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A quantification of the relationship between neuronal responses in the rat rostral ventromedial medulla and noxious stimulation-evoked withdrawal reflexes

Running title: RVM and reflex responses to noxious inputs

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Abstract

The rostral ventromedial medulla (RVM) regulates a range of involuntary behaviours but is most often associated with nociception via the action of pro-nociceptive ON cells and anti-nociceptive OFF cells. Phasic responses of ON and OFF cells determine whether or not incoming noxious signals provoke a withdrawal reflex and previous studies have suggested that reflex RVM activity patterns actively shape motor output. Here we challenge the model by using juvenile rats which are known to exhibit markedly different reflex responses compared to adults. By recording single-cell activity in the RVM and electromyographic responses of hind-limb flexor muscles to noxious thermal stimulation we found the juvenile reflex had a shorter onset latency, was larger in amplitude and exhibited a decreased rise time compared to the adult reflex. Responses of ON and OFF cells faithfully tracked the shorter onset latency of the reflex by also responding earlier and, thus, still preceded the reflex. However, neither the reflex amplitude nor the ongoing response profile was predicted by the firing rate of RVM cells in either age group. Instead we found a close correspondence between RVM activity and the reflex only during the initiation of the response. Furthermore, the short rise time of the juvenile reflex was reflected in higher rates of change of both ON and OFF cell firing. Our data suggest that the RVM is associated only with the initiation of reflexes and does not shape ongoing muscle activity which is more likely to be subserved by downstream spinal processes.

Introduction

The rostral ventromedial medulla (RVM) is a primitive brain region involved in the modulation of involuntary behaviours, especially those evoked by noxious stimuli (Mason, 2005). The RVM is the origin of a bulbospinal projection which modifies spinal dorsal horn (DH) excitability and thus facilitates or inhibits nociception (Millan, 2002; Todd, 2010). Three different cell types have been identified in the RVM of anaesthetized animals and named after their phasic response to an acute noxious stimulus: ON cells exhibit a burst in firing; OFF cells exhibit a pause in firing; and neutral (NEUT) cells show no consistent response (Fields *et al.*, 1983; Barbaro *et al.*, 1986; Heinricher *et al.*, 2009). Theories of RVM function have shifted from a focus on tonic firing of cells (Heinricher *et al.*, 1989) to their phasic response to noxious stimuli (Heinricher & Kaplan, 1991; Mason, 2012) and the RVM is increasingly implicated in central sensitisation observed in chronic pain states (Carlson *et al.*, 2007; Brink *et al.*, 2012; Khasabov *et al.*, 2012). The balance of ON and OFF cell activity is likely to play a key role in the maintenance of hyperalgesia and allodynia in such states (Leong *et al.*, 2011; Cleary *et al.*, 2014) and recent evidence suggests that even NEUT cells may play a previously under-appreciated role by adopting ON- or OFF-like functionality (Khasabov *et al.*, 2015). One current theory of RVM function, forwarded by Hellman & Mason, reiterates that tonic firing of RVM cells is not directly related to noxious stimulus sensitivity and that it is the phasic response of ON and OFF cells that determines the magnitude of evoked withdrawal reflexes, excitability of the dorsal horn (DH) and ultimate perception of pain (Hellman & Mason, 2012). Moreover, the theory makes a direct link between two specific properties of RVM activity and accompanying muscle activity: i) ON cell activity that immediately precedes the peak withdrawal reflex is correlated with the amplitude of the withdrawal, and ii) the onset latency of RVM cells is correlated with the onset latency of the withdrawal reflex. The authors use these findings to suggest that the

firing pattern of RVM cells actively shapes the ongoing withdrawal reflex (Hellman & Mason, 2012).

Here we test the above theory of RVM function to determine if the model is maintained during withdrawal reflexes that differ in their response profile; to do this, we have examined parameters of RVM cell and reflex responses evoked from juvenile (postnatal day (P)21) and adult (P40) rats. Juvenile withdrawal reflexes are larger, less coordinated and more easily evoked as sensory thresholds are lower than in adults (Fitzgerald, 2005). The use of immature animals therefore serves as a non-pharmacological, non-pathological challenge to the model proposed by Hellman and Mason. At P21 intrinsic spinal maturation is largely complete (Baccei & Fitzgerald, 2004; 2005; Baccei, 2007) as are age-dependent alterations in primary sensory neuron innervation patterns. Differences in reflex properties therefore reflect differences in supraspinal input, which principally arises from the RVM (Hathway *et al.*, 2009; Hathway *et al.*, 2012). We hypothesised that ON cell activity would predict the size of the withdrawal reflex regardless of animal age and follow muscle activity throughout the reflex, and that onset latencies of RVM cells would be correlated with the onset of withdrawal. By recording tonic and phasic RVM activity concurrently with EMG activity we have shown that juvenile withdrawal reflexes are of larger amplitude (and shorter latency) than adults and that RVM responses do indeed follow the onset of EMG responses in both ages but only during the initiation of the reflex. Furthermore, the initial shape of the reflex is reflected primarily in the rate of change of ON and OFF cell phasic responses and not the absolute level of activity as the model predicted. Finally, whilst the early period of the phasic response of ON and OFF cells does indeed follow the rise of the withdrawal reflex, only in a minority of cases do RVM cells follow the withdrawal reflex throughout its duration, which indicates an important heterogeneity within RVM cell populations.

Materials and Methods

Animals and surgical procedures

Juvenile (P21 ± 2 days; n=13) and adult (adolescent) (P38-P47 days; n=17) male Sprague Dawley rats of age were used (Charles River, Margate, UK) weighing between 52-92 g (P21) and 159-256 g (P40) were used and kept on a reversed 12-hr dark/artificial-light cycle in closed, ventilated cages in a holding room kept at a temperature of 22°C and 55% humidity; food and water were available *ad libitum*. The experiments described were approved by the local University ethical committee and all procedures were performed and specifically licensed following approval by the UK Home Office and in accordance with the Animals (Scientific Procedures) Act 1986 which incorporates Council Directive 2010/63EU of the European Parliament and the Council of 22nd September 2010 on the protection of animals used for scientific purposes.

Rats were anesthetized with urethane (0.4 g/kg) and isoflurane and a tracheostomy performed to enable artificial ventilation. One advantage of urethane is that withdrawal reflexes are present at a wide range of anaesthetic depths (Maggi & Meli, 1986). Isoflurane was vaporized in oxygen at a concentration of 1.5-2.5% during surgical preparation and 0.65-1.2% during data acquisition (juvenile: 0.79 ± 0.05 %; adult: 0.84 ± 0.17 %). Animals were artificially ventilated using a volume-controlled ventilator (Model 683 Small Animal Ventilator, Harvard Apparatus, Edenbridge, UK) with rate and inspired volume optimized by monitoring end-tidal CO_2 (typical range: 60-80 breaths per minute, 1-1.8 ml/kg; Capnovet-10, Vetronic Ltd., Abbotskerwell, UK). Body temperature was measured with a rectal probe and maintained at 37°C using a thermostatic heating pad (Harvard Apparatus, Edenbridge, UK). Animals were transferred to a stereotaxic frame (Stoelting, Dublin, Ireland) and small trepanne holes were drilled over the hind-limb area of the primary somatosensory cortex (S1HL; A*P* = -1.5 mm, ML = 2.0 mm) and left frontal cortex to allow the sub-cranial

insertion of loop-tipped silver wire EEG recording and differential electrodes respectively (∅=0.2 mm; Intracel, Royston, UK). EEG was monitored throughout the experiment to ensure stability of anaesthesia. A craniotomy was also performed overlying the RVM (A*P* = 8.0 to 12.5 mm, ML = -2.0 to 2.0mm) and the dura resected using a 31 gauge hypodermic needle (Becton-Dickinson, Oxford, UK).

Sensory stimulation

Noxious 55°C thermal stimuli were delivered with a custom-built device: briefly, a small, convex-shaped aluminium block was heated using an etched foil resistance heater encapsulated in polyamide housing running from a stabilized 24V DC supply. The area of the block coming into contact with the paw was a circular region 20 mm in diameter. Temperature was controlled via a three wire platinum resistance sensory device giving control accuracy of ± 0.5°C. The left hindpaw of the rat was secured with the plantar surface facing upwards and the heater block gently applied to the entire foot pad upon release of a pressurized pneumatic valve. The stimulus block was pre-heated to 55°C and upon contact with the paw was shown to maintain temperature within 0.1°C. The stimulus block was retracted manually upon commencement of a withdrawal reflex and stimuli were presented with an interval of at least 180 s. The heater control device communicated with a microCED1401 data acquisition unit (Cambridge Electronic Design, Cambridge, UK) so that the presentation and withdrawal of stimuli were accurately represented on acquired data and temperature of the heating block was continually monitored in this way.

