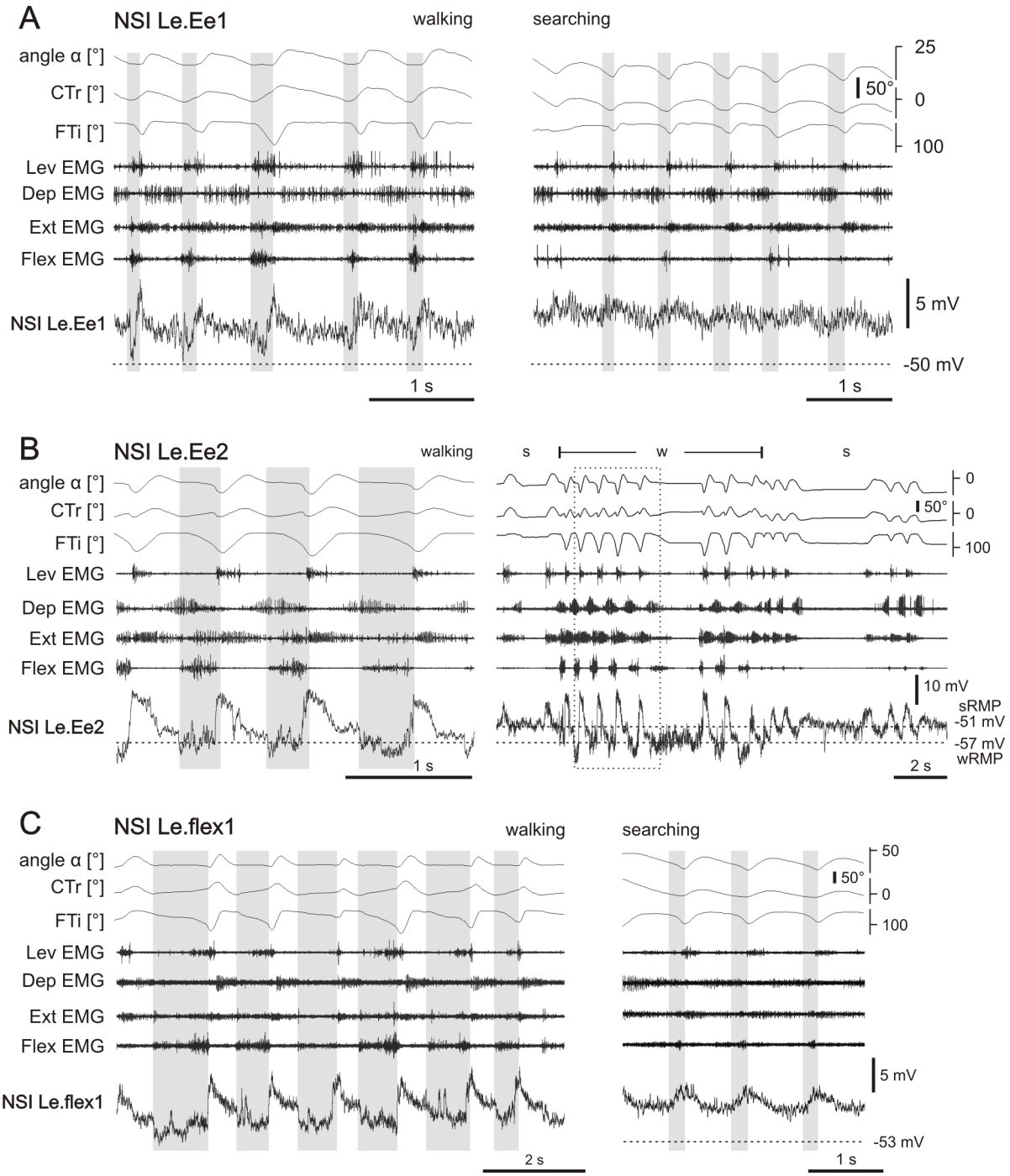

Figure 3.48 – Membrane potential modulation during searching and walking behavior of three interneurons that influence multiple leg joints; NSIs Le.Ee1 (A), Le.Ee2 (B), Le.flex1 (C). Gray bars denote stance or flexion phase, respectively. Dotted box denotes position of shown enlargement. "s" searching behavior; "w" walking behavior. "sRMP" resting membrane potential for searching behavior; "wRMP" resting membrane potential for walking behavior.

NSI	wRMP	wp-p	supp/opp	sRMP	sp-p	ΔRMP	N (w/s)
Li.De3	-48.3 (±1.5)	12.3±3.2	supp	-48.3 (±1.5)	7.6 (±1.7)	0	3/3
De8	-	15	(opp)	-55; -46	9.5; 11.5	-	1/2
de9	-	17	opp	-50; -52	11.6; 10.3	-	1/2
Le3	-	11	supp	-51	10.4	-	1/1
Le4	-	13	supp	-50	7.7	-	1/1
di3	-	10	opp	-38	3.3	-	1/1
I1	-47±1	15.7±3.8	supp	-53 (±3)	6.1 (±0.8)	+3.3 (±1.5)	3/3
I5	-36 (N=1)	22; 17	supp	-36; -57	11.8; 11.8	0 (N=1)	2/2
E2	-	17	supp	-50	11.7	-	1/1
E5	-59	20	supp	-53	7.2	-6	1/1
E10	-64 ±4	20.7±1.2	supp	-54.7 (±3.8)	11.5	-9.2 (±4.3)	3/3
E11	-57 ±9.6	19.5±2.5	supp	-53 (±5.3)	14.2 (±5.4)	-5.4 (±2.1)	4/4
E12	-	10	opp	-60	7.0 (±2.7)	-	1/1
I4	-56.4 (±4)	12.8±4.2	opp	-56.4 (±4)	11.5	0	6/9
E4	-58.5 (±3.2)	23±4.7	supp	-58,3 (±3,9)	10.6	-9.2	5/7
Le.Ee1	-56	10	supp	-56	4.7 (±2.6)	0	1/1
Le.Ee2	55.3 (±2.1)	21.3±2.9	supp	-52 (±2)	9.8 (±2.1)	-6.4	3/3
Li.Ee1	-62	19	-	-54	6.3	-8	1/1
Di.Ei1	-50 (N=1)	7.3±0.6	-	-54.7 (±6.4)	8.4 (±2.1)	-2 (N=1)	3/3
Le.Di.EF	-70 (N=1)	19.7±3.8	supp	-59.3(±1.2)	6.9	-4 (N=1)	3/3
Le.flex1	-56 (N=1)	9; 9	-	-55; -56	5.7 (±1.2)	0 (N=1)	2/2
Le.flex2	-	9	-	-63	5.8 (±0.8)	-	1/1

Table 3.2 – Summary of NSIs whose physiology was recorded during walking behavior throughout this work. "wRMP" resting membrane potential when the leg was resting on the treadwheel; "wp-p" peak-to-peak potential of membrane potential modulations during walking; "supp/opp" NSI physiology supports or opposes leg movements during walking; for comparison: "sRMP" resting membrane potential when the leg was resting in air; "sp-p" peak-to-peak potential of membrane potential modulations during searching; "ΔRMP" difference in resting membrane potential between searching and walking. Negative values indicate a more negative wRMP. "N" number of NSIs which were recorded during walking (w) and searching (s). "-" parameter could not be determined, e.g. because animal was not recorded in the resting state (RMP) or NSI influenced two muscle groups that are active during opposed searching phases (supp/opp).

stance phase. Only few were more strongly re- or depolarized (NSIs Li.De3, di3, I1, I5, E12). At the stance>swing transition some of the interneurons that had been hyperpolarized during stance were more strongly depolarized than during searching (e.g. NSIs, Le.Di.EF1, E4). Overall, phasic changes in membrane potential were more strictly coupled to phase transitions during walking than during searching. Changes in membrane potential occurred faster and their peak-to-peak value was larger. Also, some NSIs showed a different resting membrane potential depending on whether the leg was resting on the treadwheel ("walking RMP"; "wRMP") or in the air ("searching RMP"; "sRMP"). Especially, a pronounced difference was found in NSIs that excited or inhibited extensor MNs. A summary of the mentioned parameters describing NSI physiology during walking is shown in Tab. 3.2.

3.4 Physiology of nonspiking interneurons during obstacle contact and targeted searching movements

As previously mentioned, NSIs are candidates to mediate the targeted response that is shown when an animal touches an object with its leg during searching movements. Therefore, it would be interesting to investigate whether the sensory information caused by object contact is transmitted to the NSIs and reflected in their membrane potential. Also, the changes in NSI physiology during a targeted response and therefore the potential contribution of NSIs are an interesting objective for investigations. Therefore, NSI physiology was intracellularly recorded also during object contact and targeted searching movements as described on a behavioral basis in the first chapter of the results section. These experiments may be further extended but first results shall be presented here.

