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Publication Date	2016-08-09
Publication Information	Madasu, Manish K., Okine, Bright N., Olango, Weredeselam M., Rea, Kieran, Lenihan, Róisín, Roche, Michelle, et al. (2016). Genotype-dependent responsivity to inflammatory pain: A role for TRPV1 in the periaqueductal grey. Pharmacological Research, 113, 44-54., doi: 10.1016/j.phrs.2016.08.011
Publisher	Elsevier
Link to publisher's version	https://doi.org/10.1016/j.phrs.2016.08.011
Item record	http://hdl.handle.net/10379/15087
DOI	http://dx.doi.org/10.1016/j.Phrs.2016.08.011

Genotype-dependent responsivity to inflammatory pain: a role for TRPV1 in the periaqueductal grey

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Abstract:

Negative affective state has a significant impact on pain, and genetic background is an important moderating influence on this interaction. The Wistar–Kyoto (WKY) inbred rat strain exhibits a stress-hyperresponsive, anxiety/depressive-like phenotype and also displays a hyperalgesic response to noxious stimuli. Transient receptor potential subfamily V member 1 (TRPV1) within the midbrain periaqueductal grey (PAG) plays a key role in regulating both aversive and nociceptive behaviour. In the present study, we investigated the role of TRPV1 in the sub-columns of the PAG in formalin-evoked nociceptive behaviour in WKY versus Sprague-Dawley (SD) rats. TRPV1 mRNA expression was significantly lower in the dorsolateral (DL) PAG and higher in the lateral (L) PAG of WKY rats, compared with SD counterparts. There were no significant differences in TRPV1 mRNA expression in the ventrolateral (VL) PAG between the two strains. TRPV1 mRNA expression significantly decreased in the DLPAG and increased in the VLPAG of SD, but not WKY rats upon intra-plantar formalin administration. Intra-DLPAG administration of either the TRPV1 agonist capsaicin, or the TRPV1 antagonist 5'-Iodoresiniferatoxin (5'-IRTX), significantly increased formalin-evoked nociceptive behaviour in SD rats, but not in WKY rats. The effects of capsaicin were likely due to TRPV1 desensitisation, given their similarity to the effects of 5'-IRTX. Intra-VLPAG administration of capsaicin or 5'-IRTX reduced nociceptive behaviour in a moderate and transient manner in SD rats, and similar effects were seen with 5'-IRTX in WKY rats. Intra-LPAG administration of 5'-IRTX reduced nociceptive behaviour in a moderate and transient manner in SD rats, but not in WKY rats. These results indicate that modulation of inflammatory pain by TRPV1 in the PAG occurs in a sub-column-specific manner. The data also provide evidence for differences in the expression of TRPV1, and differences in the effects of pharmacological modulation of TRPV1 in specific PAG sub-columns, between WKY and SD rats, suggesting that TRPV1 expression and/or functionality

in the PAG plays a role in hyper-responsivity to noxious stimuli in a genetic background prone to negative affect.

Keywords: TRPV1; Pain; Negative affective state; Periaqueductal grey; Capsaicin; Rat

Abbreviations:

TRPV1: Transient receptor potential subfamily V member 1

PAG: Periaqueductal grey

DLPAG: Dorsolateral Periaqueductal grey

VLPAG: Ventrolateral Periaqueductal grey

LPAG: Lateral Periaqueductal grey

SD: Sprague Dawley rats

WKY: Wistar Kyoto rats

RVM: Rostral ventromedial medulla

CAP: Capsaicin

5'-IRTX: 5'-Iodoresiniferatoxin

CAP+5'-IRTX: Capsaicin in combination with 5'-Iodoresiniferatoxin

DMSO: Dimethylsulfoxide

Chemical Compounds: Capsaicin (PubChem CID: 1548943); 5'-Iodoresiniferatoxin (PubChem CID: 16219535); Formalin (PubChem CID: 712); Dimethylsulfoxide (PubChem CID: 679)

1. Introduction:

The ability to experience pain is essential for an organism's survival and to prevent potential tissue damage. The International Association for the Study of Pain (IASP) has defined pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage'. There is high comorbidity between affective disorders and chronic pain states [1–3], and it is increasingly clear that there is involvement of common neural substrates and mechanisms in the modulation of both pain and negative affective states including anxiety and depression [4–6]

Genetic background is an important moderating influence on the interaction between negative affective state and pain. The Wistar–Kyoto (WKY) inbred rat strain exhibits a stress-hyperresponsive [7,8], anxiety/depressive-like phenotype [9,10] and also displays a hyperalgesic response to a variety of noxious stimuli [11–14]. Thus, the WKY rat represents a useful model of hyperalgesia associated with negative affective state and may facilitate understanding of the underlying neurobiological mechanisms, and identification of novel therapeutic targets for pain and its co-morbidity with affective disorders.

The transient receptor potential subfamily V member 1 (TRPV1) is a non-selective ligand-gated ion-channel which can be activated by protons, capsaicin (active constituent of chilli peppers), and thermal stimuli [15]. Upon activation, TRPV1 induces release of the neuropeptides calcitonin gene-related peptide and substance P by increasing intracellular calcium levels in sensory nerve terminals within the dorsal horn of the spinal cord [16]. While TRPV1 is highly expressed in the peripheral nervous system where it plays a key role in nociception [17], a number of lines of evidence indicate that TRPV1 is expressed and functional supraspinally [4,18–20].

The periaqueductal grey - rostral ventromedial medulla (PAG-RVM) pathway plays a pivotal role in pain processing and modulation. Antinociception caused by activation of the descending inhibitory pain pathway involves the PAG-mediated activation of neurons within the RVM [21]. Expression of TRPV1 in the PAG has been demonstrated using immunohistochemistry [22–24], *in situ* hybridization [25], radioligand binding [26] and gene reporter studies [25]. McGaraughty and colleagues have shown that activation of TRPV1 in the dorsal PAG produced an initial reduction in tail-flick latency, followed later by an increase in latency likely mediated by agonist-induced desensitisation of TRPV1. These effects were associated with increased activity of ON-cells and OFF-cells in the RVM, respectively [27]. Starowicz et al. have shown that direct administration of the TRPV1 agonist capsaicin into the ventrolateral (VL) PAG leads to antinociceptive effects in the rat tail-flick test, while the combination of the TRPV1 antagonist 5'-IRTX and capsaicin resulted in pronociceptive effects. Again, antinociceptive effects were associated with an increase in RVM OFF-cell activity, while pronociceptive effects correlated with an increase in ON-cell activity [28]. Blockade of TRPV1 has also been shown to antagonise palmitoylethanolamide-induced antinociception at the level of the VLPAG in the tail-flick test, with an associated decrease in OFF cell activity in the RVM [29]. A role for TRPV1 has also been demonstrated in modulation of anxiety- and depression-related behaviour [4,30–39]. Specifically within the PAG, intra-DLPAG microinjection of capsaicin increased anxiety-related behaviour and fear-related behaviour, effects blocked by administration of TRPV1 antagonists [36–39].

Thus, while there is evidence that TRPV1 in the PAG modulates both nociceptive behaviour and anxiety-related behaviour, there is a paucity of studies examining its role in hyperalgesia associated with negative affective state such as is exhibited by the WKY rat. The aim of the present studies was to test the hypothesis that TRPV1 in the different sub-columns of the PAG

differentially regulates formalin-evoked nociceptive behaviour in WKY rats versus Sprague-Dawley (SD) rats, the most commonly used comparator strain for the WKY rat.

2. Methods

2.1. Animals

For all experiments, male Sprague–Dawley (SD) or Wistar–Kyoto (WKY) rats (260-290g) (Harlan, Bicester, UK) were used. Animals were group housed for the duration of experiment 1, and initially for experiments 2, 3, 4, with single housing following surgery. Holding rooms were maintained at a constant temperature (21±2°C) under standard lighting conditions (12:12-hour light–dark, lights on from 0800 to 2000h). Experiments were carried out during the light phase between 0800 and 1700h. Food and water were available *ad libitum*. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway, under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609. All sections of the study adhered to the ARRIVE Guidelines for reporting in animal research [40].

2.2. Experimental design

Four separate experiments were performed. In all experiments, animals were randomly assigned to treatment groups and the sequence of treatments was randomised to control for the order of testing. In experiment 1 we compared TRPV1 gene expression in WKY and SD rats, and investigated the effects of intra-plantar injection of formalin thereon. 12 male SD rats and 12 male WKY rats received an intra-plantar injection of 50µL formalin (2.5% in 0.9% saline, s.c.) or 0.9% saline (control group) into the right hind paw immediately after a 10-minute

habituation exposure to the formalin test arena and were returned to the arena for recording the formalin-evoked nociceptive behaviour. This design resulted in 4 experimental groups, as follows: SD-Saline (SD-Sal); SD-Formalin (SD-Form); WKY-Saline (WKY-Sal); and WKY-Formalin (WKY-Form) (n=5-6 per group). The data on formalin-evoked nociceptive behaviour of these rats have been published previously [Figure 1 in ref 11]. Rats were killed by decapitation at the peak of the second phase [12] of the formalin test (30 minutes after formalin injection). Brains were removed rapidly, snap-frozen on dry ice, and stored at -80°C before microdissection of the sub-columns of PAG for TRPV1 gene expression using qRT-PCR.

In experiment 2, we investigated the effects of pharmacological modulation of TRPV1 in the DLPAG on formalin-evoked nociceptive behaviour in WKY rats and SD rats. Male SD and WKY rats (n=5-6) were implanted bilaterally under isoflurane anaesthesia with stainless steel guide cannulae targeting the DLPAG. On the test day, animals received bilateral intra-DLPAG injections of either vehicle (100% DMSO), the TRPV1 agonist capsaicin (CAP; 6nmoles/0.2µL), the TRPV1 antagonist 5'-IRTX (0.5nmoles/0.2µL) or co-administration of capsaicin and 5'-IRTX, and were placed in the formalin test arena for 10 minutes before intra-plantar formalin injection (2.5%, 50µl) under brief isoflurane anaesthesia. Rats were then returned to the formalin test arena and behaviour was recorded for a period of 60 minutes. Rats were killed by decapitation following behavioural testing. A 0.3µL quantity of 1% fast green dye was microinjected via the guide cannula, and brains were rapidly removed, snap-frozen on dry ice, and stored at -80°C until injection site verification.

In experiments 3 and 4, we investigated the effects of pharmacological modulation of TRPV1 in the VLPAG and LPAG, respectively, on formalin-evoked nociceptive behaviour in WKY rats and SD rats. The methods and experimental design were identical to experiment 2 above, except the animals had guide cannulae implanted in the VLPAG (experiment 3; n = 5-8) or LPAG (experiment 4; n = 8-11) sub-columns (Fig 1).

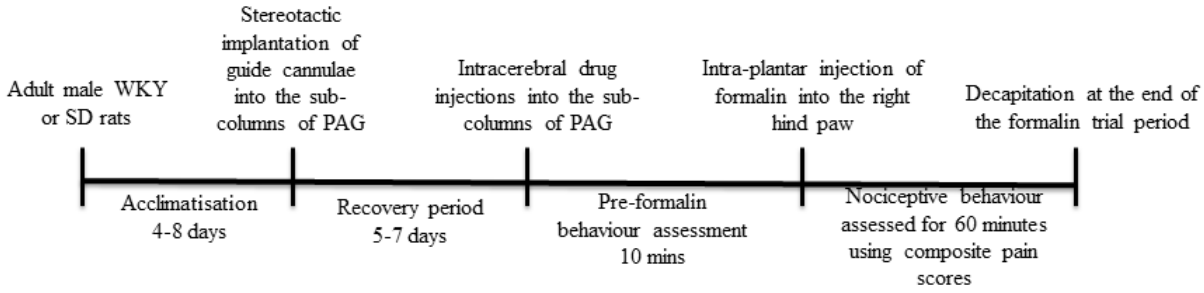


Fig 1: Design/timeline (left to right) of experiments employing drug microinjections into the sub-columns of the PAG.

2.3 Drug preparation

The TRPV1 agonist capsaicin (CAP) (PubChem CID: 1548943) was purchased from TOCRIS (Bristol, UK). TRPV1 antagonist 5-Iodo-Resiniferatoxin (5'-IRTX) (PubChem CID: 16219535) was bought from Abcam (Cambridge UK). For intra-PAG microinjections, CAP and 5'-IRTX were prepared to concentrations of 6nmol and 0.5nmol per 0.2 µL respectively in DMSO vehicle (dimethylsulfoxide, 100%). For co-administration of CAP and 5'-IRTX we prepared 2X concentrations of CAP and 5'-IRTX in DMSO and then combined them to give final concentrations equal to those of the drugs administered alone. Formalin (PubChem CID: 712) and DMSO (PubChem CID: 679) were purchased from Sigma Aldrich (Dublin, Ireland). The doses of capsaicin and 5'-IRTX were chosen based on previous studies demonstrating their efficacy following direct injection into the sub-columns of the PAG [27,28,41,42].

