Differential suppression of spontaneous and noxious-evoked somatosensory cortical activity by isoflurane in the neonatal rat

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Abbreviated Title: Neonatal cortical pain activity and isoflurane

Abstract:

Background: The effect of neonatal anesthesia and pain on the developing brain is of considerable clinical importance but few studies have evaluated noxious surgical input to the infant brain under anesthesia. Here we tested the effect of increasing isoflurane concentration upon spontaneous and evoked nociceptive activity in the somatosensory cortex of rats at different postnatal ages.

Methods: Intracortical extracellular field potentials evoked by hindpaw C-fiber electrical stimulation were recorded in the rat somatosensory cortex at postnatal day (P)7, 14, 21 and 30 during isoflurane anesthesia (n=7 per group). The amplitudes of evoked potentials and the energies of evoked oscillations (1-100Hz over 3 sec) were measured following equilibration at 1.5% isoflurane and during step increases in inspired isoflurane. Responses during and after plantar hindpaw incision were compared at P7 and P30 (n=6 per group).

Results: At P7, cortical activity was silent at isoflurane 1.5% but noxious evoked potentials decreased only gradually in amplitude and energy with step increases in isoflurane. The resistance of noxious evoked potentials to isoflurane at P7 was significantly enhanced following surgical hindpaw incision (69±16% *vs* 6±1% in non-incised animals at maximum inspired isoflurane). This resistance was age dependent; at P14-30, noxious evoked responses decreased sharply with increasing isoflurane (step 3 (4%) P7: 50±9%, P30: 4±1% of baseline). Hindpaw incision at P30 sensitized noxious evoked potentials, but this was suppressed by higher isoflurane concentrations.

Conclusions: Despite suppression of spontaneous activity, cortical evoked potentials are more resistant to isoflurane in young rats and are further sensitized by surgical injury.

Introduction

An optimal level of neonatal anesthesia achieves both hypnosis and anti-nociception while maintaining physiological stability and minimising potential neurotoxicity [1,2]. As both anesthesia and uncontrolled pain may alter cortical activity and impair neurodevelopmental outcomes [3–5], the impact of anesthetic agents upon both spontaneous and noxious-evoked neural activity in the developing brain requires further evaluation. An important aspect of neonatal anesthesia research is the effect of nociceptive sensory input on activity within cortical sensory circuits and the degree to which central nociceptive activity is modulated by anesthesia and analgesia. Both animal and clinical evidence point to long term consequences of early life procedural and surgical tissue injury upon somatosensory and nociceptive systems[6,7], highlighting the need to consider the impact of postnatal age on changes in both spontaneous and noxious-evoked cortical activity during surgery and anesthesia.

Extracellular field recording, including electroencephalogram (EEG), electrocorticogram (ECoG) and local field potentials (intracortical activity) are commonly used to monitor ongoing spontaneous brain activity and levels of anesthesia in human and rodent neonates but are also used to record specific potentials evoked by a sensory stimulus. Somatosensory potentials evoked by experimental noxious cutaneous stimulation [8–11] are commonly used to measure pain activity in the adult human and rodent brain [12]. Specific nociceptive potentials are also evoked by single, clinically required skin breaking procedures in the human infant brain[13,14] and have been used in this age group to measure the postnatal development of cortical pain processing[10]. In adult humans and rodents, nociceptive evoked potential amplitudes decrease with increasing concentrations of isoflurane [9,15] but, to date, the sensitivity of nociceptive potentials to anesthesia in infants has not been studied. In a study of the whisker barrel cortex of neonatal rats, sensory potentials evoked by whisker deflection persisted at surgical isoflurane levels (1.5-2%) that completely suppressed the EEG and silenced

spontaneous neuronal firing[16]. This suggests that noxious evoked potentials and spontaneous intracortical activity might be differentially sensitive to anesthesia in infants. Furthermore, since nociceptive potential amplitudes can be increased by peripheral C fiber sensitization in both humans and rat cortex [8,11], surgical injury may enhance evoked activity and add to this differential response to anesthesia in infancy.