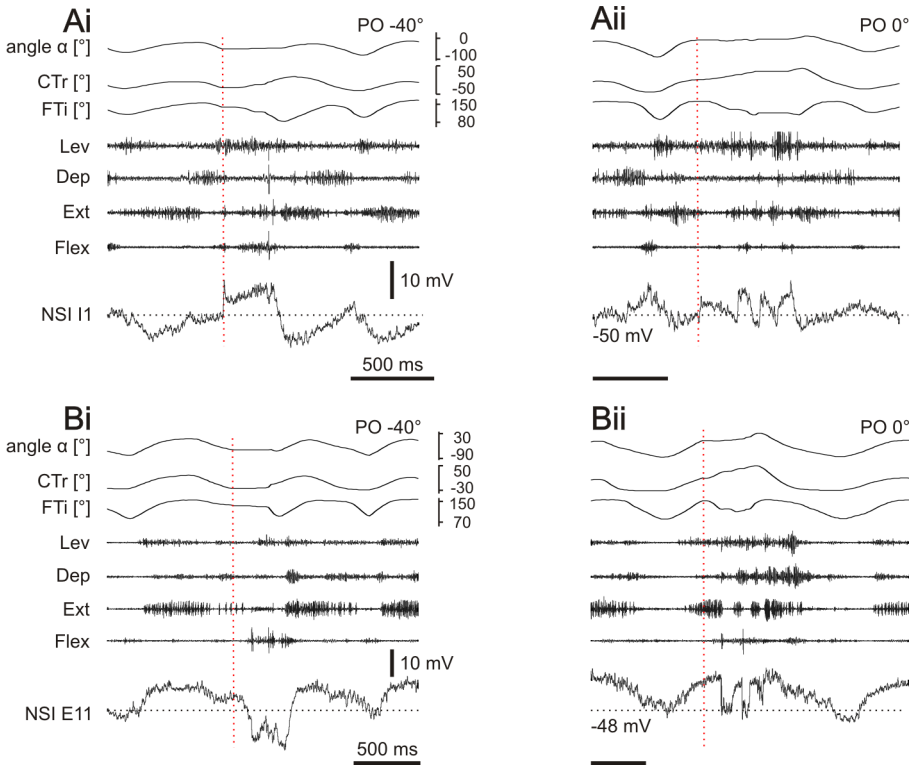

Figure 3.49 – Membrane potential modulation of NSI I1 (A) and E11 (B) when animals contact the object with the leg. Red dotted line indicates time point of initial contact. NSI I1 is depolarized both when contacting the object with the ventral leg in low positions (Ai) or dorsally in high positions (Aii). NSI E11 is hyperpolarized in both situations (Bi,Bii). Traces from top to bottom depict: overall leg movement ("angle α"), coxa-trochanter-joint movement ("CTr"), femur-tibia-joint-movement ("FTi"); electric muscle activity of levator ("Lev"), depressor ("Dep"), extensor ("Ext"), flexor ("Flex"); intracellular recording of NSIs I1 (A) and E11 (B).

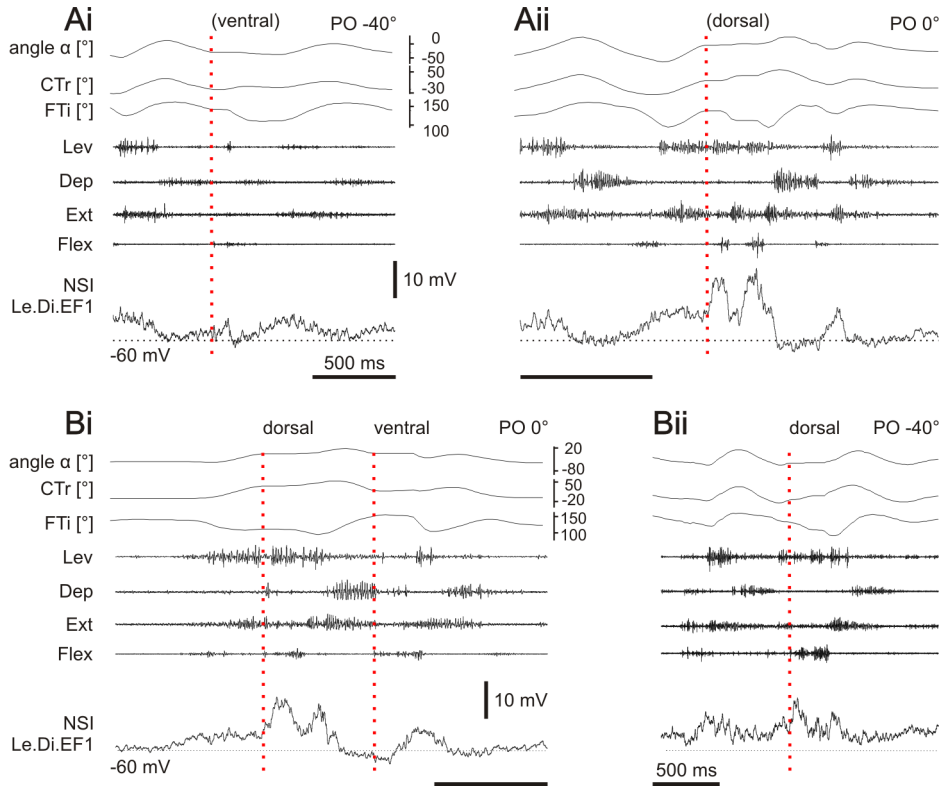

Figure 3.50 – Membrane potential modulation of NSI Le.Di.EF1 while animals contact the object with the dorsal or ventral leg in high or low positions, respectively. Red dotted line indicates initial contact. In Bi the object is first contacted with the dorsal leg and then a second time with the ventral leg. Traces from top to bottom depict: overall leg movement ("angle α"), coxa-trochanter-joint movement ("CTr"), femur-tibia-joint-movement ("FTi"); electric muscle activity of levator ("Lev"), depressor ("Dep"), extensor ("Ext"), flexor ("Flex"); intracellular recording of NSI Le.Di.EF1.

NSI	ventral contact	p-p	n	dorsal contact	p-p	n	N
I1	depol.	9.4 (±3.3)	5	depol.	10.6 (±3.4)	5	2
E11	(no change, then) hyperpol.	15 (±1) mV	13	(no change, then) hyperpol.	15 (±1) mV	14	4
Le.Di.EF1	no/weak change	4 (±2.2)	10	depol. (8)/ no change (2)	10.1 (±1.6)	10	3
Le.Ee2	hyperpol.	8.8 (±2.2)	9	short hyperpol., successive depol.	8 (±1.5)	10	3
I4	-	-	2	hyperpol.	5.5 (±1.6)	6	3
E4	hyperpol.	9.3±3.3	10	hyperpol.	5.9 (±2.1)	7	3
E10	hyperpol.	10.5 (±3.2)	9	short hyperpol., successive depol.	9.1 (±3.2)	10	3
Li.De3	depol.	4 (±1.6)	9	depol. (9)/ hyperpol. (1)	2.7 (±1.8)	10	3

Table 3.3 – The table summarizes the changes in membrane potential of several NSIs to leg contact with the object. "ventral contact", "dorsal contact": response to object contact with the ventral leg or dorsal leg, respectively; independent of the position of the object (PO). "p-p": peak-to-peak potential of membrane potential modulation. "N" number of animals, "n": number of evaluated object contacts.

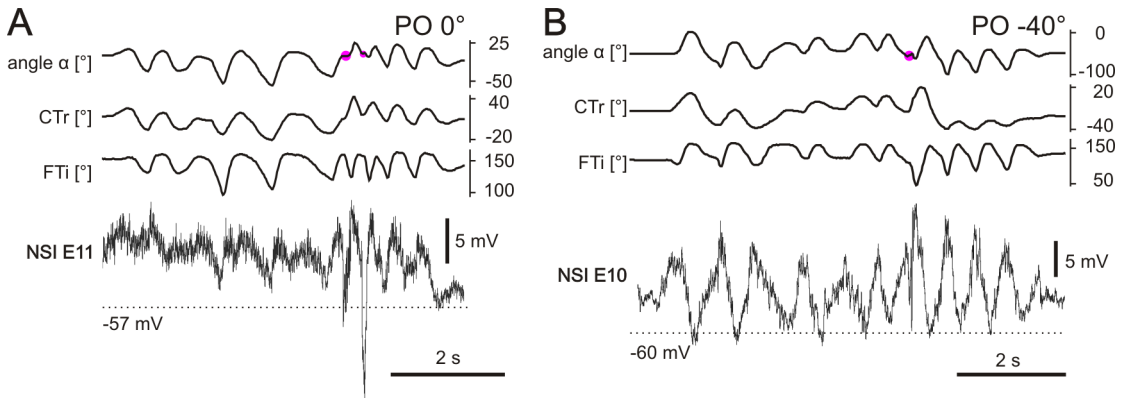

Figure 3.51 – Membrane potential modulation of NSI E11 (A) and E10 (B) during a targeted response. Magenta dot indicates time point and position of object contact. In A the object is shortly contacted a second time with the ventral leg (small magenta dot). Traces from top to bottom depict: overall leg movement ("angle α"), coxa-trochanter-joint movement ("CTr"), femur-tibia-joint-movement ("FTi"); intracellular recording of NSIs E11 (A) and E10 (B).

When the searching leg made contact with the object , a membrane potential change could be detected in all NSIs that were recorded (n = 61). The quality and quantity of the elicited change in membrane potential differed between recordings: some NSIs were clearly de- or hyperpolarized, others showed strong PSPs but without a summation to a clear de- or hyperpolarizing input.

The membrane potential changes of eight NSIs (Li.De3, I1, E10, E11, I4, E4, Le.Ee2, Le.Di.EF1) were exemplarily examined in more detail. Object contact elicited a characteristic response in each type of NSI. For example, NSI I1 was depolarized both when touching the object in a low position with the ventral side of the leg (Fig. 3.49 Ai) or in a high position with the dorsal side of the leg (Fig. 3.49 Aii). On the other hand, NSI E11 was hyperpolarized in both situations (Fig. 3.49 B). A third interneuron, NSI Le.Di.EF, was depolarized when contact was made in high positions with the dorsal leg (Fig. 3.50 Aii), but membrane potential did not change when the object was touched at low positions with the ventral leg (Fig. 3.50 Ai). The physiological response did not depend on PO but on whether the object was touched with the dorsal or ventral side of the leg. This is visible in Fig. 3.50 Bi,Bii where the response to object contact in a high position with the ventral side of the leg (PO = 0°, Fig. 3.50 Bi) and in a low position with the dorsal side of the leg is shown (PO = -40°, Fig. 3.50 Bii). This dependence on the direction of contact rather than PO was true for all NSIs that were tested for ventral and dorsal contact in high and low POs (Le.Di.EF1 (N = 2, n = 5), Le.Ee2 (n = 2), E10 (N = 2, n = 4)). Tab. 3.3 summarizes the response to object contact for several NSIs.