2.4. Formalin test

For experiment 1, rats were placed in a Perspex observation chamber (30×30×40cm; LxWxH, 30lux) for a pre-formalin habituation period of 10 mins. For experiments 2, 3 and 4 rats first received intracerebral injection of drug or vehicle into one of the PAG subcolumns and were then placed immediately into the observation chamber for 10 mins during which the effects of drug treatment on general exploratory behaviours were evaluated. After this 10 min pre-formalin trial, rats in both Experiments 1 and 2 received an intra-plantar injection of 50 µL formalin (2.5% in 0.9% saline) or 0.9% saline into the right hindpaw under brief isoflurane anaesthesia as described previously [11,12]. Rats were then returned to the same Perspex observation chamber for a period of 30 mins (experiment 1) or 60 mins (experiment 2,3,4). A video camera located beneath the observation chamber was used to record animal behaviour onto DVD for subsequent analysis. Behaviour was analysed with the aid of EthoVision XT8.5 software (Noldus, The Netherlands) by a rater blinded to treatments. Formalin-evoked nociceptive behaviour was categorized as time spent raising the formalin-injected paw above the floor without contact with any other surface (C1) and time spent holding, licking, biting, shaking, or flinching the injected paw (C2) to obtain a composite pain score [CPS=(C1+2(C2))/(total duration of analysis period)] [43].

2.5. Punch microdissection of sub-columns of PAG tissue

In experiment 1, frozen coronal brain sections (300µm in thickness) containing the PAG were cut on a cryostat (MICROM, Germany). A series of 300µm-thick sections (from AP -5.80 to -8.72mm relative to bregma) were punched using cylindrical brain punchers (Harvard Apparatus; internal diameter 0.75mm), with the aid of the rat brain atlas of Paxinos and Watson[44]. PAG sub-columns DLPAG (from AP -5.80 to -8.00mm relative to bregma), VLPAG (from AP -7.30 to -8.30mm relative to bregma) and LPAG (from AP -7.3 to -8.30mm

relative to bregma) were punched accordingly. These samples were weighed and stored at -80°C before extraction of total RNA for determination of TRPV1 gene expression in each individual sample using quantitative RT-PCR .

2.6. Quantitative RT-PCR analysis of the expression of TRPV1

qRT-PCR was carried out as described previously [11]. Briefly, total RNA was extracted from post-mortem tissue using a Machery–Nagel extraction kit (Nucleospin RNA II;Fischer Scientific, Ireland) according to the manufacturer's instructions. TRPV1 gene primers were generated using 3.0 Primer Express software and acquired from Eurofins MWG UK. The following sequences were used in generating the TRPV1-FAM labelled primers

FORWARD PRIMER: CAGCAGCAGTGAGACCCCTAA

REVERSE PRIMER: TGTCCTGTAGGAGTCGGTTCAA

PROBE: CGTCATGACATGCTTCTCGTGGAACC

VIC-labelled GAPDH (Rn_4308313; Applied Biosystems, UK) was used as the house-keeping gene and endogenous control. Expression of TRPV1 and endogenous control assessed using an Applied Biosystems 'StepOne plus' instrument (Bio-Sciences, Dun Laoghaire, Ireland). A no-template control reaction was included in all assays in order to validate the instrument and the samples in every assay. Samples were ran as duplicates and in multiplex assay. Reactions were performed for each sample and C_t values were normalized to the housekeeping GAPDH gene expression. The relative expression of the target gene to GAPDH was calculated by using the $2^{\Delta\Delta Ct}$ method. The $2^{\Delta\Delta Ct}$ values for each sample were then expressed as a percentage of the mean of the $2^{\Delta\Delta Ct}$ values for the control group (SD-SAL).

224

225 2.7. Stereotactic implantation of guide cannulae into the DLPAG, VLPAG and LPAG

226 For experiments 2, 3 and 4, rats were acclimatised to the animal unit following arrival for 4-8
227 days prior to surgery. Under isoflurane (2-3% in O2, 0.5L/min) anaesthesia, stainless steel
228 guide cannulae (9mm length, Plastics One Inc., Roanoke, Virginia, USA) were stereotactically
229 implanted bilaterally 1mm above the DLPAG (experiment 2), VLPAG (experiment 3) and
230 lPAG (experiment 4) of each rat. For experiment 2, DLPAG SD coordinates: AP = ((difference
231 from Bregma to lambda) X 0.91mm) from Bregma, ML = ±1.9mm at an angle of 10°, DV =
232 4.8mm from the meningeal dura matter; WKY coordinates: AP = ((difference from Bregma to
233 lambda) X 0.91mm) from Bregma, ML = ± 1.8mm at an angle of 10°, DV = 5.0mm from the
234 meningeal dura matter according to the Paxinos and Watson rat brain atlas [41]. For experiment
235 3, VLPAG SD coordinates: AP = ((difference from Bregma to lambda) X 0.91mm) from
236 Bregma, ML = ±1.9mm at an angle of 10°, DV = 5.3mm from the meningeal dura matter;
237 WKY coordinates: AP = ((difference from Bregma to lambda)/ X 0.91mm) from Bregma, ML
238 = ± 1.8mm at an angle of 10°, DV = 5.5mm from the meningeal dura matter according to the
239 rat brain atlas [41]. For experiment 4, LPAG SD coordinates: AP = ((difference from Bregma
240 to lambda) X 0.91mm) from Bregma, ML = ±1.9mm at an angle of 10°, DV =5.0mm from the
241 meningeal dura matter; WKY coordinates: AP = ((difference from Bregma to lambda) X
242 0.91mm) from Bregma, ML = ± 1.8mm at an angle of 10°, DV = 5.2mm from the meningeal
243 dura matter according to the rat brain atlas [41]. The 9mm cannulae were permanently fixed to
244 the skull using stainless steel screws and carboxylate cement (Durelon TM, Minnesota, USA).
245 A stylet made from stainless steel tubing (9mm, 31 G) (Plastics One Inc., Roanoke, Virginia,
246 USA) was inserted into the guide cannulae to prevent blockage by debris. The non-steroidal
247 anti-inflammatory agent, carprofen (2.5mg/kg, s.c., Rimadyl, Pfizer, Kent, UK), was
248 administered before the surgery to manage postoperative analgesia. To prevent postoperative

infection, rats received a single daily dose of the antimicrobial agent enrofloxacin (5mg/kg, s.c., Baytril, Bayer plc, Berkshire, UK) on the day of surgery and a subsequent 3 days. Following cannulae implantation, the rats were housed singly and allowed at least 5 days recovery prior to experimentation. During this recovery period, the rats were handled, cannulae checked, and their body weight and general health monitored once daily.

2.8. Histological verification of microinjection sites

For experiment 2, 3 & 4, the sites of intra-cerebral microinjection were determined before data analysis and only those rats that had cannulae correctly positioned in the relevant PAG sub-column were included in the final analysis. Brain sections with fast-green dye mark were collected on a cryostat (30µm thickness), mounted on gelatinised glass slides, and counterstained with cresyl violet to locate the precise position of microinjection sites under light microscopy.

2.9. Data analysis

The SPSS statistical package (IBM SPSS v22.0 for Windows; SPSS, Inc., Chicago, IL) was used to analyse all data. Shapiro–Wilk test confirmed that all data with the exception of defecation data were normally distributed. Further analysis of data collapsed over extended periods of the formalin trials or analysis of mRNA data was carried out using 2-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test where appropriate. Defecation (pellet number) data were non-parametric and analysed using Kruskal-Wallis test. Data were considered significant when $P<0.05$. Results are expressed as

group mean ± standard error of the mean (SEM) for parametric data and median (with interquartile range) for nonparametric data.

3. Results

3.1. Experiment 1: Formalin injection differentially regulates TRPV1 gene expression in subcolumns of the PAG in SD versus WKY rats.

TRPV1 mRNA levels were significantly lower in the DLPAG (Fig 2a SD-SAL vs. WKY-SAL, **P*<0.05), and higher in the LPAG (Fig 2c SD-SAL vs. WKY-SAL, **P*<0.05) of saline-treated WKY rats, compared with SD counterparts. There were no significant differences in TRPV1 mRNA expression in the VLPAG between the two strains (Fig 2b). Intra-plantar injection of formalin (Fig 2a SD-SAL vs. SD-FORM, *P<0.05) significantly reduced TRPV1 mRNA levels in the DLPAG of SD rats, but not WKY rats. In contrast, formalin injection significantly increased TRPV1 mRNA levels in the VLPAG of SD rats (Fig 2b SD-SAL vs. SD-FORM, **P<0.001), but not in WKY rats, and had no significant effect on levels of TRPV1 in the LPAG of either SD or WKY rats (Fig 2c).

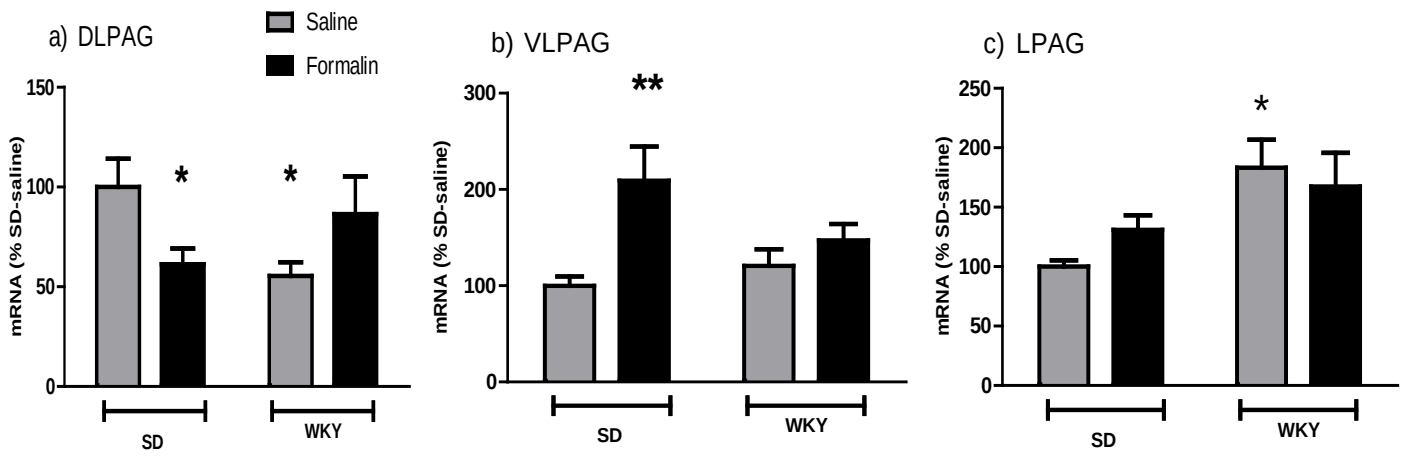


Fig 2: (a) TRPV1 mRNA levels in the DLPAG of SD and WKY rats that received intra-plantar injection of either saline or formalin. Two-way ANOVA (strain: $F_{1,18}$ = 0.646, P = 0.432; formalin: $F_{1,18}$ = 0.100, P = 0.756 and strain × formalin interaction: $F_{1,18}$ = 8.209, P < 0.05) followed by Fisher's LSD post-hoc test ($^{*}P$ < 0.05 vs SD-SAL). (b) TRPV1 mRNA levels in the VLPAG of SD and WKY rats that received intra-plantar injection of either saline or formalin. Two-way ANOVA (strain: $F_{1,19}$ = 0.840, P = 0.371; formalin: $F_{1,19}$ = 9.10, P < 0.01 and strain × formalin interaction: $F_{1,19}$ = 3.382, P = 0.082) followed by Fisher's LSD post-hoc test ($^{**}P$ < 0.01 vs SD-SAL).

(c) TRPV1 mRNA levels in LPAG of SD and WKY rats that received intra-plantar injection of either saline or formalin. Two-way ANOVA (strain: $F_{1,18}$ = 8.714, P < 0.01; formalin: $F_{1,18}$ = 0.137, P = 0.715 and strain × formalin interaction: $F_{1,18}$ = 1.346, P = 0.261) followed by Fisher's LSD post-hoc test ($^{*}P$ < 0.05 vs SD-SAL). FORM, formalin; SAL, 0.9% saline solution; SD, Sprague–Dawley; WKY, Wistar–Kyoto. Data are expressed as mean ± SEM (n = 5 - 6 rats per group).

3.2. Experiment 2: Intra-DLPAG administration of capsaicin or 5'IRTX increased formalin-evoked nociceptive behaviour in SD rats, but not in WKY rats.

63% and 73% of the injections were placed within the borders of both the right and left DLPAG of SD and WKY rats, respectively (Fig 3), with the remaining 37% and 27% of rats having one or both cannulae positioned in the LPAG, or outside of the PAG in the deep white layer of the superior colliculus. Only the results of experiments in which injections were correctly positioned in the DLPAG were included in the analysis.

