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An unbiased and efficient assessment of excitability of sensory neurons for analgesic drug discovery.

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Abstract

Alleviating chronic pain is challenging, due to lack of drugs that effectively inhibit nociceptors without off target effects on motor or central neurons. Dorsal root ganglia (DRG) contain nociceptive and non-nociceptive neurons. Drug screening on cultured DRG neurons, rather than cell lines, allows the identification of drugs most potent on nociceptors with no effects on non-nociceptors (as a proxy for unwanted side effects on CNS and motor neurons). However, screening using DRG neurons is currently a low-throughput process and there is a need for assays to speed this process for analgesic drug discovery. We previously showed that veratridine elicits distinct response profiles in sensory neurons. Here we show evidence that a veratridine-based calcium assay allows an unbiased and efficient assessment of a drug effect on nociceptors (targeted neurons) and non-nociceptors (non-targeted neurons). We confirmed the link between the oscillatory profile and nociceptors; and the slow-decay profile and non-nociceptors using three transgenic mouse lines of known pain phenotypes. We used the assay to show that blockers for Nav1.7 and Nav1.8 channels, which are validated targets for analgesics, affect non-nociceptors at concentrations needed to effectively inhibit nociceptors. However, a combination of low doses of both blockers had an additive effect on nociceptors without a significant effect on non-nociceptors, indicating that the assay can also be used to screen for combinations of existing or novel drugs for the greatest selective inhibition of nociceptors.

Keywords: Calcium imaging; veratridine; voltage gated channel blockers; mouse knockout; nociceptors; mouse DRG; capsicin

Introduction

Chronic pain has significant negative impacts at personal, social and economic levels, affecting millions of people worldwide [10; 19]. Several drug classes are used to treat chronic pain but their effectiveness is limited by either lack of potency or adverse effects. Therefore, the treatment of severe or chronic pain is a challenging clinical need.

The search for new analgesics focuses on targets in pain neurons in the dorsal root ganglia (DRG). DRG contain a heterogeneous population of neurons that can be classified according to expression of sensory markers into eleven subtypes [23], but they can be broadly classified as nociceptors and non-nociceptors (proprioceptors and touch neurons). Drug screens use cell lines expressing the nociceptive target of interest. Although this platform allows for high throughput screening it provides no information on drugs' effects on non-nociceptors. This information would weigh the potency on nociceptors against adverse effect on non-nociceptors before costly and laborious *in vivo* testing. However, screens on DRG neurons are currently not practical because they are low throughput and there is not a simple protocol to distinguish between drugs' effects on nociceptors versus non-nociceptors.

Voltage gated sodium channels (VGSCs) are critical for the excitability in DRG neurons. We discovered that the VGSC opener, veratridine, produces distinct calcium responses in cultured sensory neurons[16]. We found that most nociceptors show an oscillatory response (OS), while most non-nociceptors show a slowly decaying response (SD), Fig 1. Here we provide evidence that the OS and SD veratridine response profiles can be used as readouts in an assay to evaluate the effect of drugs on nociceptors and non-nociceptors. We first showed that the OS population is drastically reduced in a mouse model where most nociceptors are ablated. We then determined the pattern of veratridine responses in a Nav1.7 knockout mouse, a pain-free model. Subsequently, we used this pattern as a reference criterion against which two VGSC blockers, against the Nav1.7 and Nav1.8 channels, were evaluated. These channels are validated targets for analgesic drug development with several promising compounds identified in recent years [6; 25] but lack of in vivo potency and selectivity on nociceptors has contributed to failure in clinical trials [9]. The two VGSC blockers were unable to match the Nav1.7 knockout pattern when applied separately but showed an additive effect when applied together. This suggests that our assay can also be used to screen for combinations of drugs that act on different targets in nociceptors for effective pain relief.

Methods

DRG culture

C57BL6 mice were sacrificed according to Schedule 1 of the Animal (Scientific procedure) Act 1986. DRG from all spinal levels were dissected and collected in phosphate buffered saline (PBS) at room temperature. PBS was then replaced with 1 ml dissociation solution containing: Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) with Glutamax (Gibco), 1x penicillin/streptomycin mix (Gibco), Dispase (1 mg/mL, Sigma) and Collagenase Type XI (0.6 mg/mL, Sigma) and incubated at 37 ^{o}C and 5% CO_2 for 90 minutes. DRG were triturated with a P1000 pipette tip 10 times at 60 and 90 minutes. After the second trituration the cell suspension was carefully layered on top of 15% Bovine Serum Albumin (Melford), dissolved in DMEM/F12 containing penicillin/streptomycin, and centrifuged at 800 g for 10 min with the minimum deceleration speed. The cell pellet was then washed in a culture medium composed of DMEM/F12 containing 10% Fetal Bovine Serum, penicillin/streptomycin (all from Gibco). Cells were pelleted again and then re-suspended in 60 µl culture medium. A 3 µl "drop" was placed on the centre of a glass coverslip coated with D-polyornithine (20 µg/mL, Sigma). Coverslips were flooded with a 1 ml of culture medium after 15-30 minutes. Cells were imaged 24-48h after plating. DRG from 1.8-DTA, Nav1.7KO and Nav1.8KO were placed in ice-cold Hibernate-A medium (Gibco) containing penicillin/streptomycin while being transported from UCL (about 5h). DRG were then dissociated as above.

Calcium imaging

All recordings were performed at room temperature (22-25 °C). DRG neurons were loaded with 2 µM Fura-2AM (Molecular Probes) in standard Ringer solution (140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH = 7.4 with NaOH) for 30 min at 37 °C. Coverslips were then washed with ringer solution and left for 15 min at 37°C/5%CO_2 after which the Ringer was replaced. Coverslips were stored in a dark container at room temperature until imaged (within 2 hours). Cells were perfused at a flow rate of 3 ml/min with Ringer solution for at least 5 min to establish a stable calcium baseline. Ringer with 40 mM KCl was perfused at the end of recordings to identify viable neurons. Cells were imaged with a 40X objective and a Hamamatsu C4742-95 camera. Cells were excited with 350 and 380 nm for ratiometric measurement of intracellular calcium using Cairn Dual OptoLED system. *Simple PCI 6* software was used for data acquisition, background subtraction and Fura-2AM ratiometric measurement (F350/380 nm).

All drugs were made in standard Ringer solution from stock solutions of the following concentrations: Veratridine (5 mM in ethanol, Abcam ab120279), Capsaicin (10 mM in ethanol, Tocris 0462), α, β-methylene ATP (10 mM in water, Sigma M6517), allyl isothiocyanate (AITC; 100 µM, Sigma 377430), 4,9-anhydrotetrodotoxin (300uM in water, Tocris 6159), PF-04856264 (10mM in DMSO, Sigma 11916) and A-803467 (10mM in DMSO, Abcam Ab120282).

Data and statistical analysis

Neurons were included in the analysis if they respond to 40 mM KCl. On rare occasions, neurons responded to veratridine but not KCl (or the KCl response was not clear due to the calcium signal not returning to baseline after the application of last agonist). We defined a response as an increase in (F350/380) ratio of > 6 SD above the baseline. Differences in fluorescence ($\Delta F/F_0$) were calculated according to the following formula: F350/380 ratio during agonist application (F) minus the average F350/380 ratio of the 2.5 min prior to agonist application (F_0). Mean values from each experiment (each N is a culture from one mouse) were compared to each other by one-way analysis of variance (ANOVA) with Sidaks' post-test. All statistical analysis and comparisons were performed by *GraphPad Prism* software (version 7.00 for Windows).

