Pharmacological Research 202 (2024) 107111

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

In vitro models for neuropathic pain phenotypic screening in brain therapeutics

A.L. Martínez [a,b,c,1], J. Brea [a,b,c,1], D. López [a,b], N. Cosme [a,b], M. Barro [a,b], X. Monroy [d], J. Burgueño [d], M. Merlos [d], M.I. Loza [a,b,c,*]

[a] BioFarma Research Group, Centro Singular de Investigación en Medicina Molecular e Enfermidades Crónicas (CIMUS), Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[b] Instituto de Investigacións Sanitarias de Santiago de Compostela (IDIS), Santiago de Compostela, Spain
[c] Departamento de Farmacoloxía, Farmacia e Tecnoloxía Farmacéutica, Facultade de Farmacia, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[d] WeLab Barcelona, Parc Científic de Barcelona, Barcelona, Spain

ARTICLE INFO

Keywords:
Phenotypic screening
Immortalized cell lines
In vitro models
Neuropathic pain
In vitro pharmacology
Early drug discovery

Chemical compounds studied in this article:
α-lipoic acid (PubChem CID: 53259805)
Felodipine (PubChem CID: 3333)
Nicardipine (PubChem CID: 4474)
Nimodipine (PubChem CID: 4497)
Nitrendipine (PubChem CID: 4507)
Melatonin (PubChem CID: 896)
Pregabalin (PubChem CID: 5486971)
Protriptyline (PubChem CID: 4976)
Rilpivirine (PubChem CID: 6451164)
Vincristine (PubChem CID: 5978)
Zalcitabine (PubChem CID: 24066)

ABSTRACT

The discovery of brain therapeutics faces a significant challenge due to the low translatability of preclinical results into clinical success. To address this gap, several efforts have been made to obtain more translatable neuronal models for phenotypic screening. These models allow the selection of active compounds without predetermined knowledge of drug targets. In this review, we present an overview of various existing models within the field, examining their strengths and limitations, particularly in the context of neuropathic pain research. We illustrate the usefulness of these models through a comparative review in three crucial areas: i) the development of novel phenotypic screening strategies specifically for neuropathic pain, ii) the validation of the models for both primary and secondary screening assays, and iii) the use of the models in target deconvolution processes.

1. Introduction

Brain therapeutics address a broad spectrum of diseases affecting the brain, spine, and peripheral nervous system, including neurodegenerative and psychological disorders, neuropathic pain, and movement disorders [1]. The prevalence of these pathologies is increasing, partially due to population ageing [2], and they currently constitute major causes of death and disability [3]. However, developing novel brain therapeutics presents a challenge for drug discovery due to the complexity of translating preclinical results into clinical studies [4].

Brain therapeutics cover various pathologies with unmet clinical needs, and neuropathic pain is particularly noteworthy. This condition arises from damage to the central or peripheral nervous system and is characterized by spontaneous pain triggered by nonpainful stimuli (allodynia) or an exaggerated response to painful stimuli (hyperalgesia) [5]. Several factors, including metabolic disorders (e.g., diabetes), immunological mechanisms (e.g., multiple sclerosis), viral infections (e.g., HIV), mechanical nerve injuries (e.g., carpal tunnel syndrome), certain medications (e.g., cytostatic and antiretroviral drugs), or dysfunction in multiple neurotransmitter systems, can lead to

* Corresponding author at: BioFarma Research Group, Centro Singular de Investigación en Medicina Molecular e Enfermidades Crónicas (CIMUS), Universidade de Santiago de Compostela, Santiago de Compostela, Spain.
E-mail address: mabel.loza@usc.es (M.I. Loza).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.phrs.2024.107111
Received 20 December 2023; Received in revised form 2 February 2024; Accepted 18 February 2024
Available online 19 February 2024

neuropathic pain associated with peripheral nerve injury [6]. These insults induce imbalances in the pathways that transmit and process pain signals, ultimately resulting in neuropathic pain [7] (Fig. 1). This condition poses a significant health challenge, affecting approximately 10% of the global population. It incurs both direct and indirect costs and results in a decline in patients' quality of life [8–10].

Conventional pain-relieving drugs, including both steroidal and nonsteroidal anti-inflammatory drugs, as well as opioids, are often ineffective in relieving neuropathic pain. Thus, current treatment options include tricyclic antidepressants, serotonin-specific reuptake inhibitors, and anticonvulsants such as pregabalin or gabapentin. However, these drugs exhibit limited efficacy and a poor therapeutic ratio [12].

In recent decades, drug discovery research in the field has focused on various targets proposed for neuropathic pain treatment, including sodium voltage-gated channels $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$; N-type Ca^{2+} channels; hyperpolarization-activated cyclic nucleotide-gated (HCN) channels; $K_v7.2$ and $K_v7.3$ potassium channels; transient receptor potential (TRP) channels; and cytokine receptors or purinergic receptors [11,13] (Fig. 1). Despite these efforts, all attempts to develop novel drugs targeting these specific targets have failed.

One reason for the lack of effective neuropathic pain treatments stems from the low translatability of the currently employed target-based in vitro models for early drug discovery, leading to the failure of therapies in clinical trials. Current in vitro approaches for neuropathic pain drug discovery are mostly based on the expression of these reported targets in recombinant systems (mainly the HEK cell line), where drug effects are assessed by measuring either inhibition or activation of these targets outside the pathophysiological context [14,15]. Therefore, it is imperative that the drug discovery processes for developing neuropathic pain treatments consider the complexity of the mechanisms underlying the pathophysiology of neuropathic pain. Recognizing these challenges in targeting neuropathic pain highlights the urgent need for innovative approaches in early drug discovery, particularly in the context of in vitro models.

The demand for more translational assays is underscored by recent legislative changes, notably the FDA Modernization Act 2.0 enacted in December 2022, which plays a pivotal role in transforming the drug discovery [16]. This Act emphasizes the necessity for biologically relevant in vitro models with high translatability, enabling drug registration without preclinical studies in animal models [17]. Consequently, it becomes critical to develop novel in vitro models that not only allow the screening of large compound collections but also enhance the translation of in vitro screening.

This review focuses on some of the new models developed for the phenotypic evaluation of sensory neuron function with applicability to drug discovery.

