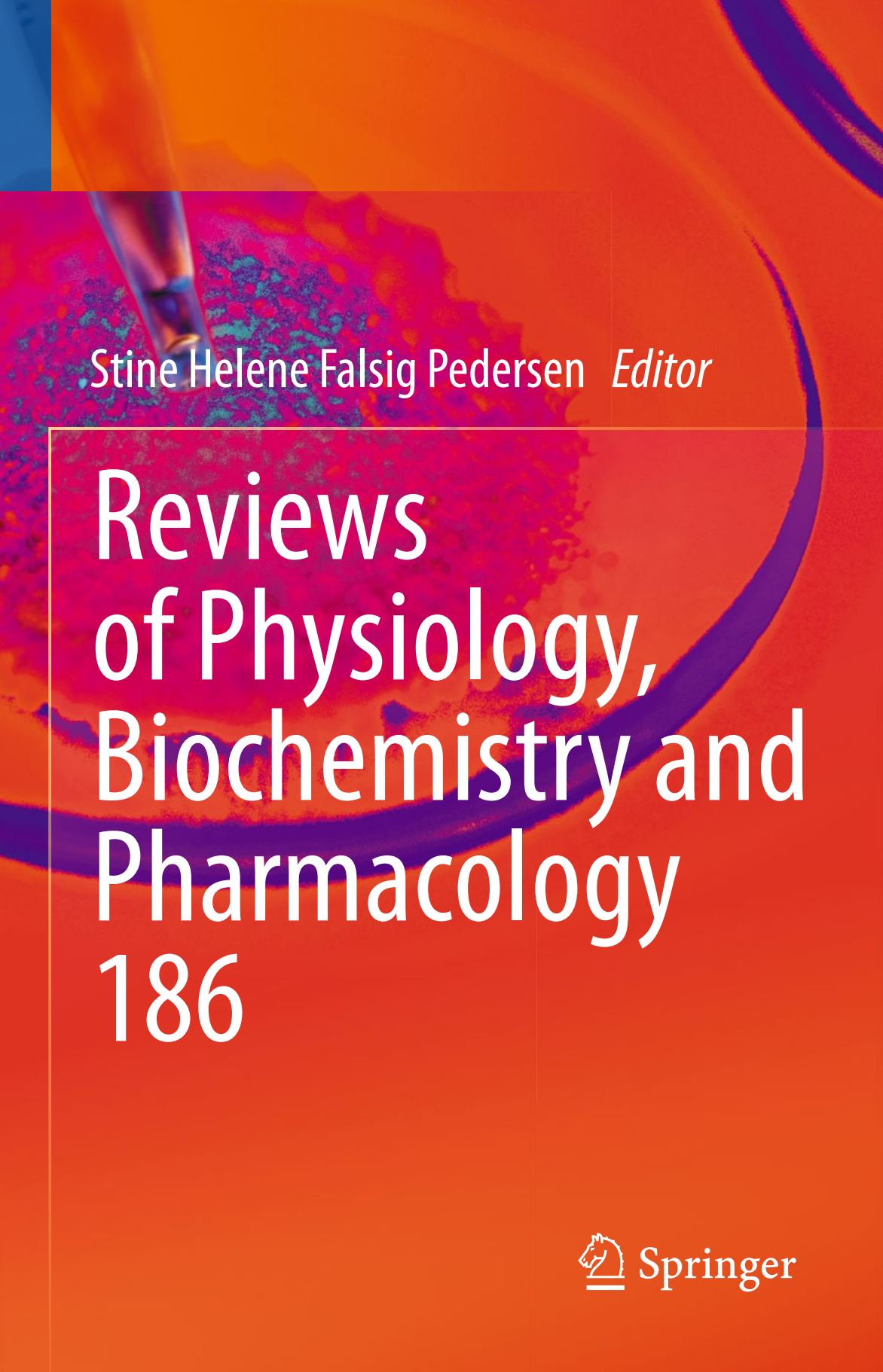
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Patch Clamp: The First Four Decades of a Technique That Revolutionized Electrophysiology and Beyond

Davide Lovisolo

Contents

Abstract Forty years ago, the introduction of a new electrophysiological technique, the patch clamp, revolutionized the fields of Cellular Physiology and Biophysics, providing for the first time the possibility of describing the behavior of a single protein, an ion-permeable channel of the cell plasma membrane, in its physiological environment. The new approach was actually much more potent and versatile than initially envisaged, and it has evolved into several different modalities that have

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radically changed our knowledge of how cells (not only the classical "electrically excitable "ones, such as nerves and muscles) use electrical signaling to modulate and organize their activity. This review aims at telling the history of the background from which the new technique evolved and at analyzing some of its more recent developments.

Keywords Biophysics · Electrophysiology · Ionic channels · Nobel Prize · Patch clamp

Abbreviations

ACh	Acetylcholine
HH	Hodgkin and Huxley
HTS	High-throughput screening
PC	Patch clamp
PCR	Polymerase chain reaction
Vm	Membrane potential

Preface

This paper aims at telling the history of a technological development, the *patch clamp*, that revolutionized the field of electrophysiology and beyond, giving us access to the description of a major set of membrane proteins, the ion-permeable channels, while performing in their physiological milieu. For the first time, "seeing" a protein at work was a reality:

> ...Here, we could watch a single biological molecule as it changed its structure by opening and closing a transmembrane pore (Sakmann 2006).

Several excellent reviews have addressed the development of this technique, setting it in the historical perspective of the development of electrophysiology and analyzing its impact on cellular and molecular physiology, mainly in reference to neuronal cells and their properties (Reyes 2019; Verkhratsky et al. 2006; Nilius 2003; Sigworth 1986). Here I will try to follow a different, more "lateral" approach: on the one hand, I will present the setting in which the new technique was developed, and on the other I will focus on how a single technological development impacted science in ways not predicted before, and how the choices made by the inventors had a "social" impact on the scientific community.

1 The Scenario

The driving force leading to the development of a radically innovative approach to electrophysiological recordings (not a simple improvement over existing techniques), that has been defined a "revolution" (Colquhoun 1991), was the search for the unitary events (single channel activities) underlying electrical signaling in cells.

2 The Emergence of the Concept of the Ion-Permeable Channel

That the surface of cells could be crossed by some inorganic ions is an old story, from the experiments of J. Bernstein at the boundary between the nineteenth and twentieth century (Verkhratsky et al. 2006; Nilius 2003). In the early 1950s of the twentieth century, an outstanding set of experiments by Hodgkin and Huxley (Hodgkin and Huxley 1952) introduced the idea of ion-selective pathways.

The physical nature of these pathways, however, was far from being understood. Even in the 1960s, different models for ionic flow across the cell surface coexisted. The process was thus quite slow and complex and was conditioned by the fact that for a membrane protein, it was necessary to have a correct description of the plasma membrane structure; even the mere existence of the plasma membrane was not completely consolidated. A detailed history of the evolution of the membrane concept can be found in several reviews (see, e.g., Lombard (2014)). Here I will only remember that the Davson-Danielli model that dominated the scene for more than 30 years contemplated proteins adsorbed on the two surfaces of the lipid bilayer: the idea of proteins that crossed the bilayer remained unfamiliar until the fluid mosaic model was proposed by Singer and Nicholson in 1972 (Singer and Nicolson 1972) and rapidly accepted as the canonical model. According to the fluid mosaic model, the lipid bilayer is the environment in which transmembrane proteins are embedded, and in this conceptual framework ion-permeable proteins could finally find their place.

Thus, along the 1950s and 1960s, ion channels had to find an identity in the absence of a coherent physical model of their localization. Hodgkin and Huxley provided evidence that the mechanism underlying the generation of an action potential in a nerve cell was based on the activation of two different conductances, one selective for Na^+ ions, the other for K^+ ions, with similar voltage dependencies but different kinetics, and produced a quantitative modelization of these mechanisms (that correctly predicted the properties of Na^+ and K^+ voltage-dependent channels decades before their identification). However, they clearly stated that, from their data, no information about the nature of these conductances could be inferred. Even 12 years after these seminal papers, in a lecture, Hodgkin referred to the issue in the following terms (Hodgkin 1964):

> At present the thickness and composition of the excitable membrane are unknown. Our experiments are therefore unlikely to give any certain information about the nature of the molecular events underlying changes in permeability...

It is remarkable that exceptionally predictive information about the behavior of unknown objects was obtained decades before they could be univocally identified!

Forty years later, the other partner in the enterprise that led to the Nobel Prize, Huxley, recalled the conceptual limits that slowed the understanding of the physical entities involved in the generation of the action potential (Huxley 2002):

> At first, we made calculations on the assumption that free K^+ and Na^+ would enter the lipid phase of the membrane from one side and dissociate from the other. It was of course known that inorganic ions are insoluble in the bulk phase of lipids but it seemed possible that an appreciable number might cross the bimolecular lipid layer that forms the cell membrane. However, we did not see any prospect of finding a basis for a major effect of membrane potential on the rate of penetration, or of selectivity between Na^+ and K^+... our final results with the voltage clamp showed clearly that the ions did not cross the membrane in combination with a lipid-soluble anion... We interpreted our results on the assumption that the ions crossed through channels that were opened or closed by alterations in the membrane potential. This has been amply confirmed...But I do not believe that we considered this possibility at any time during our speculations in 1946–1948... There are two morals that can be drawn from this story. First, showing by mathematical simulation that a theory leads to plausible results is not evidence that the theory is correct. Second, it is easy to fail to think of an idea that with hindsight seems very obvious. We felt stupid not to have considered that ions might pass through channels that are opened and closed by membrane-potential changes, just as we did for failing to think of the Na^+ theory until six years after finding the overshoot.

In 1966, in a classical textbook thanks to which a couple of generations of students had the first approach to the study of cellular neurophysiology (Katz 1966), another Nobel Prize winner, Bernard Katz, considered the difference between the terms "surface barrier" and "membrane" as a semantic dispute: physiologists could make statements about some of its properties, but not on its identity.

It is worth recalling that ion channels shared their undefined status with the other main class of membrane proteins, receptors, and it took more than 20 years of research from many laboratories around the world to isolate, purify, and characterize this family, starting from the acetylcholine nicotinic receptor of the skeletal muscle (Halliwell 2007).

Still in 1970, in a seminal paper (Hille 1970) Bertil Hille, one of the fathers of the ion channel concept, discussed the interpretation of the voltage clamp data by Hodgkin and Huxley on the selective permeability changes in the squid axon membrane in these rather tentative terms:

> ...In the following discussion the pathways for the movements are called Na channels, K channels, and leakage channels. These terms tentatively denote the concept of pathway without requiring that different ions have different pathways or even that pathways be discrete, localized structures.

In the same paper, on the other hand, the author provided strong evidence against the hypothesis of a single permeation pathway that changes selectivity during the excitation process. Actually, the data from experiments with blockers selective for

Na^+ influx and K^+ efflux (Moore and Naharashi 1967; Hille 1967) pointed to specific sites on the membrane, with openings of the same order of magnitude of the ions.

Curiously, a finding that contributed to the uncertainty about this crucial issue was the observation that at the neuromuscular junction, the nicotinic ACh receptor was permeable to both Na^+ and K^+ (Lassignal and Martin 1976).

In the 1970 paper by Hille mention is made to the first efforts to count channels and to provide an estimate for the conductance of the single channel by using the binding of the blockers to their sites (Moore et al. 1967), and more relevantly, reference is provided to the pioneering attempts to deduce unitary event properties from measurements of voltage fluctuations (membrane noise) (Verveen et al. 1967). Even if it was at the time not possible to observe unitary current events in voltage clamp measurements, some estimates of the unitary conductances could be obtained, indicating an upper limit in the 100 pS range. Some ideas were becoming widely accepted: the channels open with an all-or none mechanism, and all members of a specific channel population have the same conductance. C. F Stevens, another early proponent of the channel concept, discussed in 1972 (Stevens 1972) the unresolved issues related to the dynamics of channel opening, pointing to the importance of noise analysis to assess the validity of the different "microscopic models" still on the stage. We will return later to this issue.

Things were far from being settled, however. About 40 years later (Hille 2018) B. Hille remarked how, even in the 1970s, some of the leading researchers in membrane biophysics did not accept the ion channel concept. One of them was Kenneth Cole, the biophysicist who first realized the possibility and usefulness of the voltage clamp approach. He and I. Tasaki believed that

> . . .it was the entire membrane that became permeable to ions rather than specific structures within the membrane.

A personal recall: at the first International School of Biophysics in Erice, Sicily, in 1970, I witnessed a fierce confrontation between Tasaki and Alex Mauro, a strenuous supporter of the Hodgkin-Huxley (HH) model; the confrontation ended just short of a real physical fight.

A detailed discussion on the different interpretations of the HH data that coexisted in the literature for many years after the 1952 papers can be found in (Brenowitz et al. 2017).

3 The Consolidation of the Channel Concept

A decisive step in the consolidation of the channel concept was, from the 1970s, the isolation, purification, and characterization of some iconic channel proteins, such as the nicotinic ACh receptor (Hucho and Changeux 1973) and the voltage-dependent Na^+ channel (Noda et al. 1984).

Additional cues were provided by a different, synthetic approach: the study of the behavior of pore-forming antibiotics in artificial lipid bilayers (Mueller et al. 1962;

Marty and Finkelstein 1975). The low noise of recordings performed with macroelectrodes and the high conductance of these pores allowed to observe "unitary" electrical events; these findings provided a hint of the way a membrane pore-forming protein could behave.

Anyway, the main tool was, for about 10 years, noise analysis. By the end of the 1970s and the beginning of the '80s, a considerable amount of work had been performed on the voltage and/or current fluctuations that could be observed superimposed on macroscopic signals elicited in response to specific stimuli in different cell models. While a good deal of data came from noise analysis in electrically excitable preparations, such as axons (Wanke et al. 1974; Conti et al. 1975, 1976), due to the interest in describing the unitary events underlying voltage-dependent conductances (that had attracted the attention of biophysicists and electrophysiologists since the formulation of the HH model), many data were obtained from postsynaptic structures (specifically a large sized one, the endplate of the skeletal muscle) (Katz and Miledi 1979) and from sensory receptors, mainly photoreceptors (Minke et al. 1975; Lamb and Simon 1977; Ferraro et al. 1983).

This was in part due to the fact that these latter experimental models did not need electrical stimulation (but either chemical or light stimuli that could be accurately quantified and that did not directly affect the membrane potential), thus simplifying the analysis and allowing to gain information on voltage fluctuations in cells not maintained under voltage clamp conditions, that were difficult to arrange in many preparations. The problem with voltage noise analysis was that changes in membrane potential, V_m, are not proportional to changes in conductance, and the interpretations of the results were based on a good deal of inference and elaborate corrections (Katz and Miledi 1972); on the other hand, measuring current fluctuation in voltage clamped cells, in which it was kept constant, allowed a more direct estimate of the amplitude and time course of the unitary events (only in some cases the term "single channel" was explicitly used). From this burst of data, the first set of values for the critical parameters was obtained: from a few pS to tens of pS for the conductance, and the ms range for the duration.

4 A New Approach to the Membrane Surface

An attempt to overcome the limitation of voltage noise analysis from the muscle endplate with conventional microelectrodes led B. Katz and R. Miledi (1972) to try a quite different approach: they recorded voltage fluctuations with a focally applied extracellular pipette. While no quantitative information on the amplitude of the elementary event could be obtained, this approach allowed an evaluation of the time course of the unit conductance changes. Interestingly, this approach, while not completely new (extracellular recordings were widely used before the development of intracellular electrodes and continued to be used afterwards (see, e.g., Katz and Miledi (1965); Neher and Lux (1969, 1971)) represented a radical shift from the mainstream electrophysiological techniques, based on the use of electrodes that

penetrated the cell membrane, in favor of recordings from the extracellular surface. And it contributed to set the scene for the developments that in a few years will lead to the patch clamp technique.

5 The Preliminary Steps

It was a group of young and dynamic researchers, coordinated by Erwin Neher and Bert Sakmann, that started the trip along the new road.

Neher had worked in Dieter Lux's lab in Munich, where he met B. Sakmann, and made recordings with extracellular pipettes (Neher and Lux 1969, 1971). The contribution of D. Lux was fundamental for the training of Neher and for the emergence of the main ideas and, equally relevant, of the innovative technical procedures that later led to the development of the new technique.

Subsequently Neher went to C.F. Stevens' lab at Yale. This was the other highly formative experience at the beginning of his career, where the transition from noise analysis to the search of a direct measurement of the elementary event took shape. Sakmann went to B. Katz's lab in London, where the idea of "elementary events" was first made explicit.

After these experiences, both returned to Germany, where at Göttingen they met again, and decided to join forces and address the task of detecting the elementary components of electrophysiological responses: the unitary currents carried by single channels.

A preliminary paper appeared in 1976 (Neher and Sakmann 1976), using endplate channels and cholinergic agonists. The reference was to the 1972 paper by Katz and Miledi (Katz and Miledi 1972), but the aim was to overcome the many assumptions made to deduce values for the single channel parameters. The authors wrote:

> ...it would be of great interest to refine techniques. . .in order to resolve discrete changes in conductance which are expected to occur when single channels open or close. . .the key to high resolution. . . lies in limiting the membrane area from which current is measured to a small patch, and thereby decreasing background membrane noise. . .

The choice of relatively large muscle fibers allowed to combine the extracellular pipette with two conventional intracellular microelectrodes and thus to keep the preparation under voltage clamp. In order to obtain a better contact, an enzymatic treatment was used to remove connective tissue and basement membrane. Recordings were performed on the extrasynaptic region of denervated fibers, to increase the number of channels present on the membrane and their open times, along with other measures aimed at increasing current amplitude and duration.

The main concern was to reduce the background noise, and this was obtained by forming a close contact between the small area of the pipette opening and a patch of membrane: in order to observe discrete events, the shunt resistance had to decrease at least to one-fourth of the original value (pipette in the bath).

Under these conditions, and similarly to what had been previously observed with much larger currents in artificial membranes, the current recorded by the pipette showed discrete jumps between different levels, with square-like events in which the transition from two levels was fast as compared to event duration, thus settling the uncertainty whether unitary events had a square or exponential waveform. The analysis of the data allowed to obtain values for the average channel conductance and open time that were in strong agreement with those derived for noise analysis, and to conclude that the individual openings are statistically independent events.

The patch clamp, even in its embryonic form, was born, and single channel currents left the realm of speculation and entered the world of experimental data.

The following year, two review papers analyzed the state of the art. The first one, by Neher and Stevens (Neher and Stevens 1977), and the following by Stevens (1977) represented a turning point in channel biophysics: both not only presented what was probably the most comprehensive summary of the information on channel properties that could be obtained from noise analysis in natural and artificial systems, but for the first time mentioned the recordings of the Neher and Sakmann paper, stressing the importance of their direct observation of the actual behavior of individual channels.

The next step was a 1978 paper by the two authors, joined by H. Steinbach (Neher et al. 1978), still centered on recordings from ACh-activated endplate channels. In the Introduction, the paper is presented as a more detailed treatment of the preliminary approach ("short account") of the 1976 article. While the techniques and the experimental protocols were the same as in the previous paper, technical issues were discussed in detail. The main objective, as in the preliminary version, was to reduce as much as possible the background noise due to the high impedance of the conventional intracellular electrodes. One of the critical issues were the procedures used for obtaining glass pipettes with an optimal ratio between the resistance of the pipette (a few MΩ) and that of the seal. Interestingly, a comparison between the seal on the biological membrane and the one that could be obtained with an artificial surface made from a Sylgard resin showed that while in the former case a maximum factor of 10 could be obtained (2–4 MΩ vs. 20–40 MΩ), in the latter a seal resistance of the order of a GΩ was achieved, with a reduction of the background noise to about 0.2 pA. And this was a critical point for the subsequent efforts: the search for better, tighter seals.

The recording from the patch pipette was still performed in fibers voltage clamped by means of two intracellular electrodes; however, the authors remarked that the small currents activated in the patch membrane (a few pAs) did not significantly perturb the transmembrane voltage, and thus the patch could be considered effectively "space clamped" (i.e., its voltage kept constant and controlled) even in the absence of the intracellular clamping circuitry, evidencing another innovative feature of the approach.

The results (squared, stochastic events; Poissonian distribution; constant amplitude; linear current-voltage (I-V) relationship) were obviously in line with those of the preliminary paper.

6 The Patch Clamp Enters Adulthood

The efforts culminated in the 1981 paper (Hamill et al. 1981), the "real" patch clamp inaugural event, for which Neher and Sakmann were joined by three additional authors: in addition to F.J. Sigworth (who was the main contributor to addressing the challenges of amplifier design and single channel analysis), O.P. Hamill and A. Marty. But in the two years and half between these two papers several things had happened.

As could be expected, the new technique elicited strong interest, even if at first in a rather restricted circle of researchers already involved in trying to cope with recordings of unitary events. From 1978 to 1981 a handful of papers based on patch clamp recordings appeared in press (actually six, to my count, excluding a few ones that used previous variants of macropatch pipettes). These papers, from the labs of Neher or Sakmann, but also from other groups, made reference to the 1978 article, and were presented as applications of the new technique to different experimental preparations. However, one of them introduced an innovative development of the basic protocol, that will form the core of the 1981 article: the crucial step from a seal of a few tens of MΩ to one of several GΩ (the "gigaseal") was reported in a paper by Sigworth and Neher (Sigworth and Neher 1980). Neher had discovered that using a fresh pipette for every recording and applying a gentle suction led to a sudden and dramatic (two orders of magnitude) reduction in noise (Sigworth 2010). The reproducible and stable gigaseal was born. Another paper published in the same year by Horn and Patlak (1980) reported gigaseal recordings, making explicit reference to the results by Neher and Sigworth, and contained also the first reference to recordings from excised patches. Patlak had been in Göttingen where he could openly discuss all the preliminary results obtained by the German scientists.

Therefore, the 1981 paper can be seen, retrospectively, as the culminating point of a systematic incremental strategy that produced a mature technique that could successfully provide the long-sought answer for the description of the properties of single ion channels, and along with it, provided answers to many additional questions.

The 1981 paper presents indeed an impressive set of quantitative and qualitative improvements over the preceding publications. Every step is described in great detail. Below I will summarize the most relevant information included in the paper.

– Emphasis was put on the probabilistic nature of gigaseal formation and on the precautions needed to increase this probability. In this context, the use of a fresh pipette for each recording and the suction procedure were introduced. The critical parameters for preparation of the electrodes, along with the cleaning of the tip and of the electrode holder were meticulously described. In order to reduce the background noise, the objective was to obtain a contact as tight as possible between the electrode tip and the cell membrane. Coating with the resin Sylgard was described, in order to reduce electrode capacitance and to create a hydrophobic layer of the glass electrode surface, thus minimizing its contribution to noise. Equally accurate were the steps to improve adhesiveness on the other side

of the seal, the membrane. Enzymatic cleaning was used to remove connective tissue and other surface coating; alternatively, the use of cultured cells, that did not need enzymatic treatments (a new entry in electrophysiology), was described. Strong emphasis was put on the necessity to obtain a high degree of cleanliness of every component of the recording apparatus, from the electrode tip to the pipette and extracellular solutions.

- A key contribution to reducing background noise came from the design of the amplifier circuit, that took advantage of the use of very high-value resistors as the current-measuring elements, along with electronic compensation for the non-ideality of these resistors.
- The establishment of the gigaseal led to several radical improvements of the recording technique: the problem of the distortion of the traces due to the channels located under the rim of the seal (variations in amplitudes) was strongly reduced, if not totally eliminated; the membrane area under the pipette was efficiently isolated from the bath and thus could be effectively voltage clamped to a fixed value on an extended range of potentials; individual channel currents could be observed in voltage clamp mode without the need of two electrodes; due to the low background noise, the temporal resolution was strongly increased.

  (A side story: the establishment of gigaseals between the pipette and the membrane patch, an unprecedented event in electrophysiology, led to several efforts aimed at understanding the physicochemical nature of this complex: the data pointed to a bond between the borosilicate electrode and the phospholipid membrane, with interaction distances in the order of 1 Å; a Van der Waals bond has been proposed. A detailed description of the structure of the seal, the forces involved, and the physicochemical parameters affecting its characteristics can be found in Suchyna et al. (2009).

  The seal proved to show a high stability not only from the electrical point of view, but also from the mechanical one, and this led the authors to understand, from the beginning, that the new technique was opening the way to an extensive set of variations of the basic theme. Different recording configurations could be obtained starting from the "cell-attached" patch:

- "Inside out": if the electrode was carefully removed from the contact with the cell, a patch of membrane remained adherent to it, conserving the gigaseal, thus exposing the intracellular side of the membrane to the bathing medium, a configuration that has allowed the study of the role on intracellular messengers in the modulation of ion channels;
- "Outside out": from the "whole cell" configuration (see below), by retracting the pipette, a patch of membrane could be obtained, exposing the outer side of the membrane to the extracellular medium, thus allowing the study of the effects of extracellular agonist in cell-free conditions;
- But the more innovative finding was that when in the cell-attached mode, a further, careful suction could break the membrane patch without disrupting the seal: thus, the pipette had now access to the interior of the cell: the "whole cell" mode. Due to the low access resistance between the pipette and the cell, a small-volume cell could be considered isopotential with the electrode: therefore,

the potential imposed by the voltage clamp circuitry on the electrode could be actually the potential imposed on the whole membrane of the cell. Measuring membrane currents from cells in voltage clamp conditions was now possible with a single electrode, providing the possibility of performing quantitative electrophysiological analysis of membrane currents not only of very big cells, amenable to the insertion of two intracellular microelectrodes, but also to a myriad of small cells that were considered "off limits." Moreover, the relatively large access to the cell interior provided the opportunity to change and control the intracellular milieu by perfusing the cell with the solution contained in the pipette: not only the voltage, but also the intracellular concentration of ions and other functional relevant molecules could be quantitatively controlled. Dialysis of large cells by means of intracellular pipettes had been performed before (Kostyuk and Krishtal 1977), but it was a limited approach, restricted to a few experimental models. As we will see in more detail below, the introduction of the whole cell patch clamp marked the dawn of a new era of electrophysiology.

Looking at this impressive set of innovative and revolutionary findings presented in a single article, it has to be remarked that they did not appear by chance, but were the result of the careful, inventive, and painstaking efforts of an exceptionally talented team of young researchers.

A concluding remark of this brief review of the 1981 paper: the overall structure of the article evidences the spirit that informed the scientific effort of the authors from the beginning, and that informed the subsequent steps of their activity: the choice of a rapid dissemination of the new technique, and of an "open access," as we will say today, to the main tools and protocols necessary to implement it. All the technical details are carefully described and discussed in the text, together with the best options available at the moment.

This approach had a long-lasting impact.

A parallel, but not less relevant was the contribution of David Colquhoun and Alan Hawkes. As stated above, the behavior of a single molecule could be observed for the first time; but in order to understand the dynamics of these events, an entirely new set of statistic tools was needed, and it were Colquhoun and Hawkes that formulated the new statistics and gave a theoretical foundation to the single channel concept (Colquhoun and Hawkes 1981).

The impact of the paper was very strong and quite immediate: in almost every electrophysiology lab around the world people read the article and started to comment it and discuss how to try the new approach. However, the technique, even if excellently explained, was so new for most researchers that it was not so easy for many small and medium sized labs to implement it. In the first year or so after the publication of the paper, <20 articles based on the patch clamp technique were published, many of them by major research teams already involved in – or ready to tackle – the single channel affair. One of them deserves a particular mention: In a 1982 paper (Siegelbaum et al. 1982) S. Siegelbaum, J. Camardo, and E. Kandel, by combining patch clamp recordings and intracellular perfusion with a traditional microelectrode in Aplysia neurons, provided the first evidence for a

second-messenger regulated channel – and set the foundation for the understanding of the molecular basis of a simple form of memory, that will lead E. Kandel to the Nobel Prize Award in the year 2000.

7 The Dissemination

But in the spring of 1982 an event marked the real beginning of the era, and made it clear the actual message that the authors wanted to send to the scientific community.

Sakmann and Neher organized a course on patch clamp at the International School of Biophysics at the Ettore Majorana Centre in Erice, Italy. Courses in Biophysics had been held in Erice since 1970 and had a prominent role in disseminating knowledge among tens of young researchers from all over the world that were able to listen to and discuss with the top scientists in the field; but the 1982 course was something different. The organizers arranged for a practical course, bringing several patch clamp setups over the Erice Mount, and assembling a list of top scientists to give a complete and detailed picture of the new technique and its potentialities. I did not attend the school, due to personal problems, but a few students and former students from my lab and younger colleagues attended. They came back full of enthusiasm, and I can recall that the experience prompted many of them – and also myself – to enter this new and challenging field of electrophysiology. I have tried to reconstruct the atmosphere of this unique event by making brief interviews to some of them (I have also tried to contact the Ettore Majorana Center in order to obtain more informative material on the organization of the course, but the Center was closed and unable to provide any information – another casuality of the COVID-19 pandemic). Based on the interviews with Prof. Stefano Vicini (Georgetown University, Washington, USA) and Prof. Emilio Carbone (University of Torino, Italy), here are some of the highlights of the event – filtered through their memories:

Emilio Carbone
There were practical sessions with a couple of setups available for practicing on making glass pipettes and trying to patch clamp an isolated cell. O.P. Hamill used blood cells (to highlight the feasibility of patching and recording from small cells) and H.D. Lux collected some local Helix specimens and recorded from the snails' axon (a tough preparation). Extensive efforts were directed at explaining all the technical details and tricks necessary to obtain a successful seal (these approaches were new for electrophysiologists), such as fire polishing the pipette tip and applying negative suction to obtain a strong adhesion to the cell membrane. For the latter, Carbone recalls that E. Neher had discovered the key role of this procedure following an advice of Franco Conti, when he was at the Cybernetics and Biophysics Laboratories of the Italian National Research Council in Camogli, Italy. At that time, Franco Conti and Erwin Neher were trying to record single K^+ channels from internally perfused squid giant axons in Camogli, using a "hook-shaped" patch

Fig. 1 Credit: Emilio Carbone

pipette arranged intracellularly (see Fig. 1 in Conti and Neher (1980)). A giga-seal-resistance was impossible to achieve due to the position of the pipette and the presence of the dense cytoplasmic matrix around the internal side membrane. Franco Conti proposed for the first time to use a "suction system" inside the pipette holder to facilitate the formation of a seal and improve the resolution of K^+ current recordings. An idea that Erwin Neher developed few months later in Göttingen with Fred Sigworth to record single Na^+ channels from rat myoballs with impressively high resolution. This was possible due to the two order-of-magnitude increase of seal resistance generated by a "gentle suction" in the pipette (see Sigworth and Neher (1980)).

In parallel with the practical sessions, several lecturers introduced the basic tools for data analysis and interpretation that were as novel for most researchers as the technical details. The panel of instructors encompassed the top scientists in the field, and offered an unforgettable opportunity for the participants to become familiar with the technique that in few years would revolutionize the entire electrophysiology.

Besides Erwin Neher and Bert Sakmann at the Erice School there were Alain Marty, Owen Hamill, Fred Sigworth, Charles Stevens, Hans Dieter Lux, David Colquhoun, Luis De Felice, Fred Sachs, Steven Siegelbaum, Chris Miller, Franco Conti, Walter Stűhmer and others. The atmosphere was impressively relaxing during the whole course. In the morning there were oral presentations by the course instructors and in the afternoon brief oral presentations from the attendants and practical sections at the patch-clamp setups. There were continuous discussions, even during lunch, dinner and after dinner in a cellar with a full barrel of Marsala wine and Sicilian cookies. Figure 1 shows Erwin Neher (with his characteristic beard) explaining to Chuck Stevens (in front of him, with eye glasses) his latest

experiments on using the patch-clamp technique to detect secretory events in mast cells, with Fred Sigworth (to the left of C. Stevens) and Emilio Carbone (to the left of E. Neher) listening. It was an extraordinary experience and there was an uncommon enthusiasm among the instructors and participants. The interest for the technique and the new approach for studying ion channels was so strong that many of us asked Bert and Erwin to write a book describing all the aspects of the potency of the technique including representative examples of the results. This was actually accomplished in quite a short time (see below). The Erice School of 1982 was also for many of us the occasion to meet scientists that drastically changed our private life, by achieving new positions at different laboratories and starting an outstanding productive period of scientific publications.

Stefano Vicini

Fred Sigworth lectured and gave demos on the amplifier. I remember there were different amplifiers, comparing performance side by side. He also lectured and gave demos on programs for data analysis. Both Sakmann and Neher gave individual lectures. Sakmann on the AChR, Neher probably on voltage gated Na channels. They both gave good tips on pipette fabrication with different types of glass and on different experimental preparations.

Franco Conti was there and he also spoke. David Colquhoun and Chris Miller gave great talks on stochastic interpretations of ion channel mechanisms and the big problem of data fitting to extract transition rates.

Walter Stuhmer presented the Loose patch clamp approach and gave a demonstration. Peter Lauger dwelled into the biophysics and thermodynamics of ion channel conductance and conformational transitions. Fr Sachs gave a really entertaining lecture on the subconductance states of the AChR in muscle. I remember intense discussion with Bert Sakmann.

I don't remember if Richard Aldrich or Gary Yellen or both presented a comparison between single channel recording and noise analysis.

Louis DeFelice lectured on the Hodgkin & Huxley Model revisited with single channel data.

I think Peter Usherwood and Stuart Cull-Candy were talking about GluR channels in locust muscle.

I can't remember clearly, but I think Philip Ascher, Steve Siegelbaum, O.H. Petersen were there.

O. Hamill talked and demonstrated patching of red blood cells with the caveats of artifact introduced when clamping cells with very high input resistance. Hans Dieter Lux lectured on Voltage gated Calcium channels.

I think Alan Trautmann was also there presenting data on the influence of patch excision on AcHR channel in myotubes as it was the main topic I worked with the late Stephen M. Shutze at Columbia University in the early eighties.

The course was followed by the publication, at the beginning of 1983, of a hefty book that collected all the contributions given at the School (Sakmann and Neher 1983). The aim of the book, significantly titled "Single channel recording," to highlight what was considered the main feature of the innovative approach, was to

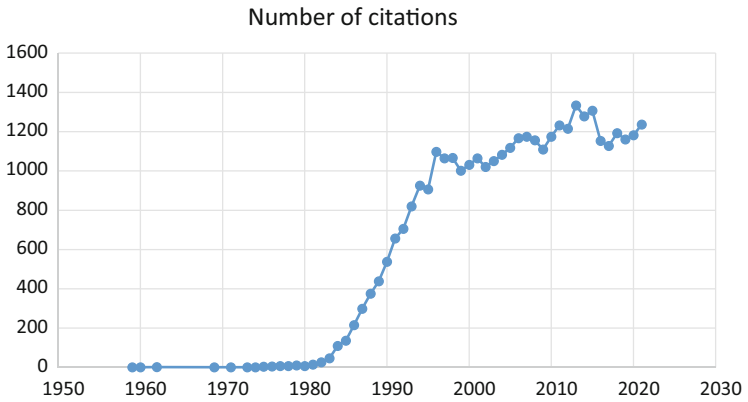

Fig. 2 From Pubmed; search terms *patch clamp*

make available the relevant information about the technique and its applications well beyond the tens of researchers who had attended the Erice course. The sentence below applies to the fabrication of patch electrodes, but was representative of the principle that inspired the organizers:

> . . .to enable those new to the technique to construct pc electrodes in their own laboratories without having to undergo a period of apprenticeship or reinvention. . . (cap 3.3 page. 59).

The technique had to be rapidly disseminated for the benefit of the scientific community and for the advancement of the knowledge on ion channel identity and properties. And the dissemination was actually quite fast. Bertil Hille gave a particularly significant contribution: in 1984, he published the manual *Ionic Channels of Excitable Membranes* (Hille 1984), that quickly became the main reference text for ion channel researchers and, through several updates, has been the most useful and comprehensive tool for several generations.

A timeline of the number of scientific articles based on the new technique (Fig. 2) shows an exponential growth in the first 15 years following the publication of the epoch-setting paper; after 1996 (year in which 1,098 related papers were published) the increase was slower, with a peak of 1,334 articles in 2013.

8 Strengths and Liabilities

The new technique virtually supplanted traditional electrophysiology with intracellular electrodes; the latter approach survived for some time in a few ecological niches, such as recording from brain slices, but even in this case the patch clamp electrode finally prevailed (see below). The only relevant niche still alive to date is the voltage clamp of oocytes (Wagner et al. 2000), where the huge dimensions of the

cell, whose voltage has to be kept under control, demand the injection of large currents and the use of two electrodes.

In my personal view, one of the factors that promoted the emergence of a whole generation of patch clamp electrophysiologists around the world was the choice by the inventors not to apply for patents (patents related to patch clamp appeared much later, and mainly refer to automated systems of relevance in applied research – see below). Looking backwards from a contemporary perspective, patenting might have been obvious and plausible. Other technical innovations in cellular and molecular biology, both before and increasingly after the patch clamp, have been quickly secured by a patenting strategy. Trying to analyze how much this approach has led to limitations in the diffusion of new techniques and in the buildup of inequalities in availability of advanced research tools between scientists in different socioeconomic milieus is outside the scope of the present article: however, I believe that the choice had its impact.

The takeover was fast and pervasive. A personal recollection from the middle 1990s: at an examination for the admission to a PhD program in Physiology, I asked the candidates to discuss the development of electrophysiological techniques. Most of them were not aware that cellular electrophysiology did not start in 1982, with the introduction of patch clamp!

On the other hand, the technique had its limitations. The protocols for preparing the electrodes, for establishing biological preparations (either dissociated cells or cell cultures) suitable for gigaseal formation and the procedures for obtaining good seals required care and patience (even if the first of these steps was strongly simplified with the introduction of capillaries with a thin glass filament inside, that significantly speeded the filling); once a good seal was formed, patch stability was not always easy to obtain, and several factors (the state of the cells, the washout of the cell interior in whole cell recordings, etc.) could prevent the attainment of a successful recording. One full day of uninterrupted (painstaking and also tiresome) work at the setup could lead in some cases to a single useful recording, or even to no one at all. The development of other techniques for the study of proteins and consequently of ion channels (PCR, immunocytochemistry, imaging, etc.) offered approaches that, at least apparently, were easier, less time-consuming, and mentally adsorbing. This was particularly appealing to researchers at the beginning of their careers, not specifically and carefully trained, and working in an environment in which the pressure to obtain results was (and is) particularly heavy. Thus, it was not surprising that patch clamp was not as popular as it could have been expected. At a Gordon Conference at the beginning of this century, a colleague from the USA asked if I had some students interested in a post-doc position in his lab; he told me that he had six setups, but only one (the one he used personally) was permanently active.

These difficulties may explain the slowing of the increase; however, the patch clamp approach, in the 40 years that followed its introduction, has consolidated as the primary and necessary tool for an unequivocal and definitive characterization of ion channel properties. In the following section I will address another major strength of the patch clamp: its versatility and the unpredicted potential it incorporated, that led to a long series of exciting applications.

9 Variations on the Basic Theme

9.1 The Loose Patch

Actually, a different version of the basic technique was already present at the Erice course and included in the resulting book: the loose patch, in which a pipette of relatively larger tip diameter (7–15 μ), as compared with conventional patch electrodes, was placed close to the cell membrane, and a seal of several tens of MΩ was obtained. This was actually the same technique as described in the 1976 paper (Neher and Sakmann 1976), before the introduction of the gigaseal. The seal was quite loose, and current leakage was relevant, but this configuration allowed to record "macroscopic" currents from a channel population; the pipette could be repeatedly moved to record from different sites of the same cell, and from muscle cells during physiological mechanical contractions without disrupting the contact (Almers et al. 1983). This approach, due to its limitations, remained confined to a niche of specialized measurements, and did not experience a wide diffusion (total cites: 166).

9.2 The Whole Cell and Its Potential

The major and at least partly unpredicted development was the rapid prevailing of whole cell over single channel recordings. Single channel citations peaked at 263 in 1996, and then declined to an average of <150 a year (total: 6002). Whole cell citations (17.375 in total) rapidly increased to a peak of 665 in 1997 and have remained around this value to date (the total citations under "patch clamp" are 32,720, likely accounting for additional papers in which more than one configuration was used).

Figure 3 compares the timeline of the citations of the two configurations.

While single channel recording has kept its absolute value for the univocal fingerprinting of channel proteins, the whole cell approach appeared soon quite attractive for it made it possible to study the electrophysiological properties of a broad set of intact and isolated cells. And this led to another revolutionary development: it opened electrophysiology to fields unthinkable before. Classical electrophysiology had been a business for neurophysiologists (and to a minor extent, for muscle and sensory receptor physiologists); moreover, quantitative measurements of membrane properties required the insertion of two electrodes in order to voltage clamp the cells. The patch pipette, enabling to record current and voltage with a single electrode and to avoid physically penetrating the cell, allowed to study the electrical properties of a whole range on "nonexcitable cells," most of them small and flat: fibroblast, endothelial and epithelial cells, endocrine and exocrine secretory cells, and many more, even red blood cells (see Fig. 3). Table 1 summarizes the rise of the number of citations in fields that were virtually impracticable to

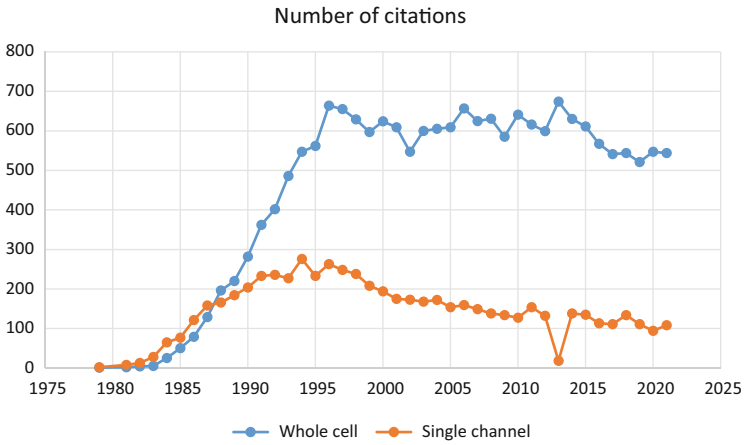

Fig. 3 From Pubmed; search terms *patch clamp, whole cell; patch clamp, single channel*

Table 1 Number of citations from Pubmed: patch clamp

No. of citations from pubmed: patch clamp	Date: 20-5-2021
Patch clamp, glial cells	980
Patch clamp, neurons	13,718
Patch clamp, sensory receptors	1897
Patch clamp, epithelial cells	3,463
Patch clamp, endothelial cells	552
Patch clamp, cardiac cells	4,878
Patch clamp, endocrine cells	359
Patch clamp, secretory cells	447
Patch clamp, mitochondrial membranes	116
Patch clamp, nuclear membranes	78

electrophysiology. Moreover, even in the realm of electrically excitable cells things were swiftly changing: most data before the patch clamp era came from "big" preparations, in most cases cells from invertebrates or lower vertebrates. Quantitative electrophysiology of voltage clamped mammalian nerve cells was restricted to a few experimental models. In 1981, the search for voltage clamp, neurons, invertebrate gave 47 citations; the same for mammalian gave 18. In 2000, the former was 38 and the latter 291. These data obscure the enormous achievements that have been possible thanks to further developments, such as multiple and in vivo recordings (see Table 1).

But another example is even more relevant: electrophysiology of vertebrate cardiocytes, an experimental model much more complex in terms of the endowment of ionic currents and quite less accessible to electrophysiological techniques (due to the dimensions of cells) than the squid giant axon, had been performed since the late

1950s, but the cites lagged in the two dozen range until the introduction of the patch clamp, after which they rapidly increased of an order of magnitude; moreover, quantitative characterization of the currents by means of voltage clamp protocols amounted to 14 papers in 1981, and soared to 154 in 1995, just to maintain a virtual plateau in the following years. It is not necessary to stress the relevance that the understanding of the complex interplay between the different ionic currents and of the intracellular pathways that modulate them has played in our approach not only to cardiac physiology, but also and more significantly to the different pathologies that affect this crucial organ.

The improvements and expansions of the basic technique went further.

9.3 The Perforated Patch

One of the strengths of whole cell recordings was that the relatively direct access of the solution contained in the pipette to the cell interior allowed to modify the intracellular concentration in terms of ionic composition, for instance introducing selective channel blockers, thus enabling to isolate a specific current component. This was particularly useful while studying cells endowed with a complex set of channels. However, this easy access had its drawbacks: the contact of a virtually infinite volume (that of the pipette) with the limited volume of the cell could lead to a more or less severe washout of the cytoplasm, with dramatic dilution of key regulators of membrane permeability, such as metabolites and second messengers. This could lead to loss of some current components particularly sensitive to cytosolic regulation. In 1989, a paper (Horn and Marty 1988) introduced a novel approach to circumvent this limitation: the perforated patch configuration, in which the pipette contained an agent, usually a pore-forming antibiotic such as nystatin. After a cell-attached seal was obtained, in a time interval ranging from some minutes to tens of minutes the agent formed ion-conducting pores in the patched membrane, thus allowing a low-resistance electrical access to the cell interior, without loss of the most relevant cytosolic macromolecules. This technique was adopted by many laboratories and has markedly improved the quantitative and qualitative analysis of several current types. The number of cites quickly rose to 98 in 1996, and the total is 1,665, even if the number declined in the following years. The reasons may be linked to the fact that perforated patch relies on a series of conditions (the cell state, the effective and controlled filling of the agent in the pipette, etc.) reserving this approach to skilled and patient researchers.

9.4 Intracellular Perfusion

On the opposite side of the whole cell pipette-cytosol affair, in some instances it has been possible to successfully change the pipette solution during the experiment, by

connecting the pipette interior to reservoirs containing different solutions, thus allowing, e.g., to study in real time the effect of second messengers (see, e.g., Hartzell and Fischmeister (1987)). Actually, the technique did not become very popular, due to the difficulty of obtaining reproducible protocols and to the fact that some of the issues this approach could answer can be addressed with excised patches; it has remained confined to a very specialized niche.

9.5 Capacitance Measurements and Vesicular Release

The whole cell configuration could be used not only to obtain information on the conductive properties of the cell, but also on its capacitative parameters. In fact, in particular in classical voltage clamp protocols, cell capacitance has to be evaluated and compensated in order to avoid transients. This was achieved thanks to circuitry included in all commercial amplifiers available and led to a relevant side benefit; the possibility to accurately measure cell capacitance, i.e. cell surface (given the almost universal value of 1 μF/cm^2 for lipid bilayers). For the first time, a quantitative and reliable measure of cell membrane area was available, far superior to all morphological evaluations, and made it possible to normalize ionic currents to the membrane area, thus allowing to compare and to average data from cells with heterogeneous morphology in a given population. Expressing currents in terms of conventional current densities (pA/pF) has become a standard in most papers. But this approach led to a more relevant development. Cell surface is not constant, and dynamically changes when the cell, either spontaneously or in response to a given stimulus, undergoes morphofunctional modifications, such as during vesicular secretion (e.g., in neurotransmitter or hormonal release). Starting from the pioneering work of Neher and Marty (Neher and Marty 1982), several refined technical approaches have been developed to monitor in real-time vesicular fusion in patch clamped cells (Lindau and Neher 1988; Carabelli et al. 2003), showing that the release of single vesicles corresponds to changes in capacitance in the fF range, and leading to a deeper understanding of the mechanisms involved in this crucial mechanism of cellular communication (463 citations).

9.6 Patching Subcellular Organelles

The versatility of the new technique offered another rather unexpected opportunity: the recording of electrical signals from intracellular organelles. A membrane is a membrane, and the patch pipette does not discriminate where it comes from: so, with the development of procedures to isolate subcellular components, it was a rather logical (even if not easy) step to try to record from them. The result has been a huge progress in our understanding of the mechanisms regulating ionic flows and homeostasis in subcellular compartments and their role in the control of key physiological

processes. Here I will only mention a few examples: recording from lysosomal vesicles (Böck et al. 2021), mitochondria (Sorgato et al. 1987), nuclear membranes (Mak and Foskett 2015) (see Table 1).