Results

The OS population is highly diminished in the Nav1.8-DTA (nociceptor-ablated) model

In order to confirm the link between neurons with the OS profile and pain behaviour we examined DRG from the Nav1.8Cre-DTA (1.8-DTA) mouse [1]. The Nav1.8 channel is expressed in 80-90% of nociceptors [5; 21]. In the 1.8-DTA mouse, Nav1.8-expressing neurons are ablated (Fig. 2a) leading to the loss of most nociceptors and a profound loss of pain [1]. Therefore, we hypothesised that the veratridine response profile that pertains to nociceptors (i.e. OS) would be reduced in the 1.8-DTA DRG. First, we determined the percentage of remaining nociceptors in 1.8-DTA DRG using the three nociceptive agonists (capsaicin, ATP and isothiocyanate) to identify all subtypes of nociceptors as described previously [10]. The percentage of neurons responding to any of the nociceptive agonists was drastically reduced from 75% in controls to just 8% in 1.8-DTA (Fig. 2b). This shows that the majority of neurons in 1.8-DTA DRG are non-nociceptors with very few nociceptors left.

The ablation of most nociceptors caused opposing changes in the percentages of the veratridine OS and SD populations. The OS population decreased from 34% in controls to 7% (Fig 2c). In contrast, the SD population increased from 17% in controls to 66%. This confirms that the OS population represents most nociceptors, while the SD population represents most non-nociceptors. Of note, the total number of veratridine-irresponsive neurons decreased from 39% in control to 23% in 1.8-DTA, (Fig 2c). The veratridine-irresponsive population can be either nociceptors (i.e. respond to the nociceptive agonists but not veratridine, yellow section in Fig 2d) or non-nociceptors (i.e. respond to neither the nociceptive agonists nor veratridine, orange section in Fig 2d). The observed decrease in veratridine-irresponsive neurons was in fact due to the loss of almost all veratridine-irresponsive nociceptors, a population that accounted for 26% of all neurons in controls but only 0.8% in 1.8-DTA.

Results from the 1.8-DTA mouse links the reduction of the OS population *in vitro* to the previously described [1] pain deficits *in vivo*. Therefore, drugs can be screened based on their ability to reduce the OS population (an indication of potency on nociceptors) without an effect on the SD population (an indication of unwanted effects on non-nociceptors), Fig 1.

A reference veratridine-response pattern for a potent and safe analgesic

Next, we established the reference criterion by which drug potency and selectivity on nociceptors can be quickly ascertained in our assay. We base this criterion on the change to the percentages of the OS and SD populations in the Nav1.7 knockout mouse where pain behaviour is lost due to a decrease in the excitability of nociceptors (as opposed to their ablation as in 1.8-DTA). Nav1.7 is a VGSC that is highly expressed in nociceptors and is a critical determinant of their excitability. Deletion of Nav1.7 in DRG neurons leads to profound loss of pain without adverse motor or central nervous system (CNS) effects [14; 17]. Therefore, the changes in the OS and SD populations in the Nav1.7 knockout represents the effects a potent and safe analgesic would have. We used the Advillin-1.7 KO mouse [14] (Nav1.7KO thereafter), where Nav1.7 is deleted in all DRG neurons. We hypothesized that Nav1.7 deletion will cause a major decrease in veratridine-responsive neurons through a decrease in the OS but not the SD population. In the Nav1.7KO, the percentage of veratridine-irresponsive neurons increased from 51% in controls (floxed Nav1.7) to 80% (Fig. 3a). This decrease in the responsiveness to veratridine was due to the reduction of the OS population, which decreased from 28% to 7% (0.25 of control). Deletion of Nav1.7 rendered three quarters of the OS population veratridine-irresponsive. Of note, there was no significant decrease in the SD population. Therefore, drugs can be screened by our assay based on the similarity of their effect on DRG neurons to Nav1.7 deletion.

To validate the use of the pattern of veratridine responses in Nav1.7KO as the criterion for a “good” analgesic we compared it to the pattern of a would be “bad” analgesic, a drug affecting non-nociceptors and CNS neurons. For this purpose, we used a Nav1.6 channel blocker. Although conditional Nav1.6 knockout suggests it contributes to pain [3] it is not a potential analgesic target because of its role in CNS and motor neurons [12; 18]. In DRG, Nav1.6 contributes more to sodium current in large (non-nociceptive) than in small (nociceptive) neurons [3]. Therefore, we hypothesised that a Nav1.6 blocker will reduce the OS and SD populations but the effect on the SD population will be greater. Low concentrations of the Tetrodotoxin metabolite, 4,9-anhydro-TTX (4,9TTX) preferentially blocks Nav1.6 (IC_{50} 120 times lower than Nav1.7)[20]. As expected, 300 nM 4,9TTX greatly increased the percentage of veratridine-irresponsive neurons from 42% to 68% (Fig. 3b). Unlike Nav1.7 deletion, 4,9TTX reduced both the SD and OS populations but the reduction in the SD population (0.48 of control) was greater than the reduction in the OS population (0.62 of control). Therefore, drugs that reduce the SD population may act on non-nociceptive neurons with potential unwanted physiological effects.

To test if our assay has the potential to detect increases in neuronal excitability (i.e., as a readout for changes that lead to hyperexcitability, Fig 1) we examined veratridine responses in the Nav1.8 Knockout mouse (Nav1.8KO). Nav1.8 is also an important determinant of the excitability of nociceptors. The global Nav1.8KO mouse, however, showed a compensatory increase in Nav1.7 expression [2]. This is the most likely reason why the Nav1.8KO mouse does not show a major loss of pain. Since Nav1.8 is expressed in nociceptors then it is logical to assume that the compensatory increase in Nav1.7 will occur in nociceptors. Therefore, we hypothesised that this compensatory increase in Nav1.7 will primarily affect the OS population. In Nav1.8KO, the percentage of veratridine-irresponsive neurons decreased from 55% in littermate controls to 37% in Nav1.8KO (Fig. 3b). This increase in veratridine-responsiveness came from the increase in the OS population from 27% in controls to 40%. The changes in the other populations were small and highly insignificant. In other words, the compensatory increase in Nav1.7 expression caused previously silent neurons to respond to veratridine and that these additional neurons had the OS profile, the profile of nociceptors. These results show that our assay is sensitive to increases in Nav1.7 and thus can be used to detect pathological changes in Nav1.7 function (e.g. gain of function mutations or painful conditions).

Collectively, our results confirm that most nociceptors respond to veratridine with the OS profile while most non-nociceptors respond to veratridine with the SD profile. Only the OS population is reduced when nociceptors are ablated (1.8-DTA) or lose a critical excitability determinant (Nav1.7KO). Importantly, the veratridine-response pattern of the Nav1.7KO can be used as the reference for potent and safe analgesic action, a reference pattern that potential analgesics can be evaluated against before detailed *in vitro* characterisation or testing.

