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Manuscript Draft

Manuscript Number: CECA-D-16-00003R1

Title: Physiology of Ca2+ signalling in stem cells of different origins and differentiation stages

Article Type: SI:Ca2+ in stem cells

Keywords: Stem cells; Ca2+ channels; Ca2+ signalling; spontaneous Ca2+ oscillations; intracellular [Ca2+]i stores; inositol trisphosphate; ryanodine receptors; glutamate; purinergic receptors; vasopressin, oxytocin; human embryonic stem cells; neural precursors; undifferentiated stem cells; bone marrow mesenchymal stromal cells; adipose tissue derived mesenchymal stem cells; human induced pluripotent stem cells; cell differentiation; cell therapy & transplantation

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Manuscript Region of Origin: CZECH REPUBLIC

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Dear Editor,

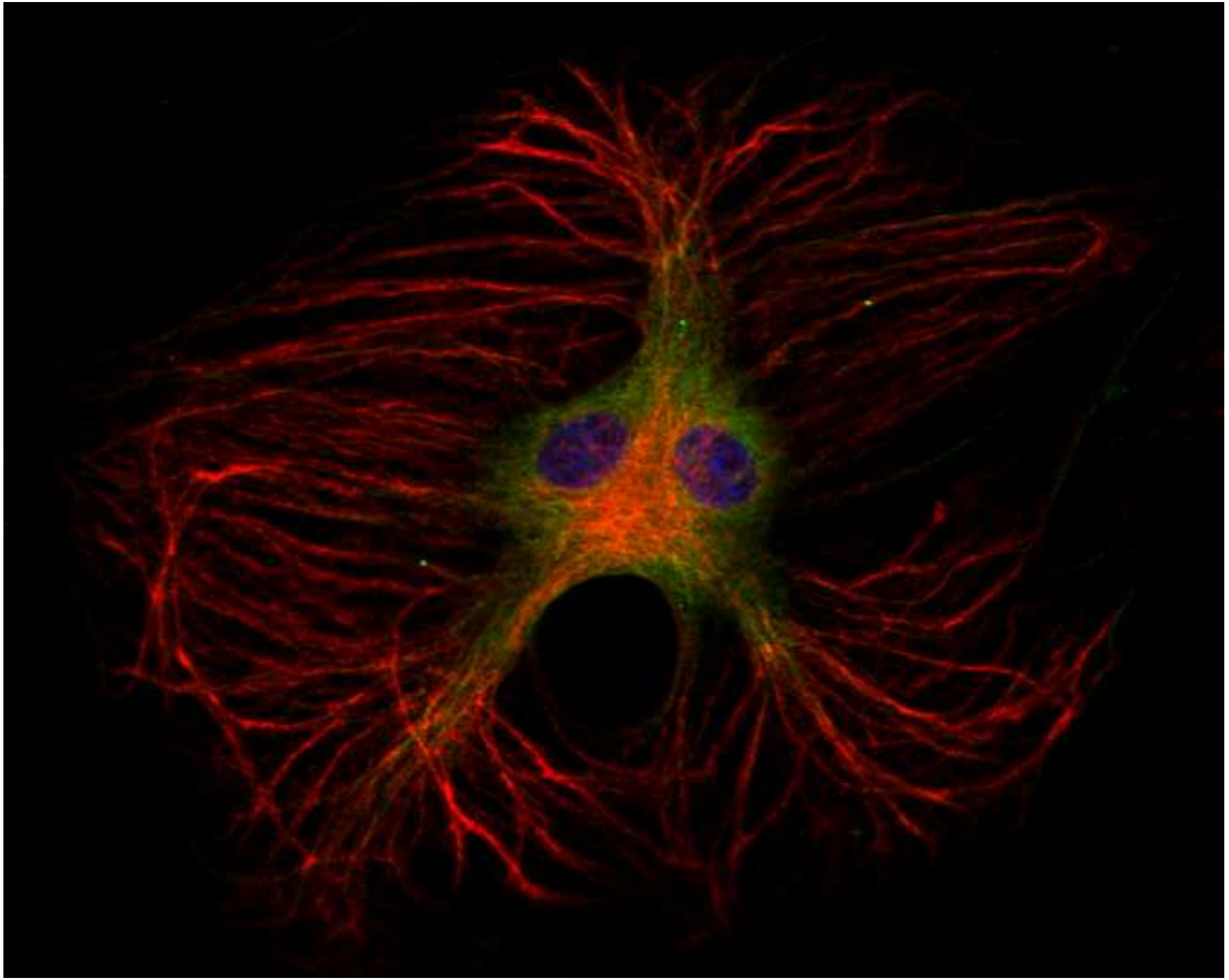
Find please enclosed attached Revised review for CECA special issue on ‘calcium in stem cells’ entitled “Physiology of Ca2+ signalling in stem cells of different origins and differentiation stages” by Forostyak et al, for publication in CECA. The manuscript has been revised according to the Referee’s suggestions.

Thank you for your consideration.

With Best regards,

Forostyak & Dayanithi

***Graphical Abstract**



Highlights

- **The fate and function of stem cells are driven by intrinsic mechanisms**
- **Stem cells express specific ion channels, receptors and signalling cascades**
- **The profile of ion channels and receptors is altered during differentiation**
- **The physiology of differentiated cells depends on the origin of their progenitors**

Physiology of Ca^{2+} signalling in stem cells of different origins and differentiation stages

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Short Title: Ca^{2+} signals in stem cells

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Abbreviations

ADSCs - adipose tissue derived stem cells; **AMSCs -** adipose mesenchymal SCs; **AVP** - arginine vasopressin; **ATP** - adenosine-5'-triphosphate; **BM** - bone marrow; **BMSCs** - bone marrow stem cells; **$[Ca^{2+}]_i$** - intracellular Ca^{2+} concentration; **CNS** - central nervous system; **CPA** - cyclopiazonic acid; **EC** - embryonal carcinoma; **ER -** endoplasmic reticulum; **ESCs** - embryonic stem cells; **GABA -** *gamma*-Aminobutyric acid; **HVA -** high voltage-activated; **hESCs** - human embryonic SCs; **hESC-NPs** - hESC-derived neural precursors; **iPSCs** - induced pluripotent stem cells; **$InsP_3R$** - inositol-1,4,5-trisphosphate receptor; **LVA -** low voltage activated; **MSCs** - mesenchymal stem cells; **NCX -** Na^+/Ca^{2+} exchanger; **NGF -** nerve growth factor; **OT**- oxytocin; **pADSCs** – pre-differentiated adipose-derived stem cells; **pBMSCs** – pre-differentiated bone marrow stem cells; **PLC -** Phospolipase-C; **PMCAs -** plasmamembrane-Ca^{2+}-ATPase; **rMSCs** - rat mesenchymal stem cells; **ROCC** - receptor-operated Ca^{2+} channels; **RyR** - ryanodine receptor; **SCs** - stem cells; **SERCA** - sarcoendoplasmic reticulum Ca^{2+}-ATPase; **SOCs -** store-operated Ca^{2+} channels; **uAMSCs** - undifferentiated adipose-derived mesenchymal stem cells; **uBMSCs** - undifferentiated bone marrow-derived mesenchymal stem cells; **uSCs** - undifferentiated SCs; **LVA -** voltage-activated; **VEGF -** vascular endothelial growth factor; **VGCC** - voltage-gated Ca^{2+} channels.

