THE DEVELOPMENT OF EVOKED POTENTIAL PROCEDURES FOR THE ASSESSMENT OF NON-FREEZING COLD INJURY IN THE RAT

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

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The development of evoked potential procedures for the assessment of non-freezing cold injury in the rat

This report focuses on the development of evoked potential (EP) methods for measuring and quantifying non-freezing cold injury (NFCI) in the rat tail. A neural volley can be elicited by electrical stimulation of the tail and recorded at various points along pathways to the brain. Through signal averaging, EPs can be derived and monitored in order to quantify the effects of cold on neural function. In the present study, the recording techniques and parameters are discussed relative to initial studies in which injuries were induced through prolonged exposure of the tail to cold water. Cold exposure produced profound changes in EP amplitudes and latencies recorded on the tail, lower back, and over the somatosensory cortex of the brain. Collectively, results indicate that the EP methodology is sensitive to neural dysfunction resulting from cold and will therefore be useful in future studies of NFCI.
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The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, DHEW, Pub. No. (NIH) 78-23.
INTRODUCTION

The effectiveness of military personnel to operate in cold, wet environments has been impeded since the Crimean War and as recently as the battle for the Falkland Islands by a condition known as trench foot or immersion foot (1). Today, this affliction, clinically and experimentally described as non-freezing cold injury (NFCI), is still a potential problem in any prolonged military exercise carried out in inclement weather conditions.

Non-freezing cold injury occurs if a limb, typically the hands or feet, is exposed to cold for a prolonged period of time. In the affected area, redness of the skin, edema, and anesthesia are evident after rewarming, as is a loss of neuromuscular function (2). The neuropathology in patients afflicted with NFCI is reflected in a numbness and tingling sensation in the extremities following rewarming, symptoms indicative of sustained damage to neural fibers. Several weeks after exposure, muscular weakness and atrophy are evident, as well as a reduction in sensory conduction velocity. Over many weeks, a gradual recovery of neural function is typical, and hypersensitivity to thermal stimuli usually develops (3).

Controlled studies in animals have yielded data on the morphological changes in neural tissue resulting from NFCI. Caudal nerves from the rat tail have been examined via phase and electron microscopy after the entire tail region was exposed to cold water for 12 hours (4). Generally, there was a marked loss of neural filaments and tubules within axons and an increase in vesicles and granular bodies one week after cold exposure. There was also a loss of the large myelinated fibers of the distal regions of the tail that persisted through four weeks post exposure. It was not until eight weeks post exposure that myelinated fiber counts increased in the distal tail region.
Gilliatt and Kennett examined the neuropathology associated with cooling of the hind limb or isolated tibial nerve in the rabbit (5). Their findings indicated a conduction block in motor fibers with axonal injury immediately after rewarming. Three to four weeks after exposure, damage was observed primarily in large myelinated axons; nonmyelinated fibers were apparently not affected by the cold exposure. Interestingly, the most prominent area of nerve injury was proximal, just below the interface of air and cold water. The finding of proximal nerve damage is intriguing because it suggests that some variable other than local cooling of the nerve may interact with cold and contribute to the neural damage. If cold were the single factor effecting neural injury, then the greatest nerve injury should be more distal since peripheral areas would presumably have the lowest temperature along the hind limb. Proximal neural degeneration in the rat tail was not observed by Peyronnard et al. (4); however, the initial examination of the tails was not until one full week after cold exposure. Clearly a more detailed examination of the time course of neural dysfunction subsequent to cold exposure is necessary.

One method for tracking nervous system integrity in a noninvasive manner over time is through the measurement of evoked potentials. Somatosensory evoked potentials reflect the averaged neural activity resulting from a given stimulus, typically mild electrical current applied to the skin (6). Stimulating a rat tail with a mild electric current produces a volley of neural activity in a variety of myelinated and unmyelinated sensory fiber types. The subsequent neural activity can be recorded at various points along the tail, spinal cord, and over the somatosensory cortex on the head (7, 8). Evoked potentials can be recorded before and immediately after cold exposure.
a-d at various points on the tail and body in order to determine the extent and distribution of the injury site. This paper documents the progress of efforts aimed at developing an electrophysiological methodology for the investigation of NFCI in the rat.

METHODS

Subjects: Fifteen male Long-Evans rats of 300 - 360 grams were used for the development of procedures detailed below. Rats were housed individually in standard hanging metal cages and received a normal light/dark cycle. Throughout the experiment, their weights were maintained at 350 grams.

