



RESEARCH ARTICLE

Tools for analysis and conditional deletion of subsets of sensory neurons [version 1; peer review: 4 approved]

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Abstract

Background: Somatosensation depends on primary sensory neurons of the trigeminal and dorsal root ganglia (DRG). Transcriptional profiling of mouse DRG sensory neurons has defined at least 18 distinct neuronal cell types. Using an advillin promoter, we have generated a transgenic mouse line that only expresses diphtheria toxin A (DTA) in sensory neurons in the presence of Cre recombinase. This has allowed us to ablate specific neuronal subsets within the DRG using a range of established and novel Cre lines that encompass all sets of sensory neurons.

Methods: A floxed-tdTomato-stop-DTA bacterial artificial chromosome (BAC) transgenic reporter line (AdvDTA) under the control of the mouse advillin DRG promoter was generated. The line was first validated using a Na_v1.8^{Cre} and then crossed to CGRP^{CreER} (Calca), Th^{CreERT2}, Tmem45b^{Cre}, Tmem233^{Cre}, Ntn1^{Cre} and TrkB^{CreER} (Ntrk2) lines. Pain behavioural assays included Hargreaves', hot plate, Randall-Selitto, cold plantar, partial sciatic nerve ligation and formalin tests.

Results: Motor activity, as assessed by the rotarod test, was normal for all lines tested. Noxious mechanosensation was significantly reduced when either Na_v1.8 positive neurons or Tmem45b positive neurons were ablated whilst acute heat pain was unaffected. In contrast, noxious mechanosensation was normal following ablation of CGRP-positive neurons but acute heat pain thresholds were significantly elevated and a reduction in nocifensive responses was observed in the second phase of the formalin test. Ablation of TrkB-positive neurons led to significant deficits in mechanical hypersensitivity in the partial sciatic nerve ligation neuropathic pain model.

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Conclusions: Ablation of specific DRG neuronal subsets using the AdvDTA line will be a useful resource for further functional characterization of somatosensory processing, neuro-immune interactions and chronic pain disorders.

Keywords

Advillin, dorsal root ganglia, primary sensory neuron, pain, somatosensation, Cre recombinase, diphtheria toxin

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Introduction

Somatosensory neurons have been classified into distinct sets first on the basis of action potential velocity, later with histochemical markers, and more recently by single cell transcriptional profiling^{1–4}. Gasser's original observations of three sets of sensory neurons (A fibres, A delta fibres and C fibres) all of which could contribute nociceptive input, demonstrated the complexity of damage-sensing in the periphery. It was only in the 1960s that Perl demonstrated the existence of functionally specialised nociceptors⁵. The debate about polymodality as opposed to modality-specific sensory neurons has been lively. It is clear that the *in vivo* properties of sensory neurons are altered when they are cultured, so that polymodality is often encountered in the dish and may be artefactual⁶. The plasticity of sensory neurons caused by inflammatory mediators is also a complication.

The existence of defined transcripts that distinguish particular sets of sensory neurons provides an opportunity to analyse their function through genetic ablation studies. Recent studies suggest that the subdivisions of sensory neuron subsets identified in mice are in the main evolutionarily conserved in primates, which is of great significance for human studies^{3,4,7}. Thus analysis of rodent sensory neuron function is a useful tool for a better understanding of human somatosensation. Here we have used established and new Cre recombinase lines to ablate subsets of sensory neurons and carried out a preliminary analysis of the consequences for acute pain behaviour. It was important to rule out effects on other tissues, and so we developed a floxed stop diphtheria toxin mouse driven by a sensory neuron specific promoter, *advillin*. *Advillin* is selectively expressed in all sensory neurons and Merkel cells⁸. This allowed us to ablate only sensory neurons with no effect on other tissue that may express the same transcripts as sensory neurons but in which *advillin* and hence diphtheria toxin is not expressed.

Methods

Ethical considerations

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 with prior approval under a Home Office project licence (PPL 70/7382). This study is reported in line with the ARRIVE guidelines⁹.

Mouse husbandry

The study took place at University College London in 2018–2021. Mice were kept on a 12-hr light/dark cycle with cage enrichment (e.g. disposable igloos) and provided with food and water *ad libitum*. Surgical procedures were performed by trained researchers and under aseptic conditions. For genotyping, genomic DNA was isolated from ear tissue for polymerase chain reaction (PCR) using GoTaq DNA Polymerase (Promega) (see *Extended data*, Supplementary Methods for primer sequences and cycling conditions⁹). Cre^{ER/ERT2} mouse lines received 200 µl doses i.p. of a 1% tamoxifen solution on five consecutive days (once per day) between 8–10 weeks of age. Tamoxifen was prepared in 15% ethanol/85% sunflower oil.

Mouse lines

Advillin Flox-tdTomato-Stop-DTA BAC transgenic (AdvDTA). We generated a floxed-tdTomato-stop-DTA bacterial artificial

chromosome (BAC) transgenic reporter line under the control of the mouse *Advillin* (*Avil*) promoter. The construct was generated using a recombineering protocol described in detail by Copeland *et al.*¹⁰ (*Extended data*, Supplementary Figure 1⁹). BAC clone RPCI23-424F19 containing part of the *Advillin* gene was used as a template to prepare a targeting construct. To construct the shuttle vector for recombineering the 5'-homology arm (432 bp) and the 3'-homology arm (274 bp) flanking exon 2 of the *Advillin* gene were cloned into a vector containing a floxTomatoDTA cassette and a Kanamycin cassette, the latter being flanked by flippase recognition target (FRT) sites. The floxTomatoDTA cassette consists of the sequence encoding tdTomato protein followed by three SV40 polyadenylation signals and flanked by the loxP sites. This sequence was fused with the sequence encoding DTA protein followed by bGH and SV40 polyA signals. The start codon in the exon 2 of the *Advillin* gene corresponds to the start codon of tdTomato. The completed shuttle construct was sequenced and the targeting cassette was isolated from the plasmid by *AscI*/*PacI* digest.