Electrophysiology

Silver wire EEG electrodes were connected to a NeuroLog head-stage (NL100AK; Digitimer, Welwyn Garden City, UK), signals amplified x2000 (NL104A), band-pass filtered between 0.5-1000 Hz (NL125) before being sampled at 2kHz using Spike2 software via a

microCED1401 data acquisition unit (Cambridge Electronic Design, Cambridge, UK). Single-unit recordings were made with either glass or glass-coated tungsten microelectrodes. Glass capillaries (GC120F-10; Harvard Apparatus, Edenbridge, UK) were heated and pulled to with a taper between 8.5-9.0 mm using a Sutter P-97 puller (Novato, CA, USA), broken back with a glass rod to reach a final tip diameter of 1.5-2.5 µm and filled with 0.5M NaCl and 20mM Chicago blue 6B (Sigma Aldrich, Gillingham, UK). Glass-coated tungsten microelectrodes were used and inserted into the RVM to reach the final co-ordinates: A*P* = 9.0 to 11.5mm, DV = 10.2 to 11.0, ML = -1.0 to 1.0mm; *in vivo* impedance of the glass microelectrodes was typically 8-14 MΩ. Microelectrode signals were amplified x100 with a NeuroLog AC-DC amplifier (NL106), filtered between 300-10kHz (NL125) and the data sampled at 20kHz. Signals from glass microelectrodes were initially passed through a NeuroData IR183 headstage and amplifier (x10; Cygnus Technology Inc., Delaware, USA). Electrodes were advanced using a motorized in vivo manipulator (IVM-1000; Scientifica, Uckfield, UK) linked to LinLab software until a responsive cell was found (i.e. in which a noxious stimulus elicited either an increase or decrease in basal firing). Typically recordings contained only an individual cell; in all cases, spikes were sorted using Spike2 software. The total number of spikes in each 500 ms time period was used as our measure of single-unit activity (SUA) and converted into an instantaneous firing rate (Hz). Electrode recording sites were marked by iontophoretically injecting Chicago Blue 6B from the glass capillary or by passing current through the glass-coated tungsten electrode (<100 µA) and sites marked on a para-sagittal atlas image (Figure 1A,B). The fur overlying the biceps femoris muscle was trimmed and a custom-built bipolar concentric needle EMG recording electrode (comprising a modified 27 gauge hypodermic needle) was inserted into the belly of the muscle. The EMG signal was amplified in the same way as EEG signals, band-pass filtered between 10-1000 Hz and sampled at 2kHz.

Data analysis

All data analyses were performed with custom-written routines in MATLAB (R2012a; MathWorks, Natick, MA, USA). Five basic peri-stimulus parameters were extracted from the SUA data: baseline firing rate (average of the 30 s before stimulus onset), maximum change in firing rate, onset latency (the time at which firing rate exceeded 10% of the maximum response amplitude), latency to the response maxima and response duration (the time between onset and the time at which firing rate returned to 10% of the maximum response amplitude). Any stimulus onset/offset artifacts were removed from the raw EMG waveform by interpolation and the data then rectified. Four basic parameters were extracted from the EMG data using the same definitions as SUA data: maximum response amplitude, onset latency, latency to the response maxima and response duration. To allow comparison with previous studies, data in most analyses were aligned to the onset of the EMG response, i.e. 0 s represents the time at which EMG activity exceeds 10% of the maximum EMG amplitude; this enables the comparison of SUA and EMG activity. It was necessary in one analysis to align the data to stimulus onset time, i.e. 0 s represents the stimulus onset time; this enables the timing of SUA and EMG activity relative to the stimulus.

To determine if the RVM may play a role in the shaping of withdrawal reflexes, two analyses were performed. The first compared the temporal response profiles of SUA and EMG activity for which baseline activity was removed, responses normalized with respect to their maxima and all activity preceding the response onset removed. The average relationship between each RVM cell and its associated EMG response was generated (over 10 s for juvenile data, 20 s for adult data) and a linear regressions performed over the entire time period (10 or 20 s) and for the period of time taken for the EMG to reach its maximum. The second analysis followed the approach of Hellman & Mason (Hellman & Mason, 2012) and performed two correlations for each RVM cell type in each age group: between the maximum EMG response

and RVM cell activity in the 1 s that immediately preceded the EMG peak; and a correlation between onset latencies of RVM cell and EMG responses (using data aligned to stimulus onset). We additionally plotted the RVM cell and EMG response profiles over time to illustrate concordance between the two responses; in order to remove high frequency components from the resultant curve, this, as well as all correlations, were performed on data down-sampled to 5 Hz.

Statistical testing was conducted using GraphPad Prism v6.00 (GraphPad Software Inc., La Jolla, CA, USA); two-way ANOVAs followed by Tukey's multiple comparison tests were performed on each of the peri-stimulus parameters described above (i.e. onset latency, latency to peak, duration) grouped by age and response-type (e.g. for onset latency data there were four response types for each age: ON cell, ON cell-associated EMG, OFF cell, OFF-cell associated EMG) in cases where comparison between ages and cell/response type was important. A chi-square test was performed on the number of cell types found in juvenile animals based on the expected number from the adult animals; linear regression was used to compare the shape of response profiles and assess the relationship between features of RVM cell and EMG responses; in all other cases, two-tailed t-tests were performed to compare between, for example pooled EMG data between ages, and are clearly stated in the text. All data are given with the mean ± s.e.m. in the text and in figures.

Results

Baseline and evoked firing of adult ON and OFF cells

A total of 14 ON cells and 12 OFF cells were recorded from 17 adult rats. Average SUA and EMG reflex responses were obtained from two to seven stimulus presentations (median = 4, mode = 4). Microelectrodes were advanced to the region of interest and the responsiveness of spontaneously active cells (detected audibly) was tested with a mixture of toe pinches and thermal stimulation to both left and right hind paws. Figure 2 illustrates representative raw data from two separate rats (juvenile OFF and ON cells along with associated EMG activity; single responses are shown in close-up on the right hand side, 2B,D,F,H). Baseline activity of responsive cells was occasionally found to fluctuate, often with depth of anaesthesia (measured using the EEG), but careful control of anaesthetic supply to maintain animals within anaesthetic state III-3/2 (using visual confirmation) ensured baseline firing rates remained stable throughout stimulation periods (Guedel, 1920; Friedberg *et al.*, 1999). There was no statistically significant difference between baseline firing rates of ON and OFF cells in adult rats although OFF cell firing was consistently higher: 12.3 ± 2.9 Hz and 21.6 ± 3.8 Hz, respectively (t_{24} = 1.943, P = 0.0639; Figure 3A,B,F,H). Average evoked ON cell activity was significantly larger (38.5 ± 8.2 Hz; paired t-test, t_{13} = 4.32, P = 0.0008), or smaller in the case of OFF cells (6.8 ± 3.4 Hz; paired t-test, t_{11} = 10.5, P < 0.0001), than baseline activity (Figure 3F,H) but the absolute change in firing rate was not different between cell types (t_{24} = 1.71, P = 0.10; 26.2 ± 6.1 Hz in ON cells, 14.8 ± 1.4 Hz in OFF cells). The smallest absolute and percentage changes observed in any individual cell from adult animals was 3.5 Hz and 32.7%.

Adult EMG reflexes and RVM responses

Data in Figure 3 have been aligned to the onset of the reflex (i.e. the time at which EMG activity exceeded 10% of the maximum response amplitude) as is the convention within

electrophysiological studies of the RVM to enable comparison with previous work. The average onset time of ON cell responses was -1.42 ± 0.21 s, with all individual responses preceding the start of the EMG reflex (Figure 3E); the difference between ON cell and EMG onset latencies was significantly different from zero (t_{13} = 6.75, P < 0.0001). Despite the earlier start point, ON cell and EMG reflex activity reached a peak at approximately the same time (Figure 3E). The average onset time of OFF cell responses was -1.24 ± 0.25 s, with the majority of individual responses preceding the start of the EMG reflex (Figure 3G); the difference between OFF cell and EMG onset latencies was significantly different from zero (t_{11} = 5.04, P = 0.0004). As with the ON cell response, OFF cell activity, on average, reached its trough at the same time as EMG activity (Figure 3G). The fact that both ON and OFF cell activity begin before EMG reflex activity yet all reach peak/trough levels of activity simultaneously suggests a role for ON and OFF cells in the initiation and shaping of reflex activity. However, the broad range of time intervals between cellular and EMG reflex activity (with respect to EMG onset, ON and OFF onset times range between -2.9 to +0.25 s) cautions against the assumption that a direct relationship exists between the onset of ON or OFF cells and EMG activity or that the former shapes the latter. Furthermore, cellular activity outlasts EMG reflex activity: the average duration of ON and OFF cell responses is significantly longer than the duration of the associated EMG reflex activity (significant effect of 'response type', $F_{3,94}$ = 24.16, P < 0.0001; ON cell: 48.97 ± 9.25 vs. 10.01 ± 1.66; P < 0.01; OFF cell: 62.71 ± 9.51 s vs. 18.85 ± 5.04 s; P < 0.001; Figure 3E,G, lower panels).