Apparatus: A Nicolet C-4 four channel electrodiagnostic system was used to generate electrical stimuli and average the neuro-electric signals. More flexible systems might be an advantage with regard to channels with individually determined sweep times and amplitude resolution since the averaged potentials from the tail, lower back, and head have varying amplitudes and latencies.

Anesthesia: Sodium Pentobarbital has been used by several laboratories investigating somatosensory evoked potentials in the anesthetized rat preparation (9, 10). Pentobarbital (Nembutal) administered i.p. at 33 to 36 mg/kg was found to maintain a level of anesthesia necessary for subdural electrode placement and evoked potential recording unconfounded by muscle artifact. To augment recovery of respiration after the evoked potential recordings, doxapram hydrochloride (Dopram-V) was administered i.p. at a dosage of 5 mg/kg.

Electrode placement: The stimulating electrodes were flat, stainless steel disks 0.5 cm in diameter, separated by 1.2 cm from edge to edge. They were fixed in a block of acrylic. The anode was placed 5 cm from the tip of the
tail. A stainless steel needle electrode placed 7 cm from the tip of the tail served as a ground. The stimulating electrode was initially located near the tip of the tail. However, the initial neural dysfunction caused by cold exposure was so profound that potentials were not detectable. Thus, the stimulating electrode was moved rostrally up the tail in order to stimulate a greater number of neurons and therefore achieve larger potentials. This served to increase the overall sensitivity of the evoked potential measure.

Recording (needle) electrodes were placed subdurally at 12 and 24 cm from the tip of the tail; reference electrodes were placed 1 cm rostral to each. The final recording electrode was a stainless steel screw placed in the skull (epidural) 1 mm caudal to bregma, 3 mm lateral (right) to the midline. This electrode, secured with dental cement, protruded through the skin, allowing the attachment of an electrode lead. The skull electrode was referenced to a clip placed on the left ear. This electrode was approximately 36-38 cm from the tip of the tail.

Stimulation: Electrical stimuli of 1 mA for 200 μsec were delivered every 1.7 seconds. Exploratory data has shown that a level of stimulation produces a stable response unconfounded by stimulus artifact; muscle twitching was evident only at much higher stimulus intensities of 2 - 3 mA. Most investigators examining somatosensory evoked potentials resulting from paw stimulation have utilized a greater stimulus current presented at a faster rate (11). In these studies curare was used, which required artificial ventilation. Since the present protocol required multiple recording sessions, we chose a protocol with a lower risk to the animal. The lower stimulating current produced no detectable muscular twitch.

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Recording parameters: Bandpass filter settings were similar to those utilized by Allison and Hume (7) and Shaw and Cant (9) for experiments on somatosensory responses resulting from paw stimulation. For the three peripheral recording electrodes, the bandpass filters were 3 - 3000 Hz. The skull electrode filters were 3 - 500 Hz. The sweep time was 60 msec for all four channels and was determined by the latency of somatosensory components examined in this study. Other recording systems might enable a separate sweep time for each channel since the evoked potentials from the tail and back recording sites have latencies under 15 msec. The sampling rate was 512 Hz. Automatic artifact rejection protected against aberrant muscle activity.

Cold exposure conditions: Prolonged cold exposures to induce neural injury were performed using the methods of Ahlers et al. (12). Briefly, rats were restrained and suspended in a 22.5 cm long cylindrical tube (7 cm diameter, adjustable) that permitted exposure of the tail and part of the torso up to the level of the hindquarters to cold (10°C) water. The cold water was produced by a circulating thermal bath. For the exposures discussed in the present paper, rats were unanesthetized for an exposure period of nine hours.

RESULTS

Tail recording site: Figure 1 displays an evoked potential record from the tail recording site. Amplitude of the evoked potential is plotted as a function of time. The potential is characterized by a substantial negative deflection occurring approximately 2.5 - 3.5 msec after stimulation. Clearly, the 60 msec sweep duration is unnecessary for this potential, but was chosen to capture the later components of the somatosensory potential recorded at the head and to compare evoked responses between each channel. Figure 2 demonstrates the effect of a nine hour exposure to 10°C water on neural
conduction in the tail. The figure displays potentials recorded before and three days after exposure. Recording sites in this figure were referenced to a needle electrode in the left flank, which seemed to cause a positive to negative slope of the averaged response. This artifact led to the use of reference electrodes within close proximity to recording electrodes, as described above. During the initial stages, a 2 mA intensity level was chosen because of the more distal location of the stimulator. Figure 2 demonstrates that after prolonged exposure to cold, the latency of the potential was slightly longer and the amplitude was reduced by over 50 percent. Figures 3 and 4 plot amplitude as a function of time (in days) for two rats in which the tail and hind flank were exposed to 10 °C for nine hours. These data demonstrate the magnitude of amplitude reduction, indicative of profound neural dysfunction resulting from cold exposure. In some instances, the tail potential was not detectable after cold exposure. This led to movement of the stimulating electrodes further up the tail.