9.7 The Patch Pipette as a Bridge Between Molecular Biology and Channel Biophysics

The introduction of the PCR technique (Saiki et al. 1985) revolutionized the field of cellular and molecular biology, allowing to amplify and analyze tiny amounts of nucleic acid material, that could thus be studied in detail. This technique allowed the study of mRNA expression of a cohort of proteins, among them ion channels. But the patch pipette contained a more intriguing possibility: after recording in the whole cell mode, the cell content could be extracted and introduced into the electrode, and subsequently it could be used for PCR analysis. The single cell PCR was born (Lambolez et al. 1992) and it has been extensively used (416 citations) to map the expression of mRNA encoding for channel proteins in heterogeneous populations (such as in the nervous tissue) and to correlate them with the currents recorded with the patch clamp approach, addressing relevant issues such as subunit composition and post-transcriptional regulation of ion channels (Sucher and Deitcher 1995; Santi et al. 1994).

9.8 Patch Clamp and Calcium Imaging

To complete the picture of the potentialities of patch clamp, only a brief mention will be made on its combination with another technique developed in the 1990s: calcium imaging. While the parallel use of the two approaches has become quite popular, in some instances it has been possible to obtain simultaneous recordings of electrical signals and changes in $[Ca^{2+}]_i$. See, e.g., Gericke et al. (1993) for an early paper, one of the first examples of multiparametric recording from living cells.

A quite different story can be told for another, not obvious development: the recording from tissue slices.

9.9 Patching Slices from the Central Nervous System

The original approach to patch clamping prescribed an obsessive attention to the cleanliness of the two partners entering in contact, the pipette and the cell surface. The idea of patching a cell incorporated into a tissue seemed impracticable at the beginning, but soon, thanks to the development of another set of inventive and delicate procedures, it was possible to clean the somata of neurons in thin brain slices

and to successfully record from the both in whole cell and in cell-attached mode (Sakmann 2006; Gray and Johnston 1985; Edwards et al. 1989). The approach, combined with the improvement of imaging techniques, the possibility to visualize cells with complex arborizations such as neurons by filling with intracellular dyes (Caccavano et al. 2020), and other refinements, led to a fast growth of the field (from six papers in 1989 to 160 in 2019, for a total of 3,443 citations) and to successive major developments. One was the possibility to perform multiple recordings from two or more cells (Rollenhagen et al. 2018), from presynaptic and postsynaptic sides of the same synapse (Clarke et al. 2016) or from different regions (up to four, to my knowledge) of the same neuron, e.g. the soma and multiple sites on the dendritic arborization (Waters et al. 2003; Williams and Atkinson 2008; Williams 2004).

10 In Vivo Patch Clamp

In 2006, a special issue of Pflügers Archiv (vol 0.453, n.3) celebrated the 25th anniversary of the inaugural patch clamp paper. After a historical introduction (Verkhratsky et al. 2006), the first research paper (Sakmann 2006) was a report from B. Sakmann of neuronal activity recorded from the brain of living rats (Emilio Carbone has drawn to my attention to the fact that the issue was the product of a meeting in Heidelberg to celebrate the anniversary, at which Bernd Nilius, then Editor-in chief of Pflugers' Archiv, proposed to collect the contributions in an issue of the journal).

This report marked a real great leap forward, that was made possible thanks to a complex combination of advancements in imaging and visualization techniques, automatic manipulation and surgical approaches, and it has opened a new window in our understanding of the role of neuronal populations in information processing in the brain as observed in the physiological context, and their relationship to higher-order functions. The number of papers reporting in vivo recordings from the animal brain has steadily increased in the years. These approaches are often combined with molecular genetics, and particularly optogenetics, techniques in order to either stimulate or inhibit specific and well-identified classes of neurons and to record their involvement in the neural circuitry (Lee et al. 2006; Luo et al. 2021; Uta et al. 2021; Wilson et al. 2018; Gao et al. 2021).

The challenges of multiple in vivo recordings have driven the search for the new approaches, such as the use of nanoelectrodes in order to cope with the spatial resolution limits of conventional multielectrode recordings (Jayant et al. 2019).

11 Two Different Approaches to Automated Patch Clamp

A radically innovative approach in the field of in vivo recordings is represented by the introduction of robotics and automated procedures (Kodandaramaiah et al. 2012, 2018; Holst et al. 2019; Suk et al. 2019). The authors of these papers have also

registered a patent for a multipatch automated system (Suk et al. 2021). These developments have been driven by different motivations: on the one side, the ability to overcome the difficulties inherent in the complex procedures for obtaining successful multiple recordings and to obtain more reproducible data, and on the other, the drive to make these procedures at least partially independent from the need of highly trained and skilled experimenters: as stated in the presentation of one of these products, from the homepage of Neuromatic Devices:

> You don't even have to be in the room, just set it up and leave, and when you come back to the lab, you've recorded about 100 cells. (Neuromatic Devices 2021)

Another company that commercializes automated patch clamp systems for neuronal recording is Neobiosystems (Neo Biosystems n.d.).

These are algorithm-driven systems, that allow also to couple the recording apparatus to an imaging system, thus facilitating detection of the target cells, and that benefit from the development of highly automated micromanipulator systems; they are designed with the purpose of allowing this delicate and highly critical field of scientific enterprise to enter the high-throughput screening (HTS) world and to be managed by less skilled operators, even if surgical techniques, on the one side, and data analysis, on the other, will still require high levels of training.

But a radically different approach to full automatization of this highly complex and sensitive technique has come from the drug screening sector, where HTS is considered a key success factor. The limitations of the standard approach were felt as particularly severe in a field subject to commercial pressures, the primary screening of hundreds of molecules of potential use as pharmacological tools targeted at ion channels. Here the requirement was for rapid, even if less precise, testing, and these profit-sensitive innovations were open to patenting. From the late '90s, a series of devices have been developed and patented. Most of them are based on the idea of a planar set of holes in which cells are directed thanks to microfluidics; after the cells have settled, a negative pressure from underneath breaks a portion of the membrane, producing a sort of whole cell patch access to the electronic circuitry incorporated in each channel.

While most of these developments have been presented as a tool for fostering diffusion of patch clamp techniques to a larger set of potential users, and some concern on the cost of these systems has been taken into account, the pressure to patenting has been relevant. Actually, from what I have found, very few companies advertise systems for either complete or partial automatization of patch clamp recording using planar setups. Presently, the two leading companies in this field are Nanion Technologies and Sophion Bioscience; reference to these systems can be found in Obergrussberger et al. (2021), Rosholm et al. (2021), respectively.

A search on the European patent database (Espacenet n.d.) using the keyword patch clamp and filtering for the time interval 1982–2020 gives 141 results. A consistent fraction (near 50%) is completely unrelated to electrophysiology, many others refer to specific and, in some cases, irrelevant technicalities; only a small fraction reports solutions describing either automatized systems or devices that can potentially be integrated in an automatic patch clamp setup.

12 A Few Final Considerations

1. Without the introduction of the patch clamp technique, entire chapters of the books relating ion channels to physiological functions and pathological states would have been left empty. An example: from the mid-1990s of the twentieth century, the discovery of the TRP superfamily of ion channels (Minke 2010) has revolutionized our understanding of a wide set of physiopathological processes, from sensory transduction (Damann et al. 2008) to cancer cell biology: had it been the same if only the traditional electrophysiological techniques had been available? The award of the Nobel Prize for Medicine and Physiology in 1991 to E. Neher and B. Sakmann, the two main inventors, was a due tribute to relevance of the new technique in inducing a radical change of scientific paradigm in the life sciences.
2. What will be the future of PC?
   Automated, HTS systems will become the standard, and the classical approach relegated to some niche? Maybe advances in optical imaging will partially provide better responses to the multicellular recording problem, but for some requirements (such as channel biophysics or molecular dissection of pathways) the good old patch pipette will still be the answer.

   I will not enter here in detail of the changes in paradigm that I have briefly outlined above, their potentialities and their flaws. One can look at these developments either as a "natural" evolution of the basic concept to adapt it to the changing role of scientific research in a different socioeconomic landscape, or as a definite departure from the idea that advanced basic science requires a mix of mental and physical attitudes and commitments that are at the foundation of the building of knowledge about the world in which we live. For sure, from an aesthetic point of view, the experimenter of the twenty-first century will be free from the toil, frustration, and fatigue of obtaining reliable data from complex objects such as neurons, cardiocytes, or endothelial cells, but he will miss the pleasure, the excitation, and the deep fulfillment of a successful experiment at the end of a long day of work.
3. What comes first? Knowledge or technique?

   What is the relationship between a technical innovation and the change in scientific paradigms? The introduction of the patch clamp technique was the consequence of a cognitive pressure that "asked for a solution"? The answer may be partly yes; but how much the new technique changed our way to look at the molecular mechanisms underlying the higher physiological processes? The new technique simply had an additive role (added new information) or also a transformative one (providing a different view of the mechanisms and of processes themselves)? This could be a nice subject for a more epistemologically-oriented article.

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Roles of Intramolecular Interactions in the Regulation of TRP Channels

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Contents

Abstract The transient receptor potential (TRP) channels, classified into six (-A, -V, -P, -C, -M, -ML, -N and -Y) subfamilies, are important membrane sensors and mediators of diverse stimuli including pH, light, mechano-force, temperature, pain, taste, and smell. The mammalian TRP superfamily of 28 members share similar membrane topology with six membrane-spanning helices (S1–S6) and cytosolic N-/C-terminus. Abnormal function or expression of TRP channels is associated with cancer, skeletal dysplasia, immunodeficiency, and cardiac, renal, and neuronal diseases. The majority of TRP members share common functional regulators such

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as phospholipid PIP2, 2-aminoethoxydiphenyl borate (2-APB), and cannabinoid, while other ligands are more specific, such as allyl isothiocyanate (TRPA1), vanilloids (TRPV1), menthol (TRPM8), ADP-ribose (TRPM2), and ML-SA1 (TRPML1). The mechanisms underlying the gating and regulation of TRP channels remain largely unclear. Recent advances in cryogenic electron microscopy provided structural insights into 19 different TRP channels which all revealed close proximity of the C-terminus with the N-terminus and intracellular S4–S5 linker. Further studies found that some highly conserved residues in these regions of TRPV, -P, -C and -M members mediate functionally critical intramolecular interactions (i.e., within one subunit) between these regions. This review provides an overview on (1) intramolecular interactions in TRP channels and their effect on channel function; (2) functional roles of interplays between PIP2 (and other ligands) and TRP intramolecular interactions; and (3) relevance of the ligand-induced modulation of intramolecular interaction to diseases.

Keywords 2-APB · Cannabionoid · Channelopathy · Cryo-EM · Intramolecular interaction · PIP2 · TRP channel

Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
ARDs	Ankyrin repeat domains
BTDM	(2-(Benzo[d][1,3]dioxol-5-ylamino)thiazol-4-yl)((3 S,5 R)-3,5-dimethylpiperidin-1-yl)methanone
CBD	Cannabinoid
Cryo-EM	Cryogenic electron microscopy
ER	Endoplasmic reticulum
HEK	Human embryonic kidney
HTH	Helix-turn-helix
MHRs	TRPM homology regions
ML-SA1	Mucolipin synthetic agonist 1
PIP2	Phosphatidylinositol 4,5-bisphosphate
RTx	Resiniferatoxin
TRP	Transient receptor potential
VSLD	Voltage-sensing like domain

1 Introduction

The transient receptor potential (TRP) channels were named after a mutant fruit fly with abnormal phototransduction (Cosens and Manning 1969). Based on amino acid sequence homology, the TRP superfamily is grouped into eight subfamilies,

including polymodal ankyrin (TRPA), calcium-selective vallinoid (TRPV), polycystic kidney disease associated polycystin (TRPP), receptor-operated canonical (TRPC), diverse function melastatin (TRPM), endomembrane mucolipin (TRPML), invertebrates and poikilothermic vertebrates exclusive NOMP (TRPN), and yeast specific (TRPY) (Li 2017). Among different subfamilies, sequence homology is modest and biophysical properties including cation selectivity differ (Li 2017). The 28 mammalian TRP channels function as polymodal cellular sensors that integrate and mediate diverse environmental and physiological stimuli in the processes of vision, somatosensation, gustation, ion equilibrium, olfaction, and audition (Damann et al. 2008). Generally, TRP channels are non-selective cation channels permeable to Ca^{2+}, Na^{+}, and K^{+} with TRPV5 and TRPV6 are the most Ca-selective (Peng et al. 2018). The gatekeepers for human magnesium homeostasis are TRPM6 and TRPM7 (Schlingmann et al. 2007). Defective TRP channels caused by mutations or altered expression result in inherited or acquired human diseases, or channelopathies (Nilius 2007). It is a valuable aim of research on TRP channels to elucidate the mechanisms underlying how stimuli-induced variations in the phospholipid composition, membrane potential, and post-translational modifications as well as natural ligands regulate the function of TRP channels.

Correct folding is critical for a protein to be fully functional (Dill et al. 2008) and requires maintenance of energetically favorable intramolecular interactions, usually through disulfide bridges, hydrogen bonds, ionic bonds, or van der Waals (hydrophobic) forces (Alberts et al. 2002). For the past decade, technical breakthroughs in cryogenic electron microscopy (cryo-EM) and improved image-processing algorithms unprecedentedly revealed the atomic details of most TRP channels (Autzen et al. 2018; Cao et al. 2013; Chen et al. 2017; Dang et al. 2019; Deng et al. 2018; Diver et al. 2019; Dosey et al. 2019; Duan et al. 2018a, b, 2019; Fan et al. 2018; Hirschi et al. 2017; Liao et al. 2013; McGoldrick et al. 2018; Paulsen et al. 2015; Ruan et al. 2021; Shen et al. 2016; Shimada et al. 2020; Song et al. 2021; Su et al. 2018; Tang et al. 2018; Wang et al. 2018). Despite the low amino acid sequence homology found among the TRPs, strikingly they share significant overall structural similarities, such as tetrameric architecture (Fig. 1), six transmembrane spans (S1–S6), and intracellularly localized N- and C-termini (Fig. 2). The S1–S4 helices together resemble the voltage-sensing domain of potassium channels, whereas S5 and S6 helices together with the extracellular loop in between form the pore region (Fig. 1). The S4–S5 linker bridges between the S1–S4 and S5–S6 domains. However, the pore-forming S5-loop-S6 protrudes to the S1–S4 domain of a neighboring subunit, rather than being vicinal to the intramolecular S1–S4 domain (Fig. 1), an arrangement called domain swapping seen in the physiologically relevant structures of TRP channels, which is important for maintaining their channel function. In comparison with TRPPs and TRPMLs, which possess an extended S1–S2 loop and relatively short cytosolic domains, TRPA, TRPVs, TRPCs, and TRPMs are evolutionally closer to each other and contain relatively long cytosolic domains (Fig. 1). Physical proximity among different parts within a TRP subunit is revealed from its structure at atomic resolutions, suggesting the presence of intramolecular interactions.

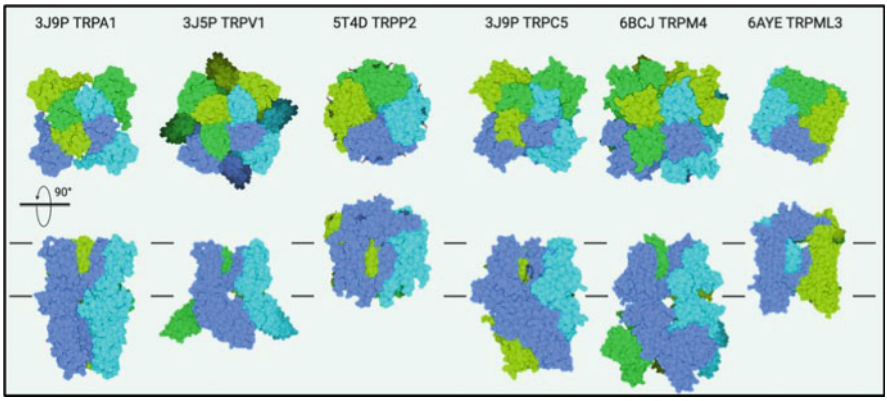

Fig. 1 Tetrameric conformation of six mammalian TRP channels. Four subunits are highlighted in separate colors. PDB numbers of corresponding TRP channels are labeled. Upper and lower panels show the view from the top and side (with 90° rotation), respectively. The lipid bilayer is illustrated by short lines in black

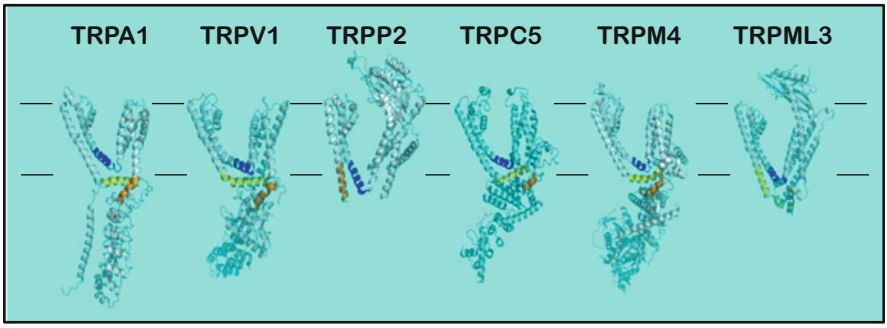

Fig. 2 Monomers of six representative mammalian TRP channels. Pre-S1 helix, S4–S5 linker, and TRP(-like) helix are in orange, purple, and yellow, respectively. "Out" and "In" stand for extracellular/luminal and intracellular side, respectively

Because crystallography and cryo-EM provide high-resolution snapshots of structures under experimental conditions they may not be able to resemble dynamics of TRP and other proteins investigated in living cells. In particular, physiological TRP intramolecular interactions should be investigated using living cells under physiological conditions. We recently characterized intramolecular interactions, functional implication and regulation by phosphatidylinositol 4,5-bisphosphate (PIP2) in several TRPs from different subfamilies including TRPV6, TRPV1, TRPP3, TRPP2, TRPC4, and TRPM8 channels (Cai et al. 2020; Zheng et al. 2018), in addition to previous studies (Nilius et al. 2008). Other research groups also reported intramolecular interactions and their functional importance in thermosensitive TRPVs (Boukalova et al. 2010; Liao et al. 2013), TRPP3 (Ng et al. 2019), TRPP2 (Vien et al. 2020), and TRPM4 (Xian et al. 2018, 2020).

The present review will summarize and provide insights into TRP intramolecular interactions and their involvements in the regulation of channel function.

2 Intramolecular Interactions in TRP Channels

Thanks to the recent cryo-EM revolution, structural details on TRP proteins at atomic levels were revealed, which otherwise are difficult to be crystalized, except for cytosolic domains (Li et al. 2011). Since the resolution of rat TRPV1 structure by cryo-EM (Liao et al. 2013), those of nearly 20 different TRPs have been mapped at near-atomic resolutions (Cao 2020), which suggested the presence of both shared and distinct intramolecular interactions.

2.1 Shared Intramolecular Interactions Mediated by Conserved Residues Across TRP Members and Species

The very first high-resolution structure resolved by cryo-EM was a truncated but functional rat TRPV1 at 3.4 Å, missing part of the N- and C-termini and S5–S6 loop (Liao et al. 2013). The structure revealed physical proximity of the TRP helix (Fig. 2, yellow helix) to the S4–S5 linker (purple helix) and pre-S1 helix (orange helix) and of the S4–S5 linker to S5 (Liao et al. 2013). Based on the structure, it was proposed that a hydrogen bonding between the side chain of W697 (in the TRP helix) and back bone of F559 (S4–S5 linker), and between R701 (TRP helix) and Q423 (pre-S1 helix) mediates the interaction of the TRP helix with the S4–S5 linker and pre-S1 helix, respectively. A subsequent functional study using over-expressed *Xenopus laevis* oocytes finds that R701 pairs with W426, rather than Q432, to mediate the TRP helix/pre-S1 helix interaction, presumably through a cation–π bonding, that is required for the channel function. The cation–π bonding is a type of non-covalent molecular interaction within an electron abundant benzene ring and positively charged side chain of two amino acids. The electronic force of cation–π bonding would stabilize the protein structure regionally. Importantly, in the capsaicin-activated state, the distance between the two residues in the W426:R701 pair is not much affected whereas Q432 moves away from R701 (Liao et al. 2013; Zheng et al. 2018). Thus, the TRP helix/pre-S1 helix interaction in TRPV1 is well-supported by both functional studies in living cells and structural studies using cryo-EM (Cao et al. 2013; Liao et al. 2013; Zheng et al. 2018).

The structures of over 20 different TRP members have now been resolved and all show close proximity of the TRP (TRP-like) helix to pre-S1 helix and S4–S5 linker (Cao 2020) (Fig. 2). By means of electrophysiology and molecular biology, the functionally important TRP/pre-S1 helix interaction has been validated in TRPV6, TRPP3, TRPP2, TRPC4, and TRPM8, and the TRP helix/S4–S5 linker interaction in

TRPV4, TRPV6, and TRPM4 (Cai et al. 2020; Xian et al. 2020; Zheng et al. 2018). Briefly, currents mediated by these TRP channels are functionally examined by two-electrode voltage clamp and patch clamp using *Xenopus* oocytes and mammalian cells, respectively. In addition, the physical association of the TRP helix with pre-S1 or S4–S5 linker has been verified by co-immunoprecipitation, co-immunostaining, and purified peptide in vitro binding. It remains to be determined whether these functionally important intramolecular interactions are shared by other TRP channels. S5, S6, and S5–S6 loop are in close vicinity and together form the so-called pore region. While the S5–S6 loop contains the selectivity filter (or upper pore gate) and the S6 helix contains the lower pore gate (or pore gate) the exact role of S5 is unclear. Our recent study showed that the TRPV6 S5 interacts with S6 through a pair of residues R532:D620 that should form a salt bridge and is critical for maintaining the basal channel function (Cai et al. 2021). In a subunit of a tetrameric TRP channel, the S1–S4 helices stay together while the S5–S6 helices extend to be adjacent to the S1–S4 helices of a neighboring subunit. This interesting arrangement is called domain swapping and may be a requirement under physiological conditions (Singh et al. 2017), but whether and how it affects channel gating remains unclear. The potential importance of the S5/S6 helix interaction in other TRP channels has not been reported yet.

Although the overall sequence similarities among the TRP channels are pretty low (~16%) (Palovcak et al. 2015) the residues that were shown by functional studies to be involved in the interactions of the TRP helix with the pre-S1 helix and S4–S5 linker are conserved among different TRP subfamilies (Fig. 3) (Cai et al. 2020; Zheng et al. 2018). For example, an aromatic residue (W or F) in pre-S1 is conserved in all TRP members (Fig. 3) and mediates interactions with the TRP helix. A cationic (K or R) or hydrophobic residue in the S4–S5 linker is also conserved in all TRP members except for TRPC1 (Fig. 3) and mediates interactions with the TRP helix. The residue in the TRP (-like) helix involved in these interactions is located within a shared motif **W/Y**XXX**Φ**, where X indicates any amino acid and Φ indicates K/R except for TRPV4 (W) and TRPV5/TRPV6 (I/V) (Fig. 3) (Cai et al. 2020; Montell 2001; Zheng et al. 2018). The functional roles of the corresponding conserved residues in other TRP channels remain to be determined. Of note, these conserved residues that were shown to mediate the interactions within TRP helix/pre-S1 or TRP helix/S4–S5 linker by functional studies are not fully consistent with structures resolved by means of cryo-EM or crystallography, possibly due to different study models and experimental conditions (Deng et al. 2018; Saotome et al. 2016). Live cells, which would preserve the native conditions for TRP channels, are used as models to examine the channel function, while structures captured by cryo-EM are snapshots of dynamic TRP channels under non-physiological conditions supplemented by artificial components. Also, in contrast to full-length constructs encoding TRP channels studied by electrophysiology, structural studies frequently utilize truncation(s) or mutations to increase the protein yield or stability and are carried out under unnatural conditions. These facts may explain the inconsistencies between the functional and structural studies.

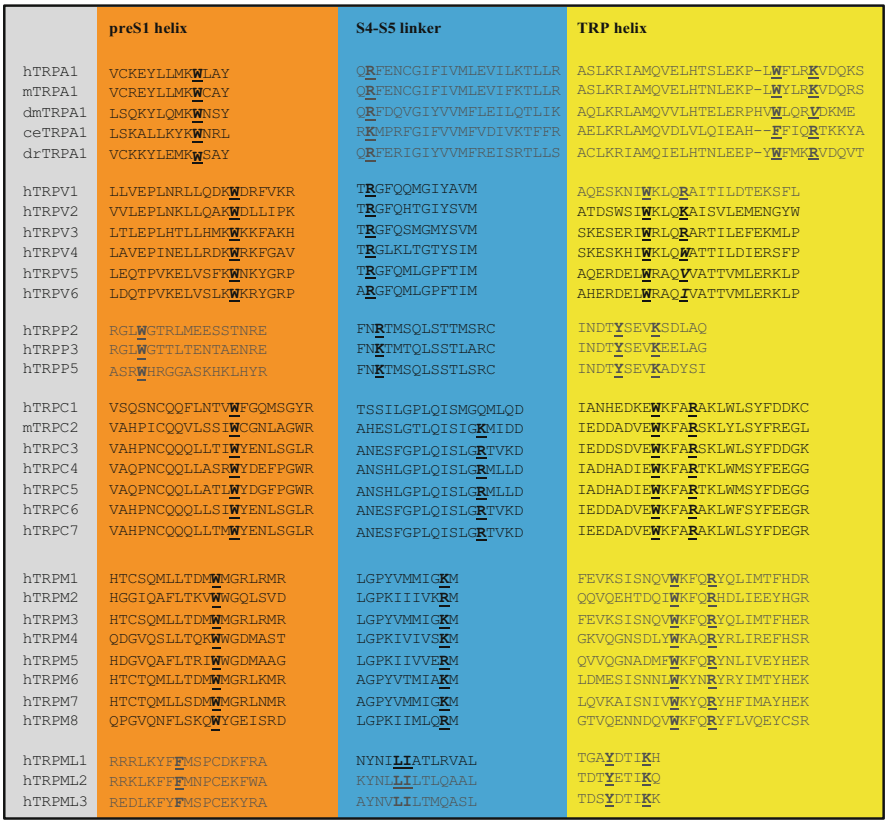

Fig. 3 Sequence alignments of pre-S1, S4–S5 linker and TRP(-like) helix across mammalian TRP channels. Conserved amino acid residues suggested to be involved in interactions are bolded and underlined. The species for TRP channels are listed as "h" for human, "m" for mouse, "dm" for *drosophila melanogaster* (fruit fly), "ce" for *Caenorhabditis elegans*, and "dr" for *Danio rerio* (zebrafish)

While similar physical interactions of the TRP helix with the pre-S1 helix and S4–S5 linker and similar residues mediating the corresponding interactions are shared among different TRP channels, their impacts on the channel function can be distinct (Fig. 4). For instance, in TRPV1, TRPP3, TRPP2, TRPC4, and TRPM8 expressed in oocytes or cultured human embryonic kidney (HEK) 293 cells, the TRP helix to pre-S1 interaction is required for the channel function or activation (Zheng et al. 2018), and the TRP helix to the S4–S5 linker interaction in TRPM4 positively correlates with the function in HEK293 cells (Xian et al. 2020). In contrast, in TRPV6, these interactions were found to be autoinhibitory, i.e. disruption of either of these interactions resulted in significant gain-of-function in *Xenopus* oocytes, cultured HEK293 cells, and zebrafish embryos (Cai et al. 2020). Similar to TRPV6, mutations in the TRPV4 S4–S5 linker that disrupts the presumable S4–S5 linker/TRP helix interaction retard budding yeast growth (Teng et al. 2015), by inducing

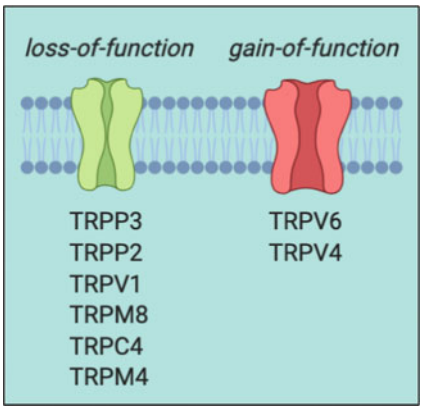

Fig. 4 Functional effects of disrupted intramolecular interactions. TRP channels are classified, respectively, based on functional studies. The TRP channels are presented as membrane-embedded with closed (left, loss-of-function) or enlarged (right, gain-of-function) channel pore

excessive ion influx (Loukin et al. 2010). Thus, whether disruption of these interactions inhibits or activates the channel function would depend on the specific conformational changes induced by a mutation.

2.2 *Subfamily- or Member-Specific Intramolecular Interactions*

Besides the structural arrangements that are shared across TRP subfamilies, there are other intramolecular interactions within transmembrane, extracellular or cytosolic domains that were reported for an individual TRP(s) or for members within a TRP subfamily (Fig. 5). Since all TRP channels are transmembrane proteins, the lipid bilayer naturally divided a TRP channel protein into three parts, namely extracellular/luminal domains, transmembrane domains, and intracellular domains based on which we here group intramolecular interactions.

2.2.1 Interactions Within Extracellular/Luminal Domains

The TRPPs are featured with the presence of a polycystin domain, a large extracellular loop between the S1 and S2 helices (Figs. 1 and 2). Several intramolecular interactions, including cation–π interaction (R322:F423), disulfide bonding (C331:C344), and hydrogen bonding (R325:T419), have been revealed by cryo-EM in an engineered human TRPP2 with N- and C-terminal truncations (198–703 vs. 1–968 for full-length protein) (Shen et al. 2016). Interestingly, mutation C331S, which presumably breaks down the C331:C344 disulfide bond, destabilizes the polycystin domain and overall protein architecture (Vien et al. 2020). Mutations disrupting the potential disulfide bonding abolish the ciliary TRPP2 function through impairment in voltage gating (Vien et al. 2020). Note that these residue pairs are conserved in the

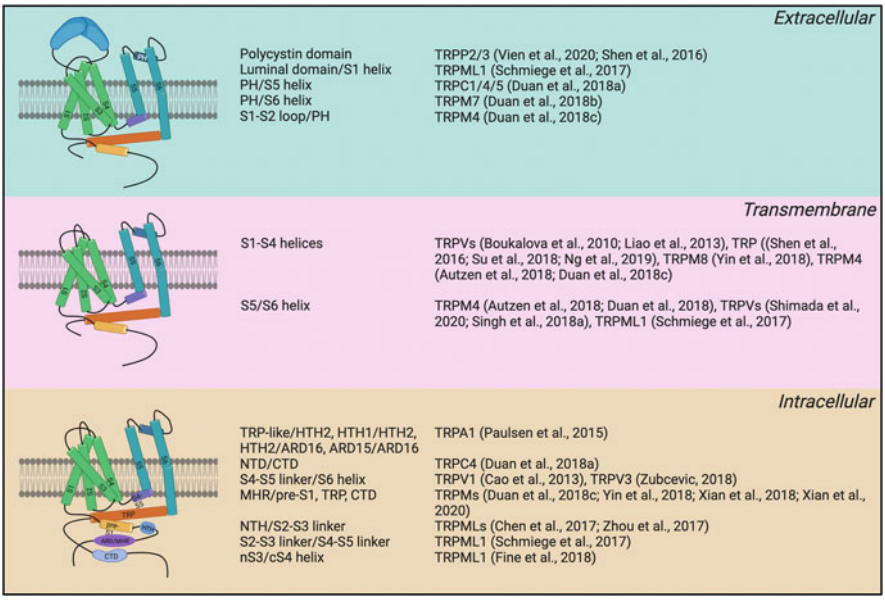

Fig. 5 Unique intramolecular interactions among different layers of TRP channels. Full names of abbreviated domains are pore helix (PH), ankyrin repeat (ARD), helix-turn-helix (HTH), N-terminal domain (NTD), C-terminal domain (CTD), melastatin homology region (MHR), N-terminal helix (NTH). The extracellular, transmembrane, intracellular domains involved in intramolecular interactions and related references are listed

other two TRPP members (TRPP3 and TRPP5) and have similar functional importance for TRPP3 (Vien et al. 2020). The TRPP2 polycystin domain was also found to bind to the extracellular S3–S4 linker and the second pore helix of the S5–S6 loop (F646-A652) through π–cation interaction (Shen et al. 2016).

TRPC4 does not have a large S1–S2 loop but its S5–S6 loop contains a disulfide bond formed by two cysteine residues (Duan et al. 2018a). This covalent interaction presumably maintains the channel in its basal state because disrupting the bond by dithiothreitol or other reducing chemicals activates the channel (and TRPC5 as well). Further, alanine substitution of both cysteines (C549A + C554A) in TRPC4 abolishes redox-related activation while retaining the englerin A-induced activation, whereas single C-to-A mutation (C549A or C554A) abolishes the function (Duan et al. 2018a). In contrast, TRPC5 with alanine substitution(s) of one or both corresponding cysteine(s) (C553A or C558A or C553A + C558A) is insensitive to DTT or englerin A (Duan et al. 2019). The underlying mechanisms are unclear. The two cysteine residues in the TRPC5 pore loop presumably form a disulfide bond and mediate its redox-induced activation, which is, however, inconsistent with a previous report that alanine substitution of either cysteine residue, which breaks the disulfide bond, constitutively opens the channel in HEK cells (Xu et al. 2008). Of note, these two cysteine residues are conserved in TRPC1, TRPC4, and TRPC5 but

not in other redox-insensitive TRPCs, indicating their distinctive regulation by redox status (Duan et al. 2018a). Interestingly, a similar cysteine pair was also found in the pore loop of all TRPMs (Duan et al. 2018c). Serine substitution of either of the two cysteine residues found in the corresponding region of TRPM7 somehow linearizes the I–V relationship from typical outward rectification (Duan et al. 2018b). A number of π–π and π–cation interactions were revealed between the S1–S2 loop and pore-helix of TRPM4 based on structural data (Duan et al. 2018c) but the functional roles of these interactions remain to be determined. The TRPML channels also possess a large luminal S1–S2 loop, consisting of four α helices and seven β strands (Figs. 1 and 2) (Schmiege et al. 2017). It was shown that its fourth α helix in the luminal domain links to the extended S1 helix through salt bridge and hydrogen bond (Schmiege et al. 2017). In the extracellular/luminal domains of TRPP, -C, -M and -ML channels, several intramolecular interactions have been characterized by functional and structural studies. More intramolecular interactions may potentially be identified in these channels and other TRPs as well.

2.2.2 Transmembrane Domains

Thermosensitive TRPVs (-V1 to -V4) possess hydrophobic residues located in the S1, S3, and S4 helices that are clustered, believed to stabilize the S1–S4 helices and are required for channel function (Boukalova et al. 2010; Liao et al. 2013); these channels also possess conserved hydrophilic aspartic acid in S5 and threonine in S6 that may be important specifically for channel opening (Shimada et al. 2020; Singh et al. 2018a). The S4 helix in TRPPs, as a voltage-sensing like domain (VSLD) reminiscent of voltage-gated channels, contains 2–3 cationic residues thought to interact with an aromatic or anionic residue located in S1, S2, or S3 (Shen et al. 2016; Su et al. 2018). Subsequent Rosetta structural modeling, a software for predictions of protein structure and function, together with the characterization of gating charge and channel function in HEK and SF9 insect cells showed that cationic residues in S4 mediate cation–π interaction with aromatic residues in S2 during polymodal activation of TRPP3 (Ng et al. 2019). Aromatic residues also form π–π interactions between S4 and S5 in TRPP2 and TRPP3 (Su et al. 2018). The thermosensitive TRPM8 S4 also acts as a VSLD and its cationic residues form salt bridges with anionic residues in S1, S2, or S3 (Yin et al. 2018). Also, a tryptophan in TRPM8 S3 interacts with aromatic residues in S4 and S5, presumably forming π–π bonds, which was thought to be important for cooling compound-induced channel gating (Yin et al. 2019). π–π interactions in S1/S4 and S5/S6 as well as salt bridges linking S4 to S2 and S3 in human TRPM4 were structurally revealed but with unclear functional implication (Autzen et al. 2018; Duan et al. 2018c). The van der Waals force formed by a valine:valine and valine:phenylalanine pair in S5/S6 of TRPML1 was thought to play an important role in stabilizing the channel in a closed state (Schmiege et al. 2017), and a V-to-P substitution in S5 resulted in gain of function of the channel, as shown by use of cell and animal models (Di Palma et al. 2002; Dong et al. 2009).

2.2.3 Intracellular Domains

The TRPA1 N-terminus contains as many as 18 ankyrin repeat domains (ARDs, a solenoid-like region mediating protein–protein interaction), which is longest in vertebrate TRP channels (Julius 2013), and extensive intramolecular interactions including TRP-like domain/helix-turn-helix (HTH) 2, HTH1/HTH2, HTH1/ARD16, and ARD15/ARD16 that are mediated by hydrophobic or polar residues and stabilize the stacking ARDs (Paulsen et al. 2015). Each of ARD16 and HTH1 is also close to, and presumably interacts with, the C-terminal coiled-coil (Paulsen et al. 2015). This interaction network possibly facilitates the channel assembly and transduces intracellular signals from ARDs and HTHs to the gating region (Paulsen et al. 2015). Similar structural arrangements among the stacking ARDs, N-linker domain (between ARD and pre-S1), and TRP helix are also present in TRPCs and TRPVs (Duan et al. 2018a, 2019; Zubcevic et al. 2016). In particular, TRPCs and TRPVs have 4 or 6 ARDs that are tightly stacked and presumably contain several intramolecular interactions (Bai et al. 2020; Cao et al. 2013; Duan et al. 2018a, 2019; Fan et al. 2018; Liao et al. 2013). In addition, the TRPC4 N-terminal region proximal to pre-S1 interacts with a distal C-terminal region, possibly via π–π interaction and hydrogen bonding (Duan et al. 2018a). In TRPV1, an aspartic acid and methionine located at the end of the S4–S5 linker and S6 helix, respectively, which are conserved in TRPVs, form a hydrogen bond mediating the binding between the two domains (Cao et al. 2013). In contrast, in $TRPV3_{apo}$, i.e. unbound state, the interaction between the S4–S5 linker and S6 helix is rather mediated by the phenylalanine:asparagine or phenylalanine:methionine pair (Zubcevic et al. 2018a).

While TRPA1 and TRPVs possess ARDs TRPMs have their N-terminus that contain four featured TRPM homology regions (MHRs, a characteristic domain shared by TRPMs) that are important for intramolecular interactions (Huang et al. 2020). In fact, A432 in the TRPM4 MHR3 interacts with residues located at MHR1, -2, and -4 (Xian et al. 2018), which may be destabilized by substitution with a bulky residue at 432 resulting in gain of function (Xian et al. 2018). The same group subsequently reported that MHR4 interacts with the TRP helix in TRPM4 (Xian et al. 2020). Further, it was reported that MHRs in TRPMs also interact with the pre-S1, TRP, and distal C-terminal domains (Duan et al. 2018c; Yin et al. 2018). In TRPMLs, the cytosolic S2–S3 linker forms a unique HTH that interacts with a cationic residue-rich H1 helix right before the S1 helix (Chen et al. 2017; Zhou et al. 2017). Cryo-EM structures of TRPML1 reveal two stable closed conformations, assigned as closed state I and II, which are distinguished by the S4–S5 linker swinging away from the channel pore (Chen et al. 2017). In closed state II of TRPML1, interaction between the H4 helix of the S2–S3 linker and the S4–S5 linker was identified (Chen et al. 2017). In TRPML1 interaction between S3 and S4 through conserved π–cation bonding has been identified (Fine et al. 2018). For TRPPs, because all constructs used for structural determinations so far lack most of their cytosolic domains (Grieben et al. 2017; Shen et al. 2016; Su et al. 2018), the

intramolecular interactions among cytosolic domains identified by the functional study (Zheng et al. 2018) would have to be verified by future structural studies.

3 Regulation of TRP Intramolecular Interactions by Chemical Ligands

The TRP channels are polymodal sensors integrating numerous signals. Some chemical ligands such as endogenous PIP2, natural compound cannabinoid, and synthetic 2-APB are known to modulate a variety of TRP channels but the underlying mechanisms are not fully understood (Muller et al. 2018). There are other chemical ligands that specifically modulate a particular TRP channel or a subset of TRP channels, such as allyl isothiocyanate (TRPA1), resiniferatoxin (TRPV1), ZINC17988990 (TRPV5), BDTM/AM-1473/AM-0833 (TRPC6), menthol/icillin/WS-12 (modulating TRPM8), ADPR (TRPM2), and ML-SA1 (TRPMLs). Of note, the natural/endogenous compounds, such as PIP2, cannabinoid, menthol, ADPR, and resiniferatoxin are more relevant to physiological or pathological modulation of the corresponding TRP member(s), in contrast to artificial chemicals that are used as pharmacological tools. Some TRP structures are resolved in the presence of a ligand molecule, which when combined with functional studies provides unprecedented opportunities to determine whether and how intramolecular interactions are involved in ligand-induced channel gating (Table 1).

3.1 PIP2

PIP2 has three isoforms, namely PI(3,5)P2, PI(3,4)P2, and PI(4,5)P2, among which PI(4,5)P2 is the most abundant phosphoinositol lipid with two phosphate anchorings in the inner leaflet while PI(3,5)P2 is enriched in the endolysosome (Vanhaesebroeck et al. 2001). PIP2 is known to modulate almost all mammalian TRP channels but with distinct effects or underlying mechanisms (Cai et al. 2020; Nilius et al. 2008; Rohacs 2014; Zheng et al. 2018). Besides regulation of TRP channels, importance of PIP2 is involved in actin dynamics, focal adhesion assembly, membrane tubulation, intracellular trafficking as well as modulation of other ion channels (Mandal 2020; Suh and Hille 2008). This review will focus on direct effects of PIP2 on TRP channels.

The structure of three TRPs have been resolved in complex with PI(4,5)P2 including rTRPV5, hTRPM8, and hTRPML1 (Chen et al. 2017; Fine et al. 2018; Hughes et al. 2018; Yin et al. 2019). In particular, the TRPV5-PI(4,5)P2 complex was resolved with a high concentration (400 μM) of PI(4,5)P2 (Hughes et al. 2018). By binding to cationic residues in the N-linker, S4–S5 linker, and S6 helix, PI(4,5)P2 induces conformational changes in the pore region thereby enlarging the lower gate

Table 1 Ligands reported to bind specific domains in corresponding TRPs and the subsequent effects on intramolecular interactions or channel gating

Ligand	TRP in complex	Bound	Effects	Reference
PIP2	rTRPV5	N-linker, S4-S5 linker, S6 helix	Enlarge lower gate	Hughes et al. (2018)
	hTRPML1	Pre-S1, S1 helix, S2-S3 linker	S4-S5 linker moves away from central pore	Fine et al. (2018)
	hTRPM8	Pre-S1, S4-S5 linker, TRP helix	Pre-S1, S4-S5 linker move towards TRP helix	Yin et al. (2019)
Cannabinoids	rTRPV2	S5/S6 helix	S4-S5 linker moves to S5 helix & TRP helix rotates	Pumroy et al. (2019)
2-APB	rTRPV6	S4-S5 linker, TRP helix	S4-S5 linker moves closer to TRP helix	Singh et al. (2018b)
	hTRPV3	S1-S4 helices	S1-S2 loop moves away from membrane & shortened S6 helix	Singh et al. (2018a)
BDTM	hTRPC6	Pre-S1, S1/S4 helix, S4-S5 linker	Maintain the closed state	Tang et al. (2018)
AM-1473		S1-S4 helices, TRP helix		Bai et al. (2020)
AM-0833		PH, S6 helix	S4-S5 linker moves away from TRP helix	
ADPR	drTRPM2	MHR1/2	Release of S4-S5 linker/TRP helix interaction	Huang et al. (2018)
RTx	hTRPV1/2	S4 helix, S4-S5 linker	S4-S5 linker moves away from central pore	Gao et al. (2016)
ZINC17988990	rbTRPV5	S1-S4 helices	Tightened S1-S4 bundle	Hughes et al. (2019)
ML-SA1	hTRPML1	S5/S6 helix	Disrupted S5/S6 helix interaction	Schmiege et al. (2017)
JT-010/BITC	hTRPA1	CTD	Translocation of a loop, stabilized by loop/TRP helix interaction	Suo et al. (2020), Zhao et al. (2020)

(Hughes et al. 2018). Residue K484 that mediates binding of the TRPV5 S4–S5 linker with PI(4,5)P2 is invariant in its closest homolog TRPV6 (as K484) and is also part of PI(4,5)P2 binding site in TRPV6 (Cai et al. 2020). Importantly, the autoinhibitory intramolecular S4–S5 linker/TRP helix interaction in hTRPV6 is mediated by the R470:W593 pair (Cai et al. 2020). Binding of PI(4,5)P2 to rTRPV5 increased the distance between R470 and W593 from 2.7 Å to 3.5 Å. Therefore, functional and structural data on TRPV5 and -V6 indicated that PIP2 attenuates the S4–S5 linker/TRP helix interaction through which it activates their channel function. TRPM8 structures in complex with icilin/PI(4,5)P2/Ca^{2+} or

WS-12/PI(4,5)P2 have been determined (Yin et al. 2019). In both complexes, a PI (4,5)P2 binding cassette is formed by residues from the pre-S1, S4–55 linker and TRP helix. Structural differences between $TRPM8_{apo}$ and $TRPM8_{icilin/PI(4,5)P2/Ca}$ indicate that the icilin/PI(4,5)P2 complex promotes the association of the pre-S1 and S4–S5 linker with the TRP helix (Yin et al. 2019), which is consistent with the functional data showing that PI(4,5)P2 strengthens the pre-S1/TRP helix interaction in TRPM8 (Zheng et al. 2018). TRPML is activated by PI(3,5)P2 in lysosome but is inhibited by PI(4,5)P2 on the cell surface (Zhang et al. 2012). PI(3,5)P2 interacts with the cytosol-facing cation-rich pre-S1 (also called H1 and H2), S1 helix and S2–S3 linker (also named H3-turn-H4). While no PIP2 density in the TRPML S4–S5 linker similar to TRPV6 was found, helix H4 can bind to the S4–S5 linker in the closed state II, which is a prerequisite for PI(3,5)P2-induced activation. Thus, the S4–S5 linker in PI(3,5)P2-activated TRPML1 moves away from the center pore and should be activated by PI(3,5)P2 indirectly (Fine et al. 2018). In summary, PI(4,5)P2 interacts with TRP channels through cytosolic domains or those proximal to the cytosol (Table 1) which further induces subsequent conformational changes. The TRP channels, underlying diverse physiological and pathological processes, with conformational changes caused by PIP2 would affect ion permeation resulting in alterations of the membrane potential and downstream signaling cascades, therefore regulating cellular functions.

3.2 Cannabinoids (CBD)

The cannabinoids are natural compounds derived from the plant *Cannabis sativa*, which have been widely used as medical interventions to alleviate diverse diseases via limiting the neurotransmitter release in presynaptic neurons (Whiting et al. 2015). In addition to the classical cannabinoid receptors (Mackie 2008), the thermosensitive TRP channels which include TRPA1, TRPV1-V4, and TRPM8 are either activated (A1 and V1-V4) or inhibited (M8) by cannabinoids and derivates and thus act as new cannabinoid-sensing integrators (Muller et al. 2018). TRPV2 has so far been the only TRP members with its structure resolved (at close-to-atomic resolution) in the presence of a cannabinoid ($TRPV2_{CBD}$) but was in a closed state (Pumroy et al. 2019). Specifically, based on the two rat TRPV2 structures resolved in the presence of 30 μM cannabidiol, the cannabidiol binding pocket was found to reside in a hydrophobic cavity formed by S5 and S6 helices from two neighboring monomers (Pumroy et al. 2019). Further, compared with $TRPV2_{apo}$, the first configuration with a bound cannabidiol ($TRPV2_{CBD1}$) only exhibits modest movements of the S4–S5 linker and TRP helix and the pore region remains unaffected. In the second structure ($TRPV2_{CBD2}$) the S4–S5 linker moves 2.6 Å toward S5 while the TRP helix rotates 8°.