After object contact, animals often stopped their searching movements or did not perform a targeted response. If searching movements continued, NSI membrane potential

continued to be phasically modulated. Two such examples are shown in Fig. 3.51. If a targeted response occurred, the frequency of membrane potential modulations was increased according to increased frequency of leg movements (Fig. 3.51 A). Also, in some recordings amplitudes of searching movements and the amplitudes of NSI membrane potential modulations correlated (e.g. Fig. 3.51 B; compare NSI and FTi joint movement amplitudes; $R^2 = 0.41$). But as yet, the number of NSI recordings during targeted responses is to low to determine the consistency and generality of such results. Generally, movement amplitudes and membrane potential modulation amplitudes during searching (pooled values of targeted and untargeted searching) could correlate strongly ($R^2 = 0.92$, NSI E11). However, the preliminary results were inconsistent even in different specimens of the same NSI and therefore are not presented here.

In summary, contact of the leg with the object seems to elicit a strong sensory signal that is transmitted and distributed to a wide array of premotor NSIs. Each NSI responded to contact of the dorsal or ventral side of the leg by characteristic de- or hyperpolarization, respectively. The response was not dependent on the position of the object (PO). The analysis of changes in membrane potential linked to a targeted response seems possible but more data has to be acquired.

4 Discussion

4.1 Kinematic analysis of targeted searching movements [1]

Throughout this thesis, I could show that stick insects performing untargeted searching movements with a leg execute a targeted response upon a one-time contact of the leg's tibia with an object that is immediately removed after contact. The targeted response consists of two components: searching movements are confined to the former position of the object (PO) by (1) a shift in average leg position and (2) a decrease in movement amplitude. Both changes appear to be independent of each other. Average position and amplitude generally regain initial values after approximately 6 s. I interpret this data as a targeted response that wanes over time. Visual sensory information is not necessary to control this behavior. In contrast, sensory information on the position of the CTr and FTi joint is essential for the targeted response. The targeted response can be mediated on a local thoracic level. The decreased amplitude of searching movements after contact coincides with an increased frequency of these movements. The shift of average leg position towards the PO is accompanied by a change in the *levator* to *depressor trochanteris* muscle electrical activity ratio, which is due to alterations in both or either one of the muscle activities. Stick insects perform targeted responses with their pro- and mesothoracic leg.

4.1.1 Undisturbed searching movements

In accordance with previous results (Karg et al., 1991; Dürr, 2001), I observed undisturbed searching movements of the intact leg to be very stereotyped and composed

[1] Major parts of the discussion section "Behavioral analysis of targeted searching movements" are already published in "E. Berg, A. Büschges, and J. Schmidt (2013) *Single perturbations cause sustained changes in searching behavior in stick insects*" J. Exp. Biol. 216, 1064-1074. The authors contribution are: EB, AB, and JS designed research; EB performed experiments, analyzed data and prepared figures; EB, AB, and JS wrote manuscript. Parts of the discussion are taken from the publication literally or with minor modifications. Additional sections were added where necessary, e.g. concerning ablation of fCO, CS, or brain; experiments with the mesothoracic leg.

of the same sequence of events in time and space as described by Karg et al. (1991). When the trochanteral hairplate (trHP), which measures the position of the CTr joint (Schmitz, 1986a; Schmitz, 1986b), was ablated, the amplitudes of undisturbed searching movements were larger compared with searching with the intact leg. This finding is in agreement with the results of Wendler (1964), which showed that walking animals lifted their legs higher during swing phase if the trHP was ablated. I did not observe a change in inter-joint coordination upon ablation of trHP as was described by Karg et al. (1991). In line with my results, Akay et al. (2001) also found no change in inter-joint coordination upon ablation of the trHP. The ability of the trHP to affect inter-joint coordination might depend on the leg's position, as legs were fixed to different angles to the body axis (60° in experiments by Karg et al. (1991), but 90° in experiments by Akay et al. (2001) and in my experiments). Upon ablation of the femoral chordotonal organ's (fCO) apodeme, I partially could confirm a change in joint coordination as described by Karg et al. (1991): Animals did not flex their FTi joint or, if flexed, the joint remained flexed for a longer time also during upward movements. Different from results by Karg (1991), no searching movements in which only FTi joint movements occurred were observed. Also, animals performed searching movements with the same coordination of CTr and FTi joints as seen with intact leg, a fact that had not been described by Karg et al. (1991).

4.1.2 Targeted movements

The initial reaction upon touching an object was to grasp for it. This grasping behavior, which was described by Bässler et al. (1991), demonstrates that the animals are not avoiding the object but instead are attracted to it. In my experiments the animals could not grasp the object because it was removed, but instead they displayed the targeted response.

As is apparent from experiments with brain-ablated animals, the modifications seen throughout a targeted response do not require the brain but are likely to be mediated on a local thoracic level. This is consistent with findings from Berkowitz and Laurent (1996b) and Matheson (1997) who showed locusts to perform site-specific grooming with their meso- and metathoracic leg in response to tactile stimuli –even after isolation of the meso- and metathoracic ganglia. Also, Berni et al. (2012) demonstrated that Drosophila larvae modify their crawling sequence according to sensory input on the level of the ventral nerve chord without the brain and SEG.

To determine the PO, the animals might use a sensory signal indicating the leg's contact with the object as a trigger to 'read out' information on the leg's position. Removal of distal sense organs by substitution of the tibia by a 'peg leg' did not affect the ability

to perform a targeted response. Thus, tibial tactile hairs or tibial campaniform sensilla (CS), sense organs in the cuticle that detect forces as cuticular strains (Pringle, 1938a; Pringle, 1938b; Zill et al., 2004), are not crucial for triggering the targeted response. In that line, Bässler et al. (1991) report that the typical leg movements preceding grasping behavior are still performed after substitution of the tibia. Also, additional ablation of all femoral and trochanteral CS did not prevent targeted responses. Therefore, making leg contact with the object seems to elicit a strong and redundantly sensed signal. This is plausible, as object contact not only exerts forces on the leg but also slows down or even blocks leg movements, which might be sensed by e.g. stretch receptors or velocity sensitive cells of chordotonal organs (e.g. tension receptor, apodeme receptor (Bässler, 1977), fCO (Borchardt, 1927; Hofmann et al., 1985)). This view is also in accordance with the intracellularly recorded reliable and strong physiological response of nonspiking interneurons (NSIs) to object contact which will be discussed later.

Shift in average leg position

The trHP appears to be an important sensor that provides position information, as ablation of trHP decreased the percentage of shift towards the PO from ~75 to 54% (Fig. 3.13 B). This view is in accordance with Schmitz (Schmitz, 1986a; Schmitz, 1986b), who furnished evidence that the trHP is the only feedback transducer in the CTr control loop, and with Cruse et al. (1984), who showed that ablation of trHP leads to false estimation of a leg's position in inter-leg targeting. However, other sense organs measuring the position of the CTr joint might also contribute to mediation of the targeted response, as ablation of trHP did not always inhibit the ability to display a shift towards the PO and also to decrease the amplitude. Such sense organs are coxal strand receptors cxtrSR1 and cxtrSR2 (Bräunig, 1982aa; Bräunig, 1982bb; Schöwerling, 1991) or coxal muscle receptor organs, as shown for Locusta (Bräunig, 1982aa). Yet, according to Schmitz (Schmitz, 1986bb), a contribution of internal sense organs to the CTr loop would depend on an intact trHP.

Considerations regarding underlying muscle activity The contribution of the CTr joint to the shift in average leg position appears to be based on a shift in the ratio of EMG activity in the *levator* and *depressor trochanteris* muscles. For example, an increase in levator activity, a decrease in depressor activity or a change in both muscles could contribute to an upward shift. All such changes were observed which indicates that there is no unitary strategy to shift the average leg position. This fits an observation made by Bässler et al. (1991), who states that *levator* and *depressor trochanteris* muscle activity is highly variable although the underlying movement is stereotypical. An alternative mechanism for a shift in average leg position could be a 'catch-like'

effect in the levator or depressor muscle. This effect describes the phenomenon that a short, high-frequency discharge of a motoneuron leads to a long-lasting increase in force production of a muscle that is maintained at a low frequency of motoneuron discharge. The effect has been described for crustacean muscles (Blaschko et al., 1932; Günzel and Rathmayer, 1994), locust tibial muscles (Wilson and Larimer, 1968) and the mesothoracic extensor tibiae in the stick insect *Carausius morosus* (Guschlbauer, 2009). If trochanteral muscles of stick insects possess catch-like properties, such an effect might hold the leg in the region of the PO. However, this explanation is a less likely alternative because we did not observe high-frequency discharges in EMGs. Furthermore, a catch-like effect perhaps triggered by a CS discharge is likely to interfere with grasping behavior and searching movements.

The average position of the CTr joint (angle β) can be set and rhythmic searching movements can be performed by activation of a single muscle (Fig. 3.8 B). In the example shown, rhythmic levator activity was working against passive forces of the depressor muscle that is not activated. As the *depressor trochanteris* muscle is innervated by only two excitatory motoneurons (Schmitz, 1986b; Goldammer et al., 2012), it is unlikely that there were EMG spikes in some remote part of the muscle that were not picked up by the electrodes. Comparable to CTr joint movements in the stick insect, rhythmic movements of the FTi joint in the locust hind leg may be controlled by the flexor or extensor tibiae muscles working against passive forces in the antagonist that is not activated by motoneurons (Berkowitz and Laurent, 1996b; Page et al., 2008).

Do passive forces determine the average leg position? Generally, small limbs assume gravity-independent rest positions without activity in leg motoneurons (Hooper et al., 2009; Ache and Matheson, 2012). The gravity-independent position of a joint depends on the passive forces of the antagonistic muscles (Hooper et al., 2009). However, it is unlikely that passive forces determine the average leg position after contact, as these positions change depending on object position and therefore differ from relatively fixed 'intrinsic' resting positions of a joint.