The primary aim of this experimental laboratory study was to test the impact of increasing isoflurane concentration upon the pattern of spontaneous and evoked nociceptive activity in the somatosensory cortex of infant rats undergoing hindpaw incision. In addition, we compared effects of isoflurane on cortical activity at different postnatal ages. We hypothesised that, in infant rats, noxious evoked brain activity is more resistant than spontaneous brain activity to isoflurane anesthesia and that this difference declines with postnatal age.

Materials and Methods

Animals

All experiments were performed under personal and project licences approved by the Home Office, London, United Kingdom under regulations of the United Kingdom Animal (Scientific Procedures) Act, 1986. Male Sprague-Dawley rats aged postnatal day (P) 7, 14, 21 and 30, were obtained from the Biological Services Unit, University College London. All animals were from the same colony, bred and maintained in-house, and exposed to the same caging, diet and handling throughout development. Rats were housed in cages of six age-matched animals (P30) or with the dam and littermates (P7, 14 and 21) under controlled environmental conditions (24–25°C; 50–60% humidity; 12 h light/dark cycle) with free access to food and water. Animals were randomly picked from litters by hand for recording and alternately assigned to an incision or no incision group. Treatment groups were distributed across multiple litters and/or adult cage groups. At the end of an experiment, the isoflurane was increased to 5% until there was no heart beat and the neck dislocated.

Cortical recordings

Rats were anaesthetised with 4% isoflurane (Abbot, AbbVie Ltd., Maidenhead, United Kingdom) in 100% oxygen via a nose cone, and following insertion of a tracheal cannula were mechanically ventilated (Small Animal Ventilator, Bioscience, Kent, United Kingdom) with 100% oxygen and isoflurane delivered from a recently calibrated vaporizer (Harvard Apparatus, Cambridge, MA). The adjustments for ventilation of rats across the ages were based on the breath rates and tidal volume. The intermittent positive pressure ventilation was achieved by using a T-type system in conjunction with a small animal lung ventilator. This system affords control of inspired gas mixture (isoflurane concentration and oxygen) inspiratory flow rate, respiratory rate and peak inspiratory pressure. A simple water manometer placed in the inspiratory limb provided a monitoring and

pressure limiting device with visual display. Using an inspiratory flow rate between 1-1.5 litres per minute and adjusting the peak inspiratory pressure between 12-15 cm water [17] allowed the tidal volume to be adjusted according to the size of animals. This mode was confirmed to be adequate in our lab by using a transcutaneous combination probe to monitor transcutaneous oxygen and carbon dioxide. Body temperature was maintained with a thermostatically controlled heated blanket and the electrocardiogram was monitored throughout (Neurolog, Digitimer, Welwyn Garden City, United Kingdom).

During anesthesia with 2.5% isoflurane, animals were placed in a stereotaxic frame and a craniotomy was performed to expose the surface of the cerebral cortex. A recording electrode (stainless steel, E363/1, Plastic One Inc. Roanoke, Virginia, USA) was inserted into the primary somatosensory cortex in the somatotopic region for the hindpaw. Coordinates for P7 and P14 rats were lateral 2.0 mm from midline and posterior 0.5 mm from the bregma; and for P21 and P30 rats were lateral 2.5 mm from midline, and posterior 1 mm from the bregma [18,19]. The reference electrode was placed subcutaneously on the surface of the skull anterior to the bregma, and the ground electrode was placed subcutaneously in the back. The inspired isoflurane concentration was then reduced to 1.5% and allowed to equilibrate for at least 40 minutes.

Continuous intracortical activity recordings were performed using a Neurolog NL100 headstage connected to a NL104 amplifier and a NL125 filter (100 Hz). The signal was digitized at 16K Hz using Axon Instruments (Digidata 1400A, Molecular Devices, Sunnyvale, CA). Data was acquired and stored using a Windows PC based programme, WinEDR v3.3.6 (John Dempster, University of Strathclyde, United Kingdom) for later analysis. The depth of the recording electrode was adjusted to optimise the somatosensory evoked potential amplitude. At the end of an experiment, the brain was removed and immediately immersed in 4% paraformaldehyde for over 24 hours then transferred to 30% sucrose post-fixation solution. Brain sections (40-µm thick thickness) were cut using a micotome

(Leica SM2000R, Leica Microsystems (UK) Ltd, United Kingdom) and stained with cresyl violet for histological location of the electrode track. This procedure verified that recordings were in layer 5-6 of the somatosensory cortex (Fig. 1). All the data recorded were included in this study and no animals were excluded.

