

## Chapter 2

# Functional Properties of C-Low Threshold Mechanoreceptors (C-LTMRs) in Nonhuman Mammals

Mark Pitcher, Claire E. Le Pichon, and Alexander Chesler

*As the methods of recording the activity of the nerve fibers now have been developed to such a degree that even the smallest afferent fibers have to yield to our curiosity, further experiments may provide more quantitative data required for the analyses of the nervous mechanism of cutaneous sensations.*

Yngve Zotterman; Touch, pain and tickling:  
An electrophysiological investigation on cutaneous  
sensory nerves (1939)

**Abstract** In humans, unmyelinated C-tactile fibers, referred to as C-low threshold mechanoreceptors (C-LTMRs) in nonhuman mammals, are found exclusively in hairy skin and preferentially respond to slow moving gentle touch, such as that produced by lightly stroking the skin. While substantial species differences exist in the proportion of C-LTMRs to the total C-fiber population, C-LTMRs appear to be expressed more densely in proximal regions of the limbs and the trunk. Functionally, C-LTMRs are specifically tuned to relatively low velocity (~0.1 cm/s) cutaneous stimulation, respond with biphasic adaptation to a single sustained stimulus and exhibit prolonged fatigue in response to repeated stimulation. While a molecular marker of the global C-LTMR population is lacking, subtypes expressing MrgprB4, VGLUT3, and TH have been identified. Considering that C-LTMRs terminate in lamina II of the spinal dorsal horn, there is increasing evidence supporting their involvement in the modulation of spinal responses to nociceptive input.

**Keywords** C-Tactile • C-Low threshold mechanoreceptor • Unmyelinated C-fiber • Sensory afferent • Touch • Pain • Hair follicle

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The purpose of somatosensory afferents and their peripheral transduction organs is to inform the central nervous system about events occurring at the interface between the surface of the organism and the outside world. At this interface, sensory afferent terminals in the skin distinguish between multitudes of different stimuli, from vibration to temperature, from gentle touch to actual or potential tissue injury. Each class of touch sensitive end organ is tuned to detect a specific type of input while the associated sensory afferents transmit this information as faithfully as possible. Since Adrian pioneered the electrophysiological techniques to record activity in sensory afferents in the early twentieth century, neuroscientists have been working ceaselessly to establish how the functional differences among the rich tapestry of sensory fibers define each of their specific roles. At the macro level, the myelinated A $\beta$  and A $\delta$  fibers generally fulfill a sensory-discriminative role in that they confer the ability to rapidly detect and faithfully transmit critical information about the physical nature of the stimulus as well as its precise location on the body, permitting prompt guidance for motor activity. On the other hand, the slow conduction velocity that characterizes unmyelinated C-fibers renders them poor discriminative sensors. In accord with the suggestion that pain, like hunger and thirst, serves a homeostatic function (Craig 2003), C-fiber-mediated signaling appears to serve an affective-motivational function (Weiss et al. 2008). An interesting example of the emotional impact of nociceptive C-fiber activation is ischemic pain. Both visceral and muscle nerves have a higher proportion of nociceptive C-fibers than cutaneous nerves (Cervero and Laird 1999; Mense and Schmidt 1974). As such, ischemic block of the forearm with a blood pressure cuff rapidly produces an ischemic block of A $\delta$  fibers followed later by C-fiber blockade. As any graduate student in an upper level pain neurophysiology course will attest, the pain from ischemic block of C-fibers is much more unpleasant than that from A $\delta$  fibers. Along a similar line, sensitivity to ischemic blockade of C-fibers is increased in depressed subjects (Suarez-Roca et al. 2003).

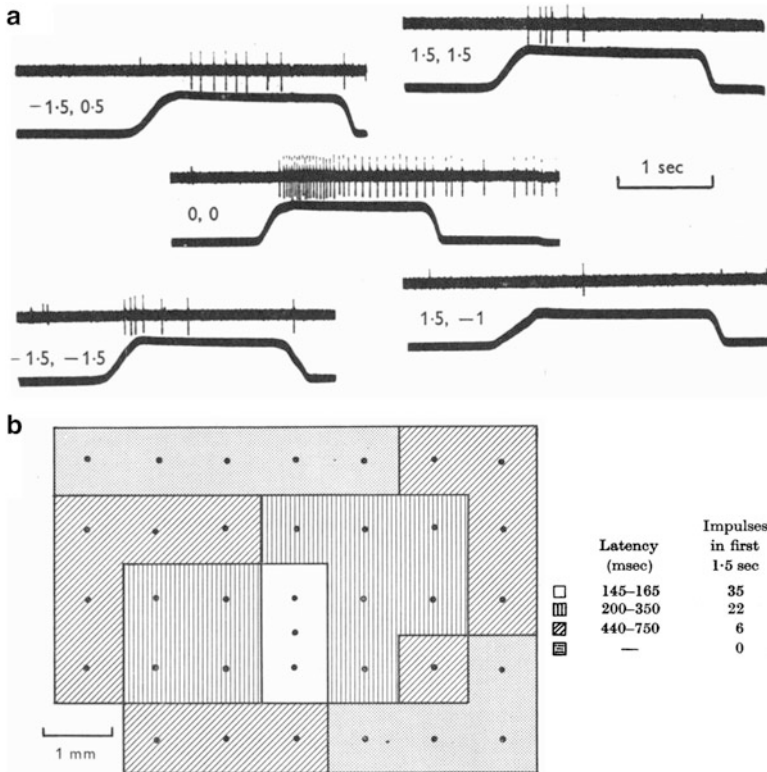
While it has been long recognized that both A $\delta$ - and C-fiber nociceptors contribute to pain perception, myelinated A $\beta$ -fibers are held to be the main nonnociceptive “touch” fibers. For these reasons, the existence of a class of unmyelinated C-fiber that appears to be specifically tuned to gentle stroking of the hairy skin (Olausson et al. 2010) with objects at body temperature (Ackerley et al. 2014) is striking. Activation of these fibers in humans evokes pleasant sensations and, as such, has been posited to subserve affective touch, consistent with the putative affective-motivational role of C-nociceptors. In fact, slow, gentle stroking evokes oxytocin and endorphin release in rodents (Uv anas-Moberg et al. 2005). To the best of our knowledge, it would seem that no other sensory fiber type is as well adapted to an affective-motivational role as the C-low threshold mechanoreceptor (C-LTMR). It appears that the survival benefits of prosocial contact (i.e., caressing/grooming) have conserved the C-LTMR, but that is a subject for another chapter.

