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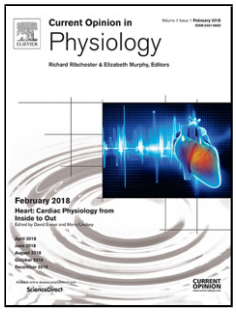

Mechanotransduction channels in proprioceptive sensory nerve terminals: still an open question?

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PII: S2468-8673(20)30156-5

DOI: https://doi.org/10.1016/j.cophys.2020.11.007

Reference: COPHYS 391

To appear in: *Current Opinion in Physiology*

Please cite this article as: Bewick GS, Banks RW, Mechanotransduction channels in proprioceptive sensory nerve terminals: still an open question?, *Current Opinion in Physiology* (2020), doi: https://doi.org/10.1016/j.cophys.2020.11.007

Mechanotransduction channels in proprioceptive sensory nerve terminals: still an open question?

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Highlights:

- Muscle spindle firing is driven by a sustained slowly adapting Na^+-dominated receptor potential.
- Piezo2, a rapidly-adapting Ca^{2+}-selective mechanosensory channel, powerfully regulates spindle function.
- DEG/ENaCs, slowly adapting Na^+-selective channels, seem dispensable for spindle responses.
- We propose a testable model, based on published literature, that may resolve this conundrum.

Abstract

Mechanosensory transduction (MST) in proprioceptors, and other low threshold mechanosensory nerve terminals (LTMT), has been debated intensely for decades. MST in muscle spindles produces a receptor potential that encodes stimulus speed and duration, is predominantly due to Na^+, a little Ca^{2+}, plus some transient, non-mechanically-gated K^+ ion fluxes. The abundant, multiple Na^+-selective DEG/ENaC channel isoforms present in all LTMTs seemed obvious Na^+ sources, perhaps supplemented with Ca^{2+}-selective TRPs, and Ca^{2+}-activated K^+ channels. However, genetic deletions of even multiple DEG/ENaC genes produces only mild functional perturbation. Conversely, deleting the more recently discovered Piezo2 mechanosensory protein profoundly impairs LTMT responses, including in muscle spindles. Yet, its transient opening, non-Na^+-selectivity and pharmacology do not reflect known receptor potential and response properties. A Ca^{2+}-dependent recycling vesicle pool that we have shown is essential for mechanosensitivity, plus other recent DEG/ENaC discoveries, may reconcile these conflicting observations. We propose the abundance of axolemmal MST complexes, comprising untested DEG/ENaC combinations, is controlled by Piezo2-gated Ca^{2+} influx that regulates their vesicular insertion and retrieval.

Key words

Muscle spindle, low threshold mechanoreceptors, synaptic-like vesicle, Piezo, DEG/ENaC, transient receptor potential TRP

Introduction

Mechanosensory transduction (MST) - the sampling and conversion of mechanical energy into neural signals typically transmitted by primary afferent neurons to the central nervous system - is a complex process. This complexity is reflected in the diversity of structures and physiological responses shown by the multitude of mechanosensory nerve endings and organs (e.g. for a recent review showing this diversity even within cutaneous endings, see[1]). The principal event of MST is the mechanical gating of one or more types of ion channel in the plasmalemma of the sensory terminal. There are many excellent recent reviews considering MST channels generally across all mechanosensory systems[2-6], so we will not do this here. Instead, we consider recent findings in the context of the proprioceptor with which we are most familiar: the mammalian muscle spindle, with reference to other low-threshold mechanoreceptors where direct spindle comparisons are not available.

The electrical response of a muscle spindle primary ending to a simple ramp-hold-release stimulus (the muscle is stretched at a constant rate, held for a period at the new length and then released at a constant rate) is complex. This is shown in the action potential firing pattern of a peroneus brevis muscle spindle in the anaesthetised cat (Figure 1a). The receptor potential, or sensory terminal depolarisation, produced by a similar stretch has been recorded by Hunt et al. (Figure 1b) and showed the complex action potential firing rates reflect the various phases of the receptor potential quite accurately. They showed this receptor potential is largely mechanically-gated throughout with two ionic components: a predominant Na^+ current, and a smaller Ca^{2+} current[7]. Both currents persist throughout the dynamic and static phases of the response (Figure 1b-d). The receptor potential is triggered immediately upon stimulation, with a very low movement threshold. The complexity of the waveform's rapidly and slowly adapting kinetics are mostly due to modifying K^+ channel components. The removal of Ca^{2+}, or blockade of the Ca^{2+} current, has little effect in the continued presence of Na^+, while removal of Na^+ alone reduces the amplitude to 30% of normal. The subsequent additional removal of Ca^{2+} produces an initially rapid, then slower, decline over several minutes to an almost 0 potential (Figure 1c). There are similar effects of Na^+ and Ca^{2+} removal on amplitude for both the dynamic and the static components of the potential (Figure 1d). In these conditions, there was no evidence of a residual K^+ current. This indicates the K^+-mediated elements are not stretch-activated. It also argues against the substantial involvement of a non-selective cation channel. This suggests the Na^+ and Ca^{2+} currents seem to involve quite ion-selective channels. Finally, Li^+ readily substitutes for Na^+, increasing the amplitude of both the dynamic and static components by 20%.

Two mechanisms are proposed for mechanical stimuli to gate mechanosensory channels: directly, by tension within the lipid membrane (force-from-lipid; FFL) or indirectly, by channel tethers intracellularly to the membrane cytoskeleton, and extracellularly, to a component such as the basal lamina (force-from-filament; FFF)[3,8]. FFL mechanisms are known to be physiologically relevant in bacterial channels, such as MscL[9], but also K_{2P} channels[10] and Piezo1[11] in eukaryotes. In eukaryotes, FFF mechanisms have been particularly studied in relation to low-threshold, slowly-adapting responses. These are perhaps best exemplified in mammals by tip-link gating *via* protocadherins in cochlear hair cells[12], but increasingly in neurites of sensory neurons in culture[13]. In muscle spindles, primary mechanosensory terminals are sandwiched between the intrafusal muscle fibres they encircle and their overlying basal laminae[14], providing ample opportunity for FFF-like interaction with both.

Synaptic-like vesicles (SLVs) are another prominent feature of all such low-threshold sensory endings[15]. We have described how SLVs recycle to and from the membrane and, like their synaptic counterparts, this is Ca^{2+}-dependent[16,17]. Some recycling is constitutive, but mechanical stimulation increases their rate of recycling, secreting glutamate. This has an excitatory action to sustain or increase both the recycling rate and the response of the sensory ending to stretch. The glutamate autoreceptor is a non-canonical metabotropic receptor with a unique pharmacology[16]. Classical blockers of canonical ionotropic or metabotropic glutamate receptors have little effect, singly or in combination, but it is specifically blockable by PCCG-13[16-19]. The effects of this system are very powerful, as autoreceptor blockade reversibly abolishes spindle firing. Similarly, intramuscular injection of tetanus toxin, which cleaves the vesicle SNARE protein synaptobrevin essential for vesicular secretion, also abolishes stretch-evoked spindle firing[20]. Importantly, these effects of SLV/glutamate-mediated modulation are not rapid, but rather their onset and persistence is over the timescale of hours. While this is in sharp contrast to the millisecond timescale responsiveness of the receptor potential, they are clearly powerful. SLVs, therefore, seem not to be important for the immediate MST response, but rather for maintaining long term sensitivity.