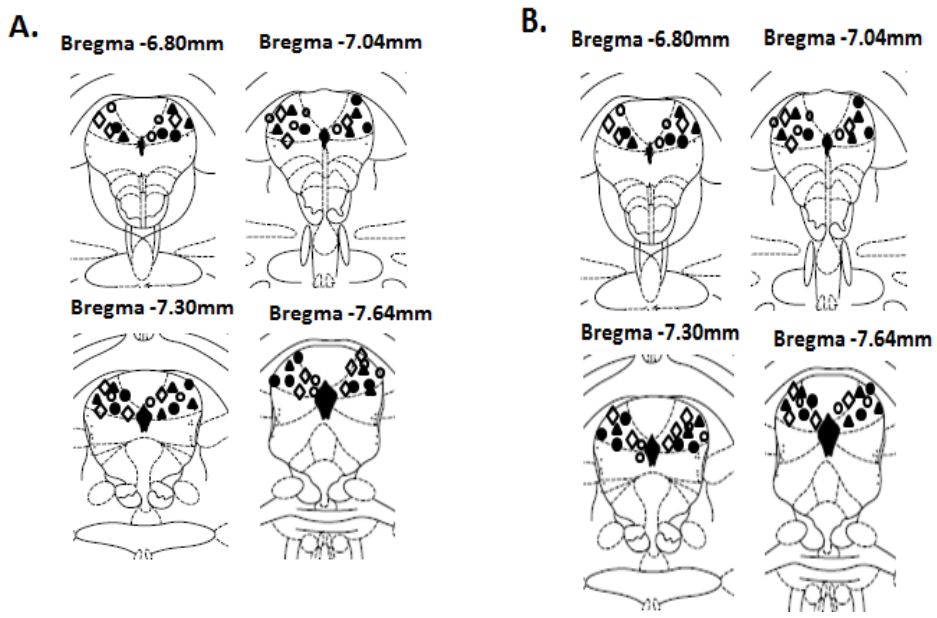
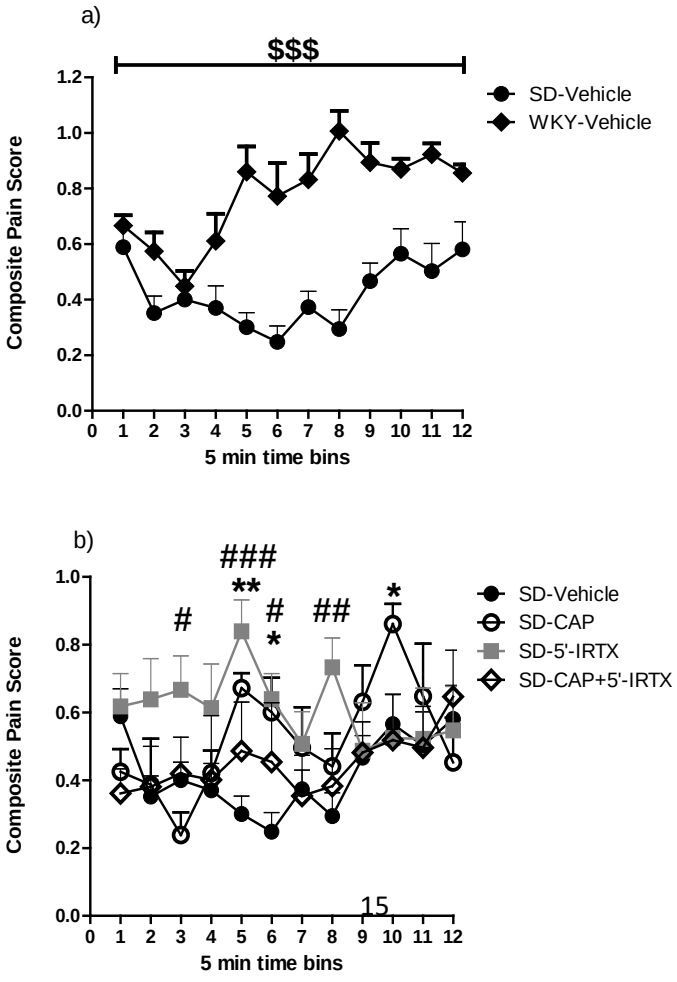


Fig 3: Schematic representation of vehicle (⭘) or capsaicin (▲) or 5'-IRTX (◇) or combination of capsaicin and 5'-IRTX (●) injections into DLPAG for (A) SD and (B) WKY rats.

WKY rats that received intra-DLPAG vehicle exhibited higher nociceptive behaviour, compared with SD counterparts (Fig 4a WKY-Vehicle vs SD-Vehicle, $^{\$\$\$}P$<0.001), confirming the hyperalgesic phenotype in the WKY strain. In SD rats, intra-DLPAG administration of

capsaicin (Fig 4b SD-CAP vs SD-Vehicle, $^{*}P<0.05$ $^{**}P<0.01$) or 5'-IRTX (Fig 4b SD-5'-IRTX vs SD-Vehicle, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$) significantly increased formalin-evoked nociceptive behaviour, in the second phase of the formalin trial, compared with vehicle-treated SD controls. Interestingly, these effects of capsaicin and 5'-IRTX were not observed in WKY rats (Fig 4c). Co-administration of capsaicin with 5'-IRTX had no effect on formalin-evoked nociceptive behaviour when compared with vehicle treatment in either SD or WKY rats (Fig 4b, Fig 4c). Intra-DLPAG administration of capsaicin or 5'-IRTX or the combination of both had no significant effect on distance moved, grooming or defecation when compared with vehicle-treated SD or WKY controls. Capsaicin and 5'-IRTX treated WKY rats exhibited lower rearing activity compared with SD counterparts (Table 1: $^{*}P<0.05$ WKY-CAP vs SD-CAP, $^{\#}P<0.05$ WKY-5'-IRTX vs SD-5'-IRTX), and similar trends were observed in WKY rats receiving vehicle or the combination of CAP and 5'-IRTX, compared with SD counterparts.



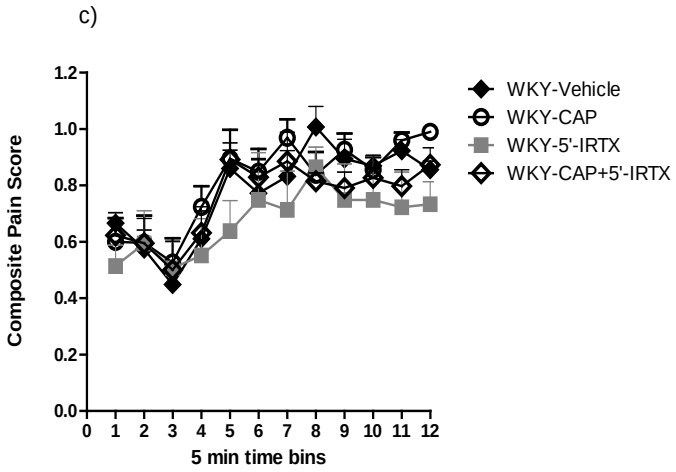


Fig 4: (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-DLPAG administration of vehicle. Intra-DLPAG administration of either the TRPV1 agonist capsaicin or the the TRPV1 antagonist 5'-Iodoresiniferatoxin (5'-IRTX) significantly increased formalin-evoked nociceptive behaviour in (b) SD rats, but not in (c) WKY rats. Repeated measures ANOVA (Time: $F_{11,781}$= 15.463, P < 0.001; time × strain: $F_{11,781}$= 2.492, P < 0.01; time × drug treatment : $F_{33,781}$= 1.818, P < 0.01; and time × strain × drug treatment interaction: $F_{33,781}$= 0.724, P = 0.874) followed by Fisher's LSD post-hoc test (Fig 2a $^{\$\$\$}P$ < 0.001, WKY-Vehicle vs SD-Vehicle; Fig 2b $^{*}P$ < 0.05, $^{**}P$ < 0.01, SD-CAP vs SD-Vehicle; $^{\#}P$ < 0.05, SD-5'-IRTX vs SD-Vehicle). Data are expressed as mean ± SEM (n = 5 - 6 rats per group).

Group	Distance moved(cm)	grooming(s)	rearing(s)	Defecation (Pellet number)
SD-Vehicle	1966.3±146.8	5.3±1.7	45.2±15.3	0 (0-1)
SD-CAP	1964.7±159.3	23.6±14.5	53.8±14.9	1 (0-1)
SD-5'-I-RTX	1847.2±111	10.2±2.8	55.5±13.1	0 (0-1)
SD-CAP+5'-I-RTX	1414.2±186.3	3.4±1.6	41±15.5	0
WKY-Vehicle	1525.3±270.4	13.5±3.5	18.3±4.1	0 (0-1)
WKY-CAP	1843.9±202.3	11.3±4.3	22.6±7.1*	0 (0-1)
WKY-5'-I-RTX	2165.1±376.7	13.4±9.3	23.9±8.5#	0
WKY- CAP+5'-I-RTX	1889.8±239.7	5.1±1.6	26.5±7.3	0 (0-1)

Table 1: Effects of intra-DLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,35}$=0.108, P =0.785); treatment ($F_{3,35}$=0.805, P = 0.5) and strain X drug interaction ($F_{1,35}$= 1.371, P = 0.268); Grooming: Two-way ANOVA effects of strain ($F_{1,35}$=0.002, P = 0.967); treatment ($F_{3,35}$= 1.353, P = 0.273) and strain X drug interaction ($F_{1,35}$=0.881, P = 0.460); Rearing: Two-way ANOVA effects of strain ($F_{1,35}$=11.792, P <0.002); treatment ($F_{3,35}$=0.243, P = 0.866) and strain X drug interaction ($F_{1,35}$= 0.272, P = 0.845) followed by Fisher's LSD post-hoc test ($^{*}P$ < 0.05 vs SD-CAP; $^{\#}P$<0.05 vs SD-5'-IRTX); Defecation: Kruskal Wallis variance of analysis by rank (X^2 = 3.174, P =0.868). Data are expressed as mean ± SEM for parametric data and median (interquartile range) for non-parametric data (n = 5 - 6 rats per group).

3.3. Experiment 3: Intra-VLPAG administration of capsaicin or 5'-IRTX reduced nociceptive behaviour in a moderate and transient manner in SD rats, and similar effects were seen with 5'-IRTX (only) in WKY rats.

71% and 64% of the injections were placed within the borders of both the right and left VLPAG in SD and WKY rats respectively (Fig 5), with the remaining 29% and 36% having one or both cannulae positioned in the DLPAG or LPAG, or outside the PAG in the deep white layer of the superior colliculus. Only the results of experiments in which injections were correctly positioned in the VLPAG were included in the analysis.

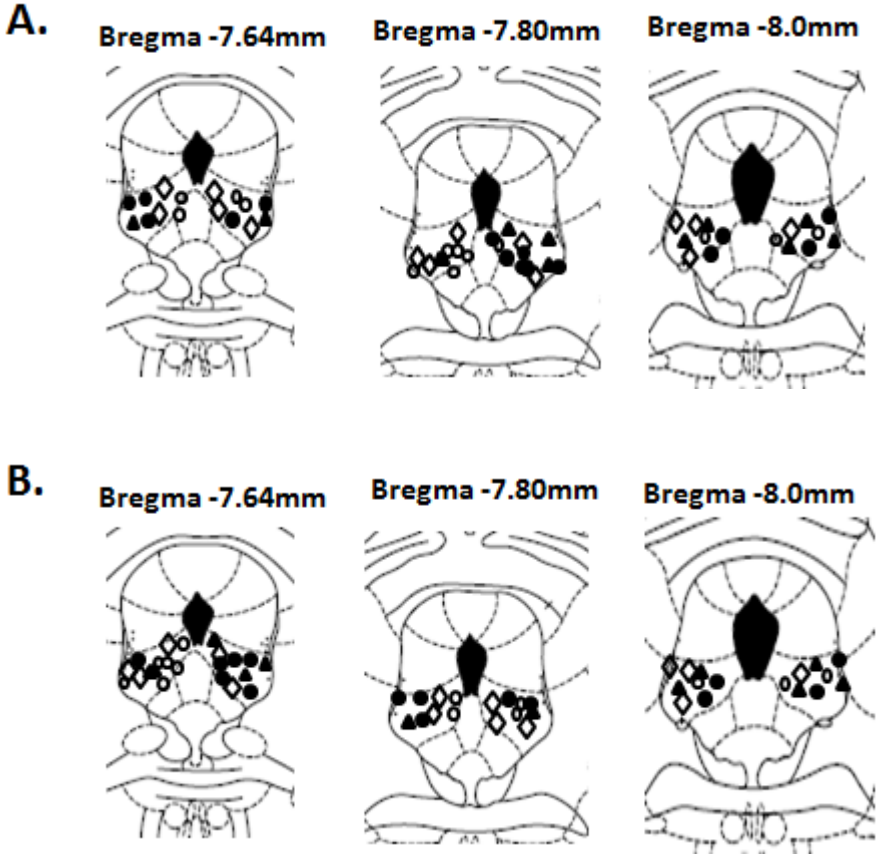


Fig 5: Schematic representation of vehicle (⭘) or capsaicin (▲) or 5'-IRTX (◇) or combination of capsaicin and 5'-IRTX (●) injections into the VLPAG of (A) SD and (B) WKY rats.

WKY rats that received intra-VLPAG vehicle exhibited higher nociceptive behaviour, compared with SD counterparts (Fig 6a WKY-Vehicle vs SD-Vehicle, $^{\$}P<0.05$, $^{\$\$}P<0.01$), confirming the hyperalgesic phenotype in the WKY strain. In SD rats, intra-VLPAG administration of capsaicin (Fig 6b SD-CAP vs SD-Vehicle, $^{*}P<0.05$) or 5'-IRTX (Fig 6b SD-5'-IRTX vs SD-Vehicle, $^{\#}P<0.05$) or the combination of both drugs (Fig 6b SD-CAP+5'-IRTX vs SD-Vehicle, $^{+}P<0.05$) significantly decreased formalin-evoked nociceptive behaviour, intermittently in the second phase of the formalin trial, compared with vehicle-treated SD rats, but not in WKY rats (Fig 6c, except for 5'-IRTX which had a similar effect at one time bin in WKY rats (WKY-5'-IRTX vs WKY-Vehicle, $^{\#}P<0.05$)). Intra-VLPAG administration of

capsaicin or 5'-IRTX or the combination of both (Table 2) had no significant effect on locomotor activity, grooming or defecation when compared to their respective controls.

