Increased activity of pre-motor network does not change the excitability of motoneurons during protracted scratch initiation

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Key points
- A single cutaneous stimulus of adjusted intensity insufficient for scratch initiation evokes scratch when repeated at subsecond intervals.
- The protracted scratch initiation shows that the spinal network is able to store information at subsecond time intervals.
- Membrane depolarization and increase in excitability of motoneurons are not necessary for protracted scratch initiation.
- The activity of the pre-motor network increases in a wind-up manner during protracted scratch initiation.

Abstract
Intrinsic response properties of neurons change during network activity. These changes may reinforce the initiation of particular forms of network activity. If so, the involvement of neurons in particular behaviours in multifunctional networks could be determined by up- or down-regulation of their intrinsic excitability. Here we employed an experimental paradigm of protracted scratch initiation in the integrated carapace–spinal cord preparation of adult turtles (\textit{Chrysemys scripta elegans}). The protracted initiation of scratch network activity allows us to investigate the excitability of motoneurons and pre-motor network activity in the time interval from the start of sensory stimulation until the onset of scratch activity. Our results suggest that increased activity in the pre-motor network facilitates the onset of scratch episodes but does not change the excitability of motoneurons at the onset of scratching.

Introduction
Some behaviours mediated by large-scale networks of neurons are initiated by brief sensory stimuli activating only a small number of afferents. In many limbed vertebrates a gentle mechanical rub to the skin within reach of a limb induces rhythmic scratching aimed at the site of stimulation (Sherrington, 1906; Stein, 1983).

Due to biomechanical constraints three different scratch forms have evolved in turtles for the scratch to reach targets in caudal, pocket and rostral receptive fields around the hind limb (Mortin \textit{et al.} 1985). The characteristic patterns of motor nerve activity of these distinct motor behaviours can be elicited in paralysed animals (Robertson \textit{et al.} 1985) and even in isolated carapace–spinal cord preparations (Keifer & Stein, 1983; Alaburda & Hounsgaard,
2003) by stimuli in the same receptive fields. Thus, brief trains of action potentials (APs) in a few sensory afferents recruit a network of an estimated 10,000 neurons (Walloe et al. 2011) that generates the scratch motor rhythms. This scratch motor circuit is multifunctional in the sense that some neurons involved are active in more than one scratch form and during locomotor network activity as well (Stein, 2005; Berkowitz, 2010). However, the steps and mechanisms that lead from stimulus to specification of the appropriate functional activity are unknown. In the small multifunctional network in the crustacean stomatogastric ganglion, modulation of the intrinsic neuronal response properties plays a crucial role in specifying the network states for each behavioural form (Blitz & Nusbaum, 2011). By analogy the intrinsic response properties of neurons and their modulation by neurotransmitters are widely believed to play a similar role in rhythm generation in spinal motor networks (Perrier et al. 2002; Grillner, 2003; Toledo-Rodriguez et al. 2005).

If so, one could speculate that a specific sensory input selects for a particular behaviour by transmitter-mediated up- and down-regulation of intrinsic excitability and rhythmic properties in key target neurons. In this way the initial activity in a small subset of neurons could funnel a large-scale network into the appropriate functional state for the selected behaviour. In support of this, the excitability of motoneurons in mammals is enhanced during fictive motor activity by hyperpolarization of the spike threshold \( V_{\text{th}} \) (Krawitz et al. 2001; Power et al. 2010).

Here we employed an experimental paradigm in which fictive scratching is initiated by a low-frequency train of stimuli in a cutaneous nerve at a stimulus intensity that does not evoke motor activity when a single stimulus is applied (Crowe & Linnartz, 1985; Currie & Stein, 1988). The protracted activation of scratch network activity allowed us to investigate the excitability of motoneurons in the time interval from the start of sensory stimulation until the onset of scratch activity recorded in the hip flexor nerve. In addition, we were able to analyse the pre-motor network activity during the protracted onset of fictive scratching.

**Methods**

**Ethical approval**

The surgical procedures complied with Danish legislation and were approved by the controlling body under The Ministry of Justice.