To investigate the role of the RVM in shaping ongoing reflex activity and compare cellular and EMG responses without the confound of timing and amplitude differences, baseline activity was removed, responses were normalized with respect to their amplitudes and all activity preceding the response onset removed (Figure 4A,C). Co-relationships of the EMG reflex and SUA responses were then examined (Figure 4B,D). Whilst both ON and OFF cells

show good agreement with the rise in EMG reflex activity, there is a divergence in the return phase. Data in Figure 3E and 3G had already shown that the duration of SUA responses was longer than the EMG reflex, but ON and OFF cells also differ in this respect: average ON cell activity returned to baseline with a shorter delay than OFF cell activity. The activity level of OFF cells was significantly larger than that of ON cells at 14.1 s (the average duration of all adult EMG reflexes; unpaired t-test, $t_{24} = 2.07$, $P = 0.049$). Thus, OFF cell activity outlasts EMG reflex activity to a greater extent than ON cell activity, seen clearly in Figures 4A and 4C. Differences in response durations resulted in a significant correlation between the EMG reflex and ON cell activity ($R^2 = 0.53$, $F_{1,99} = 112.7$, $P < 0.0001$) but not OFF cell activity ($R^2 = 0.025$, $F_{1,99} = 2.5$, $P = 0.12$) over the first 20 s of the response (Figure 4B,D). However, if only the initial period of the response is examined (up to the point at which the EMG response is maximal, because EMG invariably reaches a maximum before the cellular activity) the rise of both cell types agrees strongly with the EMG response (ON: $R^2 = 0.99$, $F_{1,12} = 1521$, $P < 0.0001$; OFF: $R^2 = 0.99$, $F_{1,8} = 1405$, $P < 0.0001$). To more accurately illustrate the range of RVM cell response types, in addition to the mean response profile, Figures 4A and 4C show the average of the two cells with the longest response and the two cells with the shortest response (shown as dashed lines). It is clear that at least a sub-population of both ON and OFF cells faithfully follow the EMG response.

Age-dependent differences in EMG reflex activity

There were marked differences in EMG reflexes evoked from juvenile and adult rats (Figure 5A). The thermal stimulus was applied until the beginning of a reflex was observed and was then manually retracted, a duration which differed between the two age groups and was effectively equivalent to EMG onset latencies. Reflexes occurred with shorter onset latencies in juvenile rats (1.57 ± 0.05 s vs. 3.05 ± 0.22 s; unpaired t-test, $t_{49} = 6.64$, $P < 0.0001$; Figure 5B) and reached maximum activity levels earlier (2.50 ± 0.08 vs. 5.60 ± 0.28 s; $t_{49} = 10.74$, P

< 0.0001; Figure 5B). Maximum response amplitude was also larger in juvenile rats (28.45 ± 3.68 μV vs. 10.73 ± 1.74 μV; t_{49} = 4.41, P < 0.0001; Figure 5C) yet juvenile reflexes were shorter than in adults (response duration: 4.96 ± 0.50 s vs. 14.09 ± 2.59 s; t_{49}=3.40, P = 0.0014; Figure 5B bottom panel).

Baseline and evoked firing of juvenile ON and OFF cells

A total of 14 ON and 11 OFF cells were recorded from 13 juvenile rats. Average SUA and EMG reflex responses were obtained from two to ten stimulus presentations (median = 5, mode = 6). Baseline firing rates of ON cells were significantly lower than OFF cells in juvenile rats: 3.71 ± 1.12 Hz vs. 18.69 ± 5.12 Hz (t_{23} = 3.19, P = 0.004; Figure 6A,B,F,H). Average evoked ON cell activity was significantly larger (19.01 ± 5.20 Hz; paired t-test, t_{13} = 3.41, P = 0.0047), or smaller in the case of OFF cells (2.33 ± 1.79 Hz; paired t-test, t_{10}=4.11, P = 0.0021), than baseline activity (Figure 6A,B,F,H) but the absolute change in firing rate was not different between cell types (15.30 ± 4.49 Hz in ON cells, 13.97 ± 4.55 Hz in OFF cells, t_{23} = 0.21, P = 0.84). The smallest absolute and percentage changes observed in any individual cell from juvenile animals was 2.3 Hz and 46.0 %.

There were no age-related differences in recording sites. In total, 85 electrode penetrations were performed in this study, 50 in the adult and 35 in juvenile rats. The proportions of ON, OFF and NEUT cell types in the different ages did not differ significantly (P > 0.05, χ^2-test; Figure 1C).

Juvenile EMG reflexes and RVM responses

The average onset time of ON cell responses relative to reflex onset time was -0.46 ± 0.09 s, with the majority of individual responses preceding the start of the EMG reflex (Figure 6E); the difference between ON cell and EMG onset latencies was significantly different from

zero (t_{13} = 5.09, P = 0.0002). As in the adult rats, and despite the earlier start point, ON cell and EMG reflex activity reached a peak at approximately the same time (Figure 6E). Individual OFF cell responses did not all begin before the reflex however; the average onset time of OFF cell responses was 0.05 ± 0.24 s (Figure 6G). The average duration of ON and OFF cell responses also follows the adult data and was significantly longer than the duration of the associated EMG reflex activity (significant effect of 'response type' ($F_{3,94}$ = 24.16, P < 0.0001; ON cell: 38.47 ± 9.52 s vs. 5.10 ± 0.73 s; P < 0.05; OFF cell: 51.77 ± 9.39 s vs. 4.81 ± 0.69 s; P < 0.001; Figure 6E,G, lower panels).

The role of juvenile ON and OFF cells in shaping ongoing reflex activity was again investigated as for adult data by normalizing ON, OFF and EMG reflex activity with respect to their maximum amplitudes and removing all activity preceding the response onsets (Figure 7A,C). Co-relationships of the EMG reflex and SUA responses were then examined (Figure 7B,D). Whilst both ON and OFF cells showed good agreement with the rise in EMG reflex activity, there was again a divergence in the return phase. Average ON cell activity returned to baseline with a shorter delay than OFF cell activity: the activity level of OFF cells was significantly larger than that of ON cells at 4.96 s (the average duration of all juvenile EMG reflexes; t_{23} = 2.68, P = 0.014). Thus, OFF cell activity outlasts EMG reflex activity to a greater extent than ON cell activity, seen clearly in Figures 7A and 7C. Differences in response durations result in a higher correlation between the EMG reflex and ON cell activity (R^2 = 0.57, $F_{1,99}$ = 129.9, P < 0.0001) than OFF cell activity (R^2 = 0.27, $F_{1,99}$ = 37.3, P < 0.0001) over the first 10 s of the response (Figure 7B,D). However, as for adult ON and OFF cells, if only the initial period of the response is examined (up to the point at which the EMG response is maximal) the rise of both cell types agrees strongly with the EMG response (ON: R^2 = 0.95, $F_{1,3}$ = 55.6, P = 0.005; OFF: R^2 = 0.99, $F_{1,3}$ = 336.9, P = 0.0004). To more accurately illustrate the range of RVM cell response types, in addition to the mean response

profile, Figures 7A and 4C show the average of the two cells with the longest response and the two cells with the shortest response (shown as dashed lines). As was found in the data from adult rats, at least a sub-population of both ON and OFF cells faithfully follow the EMG response.