Decrease in amplitude and increase in frequency

The second component of the targeted response consists of a decrease in movement amplitude accompanied by an increase in movement frequency. Both changes seem to critically depend on position signals from the CTr and FTi joint, as after ablation of either trHP or fCO the percentage of responses that showed reduced amplitude and increased frequency dropped drastically from ~83 to 34% (trHP) and 63 to 5% (fCO, lower positions). Thus, information about contact with the object without information from trHP or fCO is not sufficient to reliably induce the observed changes in amplitude and frequency.

In the stick insect, stimulation of the trHP by elevation of the leg activates depressor motoneurons (Schmitz, 1986b). Also, fCO elongation and relaxation elicits switches from depressor to levator and levator to depressor activity, respectively (Hess and Büschges, 1999). Finally, stimulation of the fCO by FTi joint flexion in the active animal first induces assisting flexor excitation and extensor inhibition, and then –when the FTi joint is in a flexed position– activates the extensor (Bässler, 1986). In my ablation experiments, these influences are missing and this might contribute to the loss of the control of amplitudes. Thus, it appears that trHP and fCO contribute to setting the movement amplitude and frequency of the pattern-generating networks that control the CTr and FTi joints during searching. Such sensory influences on the timing and magnitude of motor activity have already been shown for movement, position and load sensors in the walking system of the stick insect (Büschges and Gruhn, 2008).

Interestingly, while ablation of the trHP affected targeting in all POs, fCO-apodeme ablation only affected targeting in lower POs. This might imply that fCO integrity is especially important for signaling flexed joint positions. This fits results of Hofmann et al. (1985) who recorded from units in the chordotonal nerve and found far more units signaling a flexed position than an extended position of the FTi joint. On the other hand, fCO signals might be as important in determining extended FTi joint positions – but reduced searching movement amplitudes after fCO-apodeme ablation still result from mechanical constraints in the maximally extended FTi joint. The topic of mechanical constraints will be addressed also when discussing the impact of induced alterations in interneuron membrane potential on searching movements later on.

Overall, it seems that ablations of joint position sensors are not as easily compensated as ablations of contact sensors, which might indicate less redundancy in signaling.

4.1.3 Comparison with other systems and considerations of possible underlying cellular mechanisms

With approximately 6 s, the targeted response far outlasts the duration of the given stimulus, and the underlying mechanism might be regarded as a simple form of short-term memory as the behavior refers to an already vanished stimulus. The behavior is reminiscent of effects shown in insects for which a ‘positional memory’ has been suggested. For example, walking fruit flies show a persistence of orientation for approximately 8 s toward a landmark that disappeared during the fly’s approach (Strauss and Pichler, 1998). In walking fruit flies, the width of a gap that is being approached appears to be stored for a short time (‘subsecond memory’) to be read out upon tactile contact with the gap (Pick and Strauss, 2005). And ladder-walking locusts appear to memorize a rung’s position, as dislocation of a rung during the legs’ swing phase did

not lead to modification of the step but evoked persistent searching movements in the former rung position (Niven et al., 2010). In contrast to our experiments, the former examples of short-term memory involved visual input.

Grooming movements of a locust hind leg that are targeted towards a stimulus side on a wing involve a short-term positional memory similar to the searching movements in our experiments because grooming movements may outlast electrical or mechanical stimuli (Matheson, 1997, 1998). Because of the short-term 'learning' period and a similar time span of memory in the range of seconds, the underlying neuronal mechanisms might be similar. These mechanisms are very likely to be different from those involved in leg position learning in cockroaches and locusts, which are based on repetitive aversive stimuli, and memory in these experiments may be retained for hours (Horridge, 1962; Stowe and Leader, 1975; Hoyle, 1979). The means by which information is 'stored' for a time period of a few seconds might be a result of the intrinsic membrane properties of participating neurons. Such properties can cause long-lasting effects in neurons that alter their firing activity upon preceding excitation. Properties include plateau-like properties based on diverse inward currents (Marder, 1991; Major and Tank, 2004), slow K^+-channel kinetics (Turrigiano et al., 1996; Marder et al., 1996), slow post-inhibitory rebound mechanisms (Goaillard et al., 2010) and after-hyperpolarization depending on Na^+/K^+-pump activity (Pulver and Griffith, 2010). As time constants of these cellular 'memory' mechanisms correspond to the recovery time constants, it is worthwhile to look for such a mechanism in those premotor interneurons that control leg movements. As quite a few of those neurons have been identified (Büschges et al., 1994; Büschges, 1995; Kittmann et al., 1996; von Uckermann and Büschges, 2009; Rosenbaum, 2013), the stick insect searching leg preparation is well suited for unraveling the mechanisms of adaptation and short-term memory in a motor system. This especially refers to nonspiking interneurons which are well described in the stick insect (Büschges et al., 1994; Büschges, 1995; Kittmann et al., 1996; von Uckermann and Büschges, 2009; Rosenbaum, 2013) and provide a starting point for further investigations.

4.2 Activity of premotor nonspiking interneurons during searching and walking

In the second and third part of this thesis, I describe the activity of nonspiking interneurons (NSIs) during searching and walking behavior. NSI activity during searching was analyzed here for the first time for a larger set of interneurons. For walking, the known activity of several NSIs could be extended by the description of newly characterized interneurons.

Characterization of NSIs and description of NSI activity during searching All NSIs recorded for this thesis were shown to exert drive on one or more motoneuron (MN) pools of the coxa-trochanter (CTr) or femur-tibia (FTi) joint and therefore could elicit leg movements when artificially depolarized by current injection. The membrane potential of all recorded NSIs was shown to be modulated during searching movements. Therefore NSIs are a premotor neuronal element for the generation of searching movements. While the activity of most NSIs supported ongoing leg movements, some NSIs opposed leg movements. This is in accordance with the "parliamentary principle" (Bässler, 1993a) which will be addressed in the next section. The membrane potential modulations of some NSIs were not clearly assignable to either upward or downward movements of the leg which contrasts the situation during walking behavior and will be discussed later. No fast transitions in membrane potential were detected but rather, modulations were undulated. The peak-to-peak potential of membrane modulations varied from 2.4 to 14.2 mV in different NSIs.

NSIs were characterized and identified based on their morphology, membrane potential changes during searching and walking, their response to fCO stimulation and the drive they exert on leg MN activity when they were de- or hyperpolarized by current injection in the resting animal. The exerted drive to MNs was determined by EMG recordings from leg muscles. Setting up EMG recordings is faster and less invasive than setting up nerve recordings. This shortened the time of preparation and clearly increased the likelihood to evoke searching sequences after preparation. Leg movements were closely monitored via video recordings. This way, it could be easily determined how each NSI influenced movements in each of the leg joints. Possibly, in large muscles like the *extensor* and *flexor tibiae* muscle a part of the electrical muscle activity might not have been picked up by the EMG electrodes in the proximal third of the muscle. According to the distribution of muscle fibers (Bässler et al., 1996 and Elzbieta Godlewska, personal communication) this may mainly concern slow fibers. Indeed, artificial alteration in NSI membrane potential sometimes induced leg movements without eliciting a clear EMG signal. Nevertheless, in combination with video recordings it was possible in all but three NSIs (ext1, Le.flex1, Le.flex2) to determine the change in muscle activity underlying the elicited movement.

As another criterion to characterize NSIs, the response to fCO stimulation was recorded. In my experiments, the fCO was stimulated indirectly via passive flexion and extension of the FTi joint when the leg was resting on a treadwheel. Although this method might be rather crude because other sensory organs, e.g. campaniform sensilla measuring load (Zill et al., 2011, 2012), might be coactivated, it has been successfully used and proven to generate valid results already in previous studies by von Uckermann (2008) and Rosenbaum (2013).

Besides recordings from previously known NSIs, several other NSIs were recorded and newly characterized. This includes six NSIs influencing the MNs controlling CTr joint movements (Li.De3, De8, de9, Le3, Le4, di3), six NSIs influencing MNs controlling FTi joint movements (I5, fe1, E10, E11, E12, ext1), and six NSIs influencing MNs controlling movements of both joints (Le.Ee2, Li.Ee1, Di.Ei1, Le.Di.EF1, Le.flex1, Le.flex2). It should be noted, that only in a few cases it was possible to determine whether the influence on both joints was directly mediated by the NSIs as most experiments were done in a closed loop situation. This means, movements in one joint, which are caused by a direct influence of the NSIs, might influence the second joint via sensory feedback that cause inter-joint reflexes (Hess and Büschges, 1997, 1999). Because in the resting animal the known inter-joint reflexes rely on FTi joint movement (Hess and Büschges, 1997, 1999; Akay et al., 2001), for a few NSIs experiments with stiffened FTi joint were conducted. According to these experiments, the effects of NSIs Le.Ee1 and Li.Ee1 on both CTr and FTi joints seem to be mediated directly. These NSIs still elicited EMG activity in muscles of both joints in experiments with stiffened FTi joint. Additionally, the effect of NSI Le.Ee2 might be mediated directly, because the inter-joint reflexes known so far do not result in simultaneous levation and extension movements (Hess and Büschges, 1997, 1999). The effect of other NSIs on both joints may well be mediated via sensory feedback in form of inter-joint reflexes. Known forms of inter-joint reflexes that may apply here include flexion and extension of the FTi joint that lead to levation and depression of the CTr joint, respectively (Hess and Büschges, 1997, 1999). Thus in the future, it might be worthwhile to record from the respective NSIs when sensory feedback is prevented in order to determine the directly provided drive. Meanwhile, this does not derogate the results presented here. Quite the contrary, the experimental situation with intact sensory organs represents the natural situation and the results presented here nicely reflect the effects of NSIs on animal behavior under natural conditions.