3.3 2-ABP

The chemical 2-APB is synthesized from diphenylboronic acid generated from a reaction of methylborate with phenylmagnesium bromide (Maruyama et al. 1997). It is initially found as an antagonist of inositol trisphosphate receptor-induced calcium release (Maruyama et al. 1997). The IP3R is a ubiquitously expressed glycoprotein located on the endoplasmic reticulum (ER) membrane and its activation by IP3 releases Ca from ER and would affect intracellular Ca signaling pathways related to autophagy, cell death, or proliferation (Parys and Vervliet 2020). 2-APB is later found to exhibit functional effects on TRP channels, e.g., inhibiting TRPV6, TRPP2, TRPC1/3/5/6/7, and TRPM2/3/7/8, but activating TRPA1, TRPV1-V3, and TRPM6, and has limited or no effect on TRPV5 and TRPMLs (Clapham 2007; Colton and Zhu 2007). Several structures of the inhibited $TRPV6_{2\text{-}APB}$ and activated $TRPV3_{2\text{-}APB}$ were resolved (Deng et al. 2020; Singh et al. 2018a, b; Zubcevic et al. 2018a), which revealed that 2-APB binds to the S4–S5 linker and TRP helix of rat TRPV6 and brings them closer to each other (Singh et al. 2018b), presumably as part of channel pore closing process. This is consistent with an independent study which found that the disruption of the S4–S5 linker/TRP helix interaction is associated with TRPV6 channel opening (Cai et al. 2020). Compared with TRPV6, three putative 2-APB binding pockets were found to be distinctly located in an open TRPV3 $(Y564A)_{2\text{-}APB}$ structure (Singh et al. 2018a), among which the two pockets located within the S1–S4 helices are presumably involved in the pore gate opening. The $TRPV3(Y564A)_{2\text{-}APB}$ and $TRPV3_{apo}$ structures are basically identical, except that in $TRPV3(Y564A)_{2\text{-}APB}$ the S1–S2 loop is closer to the extracellular side and that the S6 helix is shorter and closer to S5 (Singh et al. 2018a). Based on currently available structures of 2-APB in complex with TRPV6 or TRPV3, different 2-APB binding cavities are revealed and distinct functional effects observed, presumably due to distinct conformational changes.

3.4 Specific Ligands

Apart from the promiscuous modulators discussed above, investigating regulation by member- or subfamily-specific ligands is crucial to understanding mechanisms underlying the function of TRP channels.

As the TRPV founding member, TRPV1 harbors a vanilloid-binding pocket that can interact with resiniferatoxin (RTx) and comprises residues in S4 and the S4–S5 linker (Gao et al. 2016). Compared with $TRPV1_{apo}$, the S4–S5 linker in $TRPV1_{RTx}$ moves away from the central pore which opens the lower pore gate (Gao et al. 2016) in a way that would involve in changes in the S4–S5 linker/TRP helix interaction based on related findings on TRPV6 (Cai et al. 2020). A similar vanilloid pocket is also identified in TRPV2 (Zubcevic et al. 2018b), a close homolog of TRPV1. Additionally, hydrogen bonds among S5, S6, and the pore helix in $TRPV2_{apo}$,

supposed to maintain channel symmetry, are disrupted in $TRPV1_{RTx}$ (Zubcevic et al. 2018b). The TRPV5-specific inhibitor ZINC17988990 binds with the S1–S4 helix bundle from the cytosol, which induces tighter interactions among the S1–S4 helices and thereby prevents channel activation (Hughes et al. 2019).

TRPC6 structures with a bound antagonist, $TRPC6_{BTDM}$ and $TRPC6_{AM\text{-}1473}$, where BTDM stands for (2-(benzo[d][1,3]dioxol-5-ylamino)thiazol-4-yl)((3 S,5 R)-3,5-dimethylpiperidin-1-yl)methanone a specific inhibitor, is almost identical to that of $TRPC3_{apo}$ (Bai et al. 2020; Tang et al. 2018), suggesting that these molecules may have maintained the channel in closed states. The BTDM binding pocket is composed of residues from the pre-S1, S1/S4 helices and S4–S5 linker (Tang et al. 2018), whereas AM-1473 interacts with a cavity formed by the S1–S4 and TRP helices (Bai et al. 2020). An analog of AM-1473 named SAR7334 inhibits TRPC6 based on a study using an acute hypoxic pulmonary vasoconstriction mouse model (Maier et al. 2015). In comparison with agonist-bound TRPC6 ($TRPC6_{AM\text{-}0833}$), in which AM-0833 is bound to the PH domain and S6, the major rearrangement is an upward movement of the S4–S5 linker away from the TRP helix (Bai et al. 2020). The nicotinamide adenine dinucleotide metabolite ADP-ribose (ADPR), which is a metabolite targeting ryanodine receptors in the ER and leads to Ca release underlying muscle contraction (Galione and Chuang 2020; Santulli and Marks 2015), activates TRPM2 (Huang et al. 2020). Structural comparison between EDTA-bound TRPM2 in the absence ($TRPM2_{EDTA}$) and presence of Ca^{2+} ($TRPM2_{ADPR/Ca}$) shows that upon cytosolic ADPR binding to the MHR1/2 domains, the S4–S5 linker/TRP helix association is released, which supposedly leads to upward movements of S5 and S6 and thereby opens the pore gate (Huang et al. 2018).

The TRPML-specific agonist called mucolipin synthetic agonist 1 (ML-SA1) was found to partially bind to S5, S6, and the pore helix forming an intramolecular hydrophobic cavity (Schmiege et al. 2017). In the ML-SA1-bound state, the S5/S6 helix interaction presumably is mediated by a valine:phenylalanine bond present in the apo state, is disrupted during the ML-SA1-induced pore gate opening (Schmiege et al. 2017). As abnormalities of TRPML are related to lysosomal disorders, use of ML-SA1 might provide a promising intervention (Shen et al. 2012). TRPA1 is the only TRP member sensitive to electrophiles and contains cysteine residues in its C-terminus that can be covalently modified by 2-Chloro-N-[4-(4-methoxyphenyl)-2-thiazolyl]-N-(3-methoxypropyl)acetamide (JT-010) and benzyl isothiocyanate (BITC) (Suo et al. 2020; Zhao et al. 2020). Of note, JT-010 could induce TRPA1-dependent noxious pain (Heber et al. 2019), while BITC has been linked to alleviation of lipopolysaccharides or high-fat diet induced inflammasome formation independent of TRPA1 (Chen et al. 2020; Lee et al. 2016). The electrophiles of different sizes would interact with cysteine residue(s) and occupy the cavity, thereby inducing translocation of an activation-loop out of the pocket; this rearrangement can then be stabilized through the activation-loop interaction with the TRP helix (Zhao et al. 2020).

Importantly, it should be noted that there are ligands that modulate TRP channel function but do not apparently affect intramolecular interactions. For instance, the TRPA1 antagonist A-967079 that acts as a wedge to lock S5 and S6 in a

conformation that prevents the channel from activation (Zhao et al. 2020). Another example would be the TRPV6-specific inhibitors named (4-phenylcyclohexyl) piperazine derivatives (PCHPDs) exhibit similar functional effects as calmodulin but inhibit the channel function through directly blocking the ion permeation (Bhardwaj et al. 2020). The ATP-inhibitable TRPM4 channel function is proposed to undergo conformational changes in inter-subunit interfaces of the nucleotide-binding domain and ARDs (Guo et al. 2017). Therefore, through physical blockade of channel pore, inter-molecular interactions or locking of channel, these alternative mechanisms are also important for the regulation of specific TRPs and further underlie diverse biological or pathological processes in which TRPs play roles. In addition, there are intracellular proteins such as calmodulin interacting with multiple TRPs. But how these protein–TRP interactions relate to intramolecular interactions require further investigations.

4 Implications in TRP Causing Human Diseases

Given the crucial importance of TRP channels in integrating and transducing diverse cellular signaling events such as chemical, temperature, and mechano-stimuli, it is not surprising that altered function or expression of TRP channels is associated with a range of diseases including inflammation, noxious pain, and neuronal, metabolic, and cardiovascular disorders (Kaneko and Szallasi 2014). To date, 11 channelopathies are known to be genetically caused by pathogenic mutation (s) in 9 TRP channels (Fig. 6). TRPV4 mutations are associated with multiple diseases, namely spondylometaphyseal dysplasia (SMD, characterized by short-trunk short stature), scapuloperoneal spinal muscular atrophy limb (SPSMA, with progressive muscle atrophy and weakness), and Charcot–Marie–Tooth disease type 2C (CMT2C, with weakness in limb, diaphragm, and laryngeal muscle) (Deng et al. 2010; Krakow et al. 2009; Landoure et al. 2010). A progressive disease occurred in kidney, named autosomal dominant polycystic kidney disease (ADPKD), is featured with accumulating cysts and is associated with numerous gene mutations in TRPP2 or PKD1 (Mochizuki et al. 1996). The uncontrolled growth of cysts could result in high blood pressure and kidney failure. The focal segmental glomerulosclerosis (FSGS) is a renal syndrome linked to an inheritable TRPC6 gain-of-function mutation (Winn et al. 2005). Later, 24 more mutations in TRPC6 have been identified for this syndrome (Wang et al. 2020), which has nephrotic symptoms and can lead to kidney failure. Patients with the neurodegenerative diseases, known as Guamanian amyotrophic lateral sclerosis (ALS-G) and Guamanian parkinsonism dementia (PD-G), suffer from muscle weakness and loss of motor control, which are characterized as being due to interplays between environmental lack of Ca^{2+} and Mg^{2+} and missense mutations in TRPM2 and TRPM7 (Hermosura et al. 2005, 2008). A retina-specific autosomal recessive disease called congenital stationary night blindness (CSNB) has been revealed as genetic disorder leading to night blindness and is caused by TRPM1 mutations (van Genderen et al. 2009). A missense (E to K)

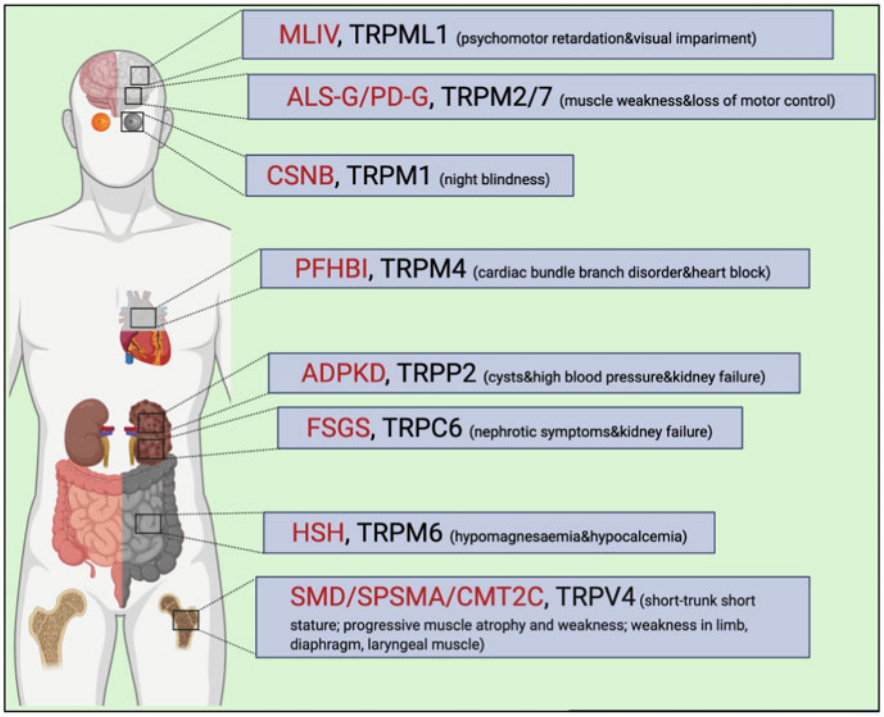

Fig. 6 TRP channelopathies in human. The diseases, namely mucolipidosis type IV (MLIV), Guamanian amyotrophic lateral sclerosis/Guamanian parkinsonism dementia (ALS-G/PD-G), congenital stationary night blindness (CSNB), progressive familial heart block type I (PFHBI), autosomal dominant polycystin kidney disease (ADPKD), focal segmental glomerulosclerosis (FSGS), hypomagnesemia with secondary hypocalcemia (HSH), spondylometaphyseal dysplasia/scapuloperoneal spinal muscular atrophy/Charcot–Marie–Tooth disease type 2C (SMD/SPSMA/CMT2C) are in red. The major organs affected by corresponding TRP(s) causing disorders are indicated as squares. The main clinical symptoms are described

mutation in TRPM4 is of gain-of-function and is observed in patients diagnosed with progressive familial heart block type I (PFHBI) (Daumy et al. 2016). Patients with PFHBI would develop into complete heart block from cardiac bundle branch disorder. The Mg^{2+} permeable TRPM6 is responsible for intestine absorption of dietary Mg^{2+}. The genetic autosomal recessive disorder called hypomagnesemia with secondary hypocalcemia (HSH) is due to mutations in TRPM6 (Schlingmann et al. 2002). Mutations in TRPML1 cause mucolipidosis type IV (MLIV), a neurodegenerative and autosomal recessive lysosomal storage disorder, and result in psychomotor retardation and visual impairment (Bargal et al. 2000). With the underlying mechanisms of pathogenesis remaining largely elusive, no effective treatment is currently available for these channelopathies.

Recent advances in cryo-EM shed light on detailed architectures at near-atomic scales of 7 out of the 9 TRPs (except TRPM1 and TRPM6) (Cao 2020). Structures of

pathogenic mutants of TRPV4, TRPP2, TRPC6, TRPM4, and TRPML1 are also available (Bai et al. 2020; Deng et al. 2018; Duan et al. 2018c; Schmiege et al. 2017; Shen et al. 2016; Zhang et al. 2017). The locations of known pathogenic mutations are clustered in interfaces between the S4–S5 linker and TRP helix, between S5 and S6, and in ankyrin repeats of TRPV4 (Deng et al. 2018); in the polycystin domain, pore helix/S6 interface and S4 of TRPP2 (Shen et al. 2016); in interfaces between ankyrin repeats and the C-terminal coiled-coil of TRPC6 (Bai et al. 2020); in the S4–S5 linker and N-terminus of TRPM4 (Duan et al. 2018c); in luminal domains, pore helix, S5 and S1–S4 helices in TRPML1 (Schmiege et al. 2017; Zhang et al. 2017). Fewer disease-causing mutations are found in TRPM2 and TRPM7, compared with other TRP channels reported with unclear mechanism. The P1018L mutation in TRPM2 (Hermosura et al. 2008) is located in the pore loop near S6 helix and may affect the stability of the S5/S6 helix interaction, given the close proximity (Huang et al. 2018). Notably, S6 helices of tetrameric TRPM2 form the channel pore and the stability of S5/S6 helix interaction potentially affects its channel function, which has been reported in TRPV6 and proposed to be conserved across TRPs (Cai et al. 2021). The TRPM2 P1080L mutant has been shown as of loss-of-function and is believed to interfere TRPM2 ion influx, causing ALS-G and PD-G diseases (Hermosura et al. 2008). The T1482I mutation in TRPM7 (Hermosura et al. 2005) is located in the C-terminus and has not been revealed in the reported structure which lacks the C-terminus (Duan et al. 2018b). These pathogenic mutations should have affected intra- and/or inter-molecular interactions in close proximity to their locations. Because these interactions maintain the physiological function of TRPs, the induced conformational changes would modulate the channel function and potentially disturb the physiological homeostasis.

In addition to the diseases caused by genetic mutations in TRP channels, there are multiple pathophysiological implications associated with certain TRP members. For instance, psoralen and ultraviolet A therapy induce pigmentation disorders through the intervention of TRPA1 and TRPV1 involved melanogenesis (Jia et al. 2021). The chondrogenesis or chondrification, which stands for mesenchymal stem cells undergoing differentiation to form cartilage, is recently proposed to rely on the presence of TRPV4 protein and its Ca permeability (Willard et al. 2021). Among all TRP channels, knockout of TRPA1, TRPV1, or TRPM3 in mice abolishes acute noxious heat sensing while retaining the sensation of cold-induced or mechanical pain (Vandewauw et al. 2018), which provides a way to alleviate chronic pain (Bamps et al. 2021). Additionally, TRPA1 and TRPV1 have been suggested to mediate nociception in the pain syndromes associated with cancer cell inoculation (de Almeida et al. 2021). Due to the involvement of multiple TRP channels in adaptive and innate immune systems, TRPA, -V, -C and -M are known to play notable roles in atherosclerosis, atopy, chronic fatigue syndrome, hypertension, inflammatory bowel disease, and myalgic encephalomyelitis, which have been reviewed in-depth (Froghi et al. 2021).

5 Discussions and Perspectives

Thanks to advances in structural determination techniques notably cryo-EM and the associated reconstruction algorithms, the majority of the TRP channels have now been uncovered at near-atomic resolutions. This review has focused on TRP intramolecular interactions and their functional importance. These intramolecular interactions are either shared by members across subfamilies or within a subfamily or unique to individual members. We have covered relationships between ligand binding and changes in intramolecular interactions as well as those between pathogenic mutations and changes in structures or intramolecular interactions. Thus, the presence of a ligand (or mutation)-intramolecular interaction relay should be a mechanism shared among all TRPs and be very important in transducing upstream signals or genetic mutations into conformational and thereby functional changes in TRPs. In fact, functional importance of intramolecular interactions has also been recognized in other ion channels including mechanosensitive Piezo1 (Lewis and Grandl 2020), calcium (Kim et al. 2018; Singh et al. 2006), sodium (Kass 2006), potassium (Sharmin and Gallin 2017), and chloride channels (Dhani and Bear 2006). Therefore, intramolecular interactions present in various types of ion channels should be important for protein stability, function, and regulation.

PIP2 is an important ligand that regulates most, if not all, TRP channels. Our recent studies allowed proposing a PIP2-intramolecular interaction relay that converts extracellular stimuli into electrical signals, which is shared by TRP members from different subfamilies including TRPV1, TRPV6, TRPP3, TRPP2, TRPC4, and TRPM8. Specifically, PIP2 affects the strength of intramolecular interactions in a TRP protein thereby regulating its channel function. It can thus be reasonably postulated that the PIP2-intramolecular interaction relay exists in most or all TRP members as a shared mechanism that mediates the functional regulation by extracellular stimuli or upstream factors. Thus, any upstream cascade, e.g. GPCR-phospholipase C (PLC) (Kadamur and Ross 2013), that affects the PIP2 level directly or indirectly, would regulate the channel function through the relay. On the other hand, hydrolysis of PIP2 by PLC produces diacylglycerol (DAG) and IP3 and would potentially affect the function of some TRP channels through PKC-dependent phosphorylation (Mandadi et al. 2011; Venkatachalam et al. 2003; Zhang and Saffen 2001).

Despite the tremendous details revealed by all the TRP structures by means of cryo-EM or crystallography, these techniques have limitations in reflecting the natural configurations of these channels. For instance, while ligand 2-APB functionally activates TRPV3 the structure of TRPV3 bound with 2-APB ($TRPV3_{2\text{-}APB}$) exhibits a closed conformation (Singh et al. 2018a). Interestingly, the $TRPV3_{2\text{-}APB}$ complex becomes an open channel in the presence of the K169A mutation to stabilize the open state or the Y564A mutation to increase the 2-APB binding affinity (Singh et al. 2018a; Zubcevic et al. 2018a). Another example is TRPV5 of which a cryo-EM structure did not reveal a PIP2 binding site until a high concentration of 200 μM diC8-PIP2 was used, which is around three folds of the PIP2 EC_{50} value for

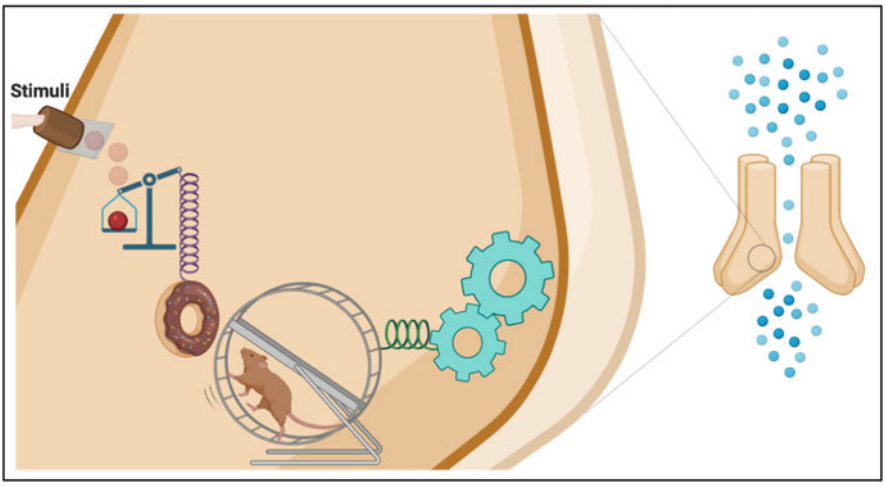

Fig. 7 Cartoon illustrates the proposed intramolecular interactions within one subunit modulating the TRP channel gating processes. The environmental stimuli induce series of subsequent changes in intramolecular interactions and protein conformation, represented by scale, donut, mice, and gear, which mediate the channel gating regulation

TRPV5 (Hughes et al. 2018). Further increase in the diC8-PIP2 dose to 400 μM enabled resolution of a $TRPV5_{diC8}$ structure (Hughes et al. 2018). In addition, although PIP2 can activate purified TRPM8 channels as shown by planar lipid bilayer electrophysiology (Zakharian et al. 2010) the $TRPM8_{PIP2}$ complex structure cannot be obtained until ligand icillin or WS-12 was added (Yin et al. 2019). Therefore, structures would have to be determined under more physiological conditions to better reconcile with functional determinations in living cells.

Based on the currently available structural and functional data, we propose that TRP channels sense extra- or intracellular stimuli, which induces conformational changes including critical intramolecular interactions and thereby results in changes in channel gating (Fig. 7). Genetic mutations associated with human channelopathies may have also altered intramolecular interactions. Notwithstanding, this hypothesized ligand-intramolecular interaction-gating scheme warrants further verifications. TRP structures resolved by currently available techniques are only a few snapshots of highly dynamic configurations during the unknown and sophisticated channel gating cycles, yet under very different experimental conditions. Structural data should be interpreted with caution when comparing or combining with functional data obtained using living cells or with data from numerical simulations. It should also be noted that although the regulation mediated through intramolecular interactions is important, other mediating routes are important parts of functional regulation. For example, a chemical antagonist can directly block the TRPV6 pore and some hydrophilic mutations can directly alter a TRP hydrophobic pore gate (Bhardwaj et al. 2020).

In summary, while the TRP superfamily has shared intramolecular interactions that act as a crucial molecular switch responding to common environmental and physiological stimuli, some TRP subfamilies or individual members have distinct intramolecular interactions or stimuli (Fig. 5). Understanding the underlying roles of these intramolecular interactions would not only help elucidating the TRP gating mechanisms but would also provide novel therapeutic targets in clinical interventions.

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The Emerging Pro-Algesic Profile of Transient Receptor Potential Vanilloid Type 4

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Abstract Transient receptor potential vanilloid type 4 (TRPV4) channels are Ca^{2+}-permeable non-selective cation channels which mediate a wide range of physiological functions and are activated and modulated by a diverse array of stimuli. One of this ion channel's least discussed functions is in relation to the generation and maintenance of certain pain sensations. However, in the two decades which have elapsed since the identification of this ion channel, considerable data has emerged concerning its function in mediating pain sensations. TRPV4 is a mediator of *mechanical hyperalgesia* in the various contexts in which a mechanical stimulus, comprising trauma (at the macro-level) or discrete extracellular pressure or stress (at the micro-level), results in pain. TRPV4 is also recognised as constituting an essential component in mediating inflammatory pain. It also plays a role in relation to many forms of neuropathic-type pain, where it functions in mediating mechanical allodynia and hyperalgesia.

Here, we review the role of TRPV4 in mediating pain sensations.

Keywords Allodynia · Inflammatory pain · Mechanical hyperalgesia · Mechanotransduction · Neuropathic pain · Pain · Sensory processing · TRPV4

1 Introduction

Transient receptor potential vanilloid type 4 (TRPV4) is a polymodal Ca^{2+}-permeable non-selective cation channel, first described by Schultz and Liedtke at the beginning of this century (Strotmann et al. 2000; Liedtke et al. 2000). Originally thought to perform the limited function of an osmosensor, this ionotropic receptor is now known to be activated by multiple disparate stimuli, as well as being constitutively expressed and capable of spontaneous activity in the absence of agonist stimulation. This feature, combined with its widespread dissemination throughout the body and its capacity to interact with other proteins, underpins TRPV4's role as a major molecular player in both physiology and pathology (White et al. 2016; Everaerts et al. 2010).

The involvement of TRPV4 as a significant mediator of pain sensations presupposes its widespread expression by primary nociceptive afferent neurons. TRPV4 is indeed strategically deployed on these neurons throughout the body and localised to serve as a responder to, if not a transducer of, several types of noxious stimuli. Thus, dorsal root ganglion (DRG) neurons and trigeminal ganglion neurons express TRPV4, with TRPV4 having been identified in primary sensory afferents in

the colon, skin, and internal elastic membrane of the tongue (Brierley et al. 2008; Sipe et al. 2008; Suzuki et al. 2003a, b; Alessandri-Haber et al. 2003, 2005; Liedtke et al. 2000). TRPV4 protein has been found to be transported distally along the axons of primary sensory afferents in the direction of the peripheral nerve endings (Alessandri-Haber et al. 2003). Osmosensitive sensory neurons of the thoracic DRG express TRPV4 and innervate the blood vessels of the liver (Lechner et al. 2011). Again, TRPV4 is found co-expressed with TRPV1 (an established important player in the generation of peripherally localised pain (White et al. 2011)) in a subpopulation of DRG neurons in rat and in their terminals located in the spinal dorsal horn (Cao et al. 2009). Likewise, trigeminal ganglion sensory neurons express TRPV4 (Liedtke et al. 2000) which innervate the *dura* (Wei et al. 2011) and are suspected of being implicated in headache and face pain (Chen et al. 2013). More generally, TRPV4 is widely expressed in multiple organs throughout the body (Liedtke et al. 2000), including the ear (Takumida et al. 2005) and skin keratinocytes (Chen et al. 2021). Given the presence of an apparent anatomical and physiological framework for the involvement of TRPV4 in pain signalling, the further question relates to the agents which are capable of activating TRPV4 for this purpose and the circumstances which enable them to do so.

In terms of activators, TRPV4 channels are activated by a multiplicity of diverse stimuli emanating from independent sources, both endogenous and exogenous, and employ varying pathways of activation of TRPV4 (Watanabe et al. 2003b; Vriens et al. 2004, 2007). Endogenous agonists include anandamide and arachidonic acid (AA) which employ epoxyeicosatrienoic acids to activate TRPV4 (Watanabe et al. 2003a, b). Recently, Liedtke and colleagues have identified lysophosphatidylcholine (LPC) as a direct activator of TRPV4 expressed in skin keratinocytes (Chen et al. 2021). The range of exogenous activators is remarkable, including even diesel exhaust particles, which comprise organic chemicals, bound to the carbon nanoparticles, which potently stimulate Ca^{2+} ingress via TRPV4 (Li et al. 2011). The range of channel activators is matched by the variety of different mechanisms of channel activation. Thus, cell swelling activates this cation channel by a different pathway from that employed by heat and phorbol esters (Vriens et al. 2004), while channel mutations have been demonstrated to exhibit different impacts on the sensitivity of TRPV4 to different agonists (Vriens et al. 2007; Berna-Erro et al. 2017). Importantly, TRPV4 also possesses the capacity to integrate diverse signals in a synergistic manner. By way of illustration, TRPV4 channels expressed in transfected cells at room temperature (22–24°C) only respond with minimal activation to stimulation by an hypotonic solution at room temperature or to challenge with the agonist, 4alpha-phorbol 12,13-didecanoate (4α-PDD). Phorbol 12-myristate 13-acetate (PMA) and shear stress likewise elicit little activation of TRPV4 in similar circumstances. Nevertheless, at 37°C (physiological body temperature), the ion channel is readily activated in response to all of these stimuli, demonstrating also the importance of temperature in the context of activation of TRPV4 (Gao et al. 2003; Liedtke et al. 2000). TRPV4 is a polymodal nociceptor and its most striking feature is its capacity to integrate the stimuli of several different inputs which, individually, may be incapable of gating the channel but which manage to effect

such gating when acting conjointly (Liedtke et al. 2000; Alessandri-Haber et al. 2003, 2005).

It is important to remember that TRPV4 is involved in normal physiological functioning in multiple domains (White et al. 2016) and may be involved at some juncture in the development or maintenance of a disease, of which one of the symptoms is pain. However, our concern here is not with the involvement of TRPV4 in the pathogenesis of disease but rather its proximate involvement in generating, or maintaining, pain as a symptom of any given pathology. In other words, we are concerned with elaborating the immediate pro-algesic roles of TRPV4.

The involvement of TRPV4, expressed by peripheral sensory neurons, in pain signalling can conveniently be considered in several connections. The first of these is pressure, or mechanical stress, applied either directly, or indirectly, to the plasmalemma of cells expressing TRPV4. This stimulus is capable of initiating a train of events which results in the gating of TRPV4 and, hence, of mediating mechanical hyperalgesia. Second, the inflammatory response constitutes another activating stimulus for TRPV4 in the context of pain generation. A multiplicity of pro-algesic activating agents of TRPV4 are to be found present amongst the diverse signalling molecules responsible for, or resulting from, inflammation, and this ion channel is fundamentally important for its role in *inflammatory-type* pain. Finally, TRPV4 has a known association with mechanical allodynia and hyperalgesia resulting from peripheral neuropathy. It is important to emphasise, however, that this categorisation is offered here for the purpose of exposition only and does not suggest the existence of pain pathways exclusive to one, or other, of these categories.

2 Cell Mechanisms of TRPV4-Mediated Pain

Several factors are of known importance in the contribution made by TRPV4 to the development of pain signalling in primary nociceptive afferents. First, various protein–protein interactions increase the capacity of TRPV4 to respond to extracellular stimuli. Second, there is the seminal role of Ca^{2+} ions, given that the current generated by activation of TRPV4 is carried by Ca^{2+} and other cations. Third, the effects of the gating of TRPV4 are modulated by various intracellular and extracellular enzymatic, and other, signalling molecules. Finally, post-Ca^{2+} influx events function to translate TRPV4 activation into pain signalling by primary nociceptive afferents.

The role of TRPV4 in generating pain signalling by peripheral nociceptors is that of a component within a dynamic system of sensory processing performed by primary nociceptive afferents which express TRPV4. The nociceptive signalling which is generated within nociceptive primary afferents is the product of a multiplicity of neurotransmitters and receptors expressed in neuronal cells which are specially adapted for the purpose of nociception. When one speaks of the role of TRPV4 as being decisive in relation to the development of pain sensations in

particular circumstances, this is in the context of its contribution within the neuronal cell to the generation of the necessary pain signal by that cell which ultimately results in the sensation of pain by the brain. These nociceptors have within themselves the ability to, and do, modulate the signal generated by their excitation by external stimuli. Thus, nociceptors modify that signal in the periphery at a point in space and time in advance of the further processing of that signal by the central nervous system (Carlton 2014).

2.1 TRPV4 and Protein–Protein Interactions

The association of Annexin A2 with TRPV4 in DRG neurons appears to function in regulating TRPV4-mediated Ca^{2+} influx and the release of substance P (Ishibashi et al. 2008; Ning et al. 2012). TRPV4 and TRPV1 are co-localised in a population of DRG neurons (Cao et al. 2009). Hypotonicity is one of the important activators of TRPV4 in its role in the development of mechanical pain. An interaction between TRPV4 and F-actin is important in sensing hypotonicity and in effecting regulatory volume decrease (RVD; Becker et al. 2009). Hypotonicity also increases the association and surface expression of aquaporin 5 (AQP5) and TRPV4, but actin depolymerisation diminishes these effects and RVD. AQP5 – not cell swelling itself – is crucial for the activation of TRPV4 by hypotonicity with TRPV4 and AQP5 acting in concert to control RVD (Liu et al. 2006).

Heteromeric TRPV4 channels can result from the association of TRPV4 monomers with other ion channel monomers. Thus, the stretch-activated transient receptor potential canonical type 1 (TRPC1) channel and transient receptor potential canonical type 6 (TRPC6) channels are often found in association with TRPV4 in DRG neurons. Here, they associate with TRPV4 channels to sensitise primary afferent nociceptors and mediate mechanical hyperalgesia (Alessandri-Haber et al. 2009). The loss of Ca^{2+} stores can cause the insertion of TRPV4–C1 heteromeric channels into the plasma membrane in human HEK293 cells transfected with both TRPV4 and TRPC1 and, also, in native vascular endothelial cells. Heteromeric TRPV4–C1 channels are preferentially translocated to the plasma membrane in comparison with homomeric TRPC1 or TRPV4 channels (Ma et al. 2010). TRPV4 can associate with transient receptor potential polycystic type 2 channels (TRPP2) or the Ca^{2+}-activated K^+ channel, KCa2.3, to form novel channel entities (Köttgen et al. 2008; Zhang et al. 2013; Saigusa et al. 2019; He et al. 2017).

Signalling microdomains allow TRPV4 to indirectly interact with other nearby Ca^{2+}-sensitive proteins. Large conductance Ca^{2+}-activated K^+ (BK) channels are found co-localised with TRPV4 in the endothelium of mesenteric resistance arteries and with calcitonin gene related peptide (CGRP) in sensory neurons (Gao and Wang 2010). The human purinergic G protein-coupled receptor $P2Y_1$ ($P2Y_1R$) activates TRPV4 expressed in a subpopulation of satellite glial cells (SGCs) (Rajasekhar et al. 2015). It also appears that inositol 1,4,5-trisphosphate (IP_3), while not activating TRPV4 directly, sensitises the channel to other agonists (Fernandes et al. 2008),

probably by interactions with the IP_3 receptor. TRPV4 and neuronal calcium sensor 1 (NCS1), which is a calcium-binding protein, functionally interact by forming a signalling complex which increases TRPV4 currents in line with increased expression of NCS1. Ca^{2+} fluxes entering through TRPV4 determine the size of TRPV4 currents. These Ca^{2+} fluxes depend on NCSI expression levels and are in line with the size of TRPV4 currents (Sánchez and Ehrlich 2021).

Interestingly in the present context, the interaction of the proline rich domain in the N-terminal of TRPV4 with protein kinase C and casein kinase substrate in neurones 3 (PACSIN3) reduces both basal activity and activation by heat and hypotonicity. This affects not only membrane expression but also channel gating, since channels remain responsive to 4-alpha-phorbol-12,-13-didecanoate (4αPDD) (Cuajungco et al. 2006; D'Hoedt et al. 2008; Garcia-Elias et al. 2013). Other molecules influence TRPV4 polyubiquitination, endocytosis, and proteasomal breakdown (Wegierski et al. 2006; Shukla et al. 2010; Saliez et al. 2008).

2.2 TRPV4 and Calcium Signalling

The most basic issue for consideration concerns how the activation of TRPV4, expressed in primary sensory afferents, is involved in the process which results in pain signalling by those nociceptors. Activation and gating of these ion channels in such circumstances results in an influx of Ca^{2+} ions and other cations which alters the electrical potential of the neuronal membrane within the vicinity of the gated ion channels. This local alteration in the neuronal membrane potential may promote action potential generation either directly, through local depolarisation, or via signalling events initiated by a local rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). It is these nociceptor action potentials which, after modulation in the spinal cord, are ultimately interpreted in the brain as a pain sensation. Alternatively, the activation of TRPV4 channels, either in the relevant primary sensory neurons or adjacent non-neuronal cells, may result in the release of an extracellular chemical signal which, in turn, activates receptors on nociceptors to initiate pain signalling. Whether, in the case of each of these nociceptive pathways, Ca^{2+} influx with a resulting heightened $[Ca^{2+}]_i$ is the decisive event consequent on TRPV4 activation which leads to pain signalling is not clear. Direct depolarisation is not dependent on the rise of Ca^{2+} per se, but rather reflects the non-specific increase in cation conductance. This is usually associated with, but is not dependent on, a rise in $[Ca^{2+}]_i$. Generation of intra- or extracellular signalling molecules, however, is almost certainly Ca^{2+}-dependent.

Baseline $[Ca^{2+}]_i$ is kept remarkably low (≤100 nM) within most cells by a variety of Ca^{2+}-removal and buffering systems (Groten et al. 2013; Schwaller, 2012). These maintain a 10^4-fold gradient between intracellular and extracellular $[Ca^{2+}]$, which means that small absolute increases in $[Ca^{2+}]_i$ as a result of agonist stimulation can be identified as an appreciable change in $[Ca^{2+}]_i$ relative to unstimulated conditions. Increases in $[Ca^{2+}]_i$ act as an intracellular messenger in almost every cell type and

function (McGeown, 2010). Such increases in $[Ca^{2+}]_i$ depend on the diffusion of Ca^{2+} ions into the cell cytosol from either intracellular stores or the extracellular fluid, with both avenues requiring the gating of Ca^{2+}-permeant channels, which allow diffusion of Ca^{2+} ions across the relevant lipid membrane (Fuertes et al. 2010). TRPV4 channels are non-selective cation channels which are 5–10 times more permeable to Ca^{2+} than to the main extracellular monovalent cation, Na^+. Activation of TRPV4 by any of its diverse array of agonists causes $[Ca^{2+}]_i$ to rise in the cell in which the channel is expressed (Plant and Strotmann 2007; Watanabe et al. 2002). Both extracellular and intracellular Ca^{2+} modify channel function in complex ways. Counterintuitively, raising $[Ca^{2+}]_o$ reduces the amplitude and accelerates the decay of 4α-PDD-induced TRPV4 currents in a HEK293 expression system. A point mutation in the sixth transmembrane domain (phenylalanine substituted with alanine at position 707) diminishes this effect, while a C terminus mutation (glutamic acid at 797 mutated to either alanine or lysine) results in a channel which is constitutively open (Watanabe et al. 2003a, b). Increasing $[Ca^{2+}]_i$ can inhibit the TRPV4 channel at physiologically relevant concentrations (IC_{50} = 406 nM), a negative feedback mechanism that may account for the transient nature of TRPV4 signals (Watanabe et al. 2002). However, $[Ca^{2+}]_i$ can also increase channel activity through an interaction with the Ca^{2+}-calmodulin binding domain on the C-terminal tail, relieving an autoinhibitory interaction with an intracellular N-terminus domain (Strotmann et al. 2003, 2010). TRPV4-initiated signals may also be amplified and propagated through Ca^{2+}-induced Ca^{2+} release from intracellular stores (Earley et al. 2005; Dunn et al. 2013). As with any channel, TRPV4-mediated influx of extracellular Ca^{2+} will be affected by rates of synthesis of appropriate sub-units, assembly, transport, and insertion into the cell membrane, rates of removal from it, as well as other processes, such as phosphorylation. Thus, the activity of TRPV4 at the cell membrane reflects not merely the extent of expression of the ion channels there but also the efficiency with which they are assembled. Channel trafficking may be disrupted either by mutation of the N-terminal ankyrin repeat domains or deletion of a C-terminal (838–857) region necessary for correct channel folding (Lei et al. 2013).

Different agonists do not necessarily activate TRPV4 by means of the same intracellular mechanisms or with the same effects; and the same agonist may engage different intracellular mechanisms and mediate different effects depending on the specific cell type in which the TRPV4 channels activated by it are expressed. GSK1016790A is a synthetic agonist which exhibits selectivity for TRPV4 and has become popular as an experimental tool. In primary endothelial cells and in HEK293 cells transfected with TRPV4, GSK1016790A rapidly activates TRPV4 channels, causing an increase in $[Ca^{2+}]_i$. TRPV4 channels are delivered to the cell surface by either "complete fusion" or "partial fusion" exocytosis. Within seconds of TRPV4 activation, there is an increase in the number of vesicles with partial fusion, while the number with complete fusion is reduced, coinciding with peak Ca^{2+} influx into the cytoplasm and localised transient increase of Ca^{2+} near to the plasma membrane. GSK1016790A-induced activation of TRPV4 results in later endocytosis of TRPV4 channels into the cytoplasm where they gather in recycling endosomes (Baratchi et al. 2019). Importantly, these investigators also noted that they found no

effect on the expression of TRPV4 at the plasma membrane when TRPV4 ion channels are activated with the agonist, 4αPDD. However, Sullivan et al. (2012) found that the majority of TRPV4 channels remain inactive even when being subjected to maximal stimulation with GSK1016790A, with GSK1016790A functioning as an agonist by recruiting previously silent channels, as opposed to increasing the level of basal channel activity (Sullivan et al. 2012). Other investigators have also identified differences in the responses resulting from GSK1016790A stimulation of TRPV4 and its stimulation by the natural agonist, 5′,6′-epoxy-8,11,14-eicosatrienoic acid (5′,6′-EET), with both of these agonists producing a sustained elevation of $[Ca^{2+}]_i$ in human umbilical vein endothelial cells, but with different kinetics of $[Ca^{2+}]_i$ increase in each case (Swain and Liddle 2020). The demonstration that the responses evoked by GSK1016790A are agonist specific is important as this means that the responses obtained by the activation of TRPV4 by this synthetic agonist are not necessarily representative of those elicited by other TRPV4 agonists.

2.3 *Modulation of TRPV4 Activity by Intracellular and Extracellular Enzymes*

Various intracellular enzymes may also play a role in modulating the activation of TRPV4. TRPV4 activation may be enhanced by the phosphorylation of specific sites – a phenomenon which requires the assembly of PKC or PKA by ankyrin kinase anchoring protein 79 (AKAP79) to constitute a signalling complex with TRPV4 (Fan et al. 2009). Tyrosine phosphorylation of TRPV4 is mediated by Src family tyrosine kinases (SFKs) (Wegierski et al. 2009; Xu et al. 2003). The SFK-induced phosphorylation sites (Tyr110 and Tyr805) are found in the NH_2-terminal and COOH-terminal cytosolic tails in TRPV4, respectively. Phosphorylation of the NH_2-terminal tyrosine by SFKs is effected in advance of the activation of TRPV4, providing evidence that tyrosine phosphorylation sensitises TRPV4 instead of activating it. Reactive oxygen species, involved in mediating inflammatory pain, strongly increase the phosphorylation of TRPV4 in the presence of SFKs which is consistent with the involvement of TRPV4 in mediating inflammatory pain (Wegierski et al. 2009). PKC can sensitise TRPV4 in central sensory and non-sensory nerve terminals, as well as in DRG neuronal cell bodies (Cao et al. 2009). In rats with chronic compression of the DRG, blockade of protein kinase G decreases thermal hyperalgesia (shown by reduction in pain-related behaviour on exposure to heat), implying that a TRPV4-NO-cGMP-PKG pathway may contribute to thermal hyperalgesia resulting from this type of neuropathic injury (Ding et al. 2010).

Proteases, which activate protease-activated receptor 2 (PAR_2), cathepsin S, and neutrophil elastase, have been identified as extracellular activators of TRPV4 which result in inflammatory-type pain. Proteolytic activation of other ion channels implicated in pain signalling is well-recognised. Thus, TRPV1 expressed in primary nociceptive afferents is sensitised by PAR_1 and PAR_2 (Vellani et al. 2010), while

PAR_2 sensitises TRPA1 expressed in DRG neurons in the context of inflammatory-type pain (Dai et al. 2007). PAR_2 is co-expressed with substance P, calcitonin, and CGRP by a population of primary afferent nociceptive neurons and mediates neurogenic inflammation and pain. Grant et al. (2007) hypothesised that PAR_2 activates a second messenger to sensitise TRPV4-dependent release of nociceptive peptides and induce mechanical hyperalgesia and that this underlies inflammatory hyperalgesia in diseases where proteases are activated and released. Activation of PAR_2 enhances the Ca^{2+} currents mediated by TRPV4 on its activation (Grant et al. 2007). This G protein-coupled receptor produces AA-derived lipid mediators, such as 5′,6′-EET, that gate TRPV4. Tyrosine phosphorylation is important because Src inhibitor 1 suppresses the gating of TRPV4 by protease activated receptor 2 (PAR_2). PAR_2 fails to activate TRPV4 with the Y110F channel mutation. Antagonism of TRPV4 inhibits PAR_2 signalling to primary afferents, while deletion of TRPV4 reduces that neurogenic inflammation stimulated by PAR_2. Activation of PAR_2 causes a signal that results in sustained activation of TRPV4, employing an essential tyrosine residue (TRPV4-Tyr-110) (Poole et al. 2013).

Cathepsin S (Cat-S) is a lysosomal cysteine protease of antigen-presenting cells. It is released in inflammatory conditions and remains active at extracellular pH. When serine proteases, such as trypsin and mast cell tryptase, cleave PAR_2 the result is a tethered ligand which is capable of exciting nociceptive neurons leading to neurogenic inflammation and pain. This tethered ligand is removed when Cat-S cleaves PAR_2 at E (56)↓T (57), thereby preventing activation by trypsin. In mouse DRG neurons, PAR_2 coupling to Gαs and formation of cAMP is stimulated by Cat-S and a decapeptide mimicking the Cat-S-revealed tethered ligand. Cat-S causes PAR_2-dependent activation of TRPV4 and contributes to neuronal hyper-excitability by adenylyl cyclase and PKA-dependent mechanisms. In mice, intraplantar injection of Cat-S results in inflammation and hyperalgesia which is reduced by deletion of PAR_2 or TRPV4 and inhibition of adenylyl cyclase. Antagonists of Cat-S and PAR_2 suppress formalin-induced inflammation and pain and this implies that endogenous Cat-S and PAR_2 are involved in mediating inflammatory-type pain (Zhao et al. 2014).

Neutrophil elastase is a biased agonist of PAR_2. It cleaves PAR_2 to result in activation of TRPV4, sensitisation of nociceptive neurons, and the development of inflammation and pain. Intraplantar injection of neutrophil elastase in mice results in the development of oedema and mechanical hyperalgesia via PAR_2-mediated and TRPV4-mediated mechanisms (Zhao et al. 2015).

2.4 Post-Ca^{2+} Influx Events in TRPV4 Pain Pathways

TRPV4 is an essential component in various pain pathways that can be initiated by a variety of activators of this ion channel, ranging from mechanical stimuli to *inflammagens*, which result in the experience of pain sensations. What remains to be elucidated, however, are the, as yet unidentified, molecules which are required to

function in these pathways after activation of TRPV4 in order to lead to the development of a pain signal in primary nociceptive afferents. Activation of TRPV4 channels may depolarise the neurone, promoting action potential generation (see under 2.2 above). Alternatively, the activation of TRPV4 channels, either in the relevant primary sensory neurons or adjacent non-neuronal cells, may result in the release of an extracellular chemical signal which, in turn, activates receptors on nociceptors to initiate pain signalling. It is not at all obvious how a localised alteration in neuronal membrane potential in the vicinity of the gated ion channels due to Ca^{2+} influx, may itself (alone or in conjunction with other inputs) be capable of initiating neuronal pain signalling. Rather it seems more likely that intracellular, or extracellular, molecules released as a result of Ca^{2+} influx consequent on activation of TRPV4 channels provide the necessary stimulation to generate the pain signal in primary afferent neurons. The elucidation of the components of this post-Ca^{2+} influx pathway ending in nociceptor excitation and signalling is of transcending importance to enable the identification of an appropriate analgesic response to TRPV4-mediated pain. The almost ubiquitous expression of TRPV4 and its importance in mediating a range of physiological processes means that the ion channel itself is a difficult therapeutic target.