Zotterman (1939) was the first to record electrophysiological activity in C-LTMR fibers. While he concluded that they subserve the sensation of tickle, more recent work suggests a grooming phenotype based on their robust response to gentle, slowly moving stimulation of hairy skin (Bessou et al. 1971; Kumazawa and Perl 1977; Li et al. 2011). Perhaps due to these unique properties they have only recently

garnered the attention of the wider neuroscience community as well as the public. In this chapter, we will review the functional characteristics of C-LTMRs. Of note is that most of the functional characterization derives from mid-twentieth-century classical electrophysiological studies in anesthetized rats, cats, and monkeys. Each section of this chapter will briefly summarize a distinct facet of C-LTMR function. We then turn to more recent work integrating genetic modification in mice in order to discuss how different subtypes fit into the classical definition of C-LTMRs, followed by reviewing the evidence supporting a potential modulating role of C-LTMRs in nociceptive processing.

## Peripheral Terminations and Receptive Field

While early studies were generally unable to study the anatomical and structural characteristics of C-LTMRs due to technical constraints, careful electrophysiological characterization showed that these fibers are associated with one or a few hair follicles (Douglas and Ritchie 1957; Iggo 1960; Iggo and Kornhuber 1977). Recent evidence suggests that at least a subset of peripheral C-LTMR terminals form longitudinal lanceolate endings in a palisade formation around the base of Zigzag and Awl/Auchene hair follicles (Li et al. 2011), rendering exquisite sensitivity to even the slightest deflection of the hair shaft. C-LTMR receptive fields (RFs) are found exclusively in hairy skin of mammals (Iggo and Kornhuber 1977; Sassen and Zimmermann 1971; Takahashi et al. 2003). A single C-LTMR fiber has multiple peripheral terminals in a relatively uniform field that is ovoid in shape and measures approximately 1 mm<sup>2</sup> in mouse (Liu et al. 2007; Seal et al. 2009) and 18 mm<sup>2</sup> in cat (Iggo 1960; Bessou and Perl 1969; Kumazawa and Perl 1977; Shea and Perl 1985). Humans appear to have larger RFs, on the order of 35 mm<sup>2</sup> (Wessberg et al. 2003). However, C-LTMR RF size may be overestimated if more intense stimuli are used (Bessou and Perl 1969; Iggo and Kornhuber 1977). For example, the center of the RFs were found to be more sensitive than the edges, hence stimulation at perithreshold intensity yielded smaller RFs than suprathreshold stimulation (Iggo 1960; Iggo and Kornhuber 1977) (Fig. 2.1). Species differences in C-LTMR expression have also been noted, where cats generally appear to have a higher proportion of C-LTMRs compared to other mammals. Specifically, in cat distal nerves C-LTMRs represent 25–50% of all C-fibers sampled (Douglas and Ritchie 1957; Iggo 1960; Bessou et al. 1971; Traub and Mendell 1988). However, in rodents such as rats, guinea pigs, and rabbits, approximately 8–15% are classified as C-LTMRs (Lynn and Carpenter 1982; Shea and Perl 1985; Fang et al. 2005; but see Takahashi et al. 2003 for a higher estimate) and approximately 10% in monkey (Kumazawa and Perl 1977). The proportion in humans is unclear. It must be noted that these estimates may not represent the actual numbers for reasons including sampling bias. In terms of C-LTMR density in skin, monkeys (Kumazawa and Perl 1977), cats (Bessou and Perl 1969), and mice (Liu et al. 2007, 2011; Delfini et al. 2013) exhibit more C-LTMRs in proximal nerves than distal.



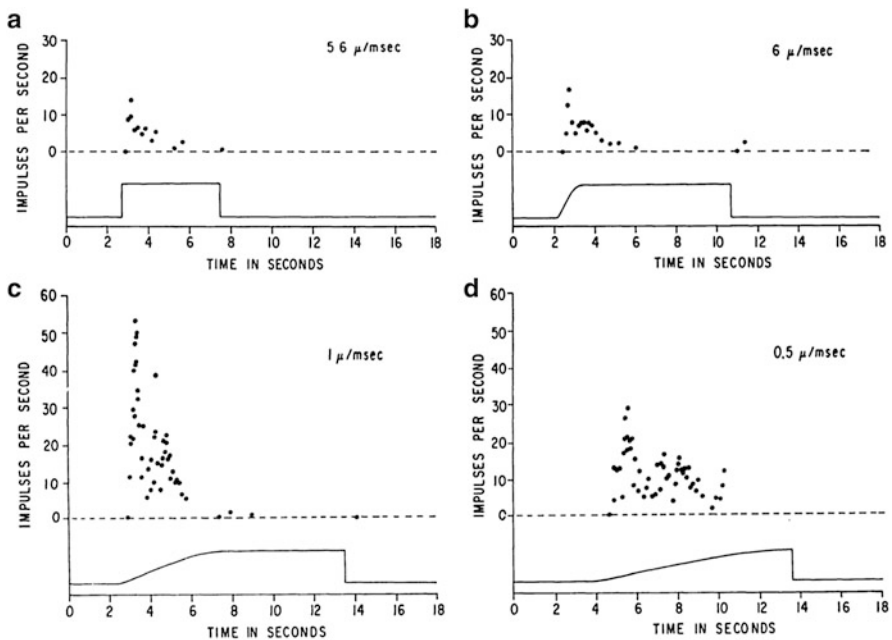
**Fig. 2.1** The receptive field of a C mechanoreceptor. (a) shows five records obtained when different spots on the skin were touched with the probe. The final load is nearly the same (2 g weight) for all positions. The distances of the probe from the most sensitive central position are indicated in millimeters. (b) is a diagram summarizing all the results for the same unit. The skin was stimulated on a 1 mm grid and each spot is indicated by a *filled circle* in the diagram. The latency of the first impulse at each position is shown by the *shading*. At the centre of the field the latency was least, the rate of firing was highest and the persistence of the discharge was longest (Adapted from Iggo 1960)