Conflict of Interest:

The authors state that they have no conflict of interest pertaining to this manuscript.

Highlights

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Abstract

Stem cells (SCs) of different origins have brought hope as potential tools for the treatment of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis. Calcium signalling plays a key role in SC differentiation and proliferation, and dysregulation of Ca^{2+} homeostasis may instigate pathological scenarios. Currently, the role of ion channels and receptors in SCs is not fully understood. In the recent years, we found that (i) the pre-differentiation of human embryonic SCs (hESCs) led to the activation of Ca^{2+} signalling cascades and enhanced the functional activities of these cells, (ii) the Ca^{2+} homeostasis and the physiological properties of hESC-derived neural precursors (NPs) changed during long term propagation *in vitro*, (iii) differentiation of NPs derived from human induced pluripotent SCs affects the expression of ion channels and receptors, (iv) these neuronal precursors exhibited spontaneous activity, indicating that their electrophysiological and Ca^{2+} handling properties are similar to those of mature neurones, and (v) in mesenchymal SCs isolated from the adipose tissue and bone marrow of rats the expression profile of ion channels and receptors depends not only on the differentiation conditions but also on the source from which the cells were isolated, indicating that the fate and functional properties of the differentiated cells are driven by intrinsic mechanisms. Together, identification and assignment of a unique ion channel and a Ca^{2+} handling footprint for each cell type would be necessary to qualify them as physiologically suitable for medical research, drug screening, and cell therapy.

1. Introduction

Public and professional interest in stem cells (SCs) has risen markedly over the last few decades. Two Nobel Prizes in Physiology and Medicine, in 2007 and 2012, were awarded for research in this field. There are many reasons why SCs capture the imagination of so many researches. Firstly, understanding the unique properties of stem cells provides a deeper insight into cell biology and embryology. Secondly, SCs represent the basis for cell replacement therapies in a multitude of degenerative and traumatic diseases. Finally, SCs can serve as an *in vitro* model of various diseases as well as a tool for drug development. SCs of different origin display many differences in molecular phenotype, growth rate, cell marker expression, and the ability to differentiate. The mechanisms underlying these differences remain poorly understood. In particular, the repertoire, major properties and the role of ion channels and receptors in SCs are the subject of intense examination. In this review, we emphasize the notion that the type of SCs used in cell replacement therapies should be carefully chosen based not only on gene expression, morphological features, or cell surface markers, but also on their origin and functional properties correlated with the type of application. Furthermore, identifying the physiological profile of stem cells is essential for assessing the suitability of these cells for their potential use.

2. Historic remarks

“Generatio spontanea” - a theory of spontaneous life generation from nonliving things pronounced by ancient Greeks existed throughout many centuries when in 17th century Francesco Redi and later in 19th century Louis Pasteur by their experiments finally disapproved it and demonstrated that life cannot emerge spontaneously but only from pre-existing life - “*Omne vivum ex vivo*" [1]. This formula was further elaborated by Rudolf Virchow to: "*Omnis cellula e*

cellula" [2], which proclaimed that all cells in the organism derive from pre-existing cells. Indeed, all the cells in multicellular organisms (including humans) arise from the fertilized egg, which effectively is the "totipotent" stem cell. The term “stem cell” or “*Stammzelle*’’ was introduced in 1868 by Ernst Haeckel [3], by which he meant the common cellular ancestor of all living forms. Somewhat later Haekel also applied this term to a fertilized egg [4] (see also [5, 6]). By the beginning of the twentieth century, the notion of SCs had been firmly established, although they were not considered a specific cell population, but rather cells that were transiently formed during development as precursors for differentiated cells. Another meaning of the term “stem cell” was introduced in 1896 by Arthur Pappenheim to describe the precursor cell of the red and white blood cells lineage [7]. Similar ideas of the common precursor for all blood cells were entertained by Alexandr Maximov, who appointed the lymphocyte to this role [8]. These discoveries not only founded the concept of haematopoietic lineages, but also described the existence of other types of SCs in the organism [5].

The simultaneous capacity of SCs to self-renew and to generate different cell types led to the development of two highly different lines of research. The first resulted as a continuation of work on the characterization and isolation of “true” haematopoietic SCs. The presence of mesenchymal progenitor cells in bone marrow has been documented since the late nineteenth century [9, 10]. Goujon was the first to show the osteogenic potential of bone marrow [10]. In 1973, Friedenstein and colleagues showed that the osteogenic potential was a feature of a specific subgroup of cells termed colony-forming unit fibroblastic (CFU-f) cells representing a heterogeneous population of stem and progenitor cells [11, 12]. All these experiments provided the theoretical basis for bone marrow transplant studies and started mesenchymal stem/stromal cells (MSCs) research.

The second line of research focused on pluripotent SCs and began in the 1950s from the studies of teratocarcinomas, the latter being malignant germ cells tumours composed of undifferentiated embryonal carcinoma (EC) cells that can include all three germ layers [13]. Subsequently, the EC cells were shown to be capable of both unlimited self-renewal and multilineage differentiation [14], providing a ground for the further generation of embryonic stem cells (ESCs). Isolation and maintenance of mouse ESCs *in vitro* was, for the first time, reported independently by Sir Martin Evans together with Matthew H. Kaufman [15] and by Gail Martin [16]. Somewhat later, in 1998, Thomson and colleagues generated ESCs from human blastocyst [17]. In 2007 Mario J. Capecci, Martin J. Evans and Oliver Smithies shared a Nobel Prize for the "discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells". The establishment of human embryonic stem cells (hESCs) stimulated the rapid rise of stem cell research as well as worldwide debates about the ethical issues of using human embryos, which waned after the discovery of induced pluripotency [18]. The newly generated induced pluripotent stem cells (iPSCs) were produced by reprogramming mature somatic cells to a pluripotent state by gene transfer and possessed properties similar to ESCs. Similar to MSCs, iPSCs can be generated from a patient's cells, allowing "personalized medicine". At the same time, due to their pluripotency, the iPSCs have broader differentiation potential and therapeutic applications compared to MSCs. The importance of this research was recognized by the Nobel Prize committee, who later awarded the 2012 Nobel Prize in Medicine and Physiology to Sir John B. Gurdon and to Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent".