Evaluating the potency and selectivity of subtype-specific VGSC blockers using the Nav1.7KO as reference

Several blockers of Nav1.7 and Nav1.8 channels have been developed and are currently in clinical trials [9; 25]. We used our assay to evaluate a candidate analgesic drug for each. PF-04856264 (PF-048) is from the arylsulfonamide class of selective Nav1.7 blockers [11]. PF-048 reduced responses to veratridine at concentrations similar to or lower than those used in standard cell line-based FLIPR assays [4; 11] and shows that our assay can identify non-pore blockers like arylsulfonamides. PF-048 at 1 µM increased the percentage of veratridine-irresponsive neurons (Fig. 4a) from 46% to 58% through a decrease in the OS population which decreased from 26% to 17% (i.e., 0.65 of control). PF-048 at 5 µM (Fig. 4b) had a greater effect, increasing veratridine-irresponsive neurons from 44% to

72% through a larger decrease in the OS population from 28% to 12% (0.43 of control). However, 5 µM PF-048 decreased the SD population from 13% to 6%. Since a reduction in the SD population was not observed in Nav1.7KO this suggests that PF-048 will affect non-nociceptors at a dose that does not even reduce the OS population to the Nav1.7KO level of 0.25 of control.

The Nav1.8 blocker A-803467 (A-80) decreases the excitability of DRG neurons [8]. At 0.1 µM it reduced the OS population from 25% to 12% (Fig. 4c) but without an overall increase in veratridine-irresponsive neurons (51% vs 55%). A-80 at 0.1 µM did not silence the OS population but rather prevented its multi-peak oscillatory behaviour, converting them to the ID and RD profiles that respond to veratridine with a single peak (Fig. 1). A-80 at 0.3 µM increased veratridine-irresponsive neurons from 41% to 66% (Fig. 4d), through significant decreases in the OS population (from 28% to 12%) and the SD population (from 19% to 12%). These data suggest that A-80 is more potent in reducing the excitability the OS population than PF-048 but it also affects the SD population at the higher concentration.

A combination of low concentrations of PF-04856264 and A-803467 have an additive effect on nociceptors.

We suggest that our assay can be used not only to screen for novel drugs, but also to identify effective combinations of existing ones. As a proof of concept, we used a combination of 1µM PF-048 and 0.1µM A-80 (Fig. 5a). When applied separately, neither reduced the SD population significantly but their reduction of the OS population is considerably less than Nav1.7 deletion. The combination reduced the OS population from 30% to 11%, greater than either alone. Importantly, the combination still did not significantly reduce the SD population. A direct comparison of the decreases in the OS and SD populations between the VGSC blockers used and Nav1.7 deletion (Fig. 5b) clearly shows that the combination produced the closest effect on the OS population (reduction to 0.38 of control) to that observed in Nav1.7KO (0.25 of control).

Discussion

Cell lines provide a high throughput platform for analgesic drug discovery. However, their use leaves a knowledge gap that must be addressed on DRG neurons using lower throughput methods like calcium imaging and patch clamping. The knowledge gap includes information on the effect of a drug on nociceptors as a whole (rather than just the molecular target of interest) and the concentrations needed for a potent effect on nociceptors with a minimal effect on non-nociceptors (as a proxy for unwanted side effects on CNS and motor neurons). We discovered that nociceptors and non-nociceptors respond to veratridine with distinct response profiles[16]. Here we show that a veratridine-based assay allows for a simultaneous assessment of drugs' action on both populations and therefore provides an efficient and more informative assay for screening for analgesics on sensory neurons. First, we discuss the results from our findings and then discuss the key advantages of our assay.

We used the 1.8-DTA mouse which has well characterised pain deficits to demonstrate the link between the loss of the OS population and loss of pain [1]. In 1.8-DTA neurons we recorded a loss of 89% of functionally defined nociceptors (Fig. 2b), which is in agreement with the expression of Nav1.8 in 80-90% of nociceptors [5; 21]. Our identification of nociceptors is based on responsiveness to one or more of three nociceptive agonists[16]. This definition will encompass six of the putative eleven molecular subtypes (the NP1-3, PEP1-2 and TH types) of sensory neurons as defined in one single-cell RNA sequencing study [23]. By our functional definition, non-nociceptors will equate to the five Neurofilament-positive subtypes (NF1-5) [23]. Ablation of nociceptors leads to the loss of 78% of the OS population, confirming that most nociceptors respond to veratridine with the OS profile. In the Nav1.7KO mouse, nociceptors are not ablated but their excitability is greatly reduced to the extent that the pain loss in the Nav1.7KO is very similar to that of the 1.8-DTA [1; 14]. Not surprisingly, deletion of Nav1.7 "silenced" 75% of the OS population (Fig. 3a), an almost identical percentage to that lost in 1.8-DTA.

Given that Nav1.7 is expressed in all DRG neurons, why was there no significant reduction in the SD population in Nav1.7KO? Veratridine primarily activates TTX-sensitive VGSCs and indeed, we previously showed that TTX silences all OS and SD neurons [16]. However, while Nav1.7 is the main TTX-sensitive channel in nociceptors, non-nociceptors rely on other TTX-sensitive subtypes, namely Nav1.1, Nav1.2 and Nav1.6. In the absence of Nav1.7, these subtypes allow veratridine to activate non-nociceptors. The predominance of other TTX-sensitive subtypes in non-nociceptors explains why deletion of Nav1.7 in all DRG neurons has no effect on touch or proprioception [14]. This is also why 4,9TTX, which preferentially blocks Nav1.6 channels, affected the SD population more than the OS population (Fig. 3b).

We propose to use veratridine responses of the Nav1.7KO as the criterion VGSC blockers need to match for *in vivo* potency and safety. The pattern of veratridine responses we observed *in vitro* is due to the deletion of Nav1.7 and are unlikely to be influenced by the reported increase in endogenous opioid agonism in the spinal cord [15]. This is because veratridine responses are recorded from disassociated neurons after one day in culture (that lacks opioid releasing spinal cord cells) and under constant Ringer flow. Our assay detected a significant increase in the number of neurons responding to veratridine in Nav1.8KO and these "unsilenced" neurons have the OS profile. Demonstrating for the first time the effect of the compensatory increase in Nav1.7 expression [2]. All of the above strongly confirms the link between nociceptors' excitability and the OS population and suggests that a reduction in the OS but not the SD population correlates with a reduction in pain without motor/CNS effects.

Our assay has several advantages over cell line-based assays and patch clamping. Firstly, a cell line-based assay examines the effect of a condition or a drug on one target at a time whereas our assay evaluates the overall effect on DRG neurons, including non-nociceptors. For example, while A-80 was potent in reducing excitability we noted clear cytotoxic effects at concentrations >0.5µM (not shown). The use of veratridine as a single “pan” activator of all sensory neurons has advantages over the commonly used potassium chloride (KCl). DRG neurons respond to veratridine with distinct profiles that can be used to identify the main populations whereas KCl produces a similar profile in all neurons. Additionally, while 80% of Nav1.7KO neurons did not respond to veratridine they were all KCl-positive (Fig. 3a), strongly suggesting that KCl responsiveness is a poor predictor of the excitability of DRG in *in vitro* assays.