## Optimal Stimuli

The specific input required for maximal action potential output by the sensory fiber is defined by response properties of both the peripheral terminal organ (i.e., Pacinian corpuscle, Ruffini ending, etc.) and the axon (i.e., unmyelinated vs. myelinated fiber, etc.). For example, for a sensory fiber to faithfully follow high frequency vibration of the skin, the response properties of the terminal ending and the associated fiber must allow for rapid adaption to the input as well as fast conduction velocity. Pacinian corpuscles and their associated myelinated A $\beta$  fibers amply fulfill these requirements, yielding increasing spike rates with increasing stimulation frequencies. Regarding C-LTMRs, considering the remarkable sensitivity of longitudinal

lanceolate endings situated around the base of the hair follicle together with the slow conduction velocity typical of unmyelinated C-fibers, human C-tactiles (Nordin 1990) and nonhuman C-LTMRs appear to be well suited to gentle, slowly moving stimulation of hairy skin (Zotterman 1939; Maruhashi et al. 1952; Douglas and Ritchie 1957; Iggo 1960; Bessou and Perl 1969; Kumazawa and Perl 1977). Single fibers can respond to barely perceptible mechanical stimuli such as the bending of a single guard or down hair (Douglas and Ritchie 1957; Iggo and Kornhuber 1977) or less than 4 mg punctate stimulation with a von Frey filament (Iggo 1960; Bessou and Perl 1969; Kumazawa and Perl 1977; Lynn and Carpenter 1982), which is consistent with C-tactile sensitivity in humans (Vallbo et al. 1999).

A remarkable characteristic of these fibers is their specific tuning to a velocity range: Using *in vivo* electrophysiological recordings from cat DRG, Bessou et al. (1971) demonstrated that C-LTMRs respond in an inverted U-shaped curve to increasingly rapid stroking of the skin with a glass probe (Fig. 2.2). Specifically, C-LTMRs show increasing spike rates to gentle stroking with a glass probe up to approximately 0.1 cm/s, with lower spike rates at higher stimulation velocities.



**Fig. 2.2** Response of a C mechanoreceptor to a smooth stimulator moving across the skin at the indicated velocities. *Upper portion* of each graph shows the evoked discharge expressed as an instantaneous frequency vs. time. Instantaneous frequencies were obtained from the time intervals between successive pairs of impulses and marked by a dot placed on the horizontal axis at the time of occurrence of the second impulse of each pair. *Lower portion* of each graph indicates movement of the stimulator parallel to the skin surface. At the beginning of the movement, the stimulator was positioned on one side of the receptive field and had traversed the field when movement ceased (Adapted from Bessou et al. 1971)

In support, Kumazawa and Perl (1977) found that C-LTMRs respond optimally to stroking between 0.05 and 0.2 cm/s and others have noted similar velocity-dependent responsiveness (Shea and Perl 1985). Maximal C-LTMR spike frequency in response to stroking can be up to 100 spikes/s, which is significantly lower than the 300 spikes/s possible in nociceptive C-fiber axons (Franz and Iggo 1968) and in stark contrast to myelinated A-fibers that can maintain an output up to 800 spikes/s (Iggo 1960; Lindblom and Tapper 1967; Merzenich and Harrington 1969). Vallbo et al. (1999) found similar spike rates in C-tactile fibers recorded from humans. Interestingly, Douglas and Ritchie suggested that C-LTMRs were only able to fire up to approximately 10 spikes/s. However, this low estimate may be related to the limited time resolution available from their experimental setup. As mentioned earlier, in addition to slow, gentle stroking, C-LTMRs also respond to punctate stimulation, exhibiting a spike threshold of approximately 10  $\mu\text{m}$  skin indentation with linear increases in spike activity up to approximately 500  $\mu\text{m}$  indentation (Iggo and Kornhuber 1977). C-LTMRs typically exhibit little or no spontaneous (“background”) activity (Bessou and Perl 1969; Takahashi et al. 2003).

## Vibration

C-LTMRs are unable to follow 1 Hz mechanical stimulation frequencies applied to the skin for more than a few stimulations (Iggo 1960; Bessou et al. 1971); however, they can follow a 0.1 Hz frequency moderately well for up to 90 s (Bessou et al. 1971). Nonetheless, Gee et al. (1996) showed that C-LTMR fibers could follow 20 Hz electrical stimulation for up to 20 s. The inability to maintain consistent spike output in response to repeated mechanical stimuli to their RF reflects fatigue, to be discussed in more detail later.

## Cooling

Kumazawa and Perl (1977) showed that while C-LTMRs do not respond to warming of the skin, they respond robustly to progressive cooling of the skin. Hahn (1971) demonstrated that cooling the skin attenuates responses to mechanical stimulation, such that cooling appears to produce cross-modal fatigue. Many other studies support C-LTMR responsiveness to cooling (Iggo 1960; Hensel et al. 1960; Bessou and Perl 1969; Hahn 1971; Iggo and Kornhuber 1977; Takahashi et al. 2003); however, the functional significance of this is not known.