Recent studies demonstrate that there are several candidates that fit the profile for downstream participation in post-Ca^{2+} influx TRPV4-mediated pain pathways. MicroRNAs are small endogenous non-coding RNA molecules that function as regulatory molecules that target mRNAs for cleavage or translational repression (Bartel 2004). Recently, in an important study, Liedtke's group identified lysophatidylcholine as a direct activator of TRPV4 in keratinocytes and showed that such activation causes the rapid extracellular release of miR-146a from keratinocytes. The miR-146a thus released activates TRPV1-expressing pruriceptor primary sensory afferents innervating the skin and thereby induces cholestatic itch (Chen et al. 2021). Some extracellular microRNA molecules may also be involved in mediating pain. Thus, TRPA1 can be activated by certain extracellular microRNAs – either independently or through TLR7 – to mediate pain (Park et al. 2014; Han et al. 2018). Moreover, at high doses, histamine, 5-HT, imiquimod, PAR_2 agonists, and AITC can cause pain rather than itch (Han et al. 2018). Activation of TRPV4 can also increase the availability of endothelin-1. Thus, UVB radiation (found in sunlight) leads to the activation of keratinocyte TRPV4 with ensuing Ca^{2+} influx, upregulation of endothelin 1 mRNA expression, secretion and binding to endothelin receptors, to result in the pain of sunburn (Moore et al. 2013). Hence, there appears to be a wide array of molecules which, if released, post-activation of TRPV4 and the resulting increase in $[Ca^{2+}]_i$, are capable of inducing the subsequent stimulation of primary nociceptive afferents to initiate pain signalling.

Adenosine triphosphate (ATP) is an energy-transporting molecule that is present in the cells of all living things. ATP is a molecule which possesses features that suggest that it may well be important in generating a pain signal in primary nociceptive afferents after the activation of TRPV4. ATP is known to be important in pain signalling and is released in millimolar amounts by cells damaged as a result of tissue injury or which are subjected to mechanical stimulation. Extracellular ATP

acts on cell-surface receptors of two types: ligand-gated ion channels (P2X receptors (P2X(R)) and G-protein-coupled receptors (P2Y receptors (P2Y(R)). Both types of receptors are found expressed on primary sensory afferents. Extracellular ATP stimulates adjacent sensory nerve endings by means of P2X and P2Y receptors (Inoue et al. 2005). P2X ion channels are preferentially permeable to sodium, potassium, and calcium. These channels are gated within milliseconds of the binding of ATP. Mihara and colleagues (2011) found that agonist-induced activation of TRPV4 increases the release of ATP from oesophageal keratinocytes in comparison with keratinocytes lacking TRPV4 suggesting that the activation of TRPV4 by heat, chemical, or mechanical agonist stimuli is involved in the release of ATP in the oesophagus and may be responsible for oesophageal pain mediated by heat or mechanical pressure (Mihara et al. 2011). The ATP transporter, vesicular nucleotide transporter (VNUT), stores ATP in secretory vesicles. Activation of TRPV4 causes the release of ATP in gastric epithelial cells (Mihara et al. 2016). TRPV4 agonists cause the release of ATP in human gastric epithelial GES-1 cells and in colonic CCD 841 cells. This ATP release can be blocked by inhibition of VNUT by clodronate (Mihara et al. 2018). Hence, the activation of TRPV4 may cause the release of ATP which may act on cell-surface receptors (P2X and/or P2Y receptors) expressed on primary sensory afferents to initiate pain signalling. A characteristic feature of rheumatoid arthritis is the hypotonicity exhibited by the synovial fluid of its sufferers. Exposure of synoviocytes to an hypo-osmotic solution activates TRPV4 channels expressed by these cells, resulting in an increase in their $[Ca^{2+}]_i$ which, in turn, causes the rapid release of ATP (Hu et al. 2017).

The $P2X_7$ receptor is a membrane cation channel gated by extracellular ATP. With prolonged activation, it forms a complex with membrane proteins that can create a broad pore that results in cell death and further release of ATP into the extracellular environment. The P_2X_7 receptor is expressed in multiple cell types, including stem, blood, glial, neural, ocular, bone, dental, exocrine, endothelial, muscle, renal, and skin cells (Sluyter 2017). ATP activation of the $P2X_7$ receptor results in the maturation and secretion of pro-inflammatory cytokines, including the interleukins (IL) IL-1β and IL-18, together with the formation of reactive nitrogen and oxygen species. This receptor also functions in the activation of caspases and the induction of apoptosis (Savio et al. 2018). Recently, Fan et al. (2021) identified a TRPV4-$P2X_7$ pathway involved in mediating pain resulting from TRPV4 activation in the chronic compression of the dorsal root ganglion (CCD) model of neuropathic pain in rat. The synthetic TRPV4 agonist GSK-1016790A, when injected intraperitoneally in rats suffering from CCD-induced neuropathic pain, causes a significant increase of mechanical and thermal hyperalgesia in these animals. It also increases the expression of the $P2X_7$ receptor. Blockade of the $P2X_7$ receptor with its antagonist Brilliant Blue G (BBG) reduces this level of mechanical and thermal hyperalgesia. Likewise, the administration of TRPV4 antagonists reduces the level of expression of the $P2X_7$ receptor. The levels of IL-1β and IL-6 are also increased by the administration of the TRPV4 agonist and reduced by the administration of its antagonists. The conclusion reached was that, in the CCD model of neuropathic pain in rat, the $P2X_7$ receptor contributes to the development of that pain which is

mediated by TRPV4 (Fan et al. 2021). Lapajne and colleagues (2020) found that TRPV4 in mouse corneal epithelial cells is activated by osmotic and thermal, but not strain, stimuli. The resulting Ca^{2+} influx following gating of TRPV4 mediates hemichannel-dependent ATP release which is thought to be responsible for corneal pain resulting from external stressors of the cornea involving the application of mechanical, osmotic, or chemical stimuli to the cornea (Lapajne et al. 2020).

2.5 Overview

Before considering the evidence for the involvement of TRPV4, expressed by peripheral sensory neurons, in pain signalling resulting from pressure/mechanical stress at the neuronal cell membrane expressing TRPV4, or resulting from the inflammatory response, or from peripheral neuropathy, respectively, it may be helpful to trace an outline of the rather complicated picture of this involvement. The accompanying chart offers an overview of how TRPV4 is implicated in mediating pain sensations in these several contexts (Fig. 1).

3 Physical Pressure/Stress-Induced Mechanical Hyperalgesia

The first category of TRPV4-mediated pain sensations comprises mechanical hyperalgesia mediated by the activation of TRPV4 expressed on primary nociceptive afferent neurons, as a result of physical pressure or stress applied *at* the neuronal membrane. This physical pressure or stress may be discrete, taking the form of extracellular hypotonicity. Alternatively, it may comprise pressure applied to the body (as in the case of trauma), or internally (as in the case of *oedema* or a tumour), which, again, in both instances translates to physical pressure or stress applied at the neuronal membrane. The term "*at* the neuronal membrane" is employed to emphasise that deformation of the lipid bilayer is not necessarily required for the activation of TRPV4. "Shear stress" describes the frictional force created by the flow of blood in the endothelium of blood vessels. Functioning endothelial TRPV4 is essential for shear stress-induced vasodilation (Hartmannsgruber et al. 2007). In vascular endothelial cells, TRPV4 can be activated by shear stress without effecting deformation of the lipid bilayer (Matthews et al. 2010; Potla et al. 2020). It has been shown that the activation of TRPV4 is elicited in both primary chondrocytes and in HEK-293T cells expressing TRPV4 by nano-scale deflections of areas of the interface between the cell and its substrate (Servin-Vences et al. 2017; Sianati et al. 2021). But TRPV4 has been shown *not* to be activated by membrane stretch (Servin-Vences et al. 2017; Nikolaev et al. 2019) or by indentation of the membrane (Servin-Vences et al. 2017).

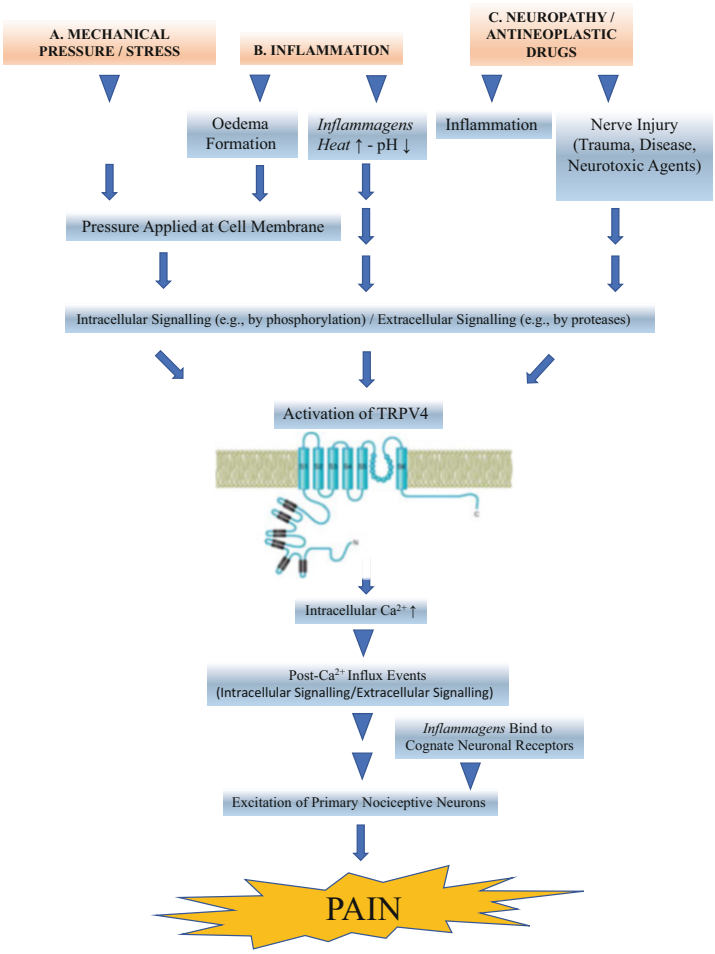

Fig. 1 TRPV4-mediated pain pathways in outline. The pathways indicated are generic and for the purposes of exposition only. Since there are multiple activators of TRPV4 which lead to the sensation of pain, it can also be said that there are as many pain pathways of which TRPV4 is an essential component. (**a**) Physical pressure/stress applied at the neuronal membrane results in the activation of TRPV4 ion channels on primary nociceptive neurones to mediate mechanical hyperalgesia. This physical pressure or stress may be discrete, taking the form of *extracellular hypotonicity*. Alternatively, it may comprise pressure applied to the body (as in the case of trauma), or internally (as in the case of *oedema* or a tumour), which, again, in both instances translates to physical pressure or stress applied at the neuronal membrane. (**b**) Oedema formation means that cellular debris-laden fluid within the interstitial space results in the application of mechanical force, or stress, against the cell membranes of local tissue while inflammatory agents are infiltrating this tissue. This can lead to the activation of TRPV4 expressed in primary afferent neurons resulting in mechanical hyperalgesia. In addition, TRPV4 can be gated by certain of the products of the local inflammatory response to injury. Such activation may also be caused, for example, by an increase in local tissue temperature and sometimes by a reduction in pH, both of which are recognised features of local inflammation. Formalin can directly activate TRPV4. Inflammatory-type pain mediated by TRPV4 includes formalin- and CFA-induced pain, pain from osteoarthritis and gout, and various types of visceral pain. (**c**) TRPV4 contributes to mechanical hyperalgesia in several specific types of painful peripheral neuropathies, including neuropathy associated with taxol, paclitaxel, vincristine, or thalidomide chemotherapy, alcoholism, diabetes, and human immunodeficiency virus/acquired immune deficiency syndrome therapy. TRPV4 is involved in the development and maintenance of mechanical allodynia in the chronic compression of the DRG (CCD) model of neuropathic pain in rat. Thermal hyperalgesia can also be caused by CCD in rat, with TRPV4 again making a contribution

The physical pressure, or stress, stimulus required to activate TRPV4-expressing primary nociceptive afferents can be provided by a diverse range of stimuli. The term *"mechanical hyperalgesia"* describes pain which has its context of origin in a mechanical stimulus. Usually, the term *"hyperalgesia"* is employed to describe pain which is disproportionate to the level of force (or other inciting stimulus) applied (Jensen and Finnerup 2014). In the present context, however, the literature sometimes applies that term to embrace even pain which is proportionate to the inciting mechanical stimulus, provided that that stimulus exceeds the minimum threshold necessary to activate TRPV4 (Suzuki et al. 2003a). *"Mechanical allodynia"* retains its standard meaning of pain which develops as a result of innocuous mechanical pressure (e.g. light touch or even a breeze) and is frequently, but not exclusively, a feature of neuropathic-type pain (Jensen and Finnerup 2014). One of the most prevalent forms of allodynia is found in the scalp allodynia of migraineurs, although migraine is not regarded as a neuropathic-type pain condition.

3.1 Discrete Pressure/Stretch at the Cell Membrane (Extracellular Hypotonicity)

The concept of TRPV4 being capable of activation by physical pressure or stress exerted at the cell membrane was prevalent as early as the ion channel's initial identification. TRPV4 was initially denominated as *vanilloid receptor-related osmotically activated channel (VR-OAC)* to reflect its capacity to respond to variations in extracellular osmolarity, i.e. the concentration of a solution expressed as the total number of solute particles per litre (Koeppen and Stanton, 2013). In isotonic media, i.e., those whose osmotic effect at the cell membrane is the same as that of normal intracellular solution, TRPV4 demonstrates spontaneous activity. Treatment with a hypotonic solution (i.e., an extracellular solution which has a lower concentration of cell impermeant solutes relative to the cytosol) results in differing pressures at each side of the membrane causing cell swelling. This results in increases in $[Ca^{2+}]_i$ as a result of activation of TRPV4 (Strotmann et al. 2000; Liedtke et al. 2000).

Levine and colleagues (Alessandri-Haber et al. 2003) showed that TRPV4 expressed by primary sensory afferents can be activated by exposure to an hypotonic solution to produce a nociceptive response. Such activation results in an increase in $[Ca^{2+}]_i$ which is dependent on the presence of extracellular Ca^{2+} but may also be amplified by Ca^{2+} from intracellular Ca^{2+} stores. Cultured DRG neurons, placed in isotonic solution, fail to generate the firing of action potentials. Perfusion with an hypotonic solution likewise fails to result in action potential firing, but a depolarisation of some neuronal membranes occurs. However, when these neurons are conditioned before such perfusion with a long depolarising pulse, or with a combination of a hyperpolarising current pulse, followed by a depolarising current pulse, this results in the same perfusion causing the firing of multiple action

potentials in 32% of neurons. TRPV4 channel blockers reduce the increase in $[Ca^{2+}]_i$ in nociceptive neurons in response to an hypotonic stimulus. Importantly, in the presence of PGE_2, the number of action potentials in C-fibres evoked by exposure to an hypotonic solution is significantly increased due, it was hypothesised, to the sensitisation of the nociceptive neurons by PGE_2. While no nociceptive behaviour was found when the authors injected rat hind-paw with a hypotonic solution (10 μl deionised water), such behaviour was evident when PGE_2 was administered prior to the same injection (Alessandri-Haber et al. 2003). Thus, a weak hypotonic stimulus can result in the activation of TRPV4 in the presence of an additional stimulus to the ion channel, and hypotonicity becomes a more efficient activator of TRPV4 when the latter has been sensitised by PGE_2.

Surprisingly, Levine's group (Alessandri-Haber et al. 2005) subsequently reported that, in an in vivo experimental model, *a mild hypertonic solution* is also capable of activating TRPV4. Intradermal injection of 2% (hypotonic) or 10% (hypertonic) saline solution into a rat's hind-paw induces a concentration-dependent pain-related behaviour, flinching. Pre-administration of PGE_2 has a sensitising effect and increases the flinching behaviour in response to 2% saline seven-fold, without any demonstrable effect on the response to 10% solution (Alessandri-Haber et al. 2005). The explanation for these findings is unclear, although given that physiological saline equates to a 9% solution, the magnitude as well as the nature of these two stimuli differs considerably.

3.2 *Pressure Applied to, or Within, the Body*

Suzuki and co-workers (2003a) hypothesised that TRPV4 might be a mechanosensitive Ca^{2+} ion channel involved in mediating the sensation of pressure in vertebrates, given its known characteristics and localisation in DRG ganglia. Mice lacking TRPV4 were found to exhibit a reduced response to the physical pressure caused by placing a weight on the animal's tail, although the response to mere touch sensation remained unaffected. The conduction velocity of myelinated nerves responding to pressure stimuli was also impaired (Suzuki et al. 2003a). This reduced response of *trpv4*$^{-/-}$ mice to noxious mechanical stimuli was soon confirmed (Liedtke et al. 2000). Such findings suggest that pain (mechanical hyperalgesia) resulting from external trauma or visceral pain, e.g., resulting from colonic distension, may be the result of mechanical stress at the neuronal membrane, activating TRPV4. TPRV4 is localised in conjunction with CGRP in colonic nerve fibres. Mice which lack TRPV4 exhibit reduced behavioural responses to noxious colonic distension (Brierley et al. 2008). Mice which lack TRPV4 and TRPA1 exhibit a marked reduction in distension-induced colonic CGRP release (Mueller-Tribbensee et al. 2015). The pain experienced by those suffering from xerostomia (dry tongue) most likely comprises a mechanical component resulting from the sufferer's need to mobilise pathologically dry oral structures. In a rat model of xerostomia, local

injection of a TRPV4 antagonist reduces the mechanical hypersensitivity of the tongue in these animals (Chen et al. 2020).

However, it is the case that noxious mechanical force, when applied to the body, results in the rapid sensation of pain. Studies in relation to the effect of shear stress on vascular endothelial cells have confirmed the capacity of TRPV4 to respond almost instantaneously to pressure at the cell membrane. These studies point to the role of focal adhesions in mechano-conversion, i.e. the translation of extracellular mechanical forces into intracellular biochemical signalling. Integrins are transmembrane receptors that function in cell-to-cell adhesion and cell-to-extracellular matrix adhesion. Focal adhesions are cell membrane-associated macromolecular assemblies that bind integrins to the actin cytoskeleton of the cell to ensure the adhesion of the cell to the extracellular matrix and to adjacent cells. These focal adhesions also engage in signalling that controls a wide range of cell processes. Matthews et al. (2010) showed that the application of force to β1 integrins causes an influx of Ca^{2+} via TRPV4 channels in intact capillary endothelial cells within 4 ms. Activation of TRPV4 is the consequence of mechanical strain in the cytoskeletal backbone of the focal adhesion, rather than as a result of deformation of the lipid bilayer or sub-membranous cortical cytoskeleton alone. These ultra-rapid responses are dependent upon the distal region of the β1 integrin cytoplasmic tail where a binding site for the integrin-associated transmembrane CD98 protein is found. These authors concluded that: "focal adhesion strengthening facilitated by binding of CD98 to the distal integrin tail in the cytoplasm appears to be critical to maintain the mechanical connectivity within the focal adhesion necessary for TRPV4 channels to *sense* stresses applied to the extracellular domain of adjacent cell surface integrins" (Matthews et al. 2010). In endothelial cells, there is a tight association and co-localisation in focal adhesions of β1 integrin, CD98hc ("transmembrane solute carrier family 3 member 2") and TRPV4. In the absence of CD98hc, Ca^{2+} influx mediated by TRPV4 in response to mechanical forces is inhibited, but without affecting the ion channel's response to chemical agonists, demonstrating the existence of a mechano-specific signalling pathway. In order to effect rapid, mechanical pressure-induced activation of TRPV4 within the focal adhesion, the forces applied to the β1 integrin must be transferred from its cytoplasmic C terminus by means of the CD98hc cytoplasmic tail to the ankyrin repeat domain of TRPV4 (Potla et al. 2020).

The capacity of TRPV4 to be activated in response to shear stress in vascular endothelial cells has also been confirmed by Swain and Liddle (2020) but with the proviso that Piezo1 governs the activation of TRPV4. Piezo1 and Piezo2 are ion channels which function in the conversion of mechanical pressure or force into physiological signalling (Coste et al. 2010). Piezo1 channels can respond to mechanical pressure by activation without having to rely on other cellular elements to detect the presence of that pressure. Indeed, mechanical disturbances of the lipid bilayer alone are capable of activating Piezo1 channels, demonstrating their inherent capacity as mechanosensors (Syeda et al. 2016). Swain and Liddle found that $[Ca^{2+}]_i$ is increased in the presence of fluid shear stress or in the presence of Yoda1 (a Piezo1 agonist). This effect is abolished by GsMTx4 (a Piezo1 antagonist), demonstrating

that, *in that specific context,* Piezo1 is essential for the occurrence of this increase in $[Ca^{2+}]_i$ which is proximally mediated by the activation of TRPV4. Piezo1 does not function in this connection to directly cause the activation of TRPV4 but rather to activate PLA_2 which, in turn, results in the gating of TRPV4. The authors concluded that neither Piezo1 nor TRPV4 alone is sufficient to transduce mechanical force into pathological conditions and propose that "functional coupling between Piezo1 and TRPV4 is a generalised process in which TRPV4 translates the mechanical force sensed by Piezo1 into pathological events" (Swain and Liddle, 2020).

At this juncture, the mechanism by which TRPV4 is activated in response to mechanical pressure or stress remains to be fully elucidated. However, it is clear that TRPV4 can be activated by such mechanical forces and that this ion channel can respond rapidly to the application of such force. Remarkably, functioning TRPV4 is also required to mediate mechanical hyperalgesia resulting from inflammation (*infra*) as well as from painful peripheral neuropathy. Levine's group (2008) showed that knock-down of TRPV4 results in a reduction in the level of mechanical hyperalgesia exhibited in animal models of painful peripheral neuropathy, regardless of whether the neuropathy was related to vincristine chemotherapy, alcoholism, diabetes, or HIV/AIDS syndrome therapy. Likewise, TRPV4 knock-out mice exhibit a major reduction in the level of mechanical hyperalgesia caused by the administration of paclitaxel, vincristine, or the development of diabetic neuropathy (Allesandri-Haber et al. 2008). TRPV4, therefore, appears to be a common molecular mechanism for mediating mechanical hyperalgesia whether resulting from inflammation, peripheral neuropathy, or physical pressure or stress applied at cells.

As regards the suggestion that TRPV4 functions as a sensory transducer in respect of mechanical pressure or stress applied at the cell membrane, it has yet to be established that the activation of TRPV4 translates the particular mechanical pressure or stress applied in the graded fashion characteristic of sensory transducers. Again, the applicability of the descriptive term "osmosensor" may also be misleading. This is because there is now evidence that TRPV4 functions as a volume-sensor as opposed to acting as an "osmosensor" (Toft-Bertelsen et al. 2017, 2019).

4 Inflammation-Induced Hyperalgesia

Inflammatory pain describes the pain that is generated by the inflammatory response resulting from wounds, surgical incisions, infections, and other forms of tissue injury or disease. Inflammation generates a multiplicity of chemical agents designed to combat infection and facilitate the repair of injured tissue. Unfortunately, this inflammatory response is poorly controlled and, typically, seriously disproportionate to the inciting stimulus, causing the development of pain that can occasionally be of such severity that it impairs recovery or, in the longer term, results in disability. Inflammation causes the release of various agents that alter both the firing pattern of nociceptive primary sensory neurons and nociceptive processing in spinal dorsal horn nociceptive neurons. These include bradykinin, eicosanoids, nerve growth

factor (NGF), artemin, glial cell-line-derived neurotrophic factor (GDNF), serotonin, histamine, anandamide, adenosine 5′ triphosphate, and cations, especially protons. These *inflammagens* constitute the so-called inflammatory soup which has been a focus of research for many years. Neuronal excitation (leading to nociception) in the context of inflammation may be reduced by elimination or pharmacological blockade of some of these inflammatory mediators (White et al. 2010).

4.1 Role of Inflammatory Mediators

Inflammagens play an important role in the development and maintenance of mechanical hyperalgesia by two quite disparate processes. First, the presence of inflammation can alter the volume and tonicity of the interstitial fluid to produce local swelling by the local accumulation of fluid comprising the products of the inflammatory response and the break down of local tissue. This phenomenon of oedema formation means that cellular debris-laden fluid within the interstitial space results in the application of mechanical force, or stress, against the cell membranes of local tissue while inflammatory agents are infiltrating this tissue (White et al. 2010). This can lead to the activation of TRPV4 expressed in primary afferent neurons resulting in mechanical hyperalgesia, as previously described.

Second, TRPV4 can be gated by certain of the products of the local inflammatory response to injury. Such activation may also be caused, for example, by an increase in local tissue temperature (Güler et al. 2002) and sometimes by a reduction in pH (Suzuki et al. 2003a), both of which are recognised features of local inflammation. Levine and co-workers (Alessandri-Haber et al. 2006) found that inflammatory mediators act directly on dissociated primary afferents in vitro. Their findings suggest that the activation of TRPV4 in the context of inflammation is more likely to be the result of multiple inflammatory agents acting in concert, that is to say, the various components of the "inflammatory soup" previously described, rather than being the result of the stimulation affected by any single specific *inflammagen* (Alessandri-Haber et al. 2006). These inflammatory mediators have various distinct avenues whereby they may effect signalling by primary sensory afferents. First, and most obviously, by their action on cognate receptors expressed on those neurons. Second – and relevant in the present context – by activating TRPV4 expressed on the same neurons or on adjacent non-neuronal cells. So the question inevitably arises as to just how important TRPV4 ion channels are in mediating the altered signalling by primary afferents which is ultimately interpreted by the brain as pain. In fact, TRPV4 has been shown to be of decisive importance in this connection. Thus, when PGE_2 and 5-HT are injected in combination into the mechanical receptive fields of C-fibres of mice which lack TRPV4 this results in significantly fewer C-fibres responding to a hypotonic stimulus than in the wild-type animals. In addition, an increase in spontaneous activity and a reduction in the mechanical threshold after such a stimulus are only found in wild-type animals (Chen et al. 2007). Hence, *inflammagens* generated may significantly contribute to a modulation of neuronal signalling.

4.2 Neurogenic Inflammation

Neurogenic inflammation is the result of the activation of primary nociceptive afferent neurons in the presence of *inflammagens* generated by the immune response. It entails an alteration in the function of the primary nociceptive afferents in the presence of inflammatory mediators, which causes these neuronal cells to produce an enhanced release of neuropeptides, including substance P and calcitonin gene-related peptide (CGRP), from their sensory nerve endings. This enhanced release of neuropeptides serves to further enhance neuronal excitation. Hence, TRPV4 may be regarded as an active component within a positive feedback mechanism which supports inflammatory conditions. This phenomenon offers a further illustration of the dynamic nature of primary nociceptive afferents which not only respond to noxious stimulation by generating pain signalling, but also by generating factors which can result in further stimulation of those neurons. Vergnolle et al. (2010) exposed TRPV4 expressed by DRG in mouse to an hypotonic solution or 4*a*-PDD and found that both cause neuropeptide release from afferent nerves and induce neurogenic inflammation in the animal. Intraplantar injection of the same agents generates oedema formation, in addition to the aggregation of granulocytes, but this is reduced by deletion, or knock-down, of the TRPV4 gene in mouse (Vergnolle et al. 2010).

4.3 Inflammation-Induced Responses Mediated by TRPV4

The characteristic feature of TRPV4 as a molecular integrator of diverse stimuli means that when stimulated by several of its multiplicity of potential agonists, the interaction with TRPV4 may be synergistic. Thus, the combination of an inflammatory mediator with an hypotonic stimulus can activate TRPV4 to induce behaviour attributable to pain in animals (Chen et al. 2007). Ablation of TRPV4 in rat eliminates the increase in the pain-induced paw-withdrawal reflex that is found on exposure to mechanical or hypotonic stimulation after intradermal injection of carrageenan or a "soup" of *inflammagens* (Alessandri-Haber et al. 2006). TRPV4 channels are found expressed on the nerve fibres of human dental pulp. An increased level of TRPV4 expression is found on nerve fibres in the pulp in inflammatory conditions and may explain the increased response of dental pulp to innocuous mechanical, thermal, and osmotic stimuli under inflammatory conditions (Bakri et al. 2018). TRPV4 is present in hamster oral mucosal Merkel cells (Soya et al. 2014), and in odontoblasts (Sato et al. 2013). TRPV4 is highly expressed by trigeminal ganglion neurons and, when activated, contributes, with TRPA1, to the trigeminal nociceptive response to formalin. Indeed, formalin can directly activate TRPV4 (Chen et al. 2014). Inflammation of the temporomandibular joint in mouse results in the activation of TRPV4 and upregulation of this channel (Chen et al. 2013). Trauma to the mucosa of the mouth is a frequent accompaniment of dental

treatment and may result in traumatic mucosal ulcers. In a rat model of this condition, the resulting symptomatology is attributed to prostanoids and PAR_2 activation which activate TRPV1 and TRPA1 to mediate the spontaneous nociceptive behaviour observed on the day of injury and also activate TRPV4 to mediate the mechanical allodynia which persists for some 3 days (Ito et al. 2017). TRPA1 is a Ca^{2+}-permeable non-selective cation channel extensively expressed in sensory neurons and in non-neuronal cells which mainly functions in the detection of a diverse array of exogenous stimuli that may result in cell damage. It also features in relation to inflammation and both acute and chronic pain (Talavera et al. 2020).

By definition, the inflammatory response to tissue injury, whether as a result of trauma or disease, is of the "blunderbuss" type, with multiple pro-inflammatory agents being brought into existence to meet the challenge presented. In the aftermath of injury to a peripheral nerve, macrophages and Schwann cells gather around the injured site and release cytokines and the growth factors necessary to facilitate nerve regeneration. Inflammation at the DRG results in an increase in pro-inflammatory cytokines and a reduction in anti-inflammatory cytokines (Zhang and An 2007). Long-term exposure of cultured rat DRG neurons to IL-17A fails to increase the expression of TRPV1, but potently upregulates TRPV4. Mice which lack IL-17A demonstrate less mechanical hyperalgesia than wild-type animals when zymosan is injected into the paw, confirming a role for IL-17A in mediating mechanical hyperalgesia (Segond von Banchet et al. 2013). However, in the specific context of the porcine choroid plexus-Riems cell line, the pro-inflammatory cytokines TNF-α, IL-1β, and TGF-β1 have inhibitory effects on TRPV4-stimulated transepithelial ion flux and permeability changes. The anti-inflammatory cytokines IL-10, IL-4, and IL-6 have no effect. NF-κB is a transcription factor which regulates the manufacture and control of various inflammatory cytokines. Its inhibition antagonises TRPV4-mediated activity (Simpson et al. 2019). Notwithstanding that it has been demonstrated that arachidonic acid (AA) itself is an activator of TRPV4 (Nilius et al. 2004), it has recently been claimed, again in the specific context of the porcine choroid plexus-Riems cell line, to have inhibitory rather than stimulatory effects on TRPV4-mediated responses (Simpson et al. 2019), although arachidonic acid's status as a substrate for the formation of multiple eicosanoids, including PGE_2, as activators of TRPV4 has not been challenged. Valverde's group (Berna-Erro et al. 2017) have identified the presence of an "EET-binding pocket" on the TRPV4 ion channel. This site is identified as mediating the activation of TRPV4 by 5′,6′-EET, by AA, or by hypotonic cell swelling. 5′,6′-EET has been found to bind directly to TRPV4 and thereby gate the channel. This binding site partially overlaps with the predicted binding site of 4α-PDD. The mutant TRPV4-K535A cannot bind EET or AA and is not activated by hypotonicity. However, the activation of the mutant channel by either GSK10196790A or 4α-PDD or temperature is unaffected. This suggests the presence of two or more structural targets within TRPV4 which gate the ion channel (Berna-Erro et al. 2017).

TRPV4 functions in mediating pain from mechanical stimulation of both the normal and inflamed knee joint. Nociceptive Aδ- and C-fibres are minimally activated by harmless stimuli applied to the joint in non-pathological circumstances,

with their responses being limited to those elicited by noxious mechanical stimuli. Inflammation of the joint changes this response profile as nociceptive Aδ- and C-fibres become sensitised with the result that their activation thresholds are reduced and their responses to both non-noxious and noxious stimuli are increased. In the result, even the application of an innocuous mechanical stimulus to the inflamed knee joint can be perceived as being painful to a greater or lesser degree. The responses of C-fibres of the *uninjured knee joint* to noxious mechanical stimulation are reduced by an intra-articular injection of a TRPV4 antagonist into the joint, while the responses of C-fibres of an *inflamed knee joint* to innocuous, as well as noxious, mechanical stimulation are reduced by the same injection. This suggests that TRPV4 functions in mediating the responses of C-fibres to noxious mechanical stimuli in the healthy knee joint, and likewise in mediating the increased responses of C-fibres to harmless, as well as noxious, mechanical stimulation in the inflamed knee joint (Richter et al. 2019).

4.4 Formalin- and CFA-Induced Pain

The "formalin test" is a test of nociception, involving the assessment of typical nocifensive behaviours of rodents that result from the injection of a dilute solution of formaldehyde in saline, given subcutaneously or intradermally, typically into the dorsal or plantar hind-paw of rat or mouse, but sometimes into the whisker-pad. It induces moderate ongoing pain unlike many other tests of nociception which rely on a brief threshold stimulus to evoke a nocifensive response (Tjølsen et al. 1992). It has an early phase, attributed mainly to C-fibre activation by the peripheral stimulus; and a late phase, attributed to the inflammation of the injured tissue and functional changes in the dorsal horn of the spinal cord. Liedtke and colleagues (Chen et al. 2014) focused on the formalin test as one which generates *"trigeminal irritant pain"* because "formalin can also irritate nerve terminals of nociceptor neurons" and "irreversibly cross-links proteins". This "trigeminal irritant pain" describes the response when the formalin is injected into the animal's whisker-pad where it is capable of activating TRPV4 expressed on trigeminal neurons. These authors identified formalin as a direct activator of TRPV4, eliciting a Ca^{2+} influx in a TRPV4-dependent manner, and found that both TRPA1 and TRPV4 contribute to the formalin trigeminal pain response, concluding that TRPV4 is necessary to mediate "trigeminal irritant" pain (Chen et al. 2014). Of course, that does not deny the contribution to the activation of TRPV4 made by the inflammatory response which necessarily also results from the formalin injection. Liedke's group (2016) successfully developed a small-molecule inhibitor of TRPV4 by modifying GSK205, a tool compound known to be selective for TRPV4. This new molecule exhibits increased potency in inhibiting TRPV4, but also enabled the development of two further compounds which concurrently inhibit *both* TRPA1 and TRPV4. These new dual channel blockers have been demonstrated to reduce pain behaviour in a model of trigeminal irritant pain which is dependent on TRPV4 and TRPA1. In

addition, they inhibit inflammation and pain-associated behaviour in a model of acute pancreatitis which they also identified as being dependent on TRPV4 and TRPA1 (Kanju et al. 2016).

Injection of Complete Freund's Adjuvant (CFA) into the plantar surface of the hind-paw of rat results in the development of inflammatory hyperalgesia. This established animal model of inflammatory pain produces an injected area of tissue which exhibits the classical signs of acute inflammation including oedema, redness, and heat which are most intense from day 1 to day 3 after injection, and last for more than 4 weeks. Following injection with 50 μl CFA, mice develop CFA-inflamed hind-paws which 24 h later exhibit both mechanical hyperalgesia and thermal hyperalgesia. When treated with local injection of suberanilohydroxamic acid (SAHA), the mechanical hypersensitivity is significantly reduced but the thermal hypersensitivity is unaffected. In established mouse models of TRPV4-mediated mechanical hyperalgesia, pre-treatment with SAHA likewise attenuates such mechanical hyperalgesia. Moreover, SAHA potently inhibits current responses evoked by the administration of the TRPV4 selective agonist GSK1016790A. Hence, local application of SAHA may be effective in attenuating TRPV4-induced mechanical hyperalgesia (Choi et al. 2019).

4.5 Pain from Osteoarthritis and Gout

4.5.1 Osteoarthritic Pain

Osteoarthritis is a progressive degenerative disease of articular cartilage and surrounding tissues. Functionally, TRPV4 responds to the mechanical load imposed on articular cartilage by generating Ca^{2+} transients within chondrocytes. Mechanical signal transduction in articular chondrocytes is dependent on TRPV4 activation (O'Conor et al. 2016). Increases in the levels of expression of TRPA1 and TRPV4 are required for the development of mechanical hyperalgesia in rats with experimental knee osteoarthritis. These rats exhibit obvious mechanical hyperalgesia from 2 weeks after induction of disease and inhibitors of TRPA1 and TRPV4 can increase the low mechanical withdrawal thresholds (Xing et al. 2017). TRPV4 is implicated in the mechano-nociception mediated by C-fibres of the normal knee joint in response to noxious mechanical stimuli and also in the C-fibre mediated mechanical hypersensitivity of inflamed knee joints (Richter et al. 2019).

Using a model of osteoarthritic pain in the knee joints of rats generated by monoiodoacetate (MIA) injection, Hinata et al. (2018) found that the expression of TRPV4 in these knee joints does not increase, but the ion channel itself does become sensitised, with increased phosphorylation of DRG neurons and increased levels of the endogenous agonist, 5′,6′-EET being found in these knee joints. MIA rats demonstrate a reduction in grip strength in comparison with sham-treated animals, a consequence which can be reversed by intra-articular injection of a TRPV4 antagonist. On the other hand, the intra-articular injection of GSK1016790A, a

TRPV4 agonist, causes an increase in the pain-related behaviours of the treated rats but not of the sham-injected animals. 5′,6′-EET and its metabolites are also found in synovial fluids of osteoarthritic patients (Hinata et al. 2018). The same group have more recently confirmed that TRPV4 ion channels are sensitised in the DRG neurons of MIA rats. Genetic ablation of TRPV4 does not prevent the development of osteoarthritis in MIA rats, and under normal conditions these animals exhibit a similar pain threshold in response to temperature and pressure as wild-type animals. However, deletion of TRPV4 totally suppresses mechanical pain behaviours due to osteoarthritis, such as reduction of grip strength, heightened mechanical allodynia, and diminished weight-bearing on the affected side. Treatment with a TRPV4 antagonist mimics the results found in the TRPV4 knock-out animals. Hence, these authors conclude that TRPV4 has a critically important role in the development of the pain of osteoarthritis (Soga et al. 2021).

4.5.2 Gout Pain

Gout is caused by hyperuricaemia, which is a condition of excessive uric acid in the body. It is a very painful form of inflammatory arthritis. Excessive amounts of uric acid lead to the development of uric acid crystals (monosodium urate) in joints, fluids, and tissues within the body. TRPV4 is functionally expressed by mouse synovial macrophages and human peripheral blood mononuclear cells (PBMCs). Expression of TRPV4 is increased by stimulation with monosodium urate (MSU) crystals and also in PBMCs of patients suffering from acute gout flares. Genetic ablation or pharmacological antagonism of TRPV4 function significantly reduces MSU crystal-induced gouty arthritis. TRPV4 drives the production of the inflammatory cytokine IL-1β. At the same time, chemical ablation of TRPV1-expressing nociceptors significantly reduces MSU crystal-induced gouty arthritis. Resident macrophages expressing TRPV4 appear to be important in MSU crystal-induced gouty arthritis (Lan et al. 2021).

4.6 Visceral Pain

Visceral pain describes pain which has its origin in an internal organ, such the stomach, bowel, bladder, or pancreas. It ranges from the discomfort of indigestion to the intense pain of renal colic (Sikandar and Dickenson 2012). Here, we shall consider the possible role of TRPV4 in the pain of irritable bowel syndrome, inflammatory bowel disease, pancreatitis, and cystitis.

4.6.1 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) occurs where there is a disturbance of the normal peristalsis of the digestive tract. Symptoms include cramping, abdominal pain,

bloating, gas, mucus in the stool, diarrhoea, and/or constipation. IBS does not result in inflammation or even permanent damage to the bowel. Biopsies of patients suffering from irritable bowel syndrome demonstrate increased levels of 5',6'-EET, an agonist of TRPV4. Supernatants from such biopsies result in visceral hypersensitivity in mouse. Knock-down of TRPV4 reduces the visceral hypersensitivity caused by supernatants from such biopsies. Polyunsaturated fatty acid (PUFA) metabolites derived from these IBS biopsies activate mouse sensory neurons in vitro, by activating TRPV4. When mouse sensory neurons are exposed to supernatants from IBS biopsies, this results in the production of 5',6'-EET, suggesting a vicious cycle in which sensory neurons are stimulated to signal pain by the activation of TRPV4 by 5',6'-EET, which, in turn, results in the generation of additional 5',6'-EET, (Cenac et al. 2015). Histamine, found in the mucosal microenvironment in patients with IBS, may sensitise TRPV4 and TRPA1 in IBS through activation of the histamine 1 receptor (H_1R), thereby increasing visceral pain sensation (Balemans et al. 2019).

4.6.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) differs radically from IBS. Here, the immune system attacks areas of the digestive tract. The diseases embraced by the concept of IBD include Crohn's disease (CD) and ulcerative colitis (UC) which involve chronic inflammation of the GI tract. Crohn's disease can affect any area of the GI tract but is often focussed in the area just proximal to the junction of the small bowel with the large bowel. All layers of the intestinal lining can become inflamed. In ulcerative colitis (UC) the inflammation is found in the large colon and the rectum, but is confined to the innermost layer of the lining of the intestine. The most common symptoms of IBD are frequent and/or urgent bowel movements, diarrhoea, bloody stool, abdominal pain, and cramping. Matsumoto et al. (2018) investigated the role of TRPV4 expressed in colonic vascular endothelial cells in a mouse model of dextran sulphate sodium (DSS)-induced colitis. Colitis is significantly reduced in animals that lack TRPV4, while it is worsened by repeated intra-rectal administration of the TRPV4 agonist, GSK1016790A. TRPV4 expression is increased in the vascular endothelia of colonic mucosa and submucosa as a result of DSS treatment, which also increases vascular permeability but not in TRPV4 KO mice. This increase in vascular permeability is heightened by *i.v.* administration of a TRPV4 agonist, but abolished by the administration of a TRPV4 antagonist. These authors conclude that increased expression of TRPV4 expressed in vascular endothelial cells, resulting in increased vascular permeability, is a factor in the development of colonic inflammation in colitis (Matsumoto et al. 2018). 5′,6′-dihydroxy-7,9,11,14-eicosatetraenoic acid (5′,6′-DiHETE) is an endogenous antagonist of TRPV4 which prevents oedema formation and leukocyte infiltration in the inflamed colon in a similar mouse model of colitis, thereby promoting healing (Kobayashi et al. 2021).

4.6.3 Pancreatitis

Pancreatitis involves inflammation of the pancreas and resulting pain. Here, both TRPV4 and TRPA1 are found in pancreatic nerve fibres and in DRG neurons innervating the pancreas in mice. The effect of agonists of TRPV4 and of TRPA1 in increasing intracellular calcium concentration, pain, and inflammation is markedly diminished or abolished in mice lacking TRPV4 and TRPA1. Both TRPV4 and TRPA1 contribute to the pain of the cerulean-induced model of pancreatitis, while TRPA1 also mediates pancreatic inflammation itself. Thus, the deletion of either ion channel suppresses c-Fos expression (a marker of spinal nociceptive processing) and pain behaviour on the part of the animals, but only the deletion of TRPA1 reduces the inflammatory response (Ceppa et al. 2010). This is only one of several animal models of acute pancreatitis and not all models will share all the same features (Hyun and Lee 2014). A recent study focussed on the molecular basis for the development of "pressure-induced" pancreatitis, and implicated TRPV4 in the development, or pathogenesis, of pancreatitis itself, rather than focussing on its specific role in the resulting pain. In this model, the activation of TRPV4 in pancreatic acinar cells was predicated upon the activation of Piezo1 channels localised in the same cells. Activation of Piezo1 gates TRPV4 which results in a sustained increase in $[Ca^{2+}]_i$ that causes intracellular organelle dysfunction. Mice lacking TRPV4 are saved from Piezo1 agonist-induced pancreatitis, as well as from pressure-induced pancreatitis. Hence, pancreatitis can result from Piezo1-induced gating of TRPV4 channels (Swain et al. 2020).

4.6.4 Cystitis

In a mouse model of cystitis induced by cyclophosphamide injection, mice lacking TRPV4 show a reduction in pain-related behaviour in cases of both acute and chronic cystitis and with, or without, an ulcerative inflammatory bladder. In chronic cystitis animals, pain-related behaviour and mechanical hypersensitivity are absent in TRPV4 knock-out mice, suggesting a direct role for TRPV4 in mediating such pain. TRPV4 antagonists reduce persistent mechanical hypersensitivity in chronic cystitis mice with an efficacy greater than that seen in mice treated with amitriptyline, gabapentin, or ibuprofen. Mice with chronic cystitis exhibit a significant increase in the level of phosphorylated TRPV4 (Kawasaki et al. 2021).

5 Peripheral Neuropathic Pain

"Mechanical allodynia" describes the phenomenon whereby innocuous contact with the sufferer's skin results in pain. The harmless stimulus may be, for example, contact with clothing or even the stimulation provided by a gust of wind against the

skin. It is a common feature of neuropathic-type pain (Jensen and Finnerup 2014), including that generated by chronic compression of the DRG (CCD) in rat. TRPV4 is involved in the development and maintenance of mechanical allodynia in this rodent pain model. Expression of this ion channel is increased from 7 days to 28 days after initial injury, being at its highest level at 7 days after injury. This mechanical allodynia is partially reversed with the administration of TRPV4 antisense oligonucleotides (Zhang et al. 2008). Thermal hyperalgesia can also be caused by CCD in rat, with TRPV4 again making a contribution (Ding et al. 2010; Wang et al. 2011; Wei et al. 2013). There is evidence that a TRPV4-NO-cGMP-PKG pathway may be involved in CCD-induced thermal hyperalgesia (Ding et al. 2010); and that NF-κB may also possibly be involved in upregulating channel expression (Wang et al. 2011). Intrathecally administered colchicine (a depolymerising agent of microtubules) causes dose-related part reduction in allodynia, both mechanical and thermal, resulting from CCD, with this reduction in allodynic pain coinciding with a dose-related reduction in the level of TRPV4 expression in CCD rats. TRPV4 currents in DRG neurons and in TRPV4-transfected HEK293 cells are also reduced by colchicine (Wei et al. 2013). Intrathecally administered colchicine also diminishes mechanical and thermal hyperalgesia post-CCD injury in rat after agonist-induced activation of TRPV4 (Ning et al. 2014). p38 mitogen-activated protein kinase (MAPK) is involved with TRPV4 in mediating the mechanical allodynia induced by CCD surgery in rat. Intrathecal administration of 4α-PDD, an agonist of TRPV4, and anisomycin (an agonist of p38) increases the mechanical allodynia induced by CCD surgery – an effect which is abolished by the administration of an antagonist of TRPV4 and an antagonist of p38 (Qu et al. 2016). Recently, a TRPV4-P2X$_7$ pathway has also been identified as being involved in mediating pain resulting from TRPV4 activation in the chronic compression of the dorsal root ganglion (CCD) model of neuropathic pain in rat (Fan et al. 2021).

It should be remembered that pain emanating from neuropathy may well have an inflammatory context of origin. Some level of inflammation may well remain in the context of an injured nerve, and that inflammation may be responsible for TRPV4 activation, as it would in other contexts. This is especially so in the context of animal models which inflict ongoing disruption of nerves using ligatures, or otherwise, for the purpose of their model. It is, on the other hand, easier to appreciate how treatment with toxins, as in chemotherapy, may (independently of inflammation) induce effects which lead to pain signalling that is dependent on the activation of TRPV4.

TRPV4 contributes to mechanical hyperalgesia in several specific types of painful peripheral neuropathies, including neuropathy associated with taxol, paclitaxel, vincristine, or thalidomide chemotherapy, alcoholism, diabetes, and human immunodeficiency virus/acquired immune deficiency syndrome therapy (Alessandri-Haber et al. 2004, 2008; Chen et al. 2011; De Logu et al. 2020). It has been proposed that α2-β1-Integrin and Src tyrosine kinase are involved with TRPV4 in mediating such mechanical hyperalgesia and that a direct interaction occurs in primary sensory neurons between these several contributors to mechanical hyperalgesia (Allesandri-Haber et al. 2008). We will consider the role of TRPV4 in a number of clinically relevant disease models.