Electrical stimulation of the hindpaw

Two stainless steel pin electrodes were placed subcutaneously 3-5 mm apart on the plantar hindpaw, contralateral to the cortical recording site. A train of 10 stimuli of 3.2 mA, 500 µs were applied at 10 sec interstimulus intervals, using a constant current stimulator (Neurolog, Digitimer, Welwyn Garden City, UK). These stimulation parameters are sufficient to recruit both A and C fibers[20] (here called 'C fiber' stimulation) and were established in pilot experiments to evoke clear potentials restricted to the somatosensory cortex. At all ages, electrical hindpaw stimulation failed to evoke visible hindlimb reflex responses at 1.5% or higher inspired isoflurane concentrations. In a separate group of P7 animals, a train of lower intensity stimuli of 0.32mA, 50 µs was also applied, sufficient to recruit only A beta fibres[20] (here called 'A fiber' stimulation), for comparison.

Plantar hindpaw incision

Plantar hindpaw incision was performed in P7 and P30 animals (*n*=6 per age group) following equilibration at 1.5% isoflurane in oxygen. A midline longitudinal incision through the skin and fascia extended from the midpoint of the heel to the proximal border of the first footpad to incise a similar relative length of paw at different ages and the underlying plantar muscle was elevated and incised [21,22]. Skin edges were closed with 5-0 nylon suture (Ethicon, Edinburgh, United Kingdom), and the procedure took 2.5 to 3 minutes. The initial skin incision evoked a brief visible muscle reflex in young animals (P7), but not in older animals (P30).

Experimental timeline

The experimental timeline is illustrated in Figure 1. Following surgical preparation and equilibration at 1.5% inspired isoflurane concentration, spontaneous intracortical activity was recorded for 100 s, followed by evoked activity during hindpaw electrical stimulation in P7, P14, P21 and P30 rats (Fig. 1, Group 1). In pilot studies, and subsequent experimental recordings, it was clear that increasing the inspired isoflurane concentration rapidly altered spontaneous activity. Therefore, the inspired isoflurane concentration was increased at 5 minute intervals (step 1 = 2%, step 2 = 3%, step 3 = 4%, and step 4 = 5%), and spontaneous and evoked potentials were measured in the 3-5 minute period after each step. The step terminology was adopted because this frequency of change would not have allowed sufficient time for equilibration at each inspired concentration. However, this protocol allowed us to assess the response to increasing isoflurane exposure without the cardiovascular compromise associated with more prolonged exposure to high concentrations.

In additional groups at P7 or P30 a plantar hindpaw incision was performed following equilibration at 1.5% isoflurane (Fig. 1, Group 2). In these experiments, spontaneous activity was measured (sampled from a 100s epoch) in the 2 minute period before, during, and 2 minutes after plantar hindpaw incision with inspired isoflurane maintained at 1.5%. Ten minutes following plantar hindpaw incision, recordings were performed during step increases in inspired isoflurane concentration as described for Group 1.

Electrocardiography confirmed that the heart rate remained stable throughout the recording periods at 1.5% isoflurane in both groups. At all ages, the heart rate was reduced by Step 4. When expressed as percentage change from baseline (as heart rate differs with postnatal age in rodents), the degree of change in heart rate was not significantly different at P7 and P30 (81±3% v. P30: 88±3%, p=0.157, Student's T test) (data not shown).

Statistical Analysis

Sample sizes (*n*=7 animals for each age) were based upon previously published group effects of volatile anesthesia on cortical activity. Statistically significant dose-dependent effects of isoflurane on evoked responses have been reported in the somatosensory cortex of adult rats (*n*=5)[9] and in the barrel cortex of neonatal (P2-7) rats (*n*=6)[16].