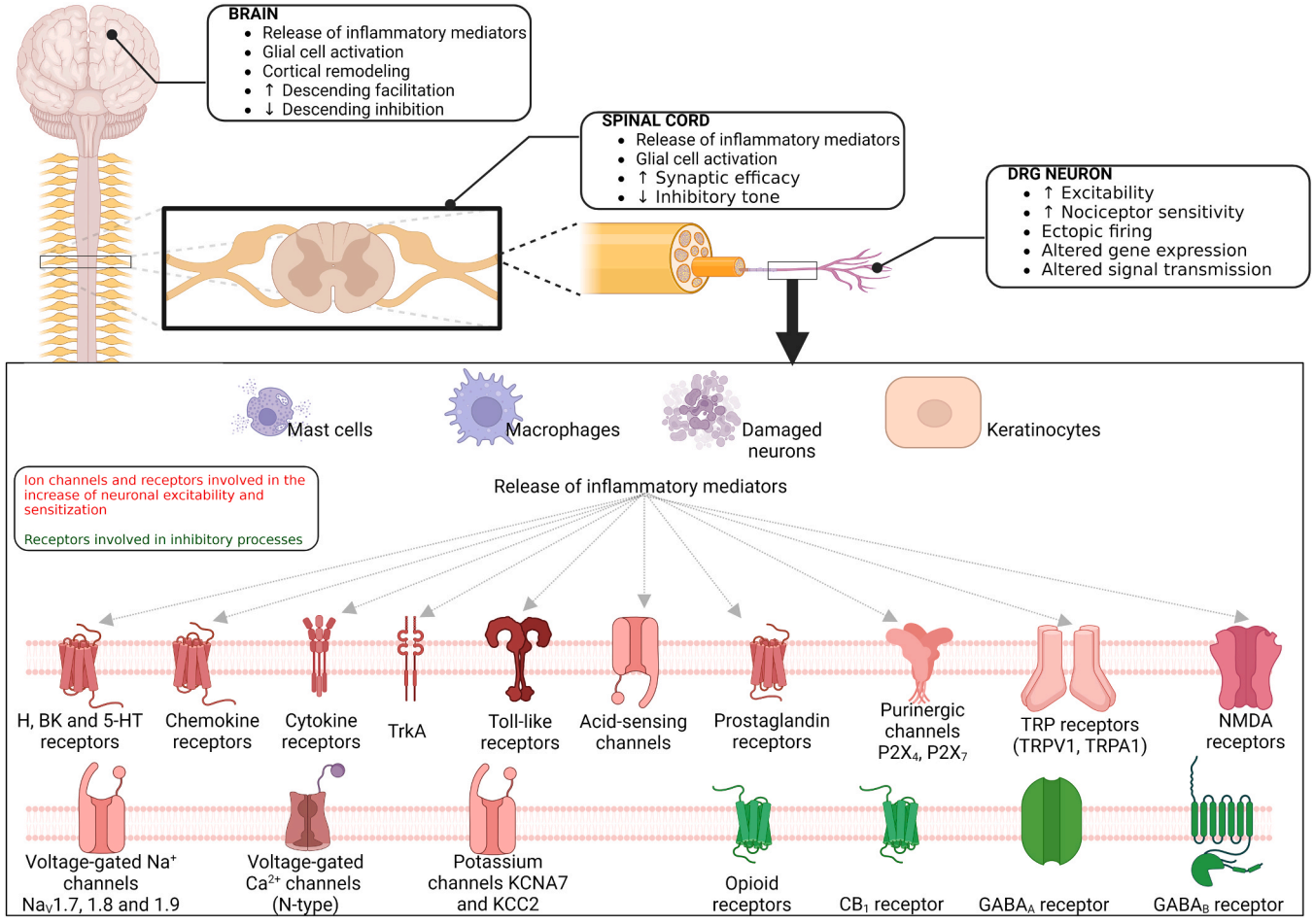


Fig. 1. Overview of the mechanisms contributing to neuropathic pain. Neuropathic pain is linked to changes in brain function, including the release of inflammatory mediators, glial cell activation, cortical remodelling, increased descending facilitation, and reduced descending inhibition. In the spinal cord, neuropathic pain involves the release of inflammatory mediators, glial cell activation, enhanced synaptic efficacy, and decreased inhibitory tone. Additionally, in DRG (dorsal root ganglia) neurons, neuropathic pain is related to increased excitability and nociceptor sensitivity, ectopic firing, and alterations in gene expression and signal transmission [7]. Peripheral neuropathic pain is connected to the release of inflammatory mediators that either activate or inhibit different targets on DRG neurons [11]. Figure created with Biorender.com.

2. In vitro phenotypic neuropathic pain models

In vitro phenotypic screening selects active compounds based on quantifiable phenotypic endpoints from cell-based disease-related assays, irrespective of prior knowledge of the drug target [18]. The phenotypic screening process typically encompasses different stages: i) primary screening of large chemical libraries to identify potential hits [19]; ii) secondary phenotypic screening with a different endpoint to deepen the understanding of pathogenic mechanisms and validate the compounds' translational potential [18], and iii) target deconvolution processes that employ molecular biology or biochemical methods to identify the molecular targets underlying the phenotypic effects elicited by the identified hits [20,21]. Thus, the development of more translational in vitro models useful for phenotypic screening is a promising tool for discovering novel mechanisms of action and treatments for neuropathic pain. Various models and technologies have been developed for this purpose in the field of neuropathic pain, such as microfluidic devices, organ-on-a-chip models, and pathophysiologically-related cellular models like cocultures, organoids, iPSCs-derived sensory neurons or differentiated cell lines (Fig. 2) (Table 1).

2.1. Microfluidic devices and organ-on-a-chip models

Both microfluidic and organ-on-a-chip models offer translational opportunities for investigating neuropathic pain treatment, enabling drug delivery to either the peripheral or central terminals of DRG neurons.