EL250 *E. coli* cells transformed with the BAC clone were co-transformed with the shuttle targeting construct and recombination was induced by heat-shock according to the recombineering protocol. The induced bacterial clones were isolated by growing on Kanamycin-containing plates. The Kanamycin resistance cassette was removed by inducing Flp-recombinase expression by arabinose. The final construct was verified by PCR and analytical restriction digests. The resulting BAC containing the targeting cassette was sent to Cyagen for pronucleus injection. The founder line was bred with wild-type C57BL/6J mice to obtain germline transmission.

Cre and floxed stop tdTomato reporter lines

The following lines were provided as generous gifts: CGRP^{CreER11}, TrkB^{CreER12} (Jackson Lab stock number 027214), Th^{CreERT213} (Jackson Lab stock number 025614), Ntng1^{Cre14} and Rosa-CAG-flox-stop-tdTomato¹⁵ (Jackson Lab stock number 007905). The Na_v1.8^{Cre} line was in house and previously described¹⁶.

Tmem45b^{Cre} and Tmem233^{Cre} lines were generated by Cyagen on a C57BL/6 background. The Tmem45b^{Cre} line is a BAC transgenic in which a fragment containing the Cre-SV40 pA together with Frt-Kanamycin cassette-Frt was introduced in exon 2 of the murine *Tmem45b* gene within BAC clone RP24-249I6. The Kanamycin cassette was removed from the recombinant BAC by Flp-mediated recombination. The recombinant clone was verified by PCR and end-sequencing and then injected into fertilized eggs for transgenic mouse production. Pups were genotyped by PCR using GoTaq DNA Polymerase (Promega) (see *Extended data*, Supplementary Methods for primer sequences and cycling conditions⁹). Founder 'R' was used for further breeding and characterization.

The Tmem233^{Cre} line is a constitutive knockin line in which the Kozak-Cre-pA cassette replaced the ATG start codon of the murine *Tmem233* gene. The Cre is under the control of *Tmem233* regulatory elements with homozygotes being knockouts of the *Tmem233* gene. The homology arms of the targeting vector were generated by PCR using BAC clones RP23-182O14 and

RP23-375117. In the targeting vector the Neo cassette was flanked by Rox sites and DTA was used for negative selection. The final constitutive KI allele was obtained after Dre-mediated recombination with C57BL/6 ES cells used for gene targeting. Pups were genotyped by PCR using GoTaq DNA Polymerase (Promega) (see *Extended data*, Supplementary Methods for primer sequences and cycling conditions⁹).

Behavioural tests

For all behavioural assays, the animals in the test groups carried both the Cre gene and the AdvDTA BAC. Animals in the control groups were negative either for the Cre gene or the AdvDTA BAC (see each figure legend for specific details). Sample sizes (1 unit=1 animal) were calculated based on our previous experience for each assay¹⁷ and animals were excluded from the study if they became unwell. All data points were included in the statistical analyses. Animals were acclimatized to the behavioural equipment for at least 2 days prior to testing; control and test groups were assayed on the same day. Observers who performed behavioural experiments were blind to the genotype of the animals. Animals were selected and placed into the apparatus randomly by an independent experimenter. The unblinding of each group followed input of the raw behavioural data into the analysis software. Experiments were conducted using both male and female adult transgenic mice and wild-type littermates (all >7 weeks in age).

Mechanical nociceptive thresholds were measured using a modified version of the Randall Selitto test for mice (Ugo Basile) that applies pressure to the tail with a 500g cut-off^{17,18}. Punctate mechanical sensitivity was measured using the up-down method for obtaining the 50% threshold using von Frey hairs (Ugo Basile stand; Aesthesio hairs), as previously described^{17,19}. Thermal nociceptive thresholds were determined by measuring paw-withdrawal latency using the Hargreaves' apparatus (IITC Life Science)^{17,20} with a ramp of 1.5°C/s and a 30-s cut-off and also by use of the 50°C hot-plate test (Ugo Basile)²¹. The response to noxious cold was measured using the cold plantar assay (Ugo Basile)²². The rotarod test (IITC Life Science) was performed as described in 23. The formalin model was performed by intraplantar injection (subcutaneously) of 20 µl of a 5% formalin solution into the left hindpaw after a 15 min acclimatization to individual observation cages. Time spent engaged in nociceptive behaviour (biting or licking the affected region) was recorded over 1 hr immediately after injection. The video recordings were sampled for 10 sec at 1 min intervals using a Python script and scored for the presence or absence of nocifensive behaviours toward the injected hind paw (licking, biting, flinching). Data is expressed as a percentage of nocifensive responses out of all responses. The early acute phase (Phase 1, 0-10 min) and the latter inflammatory phase (Phase 2, 10-60 min) were separately analysed.

Partial sciatic nerve ligation model

Surgical procedures were performed under isoflurane anaesthesia (inhalation) (2-3%). A partial nerve injury in adult mice was induced by tying a tight ligature with 6-0 silk suture around approximately 1/3 to 1/2 the diameter of the sciatic nerve, similar to the approach described in rats²⁴.

In situ hybridization (ISH) sample preparation and RNAscope

Animals were deeply anesthetized with pentobarbital (i.p.) and transcardially perfused with heparinized saline (0.9% NaCl) followed by 25 ml of cold 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Dorsal root ganglia were extracted from the lumbar area and post-fixed with the same fixative solution for 2 hours at 4 °C before being embedded in cryopreservative solution (30% sucrose) overnight at 4°C. Tissue samples were then placed in optimal cutting temperature (OCT) compound blocks for posterior sectioning by cryostat. 11 µm thick sections were mounted onto Superfrost Plus (Fisher Scientific) slides, allowed to freeze-dry overnight at -80°C, for an immediate use, or were stored at -80°C for no longer than a month for subsequent experiments.