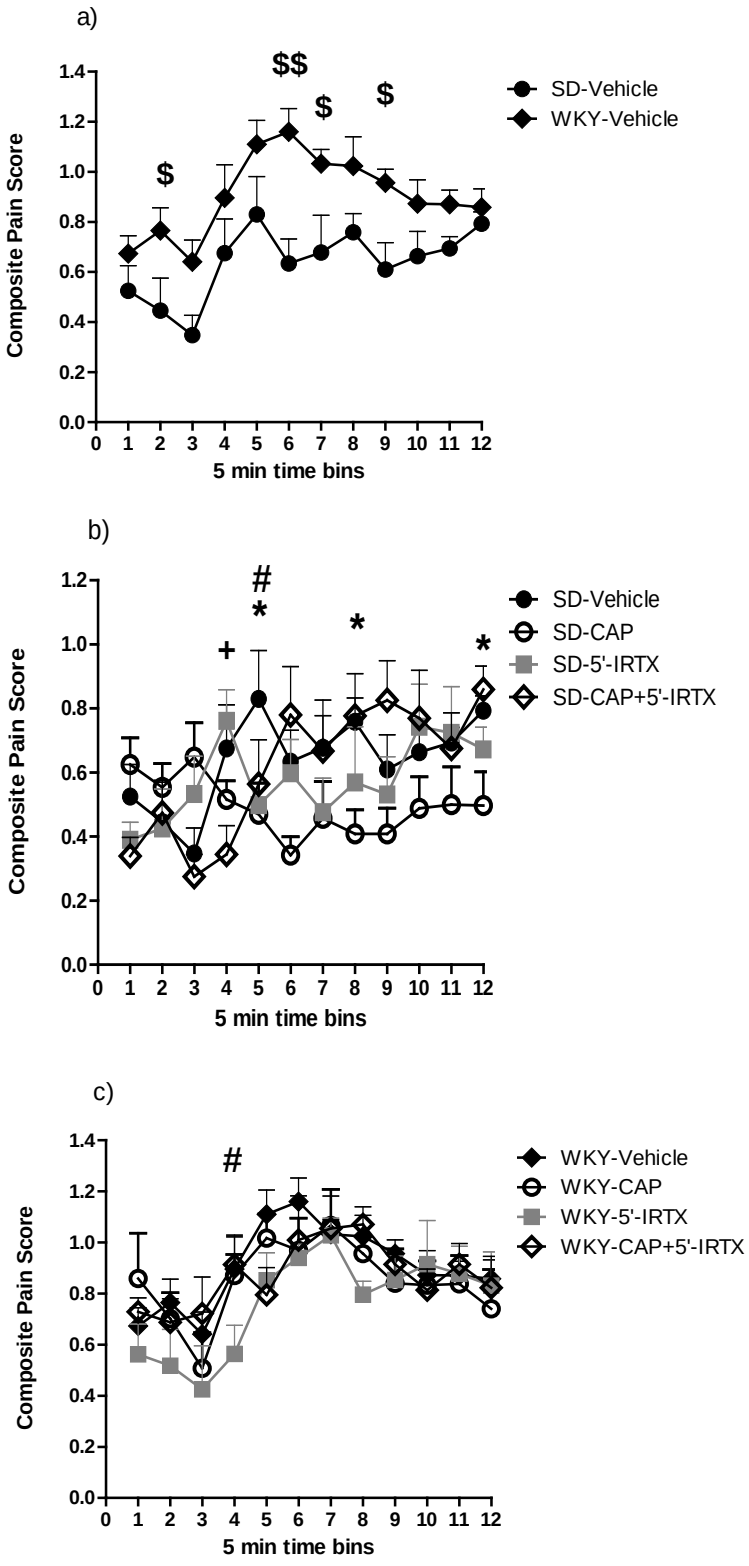


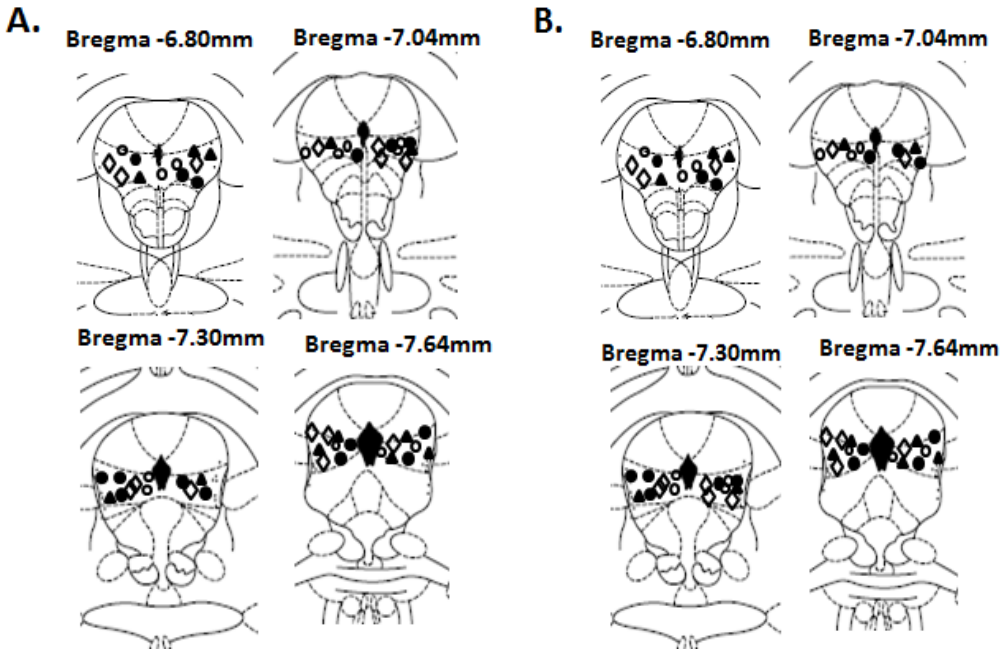
Fig 6: (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-VLPAG administration of vehicle. (b) Intra-VLPAG administration of the TRPV1 agonist capsaicin or the TRPV1 antagonist 5'-IRTX reduced formalin-evoked nociceptive behaviour in SD rats. (c) Intra-VLPAG administration of 5'-IRTX, but not capsaicin, transiently reduced formalin-evoked nociceptive behaviour in WKY rats. Repeated measures ANOVA, time: $F_{11,418}$= 9.038, $P < 0.001$; time × strain: $F_{11,418}$= 3.927, $P < 0.001$; time × drug treatment: $F_{33,418}$= 1.564, $P < 0.05$; and time × strain × drug treatment interaction: $F_{33,418}$= 1.212, $P = 0.199$) followed by Fisher's LSD post-hoc test (Fig 3a $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, SD-Vehicle vs WKY-Vehicle; Fig 3b $^{*}P < 0.05$, SD-CAP vs SD-Vehicle; $^{\#}P < 0.05$, SD-5'-IRTX vs SD-Vehicle; $^{+}P < 0.05$, SD-CAP+5'-IRTX vs SD-Vehicle; Fig 3c $^{\#}P < 0.05$, WKY-5'-IRTX vs WKY-Vehicle). Data are expressed as mean ± SEM (n = 5 - 8 rats per group).

Group	**Distance moved(cm)**	**Grooming(s)**	**Rearing(s)**	**Defecation (pellet number)**
SD-Vehicle	1860.0±57.2	13.4±3.4	65.0±4.6	0 (0-1)
SD-CAP	1785.4±143.2	21.9±5.6	58.0±10.4	0.5 (0-1)
SD-5'-IRTX	1767.6±78.7	15.2±4.5	69.6±4.2	0 (0-1)
SD-CAP+5'-IRTX	1782.7±84.9	23.2±5.7	63.8±12.3	0 (0-1)
WKY-Vehicle	1693.6±30.4	10.4±4.2	50.3±8.2	0
WKY-CAP	1736.3±106.1	28.0±8.3	75.2±6.9	0 (0-1)
WKY-5'-IRTX	1787.3±74.3	26.1±8.6	45.6±8.3	0 (0-1)
WKY-CAP+5'-IRTX	1902.6±149.1	16.6±4.1	65.6±6.7	0 (0-2)

Table 2: Effects of intra-VLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,38}$=0.076, P =0.784); treatment ($F_{3,38}$=0.27, P = 0.847) and strain X drug interaction ($F_{1,38}$= 0.741, P = 0.534); Grooming: Two-way ANOVA effects of strain ($F_{1,38}$=0.186, P = 0.669); treatment ($F_{3,38}$= 1.544, P = 0.219) and strain X drug interaction ($F_{1,38}$=0.898, P = 0.451); Rearing: Two-way ANOVA effects of strain ($F_{1,38}$=0.701, P =0.408); treatment ($F_{3,38}$=0.637, P = 0.596) and strain X drug interaction ($F_{1,38}$= 2.432, P = 0.08); Defecation: Kruskal Wallis variance of analysis by rank (X^2 = 1.329, P = 0.988). Data are expressed as mean ± SEM for parametric data and median (interquartile range) for non-parametric data (n = 5 - 8 rats per group).

444 3.4. Experiment 4: Intra-LPAG administration of 5'-IRTX moderately decreased formalin-
445 evoked nociceptive behaviour in SD rats, but not in WKY rats.

446 67% and 64% of the injections were placed within the borders of both the right and left LPAG
447 in SD and WKY rats, respectively (Fig 7), with the remaining 23% and 26% having one or
448 both cannulae positioned in the dorsolateral/ventrolateral, or outside the PAG in the deep white
449 layer of the superior colliculus. Only the results of experiments in which injections were
450 correctly positioned in the LPAG were included in the analysis.

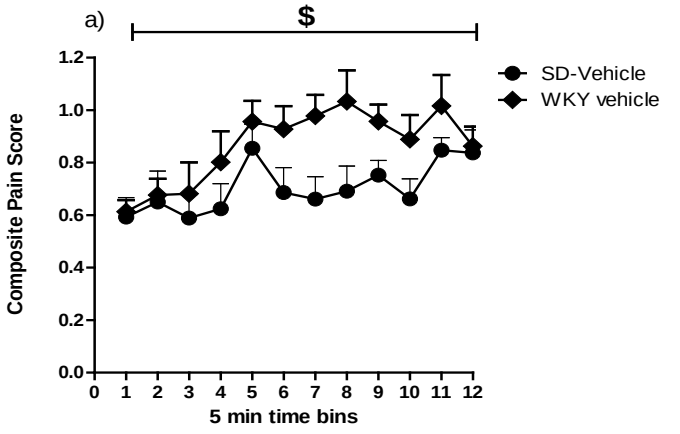


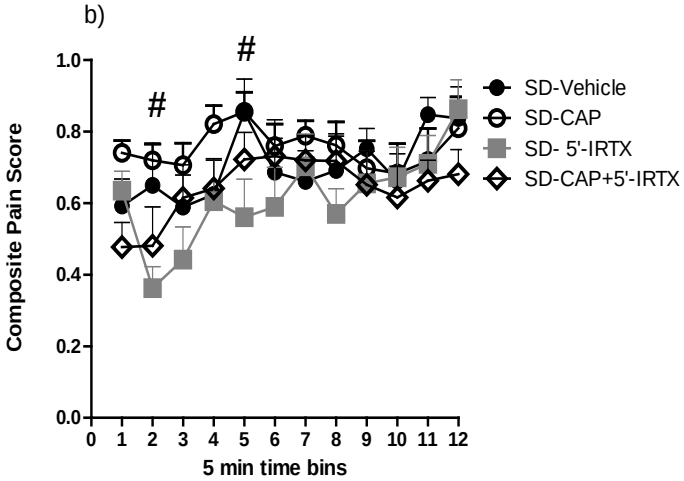
451

452 *Fig 7: Schematic representation of vehicle (○) or capsaicin (▲) or 5'-IRTX (◇) or the combination of capsaicin*
453 *and 5'-IRTX (●) injections into the LPAG of (A) SD and (B) WKY rats.*
454

455 WKY rats that received intra-LPAG vehicle exhibited higher nociceptive behaviour, compared
456 with SD counterparts (Fig 8a WKY-Vehicle vs SD-Vehicle, $^{\$}P<0.05$), confirming the
457 hyperalgesic phenotype in the WKY strain. In SD rats, intra-LPAG administration of 5'-IRTX
458 significantly decreased formalin-evoked nociceptive behaviour intermittently in the second
459 phase of the formalin trial compared with vehicle-treated SD rats (Fig 8b SD-5'-IRTX vs SD-
460 Vehicle, $^{\#}P<0.05$), an effect not observed in WKY rats (Fig 8c). Intra-LPAG administration

of either capsaicin alone or in combination with 5'-IRTX had no effect on formalin evoked nociceptive behaviour in SD (Fig 8b) or WKY rats (Fig 8c) when compared to respective vehicle-treated controls. Intra-LPAG administration of capsaicin or 5'-IRTX or combination of both drugs had no significant effect on locomotor activity, grooming or defecation in either strain (Table 3). Vehicle- and 5'-IRTX-treated WKY rats exhibited lower rearing activity compared with SD counterparts (Table 1: $^{\$}P<0.05$ WKY-Vehicle vs SD-Vehicle, $^{\#}P<0.05$ WKY-5'-IRTX vs SD-5'-IRTX).