## Conduction Velocity

Conduction velocity (CV) is a measure of the speed of action potential propagation along the axon. CV is directly related to the degree of myelination of the fiber, where the most thickly myelinated fibers exhibit the fastest CV. Measurement of CV

usually involves measuring the time to arrival of an electrically evoked action potential to reach the recording electrode from the stimulating electrode a known distance away. A cardinal property of all C-fibers is that they are unmyelinated and thus display the slowest CVs. C-LTMR CV is between 0.55 and 1.25 m/s (Iggo 1960; Gee et al. 1996; Djouhri et al. 1998; Takahashi et al. 2003); however, some have been measured from 0.4 m/s (Iriuchijima and Zotterman 1960; Fang et al. 2005) to up to 2.5 m/s (Iggo and Kornhuber 1977). Gee et al. (1999) showed that there are some species differences in C-LTMR conduction velocity: In pig, C-LTMR CV is the fastest of all C-fiber types, sampled at approximately 1.2 m/s. However, rat C-LTMR CV is similar to other C-fiber types (0.87 m/s). Interestingly, C-tactile fibers, proposed to be the human equivalent of mammalian C-LTMRs, exhibit CVs between 0.8 and 1.3 m/s (Wessberg et al. 2003). A number of studies show that C-nociceptors exhibit similar CVs as C-LTMRs (Djouhri et al. 1998; Fang et al. 2005).

A common feature of repeatedly activated C-fibers is activity-dependent CV slowing, where action potentials conduct progressively slower with increasing number of stimuli. In terms of C-nociceptors, this phenomenon has been suggested to underlie some forms of persistent pain in rodents (Shim et al. 2008). However, C-LTMRs also exhibit CV slowing. Gee et al. (1996) showed that C-LTMR CV slows by approximately 14% in response to 20 Hz electrical stimulation of the fiber for 20 s, whereas nociceptive C-fibers slow by 27–29%. Interestingly, while other C-fibers show a stable rate of slowing over time, C-LTMR CV slows more abruptly over the first 6 s followed by a plateau. This initial period of acute slowing approximates the time required for adaptation, to be discussed later in this chapter.

## Spike Properties

Using *in vivo* electrophysiological techniques in cats, Bessou et al. (1971) recorded intracellularly from C-LTMR cell bodies in the DRG. The membrane potential of C-LTMR DRG neurons was typically between 60 and 80 mV, similar to A $\delta$  cell bodies. However, in guinea pig, Djouhri et al. (1998) found that the membrane potential was approximately 40–50 mV in both C-nociceptor and C-LTMR cell bodies.

In cat, the amplitude of action potentials (APs) evoked by suprathreshold peripheral electrical stimulation averaged 90 mV, higher than APs in A $\delta$  DRGs (Bessou et al. 1971). However, in rat the AP amplitude was 61 mV in C-LTMR and 75 mV in C-nociceptors (Fang et al. 2005).

In recordings from the DRG cell bodies in cat, Bessou et al. (1971) found that C-LTMR AP duration was approximately 5 ms, much wider than the 0.6–0.8 ms width of A $\delta$  APs. However, Djouhri et al. (1998) found that AP duration was much shorter in guinea pig C-LTMR cell bodies than in C-nociceptors (2.5 ms vs. 7 ms, respectively), similar to findings in rat (Fang et al. 2005). On the other hand, Traub and Mendell (1988) found that both C-fiber types in DRG cell bodies from cat had similar AP durations of approximately 7 ms. Recording from the saphenous nerve in pigs, Gee et al. (1999) showed that C-LTMRs had narrower spikes than nociceptive C-fibers; however, spike duration in rat was similar across all C-fibers.

In terms of AP rise time, fall time and after-hyperpolarization duration, Djouhri et al. (1998) and Fang et al. (2005) demonstrated that C-nociceptors were generally longer than C-LTMRs. Specifically, AP rise time was approximately 2.5 ms in C-nociceptors and 1 ms in C-LTMRs. AP fall time was approximately 3.5–4.75 ms in C-nociceptors and 1.5 ms in C-LTMRs. Finally, duration of after-hyperpolarization to 80% of baseline was approximately 15–20 ms in C-nociceptors and 5 ms in C-LTMRs. As discussed in Fang et al. (2005), these differences may be linked to differential expression of voltage-gated sodium channels (NaVs) as well as voltage-gated calcium (CaVs) and potassium (KVs) channels. For example, nociceptive neurons, but not low threshold mechano-receptive neurons, typically express greater TTX-resistant NaV1.7, NaV1.8, and/or NaV1.9 channels that contribute to the greater rising phase and duration of the action potential.