Overall, therefore, this review uses the context of the mature differentiated terminal to consider three issues of MST: the properties of candidate MST channels, where these properties best fit with the known receptor potential properties, and what regulates their ability to respond to stimuli. We first describe recent findings regarding the candidate MST channels expressed in muscle spindle sensory endings. We make no further assumption for channel properties beyond these. We evaluate how these might produce the receptor potential characteristics in mature differentiated mechanosensitive terminals. This leads to a testable model that attempts to provide a best-fit between terminal and channel observations. Thus, the model's purpose is to be inclusive: accepting all components are expressed, and the published properties are accurate. It then proposes how they collectively might account for the different stretch-dependent components of the complex receptor potential. In so doing, it aims not only to reconcile superficially irreconcilable discrepancies in the literature but also to develop testable hypotheses.

DEG/ENaC channels

DEG/ENaCS have been widely discounted as having a major contribution to mechanical sensation in mammals. However, recent evidence challenges this conclusion, including data from our own laboratory. In 2010 we presented physiological, pharmacological and immunohistochemical evidence that the responses of muscle-spindle sensory endings to ramp-and-hold muscle stretches within the biological range depended on low-affinity, amiloride-sensitive channels[21]. Using an *ex vivo* nerve-muscle preparation, where the endings being deeply embedded and enclosed by their selectively permeable capsule, we found that responses were virtually abolished in the presence of either amiloride or its analogues benzamil and ethyl isopropyl amiloride, with an IC_{50} of only about 10 μM in each case. Hexamethylene amiloride had similar effects, although usually required higher concentrations (Figure 2). None of the drugs affected action potential propagation, even at the highest concentration, indicating they blocked a mechanism upstream of action potential propagation. Likely candidates included amiloride sensitive MST channel(s) such as α, β and γ ENaC, and ASIC2a, that display high immunoreactivity in these endings. This conclusion was also reached for several additional reasons, the principal ones being:

a) excepting ASIC1a, DEG/ENaCs are non-voltage-gated and Na^+-selective[22];

b) other DEG/ENaCs (MEC-4 & MEC-10), were already established as essential components of the MST process in *Caenorhabditis elegans*[23];

c) β and γ ENaCs and ASIC2 seem strongly expressed in all low threshold mechanosensory endings, including baroreceptors and low-threshold cutaneous receptors in the rat foot pad such as Merkel-cell receptors and Meissner corpuscles[24-26];

d) ENaCs can be mechanically gated[27,28]; and finally,

e) ASIC2a (then known as BNaC1α) is preferentially transported to the peripheral and not the central axon of the parent DRG neurons[29], indicating a sensory terminal-selective expression.

Genetic screening of touch-insensitive mutants of *C. elegans* identified other, non-ion-channel MEC proteins associated with DEG/ENaC-mediated MST. Mammalian homologues are found in large soma-diameter mechanosensory neurons indicative of low threshold afferents, including muscle spindles. One of these is stomatin-like protein 3 (STOML3), a homologue of MEC-2. It is necessary for touch responses in myelinated (A group) fibres, especially the slowly adapting ones[30], likely in a complex with stomatin[31]. STOML3/stomatin is an integral membrane-protein complex that immunoprecipitates with ASIC2, and seemingly facilitates mechanosensation by stiffening the membrane[32]. Poole *et al*. have developed a sophisticated technique for mechanical stimulation of DRG neurites in culture by substrate displacement[33], i.e. *via* the neurite filamentous attachments to the substrate. This mimicking of FFF and stimulus presentation *in vivo* reveals a STOML3-dependent nanometer displacement sensitivity, approximating a very plausible *in vivo* physiological range. These effects were again greatest on the slowly adapting components of the stretch-evoked response. This ASIC/STOML3 complex is delivered to the cell surface by vesicles, perhaps as a pre-assembled functional unit, or 'sensory transducosome'[31]. Indeed, vesicular insertion/sequestration of DEG/ENaCs is well established in transporting epithelia, where they have a half-life of around 1 hour[34,35]. Recently, further evidence has emerged for mechanical, FFF-enhanced gating of mammalian DEG/ENaC channels. In heterologous expression systems, ENaCs produce mechanically gated Na^+ currents, that are maintained for the duration of the stimulus[27,28]. Mechanical gating sensitivity is increased by contact with extracellular matrix (ECM), and the ECM-interacting glycan domains have been identified[3]. Similarly, we have shown in proprioceptor DRG cell neurites in culture that substrate indentation activates ASIC3-dependent action potentials, i.e., that are amiloride- and APETx2-sensitive (ASIC-3-specific antagonist)[36]. Further, 75% of proprioceptor neurites produced action potential trains to sustained substrate deformation, consistent with primary afferent responses. Thus, DEG/ENaC-dependent FFF mechanisms do seem relevant to mammalian MST, particularly slowly adapting mechanosensory responses such as the static response in muscle spindles.

Counter to these arguments is that mechanoclamp stimulation, i.e. depression of the cell/neurite membrane with a blunt probe, was neither ASIC-3 dependent nor amiloride/APETx2-sensitive. Indeed, DEG/ENaCs in general are not essential for responses to mechanoclamp stimulation[37]. Moreover, gene deletion of individual DEG/ENaC subunits only subtly affects mechanosensation, including our own experiments on ASIC3 in muscles spindles[36]. Indeed, triple-knock out of ASIC1a, 2 and 3 actually enhances mechanosensitivity[38]. Also, amiloride is not a particularly selective drug and can interact with other channels and transporters at the concentrations used, such as T-type Ca^{2+} channels or the Na^+/Ca^{2+} exchanger. Finally, although deletion of DEG/ENaC homologues Ppk1 and Ppk26 from *Drosophila* class IV da neurons profoundly perturbs locomotion, there are no severe proprioceptor-linked diseases associated with DEG/ENaC mutations in mammals. It is these counter observations that have historically led to the exclusion of DEG/ENaCs from continued consideration as the primary MST in mammalian systems. However, the mechanical stimulus activation threshold

in mechanoclamp systems is surprisingly large, usually around 1 μm. This could be quite damaging to sensory terminals *in vivo*. Our modelling in spindle terminals shows even an unrealistically large longitudinal strain of 20% (i.e., an elongation that exceeds flexor muscle length even in full limb extension) increases the entire membrane profile of a terminal circumference by only 1 μm, whereas much smaller strains of <1% are readily detected physiologically[39]. Thus, it is difficult to extrapolate mechanoclamp outcomes in cell culture directly to intact sensory terminals. Their high thresholds, FFL-like properties and amiloride resistance suggest they likely reflect some combination of the following: immature neurons, typically from neonates, in culture; DRG somata or expression system cells do not represent MST arrangements in highly morphologically and physiologically specialised adult terminals *in vivo*. In addition, even the triple deletion in mammals only removed two of the DEG/ENaCs expressed in proprioceptors, as ASIC1a is not present[36] still leaving all three ENaC proteins (α,β,γ) and ASIC1b highly expressed in proprioceptor neurons. In fact, ASIC1b expression is particularly high in sensory neurons[4,36]. DEG/ENaCs are well known for their promiscuous ability to form functional heterotrimeric channels in almost any possible combination. All are Na^+-selective except those containing ASIC1a, which is Ca^{2+} permeable, but this is not expressed in proprioceptors. Indeed, in mouse DRG neurons, most expressed ASIC channels are heteromeric[40]. Their ubiquity, diversity and elevated expression in mechanosensory terminals may reflect a protective functional redundancy, evolved due to the fundamental importance of mechanosensation. Moreover, in cardiac mechanosensory neurons, which co-express ASIC3 and ASIC2a, deletion of either gene leads to expression of functional homomeric channels from the remaining protein[40]. Thus, while historically discounted, many recent findings suggest DEG/ENaCs may indeed fulfil the criteria to be MST candidates in mature proprioceptor terminals. If so, they would be well suited to producing the predominant Na^+ current in the receptor potential.