3. Definition and fundamental properties of SCs

Stem cells are defined as undifferentiated, karyotypically normal cells that have the capacity of self-renewal as well as the ability to generate differentiated cells [19]. Self-renewal is the ability to generate at least one identical copy of the mother cell, and is the most important criterion of "stemness". The ability of cells to differentiate into other cell types is known as cell potency. Characteristic SCs are classified as totipotent, pluripotent and multipotent. Totipotent cells can give rise to all cell types, including cells of the trophectoderm lineage. In mammals, only zygote and early blastomeres (up to 8-cell stage) are totipotent. Pluripotent cells can generate the cells of all three germ layers as well as germline, but not the extraembryonic trophoblast. Multipotent cells can give rise to a restricted subset of tissue-specific cell types (within one germ layer). Based on the time of appearance, SCs can be further sub-classified into ESCs, which occur during embryogenesis, and somatic or adult-derived stem cells, that are present in different tissues in postnatal life. Correspondingly, SCs can be isolated from embryonic, foetal or adult tissues.

The SC lines have been characterized by their developmental potential, transcriptional and epigenetic profiles, cell-surface markers and teratomas formation in nude (i.e. immunosuppressed) mice. The criteria for these assessments include the expression of surface markers and transcription factors associated with the undifferentiated state. In addition proliferative capacity, pluripotency and euploid karyotype as well as epigenetic status are being assessed [20]. Several approaches have been used to characterize SCs, but the most widespread are analyses of the cell surface–antigen phenotype, often by flow cytofluorimetry, and gene expression studies, commonly assessed by RT-PCR or by microarray analyses. These methods are the first, and very often the only, applied to characterize SCs in the undifferentiated state and

during differentiation. However these techniques fail to characterize the functional properties of SCs.

4. Calcium as a life and death signal

It is generally recognized that Ca^{2+} is one of the most universal carriers of biological signals, controlling numerous cellular functions. It prompts conception, regulates cell proliferation and differentiation into a certain type. In differentiated cells Ca^{2+} signals regulate gene transcription, vesicular secretion, membrane excitability, muscle contraction, synaptic plasticity, etc. Finally, excessive elevations of Ca^{2+} initiate cell death either via necrotic or programmed death pathways [21-23]. Cellular Ca^{2+} is regulated by a sophisticated molecular network that has developed through evolution. This network is assembled of multiple components, many of which have several isoforms with different properties. More variations are achieved due to the interactions of Ca^{2+} with other signalling pathways. In addition, Ca^{2+} signals are able to act distinctly in the context of time, space and amplitude. Therefore each specific cell type is able to exploit this system to construct versatile Ca^{2+} signalling systems (also known as "Ca^{2+} signalling toolkits") with variable spatial and temporal properties [24].

The underlying mechanism of Ca^{2+} signalling is relatively simple and is based on the increase of cytosolic concentrations of this ion (commonly denoted as $[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ in eukaryotic cells at rest is set around 50 - 100 nM, and it can rapidly increase (to the range of 1 - 10 μM or even higher in microdomains) in response to stimulation. These increases in $[Ca^{2+}]_i$, which regulate cellular activity, can operate over a broad time and space range, therefore allowing multiple variations of this relatively simple theme. The molecular components of the

Ca^{2+} signalling network can be divided into a few groups according to their function. There are plasmalemmal Ca^{2+} channels that control Ca^{2+} entry from outside the cell. There are Ca^{2+} release channels that control Ca^{2+} release from the intracellular stores. There are Ca^{2+} buffers setting the Ca^{2+} concentration in the cytoplasm. There are Ca^{2+} pumps and exchangers that remove Ca^{2+} from the cytosol to the extracellular space or into intracellular stores. Finally there are numerous Ca^{2+} sensors that translate Ca^{2+} signals into cellular activity. Conceptually, Ca^{2+} might act both as a first and as a second messenger. Acting as a first messenger it, for example, controls axon guidance and neurite outgrowth through activation of plasma membrane calcium-sensing receptors [25]. Acting as a second messenger it regulates numerous processes in neuronal development such as proliferation, migration, differentiation, axon guidance and dendrite outgrowth [26, 27]. SCs express multiple Ca^{2+} signalling pathways, which are summarised in Fig. 1 and Table 1.

5. Ca^{2+} signals in undifferentiated SCs (uSCs)

uSCs possess a primitive Ca^{2+} signalling toolkit. They are non-excitable cells, although some voltage-activated ion channels are known to be expressed in uSCs, especially in adult uSCs [28, 29]. In recent years we performed an in depth analysis of Ca^{2+} signalling pathways in ESCs and MSCs, focusing in particular on voltage-gated Ca^{2+} channels (VGCCs), on Ca^{2+} signals controlled by receptors to glutamate, GABA, ATP, oxytocin and vasopressin, as well as on Ca^{2+} release from intracellular stores [30]. None of the undifferentiated ESCs or MSCs responded to glutamate, GABA, oxytocin or depolarization, confirming the non-excitable nature of uSCs. The majority of uSCs however, generated $[Ca^{2+}]_i$ transients in response to ATP: these were observed in 88 % of uESCs, 90 % of undifferentiated adipose-derived mesenchymal SCs (uAMSCs) and 62 % of undifferentiated bone marrow-derived mesenchymal SCs (uBMSCs). In all these cells

Ca^{2+} signals were triggered by activation of P2X (uAMSCs); or both P2X and P2Y (uBMSCs) types of purinoceptors [31]. In addition uMSCs expressed functional V_1 vasopressin receptors, the activation of which triggered Ca^{2+} release from intracellular stores [31].

uESCs have been shown to express functional purinergic receptors [32], store-operated Ca^{2+} channels [33], $InsP_3$ receptors [33], low voltage-activated (LVA) Ca^{2+} channels [34], but no high-voltage activated (HVA) Ca^{2+} channels. Undifferentiated cells of the CCTL14 hESC line were only partially sensitive to ATP and these responses were mediated mainly through $P2X_2$, $P2X_3$, $P2X_7$ and P2Y purinoceptors [30]. The expression of genes encoding $P2X_{2,5,7}$ and $P2Y_{1,2,6}$ receptors was identified in mouse ESCs [35]. In the same mouse ESCs plasmalemmal Ca^{2+} entry mainly occurs through store-operated Ca^{2+} channels (SOCs), whereas Ca^{2+} release from intracellular stores is mediated by $InsP_3$ receptors, but not by ryanodine receptors [33]. Extrusion of Ca^{2+} in mouse ESCs was mediated by both Na^+/Ca^{2+} exchanger (NCXs) and plasmamembrane-Ca^{2+}-ATPase (PMCAs) [33].