Distributed processing and the parliamentary principle The membrane potential of all recorded premotor NSIs was phasically modulated during both searching and walking behavior. No NSI could be recorded that was active in only one of the behaviors. This demonstrates NSIs to take part in the generation of several behaviors and supports the concept of "distributed processing" (review e.g. Kristan et al., 2002). This concept postulates the same interneurons to contribute to several behaviors, the respective weight of the contribution depending on the behavioral background. This allows for the generation of manifold behaviors with a relatively little set of neurons. The concept is opposed to the concept of "dedicated processing" (review e.g. Morton and Chiel, 1994) that allots a separate set of interneurons to the generation of each behavior. There have been examples of neurons that act "dedicated" (e.g. Heitler, 1985;

Ramirez and Pearson, 1988) and multifold examples of distributed networks (e.g. leech bending: Lockery and Kristan, 1990; pyloric and gastric mill rhythm in crab: Weimann et al., 1991; fictive stepping and scratching in cat: Berkinblit et al., 1978). In the stick insect, the same NSIs were found to contribute to forward and backward walking which might be regarded as two separate behaviors (Rosenbaum, 2013). Also, single NSI E4 was described to be active during searching and walking (Büschges et al., 1994).

Furthermore, Bässler (1993a) coined the term "parliamentary principle" which essentially describes the simultaneous activity and contribution of antagonistic acting pathways to the same behavior. This means, the drive that an interneuron exerts on motoneurons during rest is not necessarily reflected in its phase of activity during behavior. For example, during walking behavior NSI I4 is depolarized during swing thereby acting against leg movements (von Uckermann and Büschges (2009) and this work). In a similar way, during searching behavior NSI I4 was depolarized early during upward movements of the leg. Also, the activity of NSIs de9, di3, and E12 counteracted ongoing searching movements (and walking movements). Overall, whether the activity of single NSIs supported or opposed the behavior was not as clear during searching as during walking. This will be addressed again later on.

While theoretically the two concepts –distributed processing and parliamentary principle– are not necessarily the same because they emphasize different aspects (i.e. participation of neurons versus synergistic/antagonistic effects; e.g. there might be dedicated networks with antagonistic parallel pathways), the parliamentary principle might be regarded as the implementation of a distributed network. In this context, I would like to point out that the four NSIs that were shown to counteract searching also counteract walking. Therefore, a behavior that might be supported by these NSIs has still to be found.

4.2.1 Modulation of searching behavior by manipulations in nonspiking interneurons

Specificity of modulations in searching movements In my experiments, I found NSIs to influence several different parameters that describe searching movements, i.e. movement amplitude, position, velocity, inter-joint coordination, and general drive to searching movements. The parameters that were influenced by a given NSI were specific, as was apparent when single identified NSIs were recorded repeatedly. Different specimens of a certain identified NSI affected the same parameters in a very similar way. For example, NSI E10 when artificially depolarized decreased movement amplitudes, when artificially hyperpolarized increased movement amplitudes ($N = 3$). Differently, NSI E11 mainly decreased the velocity and changed the inter-joint coordi-

nation of searching movements when artificially depolarized, and showed weaker effects on movement amplitudes (N = 4).

The strength and reliability of the occurrence of the effect could vary between different specimens of the same NSI. There are several reasons that might contribute to this variance: first, the quality of electrode placement in the NSI is one factor that determines how well current is transmitted into the neuron and therefore how well the neuron might affect behavior. Second, although recording from the same NSI, the electrode might be positioned in different neurites. NSIs are discussed to be strongly compartmentalized (e.g. Laurent and Burrows, 1989b) due to their passive electrotonic properties and intermingled input and output synapses (Wilson and Phillips, 1982). Therefore, the position of the electrode might heavily influence the effects that can be caused by current injection. Third, manipulation of single NSIs takes place against the background of a largely intact neuronal network that might be in different states due to sensory local and intersegmental influences or neuromodulatory effects. For example, the excitability of given neurons might be different or the overall activity level in the network might vary depending on, exemplarily, the arousal of the animal. Such differences might then affect how efficiently current injections into NSIs impact the motor output. In this respect it is interesting that sensory signals –as an example of stereotypical influences on interneurons– have already been shown to be processed state dependent (Bässler, 1986; Hess and Büschges, 1997; Akay et al., 2007). Despite these sources of variability, single identified NSIs showed a typical effect on searching behavior.

Several NSIs were not able to affect ongoing searching movements in any detectable way (e.g. NSIs Li.De3, De4, E12). This might be due to issues of electrode placement and uncontrolled network state as discussed above. However, one of these NSIs (Li.De3) was recorded repeatedly (N = 3) and never showed an influence on searching movements. Therefore, the inability to change searching movements might be as characteristic for some NSIs as is for other NSIs the ability to influence certain parameters. The notion that some NSIs are not able to influence ongoing searching movements is surprising in so far, as all recorded NSIs were able to elicit leg movements in the resting animal and their membrane potential was phasically modulated with leg movements. On the other hand, leg movements in the active animal are controlled by a pool of premotor neurons that provide converging drive to MNs. Therefore, changes in the output of one NSI might simply disappear in the summed action of the network. From this point of view, it rather seems surprising to find NSIs that as an individual can change ongoing searching movements when manipulated.

Changes in general drive of searching movements Certainly, NSI I4 assumes a special role within the group of NSIs that influence ongoing searching movements. An artificial tonic depolarization of the membrane potential of this single identified interneuron was found to entirely drive or strongly facilitate the generation of rhythmic searching movements. These induced searching movements showed the usual coordination of CTr and FTi joint (not shown). At the same time, NSI I4 seems to be necessary for the generation of searching, because spontaneous searching movements stopped when the interneuron was artificially hyperpolarized.

Both facts are reminiscent of the criteria for so called "command neurons" (Kupfermann and Weiss, 1978) which demand of these neurons to be both sufficient and necessary in order to induce a certain behavior. Indeed, the two recorded specimens of NSI I4 that, when artificially depolarized, entirely drive searching movements without the need for additional "tickling" of the animal meet the strict criteria by Kupfermann and Weiss (1978). The weaker effects of other recorded NSIs I4 might be attributed to issues of electrode placement in the NSI and other sources of variability as discussed above.

On the other hand, command neurons might implicitly be expected to be located on a higher hierarchical functional level than the networks they drive. Many examples of such neurons have been shown (e.g. Wiersma and Ikeda, 1964; Pearson et al., 1985; Flood et al., 2013; Wu et al., 2014). Different from such a structure, NSI I4 was shown to be part of the central pattern generating (CPG) network for (CTr) joint control (Büschges, 1995) and therefore might be assumed to be part of the CPG also during searching behavior. This does not quite fit the notion of a command neuron. It might be added that for the generation of rhythmic activity in some animals a reciprocal interconnectivity between command neurons and rhythm-generating networks has been discussed. In these preparations command neurons are rhythmically modulated with the behavior (Davis and Kovac, 1981; Gillette et al., 1982).

Regardless of its classification, NSI I4 might drive searching movements by increasing the overall arousal in the CPG network. In this respect, it is interesting to note that during searching the membrane potential of NSI I4 is strongly tonically depolarized and only weakly phasically modulated. In MNs, a tonic depolarization was shown to underlie walking and searching sequences and was interpreted to reflect the general arousal of the motor system (Ludwar et al., 2005; Büschges et al., 1994). Accordingly, the membrane potential of NSI I4 during searching might be thought to strongly reflect the arousal state of the network and rather weakly the phasic (sensory) signals related to movement cycles.

Whether the central drive of NSI I4 alone is sufficient to induce searching or whether the contribution of sensory feedback is crucial is not clear from my experiments because of the animals' intact leg sensory organs. Related to this topic, Büschges (1995)

described the effect of NSI I4 on the central leg joint control networks in a deafferented and deefferented preparation. In such a preparation, application of the muscarinic acetylcholine agonist pilocarpine on the nerve chord induces rhythmic alternating activity in antagonistic MN pools of each leg joint (Büschges et al., 1995). This activity is interpreted as the CPG generated activity of the network for leg joint movement control (Büschges et al., 1995). In non-rhythmic preparation, such a rhythmicity could not be induced by sustained tonic depolarization of NSI I4. This suggests I4 not to function as an "endogenous oscillator" (Büschges, 1995). At the same time, this result indicates that the ability of NSI I4 to drive searching movements relies on sensory feedback.

As a second aspect, Büschges (1995) showed a sustained hyperpolarization of I4 to decrease the frequency of the pilocarpine induced rhythm. In contrast, in my experiments searching cycle frequency could not be shown to rely on the strength of current injection. Furthermore, in my experiments current puls injection into NSI I4 could not reset the searching movement rhythm. This is unexpected, because NSI I4 was shown to belong to the CPG for leg joint control and able to reset pilocarpine induced motor rhythms (Büschges, 1995). However, my results might largely be explained by the fact that two of the three NSIs I4 tested by current puls injection did not shown a "driving" effect for searching movements either.

As one possibility how searching movements might be generated in interaction of I4 activity and sensory feedback, NSI I4, when artificially depolarized, might elicit depression and flexion movements according to its known central drive on the corresponding MNs (Sauer, 1996; Büschges, 1995). The transition to levation/extension and the following upward movement could be caused by sensory feedback from the femoral chordotonal organ (fCO), a sense organ that measures FTi joint movement and position. Sensory intra- and inter-joint reflexes that fit this transition and which therefore might contribute have been described (Bässler, 1988; Hess and Büschges, 1997, 1999; Bucher et al., 2003). The transition from levation/extension to depression/flexion might be supported by known reflexes caused by signals from fCO and the trochanteral hairplate (trHP) measuring the position of the femur (Schmitz, 1986a,b; Hess and Büschges, 1997, 1999; Bucher et al., 2003). In order to test the importance of specific sensory signals, NSI I4 should be recorded and tonically depolarized in animals with (partially) ablated leg sensory organs. Particularly the trHP and fCO as the main sensory organs (Schmitz, 1986b; Hofmann et al., 1985) measuring movement and position of CTr and FTi joint, respectively, might be important.

In contrast to the prominent effect of I4, the second interneuron that is known to be part of the CPG networks for joint control , NSI E4 (Büschges, 1995), does not elicit strong effects on searching movements. Only in two recordings, a variable effect on movement

amplitude was detected. NSI E4 is known to (amongst others) excite levator and extensor MNs and to inhibit depressor and MNs and thus acts on MNs complementary to NSI I4 (Büschges, 1995). Therefore, the two phases of searching movements –upward and downward movement– might have been assumed to be generated by an interplay of NSIs E4 and I4. However, continuous de- or hyperpolarization of E4 neither facilitates nor prevents the generation of searching movements.