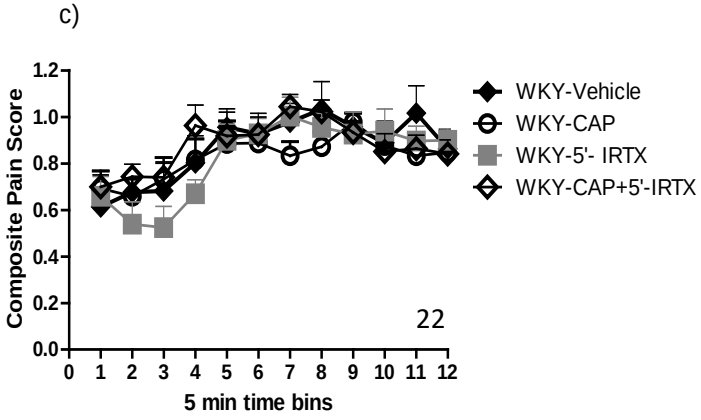


Fig 8: (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-LPAG administration of vehicle. Intra-LPAG administration of 5'-IRTX reduced formalin-evoked nociceptive behaviour in (b) SD rats, but not in (c) WKY rats. Repeated measures ANOVA (Time: $F_{11,781}$= 15.463, P < 0.001; time × strain: $F_{11,781}$= 2.492, P < 0.01; time × drug treatment : $F_{33,781}$= 1.818, P < 0.01; and time × strain × drug treatment interaction: $F_{33,781}$= 0.724, P = 0.874) followed by Fisher's LSD post-hoc test (Fig 4a $^{\$}P$ < 0.05 SD-Vehicle vs WKY-Vehicle) (Fig 3b $^{\#}P$ < 0.05, SD-5'-IRTX vs SD-Vehicle). Data are expressed as mean ± SEM (n = 8 – 11 rats per group).

Group	**Distance moved(cm)**	**Grooming(s)**	**Rearing(s)**	**Defecation (pellet number)**
SD-Vehicle	1749.9±86.4	20.1±7.4	61.04±10.1	0.5 (0-1)
SD-CAP	1687.4±75.1	31.6±4.9	55.7±6.0	0.5 (0-1)
SD-5'-IRTX	1487.6±71.3	21.1±3.5	72.0±10.6	0 (0-1)
SD-CAP+5'-IRTX	1602.1±87.7	17.7±4.4	58.7±7.0	0
WKY-Vehicle	1471.4±102.4	38.2±7.4	28.1±5.6$^{\$}$	1 (1-2)
WKY-CAP	1597.3±82.4	25.9±6.6	52.4±4.9	1 (0-1.5)
WKY-5'-IRTX	1554.3±96.0	27.3±6.0	44.5±7.8$^{\#}$	0 (0-1)
WKY-CAP+5'-IRTX	1551.5±144.0	19.8±4.9	52.6±11.3	1 (0-1)

Table 3: Effects of intra-LPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,79}$=1.710, P = 0.195); treatment ($F_{3,79}$=0.639, P = 0.592) and strain X drug interaction ($F_{1,79}$= 1.075, P =0.364); Grooming: Two-way ANOVA effects of strain ($F_{1,79}$=1.668, P=0.200); treatment ($F_{3,79}$= 1.439, P = 0.279) and strain X drug interaction ($F_{1,79}$=1.410, P = 0.246); Rearing: Two-way ANOVA effects of strain ($F_{1,79}$=8.341, P<0.01); treatment ($F_{3,79}$=0.899, P = 0.446) and strain X drug interaction ($F_{1,79}$= 1.494, P = 0.223) followed by Fisher's LSD post-hoc test ($^{\$}P$ < 0.05 vs SD Vehicle; $^{\#}P$<0.05 vs SD-5'-IRTX); Defecation: Kruskal Wallis variance of analysis by rank (X^2 = 8.562, P = 0.286). Data are expressed as mean ± SEM for parametric data and median (interquartile range) for non-parametric data (n = 8 – 11 rats per group).

4. Discussion

The data presented herein demonstrate that TRPV1 in the DLPAG and VLPAG regulates formalin-evoked nociceptive behaviour differentially in SD rats versus WKY counterparts and that alterations in TRPV1 expression or functionality might contribute to the hyperalgesic phenotype displayed by the stress-sensitive WKY strain. Formalin-injected WKY rats exhibited greater formalin-evoked nociceptive behaviour compared with formalin-injected SD rats over the 30 min trial and formalin-treated SD and WKY rats exhibited significant formalin-evoked nociceptive behaviour when compared to saline-treated counterparts which exhibited no formalin-evoked nociceptive behaviour [Figure 1 in ref 11]. In SD rats, this formalin-evoked nociceptive behaviour was associated with reduced TRPV1 expression in the DLPAG. WKY rats also had lower levels of TRPV1 expression in the DLPAG compared with SD rats and we hypothesised that this may explain their propensity to respond in a hyperalgesic manner to formalin injection. Moreover, we observed a formalin-induced increase in TRPV1 expression in the DLPAG of WKY rats and hypothesised that this may represent a compensatory change in an attempt to reduce pain behaviour in the WKY strain. To further test these hypotheses, we investigated the effects of pharmacological manipulation of TRPV1 in the DLPAG on formalin-evoked nociceptive behaviour in both strains using the TRPV1 agonist capsaicin and the TRPV1 antagonist 5'-IRTX. In SD rats, intra-DLPAG administration of either capsaicin or 5'-IRTX had a pronociceptive effect. The effect of capsaicin was likely due to desensitisation of TRPV1 in the DLPAG, given that its effects were similar to the effects of TRPV1 blockade with 5'-IRTX, and evidence from *in vivo* [27] and *in vitro* [45–47] studies that a single dose/concentration of capsaicin can desensitise TRPV1. Thus, these data are compatible with the idea that lower TRPV1 signalling in the DLPAG is associated with increased formalin-evoked nociceptive behaviour. Interestingly, the co-administration of capsaicin and 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in SD rats,

likely due to both drugs competing dynamically for binding to TRPV1, with neither drug binding for long enough to desensitise (capsaicin) or block (5'-IRTX) the channel. In contrast to their effects in SD rats, intra-DLPAG administration of capsaicin or 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in WKY rats. One possible explanation for these findings is that the formalin-evoked increase in TRPV1 expression in WKY rats serves to counteract/oppose the desensitisation or blockade caused by capsaicin or 5'-IRTX, respectively. Taken together, our data suggest that lower expression and/or functionality of TRPV1 in the DLPAG is associated with increased inflammatory pain behaviour and may underpin the hyperalgesic phenotype of WKY rats.

Formalin-evoked nociceptive behaviour in SD rats was also associated with higher TRPV1 expression in the VLPAG. Accordingly, capsaicin-induced desensitisation of TRPV1 in the VLPAG had an antinociceptive effect in SD rats. In contrast, WKY rats were non-responsive to intra-VLPAG capsaicin, possibly because formalin-treated WKY rats had lower TRPV1 expression in the VLPAG compared with SD counterparts. Such an effect on TRPV1 expression in WKY rats may constitute a compensatory change to counter the hyperalgesic response to formalin exhibited by this strain. Both strains exhibited modest and transient antinociceptive effects following intra-VLPAG administration of 5'-IRTX alone, or in combination with capsaicin. In the LPAG, although saline-injected WKY rats had higher levels of TRPV1 expression compared with SD counterparts, formalin injection had no effect on TRPV1 expression and there were no effects of intra-LPAG administration of capsaicin on formalin-evoked nociceptive behaviour, in either strain. There were some modest and transient antinociceptive effects of intra-LPAG administration of 5'-IRTX alone in SD rats, but overall the data suggest that modulation of TRPV1 activity in the LPAG has little effect on formalin-evoked nociceptive behaviour in SD rats (unlike TRPV1 in the DLPAG and VLPAG) and does not contribute to the hyperalgesic state in WKY rats. The drug treatment had no significant

effect on locomotor activity/non-pain related behaviours in either strain when injected into any of the 3 sub-columns which suggests that the effects of the drug treatments on formalin-evoked nociceptive behaviour were specific effects on nociceptive processing and were not confounded by non-specific, overt effects on locomotor activity. WKY rats exhibited less rearing behaviour when compared to SD rats as has been reported previously [10,12].

Our study is the first to demonstrate differential roles for TRPV1 in sub-columns of the PAG in regulation of formalin-evoked nociceptive behaviour in SD versus WKY rats. Previous studies have reported differential functional roles of the subcolumns in the PAG. It has been shown that DLPAG and LPAG are involved in non-opioid mediated analgesia [48–51] and the VLPAG in opioid mediated analgesia [52–57]. Previous studies focusing on thermal nociception have confirmed that TRPV1 within the PAG is involved in modulating pain behaviour. For example, capsaicin administration into the dorsal PAG of SD rats produces pronociceptive effects in the rat tail flick test, an effect associated with increased ON cell activity in the RVM [27]. Our data demonstrating that capsaicin injection into the DLPAG of SD rats was pronociceptive in the formalin test supports these results and extends them in the context of inflammatory pain. Conversely, capsaicin microinjection into the DLPAG has been shown to have an antinociceptive effect in the plantar test of thermoceptive sensitivity, an effect mediated by glutamate-induced activation of $mGluR_1$ and NMDA receptors in the DLPAG [58]. Administration of AA-5HT, a FAAH inhibitor and TRPV1 antagonist, into the VLPAG, was antinociceptive in the rat formalin test, an effect associated with reduced ON cell and OFF cell activity in the RVM. The PAG - locus coeruleus (LC) - spinal cord pathway was implicated in these effects [59]. Liao et al reported that capsacin when injected into the VLPAG, increased paw withdrawal latency in the hot plate test in Wistar rats, similar to what we have reported in experiment 3 for SD rats (capsaicin microinjection into VLPAG decreased formalin-evoked nociceptive behaviour) [60]. They suggested that capsaicin activates TRPV1 on glutamatergic

neurons in the VLPAG, resulting in mGluR5-mediated production of 2-AG which in turn results in retrograde disinhibition of GABAergic neurons with consequent activation of the descending inhibitory pain pathway and antinociception [60]. Intra-VLPAG microinjection of 5'-IRTX alone reduced the latency of the nociceptive reaction (pronociceptive effect) in the plantar test and this effect of 5'-IRTX was abolished when co-administered with capsacin [28]. The precise localisation of TRPV1 on either GABAergic or glutamatergic neurons in the PAG sub-columns is unknown, but differential effects of modulation of TRPV1 on these neuronal subtypes is likely to dictate the effects on nociceptive behaviour.

5. Conclusions

Here we have shown that modulation of inflammatory pain by TRPV1 in the PAG occurs in a sub-column-specific manner. The data also provide evidence for differences in the expression of TRPV1, and differences in the effects of pharmacological modulation of TRPV1 in specific PAG sub-columns, between WKY and SD rats, suggesting that TRPV1 expression and/or functionality in the PAG plays a role in hyper-responsivity to inflammatory pain in a genetic background prone to negative affect. These findings may have implications for the understanding and treatment of persistent pain that is co-morbid with, or exacerbated by, affective disorders.

Acknowledgements

This work was funded by grants from Science Foundation Ireland (10/IN.1/B2976), the Irish Research Council, and a PhD scholarship from the College of Science, National University of Ireland Galway.

Conflict of Interest:

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions:

Olango WM, designed experiment 1, carried out the *in-vivo* work and cDNA synthesis. Madasu MK carried out qRT-PCR for experiment 1 and *in-vivo* work for experiments 2, 3 and 4 with assistance from Okine BN, Rea K, Finn DP and Roche M. Behavioural data were generated by Lenihan R (experiement 2) and Madasu MK (experiments 2, 3 and 4). All authors contributed to study design, interpretation of the data and writing of the manuscript.

References

[1] Asmundson, G.J.G., Katz, J., Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art., Depress. Anxiety. 26 (2009) 888–901. doi:10.1002/da.20600.

[2] al Absi, M., Rokke, P.D., Can anxiety help us tolerate pain?, Pain. 46 (1991) 43–51.

[3] Atkinson, J.H., Slater, M.A., Patterson, T.L., Grant, I., Garfin, S.R., Prevalence, onset, and risk of psychiatric disorders in men with chronic low back pain: a controlled study., Pain. 45 (1991) 111–21.

[4] Madasu, M.K., Roche, M., Finn, D.P., Supraspinal Transient Receptor Potential Subfamily V Member 1 (TRPV1) in Pain and Psychiatric Disorders., Mod. Trends Pharmacopsychiatry. 30 (2015) 80–93. doi:10.1159/000435934.

[5] Burke, N.N., Finn, D.P., Roche, M. Neuroinflammatory Mechanisms Linking Pain and Depression., Mod. Trends Pharmacopsychiatry. 30 (2015) 36–50. doi:10.1159/000435931.

[6] Jennings, E.M., Okine, B.N., Roche, M., Finn, D.P., Stress-induced hyperalgesia., Prog. Neurobiol. 121 (2014) 1–18. doi:10.1016/j.pneurobio.2014.06.003.

[7] De La Garza, R., Mahoney, J.J., A distinct neurochemical profile in WKY rats at baseline and in response to acute stress: implications for animal models of anxiety and depression., Brain Res. 1021 (2004) 209–18. doi:10.1016/j.brainres.2004.06.052.

[8] Lahmame, A., del Arco, C., Pazos, A., Yritia, M., Armario, A., Brain corticotropin-releasing factor immunoreactivity and receptors in five inbred rat strains: relationship to forced swimming behaviour., Brain Res. 750 (1997) 285–92.

[9] Gentsch, C., Lichtsteiner, M., Feer, H., Open field and elevated plus-maze: A behavioural comparison between spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats and the effects of chlordiazepoxide, Behav. Brain Res. 25 (1987) 101–107. doi:10.1016/0166-4328(87)90003-9.