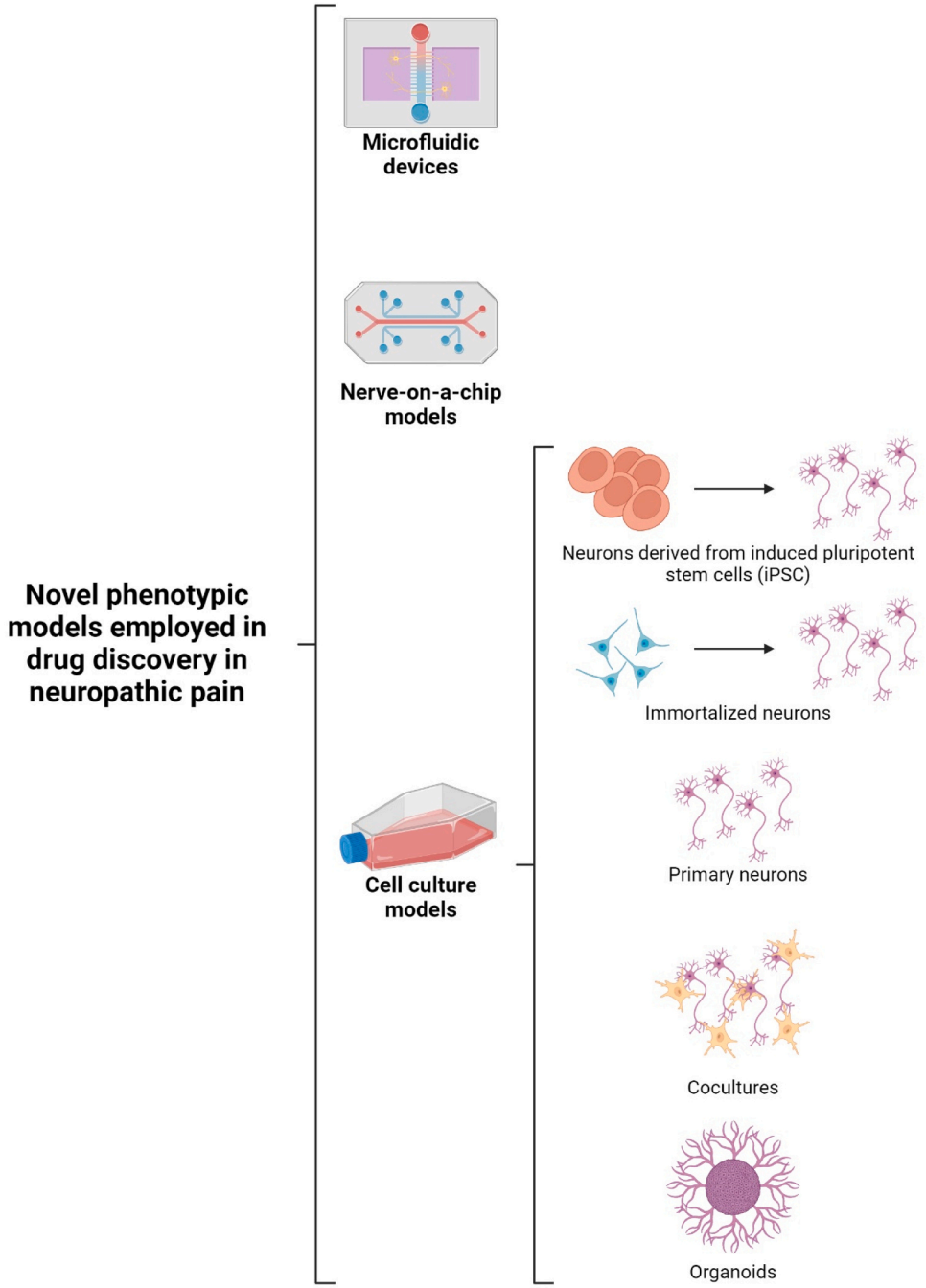


Fig. 2. Overview of novel phenotypic models employed in neuropathic pain research. Figure created with Biorender.com.

Table 1
Comparison of the reviewed phenotypic models for the discovery of new drugs for neuropathic pain.

Model	Advantages	Disadvantages
Microfluidic devices	Higher translation to the pathology Useful for mechanistic studies	Challenging preparation and culturing procedures Need of primary neurons Limited throughput
Organ-on-a-chip models	Higher translation to the pathology Useful for mechanistic studies	Challenging preparation and culturing procedures Need of primary neurons Limited throughput
Primary cells	Higher translation to the pathology Useful for hit/lead validation	Need for animal sacrifice Putative inter-species differences Limited availability Cell heterogeneity after isolation
Cocultures	Higher translation due to the incorporation of different cell types Useful for mechanistic studies	Challenging optimization for resembling the pathology phenotype Limited experience in high throughput screening in brain therapeutics
Organoids	Higher translation to the pathology Useful for hit/lead validation	Challenging to generate organoids Limited experience in high throughput screening in brain therapeutics
iPSC-derived sensory neurons	Human origin When generated from patients they can increase the model translation to the pathology	Weeks to months to reach a nociceptive state Form neuronal clusters that do not recapitulate mature DRG
Differentiated immortalized sensory neurons	High availability (high rate of proliferation) Simple differentiation process to acquire neuronal phenotype	Lower translational potency than other models

Microfluidic devices, equipped with neural cell-sized microchannels, facilitate targeted delivery of chemical compounds to specific neuronal segments [22]. For instance, Atmaramani et al. used such a model to demonstrate that IL-6 exposure sensitizes primary DRG neurons, leading to increased calcium influx and upregulation of the TRPV1 receptor when exposed to capsaicin [23]. Similarly, Giorgi et al. employed a microfluidic system to show that exposing the axonal endings of primary sensory neurons to noxious stimuli such as capsaicin, inflammatory mediators, or paclitaxel, as well as a coculture of keratinocytes, induces modifications in DRG neuron firing and cytosolic Ca^{2+} levels [24]. In these examples, the neurons most commonly used to engineer neural circuits in microfluidic platforms are typically obtained from embryonic or early postnatal animal brain tissues. While the use of primary neurons enhances translational success, challenges such as preparation and culturing procedures, heterogeneity of cellular populations obtained upon isolation, and limited available source tissues exist. Additionally, these microfluidic models exhibit a limited throughput for early stages of the drug discovery process. Nevertheless, they prove extremely useful for mode-of-action studies with advanced leads in drug discovery programs.

Organ-on-a-chip models, designed to mimic native tissue activity and physiology, replicate tissue-specific functions to enhance result translation [25]. Among the few studies carried out in the field of neuropathic pain, Pollard et al. utilized a nerve-on-a-chip platform by employing primary cells to assess the neurotoxic impact of chemotherapy drugs (cisplatin, vincristine, and paclitaxel) on DRG neuron responses to electrical stimulation and changes in neuronal morphology [26]. These models are complex, requiring a lengthy process to generate organoids from primary neurons, beginning with the formation of a spheroid and subsequent differentiation into a neuron-like organoid by modifying culture conditions. Similar to microfluidic models, organ-on-a-chip models serve as extremely useful tools for validating the effects of advanced leads before advancing to in vivo animal studies. However, they do not provide the necessary throughput for screening purposes.

2.2. Cellular models

The available microfluidic and organ-on-a-chip models developed for neuropathic pain to date are based on the use of primary neurons. While these primary neurons offer a more translatable model, they do not align with the FDA's goal of reducing animal use since they are isolated from either embryos or neonatal animals [27]. Additionally, their availability is limited once isolated, restricting their use to pathophysiological studies, new treatment investigations [28], or validating the effects of hits identified in high-throughput screening (HTS). However, they are not suitable for evaluating large chemical libraries in hit-finding processes.

Considering these factors, primary cells, whether used alone or in microfluidic or organoid models, are better suited for validating the effects of compounds identified through HTS. An example of this approach is the work of Dadi et al., who conducted an HTS to identify activators of the TREK-2 potassium channel using transfected HEK cells, and subsequently confirmed that these hits reduced calcium influx in primary DRG neurons, suggesting a potential analgesic effect [29]. Here, it's important to note that there are significant inter-species differences that alter neuronal firing [30,31]. Therefore, a human sensory neuronal model is crucial, but limited access to human DRG, especially from pain patients, has led to the need for a surrogate model mimicking human DRG neuronal properties [32,33].