Interestingly in the context of rhythm generation, current pulse injections into NSI E4 were able to shorten or prolong searching movement cycles. Such a resetting effect was already shown on pilocarpine induced rhythmic MN activity in a deafferented preparation (Büschges, 1995). There, in FTi MNs the rhythm was always reset at the end of a depolarizing stimulus. In the CTr joint, whether cycles were shortened or prolonged depended on the phase of the stimulus. In my experiments, all stimuli were applied at approximately the same phase in the middle of the cycle, therefore, I could not properly analyze phase dependency. The large variability of my results might derive from the timing of the stimuli which were presented around the phase at which a transition from shortening to prolongation effect occurs in the CTr joint (Büschges 1995). Additionally, intra- and inter-joint sensory information that was present in my experiments may impact the timing of rhythm generation (e.g. Büschges, 2005). Nevertheless, the effect on the rhythm lasted markedly longer than the current stimulus, thereby meeting the criteria for a reset (Büschges, 1995).

Changes in specific parameters Of the NSIs that were able to influence searching movements, some influenced a whole set of parameters, others influenced single parameters. Amplitudes of movement were influenced most often (by 11 NSIs), movement position (5), velocity (4), and inter-joint coordination (5) were influenced approximately by the same number of NSIs. Only one NSI, I4, provided general drive to searching movements as described above.

Generally, NSIs affected searching movements in the same joint they had been shown to provide drive to in the resting animal. However, there were exceptions; these were made by NSIs Le3, E11, and Le.Di.EF1. The NSI E11 which in the resting animal induced only FTi joint movements, also affected CTr joint movements in the searching animal. This might be explained by a prolonged extensor activity that is caused by depolarization of NSI E11 during searching and that leads to a change in CTr and FTi joint coordination (Fig. 3.28). The effect of NSI Le3 on FTi joint movement amplitudes is in accordance with a weak extension movement that was seen when depolarizing the interneuron in the resting animal. Because the extension was weak and no EMG activity could be detected, the interneuron was grouped according to its excitatory drive on levator MNs but might additionally provide weak drive to extensor MNs. In

contrast, the strong effect of NSI Le.Di.EF1 on FTi joint movement amplitude and velocity can not quite be explained by its synaptic drive. When depolarized during rest, Le.Di.EF1 elicited CTr joint movement and muscle activity. Additionally, after stimulus end, simultaneous activity was detected in extensor and flexor EMGs but never elicited FTi joint movements (N = 3). Thus, the mechanism by which Le.Di.EF1 acts on the FTi joint is unclear. Finally, another interneuron, NSI fe1, excited flexor MNs when depolarized in the resting animal. When de- or hyperpolarized during searching, this NSI affected movements in both CTr and FTi joint. The effect is likely to rely on inter-joint reflexes that are caused by FTi joint movements (Hess and Büschges, 1997, 1999; Bucher et al., 2003). A shift of FTi movements to more flexed positions during depolarization of NSI fe1, therefore might indirectly cause changed (i.e. more elevated) CTr movements. This is in accordance with a small excitatory effect of NSI fe1 on levator MNs when depolarized in the resting animal. The excitation occurs with a high latency of more than 450 ms and therefore is assumed to be mediated by sensory feedback.

Remarkably, effects of NSIs on movement amplitudes were always based on movement changes in the FTi joint. Amplitudes were always in- or decreased by in- or decreased flexion and never seen to be decreased by diminished extension. This indicates that animals might generate amplitude decreases indirectly by using the mechanical constraints of the FTi joint. In such a case, animals might change their neuronal activity as if to shift their searching movements to more extended positions. Because even in uninfluenced searching movements animals maximally extend the FTi joint in each movement cycle, such a change in activity results in less flexion but the same degree of extension (due to mechanical constraints of the FTi joint). Therefore, decreased movement amplitudes in the FTi joint might not only be achieved by reduced flexor muscle activation, but also by increased extensor muscle activation. This is in accordance with the fact that many NSIs providing excitatory drive to extensor MNs decrease FTi-movement amplitudes when artificially depolarized. The decrease might be achieved by a simultaneous decrease in flexor activation or by co-contraction during flexion movements thereby decreasing flexion. However, evidence for the latter was not found in my experiments. The observation that movement amplitudes are decreased by decreased flexion with continuing maximal extension fits the results of the behavioral experiments in the first part of the thesis: Animals more successfully targeted high POs than low POs which was mainly due to a higher occurrence of significant amplitude reduction when targeting high positions.

At the same time, the strategy to in- or decrease movement amplitudes by in- or decreasing flexion but not extension movements, means that a change in FTi amplitude automatically results in a simultaneous change in average leg position. In the overview

shown in table Tab. 3.1 such a simultaneous change was not indicated as a shift in position in order to emphasize that positional shifts occur also independent of a change in amplitude (Tab. 3.1, first column). Such independent positional shifts were induced by five NSIs. Four of the NSIs influenced the position of both CTr and FTi joint. This went along with a change in inter-joint coordination, too. In contrast, NSI Le.Ee2 elicited a shift exclusively in CTr joint position and additionally changed FTi joint movement amplitudes. Remarkably, this did not change inter-joint coordination but rather restricted searching movements to a fragment of the usual working range. Interestingly, the same change in movements occurred during targeted responses, as was described in the first part of the thesis (Fig. 3.7).

The induced changes in CTr and FTi joint coordination were in accordance with inter-joint reflexes caused by FTi joint position and movement which affected the CTr joint (Bucher et al., 2003; Hess and Büschges, 1999). Only for one NSI, Li.Ee1, the influence on both joints was shown to possibly be mediated centrally. However, its central drive works in the same direction as the known inter-joint reflexes. Additionally, one NSI, E11, induced a change in coordination without changing joint positions. Instead, changes in coordination were caused by prolonged or shortened extensor activity.

Overall, effects on joint position and inter-joint coordination often seemed to interdepend. The reason might be that a shift in FTi joint position might cause a shift in CTr joint position via inter-joint reflexes (Bucher et al., 2003; Hess and Büschges, 1999). This –as a matter of the "sign" of inter-joint reflexes– at the same time leads to a change in inter-joint coordination as compared to coordination during uninfluenced searching. Exceptions from this interdependence were NSIs E11 and Le.Ee2.

Finally, NSIs were found to also influence the velocity of searching movements. This was visible in recordings of four NSIs, E11, Le.Ee2, Li.Ee1, and Le.Di.EF1. When these NSIs were artificially depolarized, decreases in the velocity of searching movements occurred simultaneously to decreases in movement amplitude. There was no experiment in which a decrease in movement velocity occurred together with increased movement amplitudes or *vice versa*. However, the influence of NSIs on each parameter could be weighted. For example, NSI E11, when depolarized, caused a weak decrease in movement amplitudes and a strong decrease in movement velocity. This can be seen also in a resulting decreased frequency of searching cycles (the three parameters movement amplitude, movement velocity, and cycle frequency form an interdependent triangle). On the other hand, NSI Le.Ee2 when depolarized leads to a strong decrease in movement amplitudes and a comparably weak decrease in movement velocity, as can be seen by an increased frequency of searching cycles. Therefore, the two parameters movement amplitude and movement velocity are, to a certain extent, influenced independently of each other because NSIs exert a weighted influence.

In summary, each NSI that affected searching movements influenced a characteristic set of parameters. The control of the different parameters was not strictly separated as expected from behavioral experiments where changes in amplitude and average leg position could occur independently. Instead, NSIs were found to influence more than one parameter at the same time. For example, all NSIs that affected movement amplitudes (in the FTi joint) at the same time induced changes in position (of FTi joint movements). Furthermore, shifts in position (without changes in amplitude) often coincided with changes in inter-joint coordination. This might be caused by sensory feedback that was present during the experiments. Also the "driving" effect of NSI I4 is likely to depend on sensory feedback. The number of parameters that were influenced differed between NSIs and could range from low numbers (amplitude and simultaneously position) to high numbers (position, amplitude, velocity, coordination). Although NSIs influenced multiple parameters, their effectiveness on these parameters could be weighted, .i.e. they exerted a strong effect on one parameter but a weak one on others.

To my knowledge, effects of manipulations in NSI membrane potential on ongoing rhythmic behavior have been reported only occasionally in the literature. In stick insects current pulse injections into single NSIs were reported to stop sequences of walking behavior (von Uckermann and Büschges, 2009; Schmitz et al., 1991) or shorten swing movements during walking (Schmitz et al., 1991). With respect to the differential effect of individual NSIs on different movement parameters that was shown in my experiments, it is interesting to note that Büschges (1995) described different NSIs to act on different parameters of a pilocarpine induced ("fictive") motor rhythm. As another effect on the MN level, artificial depolarization of E4 was shown to increase the response of levator MNs in an inter-joint reflex (Hess and Büschges, 1997). Such an amplification of inter-joint reflexes could underlie also the changes in inter-joint coordination observed in my experiments.

4.2.2 Comparison of NSI physiology during searching and walking

As mentioned before, the membrane potential of all recorded NSIs was modulated during both searching and walking behavior. The modulations' peak-to-peak potentials during walking ranged from 10 to 22 mV which is in the same range as described by von Uckermann and Büschges (2009) and Rosenbaum (2013). They were clearly larger during walking than searching in most NSIs (2.4 to 14.2 mV during searching; for comparison in each NSI see Tab. 3.2). In the majority of NSIs the peak-to-peak potentials more than doubled. Only two NSIs, Le3 and E12, showed roughly the same peak-to-peak potential in both behaviors. Furthermore, fast transitions in membrane potential occurred at the step phase transitions during walking (this work and von

Uckermann and Büschges, 2009; Rosenbaum, 2013). This is clearly different from the modulations during searching which are "gently rolling" and without fast transitions. Overall, it seems that modulations in NSI membrane potential are closely coupled to step phase transitions.