Secondly, our assay allows changes in the population of high-threshold or “silent” sensory neurons that are observed *in vivo* [13], to be monitored *in vitro*. This is possible due to the way veratridine activates sensory neurons. Veratridine acts on open VGSC at the resting membrane potential to prevent their inactivation. Neurons with low expression of threshold VGSC channels (e.g. Nav1.7 in DRG) or/and have a hyperpolarised membrane potential will have few open VGSC channels at resting potentials and will not respond to veratridine. We observed that between 30-50% of cultured DRG neurons do not respond to three minutes of 30 µM veratridine. Interestingly, these are similar to the percentages (38-48%) of high-threshold “silent” DRG neurons that did not respond to 10-100mA current injection *in vivo* but were unmasked by inflammation [22]. Since two thirds of veratridine-irresponsive neurons responded to nociceptive markers (yellow segment in Fig. 2d),we speculate that the veratridine-irresponsive population includes the high threshold silent nociceptors observed *in vivo*. However, we have no data to support this hypothesis at present.

Thirdly, our assay allows the range of plasma concentrations for a potent action on nociceptors without an effect on non-nociceptors to be determined efficiently. Both PF-048 and A-80 reduced the OS population with A-80 being 10 times more potent than PF-048. However, at the higher concentration both reduced the SD population, an effect not observed in either Nav1.7 or Nav1.8 KOs but obtained from the Nav1.6 blocker 4,9TTX. The concentrations at which PF-048 and A-80 affected the SD population were not much higher than those at which they did not (5 fold for PF-048 and 3 fold for A-80). The reduction of the SD population by 5µM PF-048 is larger than that by 0.3μM A-80 (Fig. 4). This can be explained by the greater sequence homology between the VGSC subtypes expressed in non-nociceptors to Nav1.7 than to Nav1.8.

Finally, our assay can be used to screen for drugs combinations to obtain the most potent effect on the OS but the least effect on the SD populations. This strategy may become a necessity considering that developing potent selective Nav1.7 blockers has been a challenging task [9]; even though Nav1.7 has been a validated target since 2004 [17]. A combination strategy will be necessary when selective blockers against a single target do not produce the desired system effect. Since the pain phenotype seen in the mouse and human loss-of-function mutants [7; 14; 17] results from 100% loss of Nav1.7 channels, it remains to be determined what level of pharmacological block of Nav1.7 is needed *in vivo* for reduced pain and if selective Nav1.7 blockers can achieve this level of inhibition. Furthermore, the pain phenotype seen in the mouse and human loss-of-function mutants seems to involve changes in other systems (i.e. opioid signalling in the spinal cord [15]).In these situations, a selective Nav1.7 blocker will may not be potent enough and combination of drugs against different targets may produce the required potency on nociceptors. As a proof of concept, we demonstrate that combining low concentrations of PF-048 and A-80 produced the closest veratridine-response pattern to that of Nav1.7KO, the reference genetic model (Fig. 5b). Efficient *in vitro* screening of drugs combinations against multiple targets is not possible using cell line-based expression systems. This is because a cell line has to be engineered to express multiple targets at levels similar to their native levels in DRG neurons.

Future work will focus on adapting the assay to high throughput platforms by automating data analysis (including calculation of response onset, amplitude and area under the curve) and making use of a non-wash protocol [22]. The potential of this assay to screen for drugs for other classes of ion channels (calcium and potassium) needs to be established.

For drug screening, the assay is most useful when the primary screening criterion is the decrease in the OS population where the screening window is large, a 75% drop in the 1.7KO and 62% in the A80PF drug combination (Figure 5). Although a decrease in the total number of veratridine responsive neurons can be used as the screening criterion the screening window is narrower (A decrease of 38% in the 1.7KO). In all cases, the screening criterion should include a requirement for minimal effect on the SD population.

In summary, we described an assay to assess the excitability of nociceptors and non-nociceptors. The assay can be used in other applications (Fig. 6) in addition to drug development and screening. It can be employed for in-depth, long-term characterisation of excitability changes in knockout mice and models of pain pathologies (diabetes, cancer and aging). It can be used to optimise protocols to differentiate human stem cells into the different classes of sensory neurons [24]. Meanwhile, all this may have the added advantage of reducing the number of animals needed for *in vitro* and *in vivo* testing.

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Author’s contributions

MAN, conceived, designed and supervised experiments. ZAM and KK co-designed, carried out and analysed all experiments. MAN contributeed to preparations of DRG cultures. MAN wrote the manuscript with contributions form ZAM And KK.

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Figure 1: Assessments of the excitability of nociceptors and non-nociceptors based on veratridine response profiles.

Dorsal root ganglia (DRG) contain a heterogeneous population of sensory neurons. Nociceptors are typically small in size, express the Nav1.8 channel and respond to nociceptive compounds (e.g. ATP and capsaicin). Most nociceptors responded to veratridine with an Oscillatory (OS), Rapid decay (RD) or Intermediate decay (ID) profiles. Non-nociceptors are typically large in size, do not express the Nav1.8 channel and do not respond to nociceptive compounds. Most non-nociceptors respond to veratridine with a Slow decay (SD) profile.

The percentages of the OS and SD veratridine profiles plus the percentage of veratridine-irresponsive neurons can be used for an efficient assay of the excitability DRG neurons. Changes in the SD population reflect changes in non-nociceptors (blue, typically 15-20% of all neurons). Changes in the OS population (red, typically 30-40% of all neurons) reflect changes in nociceptors. Changes in the veratridine-irresponsive population (grey, typically 30-40% of all neurons) can reflect sensitisation of high threshold and normally “silent” neurons. The two minor profiles, ID and RD, are mostly nociceptors but can be excluded from an assay for simplicity as both account for less than 5-10% of all neurons.

Figure 2: The OS population is reduced in mice lacking most nociceptors.

A) Representative images from control and 1.8-DTA cultures loaded with fura-2AM for imaging (contrast enhanced for both). Ablation of Nav1.8-expressing neurons in 1.8-DTA mouse leaves behind mostly non-nociceptive large neurons. Scale bar is 50µm.

B) Example traces from our imaging protocol. Dashed lines indicate the periods of agonists application in recordings typically 25-35 minutes long. The four agonists and KCL are applied in the same order for all coverslips. The first row shows examples of neurons irresponsive to veratridine but respond to capsaicin and AITC, the second row shows neurons responding to veratridine and two nociceptive agonists while the third row shows neurons responding to all four agonists. Veratridine (VTD), capsaicin (CAP), α, β-methylene ATP (ATP) and allyl isothiocyanate (AITC).

C) In control DRG, 75% of neurons respond to one or more of the three nociceptive compounds and are classified as nociceptors (top) while only 8.3% do so in the 1.8-DTA confirming the loss of 89% of nociceptors.

D) Ablation of Nav1.8-expressing neurons decreases the percentage of veratridine-irresponsive neurons, decreases the percentage of OS neurons and increases the percentage of SD neurons. VTD- CTR= 38.6±3.1 vs VTD- DTA= 22.5±2.6; SDCTR= 17.11±2.8 vs SDDTA= 66.4±1.4; OSCTR= 33.7±2.1 vs OSDTA= 6.9±1.9; IDCTR= 5.9±1.1 vs IDDTA= 3.8±1.5; RDCTR= 2.6±0.9 vs RDDTA= 0.3±0.2%. One way ANOVA with Sidak's test. Pie charts represent mean percentages in the histogram.