Age-dependent relationships between RVM and EMG reflex activity

We have shown that thermal stimulation of the hindpaw produces two distinct reflex response profiles in juvenile and adult rats but if activity of RVM cells has a role in shaping the behavioural response to noxious stimuli, the firing of ON and/or OFF cells should in both ages reflect such changes in the respective reflex responses. There were four main differences between EMG reflex responses in juvenile and adult animals: juvenile EMG reflexes exhibited shorter onset latencies, took less time to reach maximum activity levels, were larger in amplitude and had shorter durations (Figure 5). Despite the shorter stimulus-aligned onset latency of EMG reflexes in juvenile animals as compared to adults (shown in Figure 5 using EMG data pooled from different cell types, and here analysed after separating into cell type-associated EMG responses; significant interaction between age and response-type, $F_{3,94} = 4.7$, $P = 0.0041$; juvenile ON cell-associated EMG: 1.48 ± 0.06 s vs. adult ON cell-associated EMG: 2.98 ± 0.31s, $P < 0.0001$; juvenile OFF cell-associated EMG: 1.67 ± 0.07 s vs. adult OFF cell-associated EMG: 3.14 ± 0.31 s, $P < 0.001$), stimulus-aligned onset latencies of ON and OFF cells did not change significantly with age nor was there any significant difference between cell type within each age (juvenile ON cell: 1.02 ± 0.12 s; adult ON cell: 1.55 ± 0.13 s; juvenile OFF cell: 1.72 ± 0.28 s; adult OFF cell: 1.93 ± 0.20 s). However, juvenile ON and OFF cell responses exhibited larger gradients than adult cells (calculated between response onset and maximum; juvenile vs. adult ON cells: $t_{26} = 4.58$, $P < 0.0001$, Figure 8C; juvenile vs. adult OFF cells: $t_{21} = 5.03$, $P < 0.0001$; Figure 8E) which corresponded to larger maximum gradients in EMG reflex responses (juvenile vs. adult ON cell-associated EMGs:

t_{26} = 7.40, P < 0.0001, Figure 8D; juvenile vs. adult OFF cell-associated EMGs: t_{21} = 5.28, P < 0.0001; Figure 8F). Despite different stimulus-aligned onset times of EMG reflex and SUA responses, SUA activity peaked at a similar time as the EMG reflex activity because the gradient of EMG reflex responses was greater than SUA responses in the respective age group (Figure 8C vs. D and E vs. F).

Parameters of RVM cells and reflex responses that have been correlated and previously used to indicate a role for the RVM in shaping muscle activity (Hellman & Mason, 2012) were also analysed: correlations between the maximum EMG response and RVM cell activity in the 1 s that immediately preceded the EMG peak; and a correlation between onset latencies of RVM cell and EMG responses. There was a significant correlation between RVM cell onset latency and EMG onset latency for both ages (Figure 8G-J); juvenile ON cell: R^2 = 0.52, $F_{1,12}$ = 13.1, P = 0.0035; adult ON cell: R^2 = 0.54, $F_{1,12}$ = 14.5, P = 0.0027; juvenile OFF cell: R^2 = 0.46, $F_{1,9}$ = 7.6, P = 0.022; adult OFF cell: R^2 = 0.35, $F_{1,10}$ = 5.8, P = 0.049 (all compared to the onset latency of their associated EMG responses). However, we found no correlation between the maximum EMG response and the ON or OFF cell activity that immediately preceded it (juvenile ON: R^2 = 0.04, $F_{1,12}$ = 0.5, P = 0.50; adult ON: R^2 = 0.15, $F_{1,12}$ = 2.1, P = 0.17; juvenile OFF: R^2 = 0.03, $F_{1,9}$ = 0.3, P = 0.60; adult OFF: R^2 = 0.03, $F_{1,10}$ = 0.25, P = 0.62; all compared to the maximum of their associated EMG responses).

Discussion

The purpose of the current study was to test the most recent theory of RVM function, forwarded by Hellman & Mason (Hellman & Mason, 2012) with a non-pharmacological and non-pathological challenge to the model by using different age rats that exhibit dissimilar reflex responses. Withdrawal reflexes in juvenile rats were found to have a decreased onset latency, decreased time to peak, increased amplitude, but decreased duration (or quicker, sharper, larger, but shorter) compared to adult responses, in agreement with previous studies (Fitzgerald, 2005). Importantly, the onset of RVM cell responses decreased in juvenile rats in accordance with the quicker withdrawal reflex and, on average, preceded or coincided with withdrawal onset. However, whilst the Hellman & Mason model predicted that the amplitude of reflexes would be predicted by preceding ON cell activity – and would follow the ongoing profile of the withdrawal reflex – we found that neither ON nor OFF cell activity predicted the size of the reflex in either age group. Whilst the overall amplitude of the withdrawal was not predicted by RVM cell activity, the rising phase of the reflex was very well matched with the rate of change of ON and OFF cell phasic responses. However, whilst both ON and OFF cell responses followed the reflex profile during the rising phase, the majority of RVM cell responses to noxious stimuli outlasted the entire withdrawal reflex, especially in OFF cells, and therefore did not follow the reflex over its entire duration. Only the ongoing activity of a minority of RVM cells reliably followed the withdrawal reflex cases in both adults and juveniles.

Only recently have the key properties of RVM cells important to the descending control of pain being identified, despite the first identification of the role the RVM plays in pain occurring over 30 years ago (Deakin *et al.*, 1977; Behbehani & Pomeroy, 1978; Fields & Anderson, 1978). ON cells were very quickly labelled as pro-nociceptive and OFF cells anti-nociceptive (Fields *et al.*, 1983) supported by the finding that morphine consistently

decreasing tonic ON cell firing (Heinricher *et al.*, 1992). Some theories suggested it was tonic firing of ON and OFF cells that were vital in determining overall sensitivity to noxious stimuli. ON and OFF cells appear to be reciprocally connected as tonic firing of each cell cycle under anaesthesia, and stimuli presented during high levels of ON cell firing (and low levels of OFF cell firing) evoked withdrawal reflexes with shorter latency than stimuli presented during low levels of ON cell firing (and high levels of OFF cell firing) (Barbaro *et al.*, 1989). However, this was largely abandoned after it was shown that localized injection of mu- opioid receptor agonists consistently decreased ON cell tonic firing but only blocked withdrawal reflexes on approximately two thirds of trials (Heinricher *et al.*, 1994). Furthermore, despite the common observation of inversely-related tonic activity between ON and OFF cells (Heinricher *et al.*, 1989) only rarely has a statistically significant difference between firing rates been reported e.g. (Guilbaud *et al.*, 1980). It was believed that phasic responses in the RVM enabled a noxious stimulus to gain access to the DH thereby triggering a protective withdrawal reflex. Indeed, the inhibition of the OFF cell pause in particular was found to be a common feature of morphine analgesia and, coupled to the finding that morphine acted solely on ON cells (Heinricher *et al.*, 1992), led to theory that ON cells were inhibitory interneurons that disinhibited GABA-sensitive OFF cells (Heinricher *et al.*, 1991). However, the theory that ON cells were inhibitory interneurons was disproved when Heinricher & McGaraughty (Heinricher & McGaraughty, 1998) found a way to selectively block the ON cell burst with the excitatory amino-acid antagonist kynurenate and observed no change in the phasic activity of OFF cells. Regardless of how it is initiated, the OFF cell pause remains vital to enabling noxious stimulus-evoked reflexes, a fact upon which the majority of researchers within the field agree. It should be noted, however, that some groups maintain the position that OFF cells are an artifact of general anaesthesia and do not exist in awake animals (Oliveras *et al.*, 1989; Oliveras *et al.*, 1991; Martin *et al.*, 1992). Further work utilizing kynurenate (Jinks *et al.*, 2007) to decrease RVM activity inhibited organized

multi-limb escape responses therefore indicating a causal relationship between the RVM and muscle activity. Our data agree with this but extend this finding by showing that it is ON cell activity that is related to the shape the entirety of the hind-limb response, from initiation to cessation, with OFF cell firing only following early stages of the reflex.

We suggest that one possible reason for so many different and conflicting theories regarding RVM function is the lack of standardization in the reporting of electrophysiological data. We have used a display method that clearly presents the overall, population response of RVM cell activity (and withdrawal reflexes) whilst not obscuring important details from what is clearly a heterogeneous population of cells (as shown by us and others). To achieve this we displayed the population response profiles of RVM cells and indicated not only the variation of this average response but the responses of cells at the 'extremes' (Figure 4A,C; Figure 6A,C). To provide individual subject data in a clear manner, we extracted and displayed basic temporal parameters from the each RVM cell response and withdrawal reflex (e.g. Figure 3E). Importantly, this enables us to show the timing parameters from all cells relative to the evoked reflex. Until recently (Cleary *et al.*, 2008), the definition of the classic phasic RVM cell response had not changed since its original description in 1983 (Fields *et al.*, 1983), i.e. "firing increased [or decreased] in cells either before or roughly concomitant with the onset of [tail] flick". Only with the reporting of population responses from ON and OFF cells by Cleary et al. and the comparison of latencies between RVM cell type and the onset of a withdrawal reflex have we begun to quantify the temporal relationship between RVM cells and reflex responses (Cleary *et al.*, 2008). It should be noted that the noxious stimulus used by Cleary et al. was not applied to the hind-paw but to the tail which precluded any comparison of RVM cell and reflex profiles; this is because reflexes in the tail manifest as brief flicks rather than temporally-broader withdrawals as in the hind-paw.