Undifferentiated MSCs are rather heterogeneous, although similar to uESCs the majority of these cells express functional purinoceptors [36]. Functional P2X ($P2X_{4,7}$) and P2Y receptors were identified in uMSCs [31, 36, 37]. The ATP autocrine/paracrine signalling pathway was involved in $[Ca^{2+}]_i$ oscillations observed in uBMSCs [38]. The uAMSCs also expressed purinergic, vasopressin, adrenergic and histamine linked to $InsP_3$ receptor-mediated $[Ca^{2+}]_i$ dynamics [31, 36, 39]. Of note, receptors expression in SCs varies among species. All three subtypes (V_{1a}, V_{1b} and V_2) of vasopressin receptors were identified in adipose mesenchymal SCs (AMSCs) isolated from mice [40], whereas human AMSCs expressed only the V_{1a} (AVP V_{1a}) subtype of vasopressin receptor [39].

6. Changes of Ca^{2+} signalling toolkit during differentiation towards neuronal phenotype

The exposure of SCs to certain environmental and differentiation conditions initiates cell maturation and their transformation into a certain cell type. These processes are accompanied by changes in the expression of various genes and proteinatious cell markers as well with functional metamorphosis. Functional properties of NPs derived from ESCs change during maintenance *in vitro* [41], which is manifested by an increased Ca^{2+} signalling during a certain time period [30, 42]. This finding demonstrates that ESCs may acquire a specific pattern of Ca^{2+} signalling for a limited period and that evaluation of Ca^{2+} signals in stem cells can be used to predict the fate of the cells during differentiation and can serve as an important criterion for assessing the quality of stem cells before their use in cell replacement therapy.

7. Voltage-gated calcium channels in SCs

Voltage-gated Ca^{2+} channels (VGCCs) represent the major Ca^{2+} entry pathway in excitable cells and thereby control a variety of cellular functions both during development and in mature cells. During embryogenesis Ca^{2+} entry through VGCCs contributes to cell proliferation and cell differentiation. Depending on biophysical and pharmacological properties in many cell types VGCCs have been classified into two major groups: high voltage-activated channels (HVA), which include L-, N-, P/Q- and R-type channels and low voltage-activated channels (LVA) or T-type channels [43-46].

The expression of L- and T-type VGCC (but not P/Q- or R-VGCC) was identified in many types of SCs. In particular they were detected by immunocytochemistry and immunoblotting in ESCs [47], AMSCs [48, 49] and in BMSCs [28, 29, 50]; however functional

VGCCs could be identified only in undifferentiated BMSCs and mouse ESCs. Small subpopulations of human undifferentiated BMSCs (~15 %) express functional L-type Ca^{2+} channels [28, 29, 50]; similarly L-type Ca^{2+} currents were recorded from rat BMSCs [51]. Unlike human cells, the undifferentiated AMSCs isolated from rat do not possess operational VGCCs [31]. These findings further confirm the distinct expression of cell markers and specificity of cell signalling pathways in SCs isolated from different species. Only T-type VGCC has been shown to be functional in mouse ESCs [34, 52]. It was claimed that the modulation of T-type channels that occurs during cell cycle progression might contribute to maintenance of ESCs self-renewal capacity [34]. Activation of LVA VGCC was shown to trigger $[Ca^{2+}]_i$ oscillations in ESC-derived neural progenitors and therefore was speculated to induce cell proliferation [52]. To the best of our knowledge there is no evidence of functional VGCCs in undifferentiated human ESCs. None of the undifferentiated human ESCs from the CCTL-14 line responded with $[Ca^{2+}]_i$ elevation to depolarisation, although during differentiation towards the neuronal phenotype almost half of the cells expressed functional L- and P/Q-type VGCCs [30]. Similarly, the uESCs lines H9 and HS181 were not responsive to depolarisation [52]. Human foetal stem cells differentiated to motor neurones have been shown to express functional L-, N-, P/Q- and T-type VGCCs [53]. The influx of Ca^{2+} through L-VGCC induces gene activation, enhances neurogenesis and differentiation. For example, it was shown that L-type VGCCs contribute to neuronal and odontogenic differentiation of dental pulp stem cells [54]. The L-type VGCCs enhanced the neuronal differentiation of ESCs as well as in adult MSCs. In particular, it has been shown that neuronal differentiation of ESCs depends on the cooperation between L-type VGCC and RyR2 intracellular Ca^{2+} release channels [55]. The L-type VGCCs play a key role in promoting neuronal differentiation of neural stem cells [56]; whereas in BMSCs L-type VGCCs regulate both neuronal and osteogenic differentiation [57, 58].

The P/Q-type VGCCs in adult neurons trigger the release of neurotransmitters at synaptic terminals [22] and the Q-type VGCCs are involved in vasopressin secretion from neurohypophysial terminals [59]. They performed the same function during development of hippocampal neurons [60]. The role of P/Q-VGCCs in SCs remains somewhat obscure, however, there are several reports showing activation of P/Q-VGCCs during differentiation toward neurons. In our experiments, we were not able to detect P/Q-VGCCs in any type of undifferentiated SCs (hESCs, rBMSCs, rAMSCs). However, after differentiation of these cells into neuronal phenotype the P/Q-VGCC could be identified both functionally and immunocytochemically in hESCs [30] and rat BMSCs [31]. Similarly, activation of P/Q-VGCC was detected in bi- and multipolar neurons derived from mESCs and a shift in channel pattern from N- and L-types in apolar cells to P/Q- and R-type channels in bi- and multipolar cells was reported [47].

The functional N-type VGCCs are present in central [61, 62] and peripheral embryonic motoneurons [63], and in the neurons of the dorsal horn of the spinal cord and of the dorsal root ganglia [64]. These channels regulate neurotransmitter release in presynaptic nerve terminals and they also contribute to pain pathways [65]. Expression of N-type channels have been analysed only in ESCs. Specific mRNA has been detected in human in neural progenitors derived from hESCs [66]; whereas in mouse ESCs N-type channels were detected at the protein and functional level [47]. Functional and mRNA expression analysis on BMSCs failed to detect N-type VGCCs [50].

8. Neurotransmitter receptors linked to Ca^{2+} signalling

8.1. P2 purinergic receptors

Evolutionary purinergic signalling systems are among the most ancient, are present virtually in every cell and control a wide variety of cell functions [67]. Purinergic receptors have been implicated in a wide variety of physiological and pathological conditions including neurotransmission, myocardium rhythm regulation, brain development, apoptosis, inflammation, pain, central nervous system injury and neurodegenerative disorders, etc. [68-71]; they also contribute to regulating cell proliferation at the early stages of brain development [72]. The P2 subfamily of purinergic receptors comprises the ionotropic P2X and metabotropic G protein coupled P2Y receptors. There are seven subtypes of P2X ($P2X_{1-7}$) and eight subtypes of P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) receptors, all of which have been identified solely or in different combinations in various SCs.