Remarkably, the differences described above are due mainly to a changed course of membrane potential in stance phase (during walking) as compared to flexion phase (during searching). The swing phase (walking) and extension phase (searching) show a similar course in membrane potential. Most NSIs (16 of 21) strongly hyperpolarized during stance phase, five NSIs depolarized.

For example, the hyperpolarization can be seen nicely in NSIs E5 and E11, where during searching the membrane potential (after depolarization during extension phase) slowly repolarizes to the resting membrane potential during flexion phase. In contrast, during walking it sharply hyperpolarizes far below resting membrane potential with the begin of stance phase. The membrane potential remains hyperpolarized throughout the entire stance phase. This can be seen nicely in NSI E10 where, during searching, the membrane potential starts to depolarize during flexion movements. In contrast, during walking the interneuron receives strong hyperpolarizing inputs until the end of stance. Some NSIs, e.g. E4, E10, Le.Di.EF1, Le.flex1, additionally show a stronger depolarization at the stance to swing transition than occurs during flexion to extension transition in searching. The stronger depolarization probably correlates with a larger and higher levation of the femur that occurs during walking and NSIs might receive stronger depolarizing inputs. Alternatively, the sudden overshooting depolarization after a hyperpolarization might be regarded as reminiscent of postinhibitory rebound effects in spiking neurons (Goaillard et al., 2010). Postinhibitory rebound spike-like events have been shown for nonspiking interneurons in the leech (Rela et al., 2009). However, in the study of Rela et al. (2009) the hyperpolarization steps that were necessary to induce postinhibitory rebound activity were much larger than the hyperpolarizations observed here during walking which are only at a scale of 5 mV. Therefore, postinhibitory rebound is unlikely to apply here.

These pronounced inputs during stance and the close coupling of modulations to step phase transitions suggest that ground contact –resulting in load– might be an important sensory signal that induces the major differences in NSI activity between walking and searching. This notion is supported by results from previous studies, in which load sensors, i.e. leg campaniform sensilla (CS) (Pringle, 1938a; Pringle, 1938b; Zill et al., 2004), were shown to provide important feedback for the control of step phase transitions (Akay et al., 2004). Furthermore, CS were shown to maximally fire in the stance phase in walking locusts (Newland and Emptage, 1996) and cockroaches (Noah et al., 2001). Finally, in the stick insect Zill et al. (2012) showed that CS are excited by load

that is generated by resisted leg movements but not by putative strains in the cuticle that might be generated by muscle contraction during unresisted movements. Therefore, CS are not excited during swing and searching movements. The connectivity of CS is not well explored. Akay (2002) showed fCS signals to cause hyperpolarization in most NSIs that provide excitatory drive to extensor MNs and depolarization in NSIs providing inhibitory drive. Whether these effects are directly mediated is not known.

Corresponding to the described stronger de- or hyperpolarization of NSIs during stance, also the resting membrane potential (RMP) of walking behavior was found to be "more positive" or "more negative" than that of searching behavior. The RMP was determined as the average membrane potential over a time course of several seconds when the animal was resting. For searching behavior, the RMP was measured when the leg was resting midair (sRMP), for walking it was measured when the leg was resting on the treadwheel (wRMP). A clear difference in RMP (up to 9.2 mV) was found especially in NSIs that excited or inhibited extensor MNs. In the NSIs recorded, the wRMP was found to be more negative than sRMP in NSIs providing excitatory drive, and more positive in NSIs providing inhibitory drive to extensor MNs. This change corresponded to the change in membrane potential during stance as compared to flexion. Also, it is consistent with the effects of fCS signals on NSI membrane potential by Akay (2002) that were described above. Therefore, the differences might be explained by CS that signal load not only when walking but also when the leg is resting on the treadwheel, thereby contributing to the resting membrane potential of NSIs. The differences in sRMP and wRMP are in accordance with a remark by Burrows (Burrows, 1996, section 3.7.4.1) who stated that the RMP of interneurons is variable because it is dependent on the context in which it is measured. Nevertheless, the resting membrane potential seems to be reproducible in certain contexts (see this work and stable RMP values in von Uckermann and Büschges, 2009; Rosenbaum, 2013) and therefore might still be used to characterize an interneuron.

The results are compatible with a hypothesis by Dürr et al. (2004) which postulates that searching movements might be continued swing movements. Indeed, upward movements during searching and beginning downward movements share a very similar pattern of muscle activation with the swing phase. This is, in both situations levator and extensor MNs are active, followed by a switch from levation to depression. Correspondingly and in favor of the hypothesis, also NSI activity was very similar for both behaviors, as was described above. However, the hypothesis is difficult to verify or falsify in so far, as certain movement phases during searching do not occur during swing and therefore can not be compared. This concerns the flexion phase during the second part of downward searching movements. This phase rather compares to the stance phase of walking but no equivalent is found during swing. Thus the activity of

the premotor network can not be compared between searching and swing for a part of the searching movement. Nevertheless, both the hypothesis and the results shown here agree that load signals provided by CS are not present during searching and that these signals might be the decisive sensory signals for the differences in membrane potential modulations of NSIs during searching and walking.

Finally, the clear difference in NSI activity between searching and walking also demonstrates how strongly sensory signals shape the activity of the (pre)motor network. This indicates the small importance of centrally generated activity in comparison to sensory induced activity; a result that was already drawn from previous work (Büschges, 1995, Bässler and Büschges, 1998) and can be confirmed here.

Interestingly, the NSI membrane potential modulations during searching and walking closely resemble the output of an artificial neuronal network (Shaw et al., 2012) that is in a state in which sensory input is ignored (when strong central drive is provided) and considered (when weak central drive is provided), respectively. Correspondingly, the activity that is recorded during searching movements might be regarded to be a rather direct reflection of the centrally generated rhythmic activity because it is unmasked from load signals provided by the CS (other sensory signals are still present though). In this regard, it is also interesting to notice that the amplitude of E4 membrane potential modulation during pilocarpine induced rhythmicity ("10 to 11 mV"; Büschges, 1995) is in the same range as peak-to-peak values during searching (9.8±2.1 mV) but not walking (23±4.7 mV in this work).

In summary, NSI membrane potential modulations during walking as compared to searching are larger and exhibit fast transitions which are closely coupled to step phase transitions. The main differences in membrane potential occur during stance phase in which NSIs are continuously strongly hyperpolarized or depolarized. This is probably caused by load signals which occur as a consequence of ground contact and are sensed by CS. NSI activity during swing phase is comparably similar to activity during extension phase.

4.2.3 NSI physiology during and after object contact

In the last chapter of the results section first data were presented describing NSI membrane potential modulations during obstacle contact of the searching leg, thereby tying up to the behavioral description of targeting in the first chapter. It shall be pointed out again that data are preliminary but were presented because they might delineate a way how to proceed with experiments.

In 24 recordings of eight NSIs, contact of the searching leg with the stick that was introduced into the plain of searching movements always elicited a clear signal. This

suggests that object contact is measured redundantly by different sensory organs and is transmitted to a large number of different interneurons. In support of this view, it has been shown in the locust that NSIs receive direct and indirect input from a wide array of sensory organs, i.e. CS (Burrows and Pflüger, 1988; Laurent and Burrows, 1988), tactile hairs (Laurent and Burrows, 1988), and proprioceptive sensors (Burrows et al., 1988). In stick insects, NSIs are known to receive input from the fCO (Büschges, 1990) and fCS (Akay, 2002). Also, sensory neurons were shown to make divergent connections with NSIs and other types of neurons (section 7.6.2.2 Burrows, 1996). As discussed previously, results of behavioral experiments with ablated sense organs suggest that object contact is sensed by multiple organs.

In response to leg contact with the object each NSI was characteristically de- or hyperpolarized. In some but not all NSIs the effect depended on the direction of contact, i.e. whether the object was touched with the dorsal or ventral side of the leg. Such a directionality of the response is reminiscent of results from previous studies which demonstrated NSIs to characteristically respond to fCO stimuli depending on the direction of the stimulus (Büschges, 1990). Different groups of CS were shown to separately encode the direction of force (Zill et al., 2012) and might project to NSIs in a specific pattern thereby providing directionality. Similarly, in the locust NSIs were shown to have tactile receptive fields on the leg (Laurent and Burrows, 1988) which lead to characteristic de- or hyperpolarization of the interneurons. According to the results so far, the response to object contact seems to be as characteristic for each NSI as is the response to fCO stimulation (Büschges, 1990) and thus might be used to identify NSIs.

When intracellular recordings were performed, after leg contact with the object, animals often stopped their movements. This is probably due to a generally decreased fitness of the animals after the preparation. The decrease was not caused by damage of the animals' leg (cuticle, muscles, nerves) or ganglion as was demonstrated by leg movements that still occurred.

In those NSIs that could be recorded during continued (targeted) searching, the membrane potential continued to be modulated in phase with leg movements as might be expected. A correlation of movement amplitudes with membrane potential modulation amplitudes was seen in some NSIs. However, due to the intact sensory feedback this does not allow to infer any causality. In the recordings so far, no apparent change in NSI membrane potential was detected that reliably could be linked to positional shifts. One reason might be that a certain behavioral output (e.g. a shift in average leg position in a certain direction) can be generated by different combinations of MN activation. This was shown in the first part of this thesis by the analysis of changes in muscle activity during targeted responses and had been observed by Bässler et al. (1991). This flexibility in the generation of stereotypical movements might be reflected

also in NSIs. As another reason, the changes in NSIs that contribute to generating a targeted response might be so subtle that they can not be detected in single recordings of NSI activity but rather the averaged data of many recordings would be necessary. In addition, the resulting (targeted) leg movement is produced by the interplay of a high number of premotor interneurons. This complicates the correlation of activity in single NSIs with the resulting movement output because it increases variability. Therefore, a critical step in the further analysis of NSIs' contributions to targeted searching movements will be to design experiments such that a high number of recordings can be gained.