In situ hybridization was performed using the RNAscope system (Advanced Cell Diagnostics) following a two-day protocol for fresh-frozen samples with 1hr post-fixing with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C and stepwise dehydration with 50%, 70% and 100% ethanol. Tissue pre-treatment consisted of hydrogen peroxide and protease IV (10 and 20 min, respectively) at room temperature (RT). Following pre-treatment, probe hybridization and detection with the Multiplex Fluorescence Kit v2 were performed according to the manufacturer's protocol.

Probes included Tmem233 (#519851), Tmem45b (#420461-c3), NEFH (#443671 or #443671-c4), Th (#317621-c4), CGRP (Calca #417961-c3), Ntng1 (#488871-C2), Ntrk2 (#423611-c3) and tdTomato (#317041-c4). Ribonucleic acid (RNA) localisation was detected with Alexa Fluor™ 488 Tyramide (Cat# B40953, green, for all target gene RNA products) and Opal 570 (Cat# FP1488001KT, red, when a probe against tdTomato was used) fluorochrome dyes (Perkin Elmer) compared to DAPI staining (nuclei) or TS-coumarin (TS405, blue, Cat# NEL703001KT, Perkin Elmer) used for neurofilament heavy chain (NEFH). *In situ* hybridisation slides were mounted using Prolong Gold (ThermoFisher Scientific #P36930).

Fluorescence was detected using Zeiss LSM 880 Airyscan microscope. Images were taken at 10x or 20x magnification with 4x averaging, airyscan processed, stitched whenever required and exported as 16-bit uncompressed tiff files for further basic editing in Adobe Lightroom v6 (Adobe) on colour calibrated iMac retina monitor. Final images were exported as jpeg files with 7,200 pix on longest side at 300 ppi.

RNA extraction and real-time qPCR

RNA was extracted from dorsal root ganglia tissue using TRIzol Reagent (Life Technologies) and Purelink RNA micro kit (Thermo Fisher) and then reverse transcribed according to standard protocols. Real-time RT-PCR using technical triplicates was performed with the BioRad CFX Connect™. PCR was carried out using the Universal SYBR Green Supermix protocol (Bio-Rad) and primers to *Scn10a* and *Gapdh* (see *Extended data*, Supplementary Methods for primer sequences and cycling conditions⁹). *Scn10a* expression was compared with that of

Gapdh measured on the same sample in parallel on the same plate, giving a CT difference (Δ CT) for *Gapdh* minus *Scn10a*. Mean and standard error were performed on the Δ CT data and converted to relative expression levels ($2^{-\Delta$ CT).

Statistics

Data was analysed using [GraphPad Prism 9](#) (GraphPad Software, Inc), and results presented as mean \pm SEM with n referring to the number of samples tested per group, as indicated in figure legends. [SOFA Statistics](#) is an alternative open access package that can be used.

Results

Single cell RNA sequencing of mouse dorsal root ganglia neurons has provided an unbiased classification of neuronal subpopulations based on their genetic profiles^{3,4}. Using these data, we selected marker genes that could be used to drive expression of Cre recombinase in restricted DRG neuron populations to enable further functional analyses of neuronal subsets (Table 1). We recruited existing lines and generated new lines for further analyses (see Materials and Methods): *Cgrp*^{CreER} (*Calca*), *Th*^{CreERT2}, *Scn10a*^{Cre} (*Na_v1.8*), *Tmem45b*^{Cre}, *Tmem233*^{Cre}, *Ntng1*^{Cre}, and *TrkB*^{CreER} (*Ntrk2*). Lines were crossed to a Rosa-CAG-flox-stop-tdTomato line and following tamoxifen administration for the CreER/CreERT2 lines, lumbar DRGs were isolated from adult mice for RNAscope testing. Figure 1 shows representative images of tdTomato expression (i.e. following Cre activity) and expression of the ‘target gene’ (i.e. an *in situ* probe against each marker gene mRNA)⁹. For each line tested, there was a large overlap between tdTomato expression and the target, indicating that Cre activity was in general consistent with expression of the endogenous marker gene. Figure 1 also shows expression of neurofilament (*Nefh*) mRNA, which is expressed in NF1, NF2, NF3, NF4, PEP1.2 and PEP2 neurons, according to single cell RNAseq data^{3,4,25}. Comparison of Cre activity (tdTomato expression) and *Nefh* expression was as predicted from single cell RNAseq data (Table 1 and Figure 1). For example, *Tmem45b* Cre activity was present in *Nefh*-negative neurons; *Ntng1* Cre was active in *Nefh*-positive neurons; and *Calca* Cre activity was in both *Nefh*-positive and -negative neurons.

Advillin Flox-tdTomato-Stop-DTA BAC transgenic

The library of validated Cre lines enables specific DRG neuronal populations to be ablated via Cre-dependent expression of Diphtheria toxin A (DTA). However, as many of the Cre lines drive expression in tissues outside of the DRG, we generated a floxed-tdTomato-stop-DTA BAC transgenic reporter line under the control of DRG-enriched mouse *Advillin* (*Avil*) promoter to enable restricted ablation²⁶ (Extended data, Supplementary Figure 1⁹). The targeting construct used to generate the line consists of a tdTomato fluorescent reporter gene and transcriptional stop sequences that are flanked by loxP sites with a DTA sequence downstream. *Advillin* is expressed in all sensory neurons and has minimal expression elsewhere in the nervous system²⁷. In Cre negative animals, the tdTomato gene is expressed with transcription of the DTA prevented by the upstream transcriptional stop sequences. In Cre positive animals, the tdTomato

and transcriptional stop sequences are excised and the DTA is transcribed leading to toxin production and cell ablation.