**The integrated carapace–spinal cord preparation**

Red-eared turtles \( (Chrysemys scripta elegans) \) of 10–15 cm carapace length were obtained from Nasco, Fort Atkinson, WI, USA. Surgical procedures were described previously (Alaburda & Hounsgaard, 2003). Briefly, turtles \( (n = 22) \) were placed on crushed ice 2 h before surgery to induce drowsiness and reduce stress and pain by hypothermia. In this way the head and neck could be protracted using minimal force. Brain functions were terminated immediately upon decapitation by crushing the head. The blood was substituted by perfusion through the heart with a Ringer solution containing \( (\text{mM}) \): 120 NaCl, 5 KCl, 15 NaHCO\(_3\), 2 MgCl\(_2\), 3 CaCl\(_2\) and 20 glucose, saturated with 98% O\(_2\) and 2% CO\(_2\) to obtain pH 7.6. Transverse cuts were made at D10 and D3 spinal cord segments. The hip flexor (HF) \( (\text{innervating puboischiofemoralis internus, pars anteroventralis muscle}) \) and cutaneous \( (\text{probably ventral posterior pocket nerve used previously to evoke scratch reflex (Currie & Stein, 1988)}) \) nerves were exposed and cut.

**Stimulation**

Mechanical stimulation for induction of the fictive scratch reflex \( (\text{rhythmic activity in ipsilateral HF nerve in response to stimulation}) \) was performed by pinching the pocket skin with a pair of tweezers \( (\text{Fig. 1Ab}) \) or with fire-polished glass rod mounted to the membrane of a loudspeaker controlled with a function generator (Alaburda & Hounsgaard, 2003) \( (\text{Fig. 1Ab}) \).

Electrical stimuli for induction of scratch episodes were applied to a cut ipsilateral cutaneous nerve \( (\text{Currie & Stein, 1988; Guzulaitis et al. 2012}) \) using a suction electrode.

**Recordings**

Intracellular recordings in current-clamp mode were performed with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Sharp glass electrodes from thin-walled borosilicate glass were filled with a mixture of \( 0.9\,\text{m} \text{CH}_3\text{CO}_2\text{K} \) and \( 0.1\,\text{m} \text{KCl} \). Resistance of electrodes was 35–50 MΩ. Intracellular recordings in bridge mode were obtained from motoneurons in ventral horn of D9 and D10 segments. Recordings were accepted if neurons had a stable membrane potential more negative than \(-50\,\text{mV}) \). Data were sampled at 10 kHz with an analog-to-digital converter \( (\text{Digidata 1440 or Digidata 1322A; Molecular Devices}) \), displayed by means of Axoscope and Clampex software \( (\text{Molecular Devices}) \) and stored on a hard disk for later analysis.

The electroneurogram (ENG) of the HF nerve was recorded with a differential amplifier ISO-DAM8 (World Precision Instruments, Sarasota, FL, USA) using a suction electrode. The bandwidth was 100 Hz–1 kHz.
Data analysis

The membrane potential ($V_m$) was quantified as the average of the instantaneous $V_m$ over 0.5 s. The excitability of motoneurons was monitored by the response to 250 ms depolarizing current pulses that evoked a single AP in the resting state prior to stimulation at 2 Hz frequency (Russo & Hounsgaard, 1994; Delgado-Lezama et al., 1997; Alaburda & Hounsgaard, 2003). A change in the number of evoked APs was taken as an indication of a change in excitability.

$V_{th}$ was defined as the membrane potential at which depolarization increased $\geq 10 \text{ V s}^{-1}$ during onset of an AP evoked by a depolarizing current pulse. At rest $V_{th}$ was determined as the average $V_{th}$ for 10 successive APs. During the stimulus train prior to fictive scratching $V_{th}$ was taken as the average value for last three APs evoked by current pulses.

Conductance of motoneurons was evaluated from $V_m$ deflections induced by hyperpolarizing current pulses. Current pulses of 250 ms were applied at 2 Hz frequency and/or current pulses of 100 ms were applied at 5 Hz frequency.

The magnitude of the synaptically induced fluctuations of $V_m$ was estimated as the standard deviation (SD) of $V_m$ (Pare et al., 1998; Destexhe & Pare, 1999) for 0.5 s at appropriate time (before the first stimulus, 1 s after the stimulus, etc.). Notch (50 Hz) and high pass (2 Hz) filters were used.