The first indications for functional P2 receptors were obtained in human BMSCs where ATP, secreted through connexin hemichannels, stimulated Ca^{2+} release from ER and $[Ca^{2+}]_i$ oscillations through activating the $P2Y_1$ receptor and the PLC-β/$InsP_3$ signalling pathway [38]. Subsequently it was argued that ATP released at the early life stages of human BMSCs modulates their proliferation rate and likely acts as one of the early factors determining their cell fate [73]. Undifferentiated AMSCs and BMSCs isolated from rat express only P2X receptors, while neuronal induction of AMSCs leads to the emergence of P2Y receptors [31]. Functional expression of $P2Y_2$ receptors in rat BMSCs depends on cell density and may correlate with cell cycle progression [74]. In humans AMSCs $P2Y_4$ and $P2Y_{14}$ receptors regulate the onset of mesenchymal differentiation, whereas $P2X_5$ and $P2X_6$ receptors are responsible for lineage

commitment [75]. Down-regulation of $P2Y_1$ and $P2Y_2$ can serve as markers for early osteogenic differentiation [75]. The release of ATP by MSCs is also able to promote osteogenic differentiation by activating $P2X_7$ receptors [76]. Expression of $P2X_4$ and $P2X_5$ mRNA was demonstrated in human uESCs [66], while in mouse uESCs expression of $P2X_{2,5,7}$ and $P2Y_{1,2,6}$ has been detected [35]. All seven P2X receptors as well as $P2Y_{1,2,6}$ receptors were expressed in GABAergic neurons differentiated from mouse ESCs, although Ca^{2+} signals were mainly associated with activation of $P2X_2$, $P2X_4$ and $P2Y_1$ receptors [35]. Functional $P2X_{2,3,7}$ and P2Y receptors were also found in neural precursors derived from human ESCs [30].

8.2. Vasopressin and oxytocin receptors

Vasopressin (AVP) and oxytocin (OT) are closely related neuropeptides synthesized in the neurons of supraoptic and paraventricular nuclei in the hypothalamus; these neurohormones control a wide range of functions [77-79]. Both AVP and OT regulate various aspects of SCs life. The AVP, for example, promotes cardiomyocyte differentiation of mouse ESCs through *Gata-4* and NO signalling [40]. In addition AVP acting through AVP V_{1a} receptors and PLC/$InsP_3$/Ca^{2+} pathways may inhibit differentiation of hAMSCs to adipocytes [39]. In our studies all BMSCs were sensitive to AVP, independent of differentiation stage. A vast majority of uADSCs (75 %) and pre-differentiated ADSCs (94 %), responded to the application of AVP by an increase in $[Ca^{2+}]_i$. The effects of AVP were dose-dependent and were mediated through the AVP V_1 receptor [31, 39]. The MSCs express OT receptors activation, have trophic and protective effects; control differentiation [80] and proliferation [81]. Treatment of BMSCs with OT reduced apoptosis increased cellular proliferation and angiogenesis, and augmented glucose uptake [82]. There is evidence that OT negatively modulates adipogenesis, while promoting osteogenesis in human ADSCs and BMSCs [80].

9. Spontaneous $[Ca^{2+}]_i$ activity

Spontaneous $[Ca^{2+}]_i$ oscillations have been observed in both excitable and non-excitable cells and are essential for embryonic development. Due to their heterogeneous spatio-temporal parameters they contribute to regulating fertilization, cell proliferation, secretion, neuronal differentiation, axonal outgrowth, radial glia proliferation and neuronal migration, etc. [83-87]. Spontaneous $[Ca^{2+}]_i$ transients occur predominantly during the early stages of neural precursor differentiation and regulate neurite outgrowth and the onset of the GABAergic phenotype [88]. Spontaneous $[Ca^{2+}]_i$ activity has been observed in 31 % of neural precursors derived from hESCs; this spontaneous $[Ca^{2+}]_i$ dynamic was mediated by Ca^{2+} influx through HVA VGCC [30]. The L-VGCC and TRPC1-dependent spontaneous $[Ca^{2+}]_i$ transients were detected in postmitotic neurones derived from hESCs. Inhibition of these spontaneous $[Ca^{2+}]_i$ dynamics lead to a significant reduction in cell proliferation [89]. Spontaneous $[Ca^{2+}]_i$ activity in human foetal neural stem cells (NSC)-derived neural progenitors was critically dependent on connexin 43-formed gap junctions [90] and VGCC. In addition gap junctions mediated electrical connectivity between these progenitors. Inhibition of the functional networks by blocking the gap junctions terminated both the spontaneous Ca^{2+} activity and the proliferation rate [52].

Undifferentiated BMSCs and neural precursors from BMSCs also exhibit spontaneous $[Ca^{2+}]_i$ transients and oscillations [31, 57, 91]. In human undifferentiated BMSCs as well as in BMSCs-derived adipocytes, spontaneous $[Ca^{2+}]_i$ transients result from $InsP_3$-mediated Ca^{2+} release from the endoplasmic reticulum (ER) stores [38, 91]. In 29 % of rat BMSCs spontaneous $[Ca^{2+}]_i$ activity was detected. This was dependent not only on Ca^{2+} release from ER, but also on plasmalemmal Ca^{2+} influx [31]. In rat AMSCs $[Ca^{2+}]_i$ oscillations were solely dependent on ER

Ca^{2+} stores and were detected in 12 – 13 % of cells [31]. Similarly, spontaneous $[Ca^{2+}]_i$ oscillations were also observed in a subpopulation of human AMSCs [36, 92]. In summary, spontaneous $[Ca^{2+}]_i$ oscillations occur in many types of SCs; in embryonic SCs they are dependent on Ca^{2+} influx through plasma membrane, whereas in adult SCs they are triggered mainly by Ca^{2+} release from ER. Without a doubt these oscillations play an important role in cell development [93]. While further research is needed, understanding the mechanisms and control of these oscillations may offer a tool to regulate cell proliferation.

10. Intracellular Ca^{2+} stores

The endoplasmic reticulum (ER) is the major Ca^{2+} storage organelle that contributes to multiple Ca^{2+} signalling pathways. There are two major Ca^{2+} release channels localized in the ER, the ryanodine receptors (RyR) and inositol 1,4,5-triphosphate ($InsP_3$) receptors; Ca^{2+} accumulation into the ER is mediated by the sarco/endoplasmic reticulum Ca^{2+}-ATPase, also known as SERCA (for details of ER handling see [94, 95]) serving to replenish the depleted stores. The metabotropic PLC/$InsP_3$ signalling cascade has been shown to be the major pathway for Ca^{2+} release from the ER in various types of SCs. The effect of ATP, histamine, vasopressin, noradrenalin and endothelin I and II on SCs is mediated via the PLC/$InsP_3$ signalling pathway [36, 38, 39, 96, 97]. Only $InsP_3$ receptors, not RyRs, are functional in undifferentiated embryonic mESC [33]; in adult hAMSCs [36, 96] and in undifferentiated hMSCs [50]. The PLC/$InsP_3$/Ca^{2+} release pathway seems to be conserved in all SCs, since it remains functional throughout the whole period independent of SC origin and differentiation stage. For example, ESCs and MSCs differentiated to cardiomyocytes, adipocytes or neurons, all possess functional $InsP_3$ receptors [38, 39, 57, 97, 98]. Ryanodine receptors have not been identified in undifferentiated SCs, although they do emerge during differentiation. For example, neural and cardiac cells derived

from ESCs, or MSCs express functional RyRs [30, 31, 57, 98]. Moreover, the functional coupling between RyR type 2 and L-VGCC is important in neuronal differentiation as demonstrated in ESCs [55] and BMSCs [57].