Figure 5 displays tail evoked response latency change before and after exposure to cold. Both subjects displayed an initial increase followed by a decrease in latency. Given the large reduction in amplitude, it is possible that cold affected the smallest of the large myelinated fiber class, resulting in only a small number of the largest neurons (with a correspondingly faster conduction velocity) responding to stimulation. This would produce an attenuated response with a slightly shorter latency. Clearly, more data is needed to adequately characterize latency changes and test such a hypothesis.

Lower back recording site: Figure 6 displays a potential recorded over the spine with a subdural electrode. The general shape of the potential is similar to spinal potentials reported in rats (10) and in humans (13).
Potentials of this type typically have latencies of 7.5 to 8.5 msec and amplitudes of 1.5 to 3 µV. Figure 7 displays recordings made before and after cold exposure with the flank reference; this montage resulted in an inverted potential and a "DC" (sloping) artifact. As shown, this spinal potential was difficult to detect after cold exposure because of its small amplitude. The use of the reference placed 1 cm rostral to the recording electrode results in a greater signal to noise ratio.

A second back recording site, 29 cm from the end of the tail (5 cm rostral to the lower back site), was originally tried in order to further define the cold injury over the pathway to the brain. Recordings made at this site were contaminated by electrical activity from the diaphragm. Automatic artifact rejection resulted in the rejection of most of the sweeps; turning the rejection mode off led to highly variable evoked potential averages. Thus, this site was eliminated from the protocol.

Head recording site: Figure 8 displays averaged evoked responses from the somatosensory cortex. The electrode used to record the evoked potential displayed in this figure was placed 1 mm posterior and 3 mm right lateral to bregma. Somatosensory evoked potentials typically display a negative-positive-negative wave form complex. The initial negative deflection is denoted N1. The original group of rats had bone screws placed 1 mm rather than 3 mm lateral to the midline. Recordings from this montage yielded an inconsistent N1 response, but a more reliable N2 response (40 - 45 msec), presumably of secondary cortical origin. The lack of a consistent N1 response may have been caused by the generator orientation, position of the electrode, or a combination of these factors. At this writing, an experiment is underway to determine the usefulness of moving the scalp reference electrode from the
left ear to the nape of the neck. Preliminary results indicate that this montage eliminates the positive to negative going artifact and leads to a much clearer resolution of the N1 response (Fig. 9). This is encouraging since the sweep time for the head electrode, and subsequently all other sites, can be reduced and enable greater temporal resolution of evoked responses. The somatosensory evoked potential (SEP) seems to be affected by the level of anesthesia (9); greater EEG activity (as seen on raw EEG input), shorter N1 latencies, and attenuated peak to peak amplitudes are observed with a lack of adequate anesthesia.

Figures 3 and 4 display changes in the P2 - N2 amplitude resulting from cold exposure. These amplitude changes generally parallel those recorded at the lower tail recording site. Changes in N2 latency as a function of cold in one subject are shown in Figure 10. The increase in latency several days after exposure has been partially replicated in another subject.

DISCUSSION

Evoked potential measures appear to be sensitive to neural dysfunction in the tail resulting from exposure to cold water of 1°C for nine hours. Reductions in the amplitudes of potentials recorded at the tail, lower back, and over the somatosensory cortex after cold exposure were substantial. Changes in latency at the three recording sites were less systematic and will require further examination. Evoked responses of the tail displayed an increase followed by a decrease in response latency several days after the cold exposure, while the latencies of the N2 component of the somatosensory evoked potential recorded on the head showed an increase in latency after the exposure. The precise distribution of the injury is unclear since the lower back potentials were rendered undetectable by the cold treatment, and thus
latency data were unobtainable. Spinal potentials recorded with the reference electrode located 1 cm anterior to the recording electrode should enable more accurate spatial characterization of the injury.