[10] Paré, W.P. Open field, learned helplessness, conditioned defensive burying, and forced-swim tests in WKY rats., Physiol. Behav. 55 (1994) 433–9.

[11] Rea, K., Olango, W.M., Okine, B.N., Madasu, M.K., McGuire, I.C., Coyle, K., Harhen, B., Roche, M., Finn, D.P., Impaired endocannabinoid signalling in the rostral ventromedial medulla underpins genotype-dependent hyper-responsivity to noxious stimuli, Pain. 155 (2014) 69–79. doi:10.1016/j.pain.2013.09.012.

[12] Burke, N., Hayes, E., Calpin, P., Kerr, D.M., Moriarty, O., Finn, D.P., Roche, M., Roche Enhanced nociceptive responding in two rat models of depression is associated with alterations in monoamine levels in discrete brain regions., Neuroscience. 171 (2010) 1300–13. doi:10.1016/j.neuroscience.2010.10.030.

[13] Moloney, R.D., Golubeva, A. V, O'Connor, R.M., Kalinichev, M., Dinan, T.G., Cryan, J.F., Negative allosteric modulation of the mGlu7 receptor reduces visceral hypersensitivity in a stress-sensitive rat strain., Neurobiol. Stress. 2 (2015) 28–33. doi:10.1016/j.ynstr.2015.04.001.

[14] O' Mahony, S.M., Clarke, G., McKernan, D.P., Bravo, J.A., Dinan, T.G., Cryan, J.F., Differential visceral nociceptive, behavioural and neurochemical responses to an immune challenge in the stress-sensitive Wistar Kyoto rat strain., Behav. Brain Res. 253 (2013) 310–7. doi:10.1016/j.bbr.2013.07.023.

[15] Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway., Nature.

389 (1997) 816–24. doi:10.1038/39807.

[16] Price, T.J., Louria, M.D., Candelario-Soto, D., Dussor, G.O., Jeske, N.A., Patwardhan, A.M., Diogenes, A., Trott, A.A., Hargreaves, K.M., Flores, C.M., Treatment of trigeminal ganglion neurons in vitro with NGF, GDNF or BDNF: effects on neuronal survival, neurochemical properties and TRPV1-mediated neuropeptide secretion., BMC Neurosci. 6 (2005) 4. doi:10.1186/1471-2202-6-4.

[17] Tominaga, M.D., Julius, D., Capsaicin receptor in the pain pathway., Jpn. J. Pharmacol. 83 (2000) 20–4.

[18] Ho, K.W., Ward, N.J., Calkins, D.J., TRPV1: a stress response protein in the central nervous system., Am. J. Neurodegener. Dis. 1 (2012) 1–14.

[19] Palazzo, E., Luongo, L., de Novellis, V., Berrino, L., Rossi, F., Maione, S., Moving towards supraspinal TRPV1 receptors for chronic pain relief., Mol. Pain. 6 (2010) 66. doi:10.1186/1744-8069-6-66.

[20] Martins, D., Tavares, I., Morgado, C., "hotheaded": The role of TRPV1 in brain functions, Neuropharmacology. 85 (2014) 151–7. doi:10.1016/j.neuropharm.2014.05.034.

[21] Bajic, D., Proudfit, H.K., Projections of neurons in the periaqueductal gray to pontine and medullary catecholamine cell groups involved in the modulation of nociception., J. Comp. Neurol. 405 (1999) 359–79.

[22] Mezey, E., Tóth, Z.E., Cortright, D.N., Arzubi, M.K., Krause, J.E., Elde, R., Guo, A., Blumberg, P.M., Szallasi, A., Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human., Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3655–60. doi:10.1073/pnas.060496197.

[23] Tóth, A., Boczán, J., Kedei, N., Lizanecz, E., Bagi, Z., Papp, Z., Edes, I., Csiba, L., Blumberg, P.M., Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain., Brain Res. Mol. Brain Res. 135 (2005) 162–8. doi:10.1016/j.molbrainres.2004.12.003.

[24] Cristino, L., de Petrocellis, L., Pryce, G., Baker, D., Guglielmotti, V., Di Marzo, V.,

680 Immunohistochemical localization of cannabinoid type 1 and vanilloid transient
681 receptor potential vanilloid type 1 receptors in the mouse brain., Neuroscience. 139
682 (2006) 1405–15. doi:10.1016/j.neuroscience.2006.02.074.

683 [25] Cavanaugh, D.J., Chesler, A.T., Jackson, A.C., Sigal, Y.M., Yamanaka, H., Grant, R.,
684 O'Donnell, D., Nicoll, R.A., Shah, N.M., Julius, D., Basbaum, A.I., Trpv1 reporter
685 mice reveal highly restricted brain distribution and functional expression in arteriolar
686 smooth muscle cells., J. Neurosci. 31 (2011) 5067–77.
687 doi:10.1523/JNEUROSCI.6451-10.2011.

688 [26] Roberts, J.C., Davis, J.B., Benham, C.D., [3H]Resiniferatoxin autoradiography in the
689 CNS of wild-type and TRPV1 null mice defines TRPV1 (VR-1) protein distribution.,
690 Brain Res. 995 (2004) 176–83.

691 [27] McGaraughty, S., Chu, K.L., Bitner, R.S., Martino, B., El Kouhen, R., Han, P., Nikkel,
692 A.L., Burgard, E.C., Faltynek, C.R., Jarvis, M.F., Capsaicin infused into the PAG
693 affects rat tail flick responses to noxious heat and alters neuronal firing in the RVM., J.
694 Neurophysiol. 90 (2003) 2702–10. doi:10.1152/jn.00433.2003.

695 [28] Starowicz, K., Maione, S., Cristino, L., Palazzo, E., Marabese, I., Rossi, F.,
696 deNovellis, V., Di Marzo, V., Tonic endovanilloid facilitation of glutamate release in
697 brainstem descending antinociceptive pathways., J. Neurosci. 27 (2007) 13739–49.
698 doi:10.1523/JNEUROSCI.3258-07.2007.

699 [29] de Novellis, V., Luongo, L., Guida, F., Cristino, L., Palazzo, E., Russo, R., Marabese,
700 I., D'Agostino, G., Calignano, A., Rossi, F., Di Marzo, V., Maione, S., Effects of intra-
701 ventrolateral periaqueductal grey palmitoylethanolamide on thermoceptive threshold
702 and rostral ventromedial medulla cell activity., Eur. J. Pharmacol. 676 (2012) 41–50.
703 doi:10.1016/j.ejphar.2011.11.034.

704 [30] Fogaça, M. V, Lisboa, S.F., Aguiar, D.C., Moreira, F.A., Gomes, F. V, Casarotto, P.C.,
705 Guimarães, F.S., Fine-tuning of defensive behaviors in the dorsal periaqueductal gray
706 by atypical neurotransmitters., Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui.
707 Médicas E Biológicas / Soc. Bras. Biofísica ... [et Al.]. 45 (2012) 357–65.

708 [31] Kasckow, J.W., Mulchahey, J.J., Geracioti, T.D., Effects of the vanilloid agonist
709 olvanil and antagonist capsazepine on rat behaviors., Prog. Neuropsychopharmacol.

Biol. Psychiatry. 28 (2004) 291–5. doi:10.1016/j.pnpbp.2003.10.007.

[32] You, I.-J., Jung, Y.-H., Kim, M.-J., Kwon, S.-H., Hong, S.-I., Lee, S.-Y., Jang, C.-G., Alterations in the emotional and memory behavioral phenotypes of transient receptor potential vanilloid type 1-deficient mice are mediated by changes in expression of 5-HT_1A, GABA(A), and NMDA receptors., Neuropharmacology. 62 (2012) 1034–43. doi:10.1016/j.neuropharm.2011.10.013.

[33] Kulisch, C., Albrecht, D., Effects of single swim stress on changes in TRPV1-mediated plasticity in the amygdala, Behav. Brain Res. 236 (2013) 344–349. doi:10.1016/j.bbr.2012.09.003.

[34] Manna, S.S.S., Umathe, S.N., A possible participation of transient receptor potential vanilloid type 1 channels in the antidepressant effect of fluoxetine., Eur. J. Pharmacol. 685 (2012) 81–90. doi:10.1016/j.ejphar.2012.04.023.

[35] Hayase, T., Differential effects of TRPV1 receptor ligands against nicotine-induced depression-like behaviors., BMC Pharmacol. 11 (2011) 6. doi:10.1186/1471-2210-11-6.

[36] Terzian, A.L.B., Aguiar, D.C., Guimarães, F.S., Moreira, F.A., Modulation of anxiety-like behaviour by Transient Receptor Potential Vanilloid Type 1 (TRPV1) channels located in the dorsolateral periaqueductal gray., Eur. Neuropsychopharmacol. 19 (2009) 188–95. doi:10.1016/j.euroneuro.2008.11.004.

[37] Mascarenhas, D.C., Gomes, K.S., Nunes-de-Souza, R.L., Anxiogenic-like effect induced by TRPV1 receptor activation within the dorsal periaqueductal gray matter in mice., Behav. Brain Res. 250 (2013) 308–15. doi:10.1016/j.bbr.2013.05.023.

[38] Almeida-Santos, A.F., Moreira, F.A., Guimarães, F.S., Aguiar, D.C., Role of TRPV1 receptors on panic-like behaviors mediated by the dorsolateral periaqueductal gray in rats, Pharmacol. Biochem. Behav. 105 (2013) 166–172. doi:10.1016/j.pbb.2013.02.012.

[39] Casarotto, P.C., Terzian, A.L.B., Aguiar, D.C., Zangrossi, H., Guimarães, F.S., Wotjak, C.T., Moreira, F.A., Opposing roles for cannabinoid receptor type-1 (CB_1) and transient receptor potential vanilloid type-1 channel (TRPV1) on the modulation of panic-like responses in rats., Neuropsychopharmacology. 37 (2012) 478–86.

740 doi:10.1038/npp.2011.207.

741 [40] Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., Improving
742 Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal
743 Research, PLoS Biol. 8 (2010) e1000412. doi:10.1371/journal.pbio.1000412.

744 [41] Maione, S., Bisogno, T., Novellis, V. De, Palazzo, E., Cristino, L., Valenti, M.,
745 Petrosino, S., Guglielmotti, V., Rossi, F., Marzo, V. Di., Elevation of Endocannabinoid
746 Levels in the Ventrolateral Periaqueductal Grey through Inhibition of Fatty Acid
747 Amide Hydrolase Affects Descending Nociceptive Pathways via Both Cannabinoid
748 Receptor Type 1 and Transient Receptor Potential Vanilloid Type-1 Re,
749 Pharmacology. 316 (2006) 969–982. doi:10.1124/jpet.105.093286.2.

750 [42] Maione, S., De Petrocellis, L., de Novellis, V., Moriello, A.S., Petrosino, S., Palazzo,
751 E., Rossi, F.S., Woodward, D.F., Di Marzo, V., Analgesic actions of N-arachidonoyl-
752 serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid
753 TRPV1 receptors, Br. J. Pharmacol. 150 (2007) 766–781. doi:0707145
754 [pii]\r10.1038/sj.bjp.0707145.

755 [43] Watson, G.S., Sufka, K.J., Coderre, T.J., Optimal scoring strategies and weights for the
756 formalin test in rats., Pain. 70 (1997) 53–8.

757 [44] Paxinos, G. and Watson, C.,, The rat brain in stereotaxic coordinates, Academic Press,
758 London,UK, 1998.

759 [45] Bhave, G., Zhu, W., Wang, H., Brasier, D.., Oxford, G.S., Gereau, R.W., cAMP-
760 Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1)
761 by Direct Phosphorylation, Neuron. 35 (2002) 721–731. doi:10.1016/S0896-
762 6273(02)00802-4.

763 [46] Docherty, R.J., Yeats, J.C., Bevan, S., Boddeke, H.W.G.M., Inhibition of calcineurin
764 inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root
765 ganglion neurones from adult rats, Pflügers Arch. Eur. J. Physiol. 431 (1996) 828–837.
766 doi:10.1007/BF02332166.

767 [47] Koplas, P.A., Rosenberg, R.L., Oxford, G.S., The Role of Calcium in the
768 Desensitization of Capsaicin Responses in Rat Dorsal Root Ganglion Neurons, J.
769 Neurosci. 17 (1997) 3525–3537.

[48] Bandler, R., Shipley, M.T., Columnar organization in the midbrain periaqueductal gray: modules for emotional expression?, Trends Neurosci. 17 (1994) 379–389. doi:10.1016/0166-2236(94)90047-7.

[49] Bandler, R., Keay, K.A., Columnar organization in the midbrain periaqueductal gray and the integration of emotional expression, Prog. Brain Res. 107 (1996) 285–300. doi:10.1016/S0079-6123(08)61871-3.

[50] Mason, P., The Nociceptive Modulatory Effects of Periaqueductal Gray Activation are Mediated by Two Neuronal Classes in the Rostral Ventromedial Medulla, in: Midbrain Periaqueductal Gray Matter, Springer US, Boston, MA, 1991: pp. 287–303. doi:10.1007/978-1-4615-3302-3_16.

[51] Lovick, T.A., Integrated activity of cardiovascular and pain regulatory systems: Role in adaptive behavioural responses, Prog. Neurobiol. 40 (1993) 631–644. doi:10.1016/0301-0082(93)90036-R.

[52] Lumb, B.M., Lovick, T.A., The rostral hypothalamus: an area for the integration of autonomic and sensory responsiveness., J. Neurophysiol. 70 (1993) 1570–7.