To validate this new reporter we crossed it with the *Na_v1.8*^{Cre} line and were able to recapitulate the behavioural phenotype observed in our earlier work in which the *Na_v1.8*^{Cre} was bred to a Rosa floxed stop DTA knockin line²⁸. We used the Hargreaves’ test to apply a latent heat stimulus to the hind paw and recorded the latency to a nocifensive response. We also used the Randall-Selitto test to apply steadily increasing mechanical pressure to the tail until a withdrawal response was observed. We showed that mice lacking the *Na_v1.8* positive population of sensory neurons have unimpaired responses to noxious heat (Figure 2A), but responses to noxious mechanical stimuli are substantially impaired (Figure 2B) suggesting that we successfully ablated *Na_v1.8* containing neurons in the BAC transgenic line. Real-time qPCR in DRG isolated from the Cre+/AdvDTA+ mice confirmed the ablation with a 97.5% decrease in *Na_v1.8* mRNA levels compared to WT DRGs (Figure 2C).

Pain behaviour tests following restricted ablation of subpopulations of DRG neurons

Following validation of the AdvDTA reporter we crossed this BAC transgenic line to five additional Cre-expressing lines with distinct expression profiles across DRG neuronal subsets (Table 1). As expected, motor co-ordination as assessed by the rotarod test, was normal for all lines compared to Cre-negative/AdvDTA+ littermates (single cell RNAseq data indicates that all five Cre lines are expressed outside of the parvalbumin-positive PSNF3 proprioceptive DRG neurons) (Figure 3A). The Randall-Selitto assay on the tail showed that noxious mechanosensation was significantly reduced in the *Tmem45b* Cre+/AdvDTA mice (Figure 3B). *Tmem45b* is expressed in a subset of *Na_v1.8* positive neurons, specifically the six non-peptidergic neuronal subsets (PSNP1-6) (Table 1). Punctate mechanical sensitivity, as assessed using von Frey filaments, was not different to controls for the *Tmem45b*, *Th*, *Ntrk2* and *Ntng1* Cre+/AdvDTA mice although surprisingly, increased sensitivity was observed in the CGRP Cre+/AdvDTA mice (Figure 3C). Noxious thermal pain thresholds were assessed by the Hargreaves’ and hot plate tests and for both assays, CGRP Cre+/AdvDTA mice showed significant hyposensitivity to noxious heat (Figures 3D and 3E). However, CGRP Cre+/AdvDTA mice did not show significant higher cold pain thresholds in the cold plantar assay, although a trend was observed (Figure 3F). The AdvDTA reporter line was also tested in inflammatory and neuropathic pain assays. In the formalin test, no significant differences were observed for all AdvDTA+/Cre+ lines in the first phase (Figure 4A). However, in the second phase, the AdvDTA+/CGRP Cre+ mice displayed significantly reduced nocifensive responses (Figure 4A).

Next, we tested the AdvDTA+/Ntrk2Cre+ mice in the partial sciatic nerve ligation model to investigate the contribution of the PSNF1 and PSNF2 neuronal populations in the development of mechanical allodynia under neuropathic pain conditions. As expected mechanical hypersensitivity developed in the control littermates at 7 days and 16 days post-surgery (Figure 4B). However, the AdvDTA+/Ntrk2Cre+ mice did not develop

Table 1. Expression profile of selected marker genes within mouse dorsal root ganglia (DRG) neurons.

	PSPEP6 TrpM8.1	PSPEP7 TrpM8.2	PSPEP8 TRPM8.3	PSPEP5 PEP1.1	PSPEP2 PEP1.2	PSPEP4 PEP1.3	PSPEP3 PEP1.4	PSPEP1 PEP2	PSNF1 NF1	PSNF2 NF2	PSNF2 NF3	PSNF3 NF4	PSNP1 TH	PSNP3 NP1.1	PSNP2 NP1.2	PSNP4 NP2.1	PSNP5 NP2.2	PSNP6 NP3
<i>Calca</i>																		
<i>Th</i>																		
<i>Pvalb</i>																		
<i>Scn10a</i>																		
<i>Tmem45b</i>																		
<i>Tmem233</i>																		
<i>Ntrng1</i>																		
<i>Ntrk2</i>																		
<i>Avil</i>																		

The PSPEP, PSNF and PSNP nomenclature of neuron types on top refers to the classification presented in Zeisel *et al.*, 2018 (available at mousebrain.org)¹. The TrpM8, PEP, NF, TH and NP nomenclature represents the relationship of these neuron types to the classification presented in Usoskin *et al.*, 2015³. PEP (peptidergic); NF (neurofilament); NP (non-peptidergic); TH (tyrosine hydroxylase). Marker genes listed in far center column. Table adapted from 25.