11. Plasticity of Ca^{2+} signalling cascades

Experiments *in vitro* and *in vivo* have shown that despite their origin all types of SCs have a broad range of regenerative capabilities acting either as neuroprotective or cell replacement agents. Early-stage oligodendrocyte progenitor cells derived from human ESCs after being grafted into the rodent model of spinal cord injury were able to secrete factors enhancing neuritis extension and resulted in decreased cavitation, remyelination and enhanced functional recovery. Eventually, these changes lead to the significant restoration of motor functions in the transplantation group compared with the control group [99]. In addition, a mixed lymphocyte reaction assay indicated that embryonic stem cell-derived neural stem/progenitor cells (ESC-NS/PCs) modulated the allogeneic immune rejection. These results demonstrated a significant cell replacement effect after implantation of ESCs and resulted in an FDA approved first phase I clinical trial involving human embryonic stem cells [100, 101]. Another study involving transplantation of nonhuman primates allogeneic ESCs demonstrated that the grafted ESC-NS/PC-derived oligodendrocytes contributed to the remyelination of demyelinated axons; moreover some transplanted neurones were myelinated by host cells whereas some grafted neurons formed synaptic connections with host tissues [102]. Thus allogeneic transplantation of ESC-NS/PCs from a nonhuman primate promoted functional recovery after spinal cord trauma without tumorigenicity.

Although the characteristics of ion channels and membrane receptors and intracellular signalling mechanisms of these cells have yet to be identified, a few attempts were made in our laboratory to demonstrate the functional differences between undifferentiated hESCs and predifferentiated cells. Undifferentiated hESCs cells were partially responsive only to ATP, whereas pre-differentiated cells expressed more sophisticated Ca^{2+} signalling mechanisms, reminiscent of a neural phenotype (functional Ca^{2+} channels, purinergic, glutamate and ryanodine receptors) and exhibited spontaneous $[Ca^{2+}]_i$ oscillations [30]. A similar approach was used to test the stem cell model - an immortalized neural stem cell line from human foetal spinal cord which preserves specific physiological and differentiation features of ventral spinal cord progenitors even after extensive *in vitro* propagation and engraftment onto a lesioned rodent spinal cord [53]. From the cell lines generated, individual SPC-01-derived neurones exhibited similar Ca^{2+} signalling patterns to what was described previously in the case of CCTL14-derived NPs; particularly the presence of functional L- and P/Q-type Ca^{2+} channels and the occurrence of spontaneous $[Ca^{2+}]_i$ oscillations.

Other studies have shown that mouse embryonic stem cell-derived neurons in the early stages of differentiation possess a complex pattern of VGCC, with a shift in channel contribution from N- and L-types in apolar cells to P/Q- and R-type channels in bi- and multipolar cells [47].

Other cell types that resemble ESCs are induced pluripotent stem cells derived from somatic cells via transduction of their properties by a cocktail of factors Oct3/4, Sox2, c-Myc, and Klf4 [103]. These cells have the potential to differentiate towards mature cell types of all three germ layers. Neural progenitor cells derived from iPSCs cells have shown changes in protein and gene expression levels during differentiation and expression of neural growth factors (BDNF and NT3)

and neuronal/glial markers [104]. Transplantation of the aforementioned cells led to stimulation of axonal ingrowth and renewal of neural tissue accompanied by a significant improvement in motor functions. The same cell-type has been shown to decrease astroglial scar formation and stimulate intrinsic growth factors (vascular endothelial growth factor, VEGF; nerve growth factor, NGF; and glia cell-line derived neurotrophic factor, GDNF [105]).

It is generally accepted that the regenerative efficacy of MSCs is based on the secretion of a wide range of substances that play a crucial role in nourishing and protecting neurons, either by host cells (stimulation of internal neurogenesis and modification of gene expression levels) or by the MSCs themselves (paracrine function) [106]. Delivery of bone marrow MSCs into rodents modulated the immune response by over-expression of some chemokines (IL-α and MCP-1) and reconstitution of T cells following transplantation [107, 108]. After transplantation of MSCs grafted cells are safely integrated into the host nervous tissue, they are capable of long-term survival and migration along cranio-caudal orientation where they form scaffolds for neurites outgrowth [109, 110]. Grafting of MSCs has also been shown to modulate adult CNS plasticity through normalization/stabilization of extracellular matrix dense structure (perineuronal nets) and by antiapoptotic effects [108]. Some studies have demonstrated that transplantation of MSCs enables the transfer of functional mitochondria from the graft into the host cells [111, 112].

12. Conclusions

Ca^{2+} signalling contributes to SCs function, proliferation and differentiation from the very early stages of development. A wide variety of functions, including the fate and overall survival of SCs, are regulated by Ca^{2+} signals. In addition, studying the functional properties of stem cells *in*

vitro may help to predict their behaviour and the fate of their physiopathological status *in vivo* and may serve as criteria to evaluate the quality of such cells. Understanding the physiology of stem cells may allow us to better control their regenerative potential, which in turn may help to improve strategies for their use in transplantation and the treatment of neurodegenerative diseases. Therefore we suggest including Ca^{2+} signalling profiles in routine laboratory practice in order to use cells of the same type and same conditional state in experiments. Detailed research of Ca^{2+} signalling pathways in stem cells would help to develop new strategies in cell therapies.

Acknowledgements

This work was supported by the grants GACR P304/11/2373, GACR 14-34077S, GACR P304/12/G069 and GACR 10504P from the Grant Agency of the Czech Republic. Govindan Dayanithi belongs to the "Centre National de la Recherche Scientifique-The Ministry of Research and Higher Education-Paris", France. We are grateful to Kip Allan Bauersfeld IEM ASCR, for critical reading of the manuscript.

