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

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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 pronociceptive ON cells and antinociceptive OFF cells. The 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 challenged the model by using juvenile rats, which are known to exhibit markedly different reflex responses compared with adults. By recording single-cell activity in the RVM and the electromyography responses of hindlimb flexor muscles to noxious thermal stimulation we found that the juvenile reflex had a shorter onset latency, was larger in amplitude and exhibited a decreased rise time compared with the adult reflex. The 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 that 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 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 sensitization 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 neutral cells may play a previously under-appreciated role by adopting ON-like or OFF-like functionality (Khasabov et al., 2015). One current theory of RVM function, put forward by Hellman & Mason (2012), 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 DH and ultimate perception of pain. 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 tested the above theory of RVM function to determine whether the model was maintained during withdrawal reflexes that differ in their response profile. We therefore examined parameters of RVM cell and reflex responses evoked from juvenile [postnatal day (P)21] and adult (P40) rats. Juvenile withdrawal reflexes were larger, less coordinated and more easily evoked as sensory thresholds were 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 & Mason (2012). 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, 2012). We hypothesized 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 electromyography (EMG) activity we showed that juvenile withdrawal reflexes were of larger amplitude (and shorter latency) than those of adults and that RVM responses did 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 was 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, whereas the early period of the phasic response of ON and OFF cells did indeed follow the rise of the withdrawal reflex, in only a minority of cases did RVM cells follow the withdrawal reflex throughout its duration, which indicated 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; n = 17) male Sprague Dawley rats (Charles River, Margate, UK) weighing between 52–92 g (P21) and 159–256 g (P40) were used and kept on a reversed 12-h 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 University of Nottingham 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 2010/63/EU of the European Parliament and the Council of 22nd September 2010 on the protection of animals used for scientific purposes. At the end of all experiments animals were killed by overdose of anaesthetic, confirmation of cessation of heart beat and this was followed by cervical dislocation.

Rats were anaesthetized 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 vapourized 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) so that the presentation and withdrawal of stimuli were accurately represented on acquired data and the 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 × 2000 (NL104A), and band-pass filtered between 0.5 and 1000 Hz (NL125) before being sampled at 2 kHz using SPIKE2 software via 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 the temperature of the heating block was continually monitored in this way.

Sensory stimulation

Noxious thermal stimuli (55 °C) 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 24-V DC supply. The area of the block coming into contact with the paw was a circular region of 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 preheated 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) to ensure stability of anaesthesia. A craniotomy was also performed overlying the RVM (anterior-posterior, 11.5 mm; medio-lateral, –2.0 to 2.0 mm) and the dura resected using a 31-gauge hypodermic needle (Becton-Dickinson, Oxford, UK).

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Data analysis

All data analyses were performed with custom-written routines in MATLAB (R2012a; MathWorks, Natick, MA, USA). Five basic peristimulus 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 the 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 the firing rate returned to 10% of the maximum response amplitude). Any stimulus onset/offset artefacts 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 represented the time at which EMG activity exceeded 10% of the maximum EMG amplitude; this enabled 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 represented the stimulus onset time; this enabled the timing of SUA and EMG activity relative to the stimulus.

Two analyses were performed to determine if the RVM may play a role in the shaping of withdrawal reflexes. 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 regression 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 (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, was performed on data downsampled 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 peristimulus 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, and OFF cell-associated EMG) in cases where comparison between ages and cell/response type was important. A Chi-squared 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 cells and EMG responses. In all other cases, two-tailed t-tests were performed to compare between, e.g. pooled EMG data between ages, and are clearly stated in the text. All data are given with the mean ± SEM in the text and 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 hindpaws. 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, Fig. 2B, D, F and H). The baseline activity of responsive cells was occasionally found to fluctuate, often with the depth of anaesthesia (measured using the EEG), but careful control of the anaesthetic supply to maintain animals within anaesthetic state III-3/2 (using visual confirmation) ensured that 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 and 21.6 ± 3.8 Hz, respectively) ($t_{24} = 1.943, P = 0.0639$; Fig. 3A, B, F and 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 (Fig. 3F and 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 were 3.5 Hz and 32.7%.

Adult electromyography reflexes and rostral ventromedial medulla responses

The data in Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3G); the difference between OFF cell and EMG onset latencies was significantly different from zero ($t_{11} = 5.04,$
As with the ON cell response, OFF cell activity, on average, reached its trough at the same time as EMG activity (Fig. 3G). The fact that both ON and OFF cell activity began before EMG reflex activity yet all reached 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 ranged between −2.9 and +0.25 s) cautions against the assumption that a direct relationship existed between the onset of ON or OFF cells and EMG activity or that the former shaped the latter. Furthermore, cellular activity outlasted EMG reflex activity, i.e. the average duration of ON and OFF cell responses 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: $48.97 \pm 9.25$ s vs. $10.01 \pm 1.66$ s; $P < 0.01$; OFF cell: $62.71 \pm 9.51$ vs. $18.85 \pm 5.04$ s; $P < 0.001$; Fig. 3E and G, lower panels).

To investigate the role of the RVM in shaping ongoing reflex activity and to 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 (Fig. 4A and C). Co-relationships of the EMG reflex and SUA responses were then examined (Fig. 4B and D). Although both ON and OFF cells showed good agreement with the rise in EMG reflex activity, there was a divergence in the return phase. Data in Fig. 3E and G had already shown that the duration of SUA responses was longer than the EMG reflex, but ON and OFF cells also differed in this respect, i.e. 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 outlasted EMG reflex activity to a greater extent than ON cell activity, seen clearly in Fig. 4A and C. 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 (Fig. 4B and 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 agreed 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, Fig. 4A and C shows 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 followed the EMG response.