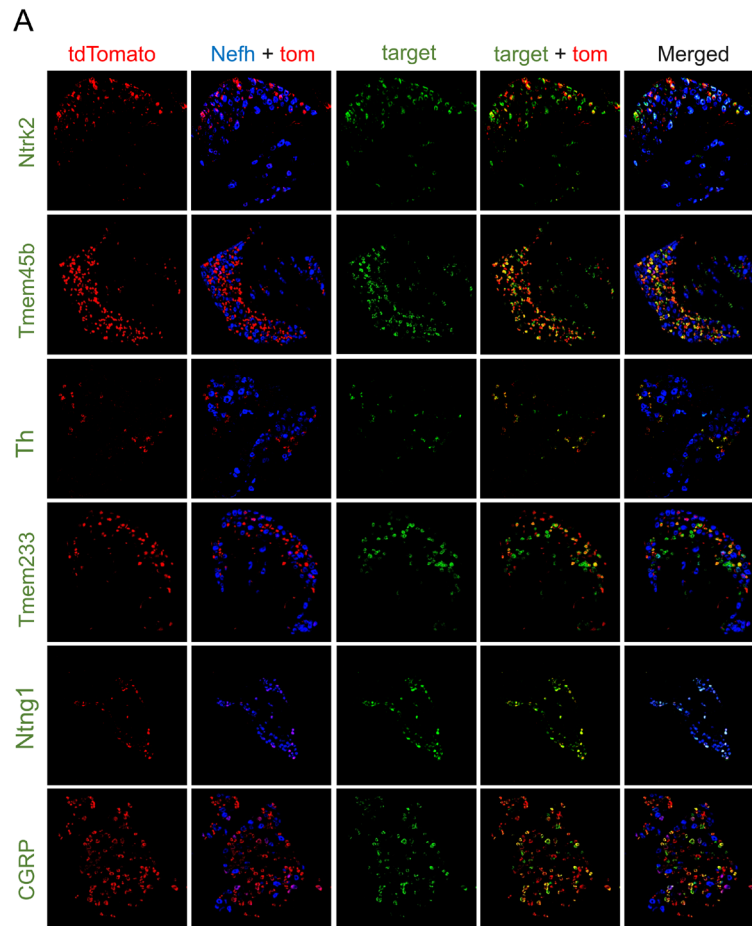


Figure 1. RNAscope analysis of Cre activity in dorsal root ganglia (DRG) neurons. Expression of tdTomato (red), Nefh RNA (detected with TS405, blue) and target marker gene RNA (detected with AF488, green). Cre lines indicated on the left: TrkB^{CreER} (Ntrk2), Tmem45b^{Cre}, Th^{CreERT2}, Tmem233^{Cre}, Ntng1^{Cre}, CGRP^{CreER} (Calca). These lines were crossed to a Rosa-CAG-flox-stop-tdTomato line and lumbar DRGs isolated from adult mice for RNAscope analyses.

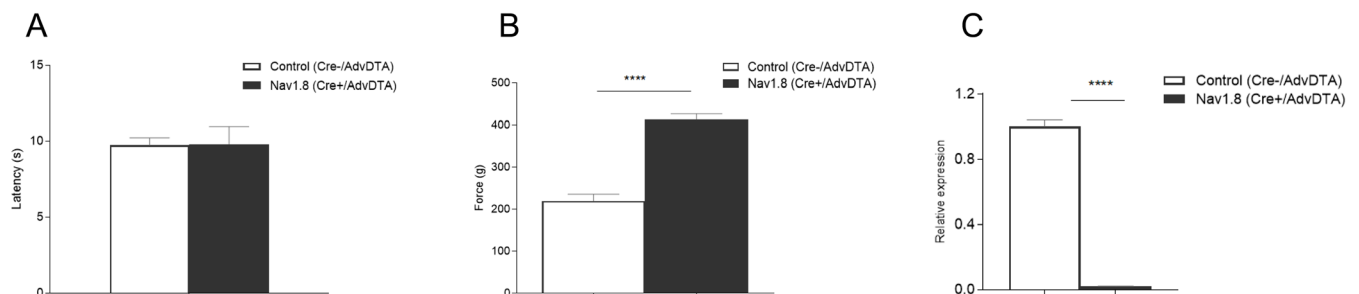


Figure 2. Validation of the *Advillin* Flox-tdTomato-Stop-DTA (AdvDTA) BAC transgenic. (A) Na_v1.8 Cre+/AdvDTA mice show normal responses to noxious thermal stimuli in the Hargreaves' test (t-test: p>0.05 (ns)); Na_v1.8 Cre-/AdvDTA n=6, Na_v1.8 Cre+/AdvDTA n=5;. (B) Na_v1.8 Cre+/AdvDTA mice show significantly impaired noxious mechanosensation in the Randall-Selitto test (t test: p<0.0001); Na_v1.8 Cre-/AdvDTA n=6, Na_v1.8 Cre+/AdvDTA n=5. (C) qPCR data shows a 97.5% decrease in levels of Na_v1.8 mRNA following ablation of the Na_v1.8-positive subset of sensory neurons in Na_v1.8 Cre+/AdvDTA mice (t test: p<0.0001). Na_v1.8 Cre-/AdvDTA n=3, Na_v1.8 Cre+/AdvDTA n=3.