Table 1. Expression of Ca^{2+} channels and receptors linked to Ca^{2+} signalling in SCs

Channel/ Receptor	Type of SCs	Species	Differentiation	Functional expression	Gene/mRNA expression	Protein Expression	Reference
Voltage-activated Ca^{2+} channels (VGCC)							
VGCC: **LVA**	ESCs	mouse	neuronal	LVA	-	-	[52]
VGCC: **L P/Q N R**	ESCs	mouse	neuronal	Yes (L-, P/Q-, N-, R-VGCC)	-	Yes (P/Q-,N-, R-VGCC)	[47]
VGCC: **L, P/Q T**	ESCs	mouse	undifferentiated	No	No	-	[33]
VGCC: **T**	ESCs	mouse	undifferentiated	Yes (T-VGCC)	Yes (T-VGCC)	Yes (T-VGCC)	[34]
VGCC: **L**	ESCs	mouse	neuronal	Yes (L-VGCC)	-	Yes (L-VGCC)	[113]
VGCC: **L**	ESCs	mouse	neuronal	Yes (L-VGCC)	-	-	[55]
VGCC: **L P/Q N**	ESCs	human	undifferentiated neuronal	No Yes (L-, P/Q-VGCC)	- -	No Yes (L-, P/Q-, N-VGCC)	[30]

VGCC	ESCs	human	undifferentiated neuronal (dopaminergic)	No Yes (VGCC)	- -	- -	[32]
VGCC: **L** **P/Q** **T** **N**	ESCs	human	neuronal	-	Yes (L-, N-, T-VGCC) No (P/Q-VGCC)	-	[66]
VGCC: **L** **P/Q** **T** **N**	Fetal SPC-01	human	motoneurons	Yes (L-, N-, P/Q-, T-VGCC)	-	-	[53]
VGCC: **L** **T**	AMSCs	human	undifferentiated	No	Yes (L-, T-VGCC)	Yes (L-VGCC)	[48]
VGCC: **L** **T**	AMSCs	human	undifferentiated neuronal	No No	Yes (L-, T-VGCC) Yes (L-, T-VGCC)	- -	[49]
VGCC: **L** **P/Q** **N**	AMSCs	rat	undifferentiated neuronal	No Yes (L-VGCC)	- -	No Yes (L-, P/Q-VGCC)	[31]
VGCC:	BMSCs	human	undifferentiated	Yes (L-VGCC)	Yes (L-VGCC)	-	[28]

L **T**							
<u>VGCC:</u> **L** **T**	BMSCs	human	undifferentiated	Yes (L-VGCC)	Yes (L-, T-VGCC)	-	[29]
<u>VGCC:</u> **L** **P/Q** **T** **N** **R**	BMSCs	human	undifferentiated	Yes (L-VGCC)	Yes (L-, P/Q-, T-VGCC)	-	[50]
<u>VGCC:</u> **L** **P/Q** **N**	BMSCs	murine	neuronal	Yes (L-VGCC)	-	-	[57]
<u>VGCC:</u> **L** **T**	BMSCs	rat	undifferentiated	Yes (L-VGCC)	Yes (L-VGCC)	-	[51]
<u>VGCC:</u> **L**	BMSCs	rat	osteogenic	Yes (L-VGCC)	Yes (L-VGCC)	-	[58]
<u>VGCC:</u> **L** **P/Q** **N**	BMSCs	rat	undifferentiated neuronal	No Yes (L-, P/Q-VGCC)	- -	No Yes (L-, P/Q-VGCC)	[31]
Intracellular Ca^{2+} stores							
$InsP_3$ R1-3	ESCs	mouse	undifferentiated	Yes ($InsP_3$)	Yes ($InsP_3$ R	-	[33]

RyR R1-3				No (RyR)	1,2,3)		
$InsP_3$	ESCs	human	cardiomyocytes	Yes ($InsP_3$)	-	Yes ($InsP_3$)	[97]
RyR2	ESCs		neuronal	Yes (RyR2)	Yes (RyR2)	Yes (RyR2)	[55]
RyR2 $InsP_3$	ESCs	human	cardiomyocytes	Yes (RyR2 $InsP_3$)	Yes (RyR2 $InsP_{3)}$	Yes (RyR2 $InsP_3$)	[98]
RyR	ESCs	human	neuronal	Yes (RyR)	-	Yes (RyR1,3)	[30]
$InsP_3$ R RyR	AMSCs	human	undifferentiated	Yes ($InsP_3$) No (RyR)	-	-	[36]
$InsP_3$	AMSCs	human	adipocyte	Yes ($InsP_3$)	-	-	[39]
$InsP_3$	AMSCs	human	adipocyte	Yes ($InsP_3$)	-	-	[96]
$InsP_3$ R1-3 RyR 1-3	BMSCs	murine	neuronal	Yes (RyR, $InsP_3$)	Yes (RyR2, RyR3, $InsP_3$R1, $InsP_3$R2, $InsP_3$R3)	-	[57]
$InsP_3$ RyR	BMSCs	human	undifferentiated	Yes ($InsP_3$)	Yes ($InsP_3$ R1-3)	-	[50]
$InsP_3$	BMSCs	human	adipocytes	Yes ($InsP_3$)	-	-	[38]
P2 purinergic receptors							
$P2X_{2,3,4,5,7}$	ESCs	human	neuronal precursors (NPs)	-	Yes ($P2X_5$, $P2X_4$)	-	[66]

			Differentiated NPs	-	Yes ($P2X_5$)	-	
$P2X_{2,3,4,6,7}$ P2Y	ESCs	human	neuronal	Yes ($P2X_{2,3,7}$, P2Y)	-	Yes ($P2X_{2,3,7}$)	[30]
$P2X_{1,2,3,45,7}$ $P2Y_{1,2,4,6}$	ESCs	mouse	undifferentiated neuronal GABAergic	- Yes ($P2X_{2,4}$, $P2Y_1$)	Yes ($P2X_{2,5,7}$ $P2Y_{1,2,6}$) Yes ($P2X_{1,2,3,4,5,6,7}$ $P2Y_{1,2,6}$)	- -	[35]
$P2X_{1-7}$ $P2Y_{1,2,4,6,11,12,13,14}$	AMSCs	human	adipogenic osteogenic	$P2Y_{1,2,4,12,13}$	Yes ($P2X_{3,4,5,6,7}$, $P2Y_{1,2,4,6,11,12,13,14}$)	-	[75]
P2X P2Y	AMSCs	rat	undifferentiated neuronal	Yes (P2X) Yes (P2X, P2Y)	- -	- -	[31]
$P2Y_2$	BMSCs	rat	undifferentiated	Yes ($P2Y_2$)	Yes ($P2Y_2$)	Yes ($P2Y_2$)	[74]
P2X P2Y	BMSCs	rat	undifferentiated neuronal	Yes (P2X) Yes ($P2X_7$)	-	- Yes ($P2X_7$)	[31]
$P2Y_1$ P2X	BMSCs	human	undifferentiated	Yes ($P2Y_1$, P2X)	-	-	[73]
$P2Y_1$	BMSCs	human	adipogenic	Yes ($P2Y_1$)	-	-	[38]
Oxytocin (OT) and vasopressin (AVP) receptors							
AVP V_{1a}	ESCs	mouse	cardiac	-	Yes (AVP V_{1a},	-	[40]