Since the first observation that phasic responses of ON and OFF cells begin immediately before withdrawal reflexes, experimenters have speculated on the possible causal relationship between the RVM and behavioural responses to noxious stimuli (Fields *et al.*, 1983). Our data supports a tight coupling between the reflex and RVM activity during the onset of a behavioural response. Indeed, regardless of the spatiotemporal profile of a withdrawal reflex, muscle activity and the ON cell phasic response reach their respective peaks at approximately the same time (Figure 8A). This coincidence of peak RVM ON cell and withdrawal reflex activity would be expected if it was indeed the rate of change of RVM cell activity which was influencing the ongoing withdrawal reflex, and suggests the ON cell may play a greater role here. It would be predicted that as soon as the rate of change of RVM ON cell firing approaches zero, the muscle would start to relax, which is indeed what we observe (Figure 8A). In the absence of a phasic ON cell response with a positive rate of change, coupling between RVM and muscle breaks down and we speculate that spinal processes and internal muscle physiology govern the duration of the relaxation period rather than medullary influences. Others have observed prolonged responses in RVM cell activity, especially in OFF cells (Chiang & Gao, 1986), a property which could allow the RVM to influence behavioural responsiveness to subsequent noxious stimuli (Heinricher *et al.*, 1989; Leung & Mason, 1998).

Previously we have shown that the consequences of broad pharmacological or electrical manipulation of the RVM in juvenile and adult rats are different, with monophasic facilitation of spinal reflexes being dominant prior to the fourth postnatal week in comparison to bi-phasic facilitation and inhibition in the adult (Hathway *et al.*, 2009; Hathway *et al.*, 2012). Our prediction from this previous work was that either or both ON and OFF cell populations would be fundamentally different between the ages. However, our data demonstrate the presence of both ON and OFF cells in the juvenile RVM in the same proportions and show

there is no difference in the magnitude of the overall response of either cell type. How they differ is in terms of the stimulus-response gradient which is tightly correlated with steeper gradient of the EMG response. These data suggest that developmentally regulated alterations in the consequences of activation of the RVM upon spinal excitability (and the overall size of reflex responses) are not the result of alterations in the properties of RVM neurons themselves but may reside in the integration of these inputs with maturing spinal networks within the DH.

In conclusion, we have shown that it is the rate of change of RVM neurons which is likely to shape spinally mediated withdrawal reflexes (at least during the initiation of the reflex). Our data also suggest that the postnatal maturation of descending control of spinal excitability probably reflects alterations in the integration of RVM projections into DH networks rather than fundamental alterations in the properties of ON or OFF cells themselves.

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Figure Legends

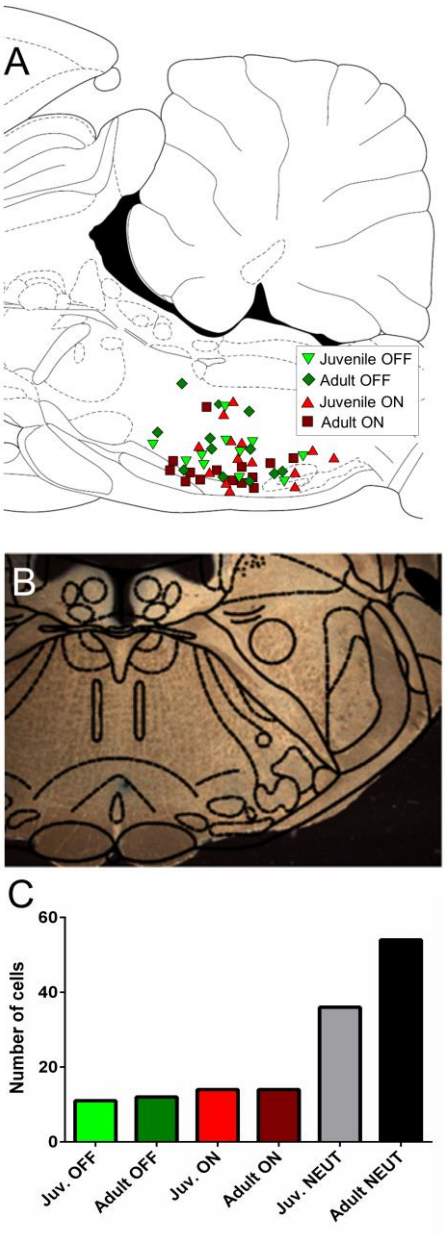

Figure 1. Location and classification of cell types in adult and juvenile RVM. A) Confirmation of recording electrode locations within the RVM of adult and juvenile rats. No differences were seen in locations of ON, OFF or NEUT cells in the two ages; all cells were within 1 mm of the midline. B) An example of the Chicago Blue deposit (arrow) in a recording site -10.04 mm behind bregma. C) Numbers of cells of each type (ON, OFF or NEUT) were the same at each age.

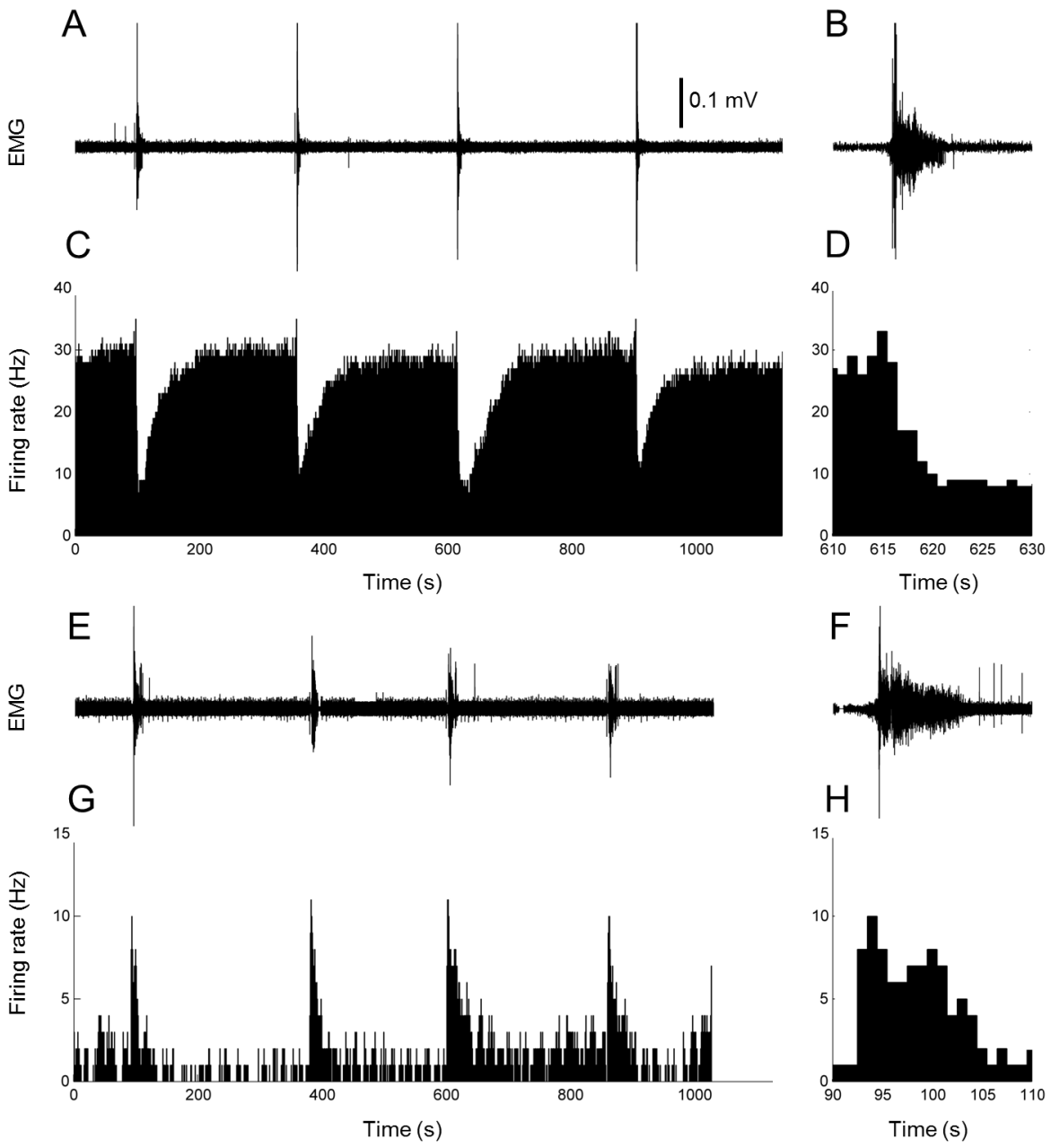

Figure 2. Example of RVM cell and EMG responses recorded from two separate animals. Multiple EMG (A) and OFF cell responses (C) evoked by thermal stimulation along with single EMG and OFF cell responses in close-up (B, D). Multiple EMG (E) and ON cell responses (G) evoked in a separate animal are shown in the lower panels along with single EMG and ON cell responses in close-up (F, H).