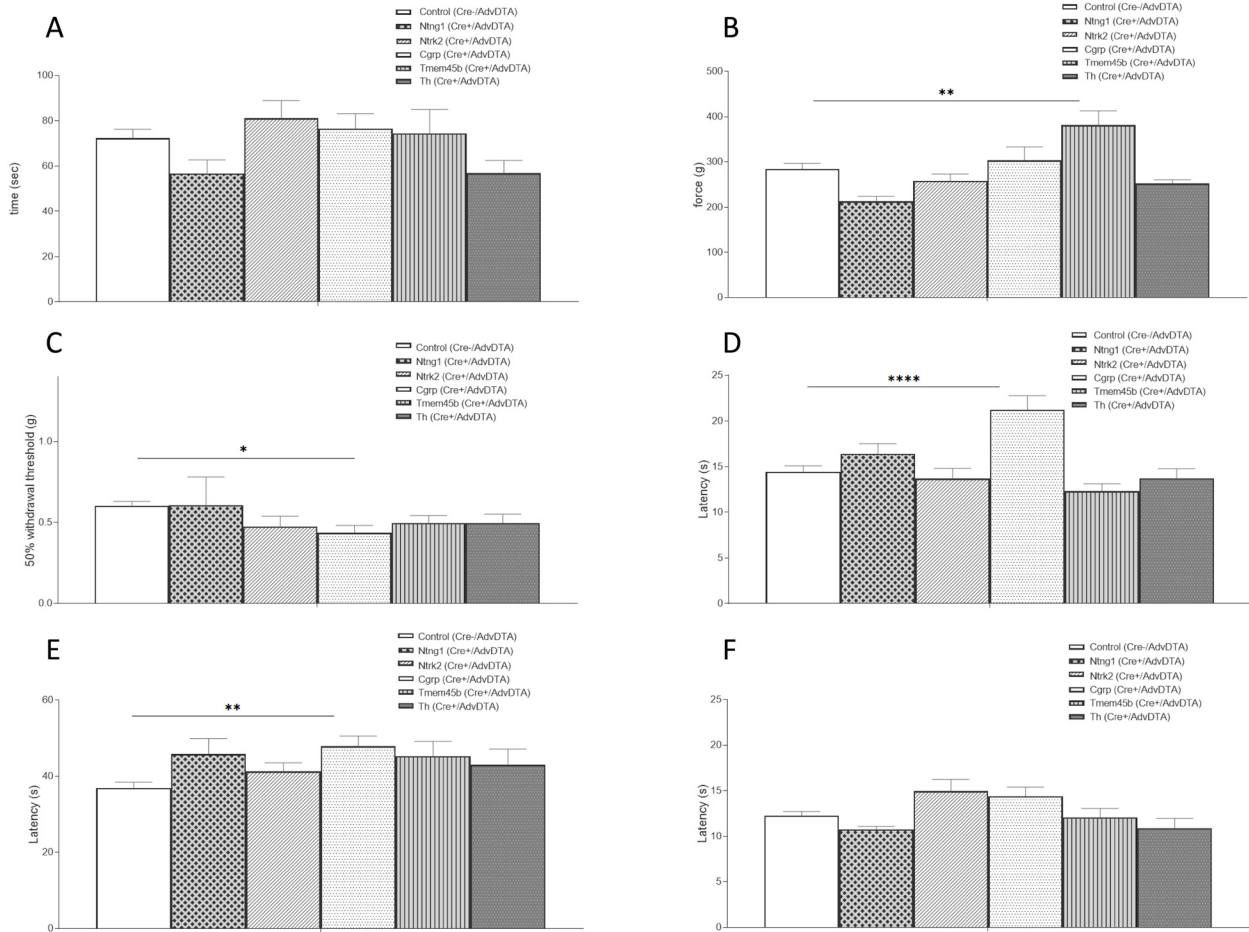


Figure 3. Behavioural analyses following ablation of subpopulations of dorsal root ganglia neurons. (A) All AdvDTA+/Cre+ mouse lines tested show normal motor co-ordination as assessed by the rotarod test (ANOVA, $p > 0.05$ (ns)); (B) AdvDTA+/Tmem45b Cre+ mice show significantly impaired noxious mechanosensation in the Randall-Selitto test (ANOVA, $p < 0.005$); (C) Increased sensitivity to punctate mechanical sensitivity was observed in the AdvDTA+/CGRP Cre+ line (ANOVA, $p < 0.05$); (D) AdvDTA+/CGRP+ mice were hyposensitive to noxious heat in the Hargreaves's test (ANOVA, $p < 0.0001$) and the (E) hot plate test (ANOVA, $p < 0.005$); (F) All AdvDTA+/Cre+ mouse lines tested show normal responses in the cold plantar assay (ANOVA, $p > 0.05$ (ns)). AdvDTA+/Cre- (n=52-54), AdvDTA+/Ntng1 Cre+ (n=5), AdvDTA+/Ntrk2 Cre+ (n=14), AdvDTA+/CGRP Cre+ (n=15-16), AdvDTA+/Tmem45b Cre+ (n=14) and AdvDTA+/Th Cre+ (n=7-8).

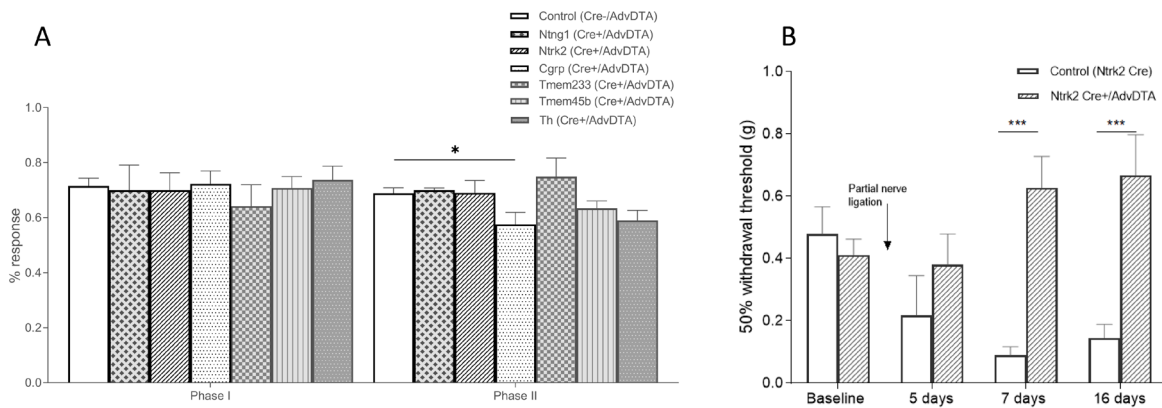


Figure 4. Inflammatory and neuropathic pain behaviour following ablation of subpopulations of DRG neurons. (A) Formalin test in all AdvDTA+/Cre+ mouse lines tested show normal phase 1 nociceptive responses to intraplantar injection of formalin. Ablation of CGRP+ neurons results in reduced responses in the second phase of the formalin test (ANOVA, $p < 0.05$). AdvDTA+/Cre- (n=43), AdvDTA+/Ntng1 Cre+ (n=4), AdvDTA+/Ntrk2 Cre+ (n=6), AdvDTA+/CGRP Cre+ (n=13), AdvDTA+/Tmem233 Cre+ (n=9), AdvDTA+/Tmem45b Cre+ (n=13) and AdvDTA+/Th Cre+ (n=8). (B) Mechanical sensitivity following the partial sciatic nerve ligation model in Ntrk2 Cre (n=9) and AdvDTA+/Ntrk2 Cre+ (n=10) mice (ANOVA, $p < 0.0005$).

mechanical hypersensitivity, highlighting the importance of the PSNF1 and PSNF2 neuronal subsets to mechanical allodynia in the partial sciatic nerve ligation (PSL) neuropathic pain model.

Discussion

Genetic tools have made a dramatic contribution to a better understanding of the nervous system. Thus human genetic studies have identified causative mutations for a variety of disorders²⁹, whilst gene and cell silencing³⁰ and in particular activity-based reporter systems such as CANE have proved extraordinarily informative³¹.