Based on the latencies of the evoked potentials, the conduction velocity of the fiber tracts eliciting the neural volley was approximately 18 m/sec, a velocity characteristic of large-myelinated fibers in the rat. At the level of the tail recording site, antidromic activation of motor fiber tracts was possible, although no muscle activity was observed. Beyond the tail, particularly at the level of the head, the evoked response was clearly of sensory origin.

The measurement of large myelinated fiber types through evoked potentials is in contrast with an experimental approach measuring thermal sensitivity of the tail in rats with NFCI (12). Tail-flick latencies in response to warm water, presumably mediated by small-unmyelinated fibers (14), reveal changes owing to cold exposure that seem independent and uncorrelated with changes in evoked potential measures. These findings suggest that the manifestation of NFCI may be dependent upon neural fiber class. Since both evoked potential and thermal sensitivity measures have clearly suggested NFCI after a nine hour exposure at 10° C, future studies will focus on the extent of NFCI at a variety of exposure times and temperatures. Therefore, a clearer understanding of the specific effects of NFCI on large-myelinated and small-unmyelinated fiber types will evolve. While the use of evoked potentials appears to provide a promising measure of NFCI in the rat tail, only through an integrated approach utilizing evoked potentials, thermal sensitivity measures, blood flow through the tail, and histological examinations will the mechanisms by which tissue damage occurs be more fully understood.
REFERENCES


FIGURE LEGENDS

Figure 1. Amplitude plotted as a function of time for an evoked potential recorded 12 cm from the end of the tail. The amplitude of the potential, approximately 34 µV, is calculated from the peak negative deflection to the following positive deflection. The latency of this potential is about 2.4 msec. The onset of all recordings is delayed by 700 µsec in order to avoid stimulator artifact. This and subsequent tracings display scales (msec and µV per division) in the upper right hand corner.

Figure 2. Pre and post cold exposure tail potentials were similar to that of figure 1 with two exceptions: active electrode was referred to the left flank, and the stimulator was located near the tip of the tail. The reference electrode and stimulator location lead to a "DC" positive to negative artifact and later potentials, respectively. Records are displaced vertically for clarity. The post exposure tracing was recorded eight days after cold exposure.

Figure 3. Evoked potential amplitudes for tail and head recording sites plotted as a function of time for one subject. The vertical dashed line denotes a nine hour exposure to water of 10 °C. Some tail responses were not identifiable and are given amplitudes of zero.

Figure 4. Head and tail evoked potential amplitudes pre and post exposure for a different subject.

Figure 5. Tail evoked response latencies for subjects shown in figures 3 and 4. Vertical dashed line denotes exposure. Graph is broken.
for subject 33 because amplitudes and thus latencies were not resolved.

**Figure 6.** An evoked response recorded from over the spinal cord, 24 cm from end of tail. These responses have approximate latencies and amplitudes of 5.5 - 7.0 msec and 2.5 - 4.5 μV, respectively.

**Figure 7.** Lower back evoked responses recorded with a flank reference and stimulator near the tip of the tail before and after cold exposure. Records are shifted vertically for clarity. Note the smaller size of the baseline, pre-cold potential compared to that of figure 6, which has a reference electrode 1 cm rostral to the active electrode.

**Figure 8.** A somatosensory evoked potential (SEP) recorded from a stainless steel screw electrode placed 1 mm caudal to bregma and 3 mm to the right of midline. The N1, P2, and N2 components are labeled. Very early components typically observed with a needle electrode reference placed at the nasal bone are not seen with the left ear reference utilized in the present protocol. The N2 response is more pronounced with this recording montage.

**Figure 9.** A typical SEP recorded in a normal (pre-exposed) rat using a needle electrode placed subdurally in the nape of the neck as the reference site. The montage, in direct line with the spinal column, produces a relatively artifact free tracing with N1 and P2 components clearly identified on a shorter time scale (30 msec).
Figure 10. SEP N2 response latency as a function of days pre/post exposure for one subject. The increase in SEP N2 latency was observed in another subject as well.
TAIL EVOKED RESPONSES

Δ—Δ subj. 33  Δ—Δ subj. 34

EVOKE POTENTIAL LATENCY (msec)

DAYS PRE/POST EXPOSURE

FIGURE 5
Figure 7: Comparison of B1 and B2 levels before and after cold exposure.