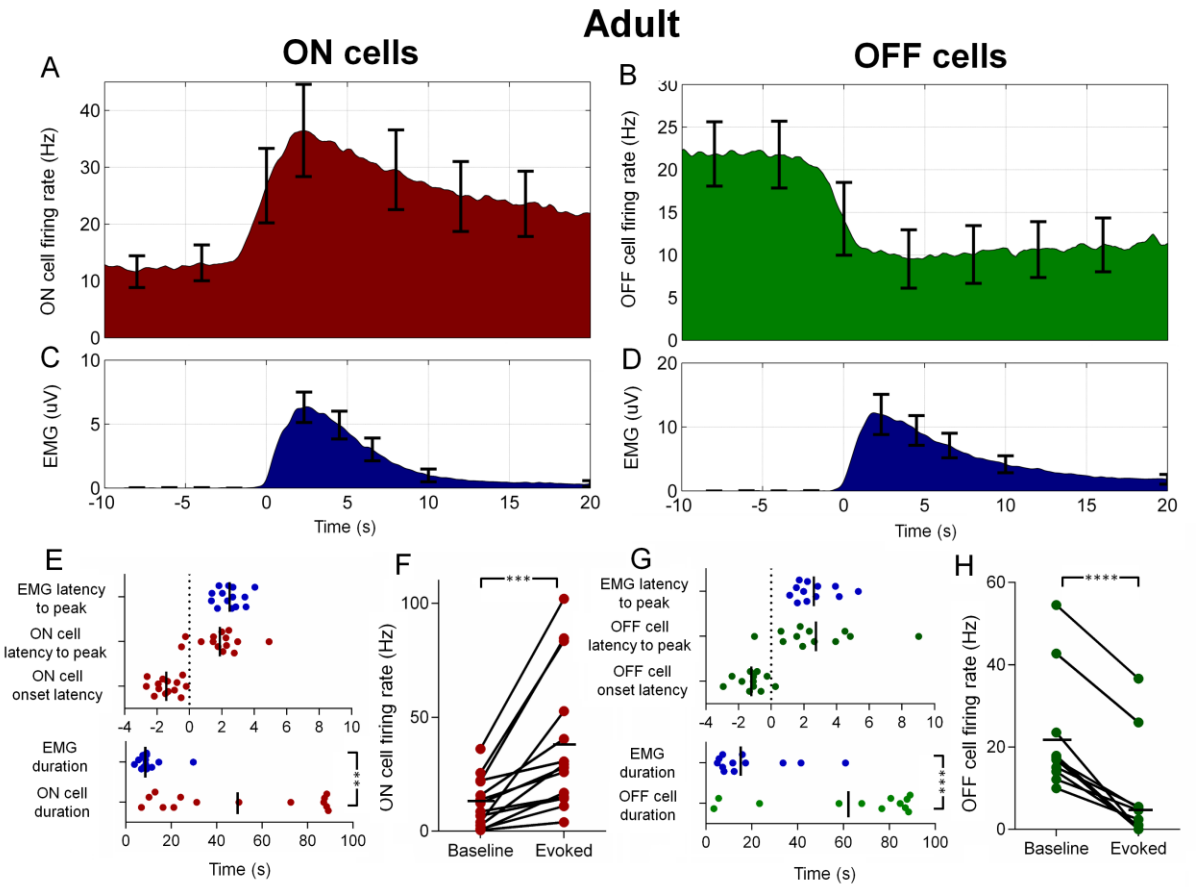

Figure 3. Adult SUA and EMG responses to noxious thermal stimulation. ON cell data are shown in panels on the left hand side, OFF cell data in panels on the right hand side. Average ON cell (A; n=14) and OFF cell (B; n=12) responses are shown alongside their associated average EMG responses (C,D) and all data have been aligned to EMG response onset (i.e. 10% of the maximum response). Duration of cellular and EMG responses (lower panels of E and G) represents the time between response onset and its return to 10% of the maximum response. Latencies of cellular and EMG responses (upper panels of E and G) are also all aligned to EMG response onset; i.e. cellular onset latencies are negative because they occur before EMG response onset (dashed line indicates EMG response onset). Baseline and evoked firing rates (F and H) were generated from the average firing rate in the 30 s preceding the noxious stimulus and the maximum response, respectively. ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$; error bars represent s.e.m.

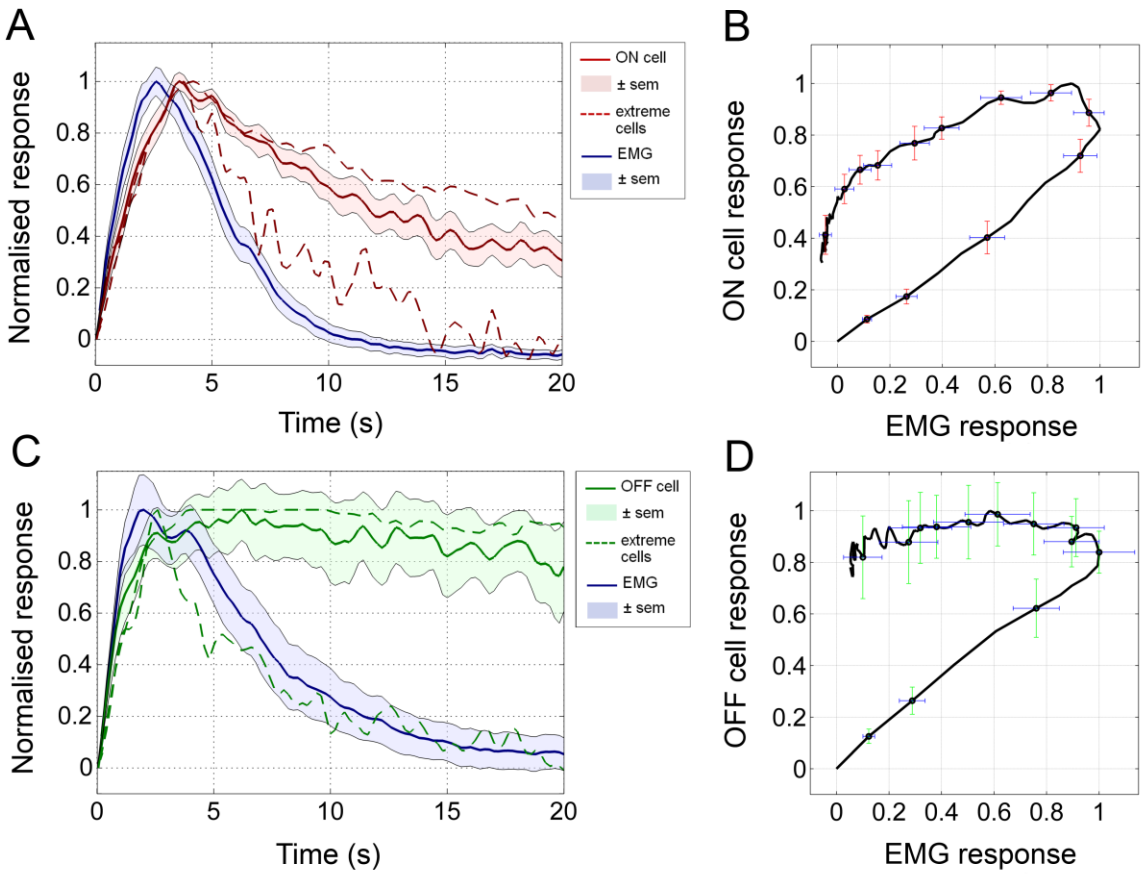

Figure 4. Temporal relationship between adult RVM cell and EMG responses. To compare response shapes without the confound of amplitude and timing differences, ON and OFF cell and EMG responses were normalised and activity preceding response onset removed (A,C). To illustrate the range of cellular response profiles, the two cells with the longest and shortest response durations have been added (dashed lines in A and C; each line is an average of the response of two cells). Co-relationship between cellular and EMG reflex responses are shown with markers indicating 0.4 s, 0.6 s and then at 1 s intervals until 10 s and a final marker at 15 s (B,D). Error bars and shaded regions represent s.e.m.