Transient receptor potential and TMEM150C/Tentonin-3 channels

There is strengthening evidence that channels of the transient receptor potential (TRP) family are expressed and functionally important in primary mechanosensory endings, particularly TRPCs and TRPVs[2,41-43]. Perhaps most studied and relevant is TRPV4, which is expressed in primary mechanosensory terminals of proprioceptors, baroreceptors and endocardium[43-46]. TRPV4 is implicated in neurodegenerative disorders, such as scapuloperoneal spinal muscular atrophy (SPSMA) and Charcot-Marie-Tooth disease type 2C (CMT2C, a hereditary motor and sensory neuropathy[47-50]. These involve progressive muscular weakness, loss of sensation and bone abnormalities, such as scoliosis. TRPV4 is FFF- but not FFL-gated[51,52]. TMEM150C/Tentonin-3 is a recently described, slowly inactivating mechanotransducer candidate, that is labelled brightly by direct immunofluorescence in terminals of proprioceptors and other slowly adapting mechanosensory neurons[RW.ERROR - Unable to find reference:1211]. Its deletion markedly impairs locomotion and perturbs, but does not abolish, stretch-evoked muscle spindle firing.

However TRPs, even TRPV4, have not been widely explored functionally in proprioceptors, while TMEM150C/Tentonin-3 has only very recently been reported. They are non-selective cation channels, which are ill-suited for the predominantly Na^+-dependent aspect of the receptor potential. Conversely, TRPs are typically associated with Ca^{2+}-driven signalling, TRPV4 in particular has a relative Ca^{2+}:Na^+ permeability ratio of 6:1[54], with Ca^{2+} being the major cation permeating under physiological conditions[RW.ERROR - Unable to find reference:1232]. Thus, TRPV4 in particular could contribute to the stretch-evoked Ca^{2+}-entry identified by Hunt *et al.*[7]

Piezo2

In strong contrast to deletion of the DEG/ENaC combinations tested to date, Piezo2 has profound effects on mechanosensory neuron responsiveness. Following its identification as a mechanically gated ion channel[55], Piezo2 was found to be expressed in DRG neuronal somata of various sizes, including large diameter proprioceptors, as well as other cell types[55]. A conditional knock out (cKO) of *Piezo2* produces behavioural abnormalities in mice consistent with loss or reduction of low-threshold mechanical responses (Figure 3). Further, in *ex vivo* skin-nerve preparations, it greatly reduces mechanosensitivity in low-threshold receptors, both rapidly and slowly adapting[56]. KO of *Piezo2* from *Pvalb+* or *HoxB8+* DRG neurons to target skeletal muscle proprioceptors, particularly spindles, led to severe motor coordination problems and abnormal limb positions, and dramatically reduced stretch-evoked firing, rendering almost all spindles insensitive despite their normal structure[57]. Both dominant and recessive mutations are now also known in the human *Piezo2* gene, including gain and loss of function. These produce complex phenotypes involving many body systems, particularly joint abnormalities (distal arthrogryposis) but weak or absent tendon reflexes also suggest this involves malfunctioning of muscle spindles[58-66]. Thus, Piezo2 is clearly extremely important for the normal function of muscle spindles and other low-threshold primary afferents. This led to the conclusion that "Piezo2 is the principal mechanotransduction channel for proprioception"[57].

However, several of its properties are difficult to map onto the slowly-adapting, Na^+-dominated receptor potential characteristics of the muscle spindle receptor potential. As for the previous channels, we will outline these inconsistencies, but our model will propose how these properties can still explain its profoundly powerful effects.

Like DEG/ENaCs, studies of Piezo2 channel properties typically present stimuli as direct membrane indentations (FFL-gated), often applied to the DRG somata. Here, Piezo2 almost invariably responds with a very rapidly adapting (τ <10 ms), amiloride-insensitive, non-specific cation current (Figure 3)[57,67,68]. Indeed, the recent discovery of the many Piezo2 splice variants shows those selectively expressed in mature proprioceptor neurons are even more rapidly adapting than variants in other tissues[69]. Unlike DEG/ENaCs, however, targeted KO of *Piezo2* from skeletal muscle proprioceptors results in the loss of the mechanoclamp-evoked Piezo2 current in cultured DRG cells[57]. The acute conditional KO of *Piezo2* from all cell types associated with cutaneous mechanotransduction, ablates mechanoclamp-evoked responses from rapidly adapting neurones but leaves MST currents unaffected in slowly adapting neurons[68].

While the arguments regarding the interpretation of direct mechanoclamp studies still apply, like ASIC2a, substrate deformation/STOML3 studies also bring Piezo thresholds within the physiological nanometer range[33]. A Piezo2 interactome complex has been shown to modify the amplitude of the rapidly adapting current. However, so far neither STOML3 expression nor the interactome complex studies have been found to greatly affect Piezo2's transient opening in response to a maintained stimulus[70,71]. Moreover, Piezo channels are non-selective cation channels, or show a small selectivity for Ca^{2+} over Na^{+}[6,55,72]. In contrast to the receptor potential, divalent cations actually impede monovalent cation permeation and Piezos show reduced permeability to Li^{+}[6]. An often-used diagnostic is blockade by the styryl dye FM1-43[67]. For many years, we have used FM1-43 to study the system of glutamatergic intracellular vesicles (SLVs) in sensory terminals of spindles and other low-threshold mammalian mechanosensory endings[16,17]. Gale *et al*. reported in isolated hair cells that when FM1-43 permeates mechanically sensitive channel(s), the internalised dye blocks them from the inside, permanently labelling the cell[73]. In contrast, in lanceolate mechanosensory endings of mouse hair follicles, even one hour of 10 μM FM1-43 does not significantly inhibit sensory-ending responses to guard hair deflection, but brightly and reversibly labels the sensory terminals - an

observation we have since confirmed in the muscle spindle[74]. Similarly, in both of these sensory terminal types, internalised FM1-43 is readily and spontaneously released and this "destain" process is accelerated by mechanical stimulation. Thus, in mature fully differentiated terminals, FM1-43's properties align more consistently with dye partitioning into the terminal membrane and being internalised *via* SLV recycling, like their synaptic counterparts, rather than channel permeation[75,76]. Similar observations have also been made for SLV-like structures in the apical region of cochlea hair cells[77].