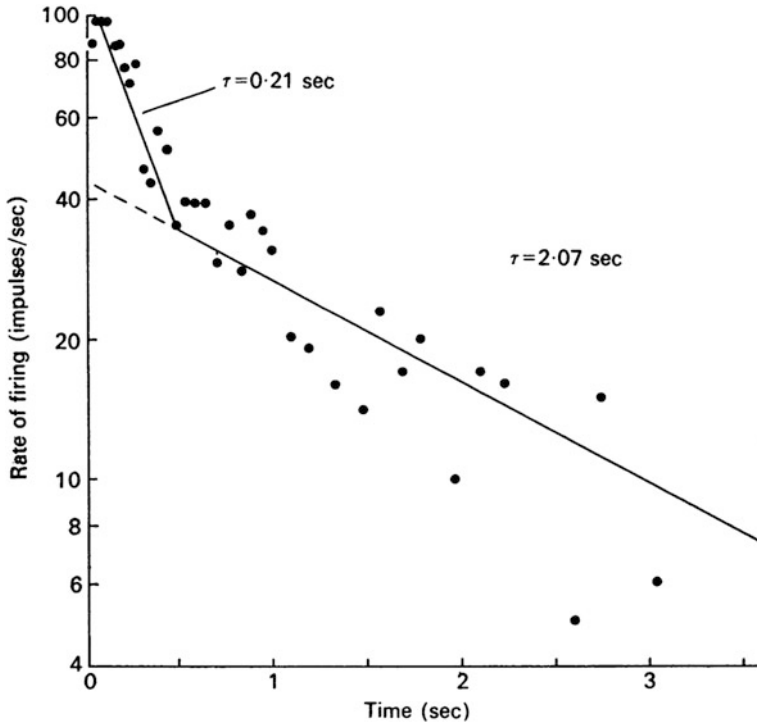
## Adaptation

Gradual decreases in neuronal output in response to a sustained, unchanging stimulus is referred to as adaptation. Rapidly adapting A-LTMRs are specially attuned to rapid movement or vibration, responding with short spike bursts that correspond to the onset/offset characteristics of the stimulus. On the other hand, slowly adapting A-LTMRs (i.e., indentation or stretch detectors) often exhibit two components of adaptation, comprised of a brief initial burst followed by relatively stable firing for the duration of the ongoing stimulus. Interestingly, C-LTMR activity in response to a sustained stimulus appears to share the two-component adaptation of slowly adapting A-LTMRs, with a brief burst of approximately 100 spikes/s followed by relatively stable spiking in the 20–65 spikes/s range (Iggo 1960; Iggo and Kornhuber 1977; Kumazawa and Perl 1977) (Fig. 2.3) that lasts approximately 5–10 s followed by virtually complete cessation of firing by 20 s (Bessou et al. 1971; Douglas and Ritchie 1957).

## Fatigue

Whereas adaptation refers to decrements in response to a single sustained stimulus, fatigue is a related phenomenon characterized by a decreased output to repeated stimuli. While A-LTMRs generally exhibit little change in spike output to repeated stimuli with interstimulus intervals (ISIs) of 2 s or more, C-LTMR output drops dramatically (Iggo 1960; Hahn 1971; Kumazawa and Perl 1977; Iggo and Kornhuber 1977), even if adaptation to the first stimuli has not yet occurred (Bessou et al. 1971). The time until recovery from fatigue, where the C-LTMR response to the second stimulus is similar to the first in terms of magnitude and duration, can take several minutes if the initial stimulus was brief (i.e., approximately 10 s). However, longer or more intense stimulation may require up to 15–30 min to recover (Iggo 1960). Considering that C-fiber receptive fields are comprised of multiple, closely





**Fig. 2.3** Adaptation of a C-mechanoreceptor to a skin indentation of 476  $\mu\text{m}$ . The rate of firing fell continuously from a maximum of 100 impulses/s and when plotted on semilogarithmic coordinates, two time constants could be fitted (Adapted from Iggo and Kornhuber 1977)

apposed terminal branches (Cauna 1969, 1973), Iggo and Kornhuber (1977) used multipoint discrimination in single C-LTMR receptive fields to determine if fatigue to repeated stimulation depends on the peripheral terminal organ or the sensory fiber axon. They showed that punctate stimulation of one point in the field did not affect responses to subsequent stimulation of another point 1 mm away within the same RF, suggesting that reduced responsiveness to repeated stimulation likely depends on end organ failure. Moreover, antidromic activation did not result in fatigue to subsequent mechanical stimulation of the RF, further supporting the end organ failure hypothesis (Iggo and Kornhuber 1977).

Similar to their observations that repeated stimuli reduce subsequent spike output, Bessou et al. (1971) observed that the minimal contact time to elicit a spike was also reduced by previous stimulation. Specifically, within 30 s of strong activation, the stimulation probe had to remain in contact with the skin for 500 ms to evoke a spike. If the receptor had not been stimulated for 5–10 min, 100–150 ms contact time was required. If 20–30 min separated the stimuli, 40–80 ms were required to evoke a spike. In contrast, A-fibers required stimulus durations of 3 ms or less to evoke robust responses (Bessou et al. 1971).

## After-Discharge

After-discharge refers to the ability of a neuron to produce impulses after cessation of the stimulus. Zotterman (1939) was the first to observe after-discharge in stroking-activated C-LTMRs. Many others have substantiated Zotterman's finding (Douglas and Ritchie 1957; Hensel et al. 1960; Lynn and Carpenter 1982). Kumazawa and Perl (1977) also demonstrated that C-LTMRs exhibit prominent after-discharge, especially when not fatigued by previous activation. C-LTMRs appear to produce lengthy after-discharge following stroking of the receptive field, whereas perithreshold punctate input may not result in any after-discharge (Iggo 1960), suggesting that this phenomenon may reflect restorative changes to the skin after indentation (Iggo and Kornhuber 1977). In other words, they interpret C-LTMR after-discharge to reflect an inverse stimulation, where it is the indented skin returning to its original position that activates C-LTMR terminals, thus producing after-discharge.

## Central Terminations

Using the plant lectin *Phaseolus vulgaris* leucoagglutinin (PHA-L) or Horseradish peroxidase (HRP) iontophoresed into the cell body, C-LTMR fibers innervating the guinea pig ear were found to terminate in lamina II of the spinal dorsal horn, similar to the termination zone of C-nociceptors (Sugiura et al. 1986; Sugiura 1996). Recent studies using modern viral vectors and genetic modification have substantiated this finding and will be discussed in the next section.

## Molecular Characterization

Until the advent of genetic modification techniques, the sole source of information on the identity C-LTMRs was based on the classical electrophysiological experiments discussed in previous sections. As such, C-LTMRs were long thought of as a homogeneous population. However, recent work has identified a number of putative markers of C-LTMRs with some degree of nonoverlapping expression. Therefore, in this section, these potentially heterogeneous C-LTMR populations will be discussed sequentially.