E) In control DRG, veratridine-irresponsive neurons can be nociceptors (yellow section, 26.4% of all neurons) or non-nociceptors (orange section, 11.6% of all neurons). Veratridine-irresponsive or “silent” nociceptors are almost completely lost in 1.8-DTA (become 0.8% of all neurons). Overall, there are less veratridine-irresponsive neurons in 1.8-DTA.

Data for C-E are from 940 neurons from three control mice and 360 neurons from four 1.8-DTA.

Figure 3: Reference veratridine-response patterns for “safe” and “unsafe” analgesic drugs.

A) Deletion of Nav1.7 causes a major loss of pain without adverse CNS or motor effects. The veratridine-response pattern of the Nav1.7KO represents that of a safe and potent analgesic. Nav1.7 deletion leads to a decrease in responsiveness to veratridine (increase VTD- population) due to a decrease in the OS population but not the SD population. VTD- CTR= 51.0±3.51 vs VTD- KO= 80.4±2.8; SDCTR= 9.1±1.9 vs SDKO= 7.5±1; OSCTR= 28.2±2.2 vs OSKO= 6.7±1.3; IDCTR = 7.4±0.4 vs IDKO= 3.2±0.6; RDCTR= 4.0±0.6 vs RDKO= 1.9±0.5%. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 1448 neurons from six floxed-control mice and 1630 neurons from six Nav1.7KO.

B) 300nM of the Nav1.6 blocker 4,9TTX, reduces responsiveness to veratridine (increases VTD-) through decreases in both the SD and OS populations. The decrease in SD is greater than that in OS. VTD- CTR= 41.6±3.1 vs VTD- 4,9TTX= 68.0±1.7; SDCTR= 20.68±2.6 vs SD4,9TTX= 10.0±1.5; OSCTR= 28.7±1.8 vs OS4,9TTX= 17.6±2.2; IDCTR= 5.4±0.9 vs ID4,9TTX= 3.1±0.5; RDCTR= 3.5±1.1 vs RD4,9TTX= 1.4±0.3 %. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 635 untreated and 927 treated neurons from six C57Bl6 mice.

C) The compensatory increase in Nav1.7 channels in nociceptors of the Nav1.8KO increases responsiveness to veratridine (decreases VTD-) due to an increase in the OS population. VTD- CTR= 55.4±3 vs VTD- KO= 37.1±3.2; SDCTR= 6.9±1.7 vs SDKO= 9.1±1.1, OSCTR= 26.7±2.0 vs OSKO= 40.3±2.7, IDCTR= 7.0±0.5 vs IDKO= 7.65±1.1, RDCTR= 3.6±0.6 vs RDKO= 5.7±0.5 %. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 1493 neurons from five littermate-control mice and 1028 neurons from four Nav1.8KO.

Figure 4: Evaluating the effect of PF-04856264 and A-803467 on nociceptors and non-nociceptors.

A) 1 µM PF-048 increases the percentage of veratridine-irresponsive neurons through a reduction of the OS but not the other three populations. VTD- CTR= 45.9±3 vs VTD- 1PF= 58.1±4; SDCTR= 16.9±2 vs SD1PF= 13.6±1.7; OSCTR= 26.2±1.7 vs OS1PF= 16.8±3; IDCTR= 7.5±1.3 vs ID1PF= 7.0±1.4; RDCTR= 4.1±1.6 vs RD1PF= 3.6±0.9 %. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 813 untreated and 681 treated neurons from seven C57Bl6 mice.

B) 5 µM PF-048 increases the percentage of veratridine-irresponsive neurons through a reduction of both the OS and SD populations. 5 µM PF-048 is equally potent on the SD and OS profiles reducing both to about 50% of control values. VTD- CTR= 44.1±2.7 vs VTD- 5PF= 71.8±1.5; SDCTR= 13.2±1.6 vs SD1PF= 6.3±1.0; OSCTR= 28.1±2.4 vs OS5PF= 12.4±1.6; IDCTR= 7.9±1.6 vs ID5PF=

5.9±1.1; RDCTR= 6.2±1.8 vs RDPF= 3.8±0.7%. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 605 untreated and 338 treated neurons from five C57Bl6 mice.

C) 100 nM A-80 does not change the percentage of veratridine responsive neurons but reduces the OS population by about 50%. VTD- CTR= 51±4.9 vs VTD- 100A80= 54.8±4.9; SDCTR= 12.9±3.2 vs SD100A80= 14.4±2; OSCTR= 25.1±2.6 vs OS100A80= 11.61±2.6; IDCTR= 5.2±1.3 vs ID100A80= 10.7±2.5; RDCTR= 6.6±0.8 vs RD100A80= 7.3±1.6%. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 607 untreated and 594 treated neurons from six C57Bl6 mice.

D) 300 nM A-80 increases the percentage of veratridine-irresponsive neurons through a reduction of both the OS and SD populations. The reduction in the OS population is slightly greater than that in the SD profile. VTD- CTR= 40.8±2.3 vs VTD- 300A80= 66.2±2.4; SDCTR= 19.1±1.7 vs SD300A80= 11.5±2; OSCTR= 28.0±1.3 vs OS300A80= 12.2±1.2; IDCTR= 8.5±1.5 vs ID300A80= 6.8±1.5; RDCTR= 4.8±0.9 vs RD300A80= 3.3±0.7%. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 1197 untreated and 1294 treated neurons from seven C57Bl6 mice.

Figure 5: The additive effects of a combination of PF-04856264 and A-803467 on nociceptors.

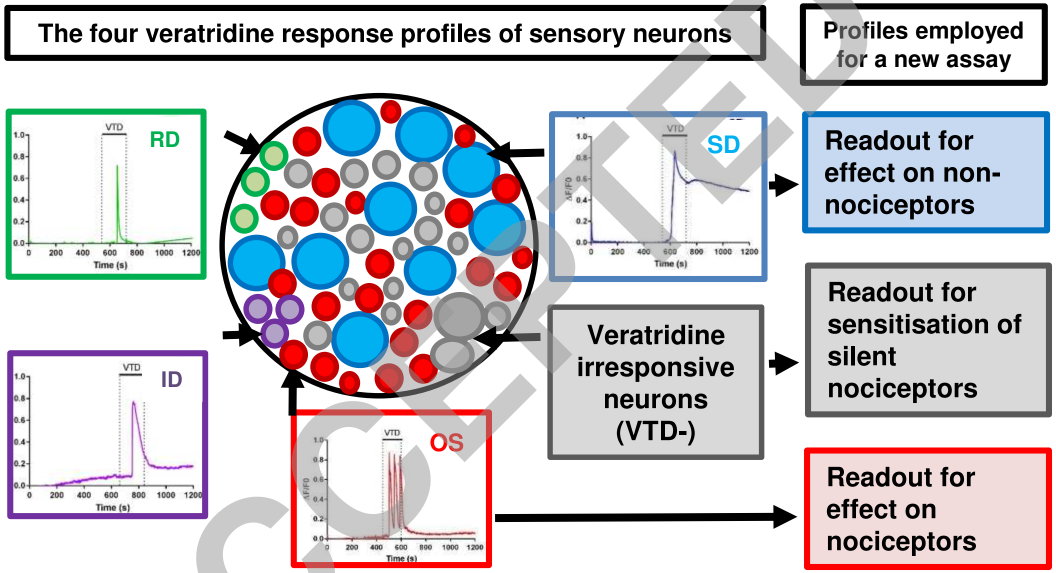
A) A combination of 1 µM PF-048 and 100 nM A-80 increases the percentage of veratridine-irresponsive neurons through a decrease of the OS population only. VTD- CTR= 40.8±3.5 vs VTD-A80PF= 71.5±3.7; SDCTR= 19.2±2.5 vs SDA80PF= 12.2±1.5; OSCTR= 30.4±2.4 vs OSA80PF= 11.5±2; IDCTR= 5.7±0.8 vs IDA80PF= 2.4±0.8; RDCTR= 3.7±0.4 vs RDA80PF= 2.1±1.1%. One way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 980 untreated and 1114 treated neurons from seven C57Bl6 mice.