Another potential issue may be protein abundance. Direct labelling with Piezo2 antibodies is readily seen in DRG cell bodies, Merkel cells and components of Meissner corpuscles, but is notoriously difficult to produce in primary mechanosensory terminals, even when adjacent in the same tissue section[78]. Rather, to label terminals antibodies against the GFP-tagged protein are generally used, presumably to provide additional signal amplification[57]. This could reflect accessibility problems or antigen masking issues peculiar to the terminals but not other cells or cell regions, although this is not found with other MSTs. Alternatively, it may suggest lower expression levels in terminals. A recent study indeed suggests Piezo2 expression in proprioceptors is not particularly enriched (<1.5-fold) compared to other sensory neurons[79]. Any model of mechanosensory function based on this evidence would need to explain how this relatively low abundance, perhaps particularly in the sensory terminals, can exert such profound functional effects.

Finally, the level of functional deficit may depend on the duration or method of Piezo2 ablation. In adult mice where targeted deletion of Piezo2 from all cutaneous mechanosensory components is induced acutely by tamoxifen administration, the frequency of action potentials in cutaneous afferents, although markedly reduced, is not totally abolished (Figure 3). Rather, the qualitative pattern of firing is similar to the wild-type, and persists for the duration of the stimulus[56], suggesting continued functionality of other mechanosensory channels. Conversely, following targeted deletion from proprioceptors throughout development, spindle function is essentially abolished[57]. This may indicate tamoxifen-induced deletion is incomplete, or that the absence of Piezo2 throughout spindle primary afferent development has additional effects. However, from the abundant data in the studies, these deletions seem entirely effective in both cases. Overall, therefore, the current model of Piezo2-mediated MST seems not to provide a ready explanation for the properties of the receptor potential.

The conundrum: channel properties in the context of the muscle spindle primary sensory terminals

Thus, there is a conundrum regarding mapping any of these various candidate MST channels onto the physiological properties of the receptor potential or firing patterns of mature differentiated spindle sensory terminals. These MST candidate channel properties are summarised in Table 1. First, none of them individually possesses all the required characteristics. DEG/ENaCs are highly expressed and have the required kinetic, ionic permeability and pharmacological properties for the persistent and predominant Na^+ component. They are also associated with a vesicle-based transducosome. However, so far, deletion of subsets of DEG/ENaCs have produced minimal deficits. TRPs and TMEM/Tentonin-3 cannot produce the predominantly Na^+ current of the proprioceptor potential, but they are slowly adapting, highly expressed in low-threshold mechanosensory neurons and deletion strongly impairs their responses. Piezo2 is clearly necessary for normal MST in the muscle spindle, but it remains unclear how its apparently modest expression levels or known kinetic, ionic permeability and pharmacological properties can reproduce the receptor potential.

A possible resolution:

We propose here a model to resolve this conundrum by matching the most appropriate properties of each sensory terminal component considered above to the most suitable aspects of the recorded responses (Figure 4). We considered various parsimonious options of ascribing all the response properties to individual channels, or sub-sets of components, but these failed on two counts – they required hypothesising additional properties, or inverting known properties, and simultaneously assuming the remaining mechanically-sensitive components had no role. This seemed both unsatisfactory and physiologically unlikely. The key, and we suggest hitherto underappreciated, aspect of the proposed model are the SLVs. These are a ubiquitous feature of primary sensory terminals of muscle spindles and all other low-threshold sensory endings[15,80]. As well as containing glutamate, which is considered further later, SLVs strongly co-localise with DEG/ENaC labelling within the annulospiral endings. Confocal imaging shows this co-localisation occurs on a pixel-by-pixel basis throughout the terminal[21] (Figure 2b). Thus, they resemble the ASIC2-containing vesicles delivering transducosomes to the membrane in DRGs in culture. SLVs undergo a similar constitutive Ca^{2+}-dependent recycling which, if blocked, reversibly abolishes spindle firing. It is important to note that this blockade requires prolonged time periods; typically tens of minutes to hours. For this and several other reasons, SLV-mediated insertion of transducosomes into the axolemma cannot provide the first step of the almost instantaneous MST response. Not least, it would be cumbersome and far too slow. Further, there is no evidence in electron micrographs for legions of SLVs docked at the membrane ready for rapid insertion[16]. And, finally, millisecond-scale, stimulus-induced insertion of MST channels allows little time for extracellular contacts necessary for FFF-gating to be established. Thus, our model proposes the SLVs provide a mechanism for regulating the long-term abundance of MSTs (transducosomes) on the surface. Rather, the immediate MST event is provided by FFF activation of transducosomes already present in the membrane. SLV recycling is constitutive: i.e., occurs even at rest[16]. This would constantly replenish or reduce the transducosome population in the axolemma. Blocking this SLV-based replenishment by receptor antagonism, PLD antagonism or tetanus toxin injection would explain the prolonged time-scale of abolition of the stretch-evoked responsiveness. SLV recycling is constitutive but also enhanced by mechanical activity, and both are Ca^{2+}-dependent. Such a mechanically sensitive, Ca^{2+}-dependent process whose blockade leads to complete terminal unresponsiveness would readily fit with the properties of a Ca^{2+}-permeable Piezo2. But, it would not fit with TRPV4 or TMEM150/Tentonin 3, whose absence impairs function but does not abolish it. Piezo2, serving as the definitive 'gatekeeper' to transducosome replenishment would place it in a pivotal place to regulate mechanosensitivity and is consistent with all the outcomes in the studies considered.

We next consider the MST channels directly responsible for the mechanically gated aspects of the receptor potential. The long-lasting, Na^+-dominated receptor potential that is enhanced with Li^+ substitution, but not permeated by K^+, is most consistent with DEG/ENaCs. Somewhat surprisingly, current evidence seems not to strongly support Piezo2 having a direct major role in the potential itself. Its modest enrichment in proprioceptors, the lack of Na^+ selectivity, its poor permeability to Li^+, transient opening even in the presence of STOML3, lack of amiloride sensitivity, blockade by FM1-43 and K^+ permeability are difficult to reconcile with the Na^+/Ca^{2+}-dominated receptor potential. It has been demonstrated that Piezo2 transient currents can produce long-lasting (100s of millisecond) depolarisation in Merkel cells[56]. This reflects the very high input resistance of Merkel cell's (REF – Woo et al). However, spindle proprioceptor terminals are about 2 orders of magnitude larger in volume and highly elongated, giving a much larger surface:volume ratio and a correspondingly very low input resistance. Thus, a high density of channels with prolonged opening

would be required for a prolonged potential. We considered if Piezo2 might contribute only the highly transient initial dynamic peak of the receptor potential but as this possesses the same ion contributions as the rest of the potential, the same reasoning argues against this. Finally, we considered if Piezo2 directly physically interacts with other candidate MST channels, enhancing their mechanosensitivity. However, the apparent discrepancy in relative expression levels is incompatible with such an arrangement. For these reasons, our model proposes Piezo2 is instead the powerful gatekeeper of MST channel availability, and not a necessary constituent of the transducosome itself. Rather, a transducosome of DEG/ENaCs with the Ca^{2+} component due to TRPV4 or TMEM150C/Tentonin-3, i.e. channels with long open times and minimal K^+ permeability, seem most appropriate.