**Age-dependent differences in electromyography reflex activity**

There were marked differences in EMG reflexes evoked from juvenile and adult rats (Fig. 5A). The thermal stimulus was applied until a response occurred and reached maximum activity levels earlier (2.50 vs. 3.05 s; unpaired t-test, $t_{23} = 6.64$, $P < 0.0001$; Fig. 5B) and reached maximum activity levels earlier (2.50 vs. 5.60 s; $t_{23} = 10.74$, $P < 0.0001$; Fig. 5B). The maximum response amplitude was also larger in juvenile rats (28.45 vs. 10.73 μV; $t_{23} = 4.41$, $P < 0.0001$; Fig. 5C) yet juvenile reflexes were shorter than in adults (response duration: 4.96 vs. 14.09 s; $t_{23} = 3.40$, $P = 0.0014$; Fig. 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 10 stimulus presentations (median, 5; mode, 6). The baseline firing rates of ON cells were significantly lower than OFF cells in juvenile rats (3.71 ± 1.12 vs. 18.69 ± 5.12 Hz; $t_{23} = 3.19$, $P = 0.004$; Fig. 6A, B, F and 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 (Fig. 6A, B, F and 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 were 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 neutral cell types in the different ages did not differ significantly ($P > 0.05$, Chi-squared test; Fig. 1C).

**Juvenile electromyography reflexes and rostral ventromedial medulla 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 (Fig. 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 (Fig. 6E). However, individual OFF cell responses did not all begin before the reflex; the average onset time of OFF cell responses was 0.05 ± 0.24 s (Fig. 6G). The average duration of ON and OFF cell responses also followed the adult data and was significantly longer than the duration of the associated EMG reflex activity (significant effect of
Age-dependent relationships between rostral ventromedial medulla and electromyography reflex activity

We have shown that thermal stimulation of the hindpaw produced 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 (Fig. 5). Despite the shorter stimulus-aligned onset latency of EMG reflexes in juvenile animals as compared with adults (shown in Fig. 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, \(F_{1,54} = 4.7, P = 0.0041\); juvenile ON cell-associated EMG: 1.48 ± 0.06 s vs. adult ON cell-associated EMG: 2.98 ± 0.31 s, \(P < 0.0001\); 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 and 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: \(t_{26} = 4.58, P < 0.0001\), Fig. 8C; juvenile vs. adult OFF cells: \(t_{21} = 5.03, P < 0.0001\), Fig. 8E), which corresponded to larger maximum gradients in EMG reflex responses (juvenile vs. adult ON cell-associated EMGs: \(t_{26} = 7.40, P < 0.0001\), Fig. 8D; juvenile vs. adult OFF cell-associated EMGs: \(t_{21} = 5.28, P < 0.0001\); Fig. 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 (Fig. 8C vs. D and E vs. F).

The 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, i.e. 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 (Fig. 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 with 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 = 0.005)\;
FIG. 6. Juvenile SUA and EMG responses to noxious thermal stimulation. ON cell data are shown in panels on the lefthand side, and OFF cell data in panels on the righthand side. Average ON cell (A; n = 14) and OFF cell (B; n = 11) responses are shown alongside their associated average EMG responses (C and 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 SEM.

**Discussion**

The purpose of the current study was to test the most recent theory of RVM function, forwarded by Hellman & Mason (2012) with a non-pharmacological and non-pathological challenge to the model by using different aged rats that exhibit dissimilar 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 with 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, whereas the model of Hellman & Mason (2012) 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. Although 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, although 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 been identified, despite the first identification of the role that 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 pronociceptive and OFF cells as antinociceptive (Fields et al., 1983), supported by the finding that morphine consistently decreased tonic ON cell firing (Heinricher et al., 1992). Some theories suggested that it was tonic firing of ON and OFF cells that was vital in

\[ R^2 = 0.03, F_{1,10} = 0.25, P = 0.62 \] (all compared with the maximum of their associated EMG responses).
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 the 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 (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 artefact of general anaesthesia and do not exist in awake animals (Oliveras et al., 1989, 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 of the entirety of the hindlimb 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) but does not obscure 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’ (Figs 4A and C, and 6A and C). To provide individual subject data in a clear manner, we extracted and displayed basic temporal parameters from each RVM cell response and withdrawal reflex (e.g. Fig. 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. (2008) 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. (2008) was not applied to the hindpaw but to the tail, which precluded any comparison of RVM cell and reflex profiles; this is because reflexes in the tail

Fig. 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 normalized and activity preceding response onset removed (A and 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-relationships between cellular and EMG reflex responses are shown with markers indicating 0.4 and 0.6 s, and then at 1-s intervals until 10 s (B and D). Error bars and shaded regions represent SEM.
manifest as brief flicks rather than temporally-broader withdrawals as in the hindpaw.

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 support 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 (Fig. 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 that was influencing the ongoing withdrawal reflex, and suggests that 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 (Fig. 8A). In the absence of a phasic ON cell response with a positive rate of change, coupling between the 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 that could allow the RVM to

Fig. 8. Comparison of response profiles between the ages. ON cell (A) and OFF cell (B) responses have been normalized and are aligned to stimulus onset time (i.e. at time = 0); EMG responses are also shown. Normalization enables comparison between age and response parameters and illustrates similarity of onset latency of all SUA responses, also highlighting the difference in response gradient between the ages. Gradients of ON cell (C), EMG associated with ON cells (D), OFF cells (E) and EMG associated with OFF cells (F) 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 SEM.

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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 biphasic facilitation and inhibition in the adult (Hathway et al., 2009, 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 that 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 the 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 that 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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Abbreviations

DH, dorsal horn; EEG, electroencephalography; EMG, electromyography; P, postnatal day; RVM, rostral ventromedial medulla; SUA, single-unit activity.

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