5.1 Chemotherapeutic-Induced Peripheral Neuropathy

5.1.1 Paclitaxel-Induced Neuropathy

Blockade of the protease-activated receptor 2 (PAR_2) by a selective antagonist inhibits neuropathic pain behaviour induced by paclitaxel in a dose-dependent and time-dependent fashion. Blockade of phospholipase C (PLC), protein kinase A (PKA), and protein kinase Cε (PKCε), which are all downstream signalling pathways of PAR_2, reduces paclitaxel-induced mechanical, heat, or cold hypersensitivity. Pain responses are also attenuated by the administration of antagonists of TRPV1, TRPV4, or TRPA1. These results show that paclitaxel-induced neuropathy is the result of several specific cell signalling pathways and involves the activation of PAR_2 and downstream enzymes PLC, PKCε, and PKA with resultant activation of TRPV1, TRPV4, and TRPA1 (Chen et al. 2011).

Mechanical and thermal hyperalgesia develop in mice as a result of paclitaxel (PTX) treatment. An important contribution to the pain induced by paclitaxel therapy is mediated by the action of the kinins (bradykinin and kallidin) and their metabolites, acting on their cognate G-protein coupled receptors, B_1 and B_2 receptors. Kallikrein enzymes act on kininogens to produce these kinins. The B_2 receptor has a higher affinity for bradykinin (BK) and kallidin peptides and is usually constitutively expressed throughout the body where it serves a "house-keeping" function. It mediates most of the effects of the kinins as well as the acute phase of inflammatory and nociceptive responses. The B_1 receptor has a high affinity for the kinin metabolites, des-Arg^9-BK (DABK) and Lys-des-Arg^9-BK. In normal circumstances, B_1 receptors are not expressed but, after injury to tissue or during inflammation, they become rapidly up-regulated, suggesting an important role for them in the context of inflammation (Beraldo and Andrade, 1997). Mice lacking one, or other, or both types of kinin receptors show a significant reduction in pain behaviour induced by paclitaxel when compared to wild-type animals. Treatment with kinin receptor antagonists significantly reduces mechanical and thermal hyperalgesia at 7 and 14 days following the initial paclitaxel injection (Costa et al. 2011). Calixto and colleagues (Costa et al. 2018) observed that antagonists of B_1 and B_2 receptors reduce PTX-induced mechanical hyperalgesia in like manner as does a selective TRPV4 antagonist. In addition, both kinin receptor antagonists inhibit the nociceptive responses caused by a hypotonic solution in PTX-injected animals. Their conclusion was that agonists of either B_1 or B_2 receptors sensitise TRPV4 to induce mechanical hyperalgesia in mice (Costa et al. 2018).

Transient receptor potential ankyrin, type 1 (TRPA1) functions in conjunction with TRPV4 to generate the mechanical allodynia caused by PTX-induced neuropathy in the mouse. Paclitaxel-induced mechanical allodynia is reduced by either a TRPA1 antagonist or a TRPV4 antagonist; and both antagonists, when administered in combination, completely abolish this mechanical allodynia. However, the cold allodynia induced by paclitaxel is completely dependent on TRPA1 (Materazzi et al. 2012). Paclitaxel administration results in neuronal degeneration of DRG coupled with increased expression of TRPV4. Goshajinkigan (a traditional Japanese

medicine, comprising 10 types of crude drugs) reduces PTX-induced hyperalgesia apparently by acting to prevent neuronal degeneration and to inhibit greater expression of TRPV4 (Matsumura et al. 2014). In PTX-mediated neurotoxicity, the inositol-trisphosphate receptor IP_3R_1 is frequently predominantly expressed at the periphery of small to medium size DRG neurons. There is also evidence of the existence of a physical interaction between IP_3R_1 and TRPV4 channels as well as some degree of co-localisation of IP_3R_1 and TRPV4 channels. By blocking Ca^{2+} influx through TRPV4 channels, cell death due to PTX-induced neurotoxicity is reduced in cultured DRG neurons. Moreover, electrophysiological and behavioural changes in mice resulting from PTX-induced neuropathy can be prevented by pre-treatment with the TRPV4 antagonist, HC067047, in advance of paclitaxel injections (Boehmerle et al. 2018). In this context, the inhibition of TRPV4 may be described as being neuroprotective. The difficulty, of course, in employing TRPV4 antagonists for this purpose in humans is the necessity to localise the action of the antagonists administered for this purpose to the TRPV4 channels which are responsible for the pathology, while saving harmless the remainder whose functioning is essential for normal physiological function.

TRPV4 and neuronal calcium sensor 1 (NCS1), which is a calcium-binding protein, functionally interact by forming a signalling complex which increases TRPV4 currents in line with increased expression of NCS1. Ca^{2+} fluxes entering through TRPV4 determine the size of TRPV4 currents. These Ca^{2+} fluxes depend on NCSI expression levels and are in line with the size of TRPV4 currents. PTX exposure increases the acute effects of TRPV4 expression, currents, and Ca^{2+} fluxes, but reduces the level of expression of NCS1 (Sánchez and Ehrlich 2021).

5.1.2 Thalidomide-Induced Neuropathy

Thalidomide, administered systemically in a single dose, results in an extended period (~35 days) of mechanical and cold hypersensitivity in the mouse hind-paw. TRPA1 and TRPV4 both contribute to this mechanical allodynia, while TRPA1 is exclusively responsible for this cold hypersensitivity. These ion channels are not activated by the drug itself but rather by hydrogen peroxide, its oxidative stress by-product. Antioxidant treatment can ameliorate both of these conditions. The cold allodynia and a component of the mechanical allodynia both result from the activation of peripheral TRPA1 by oxidative stress, but a further component of the mechanical allodynia is the result of activation of central TRPV4 by oxidative stress (De Logu et al. 2020).

5.2 Cancer-Induced Peripheral Neuropathy

Dorsal root ganglion neurons demonstrate an increase in the expression of TRPV1, TRPV4, toll like receptor 4 (TLR4) and extracellular signal-related kinase 1 and 2

(ERK 1/2) in a rat model of cancer-induced peripheral neuropathy. This phenomenon is accompanied by the development of thermal and mechanical hyperalgesia, both of which are reversed in a dose-dependent manner by the administration of TRP antagonists and a Ca^{2+} inhibitor (Maqboul and Elsadek 2018).

5.3 Diabetic Peripheral Neuropathy

Peripheral neuropathy is a not unusual accompaniment of chronic diabetes. Typically, it takes the form of a distal degenerative polyneuropathy with sensory loss. Unfortunately, from approximately 20 to 30% of patients afflicted with such sensory loss also suffer from neuropathic pain (Jolivalt et al. 2016). TRPV4 is important in insulin production and secretion. TRPV4 agonists demonstrate insulinotropic activity in pancreatic cell lines, but prolonged activation of TRPV4 leads to cell dysfunction and death (Hu et al. 2020). Recent studies using a mouse model of painful diabetic neuropathy ("streptozotocin (STZ)-induced" diabetes) have shown that TRPV4 mediates the mechanical allodynia of such neuropathy but not its cold allodynia. At 6 weeks after diabetes induction, STZ-treated animals demonstrate both mechanical allodynia and cold allodynia. Treatment of these animals with HC-067047 (1 mg/kg, s.c.), a selective TRPV4 channel antagonist, significantly inhibits the development of mechanical allodynia. This effect is independent of any reduction in blood glucose in the diabetic animals, suggesting a direct effect on downstream targets. On the other hand, the administration of HC-067047 is unable to affect cold allodynia (Dias et al. 2019). The increased expression of TRPV4 found in rats with diabetic mechanical allodynia is found mainly in the soma and central processes of neurons containing CGRP or neurofilament 200 kDa (Cui et al. 2020).

6 Headache and Temporomandibular Joint Dysfunction

About 50% of dural afferents express TRPV4 ion channels which become activated on exposure to standard activators of this ion channel, such as 4α-PDD and hypotonic solutions. Moreover, migraine-type behaviour is evoked, in the form of cephalic and extracephalic allodynia, when TRPV4 within the dura of freely moving animals is activated. This effect is inhibited by the administration of a TRPV4 antagonist. Activation of TRPV4 on dural afferents may therefore be a contributor to migraine. There is, of course, no evidence that decreased osmolarity is present in headache patients which would stimulate the activation of TRPV4 (Wei et al. 2011), but the range of activators of this ion channel is very broad (White et al. 2016). Hassler et al. (2019) found that PAR_2 is expressed by cells in the meninges and by neurons innervating the meninges in mouse. Activation of PAR_2 and mast cell degranulation in the meninges results in migraine-type pain behaviours that do not occur in mice which lack PAR_2 and these pain behaviours are also reduced by a

PAR_2 antagonist (Hassler et al. 2019). The significance of these findings resides in the previously noted finding that the activation of PAR_2 results in downstream activation of TRPV4.

Interestingly, TRPV4 has been convincingly demonstrated to be involved in another highly painful disorder, the pain from which is mediated by trigeminal ganglion sensory neurons, namely, temporomandibular joint (TMJ) disorder. In a rat model of TMJ disorder, the expression of TRPV4 and PAR_2 is up-regulated following intra-articular injection of carrageenan into the TMJ joints with resulting development of inflammation. TRPV4 agonists induce Ca^{2+} influx into trigeminal ganglion neurons and TMJ fibroblast-like synovial cells. In living animals, these agonists cause increases in plasma extravasation, myeloperoxidase activity, and mechanical allodynia which are dose-dependent (Denadai-Souza et al. 2012). TRPV4 is essential for temporomandibular joint-inflammation-evoked pain in mice and pro-nociceptive alterations in the trigeminal ganglion are dependent on TRPV4. The capacity to bite ("bite-force") of *trpv4*$^{-/-}$ mice suffering from TMJ is significantly better than that exhibited by wild-type animals. Again, wild-type animals demonstrate significant upregulation of TRPV4 and pERK in trigeminal sensory neurons of TMJ-suffering animals – features which are not found in mice lacking TRPV4 (Chen et al. 2013).

7 Conclusion

TRPV4 is the mediator of *mechanical hyperalgesia* in various contexts in which a mechanical stimulus, whether due to trauma (at the macro-level) or discrete extracellular pressure or stress (at the micro-level), results in pain. This aspect of the contribution of TRPV4 to the experience of pain sensations gives the impression of this ion channel being at the centre of an organised system aimed at directing the animal's attention to such danger as may be presented by the presence of harmful mechanical stimuli. However, TRPV4 has also been shown to be a central component in the generation and maintenance of inflammatory pain sensations, as well as functioning in mediating mechanical allodynia and hyperalgesia in many types of peripheral neuropathies. The extent of the known involvement of TRPV4 in mediating pain sensations requires that it must be borne in mind in the development of future therapies intended to treat these pain types. Since this area of study remains in its infancy, one may expect future developments which further illuminate the potential analgesic significance of controlling the activation of this remarkable ion channel, or, more realistically, of inhibiting the downstream effects of that activation by interrupting the molecular pathways to nociceptor excitation driven by TRPV4.

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Role of Oxytocin in Different Neuropsychiatric, Neurodegenerative, and Neurodevelopmental Disorders

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Contents

Abstract Oxytocin has recently gained significant attention because of its role in the pathophysiology and management of dominant neuropsychiatric disorders. Oxytocin, a peptide hormone synthesized in the hypothalamus, is released into different brain regions, acting as a neurotransmitter. Receptors for oxytocin are present in many areas of the brain, including the hypothalamus, amygdala, and nucleus accumbens, which have been involved in the pathophysiology of depression,

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anxiety, schizophrenia, autism, Alzheimer's disease, Parkinson's disease, and attention deficit hyperactivity disorder. Animal studies have spotlighted the role of oxytocin in social, behavioral, pair bonding, and mother–infant bonding. Furthermore, oxytocin protects fetal neurons against injury during childbirth and affects various behaviors, assuming its possible neuroprotective characteristics. In this review, we discuss some of the concepts and mechanisms related to the role of oxytocin in the pathophysiology and management of some neuropsychiatric, neurodegenerative, and neurodevelopmental disorders.

Keywords Neurological disorders · Neuromodulator · Neurotransmitters · Oxytocin

Abbreviations

ADHD	Attention deficit and hyperactivity disorder
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
CGRP	Calcitonin gene-related peptide
CREB	The cAMP-responsive element-binding protein
CSF	Cerebrospinal fluid
EEG	Electroencephalogram
eEF2	Eukaryotic elongation factor 2
eNOS	Endothelial NO synthase
GPCR	G-protein-coupled receptor
IN	Intranasal
iNOS	Inducible nitric oxide synthase
MAPK	Mitogen-activated protein kinase
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
OT	Oxytocin
OTR	Oxytocin receptor
PANSS	Positive and negative symptoms scale
PD	Parkinson's disease
PPD	Postpartum depression
PTZ	Pentylenetetrazole
PVN	Paraventricular nuclei
SCZ	Schizophrenia
SON	Supraoptic nuclei

1 Introduction

In 1905, Sir Henry Dale noted that extracts from the human posterior pituitary gland led to uterine contractions of pregnant cats. He fashioned the term oxytocin (OT) from the Greek words meaning "swift birth" (Magon and Kalra 2011). Oxytocin, also known as ocytocin, pitocin, oxytocinum, syntocinon, endopituitrina, oxitocina, oxytocic hormone, and orasthin, has been heavily involved in the regulation of parturition and lactation. It binds to receptors found in the myometrium, activating the hydrolysis pathway of phosphatidylinositol and diacylglycerol, leading to the liberation of intracellular $Ca^{2+,}$ which causes uterine contractions (Kabilan 2014). The drug can reach a steady state after 40 min of administration through the parenteral route. It is distributed through the extracellular fluid of the mother, and small amounts may cross the placental barrier and reach the fetus. Metabolism occurs rapidly via the liver and plasma oxytocinase enzyme and the mammary gland. Oxytocin's half-life is 8 to 3 min in the blood (Troncy et al. 2008), while in cerebrospinal fluid (CSF), it reaches 19 min in rats and 28 min in guinea pigs (Jones and Robinson 1982; Mens et al. 1983). Liver and kidney eliminate OT; its unchanged form is rarely excreted from kidneys (Kabilan 2014).

The oxytocinergic system can be approached either by directly administrating exogenous OT, or by indirectly evoking pharmacological endogenous OT release through drugs targeting another system. Such a phenomenon is a translational approach in the neuropsychiatry (Ford and Young 2020). For instance, OT release was activated in the hypothalamus of prairie voles via the administration of melanocortin (MC3/4R) receptor agonist Melanotan II (Modi et al. 2015) Furthermore, sildenafil, a selective inhibitor of the cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PEDE5) enzymes, has shown the capacity to increase electrically evoked release of OT from the posterior pituitary gland through two mechanisms. Firstly, through modulating cGMP K^+ channels in the neurohypophysis (Zhang et al. 2007). Secondly, as a result of activating the NO-cGMP signaling pathway (Matsushita et al. 2012). This can be critical regarding developing OT-based treatments for different neurological and neuropsychiatric diseases as it provides an understanding of OT endogenous release pathways, and how they can be pharmacologically leveraged.

To understand the brain impacts of oxytocin and its role in neuropsychiatric disorders, we need to elucidate the molecular cascade of events mediating different neuropsychiatric, neurodegenerative, and neurodevelopmental disorders, especially the molecular and cellular pharmacology of oxytocin and oxytocin receptor (OTR).

1.1 Neurobiology of the OT System in the Brain

The origin of central OT is the hypothalamus–neurohypophysial system (HNS), where large magnocellular OT neurons together with its related nonapeptide AVP

exist in the bilateral supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus (Armstrong 2015). Besides the magnocellular OT neurons, a few small parvocellular neurons are localized bilaterally in the dorsolateral section of the PVN. Unlike the magnocellular OT neurons, those parvocellular OT neurons do not possess projections into the neurohypophysis. Hence, they mainly connect to the midbrain, hindbrain, and spinal cord. Both magnocellular and parvocellular OT neurons form the OT brain system (Landgraf and Neumann 2004; Eliava et al. 2016). Ferretti et al. found that OT projections from the PVN to the central amygdala are responsible for OT's positive and negative emotional effects (Ferretti et al. 2019). The hypothalamic OT projections from PVN also target dopamine regions in the midbrain, affecting the dopamine-related reward circuits in animals (Xiao et al. 2017). As mentioned by Maejima et al., projections from PVN and SON OT neurons into the arcuate nucleus of the hypothalamus affect the anorexigenic action of OT (Maejima et al. 2014).

The major release of OT could occur synaptic or non-synaptic; these different patterns of OT release characterize its functional complexity within the brain (Grinevich and Neumann 2020). Although electron microscope imaging showed large dense OT vesicles pre-synaptic in the few OT synapses of the SON (Peters et al. 2008; Griffin et al. 2010), OTR was not found on the post-synaptic membrane (Son et al. 2013). Hence, the central release of OT from magnocellular neurons is functioning mainly non-synaptically. It is either released from axonal terminals in the forebrain and limbic regions or from dendrites and somata in the hypothalamic SON and PVN (Grinevich and Neumann 2020). Calculation of the effective OT concentration within the brain shows that only a radius of 55–120 μM around the site of OT release is effective. Otherwise, OT concentration is insufficient to activate OTR (Chini et al. 2017).

1.2 Pharmacological Regulation of OTR Binding and G Protein Coupling

The OTR is part of the rhodopsin-type class I G-protein-coupled receptor (GPCR) (Vrachnis et al. 2011). Most of the documented gene variations do not yield an alteration in the amino acid sequence of the corresponding protein. Nucleotide variations in non-coding regions of the OTR gene can affect OTR central expression density, influencing the central response toward OT in the brain, thus affecting behavioral responses (King et al. 2016). The expression of single G protein isoform and local OT concentrations influence the specific endogenous OTR coupling and its physiological reactions (Hurlemann and Grinevich 2018) (Fig. 1a).

Aiming to investigate the pharmacological control of OTR within the brain, three functionally selective analogs are identified that can induce a number of OTR/G protein coupling; D-Nal-OVT, carbetocin, and atosiban (Fig. 1b). However, the activation of OTRs coupled with various G protein pathways can also activate

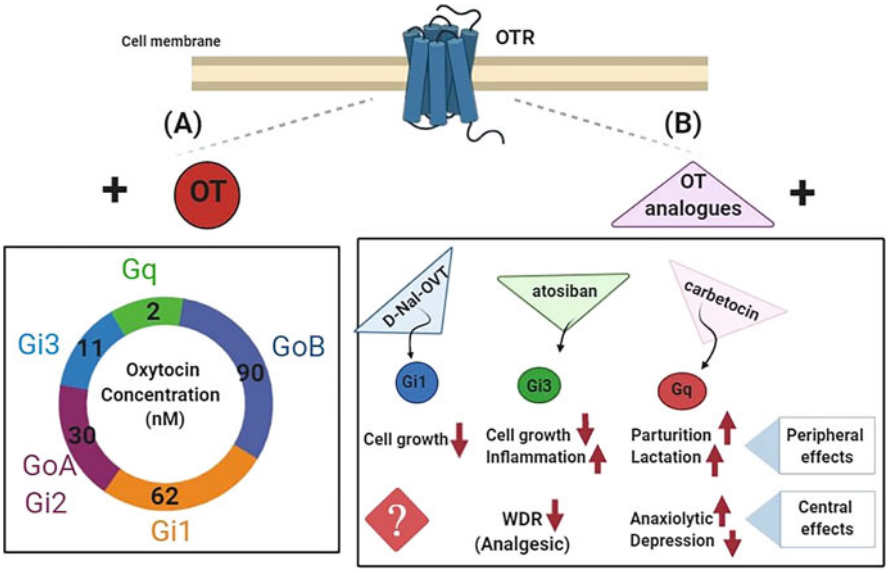

Fig. 1 The pharmacological basis of oxytocin receptor (OTR) binding and the corresponding G Protein coupling. (**a**) Various concentrations of oxytocin decide the particular binding of OTR to diverse subtypes of G protein. (**b**) "Functionally selective" analogs of oxytocin receptor actuate subtypes of single G protein. OT, oxytocin; OTR, oxytocin receptor

opposite cellular responses, e.g., OTR/Gi coupling inhibits-, in contrast, OTR/Gq coupling stimulates cell proliferation (Reversi et al. 2005). The D-Nal-OVT specifically advances OTR/Gi1 coupling and restrains cell proliferation; however, its central impacts are still questionable (Busnelli et al. 2012). As for carbetocin, it explicitly activates OTR/Gq coupling and actuates OTR internalization in the absence of β-arrestin enrolment, without recycling of OTR (β-arrestin is a cytosolic protein regulating the GPCR signaling, through receptor desensitization and internalization). Peripherally, carbetocin promotes uterine contractions and milk ejection. Centrally, carbetocin has anxiolytic and anti-depressant effects (Passoni et al. 2016). While atosiban acts as a competitive antagonist on OTR/Gq coupling, displaying agonistic properties on OTR/Gi coupling (Reversi et al. 2005), specifically activating the OTR/Gi3 pathway, it also represses cell proliferation and exhibits a pro-inflammatory effect within the central nervous system. Moreover, it represses "sensory wide dynamic range" neurons firing within the deep laminae of the spinal cord, enabling an intermediate pain-relief effect (Reversi et al. 2005).

1.3 *Intracellular OTR Effectors*

A nation of downstream pathways signaling following G protein activation, among other various OTR intracellular secondary effectors, is responsible for the rise of multiple specific neuronal cells and brain functions, as discussed below and illustrated in Fig. 2.

1.3.1 The MAP Kinase Cascade

The mitogen-activated protein kinase (MAPK) cascade is one of the essential intracellular signaling pathways stimulated by OTRs. The mammalian cell has four well-known sub-families of MAPK: ERK1/2, ERK5, p38 MAPK, and stress-activated protein kinase-1 (Sun and Nan 2016). In female mice, OTR activation causes phosphorylation of ERK and the cAMP-responsive element-binding protein (CREB), activating the spatial memory in the hippocampus to keep a memory of the

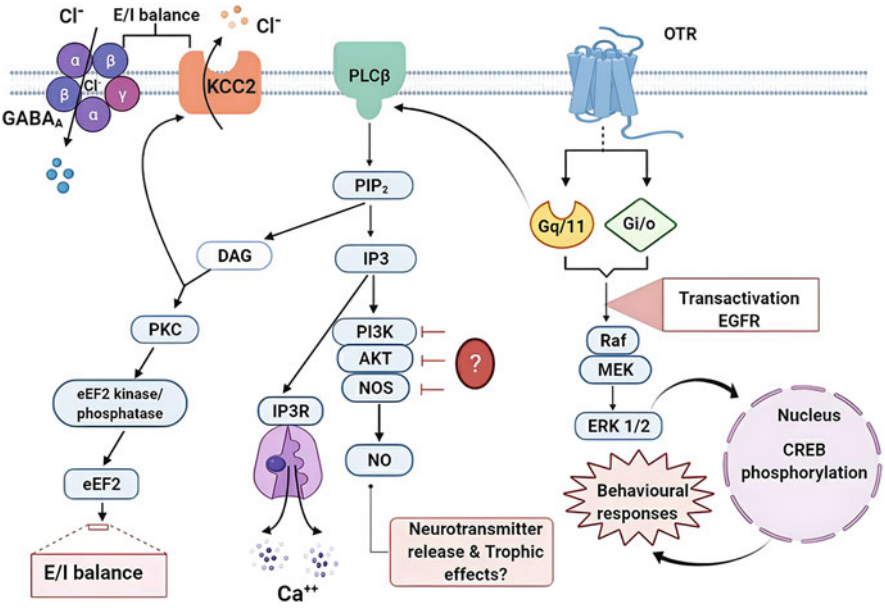

Fig. 2 Intracellular oxytocin receptor (OTR)-related signaling within the central nervous system. Different intracellular pathways are activated according to the type of G proteins stimulated by oxytocin. Black arrows indicate activation and blunt ends indicate suppression. PLCβ, Phospholipase Cβ; EGFRs, epidermal growth factor receptors; PKC, protein kinase C; IRK, inward rectifying potassium channel; eEF2, eukaryotic elongation factor 2; PIP2, phosphatidylinositol (4,5)-biphosphate; DAG, diacylglycerol; PI3K, phosphoinositide (PI)3-kinase; ERK, extracellular signal-regulated kinase; IP3, inositol triphosphate; IP3R, IP3 receptor

location of food and water to ensure the well-being of her offspring (Tomizawa et al. 2003).

However, oxytocin modulates its anxiolytic activity by OTR/ERK/MEK signaling. Blume et al. observed that intracerebral ventricular application of OT stimulated the phosphorylation of Raf-1, ERK1/2, and MEK1/2 (Blume et al. 2008), in turn stimulating MAPK inducing the translocation of ERK1 to the nucleus, activating the transcription factor CREB (Jurek et al. 2012). Lastly, it was demonstrated that the anxiolytic pathway involves the induction of extracellular calcium via transient receptor potential vanilloid (TRPV) channels. In detail, OTR stimulation in hypothalamic neurons leads to the release of PI3K and Gβγ, thus promoting the integration of TRPV channels into the plasma membrane, creating calcium influx, and resulting in MEK1/2 phosphorylation (Van Den Burg et al. 2015). The Ca^{2+} calmodulin-dependent stimulation of the EGFR is likely to be part of this system; however, the molecular players implicated are still unclear (Blume et al. 2008).

1.3.2 Nitric Oxide (NO) Production

Research on vascular endothelial cells has shown that OTR stimulation results in intracellular calcium mobilization and phosphorylation of endothelial NO synthase (eNOS) via the PI3K/AKT pathway in a Gq/PLC activity-dependent manner (Cattaneo et al. 2008). Brain cells engaging the OTR/NOS signaling pathway are still unidentified, including endothelial, neuronal, and vascular cells.

1.3.3 Eukaryotic Elongation Factor 2 (eEF2) Phosphorylation/Dephosphorylation

The eukaryotic elongation factor 2 (eEF2) is a crucial protein synthesis regulator, and the OT impact in the myometrium is mediated through the Gq/PKC modulation of eEF2 (Devost et al. 2005). Recent findings indicate that eEF2 and OTR signaling pathways are crucial to regulating the balance between inhibitory and excitatory synapses, suggesting that the two pathways could cross-talk. It was noted that lowered eEF2 action significantly decreased GABAergic synaptic transmission (Heise et al. 2017). Also, a decrease in GABAergic synapses was demonstrated in neurons obtained from mice lacking OTRs. It is assumed that the impact of OTRs on GABAergic synapses may be mediated via the eEF2 pathway (Leonzino et al. 2016).

1.3.4 GABA Transporters and the Developmentally Regulated GABA Switch

Post-natal brain growth needs a balance of excitation and inhibition (E/I) to identify its neuronal circuits accurately. The essential players in this balance are the prominent excitatory and inhibitory neurotransmitters in the CNS, glutamate, and GABA,

respectively (Hurlemann and Grinevich 2018). OTRs are essential at the molecular level for the up-regulation of the GABA switch, the chloride co-transporter KCC2. OTRs directly regulate KCC2 functional activity by stimulating its phosphorylation and its insertion into the neuronal surface employing a signaling pathway mediated by PKC. These outcomes present KCC2 as a crucial target for OT, phosphatidyl inositol, which might be involved in the pathogenesis of various neurological disorders (Leonzino et al. 2016).

1.4 The Intranasal Route of Administration

The nose represents a successful route to deliver medications to the brain. The interior surface of the nose is an easily accessible part of the body that is directly connected to the brain via a network of nerves. Medications probably completely escape the blood-brain barrier (BBB) by spraying liquid medicine into the nose. The intranasal (IN) administration of drugs follows two probable mechanisms: (1) the drug is actively taken up through the trigeminal or olfactory nerve directly into the brain and (2) through passive neuronal diffusion over the perineural clefts in the nasal epithelium, which provides a gap in the BBB, and in turn into the CSF (Quintana et al. 2021). Nasal mucosa permeability is usually low for polar molecules, such as low molecular weight drugs and exceptionally high molecular weight peptides and proteins. Consequently, the absorption of these drugs decreases (Moore et al. 1997), in addition to the mucociliary clearance of the nasal cavity, that can further dispute drug delivery through IN route (Schipper et al. 1991).

Animal studies, using LC/MS analysis of brain homogenate (Tanaka et al. 2018), and clinical investigations, using functional magnetic resonance imaging (fMRI) and behavioral testing (Quintana et al. 2016), have shown that the level of OT in the brain increases after receiving an OT nasal spray. The portion of the sprayed nasal oxytocin that does not reach the brain enters the bloodstream through the high vascularized nasal cavity (Quintana et al. 2021). This was further observed by Yeomans et al. who were able to follow the radiolabeled OT in the brains, blood, and peripheral tissues of rats after nasal delivery, confirming the olfactory and trigeminal pathways for nasal drug-brain delivery. Considerable amounts of OT were found in the blood, but interestingly very low amounts were shown in peripheral tissues (Yeomans et al. 2021).

The most efficacious IN dose of OT to use is still unknown. Generally, smaller doses are more efficient than higher doses (Quintana et al. 2015). Comparing IN and IV administration of OT in humans has shown comparable blood levels of OT, however, the social and neural manifestations were only observed after the IN-OT (Quintana et al. 2015, 2016). This outcome supports the notion that IN administration of OT does not reach the brain through the BBB, but rather directly through the olfactory route, as earlier shown by Yeomans et al.. Non-human primate study has also shown a similar result, where intranasal, but not intravenous OT, increased endogenous OT release centrally (Lee et al. 2018).

On the contrary, several neuroimaging studies and behavioral human studies showed that peripheral drug administration modulated measurable behavioral and neuronal responses (MacDonald and MacDonald 2010; Meyer-Lindenberg et al. 2011; Mottolese et al. 2014; Wei et al. 2015). However, there is still no evidence that peripheral OT penetrates the BBB and no evidence about the amount of OT that passes. No studies up till now investigated if peripheral administration of OT could trigger central OT release, highlighting the need for pharmacokinetic studies of BBB penetration in relation to intravenous or intraperitoneal OT administration. For that purpose, Lee et al. tried labeled OT administration IN and IV in a non-human primate model of rhesus macaques. The mass-spectrometry analysis did not show any advantage of one route over the other, regarding CSF concentrations, revealing that peripheral OT administration did not trigger the central release of OT (Lee et al. 2018). This offers solid evidence that OT bioavailability is not subjected to variability due to the route of administration. To put it through, IN administration of OT seems to offer an easy and more efficient way to deliver OT centrally, as IN route protects OT from metabolism and elimination.

Accordingly, direct central delivery of OT has shown promising results in multiple psychiatric disorders. The significant potential was observed in animal models of socio-cognitive behavior (Calcagnoli et al. 2015; László et al. 2016). For instance, acute IN-OT increased the connectivity mainly within the limbic system in mice. At the same time, repeated administration boosted the functional connectivity with solid coupling between the amygdala and extended cortical parts associated with a significant reduction in social communication and interaction (Pagani et al. 2020). Such differences in connectivity related to OT dosing regimen could help explain different clinical findings. In another study in titi monkeys, a socially monogamous primate revealed sex-related differences after chronic IN-OT regarding social behaviors, assessed through preference test. This showed that females had enhanced preference tests toward their parents than males, an effect attributed to the high glucose uptake across socio-cognitive neural circuitry in the brain observed by neuroimaging (Del Razo et al. 2020). Such relation between OT brain level and glucose transporters in relation to neurotransmission has recently been into focus (Ye et al. 2021).

Others also examined the chronic versus acute IN-OT administration. Daily administration of IN-OT for two weeks increased anxiety-like behavior of female rats instead of showing an anxiolytic effect. This response was reverted after OT's last dose on the fifth day (Winter et al. 2021). Winter et al. noted that chronic OT has shifted the splicing ratio toward the expression of soluble anxiogenic sCRFR2α, which was released into the CSF (Winter et al. 2021). Moreover, long-term administration of low OT dose intranasally resulted in a decrease in social behavior preference toward the opposite sex in male voles (Bales et al. 2013), an effect that was shown to be dose-dependent (Guoynes et al. 2018). Huang et al. (Huang et al. 2014) have compared short- versus long-term administration of IN-OT in wild-type C57BL/6J adult mice, where chronic overstimulation of the brain with OT for 18 months resulted in a reduction of brain OT receptors and hence a decline in the animal social behavior, a phenomenon that can be attributed to receptor

desensitization (Gimpl and Fahrenholz 2001). These findings highlight the advantage of acute OT administration over the chronic, but also triangulate the need for studies addressing the underlying molecular interactions happening at receptor level.

The OT's potential extends to human studies, as OT has displayed significance regarding numerous neuropsychiatric diseases; autism (Yamasue et al. 2020; Alaerts et al. 2021), schizophrenia (Martins et al. 2020, 2021), alcohol dependence (Melby et al. 2019, 2021), post-traumatic stress disorder (van Zuiden et al. 2017; Koch et al. 2019), and social anxiety (De Cagna et al. 2019; Sabino et al. 2020). In a randomized controlled study, chronic IN-OT administration for four weeks in healthy older men revealed no effect on cardiovascular, urine, or serum parameters (Rung et al. 2021). This spotlights the idea that chronic use of IN-OT may affect an individual's social behavior due to a change in OT brain concentration without any other health-associated side effects, with apparent discrepancy between different sexes.

2 Effect of OT in Brain Disorders

Besides being a circulating hormone, OT can also act as a neurotransmitter and a neuromodulator within the brain (Kiss and Mikkelsen 2005). Several preclinical and clinical ongoing research supports the notion that OT may play a role in regulating several brain disorders (Guastella et al. 2010; De Coster et al. 2019).

2.1 Epilepsy

Oxytocin plays a significant role in suppressing the severity of epileptic behavior. It is known to increase the onset of first myoclonic seizures and decrease the duration of seizures. Interestingly, a synergistic effect between oxytocin and benzodiazepines has also been observed in epilepsy (Erfanparast et al. 2017), as intrahippocampal OT administration in rats decreased the severity of epileptic seizure episodes. Erfanparast et al. speculated that GABA-A benzodiazepine receptor mechanism may be involved in OT proconvulsant effect, an effect that we believe to be through an indirect interaction. In this regard, the inhibitory action of OT on pyramidal neurons of the hippocampus via its indirect effect on the GABAergic interneurons is identified (Zaninetti and Raggenbass 2000), suggesting the possible involvement of OT within the pathogenesis of epilepsy.

New insight into the proconvulsant activities of sildenafil was given. In a pentylenetetrazole (PTZ) experimental model of seizures, oxytocin secretion and subsequent CREB phosphorylation initiated the proconvulsant impact of sildenafil. In addition, sildenafil's proconvulsant effects were reversed when atosiban, a selective OT receptor antagonist, was administrated (Khoshneviszadeh et al. 2016). As illustrated in Fig. 3, the indirect activation of the OTR by sildenafil initiates calcium signaling and activates the calcineurin (CN) inducible nitric oxide synthase (iNOS)

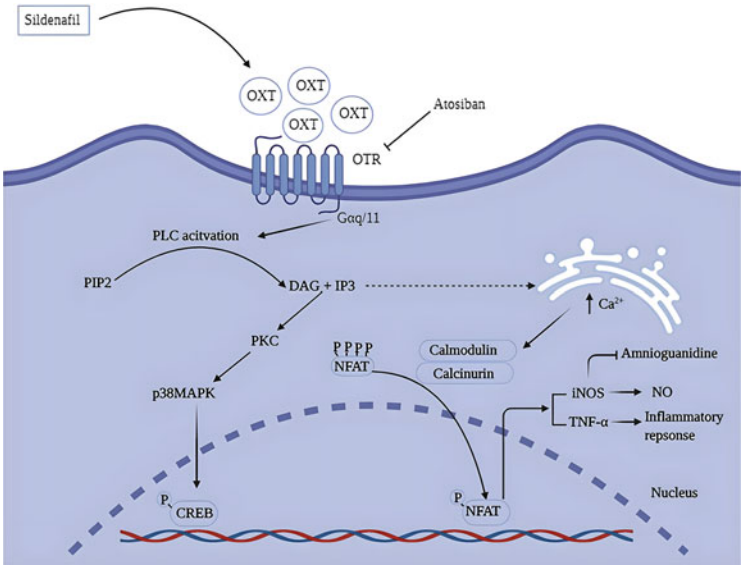

Fig. 3 The role of sildenafil in controlling the mechanism of action of oxytocin (OXT). Sildenafil stimulates the endogenous release of OXT, initiating the calcium and the p38 MAPK signaling pathways. PLC, Phospholipase C; PIP2, phosphatidylinositol 4, 5-bisphosphate; DAG, diacylglycerol; IP3, inositol trisphosphate; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; CREB, cAMP response element-binding protein; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TNF-α, tumor necrosis alpha. Black arrows indicate activation and blunt ends indicate suppression (Rahimian et al. 2020)

pathway and the p38 MAPK signaling pathway. These results introduced new insight into OT mediated mechanisms involving sildenafil in epilepsy (Rahimian et al. 2020). Another recent study by Wong et al. in a mouse model of Scn1a-derived epilepsy has shown that nanoparticle encapsulation of OT produced relatively long-lasting protection toward induced seizures and returned more normal social interaction (Wong et al. 2021). This, in turn, might have an essential role in understanding the diversity of pathways included in the pathology of epilepsy, showing a possible involvement of the endogenous OT mechanism.

The anticonvulsant effect of OT in PTZ-induced epilepsy models in rats has been questionable. In a study by Erbas et al., electroencephalogram (EEG), where the recording of thirty male Sprague-Dawley rats was used to study the effect of different doses of OT (40, 80, 160 nmol/kg) that could inhibit experimentally PTZ-induced epilepsy in rats. The EEG results demonstrated that 80 and 160 nmol/kg of OT effectively avoided PTZ-induced seizures (Erbas et al. 2013b). In contrast, Erfanparast et al. investigated the mechanism of intrahippocampal impact of OT at doses of 10 and 20 ng/site in an experimental PTZ animal model of seizure. The OT antagonist, atosiban, GABA-benzodiazepine receptor agonist and antagonist, diazepam and flumazenil, respectively, were used. Results displayed that OT terminated the severity of seizures at a hippocampal level, an effect that was

blocked by atosiban and flumazenil. In addition, OT increased the antiepileptic effect of diazepam (Erfanparast et al. 2017). Again, this outcome shows a possible indirect relation between OT and the GABA-A benzodiazepine receptor mechanism.

Aiming to optimize an antiepileptic dose of OT, A Rahimian et al. studied two doses of OT (0.25 and 0.5 mg/kg, i.p.) in PTZ-induced seizures in mice. Administration of OT one hour before determining the seizure threshold indicated that OT at a maximum dosage of 0.5 mg/kg exhibited a proconvulsant impact compared to the saline-treated group (Rahimian et al. 2020). Moreover, the effect of OT or sildenafil on cortical CN activity was studied. PTZ administration led to a significant spike in CN activity relative to the saline-treated group. Sildenafil or OT at 40 mg/kg (30 min) and 0.5 mg/kg (60 min), respectively, significantly increased the CN activity compared to the control group. The integration of atosiban (10 μg/kg, i.c.v), 30 min prior to administration of sildenafil, significantly reduced the CN activity . In contrast to the PTZ group, sildenafil and OT groups significantly increased cortical CN activity. However, co-administration of atosiban reduced the sildenafil effects on CNS activity (Rahimian et al. 2020).

OT has shown an antiepileptic effect despite insufficient clinical evidence, with solid significance toward certain brain regions, i.e., hippocampus. In addition, OT has shown a synergistic effect when combined with typical antiepileptics in various animal models. Human clinical trials investigating its role in epilepsy are strongly recommended to explore the possibility of using OT as an antiepileptic therapeutic agent, as well as exploring its outcome when combined with typical antiepileptics.

2.2 Schizophrenia (SCZ)

Preclinical and clinical studies showed that the endogenous OT circuit is dysregulated in schizophrenia (SCZ) patients and contributes to all three SCZ symptomatic domains. Hence, targeting central OT may regulate the manifestation of SCZ disorders and reveal a candidate for different therapeutic pathways (Shilling and Feifel 2016).

Brain imaging studies indicated that endogenous OT might modulate the activities of brain regions associated with social and emotional behavior, namely the amygdala, temporal gyrus, and prefrontal cortex (Bartholomeusz et al. 2015). In SCZ, the interaction between the amygdala, dopaminergic and oxytocinergic systems may be disturbed. Abnormalities related to OT are reflected in neuronal function, which causes incorrect emotional information processing and impairment of social behavior (Jusiak et al. 2017). Further research is needed to highlight the significance of endogenous OT mechanism in the treatment of psychotic disorders. Genetically, studies indicated a possible association between two variants of OT and OTR-related genes polymorphisms (rs4813625 and the rs3761248). These polymorphisms were significantly more common in cases with SCZ than in control groups (Souza et al. 2010); therefore, alteration in the OT circuit may underline the pathogenesis of SCZ.

In order to mimic the two major pathophysiological patterns of SCZ (hyperdopaminergic and glutamate hypofunction), dizocilpine, the non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptor, and amphetamine, an indirect dopamine agonist, both were used to induce deficits in prepulse inhibition, an outcome that OT administration reverted (De Bartolomeis et al. 2005; Feifel et al. 2012). In congruence with the hypothesis of reduced oxytocin-signaling in SCZ, a significant oxytocin receptor reduction was evident in the Reelin Haploinsufficient Reeler mouse (HRM) animal model of neural deficits in SCZ (Liu et al. 2005). Alongside, a study by Liu et al. has also shown that 52 patients of the first episode SCZ had a significant smaller serum OT level and OTR expression level (518.96 $\pm$ 22.22 and 174.60 $\pm$ 17.11 pg/ml) than healthy control (711.58 $\pm$ 40.57 and 252.15 $\pm$ 20.62 pg/ml) (Liu et al. 2005).

Evidence for an indirect correlation between levels of plasma OT (pOT) and severity of the symptoms suggested that OT may subsidize SCZ. For instance, low pOT levels were associated with more severe symptoms that have been involved with all three SCZ domains. This correlation is more significant concerning negative symptoms and socio-cognitive functioning conditions in SCZ patients (Shilling and Feifel 2016).

Pathophysiological, increased dopaminergic transmission is presumed to be crucial in developing positive symptoms (Winton-Brown et al. 2014). Animal studies strongly encouraged the use of OT to relieve the positive symptoms of SCZ, where OT reduced central hypoglutamatergic and excessive mesolimbic dopamine (Caldwell et al. 2009). Feifel et al. conducted a human clinical study investigating OT therapeutic possibility, where IN-OT was administrated to SCZ patients twice daily for three weeks. Patients identified with positive SCZ symptoms showed a significant reduction in their positive subscale scores on the Positive and Negative Symptoms Scale (PANSS) (Feifel et al. 2010).

Regarding the negative domain, preclinical studies investigating possible therapeutic benefits of OT are still restricted to the animal social withdrawal models. Those studies reported that the administration of OT enhanced social interaction. Lee et al. have shown that single OT infusion into the central nucleus of the amygdala could reverse the effect of phencyclidine (an NMDA receptor non-competitive antagonist) and reported a decrease in social interactions of rats (Lee et al. 2005). This study came to confirm the role of OT in SCZ pathophysiology, specifically in the amygdala region of the brain. Additionally, clinical studies by Feifel et al. (Feifel et al. 2010), Gibson et al. (Gibson et al. 2014), and Modabbernia et al. (Modabbernia et al. 2013) reported that a twice per day administration of IN-OT significantly reduced the negative score of PANSS for 3-6 weeks. As depicted in Fig. 4, brain regions involved in positive symptoms are the amygdala, nucleus accumbens, hippocampus, and ventral tegmental area. On the other hand, the dorsal striatum, prefrontal cortex, substantia nigra, and other regions are implicated in the negative symptoms (Feifel et al. 2016).

Feifel et al. observed the activity of OT and carbetocin (long-acting drug compared to OT) on low prepulse inhibition (PPI) and the acoustic startle reflex (ASR), which is an important brain function associated with SCZ cognitive dysfunction.

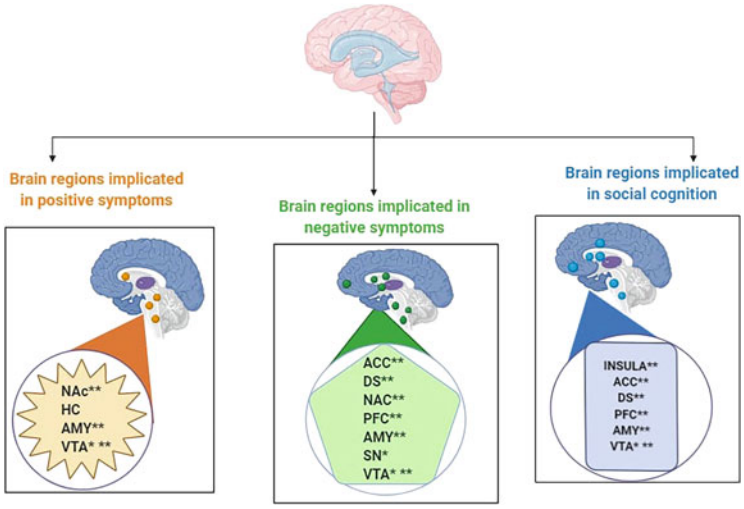

Fig. 4 Recognized brain pathways associated with oxytocin's effects on three main schizophrenia domains. Colored circles reflect the affected brain region. Single asterisk reflects areas that express OTR (oxytocin receptor) in a putative manner. Double asterisk indicates brain regions that in human functional imaging studies are supposed to be altered by single-dose intranasal oxytocin. NAc, nucleus accumbens; HC, hippocampus; AMY, amygdala; VTA, ventral tegmental area; ACC, anterior cingulate cortex; DS, dorsal striatum; PFC, prefrontal cortex; SN, substantia nigra. The figure has been developed based on the information in the following references: (Loup et al. 1991; Veening et al. 2010; Fuxe et al. 2012; Boccia et al. 2013; Kanat et al. 2015; Feifel et al. 2016)

Three doses of OT were tested (0.04, 0.2, and 1.0 mg/kg). All doses of OT increased PPI naturally present in Brown Norway rats and decreased ASR levels suggesting that OT can produce an antipsychotic effect. In contrast, three doses of carbetocin (0.04, 0.2, and 1.0 mg/kg) exhibited no impact on PPI or ASR levels, which discourages the use of carbetocin for SCZ patients (Feifel et al. 2012).

To sum up, OT is speculated to have a role in the pathophysiology of SCZ through different aspects; OT plasma concentration, OT neural circuitry dysfunction, and even genetic susceptibility. Therefore, OT administration has shown the potential to decrease the severity of SCZ in various animal models. It has also shown significance in clinical settings regarding the positive domain. Further studies are needed to assess these effects, especially in clinical settings.

2.3 Parkinson's Disease (PD)

OT can reduce cell death, act as a neuroprotective agent, and interact with neurotransmitter systems. Therefore, some studies have pointed out the role of OT on PD by more than one mechanism. In rotenone-induced PD in rats, OT administration at a dose of 160 μg/kg/day for 20 days showed a slow increase in body weight with no

significant difference between groups. (Erbaş et al. 2012). This increase in body weight is an indication of overall good health condition. Knowing that the bodyweight of PD patients decreases as the disease progresses due to muscular rigidity, anorexia, malnutrition, and depression together with low bone mineral density (Bachmann and Trenkwalder 2006; Kistner et al. 2014), an effect that improves upon dopaminergic treatment, encourages the use of OT in PD. Treatment of OT was able to prevent the dopaminergic striatal neuronal loss, as observed by Erbas et al., and hence improve the overall health condition of PD patients reflected in the slow weight gain. Maybe a higher dose of OT is required or a longer treatment period to observe a significant change in the bodyweight gain. Further study by Erbas et al. confirmed the previous finding noting to the cytoprotective, as well restorative effects of OT on dopaminergic neurons (Erbas et al. 2013a). Further studies have shown that oxytocinergic and dopaminergic systems are pretty interconnected, as dopamine levels increased when OT was administrated (Gamal-Eltrabily and Manzano-García 2018). This mechanism of OT neuroprotective effect is thought to be an inhibition of the apoptotic pathway, altering the expression of anti-apoptotic and pro-apoptotic markers, such as caspase-3, caspase-8, Bcl-2, and Bax in the striatal dopaminergic neurons. Rotenone-induced PD decreased the anti-apoptotic protein, Bcl-2, but elevated the pro-apoptotic Bax, caspase-3 and caspase-8 immuno-expression. Treatment with OT effectively increased Bcl-2 and decreased Bax expression. It also reduced the caspase-3 and caspase-8 expressions compared to the PD control group (Erbaş et al. 2012).