Returning to SLVs, our initial discovery was their role in glutamate secretion. This activates a PLD-linked glutamate receptor (PLD-GluR). A well-established role for PLD is in vesicle-mediated secretion of catecholamine from chromaffin cells[81-84]. PLD cleaves phospholipids in the inner bilayer leaflet, inducing membrane curvature to facilitate vesicle fusion. Thus, in our model, the observed enhanced sensitivity on PLD-GluR activation reflects increased transducosome insertion, increasing receptor potential amplitude and hence afferent discharge. Conversely, this could explain why anything that blocks insertion/SLV exocytosis ultimately silences the ending[16], if constitutive endocytosis continues to internalise transducosomes from the surface. This would explain the effectiveness of receptor or PLD blockade, and Piezo2 deletion. Importantly, it also explains why tetanus toxin, which blocks vesicle docking, abolishes stretch-evoked spindle firing, but is more difficult to reconcile with a purely Piezo2-driven receptor potential. The model proposes, therefore, that vesicle recycling has two functions; transducosome insertion (membrane trafficking) and glutamate sercretion (from the lumen). There are at least two vesicle populations within the terminals, so these functions may be segregated between them. However, a coupled/dual role of SLVs would enable tight coupling of the glutamate secretion and transducosome insertion processes.

Of course, by its very nature, a model is speculative. However, several lines of support for it exist. Piezos 'interact' by unspecified mechanisms with other mechanosensitive channels in heterologous expression systems[53,85,86], while Piezo1 and TRPV4 interact to produce mechanosensory responses in chondrocytes[52]. TMEM150C/Tentonin-3 may also regulate other mechanosensory channels. Studies have concluded ASICs can play an important mechanosensory function but require 'other molecular partners' [87]. For example, while ASICs are indeed acid sensitive, protons activate only a small fraction of the maximal conductance, implying other methods of more potent activation exist. Whether such additional interactions involve vesicle-based systems is unclear but one well-studied system is tantalisingly suggestive. The anomalous current in cochlea hair cells absolutely requires Piezo2[88]. However, the actual current produced is slowly adapting, amiloride-sensitive and resistant to FM1-43: classical properties of the spindle receptor potential *in vivo* but not Piezo2 *in vitro*. This may indicate Piezo2 adopts these properties in the hair cell. However, hair cells also express the DEG/ENaCs ASIC3 and ASIC1b. Moreover, ASIC1b is localised to the stereocilia insertion on the apical membrane, which is the locus of the anomalous current[89,90]. The apical membrane also has a local recycling population of SLV-like vesicles that internalise and traffic FM1-43 without blocking mechanotransduction[77,91], unlike the stereocilia tips which are permeated and blocked by FM1-43.

We have limited our speculation to how published properties of major MST candidates and SLV trafficking might reproduce the receptor potential, rather than new properties for the channels. A significant advantage of this model is its ability to be tested at a range of levels of experimental complexity. We suggest the following possibilities, but we are sure there are many other experiments that we have not thought of:

1. Since Piezo2 is blocked by FM1-43, short exposure should rapidly block Piezo2 but not impact receptor potential or afferent firing, as transducosomes would still be abundant in the membrane. Indeed, we have shown no effect up to 2 hours, despite labelling showing the FM1-43 reaches the terminal. However, prolonged dye exposure (6-12 hours) should allow constitutive SLV endocytosis to internalise transducosome complexes, eventually silencing stretch-evoked firing. Further, this should not be reversible, as FM1-43 is an internal blocker.
2. Proprioceptor-targeted expression of light-chain tetanus toxin[92,93] should block SLV recycling and transducosome insertion, by cleaving the v-SNARE synaptobrevin in proprioceptor sensory terminals. Hence, both stretch-evoked firing and recycling of FM1-43 should be blocked despite the continued expression of Piezo2. This may need to be tamoxifen-inducible to restrict effects to adult ages.
3. Affinity-columns of spindle homogenates using antibodies to synaptophysin or vGluT1 (expressed on SLVs) and/or whirlin (a putative marker of the MST complex[94]) should isolate the transducosome complex. Mass spectrometry should then identify isolated MST constituents.
4. If the model is wrong and Piezo2 produces the receptor potential by direct interaction with other channels with appropriate ion permeability, pharmacology and kinetics, Piezo2 and these channels should be of relatively equal abundance. Quantitation of protein expression in proprioceptor terminals should determine this.
5. In mice with targeted Piezo2 deletion in proprioceptors, the transducosome elements should still be expressed, but not trafficked to the surface. Thus,
    a. DEG/ENaCs should be highly expressed in spindle afferent terminals, but their expression should be entirely internal, with very limited surface expression
    b. SLV recycling should be ablated/severely reduced, i.e. there should be no FM1-43 uptake, either constitutive or during repeated stretching. However, recycling should be restored by Ca^{2+}-influx, e.g. via the optogenetically activated cation L132C mutation (CatCh) channel-rhodopsin[95]. (*NB, to avoid FM1-43 used for labelling from permeating or blocking the optogenetic channel, channel activation should terminate FM1-43 application, as Ca^{2+} effects on SLV recycling should be long-lived*).
    c. Similarly, optically-activated Ca^{2+} influx through CatCh should restore spindle stretch-sensitivity.
6. In the continued expression of Piezo2, the targeted knock out in proprioceptors of all 6 DEG/ENaCs, should:
    a. ablate/severely reduce stretch-evoked responses.
    b. allow SLV recycling to continue, evidenced by the uptake/release of FM1-43.

A simpler experiment may be deletions limited to beta and gamma ENaCs, as these are largely regarded as regulatory in function, and particularly instrumental in enabling surface expression[96].

7. The receptor potential, recorded with voltage sensitive-dyes, or the methods of Hunt et al. (1978), should be:
    a. enhanced in mice with single DEG/ENaC deletions or ASIC1a/2/3-triple deletion.
    b. ablated/severely reduced in mice with deletion of Piezo2 in proprioceptors.
    c. ablated/severely reduced in the continued presence of Piezo2 but with targeted knock out of all DEG/ENaCs, or a double deletion of β and γ ENaCs.

In summary, this model of terminal axolemmal DEG/ENaC transducosomes, perhaps including TRPs and/or TMEM/Tentonin-3, that are FFF gated and whose abundance is constantly replenished by SLV trafficking through a Piezo2-mediated Ca^{2+} influx could reconcile many of the apparently contradictory observations in the literature. This hypothesised mechanism can be tested at many

levels, both in proprioceptors and in other systems. Also, as there do not appear to be any SLVs in the cell body[97], it has the additional merit of explaining why the soma of cultured DRG cells do not behave like sensory terminals.

Acknowledgements:

We would sincerely like to thank Prof Chih-Cheng Chen, Prof Dr Stephan Kröger, Dr Rade Durbaba and Dr Susan Pyner for extremely valuable comments during preparation of this manuscript.

Funding:

The work from our laboratories in this review was supported by the Medical Research Council UK [grant number G0601253]; a Scottish Universities Life Science Alliance/Eli Lilly studentship [grant number RBZ0174]; Tenovus Scotland [grant numbers OL 532 & G13/08].

Declarations of interest: none

Author contributions:

Guy Bewick and **Robert Banks** contributed equally to this publication: Conceptualization, Visualization, Original draft preparation, Reviewing and Editing, Funding acquisition for our scientific work incorporated into the review.