B) Comparison of the changes in the OS and SD populations caused by VGSC blockers to those of the Nav1.7KO. The higher doses of PF and A80 and 4,9TTX caused a significant reduction in the SD population. Notice that the combined action of the lower doses of the PF and A80 produced the closest reduction of the OS population to Nav1.7 deletion.

Figure 6: Applications of the veratridine-based calcium assay.

The assay is a very efficient method to characterise changes in a heterogeneous population of neurons and therefore has several applications. The assay can be used to identify lead analgesic drugs either by validating hits from cell line-based screens on all types of DRG neurons, or identification of hits by a direct screen on DRG neurons. The assay can be used to assess how stem cell derived neurons compare to primary neurons of the same type more efficiently than by patch clamping. The assay can be used to compare neurons derived from patients' IPSC with known or unknown genetic mutations. The assay is suited to characterise pathologies that develop over time as in diabetes, aging or cancer. Finally, the assay can be used to efficiently characterise changes in DRG from the large number of transgenic strains generated by phenotyping consortia.

Fig 1



The four veratridine response profiles of sensory neurons

Profiles employed for a new assay

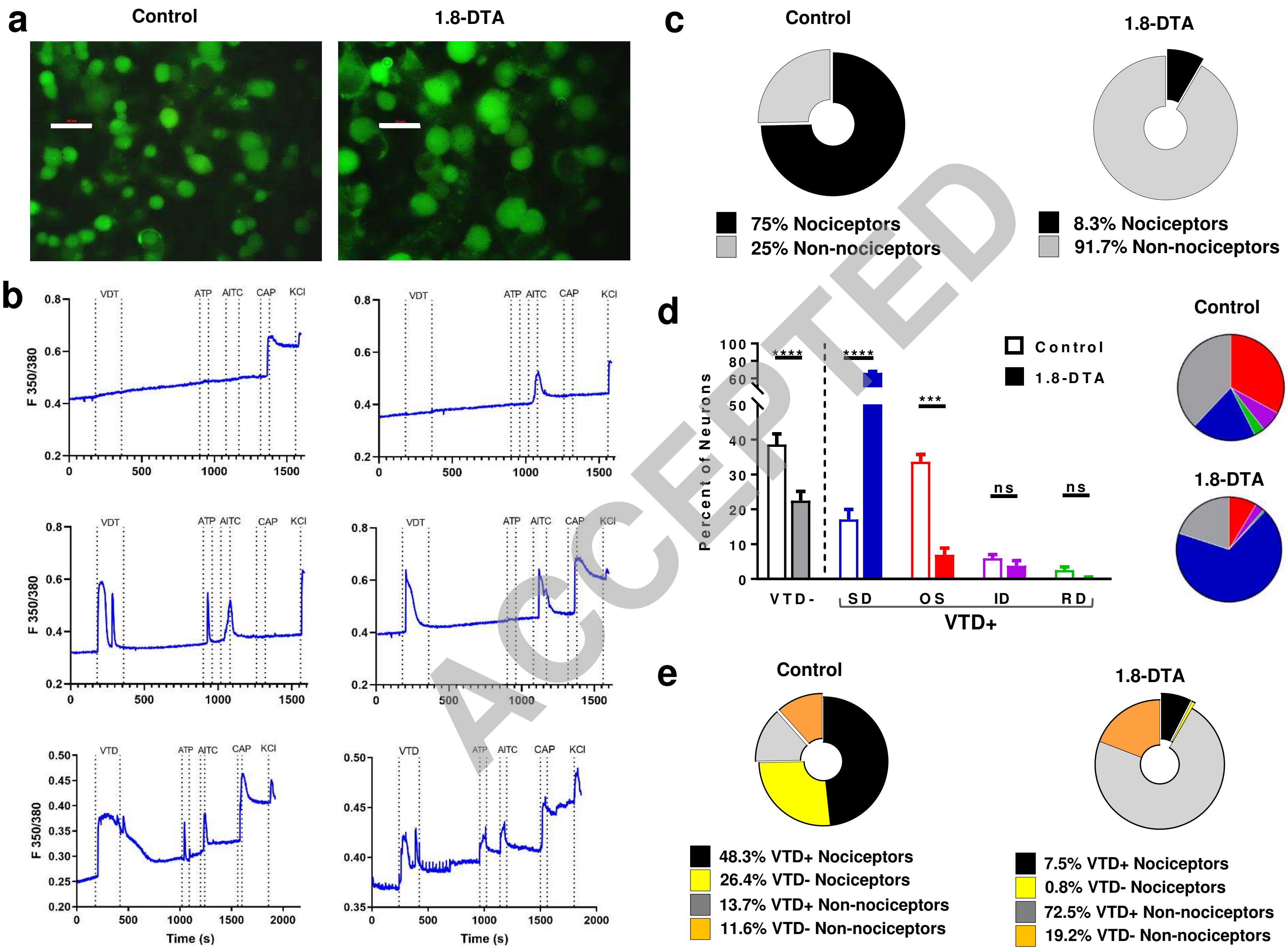
Readout for effect on non-nociceptors

Veratridine irresponsive neurons (VTD-)

Readout for sensitisation of silent nociceptors

Readout for effect on nociceptors

Fig 2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7.5% VTD+ Nociceptors
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19.2% VTD- Non-nociceptors