The complexity of sensory neurons that play a key role in pain pathways has been highlighted by single cell transcriptional analysis³. This suggests that there are at least 18 subsets for sensory neurons in mice. Importantly very similar subsets of sensory neurons are present in primates⁷, reinforcing the view that mouse genetic studies are likely to be relevant and useful for understanding human peripheral pain pathways³².

Early experiments using a Na_v1.8 Cre-recombinase to release diphtheria toxin and delete many peripherin-positive sensory neurons produced significant modality specific loss of function²⁸. Recent GCaMP studies are also consistent with the presence of a high proportion of modality-specific sensory neurons although there remains considerable debate on this topic⁶.

Linking transcriptional changes in pain states to particular sets of sensory neurons has proved problematic³³. There is clearly extensive redundancy in pain mechanisms for example in temperature sensing³⁴ and cold sensation³⁵, whilst inflammation or damage changes the expression patterns of many transcripts including those encoding neuropeptides that are known to have a role in pain pathways. In addition, cells of the skin play a role as primary sensors for many painful stimuli indirectly signalling tissue damage to sensory neurons³⁶. Neuro-immune interactions also play a major role in changing the gain of the pain system, and regulating nociceptive activity³⁷.

Despite these complexities, sets of sensory neurons with particular sensory modalities clearly exist, and we have assembled tools to address their specialised functional role. A major strength of this study is that we have exploited known and novel Cre lines that encompass all sensory neuron subsets, and generated an advillin floxed stop diphtheria toxin mouse that will release the toxin only in sets of sensory neurons in the presence of Cre recombinase. A limitation of the study is that there is random integration of the BAC into the genome of the reporter mice with the potential for leaky expression. An improvement would be to generate a knockin mouse line under the advillin promoter with expression of diphtheria toxin under the control of a Cre-dependent FLEx switch.

Expression patterns were analysed with tomato reporter mice and preliminary phenotyping has been carried out analysing acute pain in mice in which sets of sensory neurons have been

deleted. This background information should prove useful as a platform for more complete analyses of pathways involved in pain states that are clearly mechanistically different, for example cold allodynia and chronic inflammatory pain³⁸. The role of neurons normally associated with proprioception and innocuous sensation can also be addressed. Recent studies have demonstrated a significant efferent role for sensory neurons in immune responses, and conditions such as psoriasis³⁹. Dissecting out the new roles of sets of peripheral sensory neurons in regulating immune responses can thus also be addressed with the assembled mouse lines.

Data availability

Underlying data

Open Science Framework: Tools for analysis and conditional deletion of subsets of sensory neurons. <https://doi.org/10.17605/OSF.IO/KTHDU>⁹.

This project contains the following underlying data:

- Raw values for behavioural experiments (including gender-specific values) underlying Figure 2–Figure 4, in open access CSV format
- Raw unedited microscopy images for Figure 1
- Supplementary Methods.pdf (contains raw pictures of gels from PCR testing)
- Figure 2C – qPCR CT values.csv (raw Ct values for all samples and replicates from RT-PCR)

Extended data

Open Science Framework: Tools for analysis and conditional deletion of subsets of sensory neurons. <https://doi.org/10.17605/OSF.IO/KTHDU>⁹.

This project contains the following extended data:

- Supplementary Figure 1.pdf
- Supplementary Figure 2 (from v6).pdf
- Supplementary Figure Legends.pdf
- Supplementary methods.pdf

Reporting guidelines

Open Science Framework: *ARRIVE checklist for ‘Tools for analysis and conditional deletion of subsets of sensory neurons’*. <https://doi.org/10.17605/OSF.IO/KTHDU>⁹.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](https://creativecommons.org/licenses/by/4.0/) (CC0 1.0 Public domain dedication).

Acknowledgements

We thank David Ginty (Harvard), Pao-Tien Chuang (UCSF) and Fan Wang (MIT) for use of Cre lines generated in their labs.

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Reviewer Report 17 February 2022

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 **Felix Viana** 

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² Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

The authors introduce a novel transgenic mouse line that allows the ablation of subpopulations of primary sensory neurons by selective expression of diphtheria toxin. The advantage, compared to previous methods, is the fact that, by using the Advillin promoter, they can restrict the ablation to primary sensory neurons, avoiding possible confounding effects of eliminating other cell types also expressing the chosen Cre-driver. For example, it was recently shown that CGRP is also expressed in a subset of dorsal horn interneurons, and their selective activation or ablation has behavioral consequences (Löken *et al.*, DOI: 10.7554/eLife.59751).¹ The authors could cite these and other relevant literature to strengthen the point for developing this new tool. With the tool in hand, the authors explored the behavioral effects of ablating different subsets of primary sensory neurons and noted some differences. I am sure these approaches will be extended in future studies.

As a way of background, the authors briefly rant against polymodality, in the introduction and the discussion, but, I am not convinced that ablation studies can help much in establishing whether a particular neuron or a particular subpopulation is polymodal or not. The lack of a phenotype for a given submodality (e.g. mechanosensitivity) after eliminating, even a large subset of neurons, can be explained by redundancy or overlapping expression of sensors. Other approaches are needed to settle this question. A brief review on this topic, written with my colleague, Prof. Carlos Belmonte, some years ago can be found in DOI: 10.1186/1744-8069-4-14.² Depending on the lens you use, the view can be clear or blurry. Rather than simply showing that the expression of the reporter and the driver/target marker gene overlap (Figure 1) reasonably well (some additional statistics may convince the more skeptic reader), I wished the authors went directly after the important goodies: how effective is the approach in ablating the different subpopulations. To be fair, they tested it with the Nav1.8 Cre line (figure 2) and the results are quite impressive. I guess that demonstrating selectivity and lack of effect on target populations would also be an important aspect to establish in the future.