[53] Morgan, M.M., Differences in Antinociception Evoked from Dorsal and Ventral Regions of the Caudal Periaqueductal Gray Matter, in: Midbrain Periaqueductal Gray Matter, Springer US, Boston, MA, 1991: pp. 139–150. doi:10.1007/978-1-4615-3302-3_9.

[54] Bandler, R., Carrive, P., Zhang, S.P., Integration of somatic and autonomic reactions within the midbrain periaqueductal grey: Viscerotopic, somatotopic and functional organization, Prog. Brain Res. 87 (1991) 269–305. doi:10.1016/S0079-6123(08)63056-3.

[55] Depaulis, A., Keay, K.A., Bandler, R., Longitudinal neuronal organization of defensive reactions in the midbrain periaqueductal gray region of the rat, Exp. Brain Res. 90 (1992) 307–318. doi:10.1007/BF00227243.

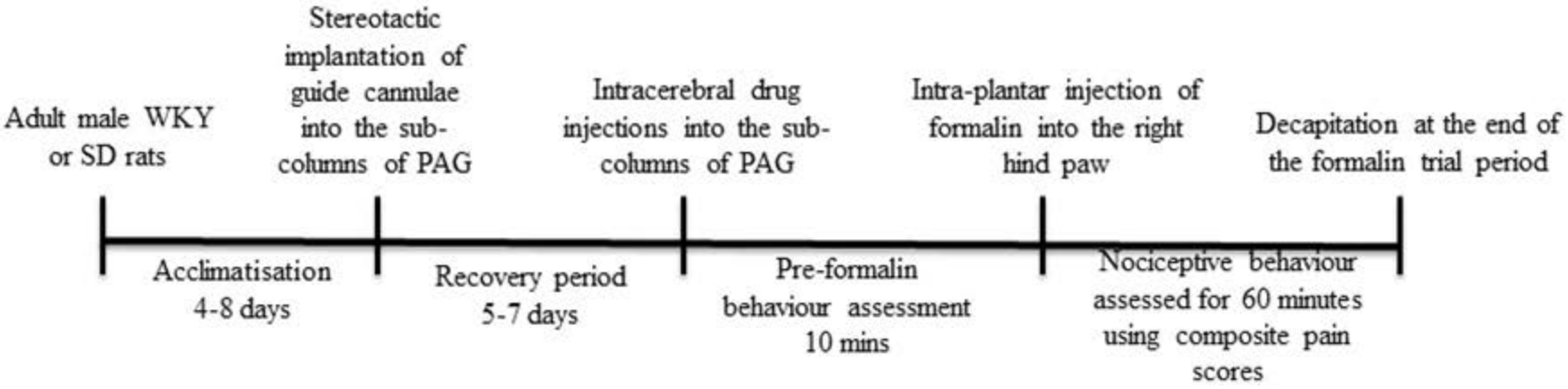
[56] Carrive, P., Bandler, R., Viscerotopic organization of neurons subserving hypotensive reactions within the midbrain periaqueductal grey: a correlative functional and anatomical study, Brain Res. 541 (1991) 206–215. doi:10.1016/0006-8993(91)91020-2.

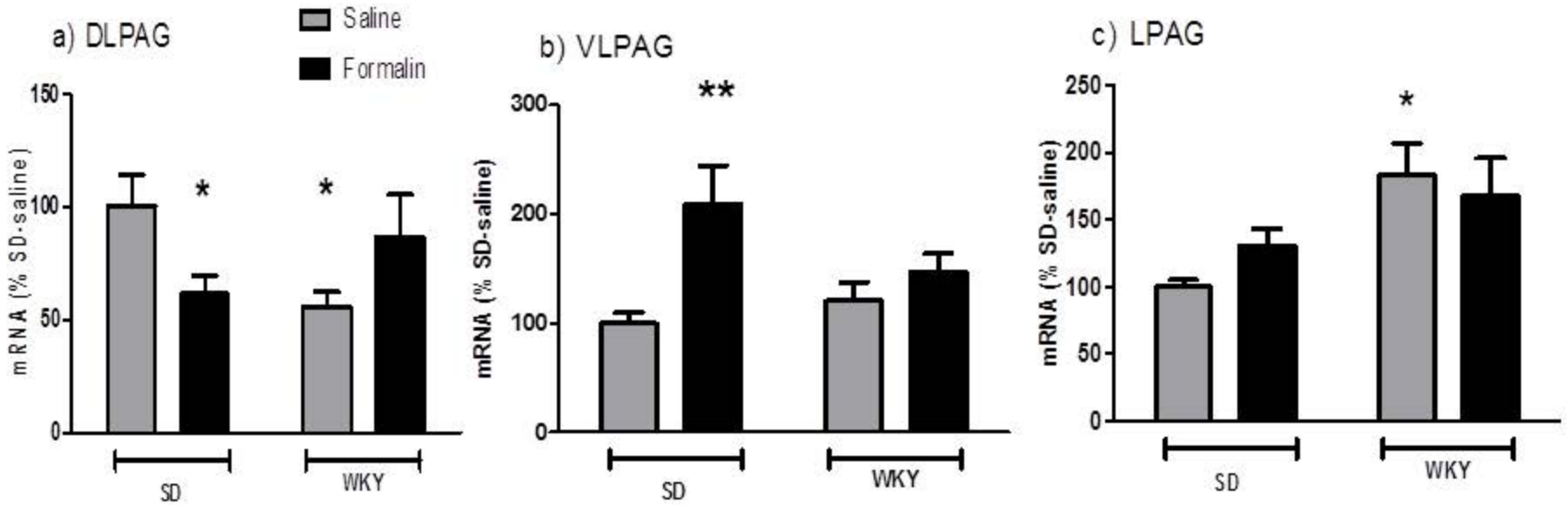
[57] Keay, K.A., Crowfoot, L.J., Floyd, N.S., Henderson, L.A., Christie, M.J., Bandler, R., Cardiovascular effects of microinjections of opioid agonists into the `Depressor Region' of the ventrolateral periaqueductal gray region, Brain Res. 762 (1997) 61–71. doi:10.1016/S0006-8993(97)00285-0.

[58] Palazzo, E., de Novellis, V., Marabese, I., Cuomo, D., Rossi, F., Berrino, L., Rossi, F., Maione, S., Interaction between vanilloid and glutamate receptors in the central modulation of nociception., Eur. J. Pharmacol. 439 (2002) 69–75.

[59] de Novellis, V., Palazzo, E., Rossi, F., De Petrocellis, L., Petrosino, S., Guida, F., Luongo, L., Migliozzi, A., Cristino, L., Marabese, I., Starowicz, K., Di Marzo, V., Maione, S., The analgesic effect of N-arachidonoyl-serotonin, a FAAH inhibitor and TRPV1 receptor antagonist, associated with changes in rostral ventromedial medulla and locus coeruleus cell activity in rats, Neuropharmacology. 55 (2008) 1105–1113. doi:10.1016/j.neuropharm.2008.06.023.

[60] Liao, H.-T., Lee, H.-J., Ho, Y.-C., Chiou, L.-C., Capsaicin in the periaqueductal gray induces analgesia via metabotropic glutamate receptor-mediated endocannabinoid retrograde disinhibition., Br. J. Pharmacol. 163 (2011) 330–45. doi:10.1111/j.1476-5381.2011.01214.x.


Adult male WKY or SD rats
Stereotactic implantation of guide cannulae into the sub-columns of PAG
Intracerebral drug injections into the sub-columns of PAG
Intra-plantar injection of formalin into the right hind paw
Decapitation at the end of the formalin trial period
Acclimatisation 4-8 days
Recovery period 5-7 days
Pre-formalin behaviour assessment 10 mins
Nociceptive behaviour assessed for 60 minutes using composite pain scores


a) DLPAG
Saline
Formalin
mRNA (% SD-saline)
150
100
50
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SD
WKY
b) VLPAG
mRNA (% SD-saline)
300
200
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SD
WKY
c) LPAG
mRNA (% SD-saline)
250
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50
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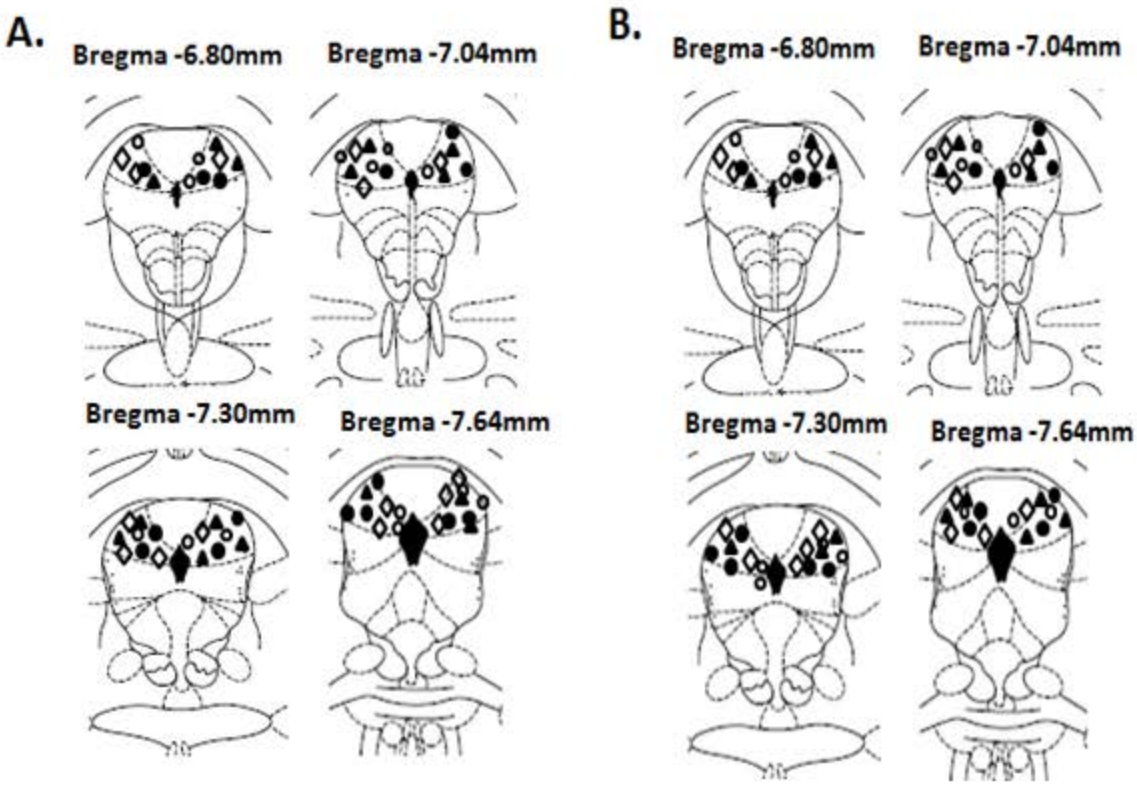
SD
WKY


A.
Bregma -6.80mm
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B.
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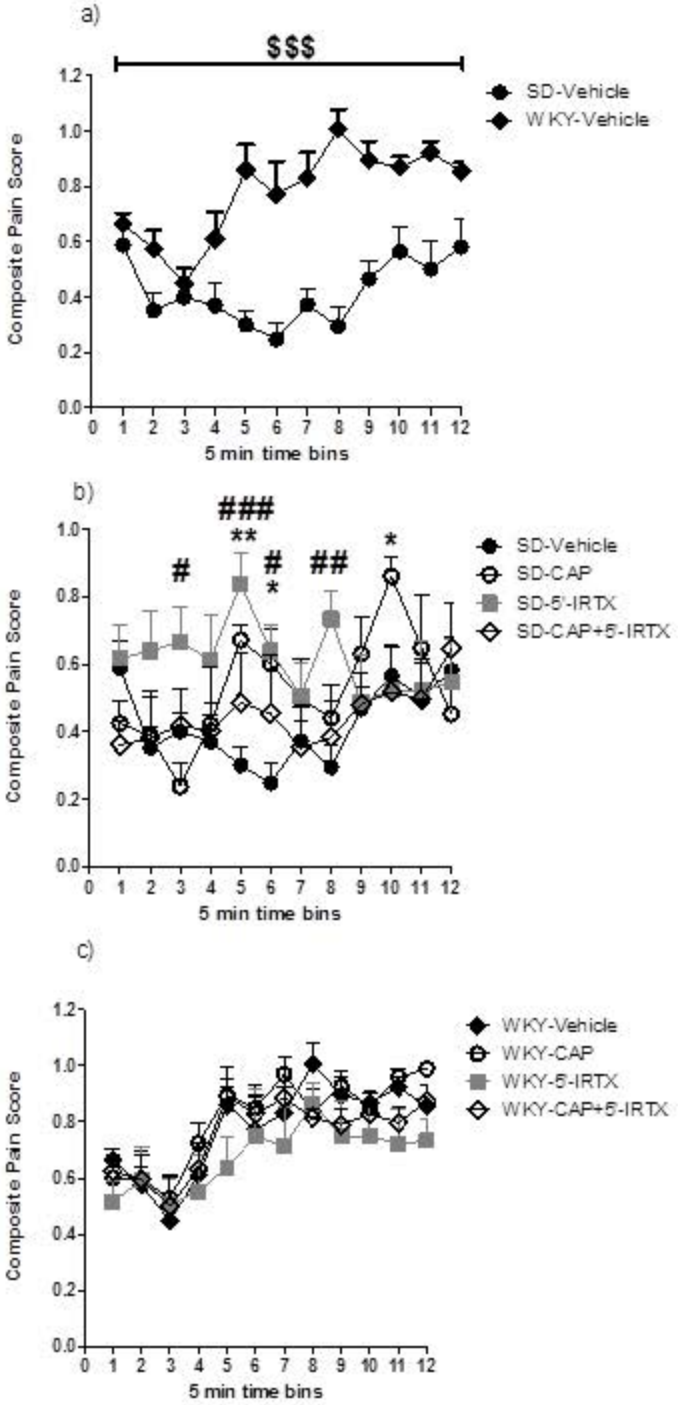
a)
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SD-Vehicle
WKY-Vehicle
Composite Pain Score
5 min time bins
b)
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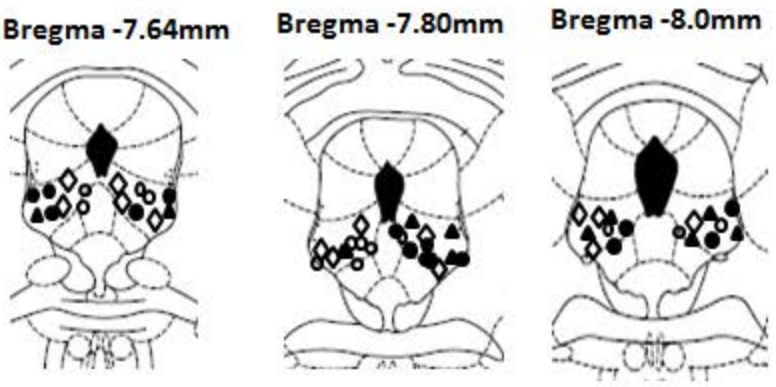
SD-Vehicle
SD-CAP
SD-5'-IRTX
SD-CAP+5'-IRTX
Composite Pain Score
5 min time bins
c)
WKY-Vehicle
WKY-CAP
WKY-5'-IRTX
WKY-CAP+5'-IRTX
Composite Pain Score
5 min time bins