Recent research has focused on novel translational cell culture models allowing the identification of active compounds through HTS that could be later validated in primary neurons. These new translational models include cocultures, organoids, human iPSCs (induced pluripotent stem cells), and differentiated immortalized neuronal cell lines.

Cocultures, varying in complexity (2D or 3D), offer a close resemblance to in vivo models [34]. They have been employed for drug discovery in neuropathic pain, particularly for characterizing the mechanism of action of drugs dependent on the interplay between DRG neurons and glial or immune cells. For example, Durante et al. identified adenosine A_3 receptor agonists as potential analgesics in neuropathic pain using various in vivo and in vitro models, including DRG neuron and $CD4^+$ lymphocyte cocultures. The efficacy of these drugs was attributed to the activation of the A_3 receptor in $CD4^+$ lymphocytes, increasing IL-10 production and consequently reducing NMDA receptor-mediated DRG neuron excitability [35]. Matsuoka et al. used cocultures of Schwann cells and DRG neurons to characterize the effects of Neurotropin®, a rabbit skin extract known for its analgesic properties in neuropathic pain. They demonstrated that the treatment promoted peripheral sensory nerve remyelination through accelerated Schwann cell differentiation [36].

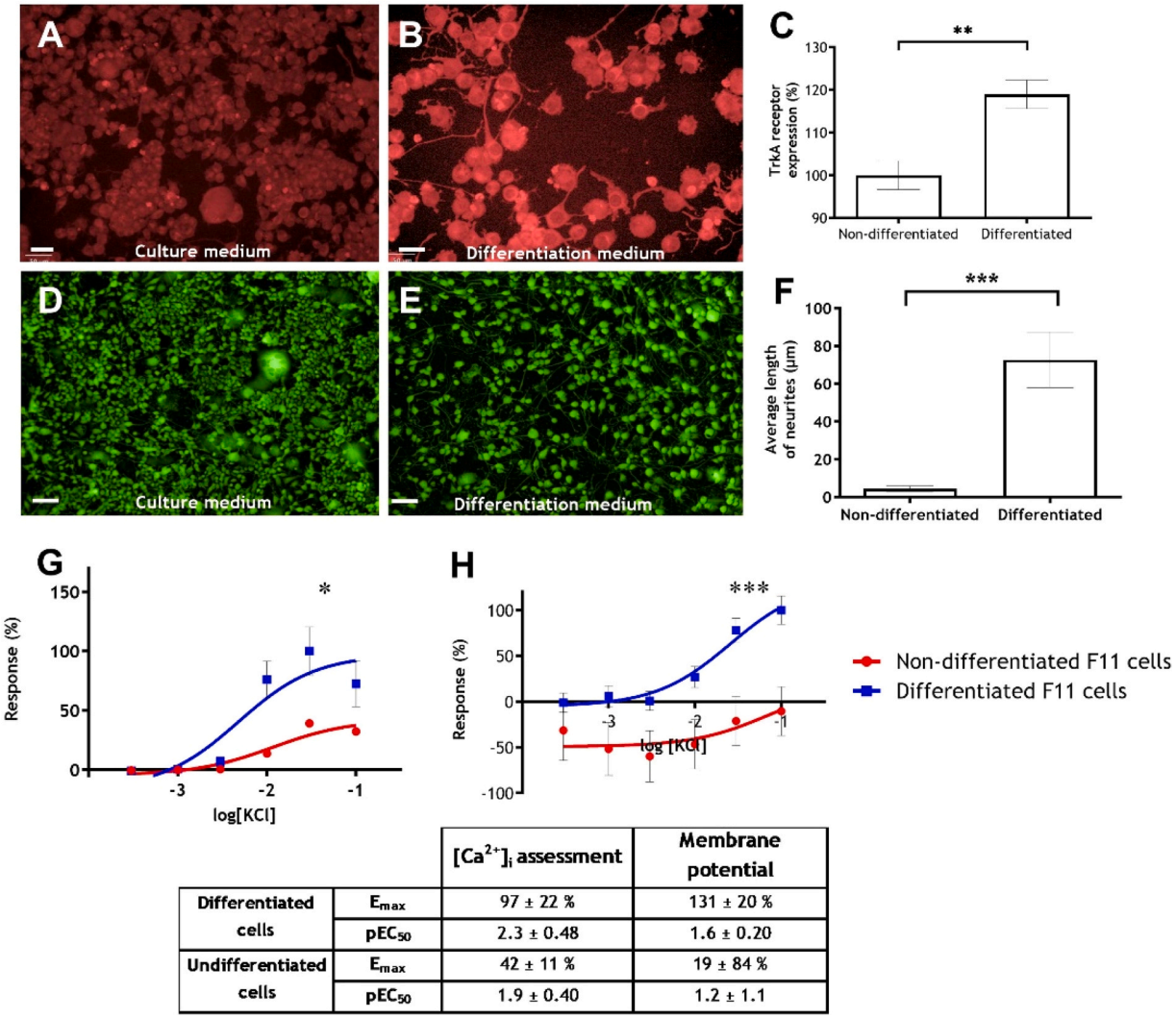
Organoids, artificially generated three-dimensional cultures derived from stem cells, attain a higher degree of translational relevance [37]. For instance, Mazzara et al. described the development of organoid models presenting features of sensory neurons and glial cells to explore the potential benefits of gene editing in the context of Friedreich's ataxia [38]. This genetic disorder is characterized by peripheral neuropathy, along with other neurological and cardiac symptoms [39]. Also, Xiao et al. presented a sensory ganglion organoid obtained from fibroblasts, exhibiting some features of sensory ganglia which could be employed in drug discovery [40].

Human iPSCs have been employed in neuropathic pain research for various purposes [41], such as exploring disease susceptibility [42,43]. A recent paper further affirms the sensory neuron-like nature of these cells [44]. The induction of neuronal differentiation of iPSCs typically

involves specific media formulations, culturing protocols, or genomic modifications [45–48]. However, the culture and maintenance of iPSCs are expensive, highly technical, and labour-intensive, requiring weeks to months to reach their nociceptive state. Moreover, the resulting neuronal clusters fail to accurately mimic DRG cellular diversity and spatial architecture [38]. For example, the majority of TRPV1-expressing sensory neurons derived from the Chambers protocol are functionally unresponsive to capsaicin [43,49,50]. This, coupled with the differential expression of Na^+ channels, suggests an immature differentiation of these iPSC-derived sensory neurons [49], altering cellular electro-responsiveness compared to mature DRG neurons [33].

Hence, differentiated immortalized cell lines have emerged as a promising option for developing translational in vitro models suitable for HTS. It has been reported that differentiated immortalized neuronal cell lines like F11, ND7/23 or 50B11 cells serve as suitable models for neuropathic pain drug discovery [51] because they fulfil two requirements for HTS [52]: i) they exhibit a high rate of proliferation, enabling the screening of thousands of compounds in a short period, and ii) they undergo a straightforward differentiation process to acquire the desired phenotype when exposed to the appropriate conditions [53]. These cell lines have been widely used in drug screening [54] and deconvolution assays [55].