Data were analysed statistically using a Student’s paired $t$ test (Origin software; Microcal Software, Northampton, MA, USA). Significance was accepted at $P < 0.05$. The level of significance is indicated as follows: n.s., $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$. Data are presented as means $\pm$ SD ($n =$ the number of neurons).

Results

Protracted initiation of scratch network activity

In the integrated spinal–carapace preparation mechanical stimulation (pinch of the hind-limb pocket skin with a pair of tweezers in Fig. 1Aa) evoked fictive scratch activity as recorded in the ipsilateral motor (HF) nerve (Keifer & Stein, 1983; Alaburda & Hounsgaard, 2003; Alaburda et al., 2005). Careful adjustment of mechanical stimulation led to protracted initiation of scratch episodes (Fig. 1Ab). The first application of a brief mechanical stimulus with a fire-polished rod did not evoke scratching but reapplication of the same stimulus after 3 s induced a scratch episode as revealed by the rhythmic motor nerve activity. This shows that the network in isolated preparation is able to store the information about sensory inputs for more than 1 s as in the spinal cord in vivo (Sherrington, 1906; Crowe & Linnartz, 1985; Currie & Stein, 1988, 1990).

To control the applied stimuli better we adopted electrical stimulation of appropriate cutaneous nerves to evoke fictive scratch (Currie & Stein, 1988; Guzulaitis et al., 2012). The induction of scratch activity during a train of electrical stimuli depends on both stimulus intensity (Fig. 1B) and stimulus frequency (Fig. 1C). With a stimulus interval of 5 s the low stimulus intensity of 15 $\mu$A (Fig. 1Ba) did not evoke spike activity in the HF motor nerve (middle sweep) during a train of four stimuli (lower sweep). At the slightly higher intensity of 20 $\mu$A (Fig. 1Bb) no activity was evoked by the first stimulus but the subsequent three stimuli of the train evoked synaptic activity in the motoneuron (upper sweep) and spike activity in the motor nerve (middle sweep). Finally, when the stimulus intensity was increased to 30 $\mu$A (Fig. 1Bc) the first stimulus evoked a full scratch episode.

Induction of scratch network activity also depended on stimulus frequency (Fig. 1C). A train of stimuli at the low intensity of 0.17 $\mu$A evoked no motor nerve activity when applied at a frequency 0.37 Hz (Fig. 1Ca). When the stimulus frequency was increased to 1.1 Hz an episode of associated increases in synaptic activity in the motoneuron and spike activity in the motor nerve was apparent (Fig. 1Cb). Finally, at a frequency of 2.2 Hz two associated rhythmic bouts of synaptic activity in the motoneuron and spike activity in the motor nerve were evoked (Fig. 1Cc).

In most preparations (15 of 22) it was possible to adjust stimulus intensity so that a scratch episode was initiated in a protracted way by the second or subsequent stimulus during a train at a stimulus frequency $<1$ Hz in the absence of evoked motor nerve activity following the first stimulus as in Fig. 1Bb. These results conform closely to the findings obtained in the spinal turtle in vivo (Crowe & Linnartz, 1985; Currie & Stein, 1988). We used this protocol of protracted scratch initiation to investigate the modulation of the intrinsic properties of motoneurons and recruitment of network activity prior to the onset of scratch activity.

$V_m$ and excitability of motoneurons prior to scratch onset

It has been suggested that motoneurons could be dedicated to changing functional needs by the metabotropic modulation of their intrinsic response properties (Delgado-Lezama & Hounsgaard, 1999). Synaptically released glutamate up-regulates the excitability of turtle motoneurons in response to brief trains of stimuli in slices (Delgado-Lezama et al., 1997) and during scratch episodes in the carapace–spinal cord preparation (Alaburda & Hounsgaard, 2003). First we examined if

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the sub-threshold stimulus caused a substantial change in \( V_m \) of motoneurons prior to fictive scratching. We found that \( V_m \) in motoneurons at rest \((-64.75 \pm 6.71 \text{ mV})\) and just before the stimulus that evoked a motor response \((-64.48 \pm 6.47 \text{ mV})\) (grey bars in Fig. 2A) differed by less than 1 mV \((n = 20)\).