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Figure Legends

Figure 1. The muscle spindle primary afferent output in response to stretch and the ionic basis of the underlying receptor potential. (a) Single unit (spindle primary afferent axon) *in situ* recording in anaesthetised cat. The output while the muscle is being stretched (dynamic response) shows at least two peaks, both higher than the approximately constant output while the muscle is held at the longer length (static response). On release of stretch the output rapidly falls to 0. Lower trace: length change approximating the same total duration as in b), with displacement applied to muscle tendon. Upper trace: action potentials recorded in primary afferent axon. Middle trace: plot of instantaneous firing frequency (1/interval between two adjacent APs). **(b)** Receptor potential of a primary ending of an isolated, decapsulated spindle of the cat, indicating dynamic peaks 1 and 2 and the static maximum phases that correspond to equivalent phases in a) and whose ionic basis is demonstrated in c) and d). Note the roles of K^+ at the start and end of the static phase, which are not further examined here. Upper trace: potential recorded from primary axon during blockade of voltage-gated Na^+ channels by TTX to prevent action-potential firing. Lower trace: length change (up = lengthening). **(c)** Successive effects of removal of Na^+ and Ca^{2+} from the external medium on dynamic peak 1 of the receptor potential. The minor contribution of Ca^{2+} was demonstrable only in the absence of Na^+, indicating that in normal conditions the receptor current is almost exclusively carried by Na^+. **(d)** Both dynamic and static components of the receptor potential are rapidly then

more slowly abolished in the absence of Na^+ and Ca^{2+}, and the effect is reversible and repeatable. **(a)** from Banks and Barker 1989[98], **(b, c, d)** Adapted from Hunt *et al.* 1978[7].

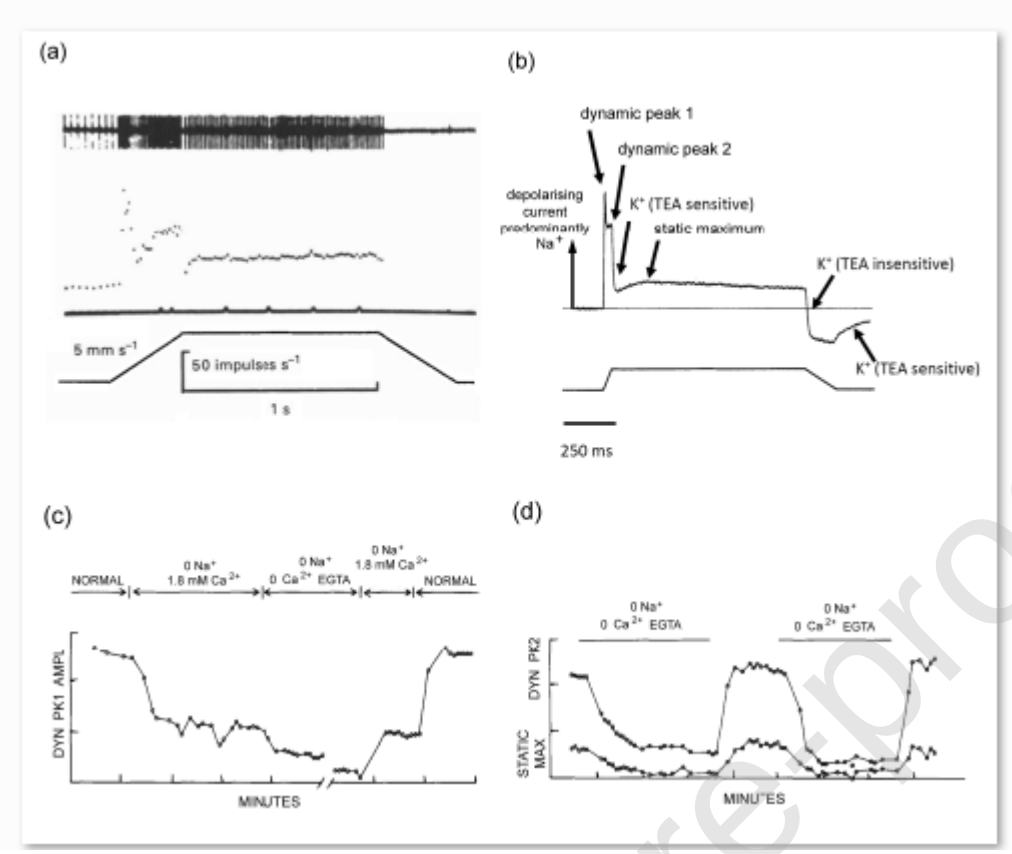

Figure 2. Muscle spindle afferent firing is amiloride sensitive, primary afferent terminals express ENaCs, and ENaCs display low-threshold mechanical gating. (a) Static-phase spindle firing frequency during 1 mm stretches of rat 4th lumbrical muscles. Amiloride and its analogues produced a dose-dependent reduction in spiking frequency. Amiloride, benzamil and EIPA had an IC_{50} of ~10 µM; HMA had little effect below 100 µM, but produced almost total abolition at 1 mM. **(b)** A spindle primary ending double labelled for anti-ENaC and anti-synaptophysin immunofluorescence analysed for intensity correlation analysis. The top two panels show ENaCα (red) and synaptophysin (green) immunoreactivity. Synaptophysin labels synaptic-like vesicles (SLVs) within the terminal. The merged image shows colocalisation as yellow/orange, which was then quantified in the PDM (product of differences from the mean) image. The PDM image indicates red/green co-localisation by intensity and by pixel – i.e. both intensity and position. PDM = (red intensity – mean red intensity) × (green intensity – mean green intensity). The image is pseudocoloured by PDM value: blue = modest colocalisation, red = higher, white = highest, black = below average in both channels. PDM is high (white) throughout the sensory terminals, indicating a strong correspondence between high expression levels of SLVs and ENaCs along the entire length of the terminal ribbon. Similar correspondence of immunolabelling with synaptophysin was found for each of the other ENaC subunits (not shown). Adapted from Simon *et al.* 2010[19]. **(c)** ENaC is activated by shear force (SF). (i) Representative recording of SF-evoked responses when αβγENaCs are co-expressed in *Xenopus* oocytes. Activating the bath perfusion (0.2 dyn/cm2, orange bar) induced a rapid increase of the transmembrane current (IM). Amiloride ('a', black bar) was applied to estimate ENaC-mediated current in the absence (0) and presence (0.2 dyn/cm^2) of SF. (ii) Membrane currents are greatly increased by SF. Values with SF normalized to current before SF application (0.2/0). Mean ± SEM; **P < 0.01, one-sample t test, two-tailed. (iii) The SF-current (ISF) increased in proportion to SF until

0.4 dyn/cm^2. (iv) SF has no effect in the presence of amiloride ('a', black bar, 10 µM), which blocks ENaCs. (v) Neither SF nor amiloride affect endogenous ion channels of *Xenopus* oocytes. Oocytes treatment was identical, except cRNA was omitted from the water used for injection (representative traces of at least 12 recordings using oocytes from at least three different animals). Adapted from Knoepp *et al.* 2020[3].