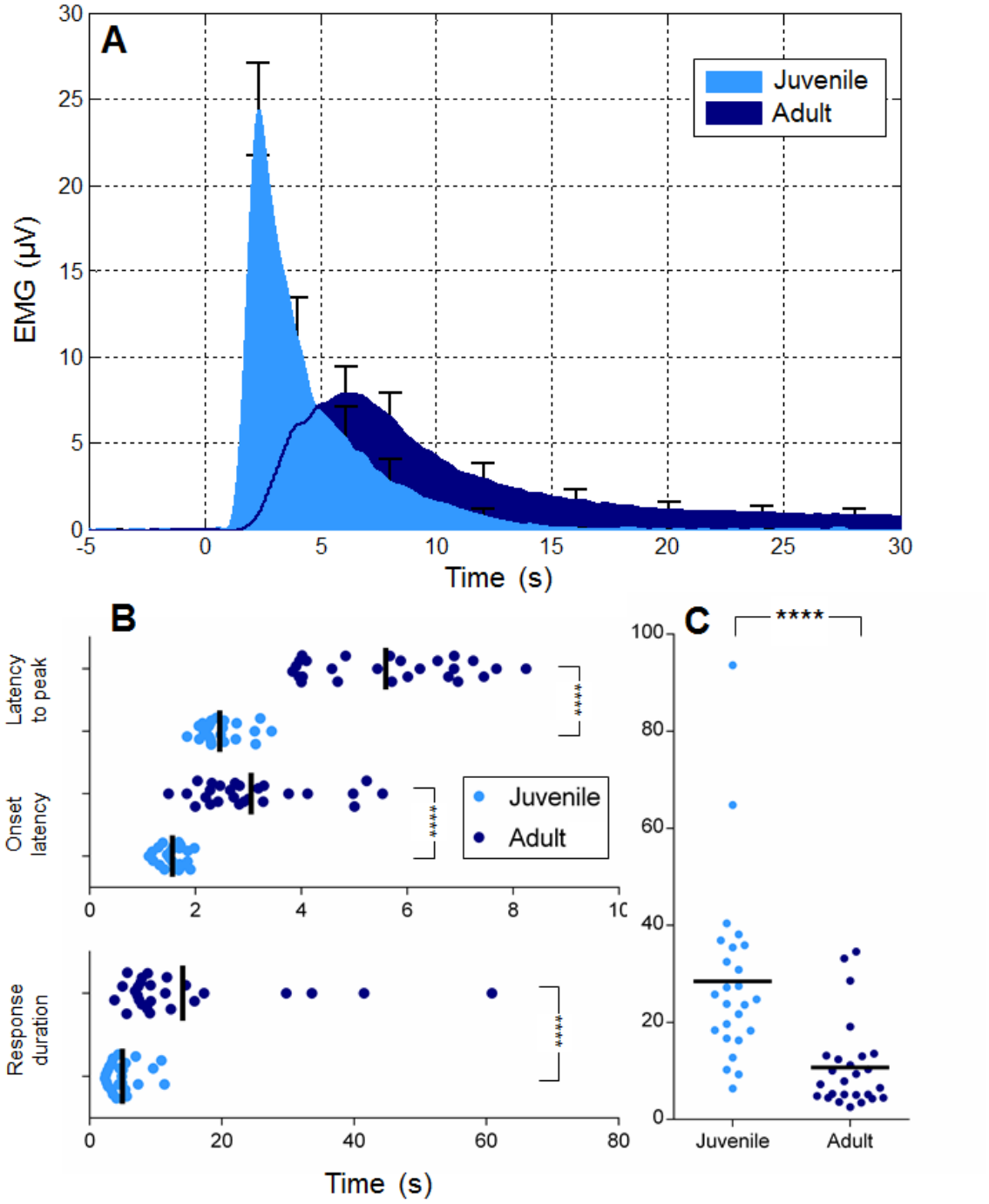

Figure 5. Juvenile and adult EMG responses to noxious thermal stimulation. A: Average EMG responses are shown aligned to stimulus onset time (0 s) for all SUA-associated responses from juvenile (n=25) and adult rats (n=26). B: Onset latency (10% of the maximum response), latency to peak and duration (between response onset and time to return to 10% of the peak response). C: Maximum EMG amplitude. **** = $P < 0.0001$; error bars represent s.e.m.

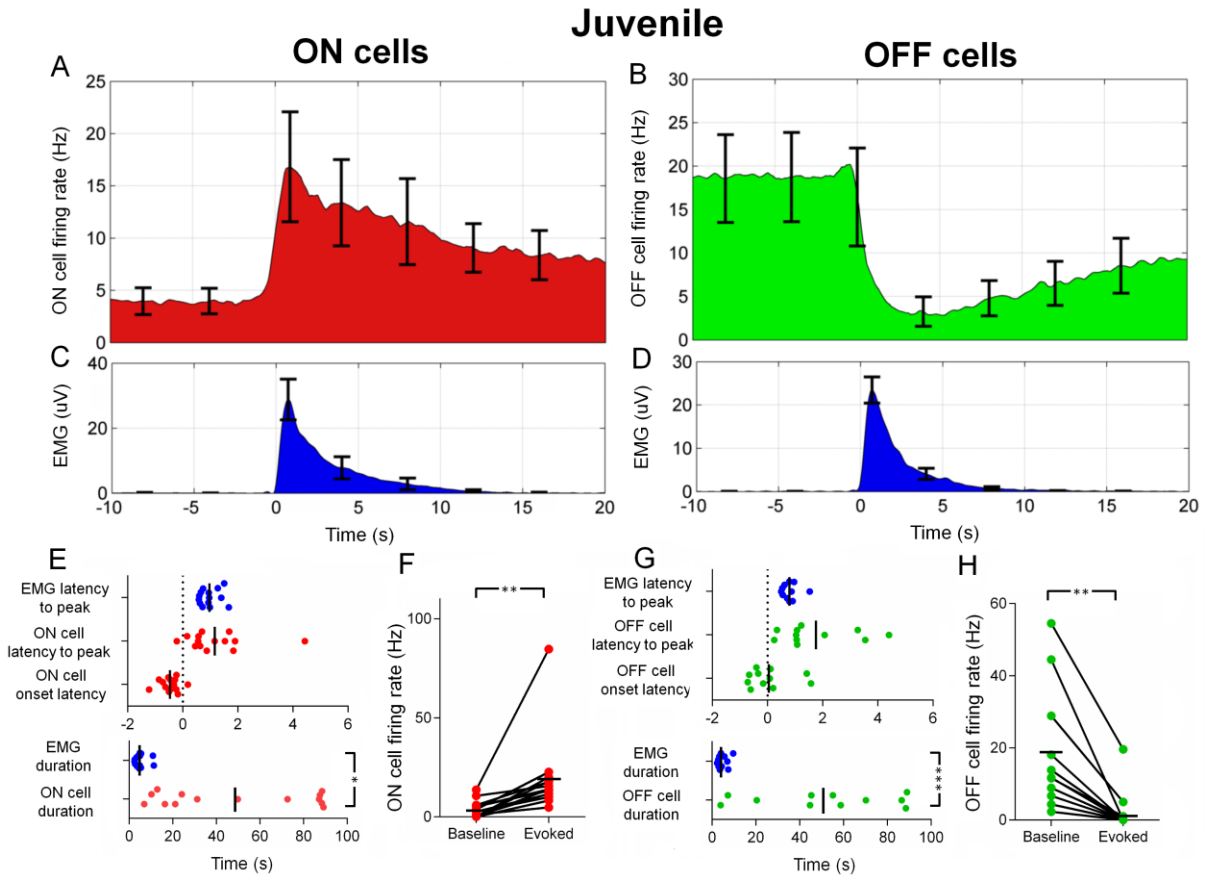

Figure 6. Juvenile SUA and EMG responses to noxious thermal stimulation. ON cell data are shown in panels on the left hand side, OFF cell data in panels on the right hand side. Average ON cell (A; n=14) and OFF cell (B; n=11) responses are shown alongside their associated average EMG responses (C,D) and all data have been aligned to EMG response onset (i.e. 10% of the maximum response). Duration of cellular and EMG responses (lower panels of E and G) represents the time between response onset and its return to 10% of the maximum response. Latencies of cellular and EMG responses (upper panels of E and G) are also all aligned to EMG response onset; i.e. cellular onset latencies are negative because they occur before EMG response onset (dashed line indicates EMG response onset). Baseline and evoked firing rates (F and H) were generated from the average firing rate in the 30 s preceding the noxious stimulus and the maximum response, respectively. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$; error bars represent s.e.m.