Administration of OT did not show any improvement in aspects and systems articulating with PD, yet it showed an anti-apoptotic effect and a protective effect, especially toward dopaminergic neurons. Further studies investigating OT in PD clinical settings are still needed.

2.4 Attention Deficit and Hyperactivity Disorder (ADHD)

A human clinical study conducted by Demirci et al. enrolled 83 male adolescents and children aged 7–18 years who were recently diagnosed with ADHD. Serum levels of OT in ADHD patients were significantly lower in male patients compared to the control group. This study proposed that ADHD and decreased OT serum levels are interrelated, especially in the hyperactive/impulsive ADHD subtype. Besides, the OT serum level in ADHD patients might be responsible for their aggression level, social withdrawal, empathy skills, and social relations (Demirci et al. 2016).

Another human clinical study by Işık et al. enrolled children and adolescents (8 to 16 years) with ADHD never exposed to treatment. Seventy-four children (17 girls and 57 boys) had ADHD with a combined presentation, 32 children (5 girls and 27 boys) showed ADHD with combined presentation and conduct disorder, 42 children (13 girls and 29 boys) served as healthy control. No significant difference was found between children with ADHD combined presentation alone and healthy control regarding serum OT level. This outcome did not support the significance

of OT in ADHD etiology. On the other hand, the finding revealed that children with ADHD combined presentation and conduct disorder had lower serum OT levels than children with ADHD combined presentation only and healthy control (Işık et al. 2018).

It is widely accepted that some psychiatric disorders, like poor empathy and impaired attachment, are associated with both conduct disorder and lower circulating OT serum levels (Buchheim et al. 2009; Feeser et al. 2015). These results suggest a correlation between low circulating OT level and the conduct disorder. The underlying mechanism of how serum OT in the case of ADHD may be linked to conduct disorder has not been yet elucidated. However, these results come to support the idea that the BBB is an endocrine tissue allowing the exchange of substance in a regulated manner between the CNS and the blood permitting the communication with peripheral tissues. In the last few years, researchers started highlighting the role of BBB in causing endocrine diseases, but also a key in treating several endocrine manifestations (Banks 2019). This besides the hypothesis that the BBB might be broken down in psychiatric and neurodegenerative diseases (Pollak et al. 2018; Sweeney et al. 2018), which could now be related not only to inflammatory-, but also endocrine abnormalities.

2.5 Migraine

Three cascading processes play a significant role in migraine: The cortical spreading depression (CSD) is defined as an intense wave of depolarization of neurons. It starts within the occipital lobe, spreads through the brain, and subsequently continues suppressing spontaneous neuronal activity. Activating the trigeminovascular system leads to the release of neuropeptides like the calcitonin gene-related peptide (CGRP) and substance P from the peripheral trigeminal nerve ending. These neuropeptides have a role in the mechanism of migraine. Finally, sensitization of central and peripheral brain areas is thought to pulsate the intensity of migraine headaches (Kowa and Nakashima 2012; Kojić and Stojanović 2013).

The CSD activating the trigeminovascular system is one of the potential mechanisms which initiate the pain process (Bolay et al. 2002). Activation of the trigeminal nerve fibers causes pain transfer to the brainstem and then to the upper brain centers, in turn leading to the release of vasoactive peptides (substance P and CGRP) from the trigeminal fibers. These peptides cause neurogenic inflammation, aggravate the existing vasodilation, and cause degranulation of mast cells (Durham 2004). The neurogenic inflammation and vasodilation further activate the sensory trigeminal fibers and resume the discharge of vasoactive peptides, including CGRP. As migraine develops, the spinal cord and brainstem are the first to collect the pain stimuli from the trigeminal nerve, then aggravate the headache pain and increase the sensitivity to other environmental stimuli (Hargreaves and Shepheard 1999).

OTR is expressed on trigeminal neurons and can inhibit the activation of the trigeminovascular system. Therefore, OT can inhibit the pain process. The use of the

IN route for delivery of OT to the brain directly exploits the olfactory pathway and constrains responses of trigeminal nucleus caudalis neurons to noxious stimulation (Wang et al. 2013; Tzabazis et al. 2017).

The effect of IN-OT (8 μg) on trigeminal nucleus caudalis (TNC) neuronal responses was investigated in the standard rodent migraine model induced by nitroglycerin injection (Sances et al. 2003, 2004), which is considered the most frequently used rodent model for migraine (Ma et al. 2008). Injection of nitroglycerin consistently stimulated C-fos expression in numerous TNC neurons compared with untreated rat TNC neurons. The IN-OT pretreatment showed the ability to significantly decrease the number of neurons displaying C-fos activity after the administration of nitroglycerin and subsequently the activation of central TNC neurons (Tzabazis et al. 2017). These results provide clear, plausible evidence of the ability of OT to decrease central pain transmission. However, there is still room for research about the molecular mechanism of OT in preventing migraine, but also other pain processes associated with multiple diseases.

2.6 Depression

The last groundbreaking event in the psychopharmacology of depression was the Selective Serotonin Reuptake Inhibitors (SSRI) and Serotonin/Norepinephrine Reuptake Inhibitors (SNRI), which did not only escalate the clinical outcome benefits of anti-depressive agents (Catena-Dell'Osso et al. 2013), but also showed effectiveness against social anxiety (Jakubovski et al. 2019). The results of a meta-analysis have shown that both SSRI's and SNRI's mechanisms were able to revert the anxiety (Jakubovski et al. 2019), as oxytocinergic systems ought to communicate, in other words, cross-talk with the monoamine system including 5-HT, norepinephrine (NE), and DA, it is thought also to affect the anxiety manifestations. Oxytocin may have a regulatory function (Vacher et al. 2002; Liu and Wang 2003), in addition to its inhibitory effect upon amygdala activation, diminishing fear and anxiety. This is indispensable in understanding both diseases, as depression usually shares shades of social isolation or withdrawal, fear, and stress.

OT decreased distress after a period of social isolation in infant rats, an effect that was diminished by administrating an OT antagonist (Insel and Winslow 1991). Alongside, maternal isolation resulted in decreased OT levels in certain parts of the brain, with observed depressive behaviors reflected in the forced swimming test as increased mobility time and decreased struggling time. An effect that was reversed through the intranasal administration of OT (Ji et al. 2016).

There is strong evidence that OT has a role in social affiliation. It is considered widely accepted that maternal and social isolation usually triggers depressive and anxiety behavior, and OT involves many tunable mechanisms that can be beneficial in reverting them (Shin et al. 2010; Ji et al. 2016). Bosch, O. J et al. elegantly expressed the neural circuitry behind passive stress as an indication of depression in male monogamous prairie vole after losing the female partner. The study reported

that partner loss compromised the OT system in many ways, with a strong emphasis on the chronic activation of Corticotropin-Releasing Factor Receptor 2 (CRFR2) in conjunction with OT striatal suppression, being the underlying cause of the aversive emotional state, also proposing that OT suppression can be a mechanism that encourages long-term relationships (Bosch et al. 2016). Oxytocin anti-depressive effect was first noticed by G. Meisenberg when 20 ul or 50 ul of OT for mice and rats, respectively, were injected through the intracerebroventricular approach. This triggered immobility, scratching, grooming behavior in the scratch mouse test and reduced immobility time in the forced swim test (Meisenberg 1982). Administration of 0.250–1.0 mg/kg of OT injected 60 min before behavioral despair test reduced the duration of immobility compared to that of imipramine (7.5–30 mg/kg); this was an acute effect. Prolonged administration was tested against the learned helplessness test, where OT (0.5 mg/kg/day) was administered for eight days. This remarkably reduced the escape failure, and this effect was more intense than that trigged by imipramine (20 mg/kg/day) (Arletti and Bertolini 1987). This could be an eye-opening result showing how potent and effective systemic OT could be, either through acute or prolonged administration.

Matsushita, H et al. have tested multiple arrays of sildenafil doses (10, 20, 30, or 60 mg/kg) in male rats one hour prior to the forced swim test, which showed a dose-dependent reduction in the duration of immobility, especially at 30 and 60 mg/kg dose level. When the sub-therapeutic dose was tested in the long run, administering 20 mg/kg/day for three days showed a significant reduction in the immobility duration. This anti-depressive effect was depleted entirely when the OT receptor blocker atosiban was used and when OT gene knocked out rats were used (Matsushita et al. 2012). This comes again to confirm that both direct and indirect release of OT helps in reverting depressive behavior and noting the role of endogenous OT in depression.

Anti-depressants have been related to neurogenesis and neuroplasticity (D'Sa and Duman 2002). Many factors used in treating behavioral symptoms of depression have also shown an increase in neurogenesis, like the electroconvulsive therapy (Scott et al. 2000), anti-depressant drugs like SSRIs (Malberg et al. 2000), and even exercise (Ernst et al. 2006). Moreover, the time taken for recovery in humans is the same time taken to induce neurogenesis in experimental rats, suggesting a strong correlation between neurogenesis and depression (Scott et al. 2000; Ernst et al. 2006). Ji H et al., previously noted that decreased OT level in rat's hypothalamus and hippocampus after a period of neonatal maternal deprivation (NMD) induced depressive-like behavior (Shin et al. 2010). Administrating IN-OT to the rats resolved this symptom; this was evident by the forced swimming test and the promoted hippocampal neurogenesis. For further investigations, an immunostaining technique of hippocampal tissue using an antibody against Ki67, a cell proliferation marker, revealed that IN-OT administration for 14 days caused a remarkable increase in Ki67 positive cells, suggesting that oxytocin has a role in progenitor proliferation of neurons (Becker and Wojtowicz 2007).

To justify, another study by Matsushita H et al. quantified the phosphorylated form of the transcriptional factor CREB. They verified that the centrally acting OT

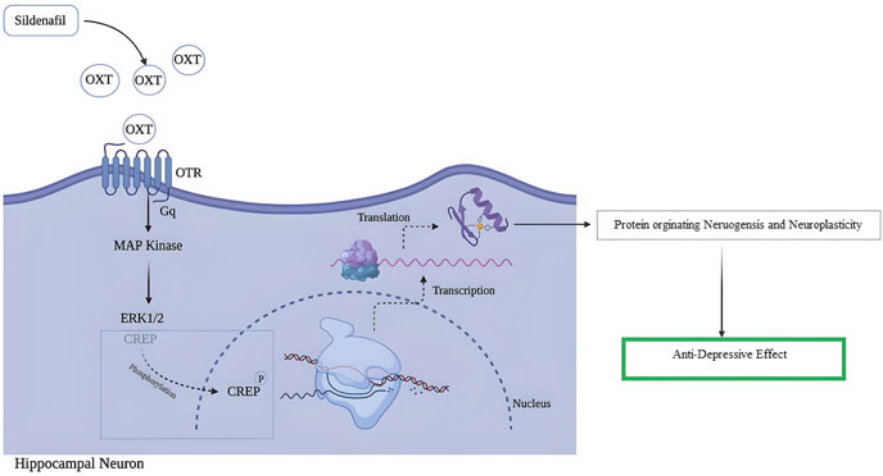

Fig. 5 The anti-depressive mechanism of oxytocin (OXT). The endogenously released OXT (with/without sildenafil stimulation) exerts an anti-depressive effect through the MAP kinase cascade inducing neurogenesis, neuroplasticity, and neural survival. OTR, oxytocin receptor; ERK1/2, sub-families of MAPK; CREB, cAMP-responsive element-binding protein. The figure has been developed based on the information in the following references: (Tomizawa et al. 2003; Duric et al. 2010; Berry et al. 2012; Matsushita et al. 2012)

released from the posterior pituitary gland resulted in the activation of the MAP kinase cascade, causing subsequent phosphorylation of the CREB transcriptional factor, thus inducing neurogenesis (Matsushita et al. 2012) (Fig. 5). This effect was also noticeable in human glioma cell lines, showing reproducibility regarding OT inducing neurogenesis (Bakos et al. 2013). Despite these results and the clear involvement of OT in the neurogenesis process, there is no human evidence proving that OT increases brain-derived neurotrophic factor (BDNF), the main indicator of neuronal growth.

Postpartum Depression (PPD) is another spectrum of depression (Guze 1995). Apart from the emotional burden on the mother, PPD can also impact the mother–infant relationship, causing a range of cognitive (Hay et al. 2001), emotional (Murray et al. 2011; Apter-Levy et al. 2013), and behavioral problems in children (Alpern and Lyons 1993). Since OT plays a role in adaptation, transitions, and adjustments at early motherhood, OT potential as an anti-depressive agent was considered in PPD research efforts (Cipriani et al. 2007; Kim et al. 2014). Intracerebroventricular injection of OT was able to induce a battery of maternal behaviors in "virgin" rats (Pedersen and Prange 1979; Pedersen et al. 1982), a phenomenon that was reproducible in mice (McCarthy 1990) and sheep (Keverne and Kendrick 1992). These findings ended when OT antagonists were infused (Van Leengoed et al. 1987; Pedersen et al. 1994). Not many human studies tackled the OT effect in PPD, yet they are promising, significantly when modulating maternal behavior. In a randomized controlled trial, administration of IN-OT has shown increased connectivity between the amygdala and regions concerned with emotional

regulation in response to infant laughter (Riem et al. 2012). Hence, OT's anti-depressive effect regarding PPD encourages more research in this regard.

Observing the molecular aspects of depression, preclinical studies revealed the underlying anti-depressive mechanisms of OT (McQuaid et al. 2014). Unfortunately, this is less certain when it comes to clinical results. De Cagna F et al. conducted a systematic review that assessed the role of IN-OT in depressive and anxiety disorders, which included seven studies with a total of 153 patients; four of the seven studies recruited post-natal depression (PND), and the remaining three recruited major depressive disorders (De Cagna et al. 2019). In all studies, patients received a single dose of IN-OT except for one study by Clarici et al., evaluating the implications of 12 days daily OT on post-natal depression (Clarici et al. 2015). The latter displayed no significant difference in depressive symptoms between OT and placebo groups. The same results were shown by the six other studies, where heart rate, blood pressure, and salivary cortisol have also demonstrated no significant difference between OT-treated group and placebo (MacDonald and Feifel 2013). Functional magnetic resonance imaging (fMRI) during Reading the Mind in the Eyes Test (RMET) showed different engagement of different brain regions in depressed patients after IN-OT, different from the baseline pattern that was established from untreated depressed patients (Pincus et al. 2010). Since most of these studies investigated the effect of single-dose OT upon depression, extending the trial duration and investigating a dosing regimen of increased frequency are strongly recommended.

2.7 Autism Spectrum Disorder (ASD)

Autism Spectrum Disorder is a neurodevelopmental disorder marked by social communication and speech impairment, limited interest, and repetitive activities (Edition 2013). Despite the high prevalence of ASD, no therapy for the treatment of social deficits or repetitive activities associated with ASD has been recognized. Major release of OT is implicated in aspects of social activity such as social memory, social recognition, pair-bond formation between different species, separation anxiety, other forms of social attachment, and stress response control (Meyer-Lindenberg et al. 2011).

Despite the highly debatable issue of the capacity of peripheral OT to accurately reflect central OT levels (Kagerbauer et al. 2013), and how OT release is dependent on social clues, questioning the validity of resting state plasma OT measurement (Andari et al. 2010; Kagerbauer et al. 2013). Plasma levels of OT provided a significant association with ASD symptom as many studies found low mean OT plasma levels in children with autism, compared to age matched healthy controls (Modahl et al. 1998; Husarova et al. 2016). Green et al. (2001) leveraged Vineland Adaptive Behavior Scale (VABS) to report elevated scores in typically developing children compared to lower scores in children diagnosed with ASD, noting increased inactive forms of OT in children diagnosed with ASD, speculating that defects in OT

processing have a role in ASD. Strikingly, in adults the OT plasma concentrations are off beat; adults with ASD fashioned increased OT plasma in comparison to healthy adults(Jansen et al. 2006). Differences in OT levels between age groups can be attributed to developmental factors, subgroup differences, or intellectual development differences. There is a plausible association between decreased OT plasma levels and ASD, in addition to evidence suggesting that dysfunctional OT processing and developmental changes can be associated with oxytocinergic system, and by turn OT plasma levels. Longitudinal studies are a necessity, with wider range population including different age groups, and different developmental stages and categories, to settle those debates, while endorsing a great asset to help in understanding ASD pathophysiology with close proximity to oxytocinergic system.

A human study by Guastella et al. was the first to demonstrate that IN-OT promotes emotion recognition in young people with ASD (Guastella et al. 2010). Another human study by Anagnostou et al. indicated that daily administration of IN-OT at 0.4 IU/kg was safe and therapeutic in children and adolescents with ASD (Anagnostou et al. 2014). Some studies have demonstrated the role of OT in adults diagnosed with autism or Asperger's disorder, where OT administration facilitated the processing and retention of social information and reduced the repetitive behavior (Hollander et al. 2003, 2007).

Single-dose intranasal OT improved performance on many social cognition-related tasks (Graustella and MacLeod 2012). Single-dose studies of OT with doses ranging from 10 to 40 international units (IU) have shown enhanced recognition of facial emotions (Averbeck et al. 2012) and greater social-cognitive work performance, e.g. assessment of tasks, empathy, and deception (Davis et al. 2013; Cacciotti-Saija et al. 2015).

The effect of multiple doses OT on neural activation of healthy adult men while processing emotional states was investigated by Bernaerts, S et al; reporting bilateral attenuation of amygdala activity, noting that amygdala attenuation persisted the intervention period of 4 weeks, and one-year post-treatment. Also, reporting bilateral posterior superior temporal sulcus increased activity, that persisted till the 1-year follow-up (Bernaerts et al. 2016). Although this effect was noticed on healthy individuals, but its shows OT multiple dose regimen ability to induce long-lasting effect on neural circuitry concerned with emotional processing, this observation should be investigated in patients with ASD, as it shows OT potential to induce a disease-modifying effect to be leveraged in ASD therapeutics. Neural effect of multiple dose regimen of OT was also investigated by Watanabe, T et al; who reported that six weeks IN-OT administration reduced autism core symptoms, through Autism Diagnostic Observation Scale evaluation, in addition to increasing connectivity between anterior cingulate cortex and dorso-medial prefrontal cortex. However, the study did not report long-lasting neural effects due to the lack of follow-up assessment (Watanabe et al. 2015). On the contrary, clinical studies recently showed no effect of single-dose IN-OT in males with ASD without intellectual disability (Mayer et al. 2021), while another revealed no effect on the social and cognitive function in ASD children after long-term (24-weeks) low-dose IN-OT treatment (Sikich et al. 2021).

Simultaneously, Siu et al. examined the involvement of OTR in the pathogenesis of ASD and ADHD. Those have shown methylated DNA in the first intron of the OTR gene associated with decreased IQ and social problems when compared to healthy control (Siu et al. 2021).

Since ASD is addressed as a group of neurodevelopmental disorders, with different shades, its pathophysiology should be investigated through OT with respect to gender, different age groups, and different neurodevelopmental milestones, aiming to fill the gaps in ASD pathophysiology, opening doors to optimize OT potential as a treatment for ASD. Principally, OT can offer a degree of social adaptation, where individuals with excessive social stress and anxiety receiving OT have shown attenuated amygdala reactivity resulting in anxiolytic effect (Labuschagne et al. 2010; Ma et al. 2016), while in case of individuals with low social engagement, OT would increase amygdala activity to enhance socio-communicative behaviors (Domes et al. 2014; Gordon et al. 2016). Taking together all those clinical investigations, one could suggest OT as a possible treatment for ASD patients. However, clinical evidences are heterogenous in effect and still lacking mechanistic molecular studies, which might explore the possible variations in the OTR gene and ASD pathophysiology. Moreover, empirical studies are needed to take the dose, frequency, the route of administration, and the interindividual variations into consideration.

3 Effect of OT During the COVID-19 Pandemic

Home quarantine and physical distancing from friends, family, and colleagues cause spectacular social isolation. This, in turn, causes stress, fear, and anxiety. Recently, there has been growing concern about the effect of social distancing on mental health, an effect of the pandemic that is not less important than the physical well-being (Krendl and Perry 2021; O'Connor et al. 2021). While social isolation decreases the socially stimulated OT release, subsides the neuronal branching, and reduces the central synthesis of neuropeptides (Ross et al. 2019; Heck et al. 2020), social support is essential for the psychological well-being with/without any disease (Kikusui et al. 2006). As the central OT neurons are in part responsible for such effects (Tsai et al. 2019; Riem et al. 2020), IN-OT administration should be put into focus during the COVID-19 pandemic to prevent serious psychological consequences associated with social isolation.

4 Conclusion

Apart from the hormonal aspect, OT has a profound role in multiple aspects of normal brain function. Oxytocinergic system dysfunction is associated with the pathophysiology of multiple neurological disorders, and administrating OT resolves

these pathologies and revert symptoms. Table 1 presents the most significant treatment outcomes of cell culture, animal, and clinical studies available for using OT in brain disorders. Figure 6 summarizes the effect of oxytocin in the pathophysiology and treatment of the major neuropsychiatric, neurodegenerative, and neurodevelopmental disorders discussed in this review and sheds light on all OT mechanistic pathways presented.

Interesting is the possible interconnection of the discussed pathways. Regarding the neuroprotective effect of OT observed in Parkinson's disease, such an effect can be beneficial in other diseases as Epilepsy and Migraine against neural insult caused by both diseases' pathophysiology. Especially in Epilepsy, as there has been an ongoing investigation about antiepileptic drugs neuroprotective potential, counting it as a disease-modifying property (Willmore 2005).

Moreover, OT hippocampal neurogenesis observed in depression can be advantageous regarding other diseases, especially neurodegenerative ones providing a mode of cell-based therapy. Animal models and postpartum studies of Parkinson's disease showed defective neurogenesis (Marxreiter et al. 2013); therefore, in addition to OT neuroprotective effect, and decreasing neuronal striatal loss, OT potential to induce striatal neurogenesis should be investigated. The effect of neurogenesis regarding epilepsy is controversial; neurogenesis after an epileptic episode showed an increase in neural progenitors, production of ectopic granule cells, and neural hypertrophy. In addition, inhibiting neurogenesis using non-pharmacological and pharmacological agents has shown the ability to decrease the severity of epileptic seizures (Cho et al. 2015). Therefore, documented neurogenesis adverse effects in epilepsy should be investigated concurrently with OT antiepileptic effect.

To sum up, OT being an endogenous peptide, having multiple roles regarding brain function, and its disruption is involved in some neurological disease's pathophysiology, should encourage considering its psychopharmacological tenets collectively, not individually on a neurological level, thus facilitating our understanding of OT and helping to make room for it in psychopharmacology. Since OT treatment also lacks significant side effects, more human studies in clinical settings are needed to fill the gaps in the paradigm of OT. Meta-analytic approaches can get good use of the data available and further influence the direction of clinical research.

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Declarations of Interest None.

Table 1 The most significant treatment outcomes of cell culture, animal, and clinical studies available for using OT in neuropsychiatric, neurodegenerative, and neurodevelopmental disorders

Disease	Animal studies					Human studies				
	First author, year	Dose/duration	Route	Sample size (n)/animal	Main findings	First author, year	Dose/duration	Route	Sample size (n)/sex	Main findings
Epilepsy	Erbas et al. (2013b)	40, 80, 160 nmol/kg OT/5 days	i.p.	6/rats	OT 80 nmol/kg and 160 nmol/kg effectively prevented PTZ-induced seizures					
	Khoshneviszadeh et al. (2016)	5, 10, 20, 40 mg/kg Sildenafil/SA (30 min before seizure threshold determination)	i.p.	8–10/mice	Administrated sildenafil induced OT release resulting in the proconvulsant effect in PTZ model, interplaying CREB transcription factor phosphorylation subsequent to OT release. Effect terminated by co-administrating Atosiban					
	Erfanparast et al. (2017)	10, 20 ng/site OT/SA (6 min before seizure induction)	ih	6–10/rats	– OT 10 ng and 20 ng/site terminated seizures at hippocampal level and					

					potentiated the effect of diazepam. – The effect was terminated by Atosiban		
	Rahimian et al. (2020)	0.25, 0.5 mg/kg OT/SA (60 min before seizure threshold determination) 5, 10, 20, 40 mg/kg mg/kg Sildenafil/SA (30 min before seizure threshold determination)	i.p.	6–10/mice	– 0.5 mg/kg i.p. OT exhibited proconvulsant impact compared to control – Sildenafil significantly increased cortical CN activity that was attenuated by administration of Atosiban prior to OT		
	Wong et al. (2021)	50 µg/50 µl OT/SA (20 min before seizure induction)	i.c.v.	10/mice	In epilepsy Scn1a mouse model, nanoparticles encapsulating OT showed sustained prophylactic effect against seizures, and revered normal social behavior		

(continued)

Table 1 (continued)

Disease	Animal studies					Human studies				
	First author, year	Dose/duration	Route	Sample size (n)/ animal	Main findings	First author, year	Dose/ duration	Route	Sample size (n)/sex	Main findings
Schizophrenia	Lee et al. (2005)	1000 ng/site OT/ SA	Central nucleus infusion	8–10/ rats	A single OT infusion into the central nucleus of the amygdala reverts negative symptoms induced by phencyclidine	Gibson et al. (2014)	24 IU OT/twice daily for 6 weeks	IN	8 Adults (6 male and 2 female)	OT reduced negative score according to the positive and negative symptoms scale
	Feifel et al. (2012)	0.04, 0.2, 1.0 mg/kg OT SA (30 min prior to behavioral testing)	s.c.	8–10/ rats	Three doses of OT increased prepulse inhibition (PPI) and decreased acoustic startle reflex (ASR) showing OT antipsychotic effect	Modabberni, Rezaei et al. 2013	20 IU OT twice daily for 1 week followed by 40 IU for 7 weeks	IN	20 Adults (17 male and 3 female)	
						Feifel et al. (2010)	20 IU twice daily, for the first week, then 40 IU for 3 weeks	IN	6 Adults	

Parkinson's disease	Erbaş et al. (2012)	160 μg/kg OT/ daily for 20 days	i.p.	7/rats	– OT administration did not show significant improvement in behavioral or postural features. Yet, It decreased dopaminergic striatal neuronal loss – OT showed neuroprotective effect by decreasing pro-apoptotic agents and increasing anti-apoptotic agents	
	Erbas et al. (2013a))	160 μg/kg OT/daily for 21 days	i.p.	7/rats	OT administration showed neuroprotective effect, in addition to restorative dopaminergic neurons capacity	
Migraine	Tzabazis et al. (2017)	8 μg OT/ SA	IN	6	Pretreatment with IN-OT in standard nitroglycerin model of migraine decreased number of neurons displaying C-fos activity and activation of central TNC neurons	

(continued)

Table 1 (continued)

Disease	Animal studies					Human studies				
	First author, year	Dose/duration	Route	Sample size (n)/ animal	Main findings	First author, year	Dose/ duration	Route	Sample size (n)/sex	Main findings
Depression	Pedersen and Prange (1979)	0.4 μg OT/SA	i.c.v.	13/rats	OT was able to induce maternal behaviors in "virgin" rats	Riem et al. (2012)	24 IU OT/SA	IN	22/female adults	OT increased connectivity between amygdala and regions concerned with emotional regulations in nulliparous women as a response to infant laughter
	Pedersen et al. (1982)	400 μg OT/ SA	i.c.v.	13/rats		De Cagna et al. (2019)	24 IU OT/SA	IN	153 adults	A meta-analysis of 7 randomized clinical trials. Single dose of OT showed no significant improvement in depressive symptoms between OT and placebo
	Meisenberg (1982)	20 μl, 50 μl OT/SA	i.c.v.	10/ mice and 10/rats	OT showed antidepressive behavior reflected in forced swimming test	Clarici et al. (2015)	16 IU OT/daily for 3 months	IN	5/female adults	No significant difference between OT and placebo
	Arletti and Bertolini (1987)	0.125, 0.25, 0.500, 1.0,	i.p.	10/ rats	– OT showed reduced duration	Bakos et al. 2013				OT induced neurogenesis in

		2.0 mg/kg OT for 10 days (60 min before behavioral testing)			of immobility – The same observed upon chronic testing of 0.5 mg/kg/day for 8 days		1 μM OT/for 48 h			human glioma cell lines
	Insel and Winslow (1991)	100, 500, 1000 ng OT/SA	i.c.v.	12/rats	OT administration reverted distress after a period of social isolation in infant rats, this effect was diminished by OT antagonists					
	Matsushita et al. (2012)	10, 20, 30, 60, and 80 mg/kg Sildenafil for 3 days.	i.p.	8/ rats	– Sildenafil showed dose-dependent effect in forced swimming test – The study shed light on OT neurogenesis mechanism; by quantifying phosphorylated transcriptional factor CREB, resulting in enhanced neurogenesis. This effect was depleted by Atosiban, and using rats with knocked out OTR					

(continued)

Table 1 (continued)

Disease	Animal studies					Human studies				
	First author, year	Dose/duration	Route	Sample size (n)/ animal	Main findings	First author, year	Dose/ duration	Route	Sample size (n)/sex	Main findings
	Ji et al. (2016)	0.1 mg/kg OT daily for 14 days	IN	5/rats	OT reverted maternal isolation depressive-like behaviors, reflected in plasma, hypothalamus, and hippocampus levels of OT, as well as forced swimming test					
Autism spectrum disorder						Hollander et al. (2003)	10 U/ml Pitocin infusion/4 hours SA	i.v.	15 Adults (14 male and 1 female)	OT in adults diagnosed with ASD or Asperger's disorder facilitated the processing and retention of social information, in addition to reducing repetitive behaviors
						Hollander et al. (2007)	10 U/ml Pitocin infusion/4 hours SA	i.v.	15 Adults (14 male and 1 female)	
						Guastella et al. (2010)	18–24 IU OT SA	IN	16 male children	OT promoted emotion recognition in young people with ASD

		Anagnostou et al. (2014)	0.4 IU/kg OT/twice daily for 12 weeks	IN	15 children and adolescents (11 male and 4 female)	OT was safe and therapeutic in children and adolescents with ASD
		Watanabe et al. (2015)	48 IU OT/6 weeks	IN	9 male adults	OT showed a reduction in autism core symptoms
		Bernaerts et al. (2016)	24 IU OT/SA	IN	23 male adults	OT enhanced emotional processing, and reported bilateral attenuation of amygdala that persisted for 1 year
		Mayer et al. (2021)	18–24 IU OT/SA	IN	11 male children and adults	No effect of single-dose OT in males with ASD without intellectual disability
		Sikich et al. (2021)	48 IU OT/daily for 24-weeks	IN	146 children	No effect on the social and cognitive function in ASD children after long-term low-dose OT

OT Oxytocin, *IN* intranasal, *s.c.* subcutaneous, *ip* intraperitoneally, *ih*. *intrahippocampal*, *i.c.v.* intracerebroventricularly, *i.v.* intravenous, *SA* single administration, *PTZ* Pentylenetetrazole, *SCZ* Schizophrenia, *PD* Parkinson's Disease, *ASD* autism spectrum disorder

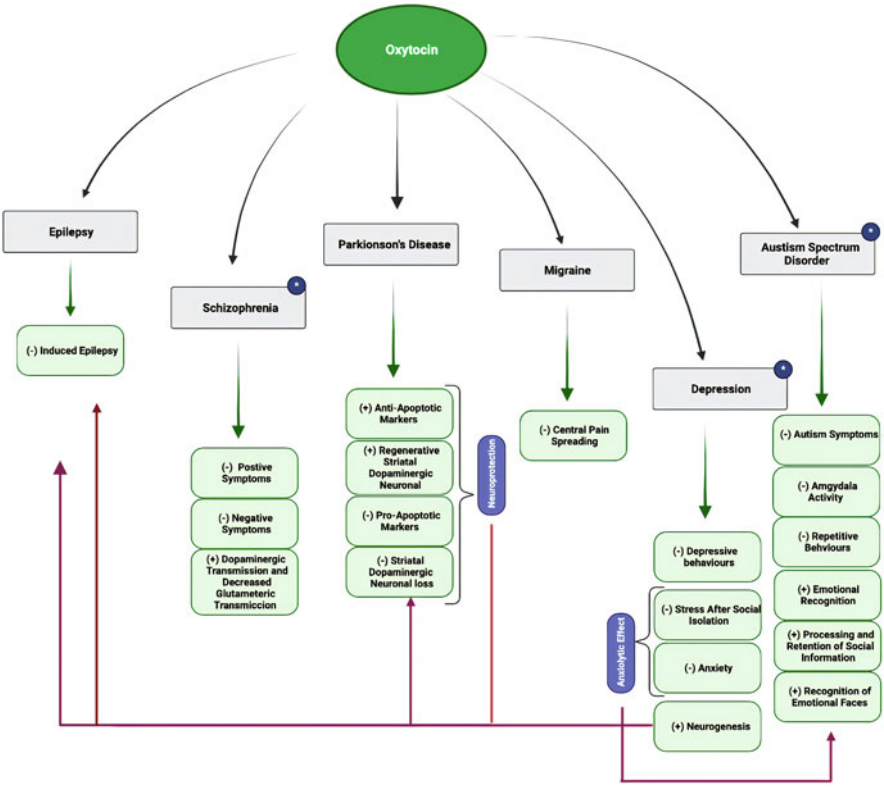

Fig. 6 Schematic diagram showing the effect of oxytocin in the pathophysiology and treatment of major neuropsychiatric, neurodegenerative, and neurodevelopmental disorders. The (+) sign indicates OT increasing/inducing the mentioned effect, while the (-) sign indicates OT decreasing the mentioned effect. The red arrows point to OT mechanisms that could be useful to understand other diseases and still need further investigation. The (*) indicates available clinical data about the disease

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Role of Distinct Fat Depots in Metabolic Regulation and Pathological Implications

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Contents

Abstract People suffering from obesity and associated metabolic disorders including diabetes are increasing exponentially around the world. Adipose tissue (AT) distribution and alteration in their biochemical properties play a major role in the pathogenesis of these diseases. Emerging evidence suggests that AT heterogeneity and depot-specific physiological changes are vital in the development of

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insulin resistance in peripheral tissues like muscle and liver. Classically, AT depots are classified into white adipose tissue (WAT) and brown adipose tissue (BAT); WAT is the site of fatty acid storage, while BAT is a dedicated organ of metabolic heat production. The discovery of beige adipocyte clusters in WAT depots indicates AT heterogeneity has a more central role than hither to ascribed. Therefore, we have discussed in detail the current state of understanding on cellular and molecular origin of different AT depots and their relevance toward physiological metabolic homeostasis. A major focus is to highlight the correlation between altered WAT distribution in the body and metabolic pathogenesis in animal models and humans. We have also underscored the disparity in the molecular (including signaling) changes in various WAT tissues during diabetic pathogenesis. Exercise-mediated beneficial alteration in WAT physiology/distribution that protects against metabolic disorders is evolving. Here we have discussed the depot-specific biochemical adjustments induced by different forms of exercise. A detailed understanding of the molecular details of inter-organ crosstalk via substrate utilization/storage and signaling through chemokines provide strategies to target selected WAT depots to pharmacologically mimic the benefits of exercise countering metabolic diseases including diabetes.

Keywords Adipomyokines diabetes · Beiging · Brown fat · Exercise · Insulin resistance · White fat

Abbreviations

AgRP	Agouti-related peptide
AKT1	A strain k thymoma/transforming protein kinase 1
ANF	Atrial natriuretic factor
ANGPTL4	Angiopoietin-like 4
ANP	Atrial natriuretic peptide
ARC	Arcuate nucleus
AT	Adipose tissue
BAIBA	β-aminoisobutyric acid
BAT	Brown adipose tissue
BCAAs	Branched-chain amino acids
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BNP	B-type natriuretic peptide
C/EBP	CCAAT/enhancer binding proteins
CAR4	Carbonic anhydrase
CD	Cluster of differentiation
CIDEA	Cell death-inducing DFFA-like effector A
CNS	Central nervous system
Cox	Cytochrome c oxidase
DAG	Diacylglycerol

dsWAT	Deep subcutaneous WAT
Ear2	Eosinophil-associated ribonuclease A-2
EBF	Empty body fat
EGR1	Early growth response 1
EN1	Engrailed-1
Epsti1	Epithelial stromal interaction 1
Eva1	Epithelial V-like antigen 1
eWAT	Epididymal white adipose tissue
EWS	Ewing sarcoma
FGF9	Fibroblast growth factor 9
FNDC4	Fibronectin type III domain-containing 4
FOXC2	Forkhead box C2
FSP27	Fat-specific protein 27
GDF15	Growth differentiation factor 15
GLUT1	Glucose transporter protein type 1
GPR	G-protein coupled receptor
Grb10	Growth factor receptor bound protein 10
GSK-3	Glycogen synthase kinase 3
HIF1	Hypoxia-inducible factor
HSL	Hormone-sensitive lipase
Hspb1	Small heat shock protein beta-1
hTBC1	Human TBC1 isoform
IKK	IκB kinase
IL6	Interleukin 6
ILC2	Innate lymphoid type 2 cells
InR	Insulin resistance
IR	Insulin receptor
IRE1α	Inositol-requiring transmembrane kinase endoribonuclease-1α
IRF 4	Interferon regulatory factor 4
IRS-2	IR substrate 2
iWAT	Inguinal WAT
JNK	c-Jun N-terminal kinase
KLF11	Kruppel-like factor 11
LHX8	LIM Homeobox8
MCP-1	Monocyte chemoattractant protein-1
MEK	Mitogen-activated protein kinase
Met-Enk	Methionine-enkephalin
METRNL	Meteorin-like hormone
MSCs	Mesenchymal stem cells
mTOR	Mechanistic target of rapamycin
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
NE	Norepinephrine
NRG-4	Neuregulin 4

OGT	O-GlcNAc transferase
P2RX5	P2X purinoceptor5
PAI-1	Plasminogen activator inhibitor 1
PAT	Phosphate acetyl transferase
PAX7	Paired box gene 7 protein
PDGFR	Platelet-derived growth factor receptor
PEDF	Pigment epithelium-derived factor
PEPCK	Phosphoenol pyruvate carboxy kinase
PERK	PKR-like ER protein kinase
PET	Positron emission tomography
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
POMC	Proopiomelanocortin
PRDM	PR domain zinc finger protein
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
RyR	Ryanodine receptor
S6K	S6 kinase beta-1
SERCA	Sarco/endoplasmic reticulum Ca2+-ATPase
SFRP4	Secreted frizzled-related protein 4
SFRP5	Soluble frizzled-related protein 5
SHIP	SH2 domain-containing inositol 5-phosphatases
SkM	Skeletal muscle
SLC25A44	Solute carrier family 25 member 44
SOCS	Suppressor of cytokine signaling
SP100	Sp100 nuclear antigen
TAF7L	TATA-binding protein-associated factor 7L
Tcf7l	T-cell-specific factor 7 like 1
Tfam	Mitochondrial transcription factor A
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TMEM26	Transmembrane protein 26
TNFSF14	Tumor necrosis factor superfamily member14
TNFα	Tumor necrosis factor α
UCP1	Uncoupling protein 1
VEGF-A	Vascular endothelial growth factor A
WAT	White adipose tissue
WISP	WNT1-inducible signaling pathway protein
WNT	Wingless-related integration site

YBX1	Y-box binding protein 1
ZFP	Zinc finger protein
ZIC1	Zinc finger protein of the cerebellum 1

1 Introduction

In the last few decades, obesity and its related disorders have taken the form of a pandemic affecting more than two billion people worldwide. Obesity is a physiological state with complex metabolic alterations impacting multiple organ systems of the body. Epidemiological studies have shown that obesity worsens the conditions of several other diseases including Type2 diabetes mellitus (T2DM), cardiovascular diseases, stroke, and even cancers (Kyrou et al. 2018). Obesity originates from a misbalance in the utilization and storage of energy substrates (lipids and sugars) that alter the canonical mechanisms in WAT and other tissues associated with the progression of T2DM and other metabolic disorders (Ormazabal et al. 2018; Romieu et al. 2017). Among mammals, the major site of energy storage is WAT which is distributed in different parts of the body as discrete depots. Apart from white, another major type of AT found in mammals is termed BAT. In addition to energy storage, WAT is shown to meet some other physiological needs such as physical protection as shock absorption and insulation as blubber layer (Choe et al. 2016). While WAT primarily serves as an energy storage organ, BAT serves as fat utilization site in converting the chemical energy of substrates into heat that is employed to maintain internal body temperature. Except for being fatty, these two AT (WAT and BAT) have nothing in common; they differ in developmental lineage, morphological appearance, texture, cellular biochemistry as well as physiological function (Billon and Dani 2012). The activities of these two tissues have been shown to greatly influence the whole-body metabolic rate of mammals including humans and as obvious this topic has attracted significant research attention. Interestingly, some recent studies have indicated the possibility of interconversion between BAT and WAT (Lee et al. 2014a), but whether this switching is partial or complete as well as its mechanism is not fully defined.

The structural and functional heterogeneity of various AT sites has also generated an idea that these adipocytes can group as distinct fat depots other than being pure WAT or BAT. One such transitional form of adipocyte cluster is termed as “Beige” adipocytes discovered in subcutaneous WAT (sWAT) depots in rodents as well as in humans (Brown 2020; Sidossis et al. 2015). Also, beige adipocyte abundance and degree of beiging depend upon physiological signals that vary across WAT depots (Romieu et al. 2017; Rabiee 2020).Their transient appearance and disappearance of beige adipocytes are highly correlated with whole-body metabolic demand (energy surplus and deficient states) (Rabiee 2020). It is suggested that the beige adipocytes are recruited for thermogenesis within WAT and are induced by external stimuli like cold, exercise (Valgas et al. 2019; Phillips 2019; Rowland et al. 2015). BAT was traditionally considered as a thermo active metabolic sink, but after the discovery of beige cells, WAT is also being proposed to provide such a site. The seesaw

equilibrium of energy storage–energy utilization lies greatly in functional capacities of AT depots. Therefore, different adipocytes including BAT, WAT, and beige have been targeted by pharmacological agents to enhance energy consumption and counter metabolic diseases (González et al. 2017; Thyagarajan and Foster 2017). In mammals, WAT depots govern metabolic homeostasis by influencing nutrient mobilization and thermogenesis mediated by several signaling pathways including insulin (Chait and den Hartigh 2020). Interestingly, exercise or elevated physical activity status directly influences WAT physiology including its beiging in different depots (Dewal and Stanford 2019). Recent studies have identified several adipocytes, myokines, and hepatocytes mediating functional crosstalk between fat tissue and muscle during various physiological and/or pathological states (Dewal and Stanford 2019; Rodríguez et al. 2017). It has been proposed that pharmacological activation of WAT or skeletal muscle (SkM) function mimicking exercise can retard metabolic diseases (Yu et al. 2021; Piccirillo 2019; Olesen et al. 2014; Cabrero et al. 2001). However, WAT heterogeneity is an important aspect that can affect the outcome by a pharmacological agent and may be a major cause of not being able to effectively enhance whole-body energy status. In obese individuals, these depot-specific differences transform into fat distribution patterns that also display gender-based variations implying T2DM (Jensen 2008; Karastergiou et al. 2012). Therefore, the differential role of WAT depots needs more detailed investigation to gain insight in selective targeting of some individual WAT depots. Here, we are trying to highlight the structure/function of different WAT depots and their biochemical and physiological roles in metabolic diseases.

2 Heterogeneity of Fat Depots: Morphology, Molecular Variability, and Differentiation

Adipocytes are localized distinctly as aggregated masses termed as AT or depot in different parts of the mammalian body as shown in Fig. 1. Moreover, in the AT the relative abundance of components such as preadipocytes, endothelial cells, macrophages, lymphocytes, blood vessels, and loose connective tissue varies across the different depots (Frese et al. 2016). Due to this, each depot displays uniqueness in their protein expression profile, texture, shape, and 3-dimensional arrangement. This process is regulated developmentally as well as in a gender-specific manner (Rodgers and Sferruzzi-Perri 2021; Keuper and Jastroch 2021).The initial biogenesis of the major adipocytes (white, brown, and beige) occurs during perinatal development from the mesenchymal stem cells (MSCs) regulated by a set of common transcription factors (TFs) (Chooi et al. 2019; Harms and Seale 2013). MSCs undergo differentiation and become committed preadipocytes that are unique for white and brown lineages (Fig. 2). This initial adipogenic differentiation program is regulated by PPARγ that is induced by C/EBP family members, especially C/EBP-β and C/EBP-δ (Ambele et al. 2020). During the latter part of adipogenesis, C/EBP-α

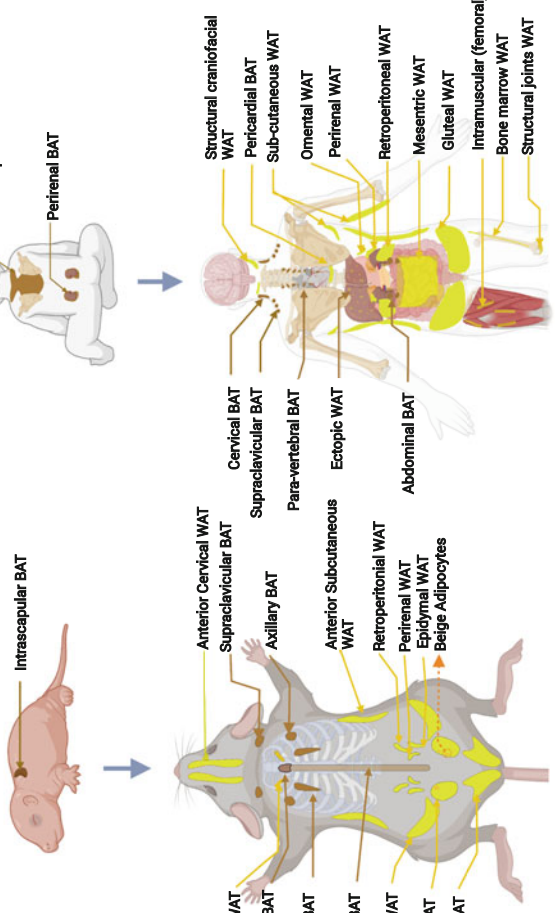

Fig. 1 Temporal and spatial heterogeneity in AT depots in rodents and humans. In the neonatal stages of both cases, BAT is prominent (interscapular BAT being the most conspicuous one) and WAT is a minor component, just starting to grow. However, in the adult stages, WAT depots are more abundant and distinct. In human adults, BAT is qualitative and quantitatively much minor than in adult rodents. WAT is broadly categorized into visceral AT (vWAT, primarily around visceral organs) and subcutaneous AT (sWAT, located beneath the skin). The vWAT can be subdivided into gonadal (as epididymal in males and periovarian in females), mesenteric, perirenal (prWAT, around kidneys), and retroperitoneal. The sWAT can be further classified as anterior sWAT, found between the cervical and axillae regions including the interscapular WAT (isWAT) and posterior sWAT, spreading from dorsolumbar to the gluteal region encompassing inguinal (iWAT). Other minor BAT depots are distinctly shown, although in most cases these sites are closely integrated with the surrounding organs. Created withBioRender.com

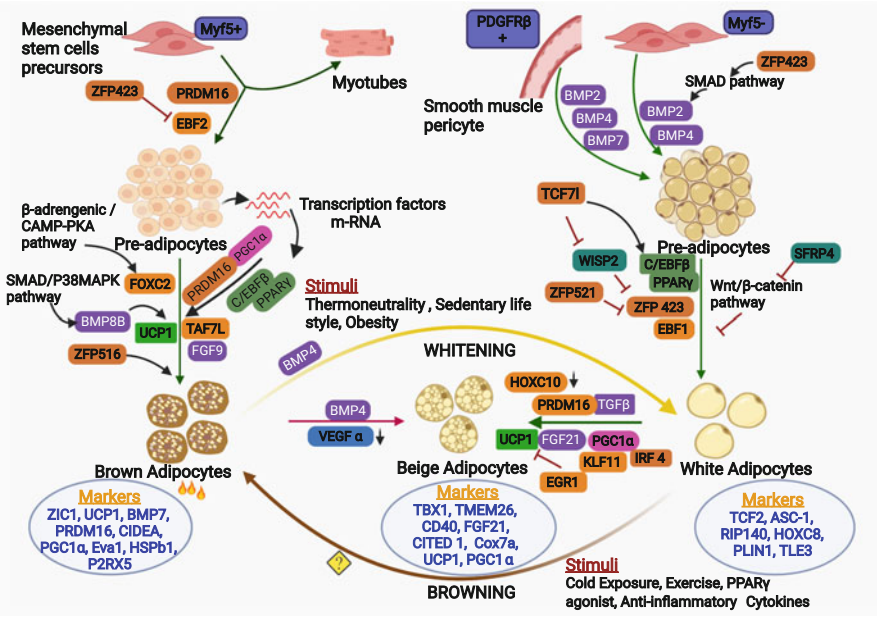

Fig. 2 Scheme showing pathways and processes involved in white-beige-brown fat/adipocyte interconversions. The BAT and WAT depots have a distinct developmental origin. While BAT is close to skeletal muscle, WAT is close to smooth muscle in their cellular lineage. PRDM16 is the key transcription factor that determines the differentiation of mesenchymal stem cell precursors into BAT lineage. Another transcription factor "ZFP423" helps in the induction of commitment toward WAT cellular lineage. The molecular markers of mature BAT, WAT, and beige adipocytes are shown in blue fonts. The possibility of interconversion of BAT to WAT and vice versa has been proposed and several stimuli and transcription factors/cytokines are shown suggested to mediate this process. Created withBioRender.com. Factors regulating adipocytes differentiation are represented in colored box as such: Zinc finger proteins, Cytokines, angiogenesis factor, thermogenic gene, transcription coactivators for energy metabolism, transcription factor for differentiation, cell signaling pathway regulators, and precursor cell markers

plays a key role in maintaining PPARγ expression and both these TFs cooperatively modulate the expression of adipocyte-specific genes involved in lipid metabolism, storage, and cytokine secretion (Moseti et al. 2016). Despite the similarity in early adipogenesis, each of the adipose depots shows remarkable variability in several attributes.