Although cocultures and organoids provide translational models for low to medium throughput screening and have been employed for that aim in pathologies such as cancer [56], their application in drug screening specifically for neuropathic pain has not yet been explored to the best of our knowledge. Therefore, the only available models, up to date, allowing a disease-related in vitro phenotypic screening are those



		$[Ca^{2+}]_i$ assessment	Membrane potential
Differentiated cells	E_{max}	97 ± 22 %	131 ± 20 %
	pEC_{50}	2.3 ± 0.48	1.6 ± 0.20
Undifferentiated cells	E_{max}	42 ± 11 %	19 ± 84 %
	pEC_{50}	1.9 ± 0.40	1.2 ± 1.1

Fig. 3. Differentiation induced the acquisition of neuronal characteristics in F11 cells. Representative images of TrkA receptor staining in (**A**) undifferentiated F11 cells and (**B**) F11 cells differentiated using forskolin and dibutyryl-cAMP. Images are representative of three assays (N=3) with ten replicates per condition (n=10), 20X. Scale bar = 50 µm. (**C**) Percentage increase in mean fluorescence intensity of CFL647 (corresponding to TrkA receptor expression) in undifferentiated and differentiated F11 cells, relative to the fluorescence intensity in undifferentiated F11 cells. The values shown are mean ± S.E.M. of three independent assays (N=3) with ten replicates per measurement (n=10). **$p < 0.01$ (Student's *t-test*). Representative images of F11 cells (**D**) undifferentiated and (**E**) differentiated with forskolin and dibutyryl-cAMP. Images are representative of two assays (N=2) with eleven replicates per condition (n=11), 10X. Scale bar = 100 µm. (**F**) Variations in neurite length after three days of exposure to differentiation medium and to culture medium. Values shown are mean ± S.E.M. of two independent assays (N=2) with eleven replicates per measurement (n=11). ***$p < 0.001$ (Student's *t-test*). (**G**) Changes in $[Ca^{2+}]_i$ when differentiated and non-differentiated F11 cells were exposed to different concentrations of KCl. (**H**) Changes in membrane potential with $Dibac_4(3)$ when differentiated and non-differentiated F11 cells were exposed to a dilution series of KCl. Points in **G** and **H** represent the mean values ± S.D. of four independent assays (N=4) with three replicates per measurement (n=3) *$p < 0.05$, ***$p < 0.001$ (extra sum-of-squares F test). Data are normalized to the maximum response elicited by KCl in differentiated cells. Adapted from SLAS Discovery, 24, Martínez AL et al. A New Model of Sensorial Neuron-Like Cells for HTS of Novel Analgesics for Neuropathic Pain, 158–68, 2019 with permission from Elsevier.

employing either iPSCs or differentiated cell lines.

Thus, we developed a phenotypic model for drug screening in neuropathic pain at the Innopharma Drug Screening and Pharmacogenomics Platform, a high-capacity screening platform within the European Research Infrastructure Consortium (ERIC) EU-OPENSCREEN. We employed the F11 immortalized DRG neuronal cell line. These cells, hybrids of rat DRG neurons and murine neuroblastoma cells [57], were differentiated into a DRG-like phenotype using 30 μM forskolin and 1 mM dibutyryl-cAMP [52]. Differentiation resulted in increased expression of the nociceptive neuron marker TrkA receptor, longer neurites (Fig. 3**A-F**), and an enhanced response of F11 cells to a depolarizing stimulus such as KCl, both in terms of intracellular calcium concentration and membrane potential changes (Fig. 3**G-H**).

The achievement of a DRG phenotype through differentiation was confirmed by transcriptomics, observing an increased expression of genes encoding voltage-dependent sodium (*Scn5a*) and calcium channels (*Cacna1c*, *Cacna2d2*, and *Cacna2d3*), as well as neuron-related genes *Shc2* and *Neurog* (Suppl. Fig. 1A-**C**) [58].

3. HTS employing in vitro phenotypic neuropathic pain models

The employment of iPSCs-derived DRG in HTS campaigns is still scarce, likely due to the high cost of reproducibly scaling up differentiation protocols and cell production [41], poor efficiency of replating into microtiter assay plates at the appropriate state of maturation, and challenges in identifying a disease-relevant phenotype suitable for assay development [59–62]. Indeed, to our knowledge, there is just one report of phenotypic in vitro screening by employing iPSCs-derived DRG neurons by Stacey et al., who used differentiated sensory neurons from iPSCs in an 11-day protocol to screen a library of 2746 compounds [63]. They mimicked membrane depolarization induced by nociceptive stimuli by employing veratridine, a nonspecific opener of Na_V channels and measured the neuron excitability by quantification of the consequent increase in intracellular Ca^{2+} via Ca_V channel activation through Ca^{2+}-sensitive fluorescent dyes. They validated the method with a subset of compounds with known analgesic activity and observed that maturation times of 2–28 days did not yield greater differences in the nature of the hits identified, despite a trend toward loss of both potency and number of active compounds with maturation. TrkA blockers were inactive, and the potency of active compounds was right-shifted relative to known target-based potency, which the authors ascribe to the different endpoint measured or the "harsh" nature of the veratridine stimulus. The 384-well calcium flux assay allowed the clear identification of known blockers of Na_V activity through antagonism of the veratridine-induced depolarization. They identified 13 hits that targeted pain-related pathways, effectively