AVP V_{1b} **AVP V_2**					AVP V_{1b}, AVP V_2)		
AVP V_{1a} **AVP V_{1b}** **AVP V_2**	AMSCs	human	adipogenic	Yes (V_{1a} R)	Yes (V1a R)	-	[39]
OT R	AMSCs	mouse	neuronal	-	Yes (OT R)	-	[81]
OT R **AVP-V_{1a}** **AVP V_2**	BMSCs	rat	undifferentiated	Yes (OT R)	Yes (OT R, AVP-V_{1a} R	Yes (OT R)	[82]
OT R **AVP-V_1**	BMSCs	rat	undifferentiated neuronal	Yes (AVP V_1) Yes (OT R, AVP V_1)	-	Yes (AVP) Yes (OT, AVP)	[31]
OT R	AMSCs BMSCs	human	adipogenic osteogenic	Yes (OT R)	-	-	[80]

Abbreviations: - **AMSCs**, adipose tissue derived mesenchymal stromal cells; **AVP**, vasopressin; **BMSCs**, bone marrow mesenchymal stromal cells; **ESCs**, embryonic stem cells; **InsP3**, Inositol 1,4,5-trisphosphate receptor; **LVA**, low voltage activated Ca^{2+} channels, **OT**, oxytocin; OT R - oxytocin receptor; **RyR**, ryanodine receptor.

Figure legend

Figure 1. Schematic drawing showing the functional expression of Ca^{2+}-sensitive channels and receptors in ESCs, ADSCs and BMSCs. Growth, lineage commitment and maturation demands SCs develop more various and sophisticated signalling pathways. The universality of the Ca^{2+} signal makes it an ideal tool for this purpose. The recent reports on the functional expression of Ca^{2+}-sensitive channels and receptors in ESCs, AMSCs and BMSCs are schematically presented in the figure. In particular, voltage-operated Ca^{2+} channels (VGCC), $InsP_3$, inositol trisphosphate receptors ($InsP_3R$), ryanodine receptors (RyR), P2 purinergic, vasopressin and oxytocin receptors, as well as spontaneous Ca^{2+} oscillations and sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA pump) are shown.

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Figure 1

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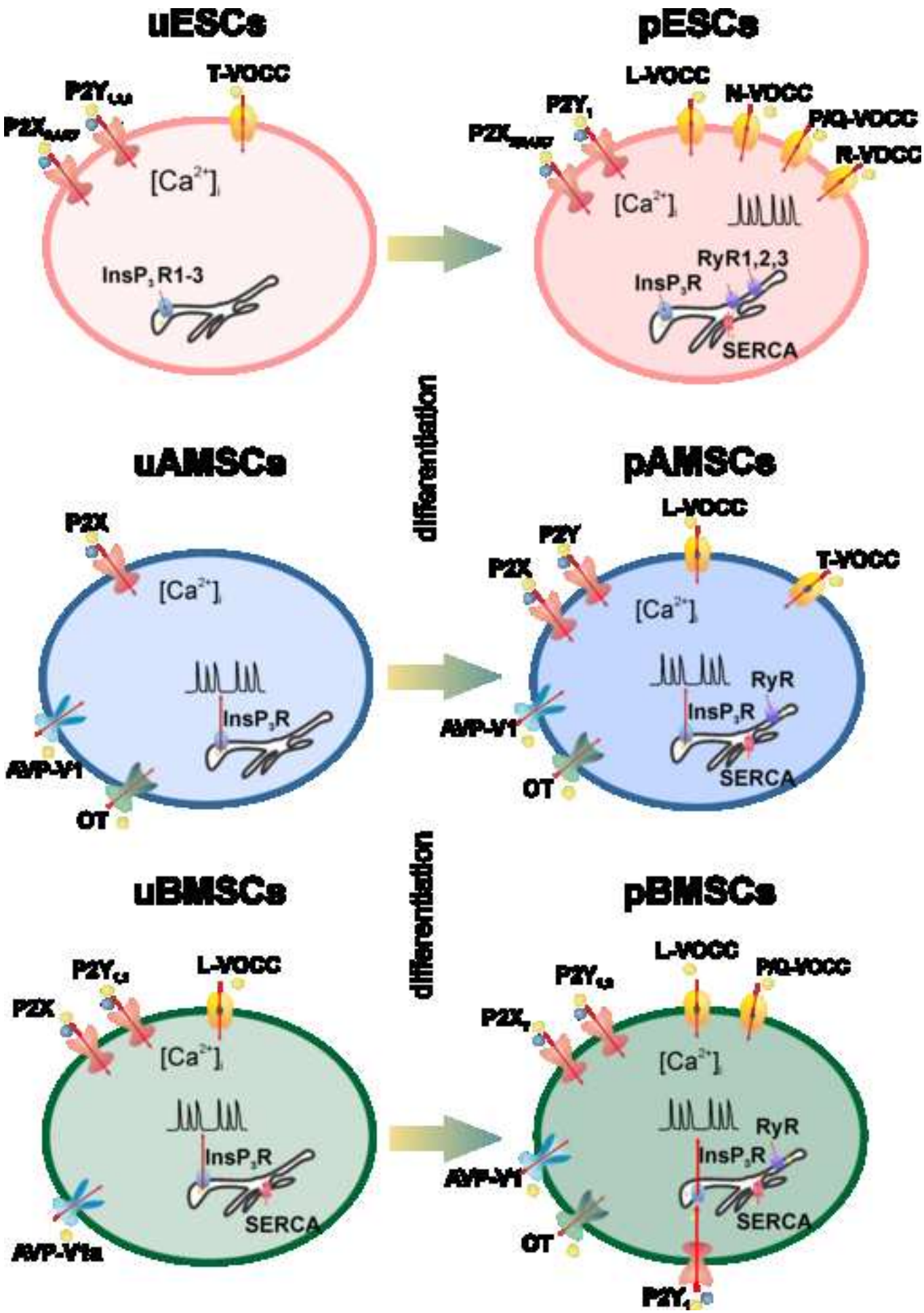

*Co-author Approval Statement

We have an approval from all co-authors of the manuscript “Physiology of Ca2+ signalling in stem cells of different origins and differentiation stages” submitted to CECASI on calcium in Stem Cells for publication.

Best regards

Forostyak and Dayanithi

*Conflict of Interest Statement

All co-authors of the manuscript "Physiology of Ca2+ signalling in stem cells of different origins and differentiation stages" submitted to CECASI on calcium in Stem Cells for publication have 'NO CONFLICT OF INTEREST"

Best regards

Forostyak and Dayanithi

*Detailed Response to Reviewers

We thank the referee for the valuable critics on this m.s.

All suggestions were considered including the language and m.s has been revised accordingly.