Fig 3

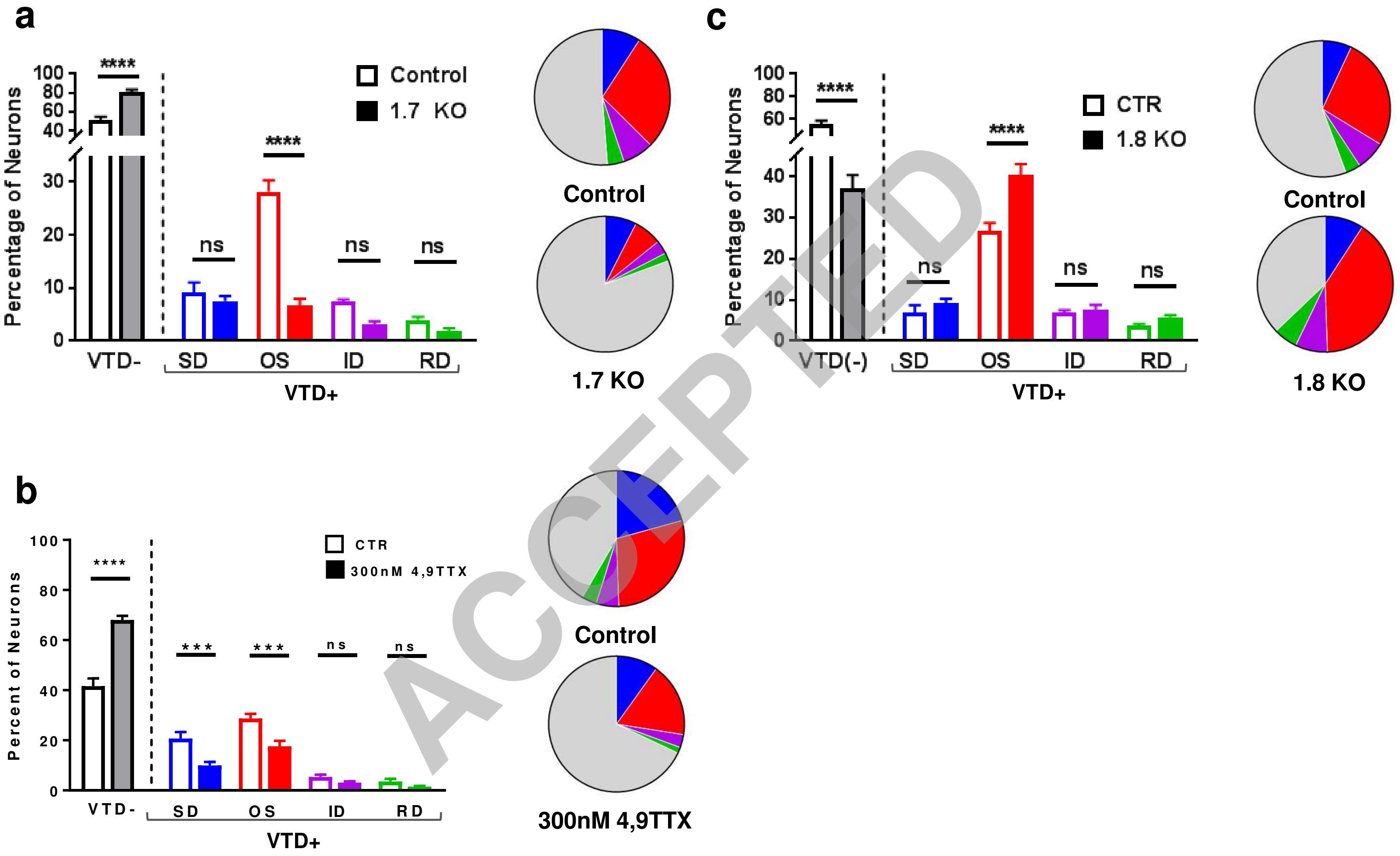
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Fig 4