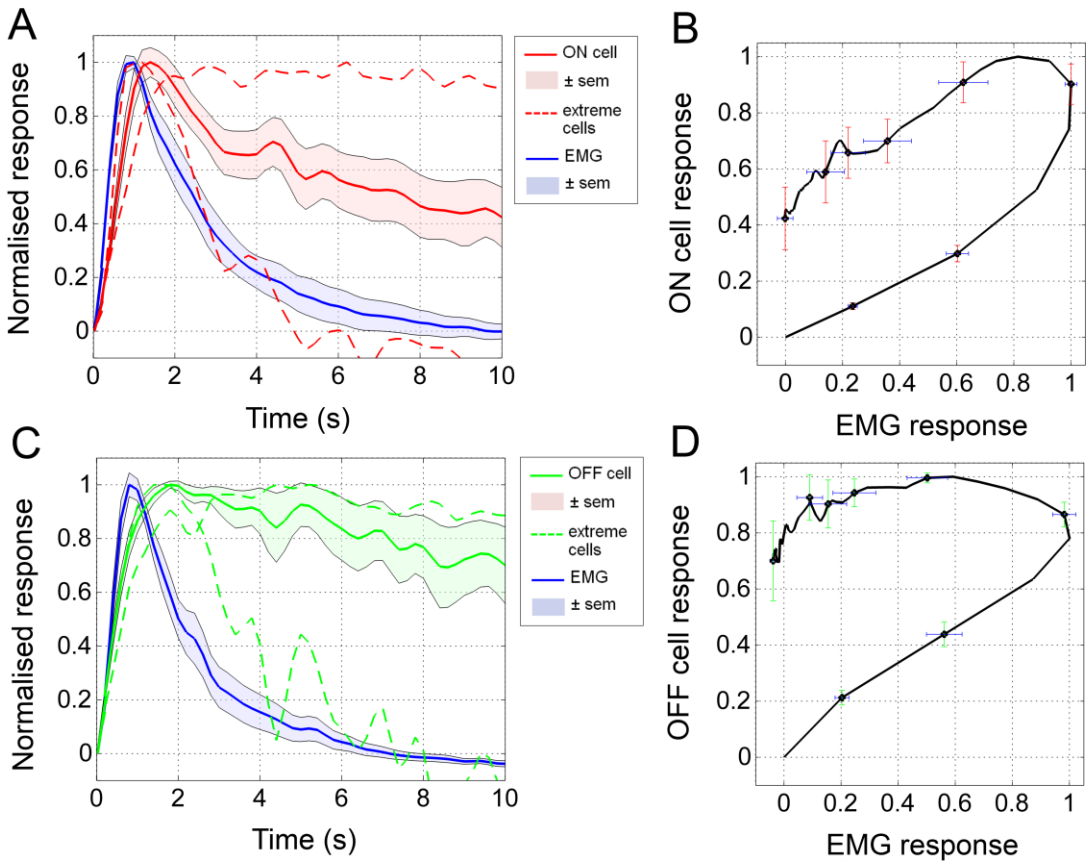

Figure 7. Temporal relationship between adult RVM cell and EMG responses. To compare response shapes without the confound of amplitude and timing differences, ON and OFF cell and EMG responses have been normalised and activity preceding response onset removed (A,C). To illustrate the range of cellular response profiles, two cells with the longest and shortest response duration are added (dashed lines in A and C; each line is an average of the response of two cells). Co-relationship between cellular and EMG reflex responses are shown with markers indicating 0.4 s, 0.6 s and then at 1 s intervals until 10 s (B,D). Error bars and shaded regions represent s.e.m.

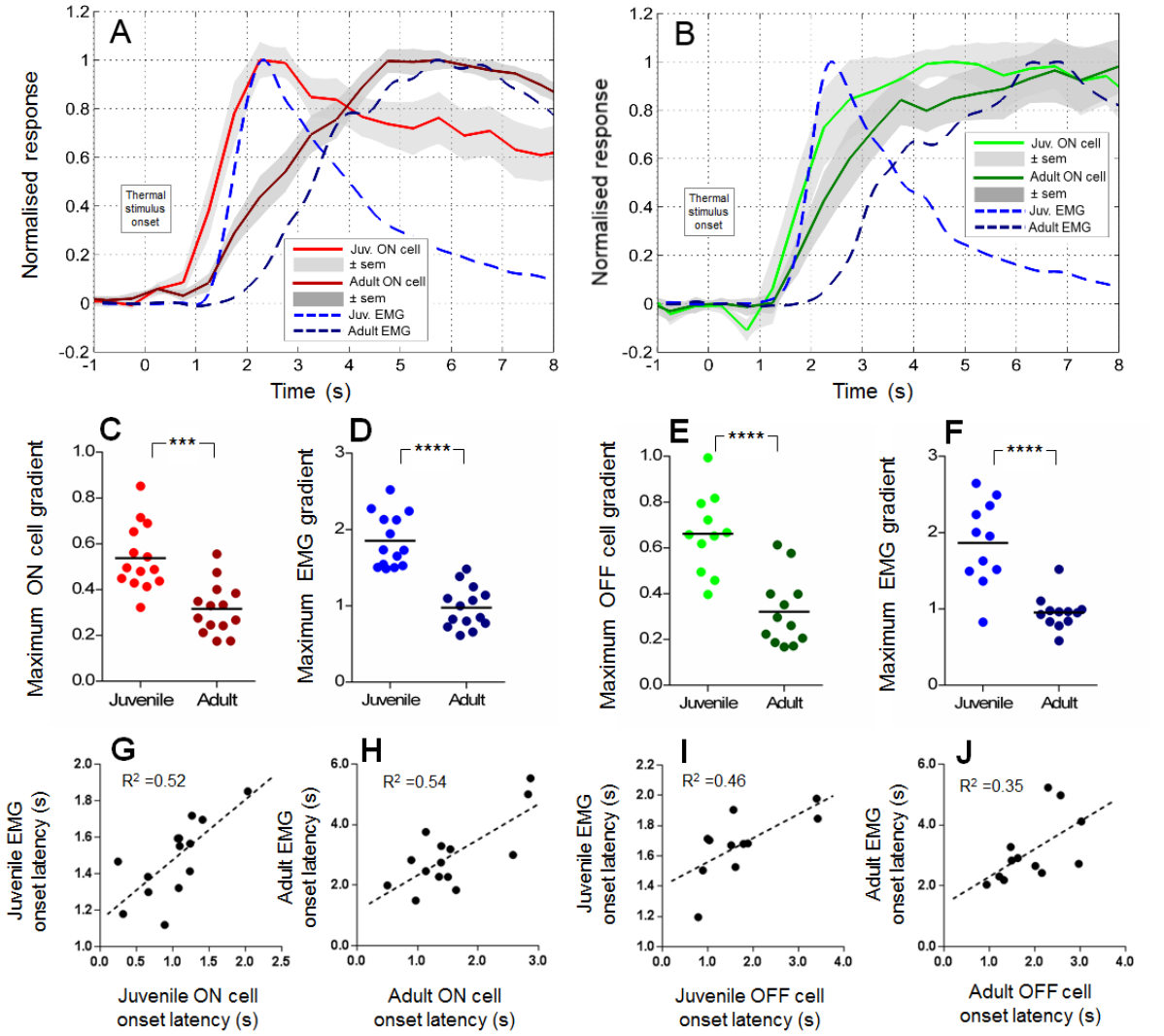

Figure 8. Comparison of response profiles between the ages. ON cell (A) and OFF cell (B) responses have been normalised and are aligned to stimulus onset time (i.e. at time = 0); EMG responses are also shown. Normalisation enables comparison between age and response parameters and illustrates similarity of onset latency of all SUA responses whilst highlighting the difference in response gradient between the ages. Gradients of ON cell (C), EMG associated with ON cells (D), OFF cells (E) and EMGs associated with OFF cells are also shown. ON, OFF and EMG responses have larger gradients in juvenile rats. Correlation between onset latencies of RVM cells and EMG responses are shown for juvenile ON cells (G), adult ON cells (H), juvenile OFF cells (I) and adult OFF cells (J); see text for results of correlations. *** = $P < 0.001$, **** = $P < 0.0001$; error bars and shaded regions represent s.e.m.