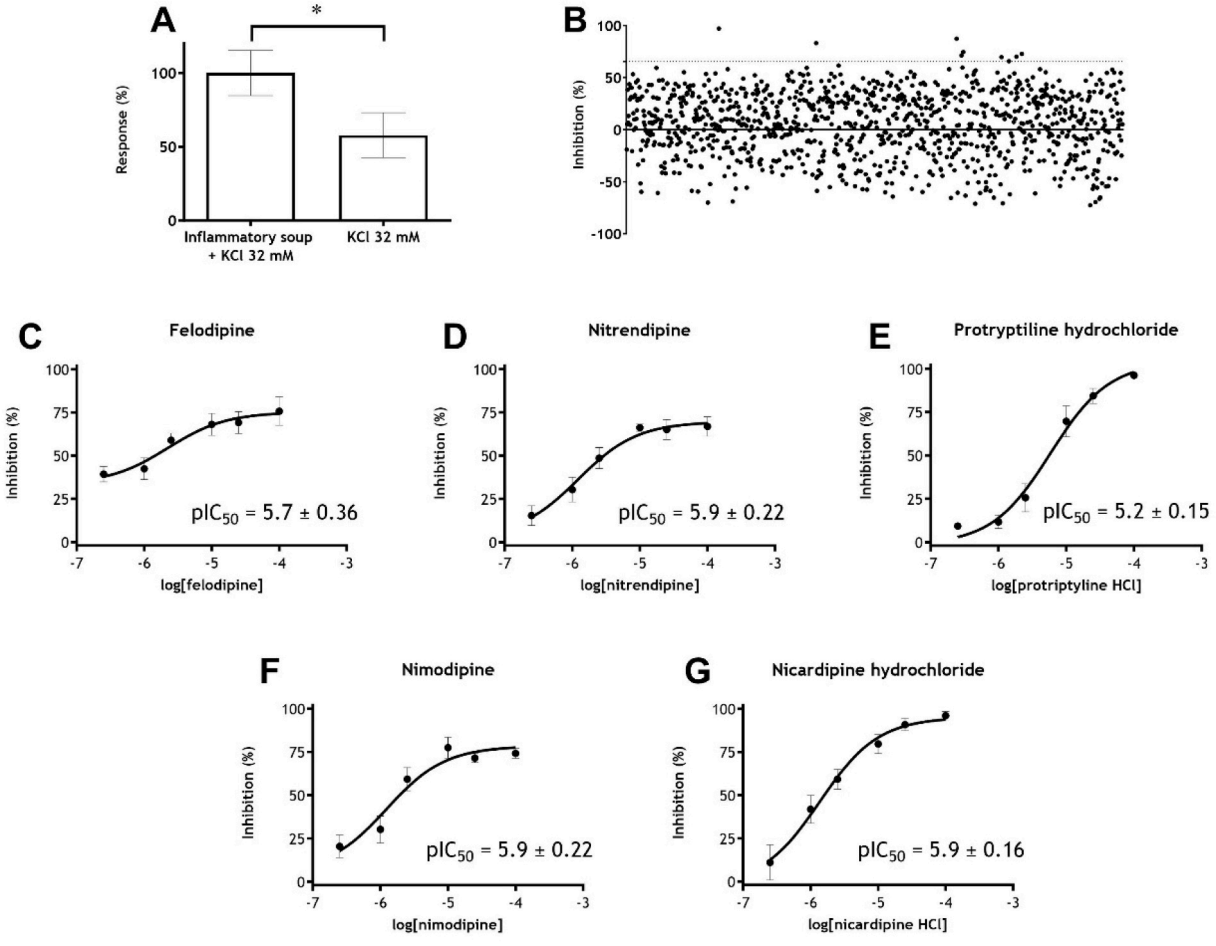


Fig. 4. Five hits demonstrated a concentration-dependent reduction in the excitability of differentiated F11 cells to KCl after exposure to *inflammatory soup*. (**A**) Response of F11 cells to 32 mM KCl with and without previous exposure to inflammatory soup for 10 minutes. Mean values ± S.D. of triplicate measurements (n=3) of representative results from one of three assays (N=3) are shown, *$p < 0.05$ (Student's *t-test*). (B) Inhibition of the response to 32 mM KCl after exposure to inflammatory soup for 10 minutes by 1120 compounds of the Prestwick Chemical Library at a concentration of 10 μM. (C-G) Inhibition of effects of 32 mM KCl treatment by the five confirmed hits. Points represent the mean ± S.D. of two assays (N=2), each with triplicate measurements (n=3). Reprinted from SLAS Discovery, 24, Martínez AL et al. A New Model of Sensorial Neuron-Like Cells for HTS of Novel Analgesics for Neuropathic Pain, 158–68, 2019 with permission from Elsevier.

inhibiting veratridine-induced calcium transients at a concentration of 1 μM [63].

Differentiated cell lines were also useful for HTS campaigns searching for novel analgesics. To mimic hyperalgesia in neuropathic pain following nerve injury, we exposed our differentiated F11 cell model to a previously reported *inflammatory soup* containing 10 μM histamine, 1 μM bradykinin, 10 μM serotonin and 10 μM prostaglandin E_2 [64]. Exposure to this *inflammatory soup* resulted in an augmented influx of calcium into the cytoplasm in response to the depolarizing agent KCl at a concentration of 32 mM (Fig. 4**A**), compatible with an increase in neuron excitability and validating the model for its application to drug screening with the Prestwick Chemical Library, comprising natural substances and off-patent drugs with known mechanisms of action [65] (Fig. 4**B**). Nine compounds were identified as hits: N6-methyladenosine, nicardipine, felodipine, nitrendipine, protriptyline, nimodipine, promethazine, piribedril, and prenylamine. Five of those compounds (felodipine, nicardipine, nitrendipine, nimodipine, and protriptyline) showed a concentration-dependent inhibition of differentiated F11 cells' response to KCl in the presence of the *inflammatory soup* with IC_{50} values in the range between 1 and 10 μM (Fig. 4**C-G**).

The differentiated cell line model offers several advantages for primary screening compared to iPSC-derived models. One key benefit is the ability to generate large quantities of cells for high throughput screening within a short time frame (3 days) without the lengthy and complex differentiation and generation protocols required for iPSCs, often extending beyond one week.

Furthermore, the developed model demonstrated high versatility, being suitable not only for neuronal excitability assays but also for employment in fluorescence microscopy to quantify morphological change as happens in iatrogenic neuropathic pain [66]. Iatrogenic neuropathic pain, caused by neurotoxic effects of antitumor or antiviral drugs on peripheral neurons, leads to sensory nerve degeneration, resulting in sensory disturbances like numbness and neuropathic pain [67]. This condition typically begins in the extremities, following a glove-and-stocking pattern [68]. Its exact pathophysiology remains unclear, although mechanisms like oxidative stress and calcium signaling are thought to be involved (Fig. 5) [69].

High Content Screening of the differentiated cell lines was envisioned as a secondary assay for validating hits capable of counteracting the deleterious effects of antiviral and antitumor drugs on DRG neurons [70]. Treatment of differentiated cells with 1 nM vincristine and 100 nM rilpivirine notably reduced neurite length compared to control cells (Fig. 6). The concentrations employed are within the range of therapeutic plasmatic concentrations achieved by both drugs [71,72].

To validate the model's capability of identifying neuroprotective compounds, we tested different drugs that are already used in the treatment of iatrogenic neuropathic pain in clinical practice or that were identified in in vitro models. We found that 10 μM α-lipoic acid, 100 μM pregabalin and 1 μM melatonin partially reversed the neurite damage induced by both vincristine and rilpivirine (Fig. 6). The three studied drugs act through different mechanisms of action: α-lipoic acid is an antioxidant drug [73], pregabalin blocks calcium channels with a neurite growth promoter effect [74], and melatonin counteracts the toxic effects of vincristine and rilpivirine on the mitochondria [75]. Thus, our in vitro model successfully identified neuroprotective compounds acting on different signaling pathways, validating it as a suitable model for phenotypic screening for iatrogenic neuropathic pain.