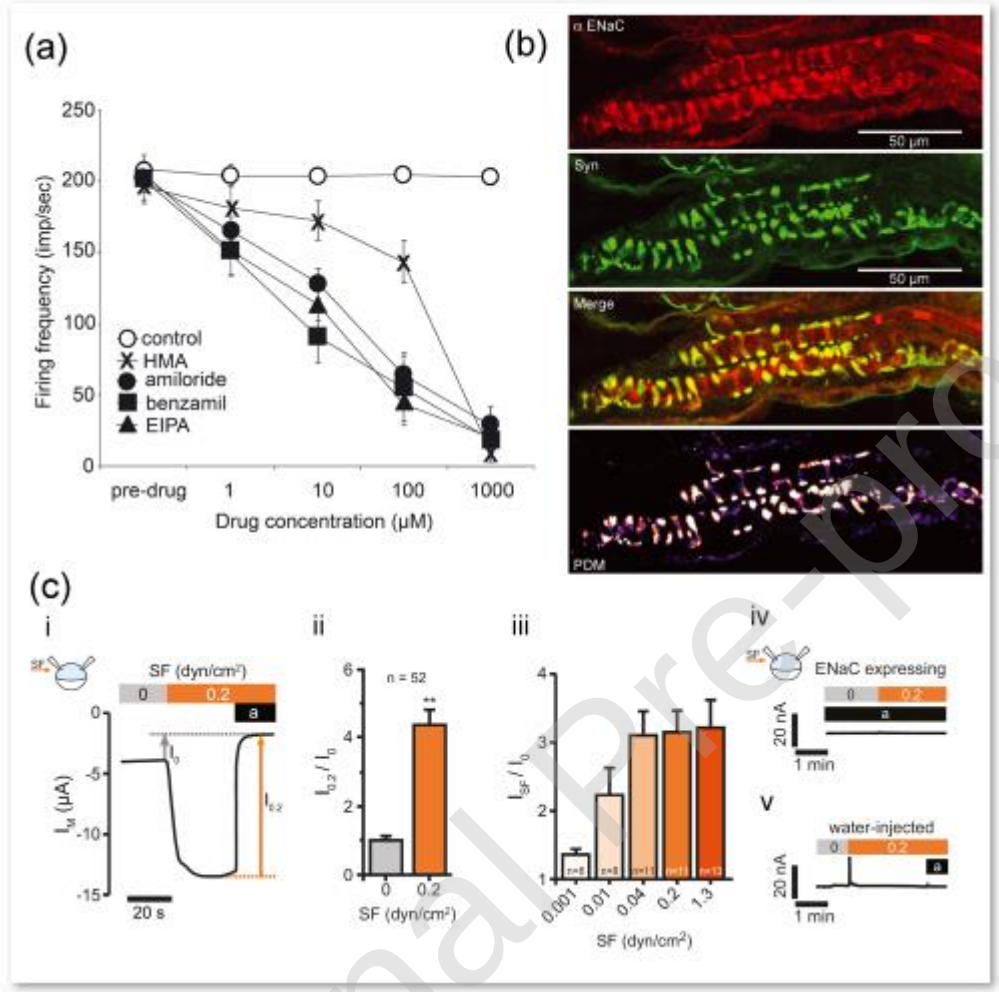

Figure 3. Recordings of mechanically-evoked responses in skin and cultured DRG neurons from mice after deletion of Piezo2 in all cell types either proposed or known to be relevant to somatosensory transduction. (a) *Ex vivo* skin-saphenous nerve recordings in WT and Piezo2cKO mice. Typical examples of slowly adapting mechanoreceptor (SAM) I responses from Piezo2WT (left) and Piezo2CKO (right) mice. Note in SAM I's recorded from Piezo2CKO mice, that while the frequency of action potentials is much reduced, the pattern of firing is largely conserved and persists throughout the stimulus. **(b)** Mechanically activated currents elicited in DRG neurons from Piezo2WT and Piezo2CKO mice by indenting the soma with a blunt probe. RA recordings, representative traces of rapidly adapting (RA) currents in Piezo2WT (top) and Piezo2CKO (bottom). Piezo2WT DRG neuron somata show characteristic transient currents, a sub-population of which were activated with lower thresholds (right; 3.5% of cell diameter). In Piezo2CKO mice, a few cells produced RA type responses but none had low thresholds, indicating Piezo2 was usually necessary for RA responses. IA and SA recordings, representative traces of IA (intermediately adapting) and SA (slowly adapting) currents, respectively. No observable effect was seen of deleting Piezo2 in the more slowly adapting IA and SA

mechanoreceptors. Piezo2WT: RA, left: 20µm diameter, 5 µm apparent threshold; RA, right: 28µm diameter, 1 µm apparent threshold; IA: 23µm diameter, 6 µm apparent threshold; SA: 20µm diameter; 2 µm apparent threshold. Piezo2CKO: RA, left: 23µm diameter, 8 µm apparent threshold; RA, right: none found; IA: 20µm diameter, 5.5 µm apparent threshold; SA: 30µm diameter, 8 µm apparent threshold. Lower right: example of probe displacement protocol (stimulus). Thus, no displacements are in the nanometre range, depression was from 3-35% of cell diameter, and typically ~25% for most RA, and all IA and SA DRGs. Finally, SA and IA responses were unaffected by the absence of Piezo2. Adapted from Ranade *et al.*, 2014[57].