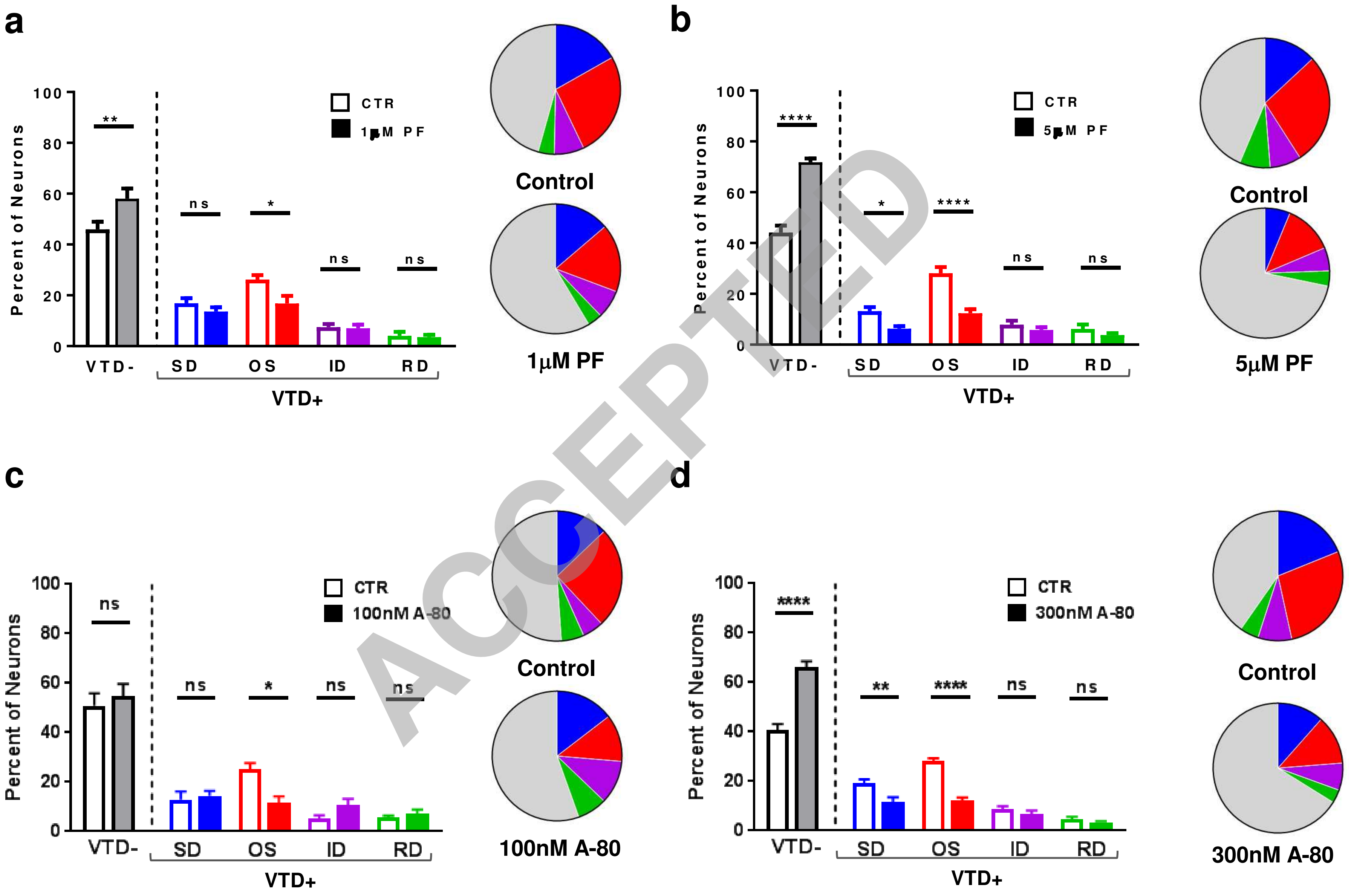
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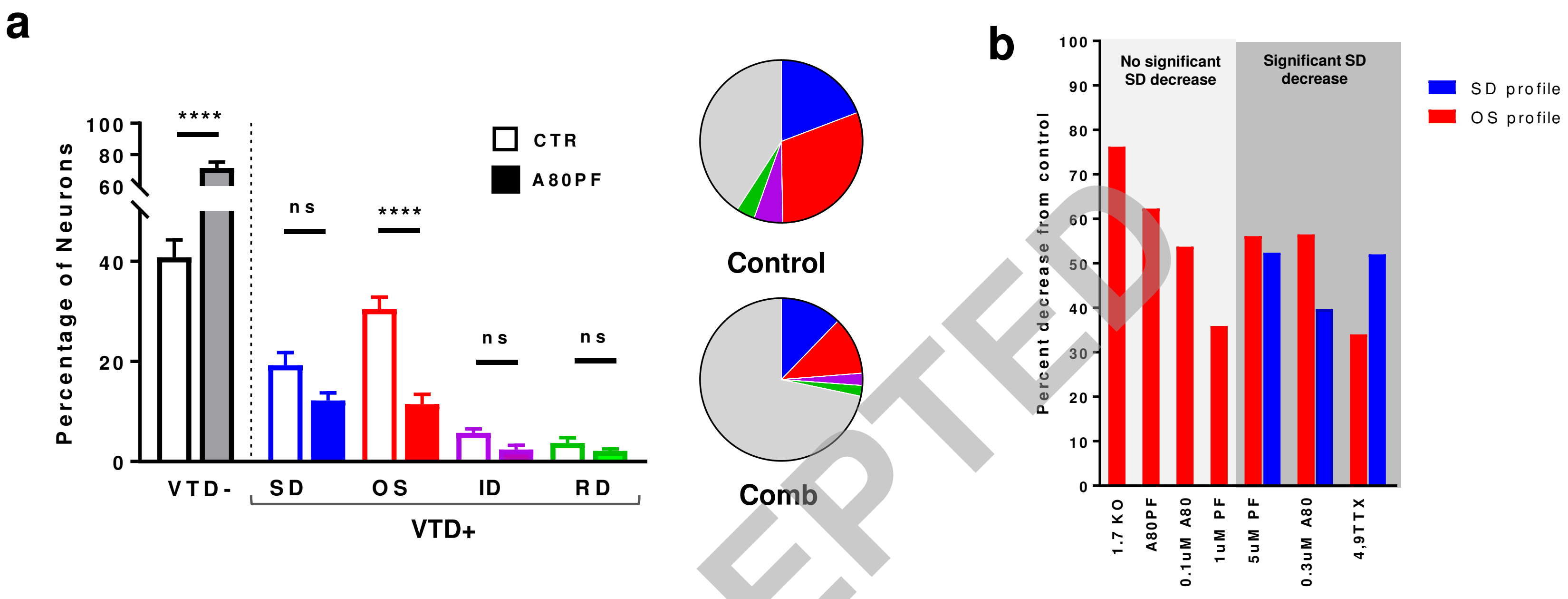
Fig 5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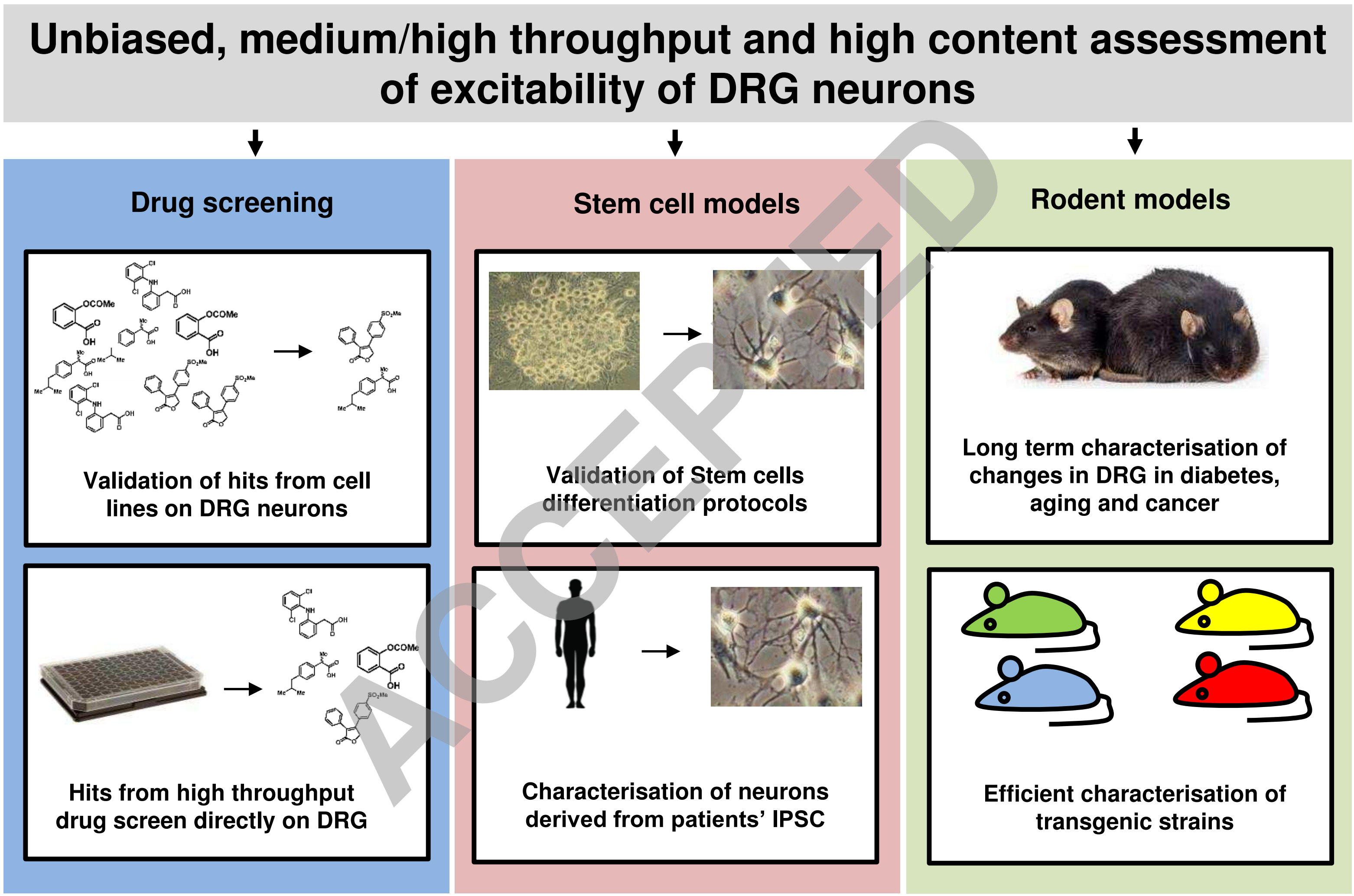
0.3uM A80
4,9TTX

Fig 6



Unbiased, medium/high throughput and high content assessment of excitability of DRG neurons

Drug screening

Validation of hits from cell lines on DRG neurons

Hits from high throughput drug screen directly on DRG

Stem cell models

Validation of Stem cells differentiation protocols

Characterisation of neurons derived from patients' IPSC

Rodent models

Long term characterisation of changes in DRG in diabetes, aging and cancer

Efficient characterisation of transgenic strains