Therefore, we employed this microscopy assay to determine the protective efficacy of the hits identified from the primary screening, observing that nitrendipine counteracted neurite shortening induced by both vincristine and rilpivirine, while felodipine was effective only against vincristine-induced shortening (Suppl. Fig. 2).

4. Target deconvolution from neuropathic pain phenotypic model

As described earlier, one of the needs when conducting phenotypic screening may arise from the identification of targets/pathways that are

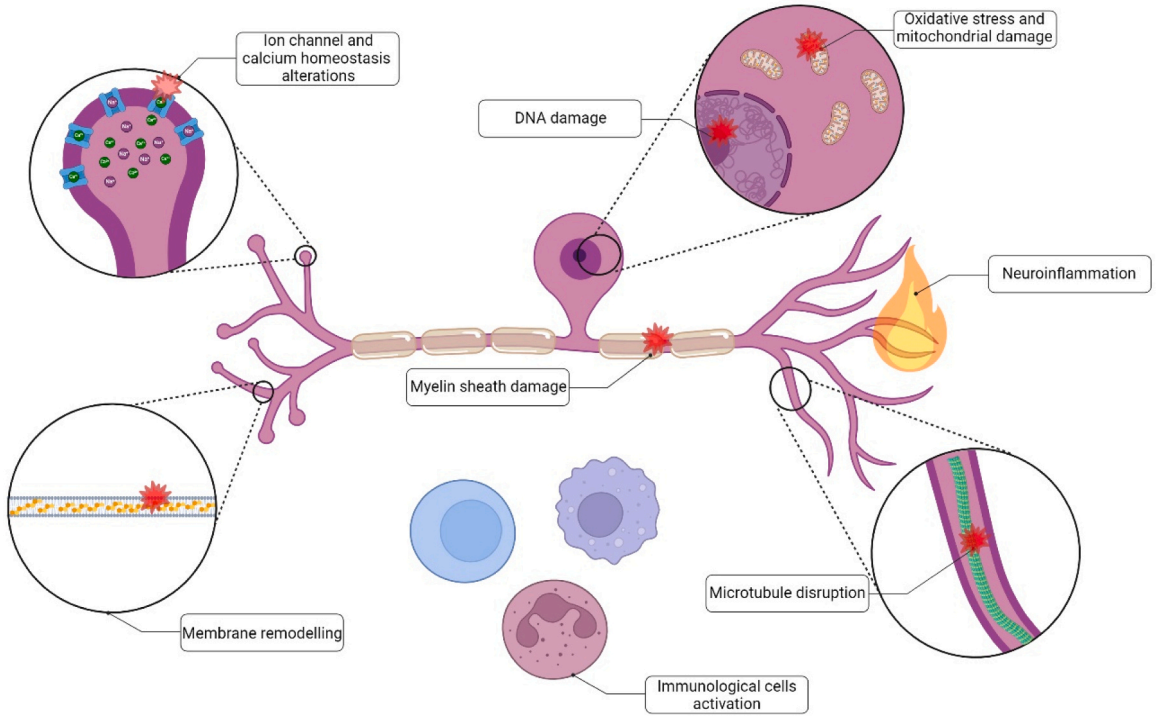


Fig. 5. Mechanisms of iatrogenic neuropathic pain induction by antitumor drugs. Antitumor drugs can induce peripheral neuropathic pain by acting on peripheral neurons. The mechanisms reported include changes in ion channel conductivity and ion homeostasis, DNA damage, membrane remodelling, microtubule disruption, increased oxidative stress and mitochondrial damage, activation of immunological cells and neuroinflammation, and damage to the myelin sheath [69]. Figure created with Biorender.com.