The excitability of neurons can be changed by synaptic modulation even without changes in \( V_m \) (Delgado-Lezama et al. 1997; Russo et al. 1997). Therefore, we compared the response of motoneurons to a supra-threshold depolarizing current pulse at rest and during protracted initiation of scratch network activity. The excitability after cutaneous nerve stimuli increased in 7 of 12 motoneurons, decreased in one and did not change in the remaining four cells. The changes of excitability just after the stimulus could be due to increased synaptic inputs from the pre-motor network. However, the response to the depolarizing test pulse was unchanged in all 12 neurons when applied just prior to the stimulus that preceded the motor activity (grey bars in Fig. 2B).

The excitability of mammalian motoneurons increases during fictive locomotion (Krawitz et al. 2001) and scratching (Power et al. 2010) due to hyperpolarization of \( V_{th} \). We evaluated changes of \( V_{th} \) during protracted scratch initiation. However, we did not observe any differences between \( V_{th} \) at rest \((-41.6 \pm 5.19 \text{ mV})\) and prior to onset of fictive scratch \((-41.75 \pm 5.46 \text{ mV})\) \((P > 0.05, n = 12)\). These findings revealed no detectable sustained changes in \( V_m \) and excitability in motoneurons during protracted initiation of scratch network activity.

**Figure 1. Protracted scratch initiation by repeated subthreshold mechanical and nerve stimulation**

*Ab* mechanical stimulation of the pocket skin evokes an episode of scratch network activity. *Ab* fine adjustment of stimulus intensity and duration leads to protracted activation of a scratch episode – activity in the motor nerve starts only after the second brief stimulus. *B* and *C*, inductions of scratch network activity by electrical stimulation of cutaneous nerve depends on stimulus intensity (*B*) and frequency (*C*). A weak stimulus does not induce activity in HF nerve ENG (*Ba*, *Ca*). At appropriate intensity even a single stimulus initiates scratching (*Bb*). At intermediate intensities stimulation induces protracted initiation of scratch episodes (*Bb*). The recordings in *A* are from a different preparation than *B* and *C*. From top: \( V_m \) of motoneuron; ENG activity from HF nerve; stimulus.
Pre-motor network activity during protracted scratch initiation

The evoked synaptic activity in motoneurons prior to scratch onset signals the recruitment of the pre-motor network. In the example shown in Fig. 3A, the first electrical stimulus induced a long-lasting (>1 s) sub-threshold barrage of synaptic fluctuations in $V_m$ in a motoneuron (upper trace) without activity in the HF nerve (middle trace).

The intensity of the prolonged synaptic activity recorded in motoneurons was evaluated as SD of $V_m$. We compared SD of $V_m$ in motoneurons at rest prior to the first stimulus (I), 1 s after first stimulus (II) and just before the stimulus that evoked activity in HF nerve (III) (grey bars in Fig. 3A). SD of $V_m$ at rest was $0.25 \pm 0.08 \text{ mV}$, increased to $0.49 \pm 0.23 \text{ mV}$ 1 s after the first stimulus and remained elevated at $0.36 \pm 0.18 \text{ mV}$ in the 0.5 s time period just prior to the onset of scratch motor activity ($n=20$) (Fig. 3B). A single electrical stimulus increased pre-motor network activity for more than 1 s without any motor response. The SD of $V_m$ 1 s after the first stimulus and just before the scratch episode was significantly higher than at rest ($P < 0.01$). Moreover, the SD of $V_m$ 1 s after the first stimulus was significantly higher than immediately before the onset of scratch network activity i.e. the activity of the pre-motor network during protracted activation of fictive scratch gradually decreased over time. In addition, we did not observe any scratch-like rhythmicity in fluctuations of $V_m$ during protracted scratch initiation. Thus the pattern of activity of the pre-motor network during protracted scratch initiation is tonic and gradually decreases over time from an early peak after each stimulus.