Before discussing C-LTMRs, a brief description of C-nociceptors is in order. C-nociceptors terminate in the epidermis with free nerve endings that respond preferentially to noxious mechanical or thermal stimuli (Bessou and Perl 1969; Cain et al. 2001) and can be found in both glabrous and hairy skin. C-nociceptors are neurochemically classified by their neuropeptide content: Peptidergic C-nociceptors terminate deeper in the epidermis and contain substance P and CGRP, while nonpeptidergic C-nociceptors (often identified as isolectin B4-positive) terminate in more superficial layers of the epidermis (Perry and Lawson 1998; Ribeiro-da-Silva et al. 1989). Functionally, nonpeptidergic C-nociceptive fibers are associated with transduction

of noxious mechanical stimuli (Cavanaugh et al. 2009; Zylka et al. 2005) whereas peptidergic fibers transduce thermal stimulation in the noxious range (Cavanaugh et al. 2009). Mas-related G protein-coupled receptors (Mrgprs) comprise a family of receptors that are found specifically in small diameter sensory neurons (Dong et al. 2001; Lembo et al. 2002; Han et al. 2002; Zylka et al. 2003). Mrgprs are differentially expressed in subsets of sensory afferents suggesting functional specificity of these subsets.

Liu et al. (2007) demonstrated that MrgprB4-expressing neurons represent less than 2 % of dorsal root ganglion (DRG) neurons and do not coexpress CGRP, P2X3, or TRPV1. However, they do express isolectin B4 (IB4) and c-RET, indicating that MrgprB4<sup>+</sup> neurons are indeed nonpeptidergic but distinct from the nociceptive nonpeptidergic C-fibers. Consistent with findings from classical studies described earlier, peripheral terminations of MrgprB4<sup>+</sup> neurons are found only in hairy skin and are less dense in skin of distal limbs. The RFs in these mice are small, roughly 1 mm<sup>2</sup> with 1–3 arborization fields per terminal. These terminations are closely apposed to hair follicles and are absent from structures such as blood vessels and muscle. Centrally, MrgprB4<sup>+</sup> neurons coterminate in Lamina II<sub>outer</sub> with other IB4<sup>+</sup> neurons. Using two-photon calcium imaging of genetically labeled MrgprB4<sup>+</sup> neurons in the mouse spinal cord, Vrontou et al. (2013) observed increased fluorescence following application of  $\alpha,\beta$ -methylene (Me) ATP to hairy and, unexpectedly, to glabrous skin of the hind paw. Importantly, gentle stroking of the hairy skin at 0.5–2 cm/s (0.2–0.5 Hz) also enhanced calcium transients whereas punctate stimulation with von Frey filaments and noxious pinching did not. Considering the pleasant sensations evoked by C-tactile input in humans (Olausson et al. 2010), Vrontou et al. (2013) used the DREADD (Designer Receptor Exclusively Activated by Designer Drugs) approach to activate MrgprB4<sup>+</sup> neurons in the conditioned place preference (CPP) assay to find that MrgprB4<sup>+</sup> neuronal activation is indeed positively reinforcing, suggesting a role in motivational reward processing.

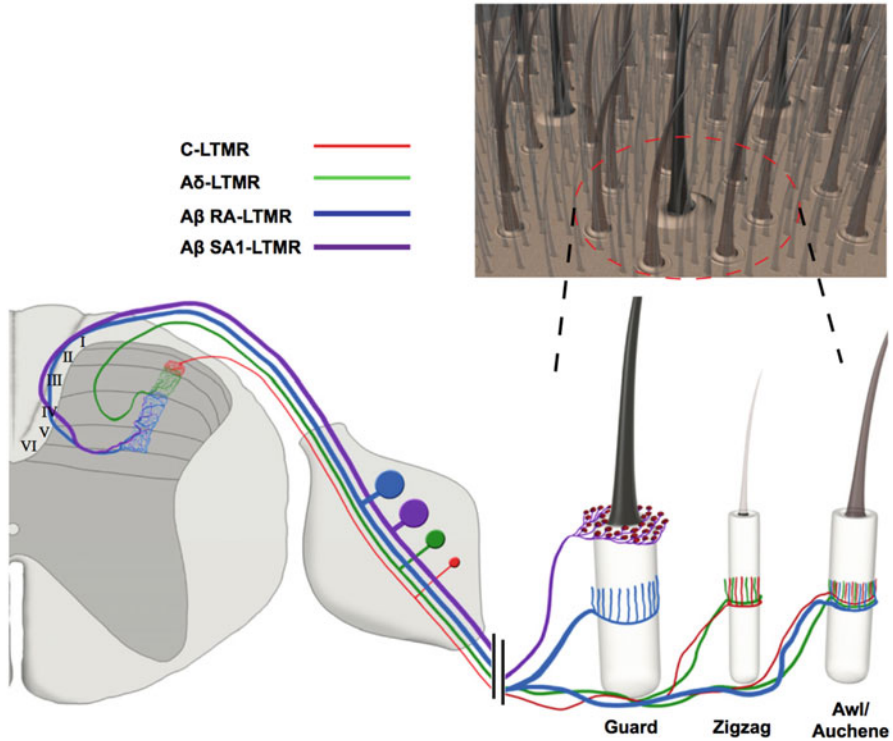
The vesicular glutamate transporter (VGLUT) family is comprised of three isoforms (VGLUT1–3), involved in intracellular transport of glutamate to synaptic release sites. VGLUT3 is the least abundant isoform, found in sensory afferents terminating in spinal lamina II<sub>inner</sub> as well as some projections to lamina I and III (Seal et al. 2009). Specifically, VGLUT3<sup>+</sup> terminals correspond closely to PKC $\gamma$ -expressing interneurons but not nonpeptidergic IB4 afferents, suggesting that VGLUT3<sup>+</sup> neurons represent a different population than MrgprB4<sup>+</sup> neurons. VGLUT3<sup>+</sup> neurons belong to the small/medium sized, unmyelinated neuronal population, and account for approximately 10 % of DRG neurons in mice. These cells are largely IB4-negative and do not express CGRP, SP, or TRPV1. Conduction velocity of VGLUT3<sup>+</sup> axons is approximately 0.6 m/s, consistent with C-fibers. In addition, these neurons are more responsive to slowly moving stimuli and exhibit clear adaptation to ongoing stimulation. RF size of VGLUT3<sup>+</sup> peripheral terminals is identical to MrgprB4<sup>+</sup> RF's at approximately 1 mm<sup>2</sup> with 1–3 sensitive spots. In contrast to findings from Liu et al. (2007) and others (Kumazawa and Perl 1977; Bessou and Perl 1969) showing reduced C-LTMR density in distal nerves, VGLUT3<sup>+</sup> neuron density appears to be equal in thoracic and lumbar regions of the spinal cord (Seal et al. 2009).