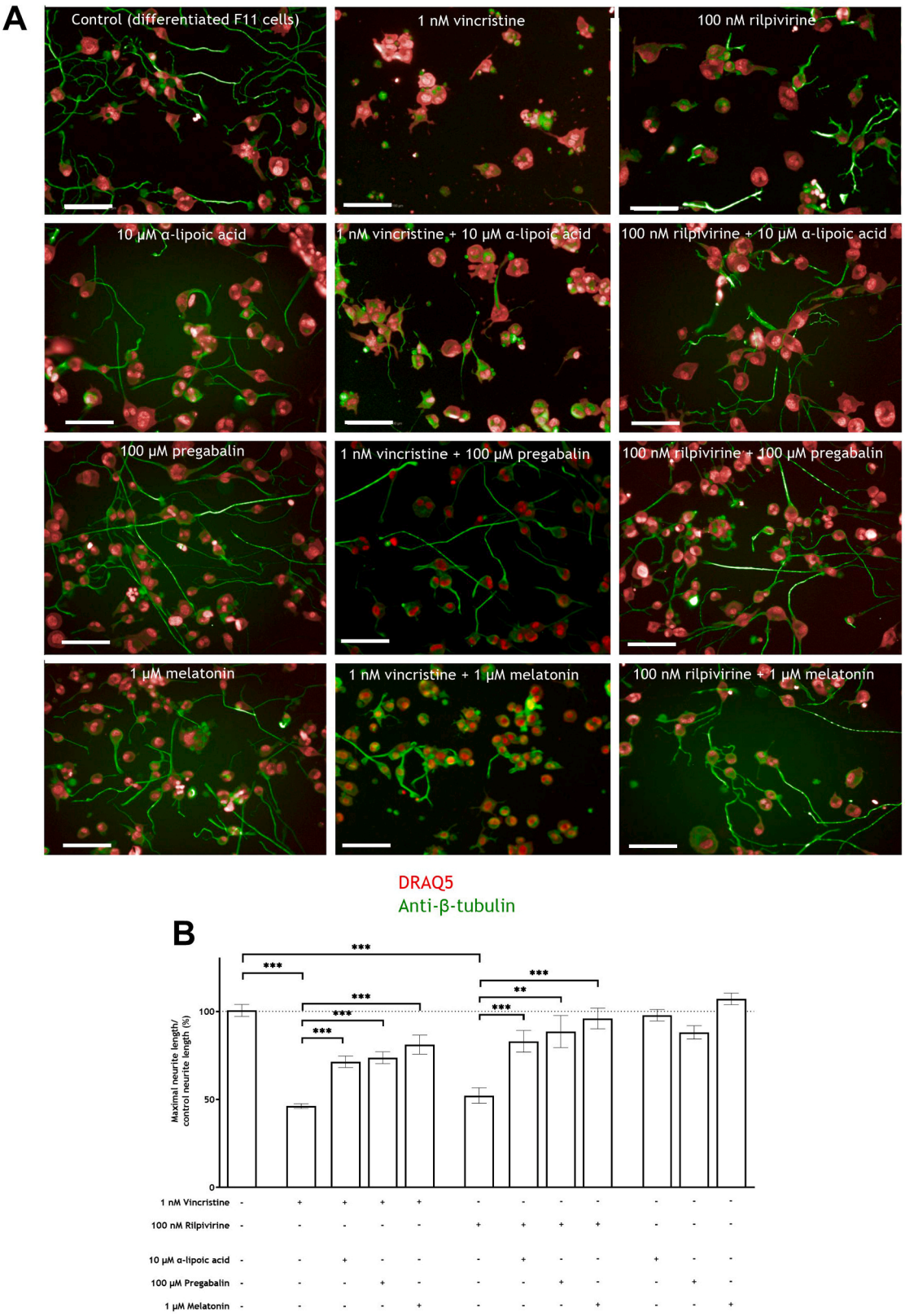


Fig. 6. α-lipoic acid, pregabalin and melatonin significantly attenuated the neurite shortening induced by vincristine and rilpivirine. (**A**) Representative images of the effect of 1 nM vincristine and 100 nM rilpivirine on F11 cells and of 10 μM α-lipoic acid, 100 μM pregabalin and 1 μM melatonin on control F11 cells and on F11 cells exposed to 1 nM vincristine and 100 nM rilpivirine. Images are representative of four assays (N=4), each with six replicates (n=6), 20X. Scale bar = 100 μm. (**B**) Graph depicting the average length of the neurites of differentiated F11 cells exposed to 1 nM vincristine and 100 nM rilpivirine after treatment with 10 μM α-lipoic acid, 100 μM pregabalin and 1 μM melatonin. The values represent the mean ± S.E.M. of four independent experiments (N=4) with measurements performed in six replicates (n=6). **p<0.01; ***p<0.001 (ANOVA test followed by Dunnett's post hoc analysis). Adapted from [70]. CC-BY 2021 license.

being modulated by the hits. In this way, microfluidics has been extensively used for separating and sorting both primary and cultured neurons, which have subsequently been used in single cell transcriptomic studies [76–79]. The findings of these studies are often the starting point for identifying molecular drivers of differentiation and can be used to produce neurons in vitro from ESCs (embryonic stem cells) or iPSCs [80].

Organoids are also employed in transcriptomic studies, using either bulk or single-cell transcriptome profiling. By employing both approaches, Xiao et al. evaluated the target expression of sensory ganglion organoids by means of bulk-RNAseq observing up-regulated genes that are relevant to neural function and development such as synaptic signaling, synaptic vesicle, synapse organization, neurotransmitter transport, regulation of neurotransmitter levels, exocytosis, calcium ion binding, ligand-gated channel activity, neuron projection, axon, and nervous system development [40]. These results are consistent with the induction of functional sensory ganglion and retinal ganglion neurons. Single cell RNA-seq analysis of single induced sensory ganglion cells showed expression of *NF200, peripherin, p75NTR, TrkB, TrkC, Trpv1, Trpv2, P2×3, Accn2, Kcnq2, Cacna1a*, and *CGRP* in various clusters of sequenced cells, indicating their expression in mature induced sensory ganglion neurons and their expression specificity. The possibility of running both bulk and single cell transcriptomic analysis in organoids paves the way for future target deconvolution of compounds that show activity in these models.

DRG neurons derived from iPSCs can also be subjected to transcriptomic analysis to deconvolute the targets modulated by the active compounds by applying the same technologies as used with organoids. However, there are no works on neuropathic pain target deconvolution from ligands acting on sensory neurons derived from iPSCs. The only target deconvolution approach identified was the one carried out by Stacey et al. with the hits identified in their phenotypic screening [63]. In this work, they were evaluating an internal chemical library where the compounds have been previously annotated with different targets, and therefore, the target deconvolution was carried out using this information. Among the targets included in the painful phenotype, they identified 5-HT_6 receptor, μ opioid receptor, voltage-gated potassium channels KCNC2 and KCNA4, voltage-dependent N-type calcium channel CACNA1B, integrins α4 and β1, farnesyltransferases CAAX box α and β, neuropeptide FF receptor 2, tachykinin receptor 1, interleukin 2, and chemokine (C-C motif) receptor 1 (CCR1). Nevertheless, it must be considered that they evaluated the compounds over veratridine-induced neuron excitation, which could condition the targets identified, and maybe if different stimuli were employed, a different outcome could be obtained.

We performed a transcriptomic characterization of the effect of zalcitabine (ddC) on F11 cells. Zalcitabine is a reverse-transcriptase inhibitor (NRTI) associated with severe neuropathic pain in HIV patients [81] that does not induce neurite shortening like other antiretrovirals [82]. The transcriptomic study allowed the identification of *Nqo1* and *Atp2a3* as targets associated with the increase in neuron excitability (Suppl. Fig. 3). The involvement of these genes in zalcitabine-induced oxidative stress and calcium regulation was confirmed by independently overexpressing each gene in differentiated F11 cells (Suppl. Fig. 4).

5. Conclusion

In conclusion, we review here the different in vitro translational approaches available for phenotypic screening in neuropathic pain. Within the array of available models, a screening cascade can be established, utilizing either iPSCs-derived neurons or differentiated cell lines as primary screening assays in HTS-compatible formats. Both models have demonstrated their usefulness for finding compounds able to decrease neuron excitability in the presence of painful stimuli. Differentiated cell lines have also demonstrated being useful to detect compounds able to revert the neurotoxic effect of either cytotoxic or antiretroviral drugs. Furthermore, differentiated cell lines showed several advantages to be prioritized as primary screening assays based on the lower cost maintenance of the cell culture, as well as the shorter and simpler differentiation process. F11 cells have also demonstrated ease of transfection using nonviral methods, which can sometimes be inefficient for transfecting primary cells [83]. A challenge arises with F11 cells due to their hybrid nature, combining mouse and rat cells. This necessitates additional characterization efforts for changes in both mouse and rat genes compared with the greater translationality of primary cells.

Both iPSCs-derived and differentiated cell lines facilitated the acquisition of a sensory neuron phenotype mimicking that of primary cells. However, variations exist in the expression and function of nociceptive receptors, which may impact the identified hits. Therefore, it is advisable to use primary cells in subsequent stages to validate the effects of the identified compounds. The use of complex models like organoids, co-cultures, organ-on-a-chip or microfluidics offer the possibility of increasing the success of results translation from in vitro to preclinical studies allowing the validation of the compounds in a phenotype closer to that of the pathology.

Funding sources

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The research for this work was supported by the Agencia Estatal de Investigación (PID2020-119428RB-I00) and Xunta de Galicia (ED431C 2022/20) and European Regional Development Fund (ERDF). NC was in receipt of a predoctoral grant (ED481A 2021) from Xunta de Galicia and MB was in receipt of a predoctoral grant (ED481A-2020) from Xunta de Galicia.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgement

Authors would like to acknowledge Geert Daudey for reviewing the manuscript wording and grammar.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2024.107111.

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