In my opinion, the authors should provide further details in the description of their behavioral methods. Some of their descriptions are too sparse and would not allow the proper replication of the experimental conditions. For example, when describing their noxious cold test, they simply refer to a published article (Brenner *et al.*, 2012, DOI: 10.1371/journal.pone.0039765).³ However, in that study, the authors used three different glass thicknesses, which has a notable impact on the cooling rate of the surface and the withdrawal latency. Are their results comparable? Difficult to judge without knowing the thickness of the glass.

Another aspect of the behavioral analysis used I find a bit trickier. They state in the methods that "control and test groups were assayed on the same day". Presumably, this is important to minimize uncontrolled sources of variability in pain assessment in non-verbal species (DOI: 10.30802/AALAS-CM-19-000042).⁴ However, in the analysis performed on figure 4 this is no longer considered and the group of control animals includes, apparently, all the controls (this group is much larger than the rest!), and the mean and SD obtained is the average for all the groups. Would the results/conclusion obtained be different if each of the groups was evaluated against the corresponding control? I don't know but it would be relevant information. An expert in statistics, which I am not, can probably provide some advice.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Sensory transduction, pain neurobiology, role of ion channels in neuronal excitability

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 08 February 2022

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Cruz Miguel Cendán Martínez 

Department of Pharmacology, Faculty of Medicine, Biomedical Research Center, Institute of Neuroscience, Parque Tecnológico de Ciencias de la Salud, University of Granada, Granada, Spain

Santana-Varela and colleagues have established new tools to study the specialised functional role of sets of sensory neurons. The authors have performed a series of elegant experiments to delete sensory neuron-specific Advillin which is expressed in all sensory neurons. The Advillin DRG promoter has been crossed with several new inducible Cre recombinase lines and behaviourally studied. The types of pain models used are wide and appropriate and explore some of the most common acute pain modalities such as mechanical and thermal pain. Also, the new Cre lines has been tested in the formalin test (inflammatory pain) and one of these lines (Ntrk2) was evaluated for neuropathic pain. However, as the authors mentioned in the manuscript, a more complete analyses of phenotyping of these mice including cold allodynia or chronic inflammatory pain would be necessary to characterize these lines. Nevertheless, these genetic tools will be useful and important for understanding the peripheral pain pathways.

Comments for the Authors:

- It is not clear if the same animal was tested once in only a pain model or in several of them. If the latter, it would be relevant to describe how much time passed between behavioural measuring.
- Since the legends in figure 3 are the same for all graphs and are very difficult to see because they are so small, perhaps putting a larger general legend above the figure would make it easier to read.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Behavioural pain research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 04 February 2022

<https://doi.org/10.21956/wellcomeopenres.18877.r47926>

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Uhtaek Oh 

Korea Institute of Science and Technology, Seoul, South Korea

Dorsal-root ganglion (DRG) neurons are very heterogeneous in size as well as in physiological functions including touch sensation, proprioception, pain, and itch. To dissect the roles of subsets of DRG neurons, the authors developed mouse lines that lacked specific subsets of DRG neurons. They developed Cre recombinase mouse lines in key marker genes that define subsets of DRG neurons. These Cre mice were crossed with transgenic mice that overexpressed DTA in DRG neurons. The authors confirmed the specific targeting of DTA to subtypes of DRG neurons. Then, the authors applied several pain tests to these mice lines, such as CGRP(Cre+/AdvDTA), Th(Cre+/AdvDTA), Tmem45b(Cre+/AdvDTA), Ntn1(Cre+/AdvDTA), and Ntrk2(Cre+/AdvDTA) to characterize the pain modality of the subsets of DRG neurons. In this study, a specific group of DRG neurons was also nicely ablated. This study contributes to dissecting the diverse roles of subsets of DRG neurons. The molecular and genetic techniques are good to test the hypothesis. Experiment results are significant. There are some points that I would like to comment on:

1. It would be nice if the authors discuss and compare these results with previous reports. For example, CGRP was known to play an important role in noxious heat sensitivity or inflammatory pain^{1,2}. TrkB-positive neurons were also ablated to investigate its role in mechanical pain³.

2. The labels and numbers in the figures are too small to read. It would be better if the labels are enlarged.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pain signalling mechanisms, Anoctamins, Tentonins

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 01 November 2021

<https://doi.org/10.21956/wellcomeopenres.18877.r46341>

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**Mohammed A. Nassar**

Department of Biomedical Science, University of Sheffield, Sheffield, UK

The authors developed several new inducible Cre lines for targeting specific subsets of sensory neurons *in vivo*. The choice of driver promoters is based on genes that were found to be markers of specific subsets of sensory neurons by single cell sequencing of DRG neurons. The generated Cre lines were all inducible allowing genetic manipulations in adult mice. Inducible Cre expressed was demonstrated by a dTomato reporter line. The pattern of Cre activity was then compared to the mRNA expression of the driver genes. To demonstrate the applicability of the Cre lines for pain research, the authors used them to ablate the targeted neuronal populations. They generated a new DTA mouse line that is restricted to DRG neurons to reduce the possibility of cell death in any other tissue. Double transgenic mice were subjected to pain testing. Undoubtedly, the generated Cre lines are important for expanding our understanding of the role of the various subtypes of sensory neurons in plain conditions. The authors used an inducible Cre which is very appropriate to avoid the well-known disadvantages of genetic manipulations through development. It was great to note that the behavioural testing was carried out by researchers blind to the mice's genotype.

Comments for the Authors:

1. The panels in figure 2 are too small, I had to enlarge the PDF to 400% to be able to clearly see the labelling. Would be it be possible to enlarge the panels?
2. In the first results paragraph, the authors state that there is a "large overlap" between the mRNA expression of the target and dTomato expression. It would be better if this was quantified as the percent of dtomot+/target+. The panels indicate that Cre may not be active in all the target neurons.
3. It is not clear why only the Ntrk2 line was tested for neuropathic pain development.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Using transgenic mice to study pain

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