2.1 *Not All White Adipose Tissue (WAT)s Are Physiologically Identical*

The unique MSCs that generate white adipocytes during development do not express the key TF, Myf5 (Fig. 2). The commitment in these MSCs is induced by bone

morphogenetic protein (BMP) family cytokines (especially BMP2 and BMP4) by the modulation of the SMAD pathway, converting them into preadipocytes. It is interesting to point out that BMP4 is capable of the conversion of brown adipocytes to white in the BAT (Qian et al. 2013; Denton et al. 2019). While zinc finger protein (ZFP) 423, T-cell-specific factor 7 like 1 (TCF7l), and early B cell factor (EBF) 1 positively induce white adipogenesis; contrastingly ZFP521 and WNT1-inducible signaling pathway protein (WISP) 2 negatively regulate the process (Addison et al. 2014; Shao et al. 2016a; Cristancho et al. 2011; Hammarstedt et al. 2013; Gupta et al. 2010). ZFP423 drives early-stage adipogenesis by SMAD and BMP pathways and suppressing factors like EBF2, Prdm16 in the white adipocyte precursors (Addison et al. 2014; Shao et al. 2016a; Gupta et al. 2010). ZFP423 also regulated EBF1 by the formation of a heterodimer, which is inhibited by ZFP521 and WISP2 retarding the adipogenesis process. TCF7l1 promotes adipogenesis by repressing the WNT pathway and cell structural genes while enhancing the expression of PPARγ (Cristancho et al. 2011). C/EBPβ works in close association with PPAR to promote WAT adipogenesis (Rosen et al. 1999). Another protein called secreted frizzled-related protein 4 (SFRP4) reduces commitment toward brown adipocyte lineage and mediates white adipogenesis in a depot-specific manner; while positively in vWAT and eWAT, negatively in iWAT (Guan et al. 2018, 2021). White adipocytes also express receptors for several hormones like insulin, glucagon, catecholamines, and glucocorticoids mediating interorgan-crosstalk regulating energy homeostasis (Kuo et al. 2015). The mature white adipocytes are typified by the expression of some transcriptional genes and genes for lipid droplets associated proteins (TCF2, ASC-1, RIP140, HOXC8, PLIN1, and TLE3) (Giordano et al. 2016; Shijun et al. 2020; Ussar et al. 2014; Onogi et al. 2020; Inagaki et al. 2016; Nanduri 2021; Ma et al. 2015).

Depending on the location in the body WAT has been categorized as visceral AT (vWAT, primarily around visceral organs) and subcutaneous AT (sWAT, located below the skin). Types of WAT are shown in Fig. 1. Rodents, especially mice have been used as a model for studying whole-body energy homeostasis. Interestingly, the distributions of WAT, as well as its sexual dimorphism, do differ between humans and rodents. In humans, the anterior sWAT has been distinguished based on depth as superficial subcutaneous WAT (ssWAT) or deep subcutaneous WAT (dsWAT), which is absent in rodents (Chusyd et al. 2016). The posterior sWAT in the human body is mainly localized in the abdomen, buttocks, and thighs and has been considered to be analogous to the iWAT of rodents. In women, sWAT is more conspicuous and vWAT is lesser than men (Demerath et al. 2007; Després et al. 2000). Further, in obese women, the adipocytes in anterior sWAT undergo hypertrophy, as opposed to hyperplasia in the posterior sWAT during weight gain (Jensen et al. 1989). Due to this, women and men after becoming obese display characteristic pear and apple body shapes, respectively (Karastergiou et al. 2012; Bloor and Symonds 2014). In contrast, rodents do not exhibit a clear sexual dimorphism (Chusyd et al. 2016). Additionally, rodent sWAT is separated from dermal AT by a smooth muscle layer whereas; in humans, the sWAT is continuous with dermal AT (Luong et al. 2019). The mass of perigonadal and peritoneal vWAT depots in

comparison to body weight is higher in rodents than humans. It has been observed that rodent females tend to accumulate more fat in posterior sWAT while women accumulate more in anterior sWAT (Chusyd et al. 2016). So, the different WAT depots show distinct features which can imply metabolic pathogenesis in a gender-specific manner (Keuper and Jastroch 2021).

2.2 *Brown Adipose Tissue (BAT): House of Futile Mitochondria*

In contrast to WAT, the BAT is a primary thermogenic organ that is highly vascularized with a lesser amount of loose connective tissue. BAT is abundant in several clades of eutherian mammals especially during neonatal stages and hibernation (Tapia et al. 2018); but, is found in most rodents throughout their life (Cannon and Nedergaard 2004). The brown adipocytes are characterized by their multilocular appearance due to small lipid droplets and the presence of numerous cristae-dense mitochondria that express UCP1 abundantly in the inner membrane (Ikeda et al. 2018; Michurina et al. 2021). Its function is coordinated through β-adrenergic stimulation and synergistic inputs from various endocrine mediators, especially the thyroid and steroids. The other proteins hallmarking BAT are FGF21, ZIC1, BMP7, PRDM16, CIDEA, PGC1α, Eva1, EBF3, Hspb1, P2RX5, and PAT2 (Harms and Seale 2013; Ussar et al. 2014; Rockstroh et al. 2015; Sharp et al. 2012; Waldén et al. 2012). The BAT in neonatal rodents can be considered as classical BAT with relatively uniform small lipid droplets and numerous mitochondria with highly abundant UCP1 expression. Postnatally, however, UCP1 and Tfam expression is gradually decreased, indicating the reduction of mitochondrial abundance, along with the increase in lipid droplet size (Liu et al. 2020). Moreover, mitochondrial activity, protein synthesis, and metabolism were higher in neonates compared to adult BAT (Liu et al. 2020). In rodents, the major BAT depot is located interscapular under the skin, whereas in humans its abundance is reduced during early life (Nedergaard et al. 2007). Interestingly, recent PET studies revealed the presence of BAT in limited quantities discretely in interscapular, supraclavicular, cervical, axillary, periaortic, peri-vertebral, and suprarenal areas in the human adults and that even respond to cold exposure (Ogawa et al. 2018).

The BAT adipocytes originate from myogenic precursor MSCs that express Myf5, EN1, and PAX7, thus a distinct cellular lineage than WAT (Fig. 2) (Wang and Seale 2016). During early gastrulation, in these progenitor cells, two factors, namely, Ewing sarcoma (EWS) and its binding partner Y-box binding protein 1 (YBX1) are upregulated, which activate BMP7 expression inducing commitment for BAT adipogenesis by modulating the key TFs such as PRDM16, PGC1α, PPARγ, C/EBPβ (Park et al. 2013). PRDM16 in partnership with EBF2 repress myogenesis by downregulating the expression of MyoD, myogenin in Myf5-expressing preadipocytes (Wang et al. 2014). Post-natal development and regulation

of BAT are much more complex due to the involvement of several reported factors such as FOXC2 (Forkhead box C2, a member of forkhead family protein), BMP8B, TAF7L (TATA-binding protein-associated factor 7L), FGF9 (fibroblast growth factor) (Xue et al. 2008; Zhou et al. 2013, 2014; Whittle Andrew et al. 2012; Cederberg et al. 2001; Mueller 2016; Shamsi et al. 2020). This early differentiation of BAT requires sympathetic activation and is reliant on mitochondrial biogenesis and the expression of thermogenic genes. While FOXC2 works through β-adrenergic-cAMP-Protein kinase A (PKA) signaling cascade (Cederberg et al. 2001), BMP8B mediates its effect through the SMADs/p38 MAPK pathway (Whittle Andrew et al. 2012). Interestingly, loss of TAF7L has been shown to cause activation of myocyte factors in BAT (Zhou et al. 2014), whereas loss of FGF9 affects UCP1 expression leading to impaired BAT development and thermogenesis (Shamsi et al. 2020). Further, differentiated BAT is highly responsive to caloric availability and plays a critical role in metabolic disorders.

2.3 Beige Fat: A Recent Discovery

In the last decade, exciting discoveries identified specialized preadipocytes in some of the WAT depots that can acquire BAT-like features and have been termed as "Beige adipocytes" (Fig. 2) (Wu et al. 2012). The stimuli for inducing beige fat can be external like cold, exercise, PPARγ agonists or internal such as immune function, chronic β-adrenergic response, and cancer cachexia (Arroyave et al. 2020; Chang et al. 2019; Markussen et al. 2017; Petruzzelli et al. 2014). The appearance of beige adipocytes and increased vascularization are suggested as the two major attributes of WAT-to-beige conversion (Harms and Seale 2013). These adipocytes express LHX8, Cox7a, PAT2, and P2RX5 similar to BAT and show low-level UCP1 expression associated with accelerated mitochondrial biogenesis as well as other thermogenic proteins like PGC1α (Ussar et al. 2014; Di Franco et al. 2014; Fang et al. 2020). In addition, beige adipocytes are unique in expressing proteins specific to themselves, not found in BAT or WAT. Such beige markers are TBX1, TMEM26, CD137, Epsti1, Ear2, SP100, CD40, CITED1, and CAR4 (Wu et al. 2012; Garcia et al. 2016; Wang et al. 2016; De Jong et al. 2015). The beige adipocytes cluster predominantly in the iWAT and anterior sWAT in mice, whereas gluteofemoral sWAT and supraclavicular area in humans (Luong et al. 2019).

The origin of beige adipocytes has been a topic of hot debate. Studies have also claimed that these cells are derived from (1) smooth muscle cell precursors ((Long et al. 2014), (Tran et al. 2012)) or (2) white adipocyte precursors (Wu et al. 2012; Garcia et al. 2016) or (3) transdifferentiated directly from existing white adipocytes (Barbatelli et al. 2010). Cold exposure is suggested to induce beiging in rodents by the activation of pro-opiomelanocortin (POMC)-expressing neurons increasing sympathetic tone in WAT that recruit PRDM16, PGC1α (Zhu et al. 2016; Contreras et al. 2014; Lee et al. 2014b). Cold exposure and cAMP upregulated a transcriptional co-partner of PGC-1α named interferon regulatory factor (IRF) 4. IRF 4 acts as a

dominant transcriptional effector of thermogenesis and beiging in adipocytes (Kong et al. 2014). Adding to this, in vitro studies and shotgun proteomics analysis revealed that cold exposure also induces PKA-dependent proteasomal degradation of homeobox protein 10 (HOXC10, negative regulator of beiging) thereby promoting browning in white adipocytes (Tan et al. 2021). Similarly, Kruppel-like factor 11 (KLF11) is a target of PPARγ, as well as a cofactor of PPARγ super-enhancers of beiging, leading to increased mitochondrial oxidative capacity in rosiglitazone-induced beiging in human adipocytes (Loft et al. 2015). Whereas Foxp1 directly represses β3-AR transcription thereby playing the role of the master repressor of browning and thermogenesis (Liu et al. 2019). Interestingly, several immunomodulators (both cytokines and cells) have been shown to influence beiging thereby linking energy metabolism with immunity (Ding et al. 2016; Villarroya et al. 2018; Lv et al. 2016; Lee et al. 2015; Rao et al. 2014). WAT browning is determined by an equilibrium between pro-inflammatory [inducible nitric oxide synthase (iNOS), TNFα, IL6, and MCP-1] and anti-inflammatory cytokines (Rao et al. 2014; Cawthorn et al. 2007). Other immune cells (eosinophils and ILC2) induce M2 macrophage to produce anti-inflammatory cytokines mediating beiging, which is also modulated by meteorin-like hormone (METRNL) secreted from SkM and adipocytes (Lee et al. 2015; Rao et al. 2014). ILC2 cells induce beiging by the differentiation of PDGFRα^{+} (smooth muscle) precursors through recruitment of eosinophils or via the secretion of methionine-enkephalin (Met-Enk) in white adipocytes (Lee et al. 2015; Brestoff et al. 2015; Man et al. 2017). A recent study shows that a pro-inflammatory cytokine, TNF super family protein 14 (TNFSF14) attenuates WAT adipogenesis and beige adipocyte differentiation by blocking JNK signaling, thereby playing a key role in diverting energy in favor of immune activation. Its deficiency caused diet-induced obesity, glucose intolerance, InR in the KO mouse model suggesting it as a regulator of AT homeostasis (Kou et al. 2019). Other cytokines originating from different organs can also influence beige adipocyte development include BDNF (CNS), TGF-β (immune cells), FGF21 (liver), betatrophin (WAT), ANF (heart), suggesting that beige fat tissues are versatile regulators of body energy equilibrium (Kajimura et al. 2015; Liao et al. 2020; Luce et al. 2020; Kleiner et al. 2012; Wang and Yang 2017). Therefore, inducing beiging to treat metabolic disorders has been an attractive weapon and several different approaches for its application are being tested.

3 BAT as a Coordinating Center of Metabolism

Since the discovery of activatedable BAT in adult humans' extensive studies have been performed to define its role in health and disease. However, the mechanistic understanding of the BAT function comes primarily from studies in rodents, where BAT is abundant during adulthood (Cannon and Nedergaard 2004). BAT mainly relies on UCP1-mediated heat production in mitochondria as shown in Fig. 3. Recent studies suggest BAT also possesses noncanonical futile cycling mechanisms like

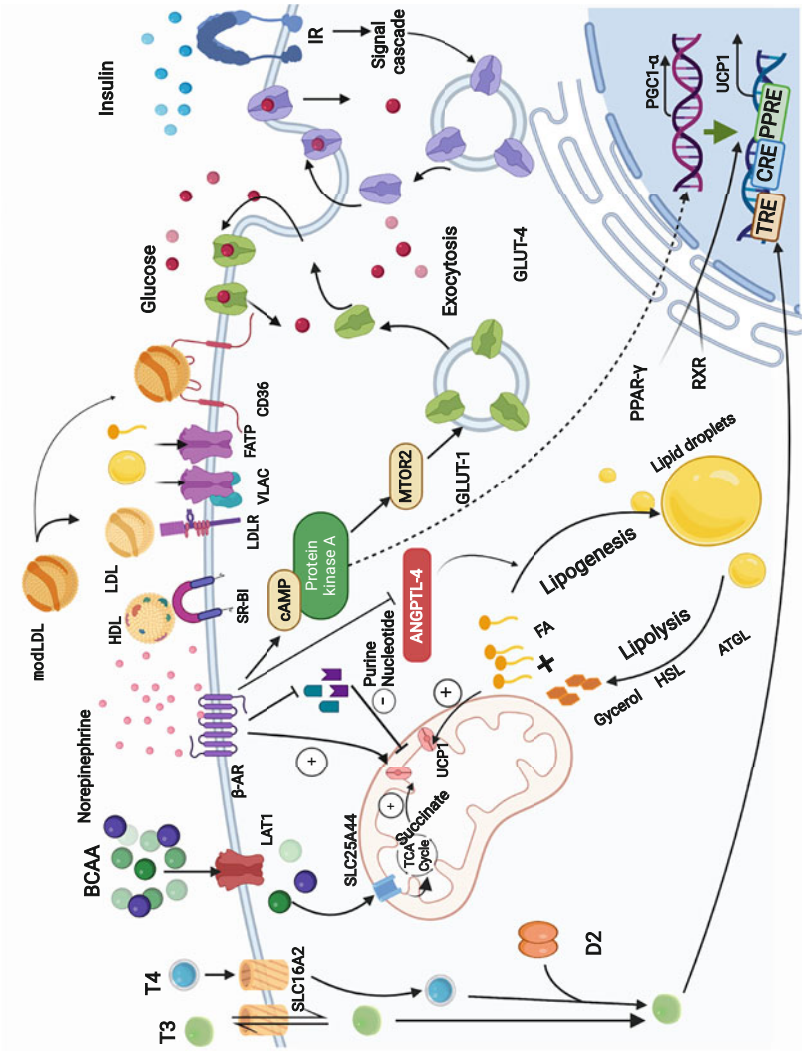

Fig. 3 BAT as a playground of substrate utilization and molecular metabolic processes. In the BAT depots, receptors for glucose, lipid, and BCAA uptake are abundantly expressed mediating substrate uptake and utilization. Glut1 (induced by mTOR pathway) and Glut4 (induced by insulin signaling) mediate glucose uptake, while several receptors for different lipids are found on brown adipocyte membrane such as SR-BI (scavenger receptor class B type I), LDLR (LDL Receptor), FATP (Fatty acid transporters), CD36 (cluster of differentiation 36). BCAA utilization is mediated by LAT1 (L-type amino acid transporter 1) located

creatine and Ca^{2+} (via SERCA2b) (Kazak et al. 2015; Bertholet et al. 2017). BAT function is mainly regulated by norepinephrine (NE) and thyroid both during cold and diet-induced thermogenesis. Interestingly, UCP1 is activated in the BAT by free FA and succinate while it is inhibited by purine nucleotides (Fromme et al. 2018; Fedorenko et al. 2012). In addition to thermogenesis, BAT is important in the regulation of energy expenditure, glucose (substrate) utilization, reliving oxidative stress thereby protecting against obesity and diabetes (Jung et al. 2021; Carpentier et al. 2018; Fernández-Verdejo et al. 2019; McNeill et al. 2020; Lee et al. 2019). In rodents, BAT has been demonstrated to have a very high capacity for utilizing both lipid and glucose. BAT also has high rates of de novo lipogenesis with some lipid storage capacity (Sanchez-Gurmaches et al. 2018; Townsend and Tseng 2014). The lipid reserve in the BAT can be mobilized by NE and recruited for NST in coordination with several factors like PGC, insulin, thyroid. PGC1 (α and β) downregulates lipogenesis and promotes mitochondrial biogenesis priming the BAT for NST and energy utilization (Kim et al. 2018; Worsch et al. 2018). Insulin also plays an important role in substrate fluxes into the BAT and this response is blunted during metabolic disorders (Smith et al. 2018).

3.1 Amino Acids as Substrate

During the scarce availability of sugars, amino acids play a major role in energy metabolism. Studies in rodents show that amino acids can be used as metabolic (anaplerotic) substrates by the BAT (Carpentier et al. 2018). During cold exposure, a specialized protein called SLC25A44 is expressed in BAT mitochondria that facilitate uptake of amino acids, more specifically the branched-chain amino acids (BCAAs) (Yoneshiro et al. 2019, 2021). BAT mitochondria can also use the BCAAs to generate heat (McNeill et al. 2020; Cannavino et al. 2021). Reduced BCAAs uptake by BAT has been suggested to be associated with obesity and T2DM (White et al. 2021; Bloomgarden 2018).

Fig. 3 (continued) on the plasma membrane and SLC25A44 (Solute Carrier Family 25 Member 44) located on the mitochondria. Thyroid, NE, and insulin are major hormones that influence the substrate metabolism of brown adipocytes. Both thyroid and NE induce UCP1 expression via transcriptional upregulation and function in the BAT. Insulin signaling, on the other hand, enhances glucose uptake via Glut4, which is essential to support elevated BAT metabolism and UCP1-mediated heat production. Abbreviations: D2: type 2 deiodinase; TRE: a thyroid or T3 response element; CRE: cAMP response element; PPRE: PPAR response element; ATGL: Adipose triglyceride lipase; modLDL: modified LDL; VLSC: very long-chain acyl-CoA-synthetase. Created with BioRender.com

3.2 BAT as a Sugar Sink

Activated BAT has been shown to uptake a significant amount of glucose and reduces serum glucose both in rodents and humans serving as a "Sugar sink" (Bloomgarden 2018; Sandoval and D'Alessio 2015). Studies suggest NE-induced β3-adrenoceptor-stimulated acute glucose uptake to depend on cAMP-mediated rapid de novo synthesis of GLUT1 and its translocation to the plasma membrane by mTORC2 (Chernogubova et al. 2004; Mukaida et al. 2017). Other studies report that glucose disposal into BAT is via postprandial activation of the RalA-glut4-axis, which might be altered in obesity and diabetes (Karunanithi et al. 2014; Olsen et al. 2014). Depending on the physiological state glucose inside the brown adipocytes can enter either anabolic or catabolic pathways (McNamara 1991). While at rest, it might enter anabolic pathways such as lipogenesis, during the cold challenge it would enter catabolic pathways leading to heat production via UCP1 in mitochondria (Schlein et al. 2021; Boon et al. 2014). Interestingly, the glucose uptake in BAT is enhanced by hypothalamic nuclei (ARC and POMC) via the secretion of α-MSH that exerts its effects by acting on the sympathetic innervations in the BAT (Han et al. 2021; Labbé et al. 2015; Timper and Brüning 2017). Further, BAT glucose utilization is closely associated with Rev-Erbα circadian rhythm that regulates Glut4 and UCP1 functions (Heyde et al. 2021; Lee et al. 2016). Other studies suggest that expanded BAT mass can provide a sink for the excess of glucose in the body and compensate for InR (Virtanen et al. 2005; Mitrou et al. 2009; Bernardis 1985).

3.3 Lipid Clearance by BAT

The BAT has also been proposed as a sink for lipid substrates, as it has been found that the rate of lipid uptake into BAT coincides with plasma lipid metabolism and clearance of triglycerides (Hauton et al. 2009; Hoeke et al. 2016). Studies have demonstrated that BAT-mediated lipid utilization is regulated at two levels: one, by plasma levels of NEFA, triglyceride-rich lipoproteins (TRL) like chylomicrons, VLDL those are mostly synchronized with circadian rhythm; second, by the activity of local mediators of lipid utilization in BAT-like LPL activity, CD36, and angiopoietin-like 4 (ANGPTL4) (Hoeke et al. 2016; Singh et al. 2018; Bartelt et al. 2011). Higher BAT lipid uptake affects vascular lipoprotein homeostasis protecting hyperlipidemia and the development of other cardiovascular diseases (Shao et al. 2016b; Berbée et al. 2015). Many studies confirm that the reduction of intracellular triglyceride content in the BAT during acute cold exposure is independent of age and diabetic status, influencing body insulin sensitivity (Remie et al. 2021; Iwen et al. 2017; Hanssen et al. 2016). Based on these observations it has been suggested that browning can be recruited for clearance of FFA in systemic circulation to ameliorate the progression of the T2DM phenotype (Crandall and Wahl 2021).

4 WAT: More Than an Inert Fat Storage Site

Different WAT depots have distinct functions not necessarily energy storage, like the fat layers in the skin and around internal organs are primarily intended to be shock absorbers and/or connective tissue. But, it is true in mammals that WAT is the major organ of fat storage capable of up-taking both FFA and glucose from the plasma and reserve fat during energy surplus (fed) states. During conditions of high energy demand such as cold, exercise, and low energy intake (starvation), WAT releases FFA. So, switching from fed to the fasted state, WAT becomes a lipid buffering site: during fed state lipid flux into WAT increases, whereas in fasted state lipid efflux predominates (Ruge et al. 2009). In addition to lipid storage and remobilization, WAT has several important functions such as shock absorption, insulation, hormone/cytokine secretion (Rondinone 2006; Zwick et al. 2018). Through the cytokines (adipokines), WAT influences the function of many organs including the brain, heart, and liver (Rondinone 2006; Castillo-Armengol et al. 2019). Therefore, WAT metabolism is closely associated with whole-body energy status and plays a critical role in InR and the progression of metabolic syndrome.

4.1 Fat Remobilization

Retrieval of stored lipids in WAT is facilitated largely by perilipin 1 and hormone-sensitive lipase (HSL) regulated by insulin and catecholamines (Frühbeck et al. 2014). Perilipin 1 coats the lipid droplet in adipocytes and serves as a physical barrier protecting them from breakdown by HSL, thereby regulating lipid metabolism (Moore et al. 2005). Studies show that loss of perilipin 1 action leads to increased basal lipolysis and reduction of WAT size. The phosphorylation of perilipin 1 by cAMP-dependent PKA facilitates HSL translocation to the lipid droplet promoting lipolysis and release of FA (Holm 2003). Elevation of perilipin1 expression has been found in people with obesity, without significant correlation with peripheral InR (Pinhel et al. 2017). Reduced adipose O-GlcNAc transferase (OGT) increases O-GlcNAcylation of perilipin1 that inhibits lipolysis in eWAT and promotes diet-induced obesity (Yang et al. 2020). On the other hand, reduced HSL function (haploinsufficiency or inhibitor treatment) improves insulin-stimulated lipogenesis in WAT in mice models and human-derived primary adipocytes (Girousse et al. 2013). This de novo lipogenesis along with reduced lipolysis reshapes FA uptake in the WAT, which also increases glucose uptake thereby minimizing the systemic load inducing whole-body insulin sensitivity in coordination with other peripheral organs like the liver and SkM (Solinas et al. 2015). Lipolysis of WAT and subsequent release of NEFA is also dependent on the action of LPL and ANGPTL4. LPL located at capillary endothelium hydrolyzes triacylglycerol (TAG)-rich plasma lipoproteins to glycerol and NEFAs depending on tissue nutritional status and also is regulated by hormones. ANGPTL4 inhibits

LPL and its expression correlates with alterations in circulating lipids both in mouse models and humans. Further, fat remobilization from the WAT is regulated via CNS-derived hormones like the GH, which modulates FA metabolism in two ways: (1) by increasing glycerol production and (2) through decreasing the amount of FA reconversion to triglyceride (Goodman 1988; Møller and Jørgensen 2009). Overall, blunted lipolysis and lipogenesis from glucose in WAT are major factors in protecting against InR and pathogenesis of T2DM.

4.2 Vascularization of WAT

According to the bodily energy demand, the lipid fluxes from/to the WAT require appropriate vascularization (Choe et al. 2016). It is observed mostly that sWAT has a higher vascularization capacity than that of vWAT in humans (Caputo et al. 2021). The original blood vessels in the WAT depots are formed during embryonic stages by vasculogenesis from the mesodermal angioblasts. In contrast, neovascularization of adult WAT is more closely regulated by the involvement of pro- and anti-angiogenic factors, which is tuned to lipid flux to the WAT. This process involves primarily two types of progenitor cells; one for new endothelial cell generation and the other (pericytes) for generation of smooth muscle and supporting cells blood vessels in the WAT depots are formed during embryonic stages by vasculogenesis from the mesodermal angioblasts (Corvera and Gealekman 2014). While, physiological stresses that cause lipid efflux from WAT like cold, exercise, starvation promote neovascularization; conditions of lipid influx like obesity induce the reduction of capillary density in both vWAT and sWAT (Fan et al. 2021; Fuster et al. 2016). The factor most highlighted as WAT angiogenesis regulator is VEGF-A, which is also suggested in WAT browning independent of IL-4R activation (Park et al. 2017). While adipocyte-specific overexpression of VEGF-A promotes vascularization, the depletion of VEGF-A in the adipocytes reduces vascularization in mouse model leading to impaired insulin sensitivity inducing inflammation (Corvera and Gealekman 2014; Sun et al. 2012). Intriguingly, it has been observed that upon significant reduction of capillary density in the WAT, larger blood vessels are upregulated potentially due to the elevated local hypoxia and induction of HIF1 (Gaspar and Velloso 2018). In fat from obese individual and mouse models, capillary density is often reduced which is also associated with increased levels of endogenous angiogenic inhibitors in WAT such as pigment epithelium-derived factor (PEDF), angiostatin, endostatin (Cheng and Ma 2015). In addition, endothelial cell activation in the capillaries of WAT is observed during obesity, which catalyzes the recruitment of immune cells like macrophages and T-cells (Cho et al. 2007; Leung et al. 2018). Therefore, WAT vascularization serves as a connecting link between the pathogenesis of metabolic diseases and the immune system of the individual.

4.3 Browning of WAT

It has been reported that selective WAT depots are prone to browning; while, abdominal omental vWAT (oWAT) in humans, sWAT in rodents (Cleal et al. 2017). Several physiological stimuli have been proposed to induce browning such as exercise and cold adaptation (Wang and Seale 2016; Arroyave et al. 2020; Chang et al. 2019; Markussen et al. 2017; Petruzzelli et al. 2014). Recent studies have identified several exercise-induced myokines such as irisin, myostatin, METRNL, and β-amino isobutyric acid (BAIBA) that are suggested to cause WAT browning mostly in mice models (Rodríguez et al. 2017; Rao et al. 2014; Murphy et al. 2020; Roberts et al. 2014; Maalouf and El Khoury 2019). Metabolic benefits of WAT browning have been highlighted, which include increased glucose utilization and reduction in adiposity. A major regulator of WAT browning, PRDM16, has also been shown to influence the metabolic demand of the beige adipocytes via promoting futile Ca2+-cycling through the SERCA2b-RyR2 pathway (Ikeda et al. 2017). WAT browning induced by cold adaptation has been difficult to be defined as lipolysis-mediated changes can also produce similar WAT phenotypes (Schreiber et al. 2017). Studies show that WAT browning is controlled by neurons in the hypothalamus involved in the regulation of the caloric status of the body. While POMC and RIP-Cre neurons promote, agouti-related peptide (AgRP) neurons usually retard the browning of iWAT and vWAT (Wang et al. 2018; Bi and Li 2013; Dodd et al. 2015; Ruan et al. 2014). Stress-induced WAT browning is complex as well as interesting because of its association with neuro-hormonal factors and metabolic diseases. The HPA and HPT axes along with cytokine regulators like IL4, IL6, and IL13 have been suggested to critically influence WAT browning (Stephens and Wand 2012; Fekete and Lechan 2014; Reinehr 2010). This process of browning is more complex due to the further involvement of hormones such as insulin, leptin, IGF1, catecholamines (Dodd et al. 2015; Boucher et al. 2016; Yasmeen et al. 2018). During the progression of obesity, most of these factors are altered reducing WAT browning (Chen et al. 2017; Ye 2013; Bose et al. 2009). Chronic β-adrenergic stimulus enhances glycogen accumulation, glycogen turnover in sWAT, which is driving UCP1 expression and thermogenesis via the ROS mediated p38MAPK pathway (Keinan et al. 2021). Interestingly, an experiment mimicking WAT browning by ectopic overexpression of UCP1 in sWAT was shown to improve insulin sensitivity and whole-body glucose homeostasis providing evidence for beneficial effects of browning (Poher et al. 2015).

4.4 Pathological Changes in WAT Distribution

Different WAT depots having discrete functions can have differential fat storage capacity. It is believed that metabolic disorders during energy surplus start after storing capacity of preferentially fat-storing WATs is exhausted causing the

recruitment of the alternate sites not primarily meant for energy storage (Lanktree et al. 2010; Akinci et al. 2018; Fiorenza et al. 2011). The altered WAT distribution also has significant metabolic consequences and is classified into two main types: lipodystrophy (including lipoatrophy) and ectopic adiposity. Lipodystrophy is defined as a lack of adipocyte expandability with reduced lipid accumulation capacity in the adipocytes; ectopic adiposity describes the condition when fat accumulation is found in tissues other than WAT like SkM, liver, kidney, and pancreas (Bombardier et al. 2013; Purcell and Taylor 2019; Guebre-Egziabher et al. 2013; Chung and Qi 2019; Singh et al. 2017; van der Zijl et al. 2011). Lipodystrophy can be observed in any fat depot iWAT, vWAT, etc. These conditions may arise due to either the dysregulation of storage (including substrate uptake) or the secretion of fat from the adipocytes (Lim et al. 2020). Obviously, in most obese individuals the fat-storing capacity of adipocytes is already exhausted leading to hypertriglyceridemia along with higher fatty substances in circulation that induce ectopic adiposity (Laclaustra et al. 2007). On the other hand, lipodystrophy is closely associated with altered adipokine (leptin and adiponectin) production leading to impaired InR in the skeletal muscle and liver that is often associated with reduced energy expenditure (Fiorenza et al. 2011). The hepatic and SkM lipodeposition shows similarity while the deposition of lipid in the pancreas during pathogenesis slightly differs in humans compared to rodents (Pajed et al. 2021; Yki-Järvinen 2002). The pancreas is more susceptible than the liver as 20-folds higher lipid infiltration is observed after 15 weeks of HFD feeding in mice. This fat infiltration to the pancreas is associated with de novo lipogenesis and the accumulation of unsaturated fatty acids. In contrast, the fat deposition in the human pancreas is more extensive encompassing both exocrine and endocrine parts. This differential fat accumulation enriches the paracrine effects of leptin and adipokines in the proximity of pancreatic islet leading to altered insulin secretion (Pinnick et al. 2008). Lipodystrophy patients usually have a lower circulating level of leptin and beneficial effects of leptin replacement have been reported (Oral et al. 2002). The increased ectopic adiposity is often associated with an increase in systemic FFA, diacylglycerol (DAG), and ceramide that promotes T2DM (Pararasa et al. 2015).

5 Altered WAT Function in Diabetic Pathogenesis

During diabetic pathogenesis, the various WAT depots undergo several key alterations both in human and animal models. This as a cause or an effect can be debatable, but the changes in WAT overlap with the progression of diabetic pathogenesis from a quite early stage indicating a cause.

5.1 Insulin Signaling Is a Major Determiner of WAT

Obviously, the insulin signaling pathway is heavily impacted; several other cytokines also exhibit marked changes that lead to altered glucose and lipid homeostasis in WAT. Interestingly in humans, the expression of IR, IR substrate 2 (IRS-2), p85, Glycogen synthase kinase 3 (GSK-3), mitogen-activated protein kinase (MEK), and ERK is higher in vWAT than sWAT, whereas IRS-1 and AKT show equivalent expression (Laviola et al. 2006). Negative regulators of insulin signaling such as inositol phosphate (IP) 7 and mTOR working in concert with S6K and Grb10 also play a critical role in WAT function (Hsu et al. 2011; Yu et al. 2011). Insulin along with IGF-1 increases IP7 that reduces AKT-signaling, while mTOR along with S6K reduces insulin signaling by altering phosphorylation of IRS and Grb10. The action of these pathways is profoundly altered during metabolic imbalance due to stress, high-fat-diet (HFD), high sugar diet, and obesity that impacts the pathogenesis of T2DM. It has been shown that insulin-mediated anti-lipolytic effects differ in various fat depots; sWAT being more responsive than vWAT, suggesting a less robust intracellular insulin signaling pathway (Perrini et al. 2003; Giorgino et al. 2005; Perrini et al. 2008). Insulin signaling is initiated in WAT adipocyte upon insulin binding to its receptor via IRS-1 and IRS-2 along with the Shc proteins (i.e., p66Shc, p52Shc, and p46Shc) that recruit downstream signaling cascade as shown in Fig. 4 (Li et al. 2019). The speed of insulin action varies across WAT depots. It has been shown that intravenous insulin administration induces phosphorylation of IR and the p85 regulatory site of PI3K within 6 min in vWAT much higher than in the sWAT. While receptor phosphorylation comes back to baseline in vWAT within 30 min, it remains high in sWAT (Li et al. 2019). The next signaling protein AKT undergoes differential phosphorylation upon insulin administration. In the vWAT (especially oWAT), Ser-473 and Thr-308 sites of AKT become phosphorylated at a faster rate than in the sWAT on insulin injection (Li et al. 2019). Similarly, phosphorylation of GSK-3α, ERK-1, and ERK-2 was found to be higher within a few minutes of insulin injection in the vWAT compared to sWAT. In addition to phosphorylation, an increase in protein expression of insulin signaling intermediates like PI3K, MEK was shown to be more pronounced in vWAT than sWAT.

It is commonly observed that WAT adipocytes from obese people and mice models exhibit impaired insulin signaling resulting in poor Glut4 translocation, thus glucose uptake (Freidenberg et al. 1988). Surprisingly, the initial signaling events of insulin receptor tyrosine kinase activity in adipocytes from obese insulin-resistant patients are normal (de Mutsert et al. 2018), suggesting an alteration in downstream intracellular signaling. Weaker association of IRS-1 to PI3K in obese individual-derived adipocytes upon insulin action is suggested as a major cause of impaired insulin signaling. Few other alterations suggested for insulin signaling in the adipocytes upon obesity are; changes in protein expression of p85α subunit, impaired AKT phosphorylation. Interestingly, gender-specific differences in WAT depot insulin signaling were reported. Epidemiological studies showed that in men, Swat in the abdominal part and vWAT are associated with InR to a similar extent;

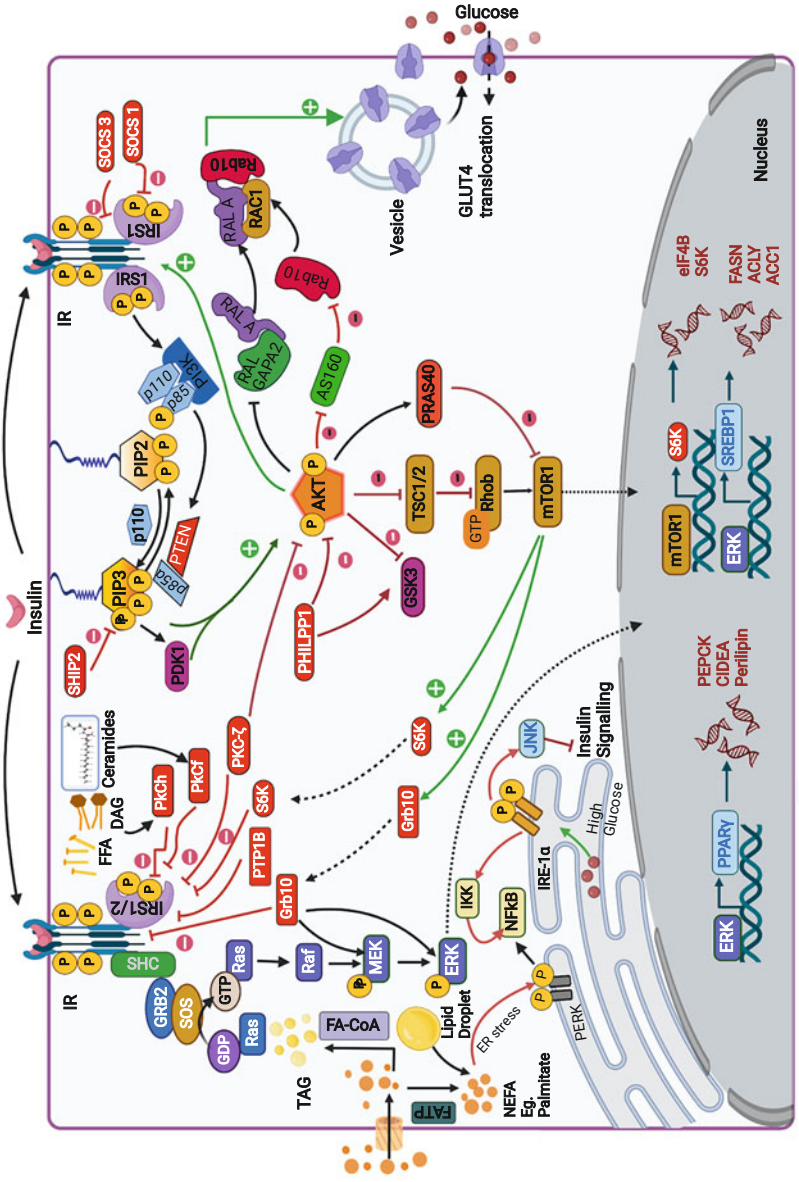

Fig. 4 Schematic diagram showing pathways and their alterations in the WAT adipocyte. In the WAT depots, insulin signaling ensures glucose uptake via transcriptional activation in the nucleus as well as the regulation of Glut4-mediated glucose uptake by regulating the function of the RAL-A/RAC1/Rab10

whereas, in women, it is particularly vWAT (Björnholm et al. 2002). Adipocytes isolated from sWAT of obese women exhibit markedly impaired IRS-1 associated PI3K activity, while increased IRS-2 associated PI3K activity. Further, a reduction in protein expression of Glut4 (37%) and p85α-subunit of PI3K (55%) was observed in obese women compared to lean subjects (Boura-Halfon and Zick 2009).

5.2 *Signals Opposing Insulin Action Are Equally Important*

Inhibitory regulation of insulin signaling is also equally important that is primarily governed via inhibitory Ser/Thr phosphorylation of IR, IRS-1, and -2. These pathways are recruited in the adipocytes by factors like cytokines, fatty acids, hyperglycemia, and insulin itself via the activation of multiple kinases (JNK, hTBC1, and MAPK) (Davis et al. 2000; Gao et al. 2002; Zhang et al. 2008; Geraldes and King 2010; Hilton et al. 2000). Atypical PKC-ζ also reduces insulin signaling via Ser-phosphorylation of IRS-1 and Thr-34 phosphorylation of AKT, thereby blocking its translocation to the plasma membrane (Geraldes and King 2010; Goldstein et al. 1998). Some transmembrane phosphatases including protein tyrosine phosphatase (PTP) 1B dephosphorylate activated IR and IRS proteins thereby deterring insulin signaling (Emamgholipour et al. 2020). The role of PTP1B is demonstrated by the finding that PTP1B KO shows improved IR phosphorylation and resistance to HFD-induced obesity and associated InR (Holt and Siddle 2005). Some cytoplasmic adaptor proteins like Grb10 and Grb14 have been shown to decrease IR activity by preventing access of substrates to the activated receptors (Youngren 2007; Smith et al. 2007; Liu et al. 2014). Grb10 overexpression in adipocytes results in impaired growth, glucose intolerance, and InR. Upregulation in Grb14 expression was found in AT of insulin-resistant animal models and type-2 diabetic patients (Béréziat et al. 2002; Errico 2018). Interestingly, insulin signaling is down-regulated by the suppressor of cytokine signaling (SOCS) proteins, especially SOCS1 and SOCS3 (Hilton et al. 2000; Rui et al. 2002). Their expression is increased in WAT during obesity and they induce InR via either the inhibition of

Fig. 4 (continued) complex. Upon insulin binding to its receptor on the WAT adipocyte two major pathways, namely, AKT and MAPK pathways are activated. The AKT pathway is the most abundant in WAT insulin signaling that is regulated mostly via phosphorylation and substrates mainly glucose. Lipid load on WAT adipocyte also influences AKT pathway and some lipids like palmitate induce ER stress that may have multiple effects such as transcriptional changes, inflammation, autophagy. Points of dysfunction during T2DM are shown by "red circle with a white minus sign (⛔)" and blunted (red) arrows, while that are points of negative regulation during physiological states is shown by "red circle with a black minus sign (⛔)" and blunted (black) arrows. The signaling steps that are activated during normal physiological states are shown by "green circle with a black plus sign (⊕)." Abbreviations: AS160: AKT substrate of 160 kDa, RAC1: Rac Family Small GTPase 1, TSC1/2: Tuberous sclerosis proteins 1/2,4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1. Created with BioRender.com

the tyrosine kinase activity of the IR or targeting the IRS proteins to degradation (Rui et al. 2002; Palanivel et al. 2012). However, the overexpression of SOCS3 alone in pre-eWAT causes local InR, but not sufficient to cause systemic InR (Shi et al. 2006; Sleeman et al. 2005).

Obesity and InR in WAT are also found to be initiated via some other intermediary signaling pathways. Enhanced PERK and IRE1α activity in WAT of obese mice is suggested to cause JNK and IKK activation inducing Ser^{307}-phosphorylation of IRS-1. Protein phosphatases (PPs) are important regulators of rate-limiting enzymes in glucose and lipid metabolism in the WAT, including glycogen synthase, hormone-sensitive lipase, acetyl CoA carboxylase. A protein phosphatase – PHLPP1 impairs AKT and glycogen synthase kinase 3 (GSK3) activities in adipocytes, resulting in decreased glycogen synthesis and glucose uptake. Upregulated PHLPP1 has been observed in WAT from obese and diabetic patients that correlate with reduced AKT2 phosphorylation. Lipid phosphatases regulate insulin signaling by modulating PIP3 levels which are dephosphorylated by PTEN, thus antagonizing PI3K signaling in adipocytes. Consistently, the deletion of PTEN in mice AT increases insulin sensitivity. A subunit of PI3K called p85α has been shown to enhance PTEN activity regulating both generation and degradation of PIP3. Another phosphatase called SH2 domain-containing inositol 5-phosphatases (SHIP) 2 is ubiquitously expressed and plays a role in insulin signaling in WAT through the AKT pathway (Tang et al. 2005).

5.3 Metabolites May Have a More Critical Role in WAT Regulation

The regulation of WAT function by substrates (glucose and lipids) and metabolites has also been studied in recent decades. Elevated circulating levels of FFAs are observed in obesity and induce activation of JNK, IKK, PKC, and IRS-1 Ser^{307} phosphorylation in the WAT (Davis et al. 2009). Among the FFAs, palmitate (16:0), DAG, and ceramide have a critical role in InR. In WAT, palmitate causes InR by inducing JNK activation and ER stress (Guo et al. 2007; VandeKopple et al. 2017), while DAG by inducing the activation of PKCh which inhibits PI3K, whereas ceramides by activating PP2A and PKCf that inhibit insulin signaling. Interestingly, the induced anomalies of fat metabolism in the WAT depots increase FFA flux to non-adipose tissues that amplify dyslipidemia, hepatic steatosis, and peripheral tissue InR. Recent studies in cultured adipocytes suggest that NF-κB signaling downregulates PPARγ that impairs triglyceride storage. This can occur through the expressional regulation of triglyceride metabolism enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), fatty acid synthase (FAS), Acyl-CoA synthetase (ACS), lipoprotein lipase (LPL), and proteins associated with lipid droplet including CIDEA, FSP27, perilipin, and HILPDA (Shijun et al. 2020; Ahmadian et al. 2013; Morigny et al. 2021; Foretz et al. 2005).