A somewhat distinct subset of mouse C-LTMRs specifically expresses tyrosine hydroxylase (TH; Li et al. 2011). TH<sup>+</sup> neurons comprise a large group of small caliber, unmyelinated neurons (Brumovsky et al. 2006; Rice and Albrecht 2008) that do not express common markers of peptidergic nociceptors, yet are also Mrgpr negative (Dong et al. 2001; Molliver et al. 1997) and do not bind isolectin B4 (Li et al. 2011). On the other hand, virtually all TH<sup>+</sup> neurons express cRet and Gfr $\alpha$ 2, markers of nonpeptidergic nociceptors (Molliver et al. 1997) and over 80 % also express VGLUT3 (Li et al. 2011). Using the skin-nerve electrophysiological preparation, Li et al. (2011) demonstrated that functional characteristics of TH<sup>+</sup> neurons are consistent with C-LTMRs. Specifically, they exhibit CVs of approximately 0.6 m/s, respond to low intensity mechanical stimulation (1–5 mN), adapt to stationary mechanical stimulation and respond to cooling but not warming. Moreover, peripheral terminations of TH<sup>+</sup> neurons form longitudinal lanceolate endings on zigzag and awl/auchene hairs, but not guard hairs (Fig. 2.4). The RF size was small, approximately 0.2–0.4 mm<sup>2</sup>, and found only in hairy skin. Finally, TH<sup>+</sup> DRG neurons were more prominent in nonlimb regions (i.e., trunk and genitalia). As such, both VGLUT3 and TH appear to label overlapping subsets of C-LTMRs. Lou et al. (2013) showed that Runx1, a transcription factor involved in the development of a wide variety of unmyelinated fibers, controls VGLUT3 and TH expression in C-LTMRs, as well as the formation of their longitudinal lanceolate terminations and, via Piezo2, mechanosensitivity.

In another study, Delfini et al. (2013) identified a putative marker of C-LTMRs that predominantly coexpresses both VGLUT3 and TH. TFAFA4, a chemokine-like secreted protein, is found in approximately 19% of thoracic and 8% of lumbar DRG neurons that do not express TRKA nor bind IB4. In addition, these neurons contain neither MrgprD nor MrgprB4. Their central terminations are found in Lamina II<sub>inner</sub> while peripheral terminations are exclusive to hairy skin. TFAFA4 neurons exhibit electrophysiological properties similar to C-nociceptors such as small cell capacitance, high input resistance, short AP duration, and expression of Nav1.8 and a number of low threshold-type currents. However, these neurons do not respond to capsaicin, menthol, or other agents known to activate nociceptors. They show slowly adapting mechanosensitive currents that best respond to slow moving stimuli (Delfini et al. 2013). Taken together, VGLUT3- and TH-containing neurons appear to be strong candidates for specific markers of most C-LTMRs (Seal et al. 2009; Li et al. 2011; Lou et al. 2013; Delfini et al. 2013); however, MrgprB4 seems to define a separate class (Vrontou et al. 2013).

## Nociceptive Processing

The spinal termination pattern of C-LTMRs places them in a privileged place to be involved in nociceptive processing: Lamina II neurons are well known to contribute to processing injury-induced hypersensitivity (Malmberg et al. 1997). In humans, inhibition of C-tactile input has been suggested to promote tactile



**Fig. 2.4** The organization of LTMR endings in hairy skin and the spinal cord dorsal horn. The peripheral endings of Abeta-LTMRs, Adelta-LTMRs, and C-LTMRs associate with either one or two of the three types of hair follicles of trunk and proximal limb hairy skin. At zigzag hair follicles, C-LTMRs (*red*) and Adelta-LTMRs (*green*) form interdigitated longitudinal lanceolate endings; At awl/auchene hair follicles, Abeta RA-LTMRs (*blue*), Adelta-LTMRs (*green*), and C-LTMRs (*red*) form inter-digitated longitudinal lanceolate endings; Guard hair follicles are associated with longitudinal lanceolate endings formed by Abeta RA-LTMRs (*blue*) and clusters of Merkel cells, or touch domes and thus Abeta SA1-LTMRs (*purple*). The central terminals of LTMRs that innervate the same or adjacent hair follicles within a peripheral LTMR unit are aligned to form a narrow LTMR column in the spinal cord dorsal horn (Adapted from Li et al. 2011)

allodynia (Kramer et al. 2007; Linde et al. 2004), and activation of C-tactile fibers attenuates experimental pain (Kramer et al. 2006) in a manner reminiscent of Melzack and Wall's gate control theory (Melzack and Wall 1965). However, as described later, recent rodent studies suggest that C-LTMRs may play either pronociceptive or antinociceptive roles in persistent pain states (Seal et al. 2009; Lou et al. 2013; Delfini et al. 2013).