A.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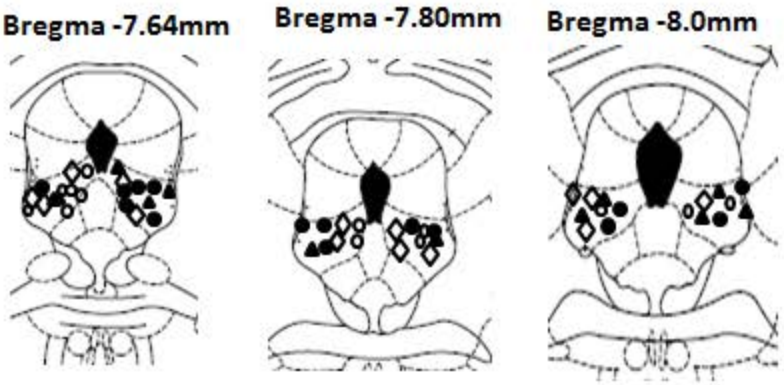
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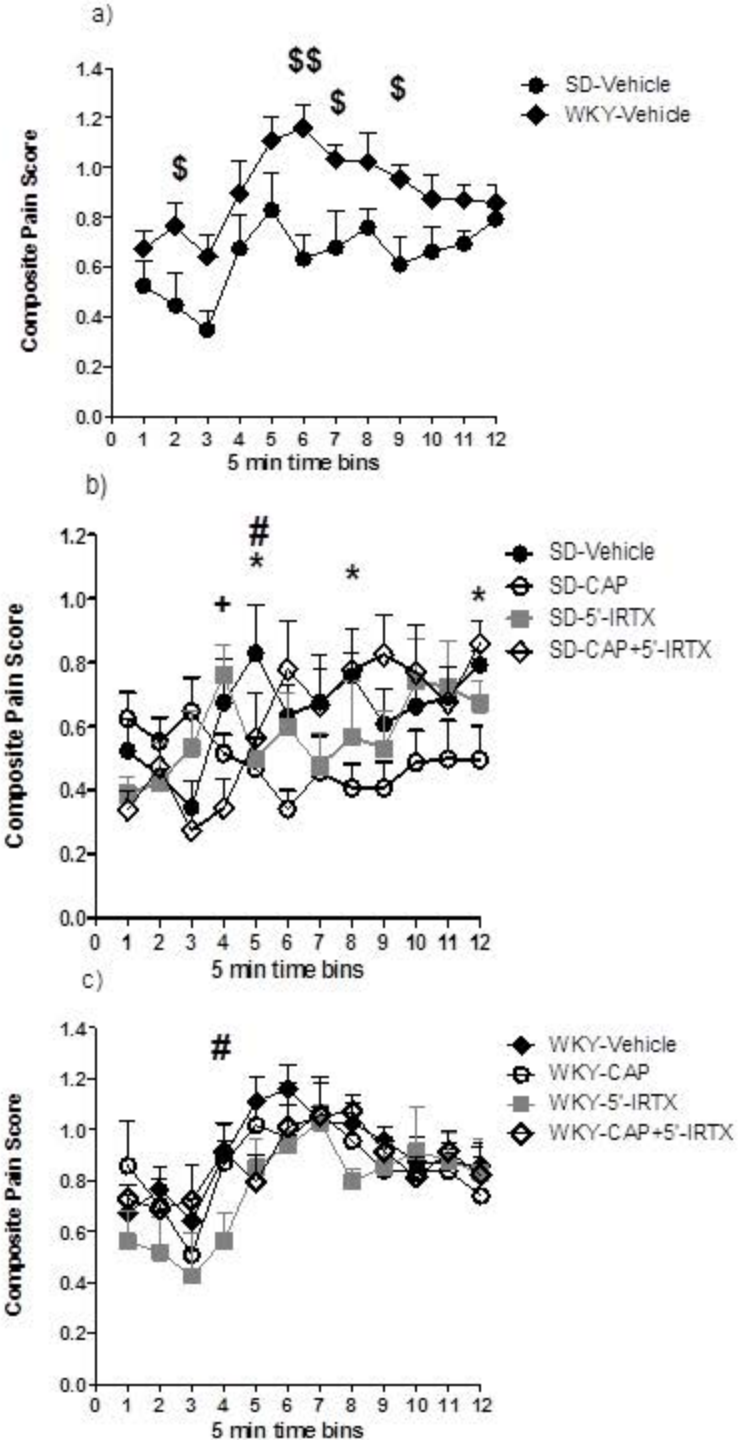
a)
Composite Pain Score
5 min time bins
SD-Vehicle
WKY-Vehicle
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b)
Composite Pain Score
5 min time bins
SD-Vehicle
SD-CAP
SD-5'-IRTX
SD-CAP+5'-IRTX
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WKY-Vehicle
WKY-CAP
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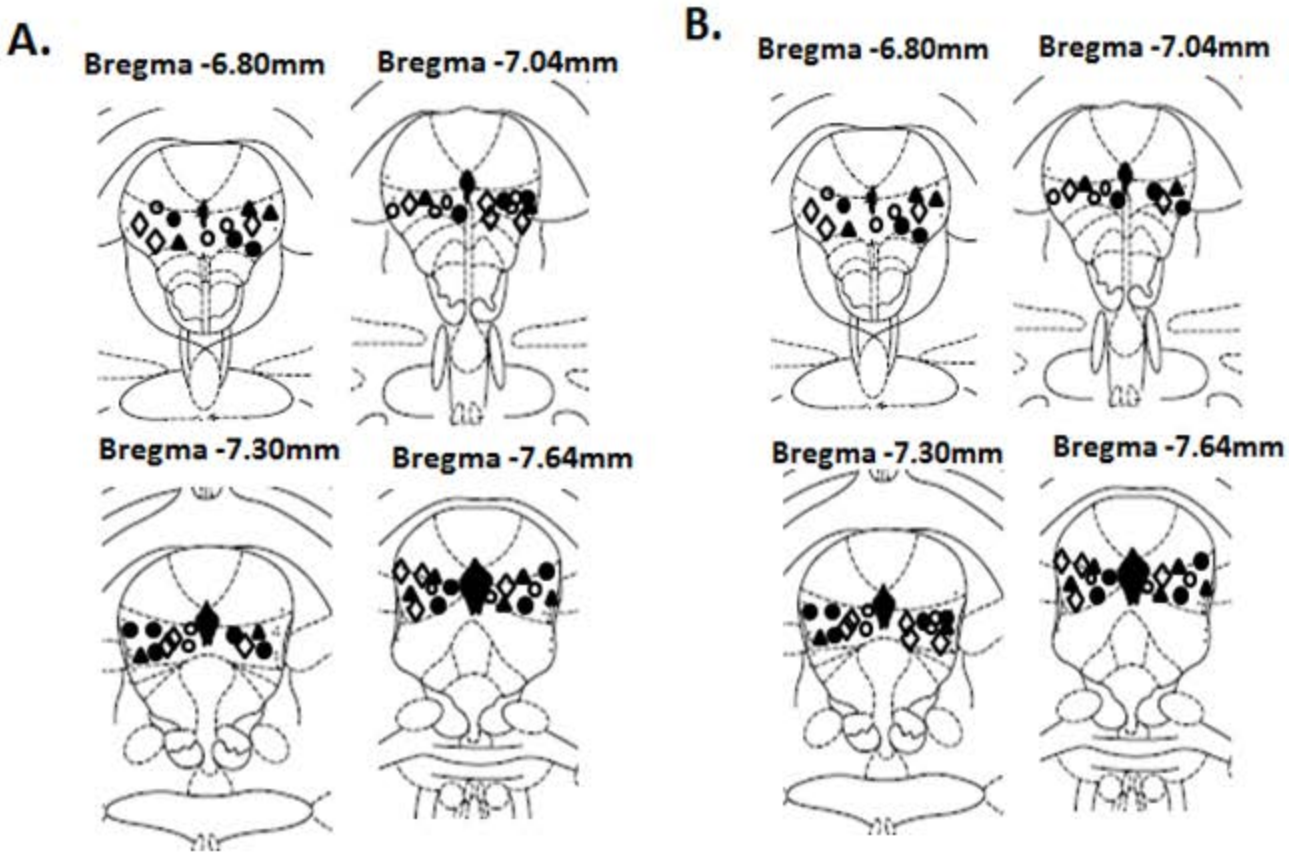
A.
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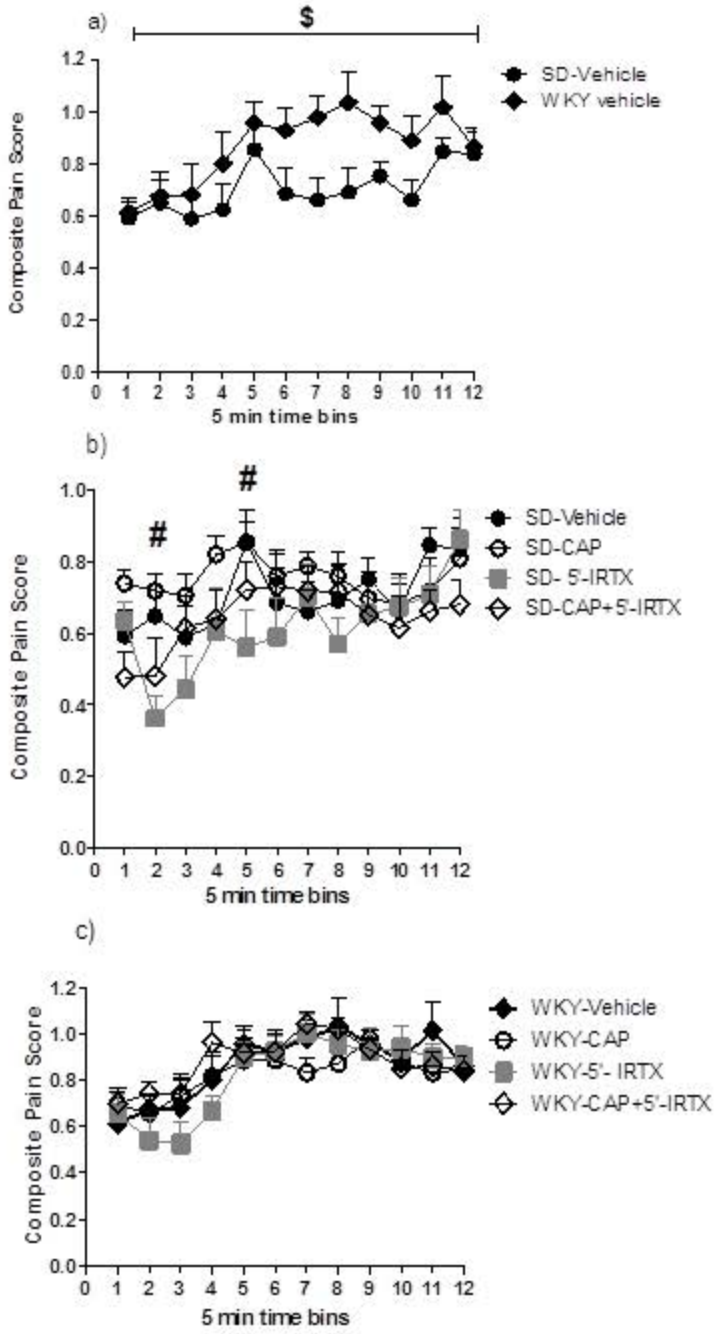
a)
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SD-Vehicle
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SD- 5'-IRTX
SD-CAP+5'-IRTX
Composite Pain Score
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WKY-CAP
WKY-5'- IRTX
WKY-CAP+5'-IRTX
Composite Pain Score
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