5.4 Altered Chemokines and Adipomyokines in Diabetes

Many adipokines have been identified in recent decades and shown to affect WAT pathophysiology including the progression of T2DM. Several types of immune cells reside within WAT and contribute to adipokine secretion that plays a critical role in pathological conditions. Adiponectin (primarily produced by sWAT) enhances glucose and fat use in SkM as well as adipocytes and its reduced circulatory level is associated with obesity (Cătoi et al. 2014). Omentin-1 is produced primarily by vWAT correlates with InR, oxidative stress, and chronic inflammation in morbidly obese patients (Li et al. 2008). Vaspin (visceral adipose tissue-derived serine protease inhibitor) is another newly defined adipokine that reduces InR and metabolic disorders (Ruigrok et al. 2021). Another adipokine, Leptin, is known to affect substrate utilization in the SkM and nutrient sensing in the brain, thereby influencing whole-body energy consumption and InR (Gerrits et al. 2012). Apart from these adipokines, several cytokines are also produced by several other tissues but still are considered to be adipokines and contribute to InR in the WAT. Such ubiquitous adipokines are PAI-1, resistin, BMP, NRG-4, FGF21, SFRP5, visfatin; which can affect the function of other tissues in addition to WAT (Feijóo-Bandín et al. 2020; Christian 2015). Interestingly, resident immune cells (macrophages, T-cells, neutrophils, etc.) secrete cytokines like MCP-1, IL1β, TNF-α that affect the inflammation of WAT during obesity and associated T2DM (Panee 2012; Mazur-Bialy et al. 2017). These cytokines act through several pathways in the WAT including the activation of Ser/Thr phosphatases and SOCS3, decreasing IRS-1, expressional regulation of GLUT4, and PPARγ. MCP-1 secreted by macrophages attracts monocytes into WAT causing macrophage accumulation and InR (Mazur-Bialy et al. 2017). Further, some adipokines are also substantially secreted by muscles and are now classified as adipomyokines such as IL-6, TNF-α, irisin (Luo et al. 2020; Bal et al. 2017a). These muscle-derived cytokines affect substrate (glucose and fatty acid) fluxes into/out of adipocytes, influence mitochondrial metabolism, and modulate insulin action in WAT. The role of adipomyokines in muscle-AT crosstalk during physiological challenges (Bal et al. 2017b; Sahu et al. 2019), like cold, exercise, starvation, and pathological states such as obesity and T2DM needs more detailed understanding.

6 Why Does Exercise Improve WAT Metabolism?

It is well documented that exercise, both acute and chronic, enhances cardiac output and muscular activities. However, the way different forms of exercise work on WAT metabolism is still not fully explained (Pedersen 2017a). Exercise may impact WAT function in two ways: first, by creating an energy demand it stimulates WAT to undergo lipolysis releasing of FFAs; second, by affecting other organ function it

modulates circulatory cytokines (hepatokines and cardiokines) levels that indirectly affect WAT physiology.

6.1 Exercise-Induced Myokines

Acute bout of exercise increases the secretion of Interleukin-6 (IL-6), irisin, BAIBA, IL-15, and METRONL that signals to the WAT. Acute exercise of 60 min increases IL-6 output depending on the intensity and continues to be released post-exercise (Carey et al. 2006). In WAT (mainly sWAT), IL-6 is involved in glucose utilization and AMPK-mediated fat remobilization (Steensberg et al. 2003). Intriguingly, IL-6 induces mononuclear immune cells to produce IL-10 (Opp et al. 1995), which retard the synthesis of pro-inflammatory cytokines such as TNF-a thereby reducing the IR of WAT (Tsuchiya et al. 2014). The high-intensity acute exercise was shown to induce higher irisin production compared to low-intensity (Löffler et al. 2015). Irisin is suggested to cause mitochondrial biogenesis in WAT and PGC1α-dependent browning in both mice and humans. Reports showed a positive association between circulating irisin and BMI along with improved glucose homeostasis by both acute exercise and training (Crujeiras et al. 2014; Stengel et al. 2013; Boström et al. 2012). A study by Rodríguez et al. showed that leptin crosstalk with irisin differentially in fat and SkM. This antagonizes the thermogenic mechanism of irisin in sWAT while promoting SkM myogenesis during exercise. It suggests that higher leptin concentration in obesity hinders irisin's role in sWAT although physical exercise is applied (Rodríguez et al. 2015). Interestingly, the rate of IL-6 release from muscle is retarded upon long-term exercise; other myokines are suggested to mediate the beneficial effects of chronic exercise in WAT. Different exercise training has been found to have differential effects on WAT in various individuals, which may depend on myokines secretion. Basal irisin level was increased following long-term resistance training, while simple aerobic training had no effects (Kim et al. 2016; Stautemas et al. 2019). A novel myokine, BAIBA, was shown to increase following 30 min of acute exercise (Stautemas et al. 2018; Riechman et al. 2004) as well as 16–20 weeks of aerobic exercise training only in the normal subjects compared to the sedentary and obese individuals (Roberts et al. 2014; Stautemas et al. 2019). However, sedentary subjects can also increase circulating BAIBA upon regular exercise reducing WAT mass (Roberts et al. 2014). The secretion of other myokines, IL-15, in acute vs. chronic physical activity is unclear as opposing results have been published. While Riechman et al. showed transient increase following acute resistance exercise and no change with age training, few other studies showed no change upon sub-maximal acute exercise and increase in basal IL-15 level following long-term endurance training (Riechman et al. 2004; Rinnov et al. 2014; Ostrowski et al. 1998). The elevated level of IL-15 in trained humans has been suggested to induce lipolysis of visceral fat thereby regulating abdominal obesity (Pedersen 2017b). IL-15 influences WAT physiology by decreasing lipid deposition in preadipocytes, adiponectin secretion, and TNFα secretion (especially in patients

with low-grade chronic inflammation) (Carbó et al. 2001; Sánchez-Jiménez and Alvarado-Vásquez 2013). METRNL production in muscles was shown to be increased by a single bout of downhill treadmill-running exercise in both mice and humans (Rao et al. 2014). Both aerobic and resistance training increase circulating METRNL levels that induce WAT browning and reduce adiposity (Rao et al. 2014; Bae 2018; Amano et al. 2020).

6.2 Exercise-Induced Chemokines from Other Organs

Exercise affects the function of other organs like the adrenal, heart, liver, and pancreas to produce factors that have been shown to indirectly influence WAT physiology. Acute resistance exercise induces epinephrine production that is known to cause lipolysis of WAT, especially ipWAT and sWAT/ ingWAT in both humans and mice. Studies in humans have identified follistatin as an exercise-inducible hepatokine that is produced during recovery from an exercise bout. Follistatin enhances the expression of thermogenic markers in WAT and also reduces the production of myostatin that lowers WAT mass (Braga et al. 2014; Allen et al. 2008). The liver also produces FGF21 and Soluble Fibronectin type III domain-containing 4 (sFNDC4) upon both acute and chronic exercise. FGF21 is known to decrease body weights by a reduction in WAT mass leading to improved whole-body insulin sensitivity (Sarruf et al. 2010; Coskun et al. 2008). On the other hand, sFNDC4 binds to its G-Protein coupled Receptor (GPR) 116 in the WAT (mostly iWAT) that is suggested to improve insulin tolerance in prediabetic mice (Georgiadi et al. 2021). Recent studies have described a few other hepatokines like Activin-E, Growth differentiation factor 15 (GDF15), ANGPTL6, Lipocalin 13; that modulate WAT metabolism-regulating fat mass and weight gain. Heart with the greater load during exercise secretes cardiokines like atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). Acute exercise with the highest workload causes a twofold increase in ANP, while ~30% increase in BNP secretion (Barletta et al. 1998). Both ANP and BNP are shown to induce lipolysis and AT remodeling enhancing lipid mobilization in human sWAT. Surprisingly, long-term exercise training induces no rise in BNP secretion, whereas rise in ANP continues although at a reduced rate. The physiological rise in ANP and BNP levels is impaired in the case of overweight and obese individuals, which is however improved by endurance exercise training (Lafontan et al. 2005). Interestingly, the kidney also participates in this exercise-induced inter organ-crosstalk by secreting erythropoietin that promotes vascularization in sWAT in trained runners after prolonged exercise (Bodary et al. 1999; Schwandt et al. 1991). Hence, increased physical activity exerts its beneficial effects by decreasing total, truncal, and limb AT, reducing triglyceride levels, increasing HDL cholesterol levels that in turn improve peripheral insulin sensitivity in humans, especially important for obese patients.

7 Outlook and Future Direction

In humans, adipose depot-specific differences are documented that are influenced by age, gender, genetic predisposition, and environmental factors. These inter- and intra-depot heterogeneity also drastically modulate embryonic development, cellular composition, and whole-body phenotype of the offspring. Studies have shown a depot-dependent disparity in physiology (vascularity), metabolic function (nutrient uptake and clearance), and endocrine function. The sWAT is lipolytically less active with high insulin sensitivity unlike vWAT excepting oWAT. Hence, the sWAT plays a protective role in the nutrient surplus state, whereas vWAT is associated with central obesity and InR. The literature supports the differential role of WAT depots in physiological and pathological conditions that can be targeted for weight management and potentially counter metabolic disorders. In light of emerging research on the impact of different forms of exercise on different fat depots as well as ectopic fat accumulation can be expected to be beneficial to halt the progression of T2DM. Further understanding into molecular details of exercise as an antioxidant and anti-inflammatory agent can provide better targets to future pharmacological agents.

Unlike appreciated by many in the field WAT depots can be categorized into more subtypes than merely sWAT and vWAT. In most mammals including rodents and humans, the unique anatomical and physiological roles of epicardial WAT, eWAT, and oWAT depots are being unraveled by recent discoveries. One of the interesting aspects is that different WAT depots originate from diverse precursor cells, which are regulated by many endocrine agents and growth factors. The mechanistic details of preadipocytes differentiation in different WAT depots are not well understood and insight on this aspect will help in defining the role of distinct WAT depots in the progression of metabolic syndrome. Similarly, the post-natal expansion of WAT should be studied to understand the role of vascularity and epigenetic effect in the different WAT depots. These studies will help in better delineating the mechanism behind lipodystrophy and lipoatrophy.

Increasing energy expenditure has been proposed as an attractive target to counter obesity and to some extent T2DM. Although classically energy-dispensing properties of BAT were being suggested as the main target, the discovery of beige adipocytes has brought WAT onto the center stage. However, distinct WAT depots display the differential ability to undergo beiging, which may mean that not all WAT can be pharmacologically targeted to similar extents. Further, health outcomes (both obesity and T2DM) of pharmacological targeting of different WAT depots need to be carefully evaluated. In traditional medicine, plant-derived agents have been used to target different WAT depots especially for T2DM that needs to be reassessed with modern biomedical research approaches.

In the literature, the two terms beiging and browning of WAT have been used very loosely and, in many cases, interchangeably. However, "Beiging" is a transitional state due to pharmacological intervention and/or external stimuli, while browning of WAT is a more durable conversion primarily due to sustained pathological state. Hence, the requisite conditions and therefore the molecular mechanism

must be different. Research should be conducted for clarification of the distinction between beiging and browning of WAT to understand whether beiging can be used to modulate nutrient metabolism in a regulated manner.

Emerging facts suggest obesity-associated inflammation is mainly caused by cytokines released by resident immune cells in AT; integrating the immune system with glucose utilization. The imbalances of pro-inflammatory and anti-inflammatory cytokines worsen the insulin signaling in various fat depots and SkM. A vivid understanding of these cytokines will provide much-needed insight into the genesis of metabolic imbalance leading to obesity. Further, cytokines from other organs (especially hepatokine and cardiokine) describe the influence of other organs in the WAT substrate cycling. Future studies should be addressed to unravel the molecular details of inter-organ cytokine crosstalk and might provide strategies to target lipid mobilization in selected WAT depots and the suppression of ectopic fat deposition.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Author Contributions BS and NCB conceived the idea. BS, OT, and the US prepared the first draft and figures. All authors critically discussed and edited the manuscript.

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Autocrine, Paracrine, and Endocrine Signals That Can Alter Alveolar Macrophages Function

Yue Yang and Yun Wang

Contents

Abstract Alveolar macrophages (AMs) are extremely versatile cells with complex functions involved in health or diseases such as pneumonia, asthma, and pulmonary alveolar proteinosis. In recent years, it has been widely identified that the different functions and states of macrophages are the results from the complex interplay between microenvironmental signals and macrophage lineage. Diverse and complicated signals to which AMs respond are mentioned when they are described individually or in a particular state of AMs. In this review, the microenvironmental signals are divided into autocrine, paracrine, and endocrine signals based on their secreting characteristics. This new perspective on classification provides a more comprehensive and systematic introduction to the complex signals around AMs and is helpful for understanding the roles of AMs affected by physiological environment.

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The existing possible treatments of AMs are also mentioned in it. The thorough understanding of AMs signals modulation may be contributed to the development of more effective therapies for AMs-related lung diseases.

Keywords Alveolar macrophage · Autocrine · Endocrine · Immune · Macrophage · Paracrine

1 Introduction

There are at least two different types of macrophages in lung: Alveolar macrophages (AMs) and interstitial macrophages (IMs). AMs exist in the alveolar cavity and are specialized in recycling of surfactant molecules and removal of debris. AMs are highly abundant immune cells located in the airway lumen and play important roles in lung homeostasis and immunity (Lambrecht 2006). IMs are found in the interstitium and play an important part in immune functions, including maintaining lung homeostasis and preventing immune-mediated allergic airway inflammation (Schyns et al. 2018). The two macrophages and their roles in lung diseases have attracted attention in recent years (Hou et al. 2021). Recently, AMs are proved to be greatly involved in inflammation, tissue repair, and so on (Cheng et al. 2021).

The varied functions of AMs may associate with their complex origin and environmental influences. Most tissue macrophages and circulating monocytes are considered as bone marrow (BM)-derived cells (van Furth et al. 1972). But it has proven that most steady-state AMs are derived from embryonic progenitors recently. In steady state, AMs are able to keep self-maintenance locally and don't contribute to circulating monocytes (Hashimoto et al. 2013; Epelman et al. 2014). During lung injury or inflammation, resident AMs could be depleted and additional monocytes-derived cells are recruited to the infection sites and conduce to the immune response (Aegerter et al. 2020). In the alveolar microenvironment, the differentiation of recruited monocytes into AMs depends on the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Guilliams et al. 2013). Notably, both tissue-resident and monocyte-derived alveolar macrophages respond to the changes in the local environment (Abdala Valencia et al. 2016).

Comparing to macrophages in other tissues, AMs reside in a very unique micro-environment with the remarkable environmental fluctuations (Tschumperlin and Drazen 2006), which has a considerable influence on the cell populations living there. To adapt the changeable dynamic environment, AMs exhibit the marked phenotypical and functional plasticity to allow the efficient responses to environmental signals and rapid alterations in phenotype and functions (Hussell and Bell 2014).

Besides early recognized functions of AMs in the clearance of particles, microbes as well as apoptotic cells and cellular debris in health, AMs play vital roles in immunological responses, especially in the progression of many inflammatory

diseases. The phagocytosis of AM is equally important and closely interrelated with immunologic regulation of AMs, for example, whether the cells cleared by AMs are apoptotic or necrotic will determine whether AMs has an anti-inflammatory or pro-inflammatory response (Zhang and Mosser 2008).

These different functions lead to their different classification. Macrophages are roughly divided into classically activated macrophages and alternatively activated macrophages, commonly termed M1 and M2, which are induced by interferon-γ (IFN-γ)/lipopolysaccharide (LPS) and interleukin-4 (IL-4) in vitro, respectively (Orecchioni et al. 2019). M1 macrophages are involved in the development of inflammations, while M2 macrophages have been involved in tissue repair and fibrosis. M1 macrophages produce pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-12, and tumor necrosis factor-α (TNF-α). They can also express CXC-chemokine ligand 9 (CXCL9), CXCL10, CXCL11, and CC-chemokine ligand 5 (CCL5). M2 macrophages typically express the mannose receptor (also known as CD206), the tyrosine protein kinase MER, growth arrest-specific protein 7 (GAS7), CD163, arginase and produce anti-inflammatory cytokines such as IL-10 and tumor necrosis factor-β (TGF-β) (Shapouri-Moghaddam et al. 2018). It should be noted that M1/M2 is the extreme of the intermediate cell continuum, therefore, the macrophages in vivo may not strictly be divided into M1 or M2 (Martinez and Gordon 2014). The existing AMs subtypes have been mostly classified by recombinant cytokines combined with in vitro (Murray et al. 2014). The properties of AMs in vivo are predicted by in vitro experiments.

As mentioned above, AMs have sufficient plasticity to integrate multiple signals and their activation is tightly controlled by general tissue-specific influences or some normal/pathological processes, including cell–cell interactions and many other soluble mediated factors (Murray 2017). In recent years, a large number of studies have focused on the polarization/activation/phenotypic changes of macrophages, in which were found a variety of stimulus signals, including cytokines, extracellular vesicles, miRNAs as well as physical signals in health and disease state (Hamidzadeh et al. 2017). In this review, it is focused on a new perspective of the diverse and complicated signals that could regulate AMs function by characterizing autocrine, paracrine, and endocrine signals. These signals can affect AMs status, and sometimes the same factors may act in different manners. This new insight can provide a novel framework for signals remodeling AMs function and the unique role of AMs in immunity.

2 Autocrine Signals of Alveolar Macrophages

There is a lot of evidence that AMs can self-regulate their function to produce an appropriate immune response. Autocrine signals can be described as extracellular mediators produced or secreted by a certain cell and then bind to the receptor on that cell. Autocrine stimulation often operates in autocrine circulation. AMs can release

many cytokines and chemokines. However, it is rarely mentioned that these signals may act on macrophages in an autocrine manner.

2.1 Interferons

Interferons (IFNs) are a broad class of cytokines that can challenge the host defenses and mobilize the immune responses. IFNs are divided into three categories based on their receptor usage, induction mode, biological activity, and amino acid sequence. Type I IFNs consist of a polygenic family with multiple subtypes, the two most typical families are IFN-α and IFN-β. Type II IFN constitutes the only type IFN-γ, which has antiviral activity induced by activated immune cells, typically by natural killer (NK) and T cells. Type III IFN or IFN-λ is structurally similar to type I IFNs and acts predominantly at epithelial surfaces (Negishi et al. 2018).

IFN-γ is one of the most important cytokines that affect macrophages through transcriptional regulation of immune-related genes, resulting in enhanced immune processes (Schroder et al. 2004). Macrophages stimulation by IFN-γ is often classically activated, and M1 macrophages' response to IFN-γ is essential for full systemic cytokine storm and alveolar inflammation (Gao et al. 2021). T lymphocytes and NK cells are the main source of IFN-γ. It is reported that in vitro infection of AMs with M. tuberculosis induces both the release of IFN-γ protein and a transient increase in IFN-γ mRNA levels (Fenton et al. 1997). Some studies have suggested that macrophages (including but not limited to AMs) can secrete IFN-γ in response to various stimuli, which led to the idea of autocrine macrophage activation in innate immunity (Bogdan and Schleicher 2006; Zhang et al. 2020). IFN-γ also activates TNF-α produced by AMs (Fig. 1), potentiating macrophage-derived chemokine/macrophage-derived C-C motif chemokine ligand 22 (MDC/CCL22) via an autocrine mechanism (Beckmann et al. 2020b).

Except IFN-γ, secreted type I IFNs (IFN-α, IFN-β) play a role in the initial control of viral replication especially in influenza virus infection (Helft et al. 2012), and AMs are the main producers of type I IFNs during pulmonary viral infection (Kumagai et al. 2007). IFN-β signaling triggers a macrophage phenotype similar to that induced by IFN-γ (Lawrence and Natoli 2011), which activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway and generates a positive feedback loop that prolongs activation of IFN-stimulated genes (Li et al. 2009). IFN-β autocrine feedback loop is required to sustain the production of monocyte chemotactic protein-1 (MCP-1) induced by Toll-like receptor (TLR), which is crucial to recruit some other immune cells such as memory T lymphocytes and NK cells (Pattison et al. 2013). These results indicated that autocrine type I IFN provides important support for the effective immune response to AMs.

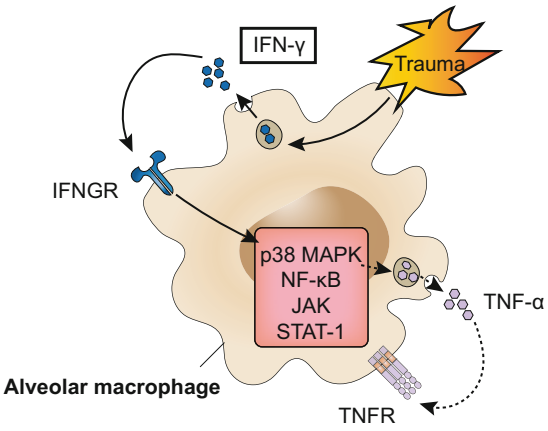

Fig. 1 IFN-γ as an autocrine signal of alveolar macrophages. Alveolar macrophages release certain factors upon stimulation to initiate signaling and then act on alveolar macrophages. Trauma induces alveolar macrophages to secrete IFN-γ firstly, then the autocrine IFN-γ binds to IFNGR on the surface membrane of alveolar macrophages and activates the related pathways including p38 MAPK, NF-κB, JAK, and STAT-1. The activation of these pathways leads to TNF-α production from alveolar macrophages. This process is a paradigm of autocrine signaling of alveolar macrophages, in which IFN-γ acts as an autocrine signal to regulate alveolar macrophages' function. IFN-γ, interferons-γ; IFNGR, interferon-γ receptor; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor; p38 MAPK, p38 mitogen-activated protein kinase; NF-κB, nuclear factor-κB; JAK, Janus-like kinase-1; STAT-1, signal transducer and activator of transcription-1

2.2 Interleukins

IL-10 is a key anti-inflammatory cytokine produced by a variety of cells among which monocytes/macrophages are the main sources, and it can alleviate the inflammatory responses (Moore et al. 2001). For instance, IL-10 exerts strong modulatory effects which can induce the conversion of neonatal AMs from an antiviral phenotype to a new pro-type 17 immune response phenotype in respiratory syncytial virus (RSV) infection (Laubreton et al. 2020). LPS induces human AMs to produce IL-10 via mitogen-activated protein kinase and transcription factor Sp1-dependent mechanisms (Chanteux et al. 2007). It is demonstrated that AMs express IL-10 receptors (Lim et al. 2004) by which IL-10 inhibits the production of pro-inflammatory cytokines (such as IL-1β, TNF-α, and GM-CSF) on monocytes and macrophages (Raychaudhuri et al. 2000). IL-1α induces the proliferation of CD11b (low) alveolar macrophages and differentiates these cells into CD11b (high) macrophages which play critical phagocytic functions and organize granuloma (Huaux et al. 2015). IL-1 signaling was proved to prevent AMs depletion and was essential for maintaining antibacterial immunity during post-influenza pneumococcal infection (Bansal et al. 2018).

There is evidence that IL-12 p70 produced by AMs appears to be tightly controlled by the autocrine release of IL-10 when stimulated by LPS/IFN-γ (Isler

et al. 1999). IL-13 in human AMs from normal and fibrotic lung is proved to promote the initiation and progression of pulmonary fibrosis in a macrophage-dependent manner (Li et al. 2014). Besides, autocrine IL-33 in AMs upregulated the level of matrix metalloproteinase 2 (MMP2) and MMP9 through activating STAT3 in LPS-induced acute lung injury (Liang et al. 2018).

2.3 Tumor Necrosis Factor

TNF-α is a key inflammatory cytokine produced by macrophages and it is extensively taken as an autocrine regulator of TLR-induced inflammatory signaling (Xaus et al. 2000). Autocrine secretion of TNF-α plays an important role in LPS-induced macrophage apoptosis (especially in early apoptotic events) (Xaus et al. 2000). In other words, LPS induces TNF-α production and that TNF-α induces apoptosis of macrophages simultaneously. Recently, Santos and colleagues showed that autocrine TNF mediates AM necroptosis during RSV infection. They use infliximab (a therapeutic TNF-specific neutralizing antibody) to neutralize TNF, which abolishes RSV-induced macrophage necroptosis in vitro. Macrophages respond to ex vivo RSV infection by releasing TNF (Santos et al. 2021). It was found that TNF-α and IL-10 control the expression of chemokine CXCL13 which could predict the progression and severity of idiopathic pulmonary fibrosis (IPF) in monocyte-derived macrophages and AMs by activating the nuclear factor-kappa B (NF-κB) and JAK/STAT pathways, respectively (Bellamri et al. 2020).

2.4 Transforming Growth Factor-β1

TGF-β1 is highly expressed in AMs as a crucial differentiation factor for the development and homeostasis of AMs. It is secreted as an inactive form termed latent TGF-β1 (L-TGF-β1), and the activation of L-TGF-β1 occurs at the cell surface of activated alveolar macrophages by the interaction with thrombospondin 1, CD36, and the protease plasmin (Yehualaeshet et al. 1999). It was shown that the number and frequency of AMs were significantly reduced in $Itgax^{Cre}Tgfb1^{fl/fl}$ mice in which Tgfb1 is deleted in CD11c + cells (AMs), and the remaining AMs exhibited a foam-cell-like phenotype, characterized by enlarged cytoplasm and lipid accumulation (Yu et al. 2017). To sum up, TGF-β1 controls AMs in an autocrine manner.

2.5 Other AM-Derived Signals

GM-CSF is viewed as a major regulator of macrophage functions at all stages of proliferation and differentiation, and it has been shown to be secreted by several

different cell types including macrophages themselves (Hamilton 2002). Autocrine motor factor receptors have been reported to drive the development of allergic asthma by promoting the production of AM-derived GM-CSF (Zhang et al. 2022). In addition, mechanistic studies demonstrated that autocrine GM-CSF released by tumor-stimulated macrophages could synergistically enhance expression and function of relevant receptor in macrophages (Wang et al. 2021). AM-derived GM-CSF may play an important role in AM function. Macrophages with high SPP1 expression play an important role in idiopathic pulmonary fibrosis (Morse et al. 2019).

3 Paracrine Signals of Alveolar Macrophages

Paracrine signaling functions are key to the amplification and control of an inflammatory response in a tissue or organ and to the activation of a coordinated immune response of various cell types involved. Here we introduce diverse signals which modulate AMs in a paracrine manner. Paracrine signaling from alveolar epithelial cells, T cells, and other cells is critical for altering the functions of AMs (Fig. 2).

3.1 Alveolar Epithelial Cells

AMs resided in the airspaces juxtaposed with alveolar epithelial cells and these two types of cells are often described as the most important cells in the maintenance of lung homeostasis (Lambrecht 2006). Numerous researches have focused on the complex cross talk between the airway epithelium and the AMs via cell surface-expressed receptors and secreted products (Bhattacharya and Westphalen 2016). It should be noted that the contiguity of these two cell types may also allow the paracrine communication.

Alveolar epithelial cells could alleviate AMs activation and limit inflammations by their interactions with CD200 (Snelgrove et al. 2008), which is expressed by type II alveolar cells. TGF-β is connected to the epithelial cell surface by αvβ6 integrin (Munger et al. 1999), is used by alveolar epithelial cells to regulate AMs to attenuate endogenous inflammatory signals in alveolar inflammation (Mu et al. 2020). These interactions can also take place in the larger airways (Mayer et al. 2008), where CD200 and αvβ6 integrin are also expressed by the bronchial epithelium. Besides surface protein interaction, these two cells can communicate via secreted cytokines such as IL-10 (Bonfield et al. 1995; Lim et al. 2004) which exerts anti-inflammatory effects on AMs. The epithelium is also a main source of GM-CSF which is required for the differentiation of fetal monocytes into AMs (Schneider et al. 2014). AMs from GM-CSF-deficient mice are not fully differentiated and exhibit reduced responsiveness to the bacterial TLR4 agonist LPS and impaired phagocytosis (Trapnell et al. 2009). The stringent requirement for GM-CSF derived from alveolar epithelial

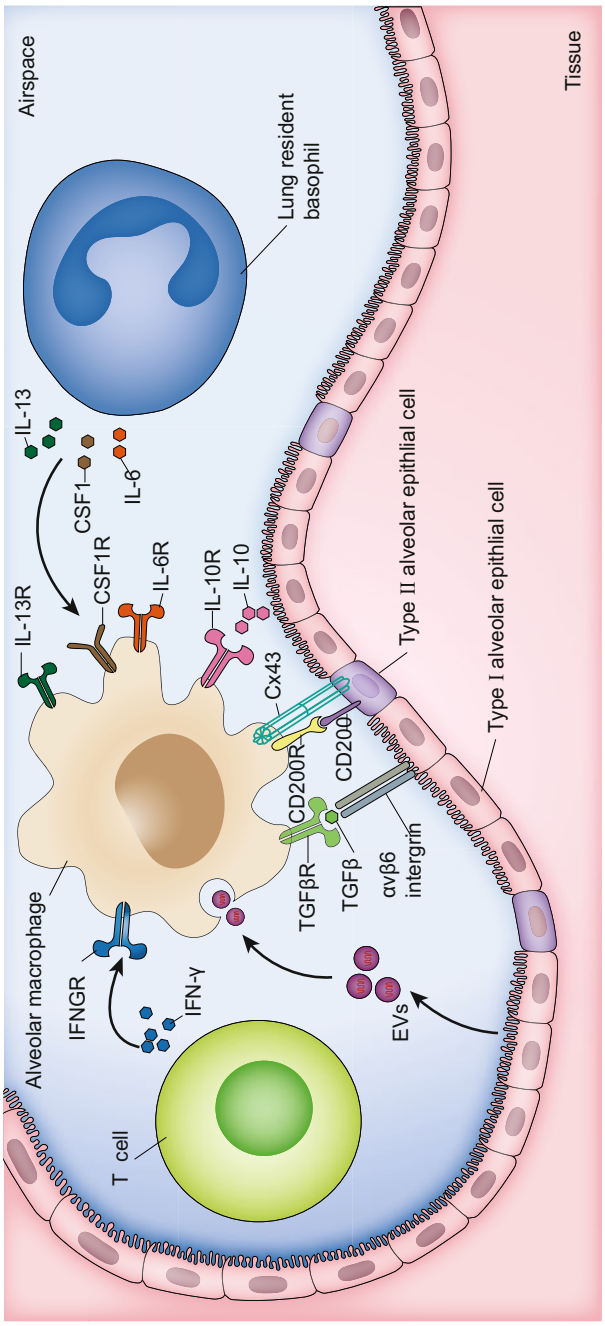

Fig. 2 Paracrine signaling of alveolar macrophages. Alveolar macrophages locate in the lumen of the airways where also reside many other cells including T cells, basophils, and alveolar epithelial cells. Alveolar macrophages can be regulated by neighboring cells in a paracrine manner. Alveolar macrophages are restricted by cell–cell interactions and soluble mediators from neighboring cells. Intercellular communication between alveolar macrophages and alveolar epithelial cells is mediated by CD200 and gap junctions containing Cx43. Besides, alveolar macrophages could receive the paracrine signals including IL-10, IFN-γ, IL-13, CSF-1, IL-6, TGF-β which is tethered to the epithelial cell surface by αvβ6 integrin, and EVs including exosomes and microvesicles. CD200R, CD200 receptor; Cx43, connexin 43; IL, interleukin; IFN, interferon; CSF1, colony-stimulating factor 1; TGF-β, transforming growth factor-β; EV, extracellular vesicles; IL-10R, IL-10 receptor; IFNGR, interferon-γ receptor; IL-13R, IL-13 receptor; CSF1R, colony-stimulating factor 1 receptor; IL-6R, IL-6 receptor; TGF-βR, TGF-β receptor

type 2 cells in AM development and maintenance was recently demonstrated (Gschwend et al. 2021).

AMs and alveolar epithelial cells functions can also be regulated by the release of extracellular vesicles (EVs) – a heterogeneous group of cell-derived membranous structures – which have inflammatory or anti-inflammatory functions by EV surface proteins, cytokines, or miRNAs. Extracellular vesicles comprising exosomes and microvesicles (MVs) are now considered as an additional mechanism for intercellular communication. Under hyperoxia-associated oxidative stress, epithelial cell-derived MVs promote macrophage to regulate lung inflammatory responses via certain specific miRNAs, such as the miR-320a and miR-221 (Lee et al. 2016). Salidroside provides a protective effect on LPS-induced acute lung injury through influencing lung epithelial cells-secreted and miRNA-146a-invovled exosomes which regulate inflammatory pathway of AMs (Zheng et al. 2020). MiR-92a-3p-enriched exosomes derived from alveolar epithelial cells could actuate AMs through activating the NF-κB pathway and downregulating phosphatase and tensin homolog (PTEN) level to significantly aggravate pulmonary inflammation and alveolar permeability in sepsis-induced acute lung injury (Liu et al. 2021). Treating AMs with hyperoxia-induced, epithelial cell-derived and caspase-3 enriched EVs led to an increased secretion of pro-inflammatory cytokines and mediated the inflammatory lung responses (Moon et al. 2015).

It has been demonstrated that AMs could attach to the alveolar wall to form gap junction channels containing connexin 43 (Cx43) with the epithelial cells using real-time alveolar imaging in situ and the two cells communicate with each other through synchronized Ca (2+) waves during LPS-induced inflammation (Westphalen et al. 2014). The gap connexins expressed in these different cell types form spacer regions for cell-to-cell communication (Losa and Chanson 2015). In humans, the alveolar Cx43 protein has also been detected in cocultures, generating evidence for a direct interaction between AMs and alveolar epithelial cells (Beckmann et al. 2020a).

Collectively, regulation of AMs function by alveolar epithelial cells is common but complex, and paracrine secretion of mediators is an important component and is essential to maintain lung homeostasis and inflammatory response.

3.2 T Cells

Another population of immune cells, T cells reside in the lung airway or bronchial lumen (Kaur et al. 2012) under both steady-state and disease conditions. T cells-mediated immune responses are critical for adaptive immunity in both mice and humans (Cooper 2009). Target-mutant mice incapable of making T cells manifested necrotic, neutrophil-dominated lung pathology and dead after a median survival time (MST) of 48 d in Mycobacterium tuberculosis, while wild-type mice manifested macrophage-dominated alveolitis at sites of infection and acquired the ability to control infection with an MST of 258.5 d (Mogues et al. 2001). Both human and animal studies confirmed that T cells are recruited to the lung in various

inflammatory pulmonary diseases, where they coordinate inflammatory responses (Medoff et al. 2005).

It is demonstrated that lung-resident memory T cells colocalize with lung-resident macrophages around the human airways, meanwhile AMs provide co-stimulatory signaling to T cells to construct their effector functions (Snyder et al. 2021). Therefore, T cells may participate in AMs phenotype changes in a paracrine way.

Interactions between AMs and T cells have been investigated extensively in recent years. Grant and colleagues found that circuits exist between infected AMs and T cells in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pneumonia. SARS-CoV-2-infected AMs promote T cells activation, and then these T cells produce IFN-γ to induce AMs to release inflammatory cytokines (Grant et al. 2021).

In innate immune memory or trained immunity, effector CD8 T cells significantly start long-term memory AMs, which is induced by respiratory adenoviral infection in an IFN-γ- and contact-dependent manner where AMs are characterized by high major histocompatibility complex II (MHC II) expression, a defense-ready gene signature, increased glycolytic metabolism, and heightened chemokine production (Yao et al. 2018).

3.3 Paracrine Signaling from Other Cells

Merav Cohen and colleagues identified the spatial localization of lung-resident basophils which reside within the tissue parenchyma, specifically localize near the alveoli and highly express IL6, IL13, and colony-stimulating factor 1 (CSF1), three important myeloid growth factors. Antibody-depletion strategies, diphtheria toxin-mediated selective depletion of basophils, and co-culture studies show alterations in differentiation, compartmentalization, and phagocytic properties of AMs derived from basophil-depleted lungs. These alterations include reduction in gene expression like mature AMs, reduced AMs fraction within the macrophage compartment, and impaired phagocytosis on inactive bacteria (Cohen et al. 2018).

It is also reported that regulatory CD5+ B lymphocytes colonize the respiratory tract of neonatal mice during the early period of life and produce IL-10 to modulate immune responses of AMs to respiratory syncytial virus infection (Laubreton et al. 2020). IL-13 derived from lung eosinophils promoted MMP-12 (a mediator of emphysema) production by AMs (Doyle et al. 2019).

4 Endocrine Signals of Alveolar Macrophages

Many classical hormones secreted by endocrine glands in immune system affect the differentiation, proliferation, activation of the immune cells (Muthusami et al. 2020; Xia et al. 2019). Some other systemic factors including inflammatory cytokines from

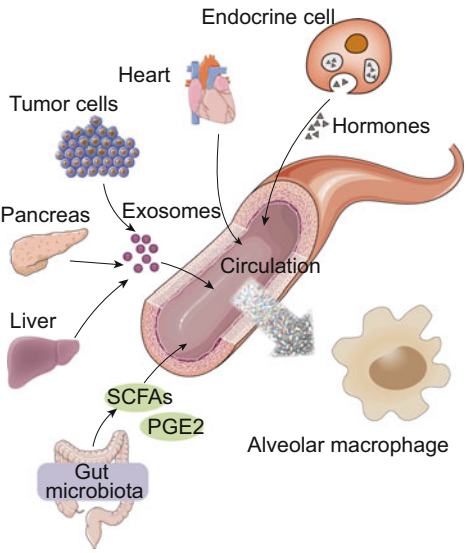

Fig. 3 Endocrine signaling of alveolar macrophages. Alveolar macrophages can be affected by multiple systemic factors (hormones, inflammatory cytokines, and exosomes of systemic origin) throughout the body in an endocrine manner. Gut microbiota, endocrine system, and the remote organs including heart, liver, and pancreas play a role in alveolar macrophages states. And the cross talk between alveolar macrophages and distant cells (mesenchymal stem cells) rather than nearby cells has been reported. Alveolar macrophages respond to various signals derived from peripheral blood in both disease states and homeostasis. PGE2, prostaglandin E2; SCFAs, short-chain fatty acids

remote organ and gut microbiome are highly involved in the endocrine system (Banks 2019; Garcia-Reyero 2018). Here, hormones and cytokines or exosomes of systemic origin which could affect AMs function in a circulating endocrine manner are referred to as endocrine signals (Fig. 3).

There is few research focused on the interaction between endocrine hormones and AMs. Loss of hepcidin (an iron regulatory peptide hormone) in vivo results in blunted functional responses of AMs and exaggerates responses to Streptococcus pneumonia infection (Perez et al. 2020). In vitro experiments in mice, corticosteroids altered the AM control of lichtheimia corymbifera spores by inhibiting AM phagocytosis and decreasing oxidative burst (Brunet et al. 2021). Androgen enhanced IL-4-induced M2 polarization of AMs in vitro, and the deficiency of macrophage-specific androgen receptor diminished M2 polarization of lung macrophages in vivo (Becerra-Díaz et al. 2018). Therefore, the AMs respond to hormones, which makes them as an endocrine target.

As mentioned above, many cytokines or exosomes can affect AMs in an autocrine/paracrine manner, and those carried in the blood can also act in an endocrine-like manner, although they are not considered as traditional hormones. AMs can respond to distant injury such as myocardial infarction and then have a survival benefit to lung infection by removing bacteria more efficiently (Hoyer et al. 2019).

Injured livers release large amounts of miR-122 in an exosome-independent manner into the circulation. Compared with normal livers, circulating miR-122 is then preferentially transported to lungs and taken in by AMs in which it binds TLR7 and activates inflammatory responses (Wang et al. 2019). Plasma-derived exosomes from acute pancreatitis mice can trigger the NOD-like receptor protein 3 (NLRP3)-dependent pyroptosis in AMs and are responsible for the lung injury secondary to acute pancreatitis (AP). Inhibiting the release or uptake of exosomes in vivo substantially suppresses AMs pyroptosis to alleviate AP-induced pulmonary lesion (Wu et al. 2020). Exosomes from metastatic osteosarcoma cells can modulate cellular signaling of AMs, thereby promoting the M2 phenotype and decreasing phagocytosis, efferocytosis, and macrophage-mediated tumor cell killing, which produce an immunosuppressive and tumor-promoting microenvironment via TGF-β2 (Wolf-Dennen et al. 2020).

The cross talks between AMs and nonclassical monocytes/(myo) fibroblasts/mesenchymal stem cells have been reported. For example, the cross talks between nonclassical monocytes and AMs mediate transplantation ischemia-reperfusion injury through classical monocyte recruitment, whereas nonclassical monocyte-derived IL-1β promotes CCL2 production by donor AMs (Kurihara et al. 2021). Lung myofibroblasts may regulate the pathogenic phenotype of AMs through lactate-induced histone acetylation (Cui et al. 2021). In the context of acute respiratory distress syndrome (ARDS), mesenchymal stem cells promote an anti-inflammatory and hyperphagocytic AMS phenotypes through EV-mediated mitochondrial transfer (Morrison et al. 2017). Mesenchymal stem cells can alleviate experimental asthma by inducing AMs polarization (Song et al. 2015).

Besides, the gut microbiota is proved to have a marked influence on AMs function. AMs derived from gut microbiota-depleted mice showed a diminished capacity to phagocytose S. pneumonia. Whole-genome mapping of AMs represented the upregulation of metabolic pathways in the absence of a healthy gut microbiota, which was correlated with an altered cellular responsiveness toward lipoteichoic acid and lipopolysaccharide (Schuijt et al. 2016). Gut dysbiosis during influenza could decrease the bactericidal activity of AMs through gut microbiota-derived short-chain fatty acids (SCFAs) (Sencio et al. 2020). Antibiotic treatment resulted in the overgrowth of a commensal fungal Candida species in the gut and increased plasma concentrations of prostaglandin E2 (PGE2), which induced M2 macrophage polarization and promoted systemic responses including allergic inflammation (Kim et al. 2014). Intestinal dysbiosis induced by anti-tuberculosis therapy (ATT) may have adverse effects on the bactericidal activity of AMs (Khan et al. 2019). It is revealed that probiotics protect against RSV infection in neonatal mice by AM-derived IFN-β via a microbiota-AM axis (Ji et al. 2021).

5 Therapeutic Manipulation of Alveolar Macrophages

Imbalance of AMs was correlated with many pathological processes (Table 1), which makes AMs as an important possible target in the treatment of lung diseases. With the increasing understanding of AMs signaling, manipulation of macrophage function during diseases may become an attractive therapeutic strategy. Pulmonary macrophage transplantation (PMT) is a promising new cell therapy of pulmonary alveolar proteinosis, which is efficacious, durable, and well tolerated. Pulmonary alveolar proteinosis is a disease characterized by abnormal accumulation of surfactant in the alveoli. Most cases are associated with an autoantibody against GM-CSF that prevents clearing of pulmonary surfactant by AMs. In a randomized, controlled trial, inhaled recombinant human GM-CSF was associated with a modest salutary effect on the laboratory outcome of arterial oxygen tension, and no clinical benefits were noted (Tazawa et al. 2019). PMT therapy resulted in correction of alveolar macrophages GM-CSF signaling and long-term persistence of gene-corrected alveolar macrophages carrying functional GM-CSF receptors (Arumugam et al. 2019). As PMT-derived AMs have been persisted for at least 1 year, the therapeutic effects are characterized by alleviating systemic manifestations, normalizing disease-related biomarkers and preventing disease-specific mortality (Suzuki et al. 2014).

Positive effect of Dioscin on crystalline silica-induced pulmonary inflammation and fibrosis was demonstrated to be linked with promoting autophagy of AMs, thus leading to reduced apoptosis and cytokine production (Du et al. 2019). Exosomes mentioned above are considered as therapeutic prospects. For example, mesenchymal stromal cell exosomes were proved to ameliorate experimental bronchopulmonary dysplasia and restore lung function through modulation of AMs phenotype, by suppressing the pro-inflammatory M1 phenotype and promoting an anti-inflammatory M2-like phenotype in both vitro and vivo (Willis et al. 2018). Timely treatment with exosomes derived from endothelial cells and type II alveolar

Table 1 Functional changes in alveolar macrophages (AMs) after challenges

Challenges	Functional changes
Respiratory syncytial virus (RSV) infection	AM necroptosis (Santos et al. 2021)
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection	AM dysfunction (Grant et al. 2021)
Hereditary pulmonary alveolar proteinosis (herPAP)	Disturbed AM differentiation (Happle et al. 2014)
Influenza A virus infection	Increased IL-6 production and prolonged antibacterial protection (Aegerter et al. 2020)
Cigarette smoke exposure	Increased expression of CD14 and impaired bactericidal and phagocytic processes (Lugg et al. 2022)
Adenovirus infection	Increased production of neutrophil chemokines; increased glycolysis; increased protection (Yao et al. 2018)

epithelial cells significantly improved endotoxin-induced acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and bleomycin-induced pulmonary fibrosis (PF) in mice (Feng et al. 2021).

Coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 has become a public health emergency of international concern (Bao et al. 2020). Basic pathologic examination of the lungs of COVID-19 deaths has revealed dysfunction of AMs and AMs infection by SARS-CoV-2 may be the drivers of the "cytokine storm" that can lead to cardiopulmonary damage and even multiple organ failure (Wang et al. 2020). EVs-based therapies hold great potential benefits on COVID-19-related lung injuries, and the modulation of EVs was primarily achieved by reduction of pro-inflammatory cytokines and AM polarization to the M2 reparative phenotype (Khalaj et al. 2020). GM-CSF-based treatments are currently being therapeutically tested in COVID-19 clinical trials. GM-CSF targeting may be due to stabilization of AMs and epithelial cell functions (Lang et al. 2020).

6 Perspectives

AMs are the most abundant innate immune cells in the distal lung parenchyma, located on the luminal surface of the alveolar space. They are the first to encounter incoming pathogens and pollutants and to help orchestrate the initiation and resolution of the immune response in the lung. In vivo, both AMs and other tissue macrophages are exposed to complex microenvironmental signals influencing their essential functions and responses to danger signals, which results in further distinct inflammatory gene-expression programs in different tissues (Glass and Natoli 2016).

On the one hand, AMs have a much lower capacity to respond to the typical type 2 cytokine IL-4, which underlies allergic disease and parasite infection, as compared to macrophages derived from the lung tissue or peritoneal cavity (Svedberg et al. 2019). On the other hand, it is necessary to study tissue-specific macrophages more systematically and to take the complex tissue-specific microenvironment into account. Bone marrow progenitors' cells transferred into the alveolar space have acquired an AM phenotype and have also been shown to ameliorate LPS-induced lung inflammation (Happle et al. 2014). The hyporesponsiveness of AMs to IL-4 may also be associated with the lung microenvironment (Svedberg et al. 2019). Unfortunately, many functional and nearly all molecular studies of macrophages so far focus on primary macrophages and cell lines exposed to single polarizing cytokines in vitro, of which LPS, IFN-γ, and IL-4 are most widely used. It is appreciated to view them based on their unique microenvironment rather than classifying them as M1/M2 paradigms.

In addition, the differences between human and mouse AMs are noteworthy. In contrast to laboratory mice housed in specific pathogen-free conditions, humans are continuously exposed to respiratory pathogens. The ontogeny of human lung macrophages remains poorly understood due to the lack of suitable in vivo models.

Many markers used for murine macrophages were not directly converted to human macrophages directly. Lung transplantation studies indicate that human interstitial and alveolar macrophages are predominantly derived from recruited hematopoietic precursors, possibly circulating monocytes (Byrne et al. 2020). Understanding the differences and similarities between species will help us develop preclinical therapies based on mouse models (Murray and Wynn 2011).

Recent findings show that the immune, nervous, and endocrine systems seem to be closely interacted, for instance, the immune-pineal axis is observed when exposed to polluted air, the synthesis of melatonin switched from the pineal gland to AMs, enhancing phagocytosis of PM2.5 and reducing necroptosis (Carvalho-Sousa et al. 2020). Endocrine-neural signals on AMs are interesting aspects in the future research.

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