During scratch network activity motoneurons enter a high conductance state due to intense synaptic activity (Alaburda et al. 2005; Berg et al. 2007). To evaluate the intensity of synaptic input during the protracted initiation of fictive scratch we calculated the input conductance of motoneurons. The conductance at rest ($73 \pm 36.53 \text{ nS}$) and immediately prior to the stimulus that evoked a motor response ($73.59 \pm 37.16 \text{ nS}$) did not differ in motoneurons with long-lasting (>1 s) synaptic input during protracted onset of fictive scratch ($n=15$). We conclude that synaptic input to motoneurons during protracted initiation of fictive scratch is too sparse to increase input conductance. With proper adjustment of stimulus intensity the onset of fictive scratching could be delayed to occur with the third or subsequent stimuli during the train (Fig. 4Aa and Ab). Under these conditions the pre-motor network...
activity as reflected in the synaptic input to motoneurons increases (winds-up) with repeated stimuli. Note that not only excitatory input winds up prior to the onset of scratch network activity. In 3 of 8 motoneurons there was a net increase of inhibition during synaptic wind-up, as illustrated in Fig. 4Ab.

We compared SD of $V_m$ in motoneurons at rest (I), 1 s after the first stimulus (II) and 1 s after the second stimulus (III) (grey bars in Fig. 4Aa and Ab). SD was 0.24 ± 0.09 mV at rest, 0.25 ± 0.06 mV after the first stimulus and 0.66 ± 0.33 mV after the second stimulus ($n=8$) (Fig. 4B). The pre-motor network activity was significantly higher after the second than after the first stimulus or at rest. We conclude that sustained pre-motor network activity winds-up during protracted scratch initiation.

**Discussion**

In this study we used the isolated carapace–spinal cord preparation of the turtle to investigate the initiation of functional network activity. The initiation of scratching depends on stimulus amplitude and timing. Even a single electrical stimulus of sufficient amplitude can induce long-lasting functional network activity (as illustrated in Fig. 1Bc). If the stimulus amplitude is lower, a single stimulus does not induce scratch. However, the spinal network ‘remembers’ the sub-threshold event for a few seconds so that subsequent stimuli may induce a scratch episode (Fig. 1Ab and Bb) (Sherrington, 1906; Crowe & Linnartz, 1985; Currie & Stein, 1988, 1990). Here we took advantage of the protracted initiation of scratch episodes achieved by proper adjustment of intensity and frequency of electrical stimuli applied to a sensory cutaneous nerve from the receptive field of the pocket scratch (Currie & Stein, 1988). In this way we were able to follow the evolution of the synaptic activity from the pre-motor network to motoneurons and how the synaptic activity affected excitability of motoneurons prior to the onset of fictive scratching.

We find a long-lasting (>1 s) increase of pre-motor network activity after a single nerve stimulus. Moreover, excitatory and inhibitory synaptic activity in motoneurons winds-up with each sub-threshold stimulus prior to the onset of fictive scratching. However, this synaptic activity did not induce noticeable lasting changes in the excitability of motoneurons.

The persistent activity of neurons to brief stimuli observed in many regions of the central nervous system is believed to serve as a working memory mechanism (Major & Tank, 2004). Intrinsic plateau properties of individual neurons (Russo & Hounsgaard, 1994; Bennett et al. 1998; Di Prisco et al. 2000) or recurrent networks (Li et al. 2006) are likely mechanisms underlying long-lasting spiking after short stimuli.

The timescale of increased spinal network excitability following sub-threshold stimulation (Currie & Stein, 1988) is compatible with the duration of increased pre-motor network activity reported here. It is also similar to the frequency range in which temporal summation is observed in long-afterdischarge

![Figure 4](https://example.com/image.png)

**Figure 4. Wind-up of pre-motor network activity evoked by repeated sub-threshold stimuli**

A, wind-up of net excitatory (Aa) and net inhibitory (Ab) synaptic activity in motoneurons in response to repeated sub-threshold stimuli. B, $V_m$ fluctuation evaluated as SD of $V_m$ at rest (I), 1 s after first stimulus (II) and 1 s after second stimulus (III) (grey bars in Aa and Ab). $V_m$ fluctuations increase (wind up) with repeated sub-threshold stimuli (B). Recordings in Aa and Ab are from different motoneurons. Aa and Ab from top: $V_m$ of motoneuron; ENG activity from HF nerve; stimulus. Differences significant at n.s., $P>0.05$; **$P<0.01$, $n=8$. **