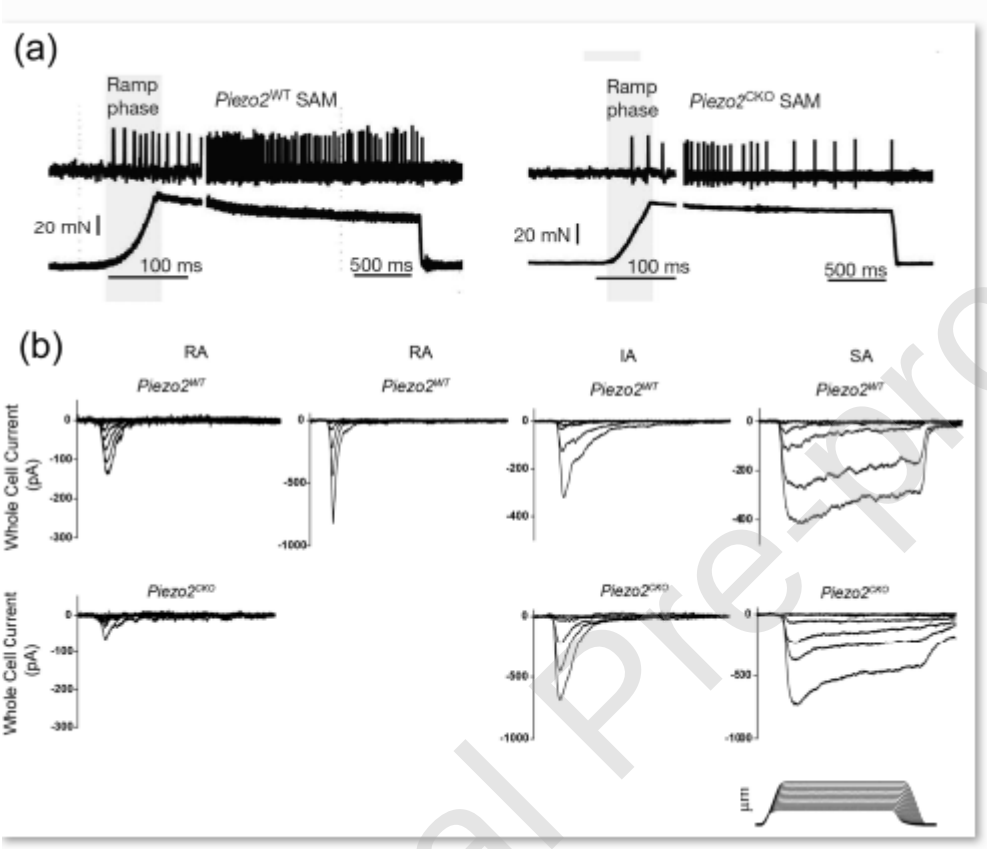

Figure 4. Further development of a model proposed for the events and channels in mechanosensory transduction of the muscle spindle. Progressive geometrical abstraction of a spindle primary-ending terminal, leading to a flow-chart summary of MST. Green block arrows in **(a-c)** indicate the direction and distribution of stretch applied to the terminal when the primary ending is lengthened. **(a)** A single annulospiral from a primary ending, several of which typically enclose a single intrafusal muscle fibre. The terminal is connected to its associated heminode by a short, unmyelinated preterminal axonal branch at the point shown. **(b)** The terminal unrolled and turned through 90°. Individual terminals may be repeatedly branched and the direction of stress during stretch is orthogonal to the long axis of the terminal. **(c)** A terminal and its associated unmyelinated preterminal branch shown in abstract cylindrical form, indicating their relative diameters. The smaller preterminal branch to the right is about 1 µm diameter. The lengths, especially that of the much larger terminal to the left, are highly variable. **(d)** Summary to illustrate the main events of MST, as described in this review. The principal feed-forward pathway from stimulus (stretch) to output (action potentials) is shown by the white block arrows. The receptor potential is due mainly to an influx of Na^+, with a minor contribution from Ca^{2+}. We envisage that the overall gain of this pathway is controlled by several feedback mechanisms: *negative feedback 1* is at present

hypothetical and is included to account for the reversible silencing of the primary ending by inhibition of the PLD-linked mGluR; the *positive feedback pathway* is the well-established SLV/glutamatergic loop. This is Ca^{2+}-dependent and it is this key step that we propose may be controlled by Piezo2, possibly with TRPV4. As SLVs are present in all low-threshold mechanosensory terminals, Piezo2 would then be similarly important in them all. *Negative feedbacks 2 and 3* involve different kinds of K[Ca], one located in the terminal, the other in the heminode, with at least the latter triggered by afferent action potentials opening voltage-gated Ca channels. *Green* lines and arrowheads indicate enhancing/excitatory actions; *red* lines and circles indicate reducing/inhibitory actions. Adapted from Bewick and Banks, 2015[66].

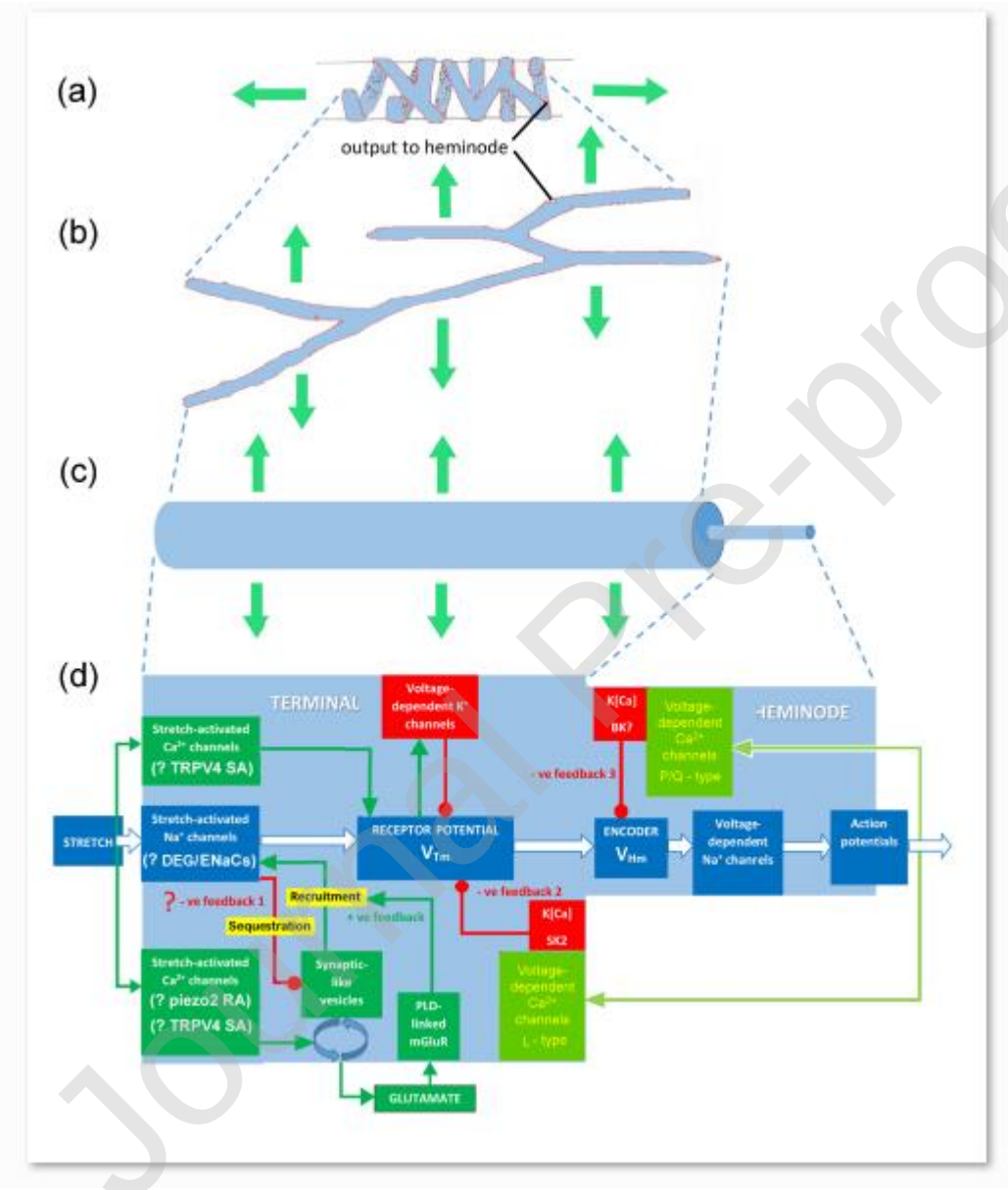

Table 1. Summary of candidate MST channels expressed in muscle spindle sensory terminals. Abbreviations: Mech sens = mechanically sensitive, sel = selective, perm = permeable, expr/n = expression, FFF = force from filament gated, FFL = force from lipid gated, OT = open time. ✓ = present, X = not present, ? = unknown/not established.

Channel\Property	Mech sens	Na-sel	Na-perm	Ca-sel	Ca-perm	K-perm	Li-perm	High expr/n	FFF	FFL	Long OT
DEG/ENaC	✓	✓	✓	X	X	X	hi	✓	✓	X	✓
Piezo2	✓	X	✓	X	✓	✓	low	?	?	✓	X
TRPV4/Tn-3	✓	X	✓	hi	✓	✓	?	?	✓	X	✓