VGLUT3 is selectively expressed in unmyelinated sensory fibers that do not express markers of nociceptive neurons. Considering their role in synaptic glutamate release, mice lacking VGLUT3 would be expected to show deficits in sensory transmission. Seal et al. (2009) showed that mice in which VGLUT3 has been genetically deleted (VGLUT3<sup>-/-</sup>) are indistinguishable from wild-type littermates in

terms of innocuous thermal and mechanical sensitivity; however, responses to higher intensity mechanical stimuli are blunted. Spinal wide dynamic range (WDR) neurons are well known to respond in a graded fashion to graded mechanical stimuli (Pitcher and Cervero 2010). In VGLUT3<sup>-/-</sup> mice, WDR neuronal responses to mechanical stimulation of the RF mirrored behavioral findings in that they exhibited normal responses to innocuous stimuli but had reduced firing to more intense mechanical stimulation. Accordingly, in mice with intact VGLUT3, VGLUT3<sup>+</sup> fibers responded more intensely to higher intensity than lower intensity mechanical stimulation. In response to carrageenan-induced inflammation of the hind paw, nerve injury as well as a hind paw model of postsurgical pain, VGLUT3<sup>-/-</sup> mice exhibited attenuated mechanical hypersensitivity but normal thermal hypersensitivity. In contrast, after intraplantar formalin they showed similar responses to wild-type littermates (Seal et al. 2009). Based on this data, Seal et al. suggest that the C-LTMR activity may have pro-nociceptive effects, particularly in persistent pain states. As aforementioned, Runx1 controls VGLUT3 and TH expression in neurons with unmyelinated axons. Behavioral findings based on the global VGLUT3 knockout approach used by Seal et al. (2009) are difficult to interpret due to the wide expression pattern of VGLUT3 in other tissues. Using mice deficient in VGLUT3 specifically in Runx1-lineage neurons, Lou et al. (2013) explored C-LTMR involvement in nociceptive processing. In contrast to Seal et al. (2009), they found no changes to heat or mechanical sensitivity at baseline, after intraplantar capsaicin, CFA, and following spared nerve injury. However, similar to Seal et al. (2009), these mice had subtle yet statistically significant increases in mechanical thresholds (i.e., reduced mechanical hypersensitivity) after carrageenan-induced inflammation of the hind paw. Together, these studies suggest that C-LTMRs may play pro-nociceptive role in persistent inflammatory pain signaling.

Other studies have also focused on molecular markers related to VGLUT3-expressing DRG neurons. TAF4 is strongly coexpressed with VGLUT3 and TH, and completely distinct from MrgprB4 neurons (Delfini et al. 2013). TAF4-deficient mice have normal baseline thermal responsiveness but baseline mechanical sensitivity was not tested. Interestingly, in response to intraplantar formalin, while TAF4 nulls exhibited a similar number of nociceptive behaviors during the first 5-min period (first phase) compared to wild types, they had increased behaviors during the second phase (10–60 min postformalin). Similarly, in both carrageenan-inflamed and nerve injured TAF4 null mice, mechanical hypersensitivity was dramatically prolonged, an effect that was reversed with exogenous TAF4. Moreover, excitability of lamina II<sub>inner</sub> neurons in TAF4-deficient mice was increased, suggesting that under normal conditions endogenous TAF4 is antinociceptive.

To reiterate, mice deficient of VGLUT3 in C-LTMRs exhibit blunted nociceptive responses whereas mice deficient in TAF4, found mainly in VGLUT3 neurons, show enhanced nociceptive responses. The same neurons appear to express molecules that promote nociceptive signaling (VGLUT3) and resist nociceptive signaling (TAF4). Delfini et al. (2013) address this apparent contradiction by proposing that C-LTMRs may corelease glutamate and TAF4, and it is the balance created by this corelease that tips either in favor of pro-nociceptive or antinociceptive effects.

They go on to suggest that under pathological conditions, increased C-LTMR activity may result in elevated TAF4 release that suppresses nociceptive output to nociceptive neurons in the superficial dorsal horn. There is evidence to support increased C-LTMR activity in persistent inflammatory pain states: Using the intraplantar CFA model of peripheral inflammation, Takahashi et al. (2003) showed that electrophysiologically characterized C-LTMRs exhibit an enhanced responsiveness to cooling the skin as well as increased spontaneous activity. Considering the complex tangle of excitatory and inhibitory interneurons, intrinsic spinal neurons as well as peripheral terminals of sensory afferents, more work is required to understand just how C-LTMRs contribute to pain processing.

## Final Statements

C-LTMRs are clearly involved in detecting gentle, slowly moving mechanical stimuli and, in contrast to myelinated A-fibers, are not ideally suited to sensory-discriminative functions. Thus, under normal conditions, C-LTMRs may serve affective-motivational purposes underlying social bonding. Considering the relatively small number of studies addressing C-LTMRs in pain processing as well as the technical differences between these studies, it is difficult to draw conclusions. An added confound may be related to the virtually universal approach to testing pain in rodents: The hind paw. C-LTMRs are not found in glabrous skin. As such, assessing C-LTMR involvement in pain processing using hind paw injections of irritants or nerve injury to distal limb nerves may not be optimal. For these reasons, Lou et al. (2013) also injected capsaicin into the hairy skin of the dorsal hind paw in VGLUT3-deficient mice, but found no difference compared to wild types, perhaps due to the generally smaller numbers of C-LTMRs in distal nerves innervating the limbs. With this in mind, as well as the generally greater number of C-fibers in visceral and deep muscle tissues, perhaps future studies using visceral or muscle pain models may be more fruitful. Moreover, considering the putative role that C-LTMRs play in social bonding, as well as mesolimbic pathway involvement in both reward and analgesia, it is surprising that only one study has probed their role in reward: Vrontou et al. (2013) demonstrated that DREADD-induced activation of C-LTMRs produced conditioned place preference. Future studies may also incorporate potential C-LTMR-induced changes in reward-motivational circuitry. Overall, C-LTMRs represent a small but fascinating class of sensory fibers that require additional experimentation.

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