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interneurons in spinal segment D4 (Currie & Stein, 1990). It is tempting to hypothesize that temporal summation in long-afterdischarge interneurons is the common parsimonious underlying cause for synaptic wind-up and protracted activation of scratch episodes. In support of this, temporal summation in long-afterdischarge interneurons and protracted scratch initiation is reduced by N-methyl-D-aspartate (NMDA) receptor antagonists in vivo (Currie & Stein, 1992). However, the lowered responsiveness may be due to non-specific reduction of synaptic excitation rather than elimination of the mechanism for the multisecound storage of excitability. Plateau-generating spinal interneurons in the turtle (Hounsgaard & Kjaerulf, 1992; Russo & Hounsgaard, 1996) provide a candidate for the long-afterdischarge interneurons. In response to brief depolarization these cells generate a long-lasting afterdischarge of APs due to activation of a plateau potential. In addition, the plateau mechanism promoted by voltage- and transmitter-dependent facilitation (Russo & Hounsgaard, 1994, 1996; Russo et al. 1997; Perrier et al. 2002) displays temporal summation on a timescale appropriate for a unifying common explanation. We note that wind-up of this kind is evoked by NMDA receptors activating L-type Ca\(^{2+}\) channels in plateau-generating wide dynamic range neurons during central pain sensitization (Fossat et al. 2007). It is possible that these mechanisms in wide dynamic range neurons also account for temporal integration of non-nociceptive sensory input observed here.

Intracellular recordings from the interneurons that generate afterdischarges to brief sensory stimuli could help to uncover the mechanism underlying temporal integration of sensory input. However, direct experimental test awaits conditional knock-outs targeted to L-type Ca\(^{2+}\) channels or NMDA receptors in relevant subpopulations of spinal interneurons. This approach will be needed to test if the activity of the pre-motor network is (a) part of the network mechanism that leads to the particular behavioural form in response to stimulation or (b) just an unspecified arousal that merely serves to reach threshold for the ‘real’ scratch mechanism.

If the intrinsic response properties of motoneurons select for ‘useful’ response patterns (Delgado-Lezama & Hounsgaard, 1999) they would seem an appropriate target for regulation. Metabotropic facilitation of L-type Ca\(^{2+}\) channels increase excitability in turtle motoneurons (Perrier et al. 2002). Glutamate released during scratch network activity produces robust facilitation of motoneurons that outlast the scratch by tens of seconds (Alaburda & Hounsgaard, 2003). In transverse slices a similar effect is produced even with a brief train of APs in glutamatergic axons of dorsolateral funiculus (DLF) in the absence of network activity (Delgado-Lezama et al. 1997). Nevertheless, the experiments reported here did not reveal changes in the excitability of motoneurons prior to the onset of scratch episodes. This suggests that the intensity of glutamate release during protracted activation of scratch is limited despite the wind-up of synaptic activity. The undetectable increase in conductance during protracted scratch initiation supports this possibility, particularly compared with the dramatic increase in synaptic conductance during scratch network activity (Alaburda et al. 2005; Berg et al. 2007). It is also possible that DLF and afferent nerve stimulation activate different groups of glutamatergic synapses with and without the ability to facilitate L-type Ca\(^{2+}\) channels.

Protracted rostral (Currie & Stein, 1990) as well as pocket scratch can be evoked by electrical stimulation of the appropriate receptive field. Moreover, fine adjustment of mechanical stimulation also leads to protracted activation of scratching (Fig. 1Ab). This long-lasting increase in the activity of interneurons may store information about sensory specificity for several seconds for each scratch form (Currie & Stein, 1988, 1990). Because our results only involve spinal mechanisms they cannot be generalized to locomotor network activity that also involves descending pathways.

In conclusion, our findings suggest that increased activity of the pre-motor network may contribute to scratch initiation but leaves the excitability of motoneurons unchanged at the onset of scratching.

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Author contributions

Experiments were performed in the Neuronal Signalling Laboratory at the Department of Neuroscience and Pharmacology, University of Copenhagen. All authors designed the study. R.G. conducted the experiments and analysed the data. All authors evaluated the data, wrote the manuscript and approved the final version of this manuscript.

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