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Appendix

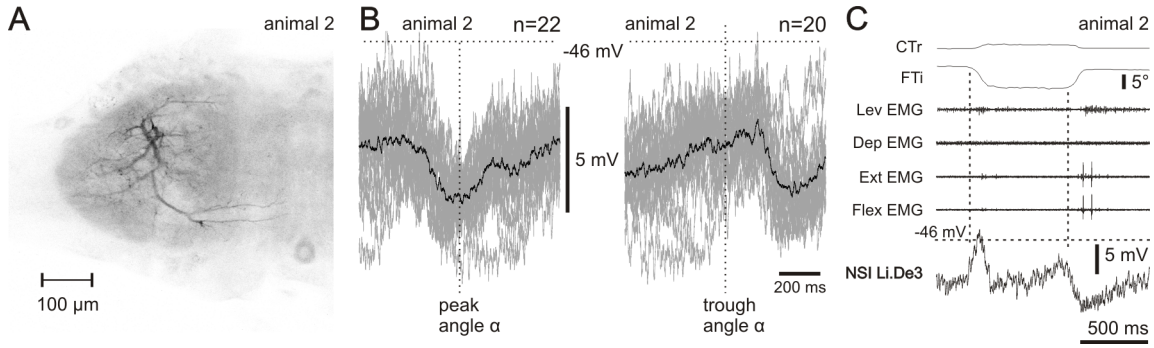

Figure 4.1 – (A) Morphology of NSI Li.De3 from a dorsal view. (B) Overdraws of membrane potential modulation during searching movements; triggered by peak (left side) or trough values (right side) of angle α. Gray traces depict single sweeps, black trace depicts average potential. n = number of sweeps (C) Response of NSI Li.De3 to fCO stimulation. Time points of start of passive flexion and extension are indicated by dotted line. The two top traces depict leg movements in the CTr and FTi joint; four middle traces depict EMG recordings of levator, depressor, extensor and flexor muscle. Bottom trace shows membrane potential of NSI Li.De3.

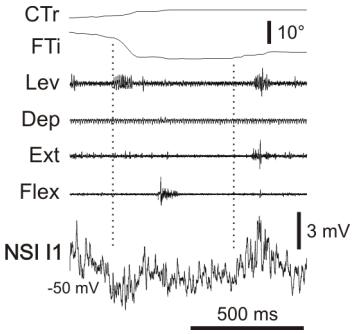

Figure 4.2 – Response of NSI I1 to fCO stimulation. Time points of start of passive flexion and extension are indicated by dotted line. The two top traces depict leg movements in the CTr and FTi joint; four middle traces depict EMG recordings of levator, depressor, extensor and flexor muscles. Bottom trace shows membrane potential of NSI I1.

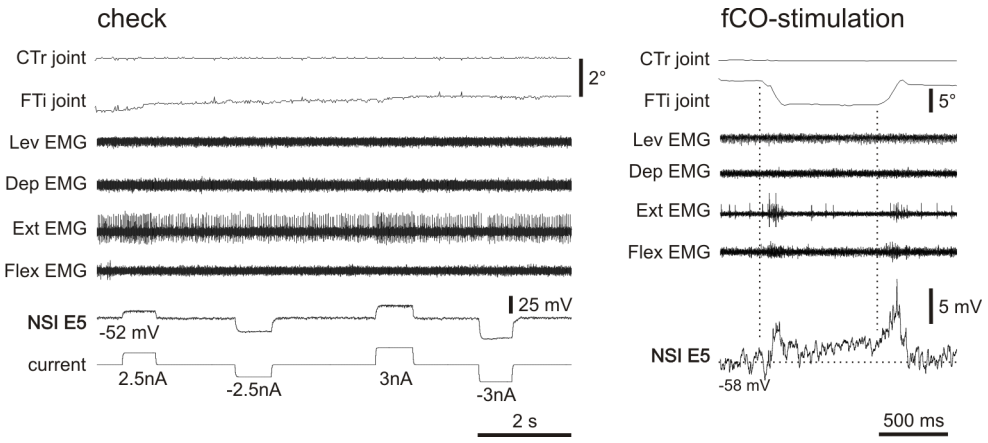

Figure 4.3 – Left panel: Current injection into NSI E5 increased extensor muscle activity and elicited small extension movements. The two top traces depict leg movements in the CTr and FTi joint; four middle traces depict EMG recordings of levator, depressor, extensor and flexor muscles. Bottom traces depict recorded membrane potential of NSI E5 and the injected current. Right panel: Response of NSI E5 to fCO stimulation.

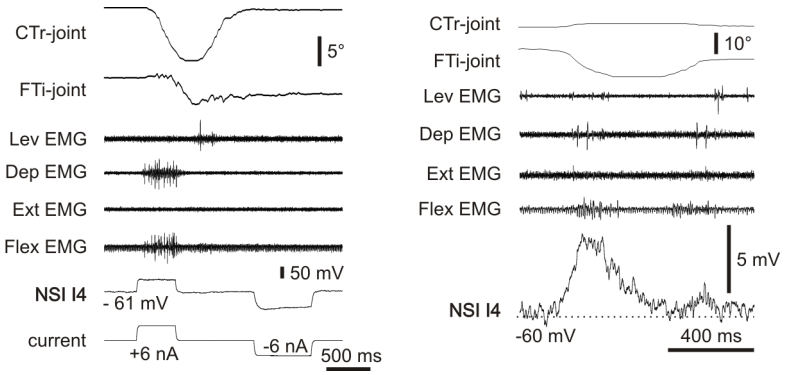

Figure 4.4 – Left panel: Current injection into NSI I4 excites depressor and flexor muscles and elicits depression and flexion movements of the leg. Two top traces depict leg movements in the CTr and FTi joint; four middle traces depict EMG recordings of levator, depressor, extensor and flexor muscles. Two bottom traces depict recorded membrane potential of NSI I4 and the injected current. Right panel: Response of NSI I4 to fCO-stimulation.

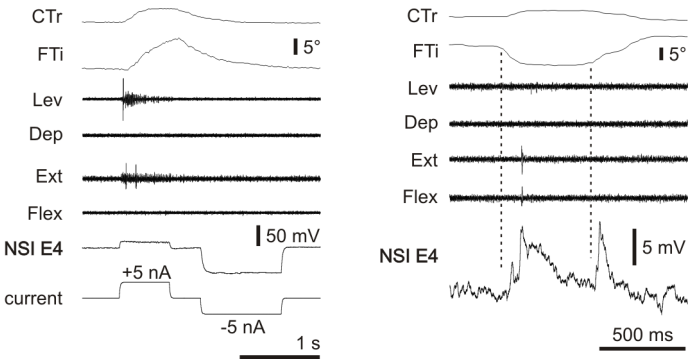

Figure 4.5 – Left panel: Current injection into NSI E4 excites levator and extensor muscles and elicits levation and extension movements of the leg. Two top traces depict leg movements in the CTr and FTi joint; four middle traces depict EMG recordings of levator, depressor, extensor and flexor muscles. Two bottom traces depict recorded membrane potential of NSI E4 and the injected current. Right panel: Response of NSI E4 to fCO-stimulation.

original name	reference	N	new name
Le1	Hess 1998	7	Le1
le2	Hess 1998	1	-
le3	Hess 1998	2	-
le4	Hess 1998	1	-
Li1	Hess 1998	11	Li1
Li2	Hess 1998	15	Li2
li3	Hess 1998	1	-
li4	Hess 1998	1	-
li5	Hess 1998	1	-
de1	Hess 1998	1	-
Di1	Hess 1998	2	Di1
di2	Hess 1998	1	-
I3	Akay 2002	1	I3
I8	Akay 2002	3	I8
E9	Akay 2002	3	E9
E10	Akay 2002	1	-
De2	Rosenbaum 2013	5	De.Li2
Ee.Le1	Rosenbaum 2013	1	Le.Ee1

Table 4.1 – Corresponding old and new names of NSIs which were renamed from previous work. N is number of recordings of the NSI in the original work (reference).

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Teilpublikationen und Vorträge

Publications

E. Berg, A. Büschges, J. Schmidt (2013). Single perturbations cause sustained changes in searching behavior in stick insects. *Journal of Experimental Biology* 216: 1064-1074.

Highlighted in: Current Biology, 2013, Vol. 23(8), R324-R326

Conference abstracts

1. **E. Berg**, J. Schmidt, A. Büschges (2012) "Short term memory in the leg muscle control system of the stick insect Cuniculina impigra", *Annual Meeting of the Society for Neuroscience 922.03/EEE58*
2. **E. Berg**, J. Schmidt, A. Büschges (2011) "Working memory in the leg muscle control system of the stick insect Cuniculina impigra", *Interdisciplinary College Günne*
3. **E. Berg**, J. Schmidt, A. Büschges (2011) "Working memory in the leg muscle control system of the stick insect Cuniculina impigra", *Annual Meeting of the German Neuroscience Society T21-13C*
4. **E. Berg**, A. Büschges, J. Schmidt (2010) "Working memory in the stick insect Cuniculina impigra", *9th International Congress of Neuroethology P110*

Talks

1. "Modification of behavior and its neural control: targeted leg searching movements in the stick insect" *Neurobiological Seminar, Ludwig-Maximilians-Universität Munich (2013)*
2. "(Aiming of) searching movements in the stick insect. In search of the neural correlates" *Biological Cybernetics Department, University Bielefeld*
3. "Single perturbations cause sustained changes in searching behavior of stick insects" *Young Investigator Presentation at the Annual Meeting of the German Neuroscience Society (2013)*
4. "(Targeted) searching behavior and its neural control in the stick insect Cuniculina impigra." *Janelia Farm Research Campus/ USA (2013)*
5. "Single perturbations cause sustained changes in searching behavior of stick insects – memories of a phasmid" *Annual Meeting of the German Zoological Society (2012)*

6. "Single perturbations cause sustained changes in searching behavior of stick insects" *Neurobiology Doctoral Students Workshop (2012)*

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Köln, den 05.05.2014

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von oben angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Ansgar Büschges betreut worden.

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