Ongoing spontaneous intracortical activity was analysed in 100 second epochs at each isoflurane step and before, during, and after plantar hindpaw incision. Frequency domain analysis was performed by Fast Fourier Transformation (FFT) (Spectrum type: one-sided) using the 'Welch Window' [23]. Intracortical responses were converted from the time domain to the frequency domain and the Fourier transform components amplitude (millivolts per Hertz, mV/Hz) was computed using OriginPro 9 (OriginLab Corporation, Northampton, Massachusetts, USA). Brain waves have been categorized into five frequency bands: delta (δ=2-4 Hz), theta (θ =4–8 Hz), alpha (α =8–12 Hz), beta (β= 13–30 Hz), and gamma (γ>30 Hz)[24]. Network oscillations with a range of frequencies are thought to control the flow of information through anatomical pathways, and communicate among local networks. Changing patterns of network oscillation are tightly correlated with behavior or features of sensory stimuli[25]

Individual noxious evoked potentials were averaged (10 stimuli per animal) and peak amplitudes were computed for each animal. Data are given as grand mean ± standard error of the mean (SEM) (number of stimuli * number of animals). In addition evoked potentials at Steps 2-5 were normalised to baseline (1.5 % of inspired isoflurane) and presented as % of baseline. Statistical analysis of peak amplitudes was performed using one way repeated measures ANOVA (RM ANOVA) followed by Dunnett's *post-hoc* comparison tests.

Time frequency (TF) analysis was also used to detect the energy of the oscillations in the brain evoked by the noxious electrical stimulus. Time frequency analysis has been extensively used in functional studies of brain activity in humans and in animal models [10,26,27]. First, continuous

intracortical activity was high-pass filtered at 0.5 Hz with a zero phase 2nd order Butterworth filter. TF analysis was then performed using a complex Morse wavelet transform [26,28]. This allowed us to calculate a complex time-frequency spectral estimate W(a,b) of the intracortical activity at each point (a,b) of the time-frequency plane from 3s before the stimulus to 3s after stimulus in the time domain, and between 0.5 and 100 Hz (in logarithmic steps) in the frequency domain. The changes in time-frequency spectral energy (i.e., modulus square) in the intracortical activity in response to the stimulation were estimated by normalizing to the mean energy content of the baseline period (a period of quiescence of 1 sec) at each frequency [10]. The time-frequency energy, time-locked to the stimulation in each group (anesthetic step, age, incision), was presented as a group median. The stimulus-induced energy changes, time-locked to each stimulus, were estimated separately at each postnatal age and anesthetic step.

Results

Noxious evoked somatosensory cortical activity is resistant to isoflurane anesthesia in neonatal rats

We first recorded intracortical spontaneous activity in the primary somatosensory cortex of P7 rats following 40 minutes equilibration at 1.5% isoflurane and through subsequent step increases in inspired concentration (*n*=7 animals). Figure 2A shows typical traces of S1 intracortical activity at each inspired isoflurane concentration. No spontaneous activity was recorded in any frequency band in the P7 primary somatosensory cortex at 1.5% inspired isoflurane or at higher concentrations (Figure 2B)

Despite the lack of spontaneous activity, hindpaw electrical stimulation evoked clear low amplitude evoked potentials, with simple positive-negative voltage waveforms, in the P7 somatosensory cortex (Figure 3). A significant decrease in the mean evoked potential amplitude did not occur until step 3, where it dropped to half the amplitude recorded at 1.5% inspired isoflurane (50±9%) (Figure 3A & B). At the maximum inspired isoflurane concentration (step 4), evoked potentials were greatly diminished (6±1%) but importantly, were still clearly detectable in five of the seven animals studied. A time frequency analysis of this evoked activity is shown in (Figure 3C) to demonstrate the mean energy across different frequencies (1-100Hz) evoked in the 3 seconds following noxious electrical stimulation. At 1.5% inspired isoflurane, a strong increase in energy at all frequencies is observed in the first 500 milliseconds (ms) post stimulus, but this declines with increasing levels of inspired isoflurane, notably at Step 3 and 4 (Figure 3C). At Step 4 the oscillation energy is greatly reduced, but still present.

We next tested whether hindpaw incision influenced the sensitivity of plantar C-fiber evoked potentials to step increases in anesthesia. The immediate effect of the plantar incision is shown in Figure 4. In P7 rats, spontaneous activity was increased during, and immediately after, the incision,

(delta band activity (0.02±0.01 µV/Hz to 0.10±0.02 µV/Hz; RM ANOVA $F_{(3, 15)}$ = 13.5, P=0.0002; post hoc Dunnetts P<0.001), and beta band activity (0.01±0.00 µV/Hz to 0.02±0.01 µV/Hz, RM ANOVA, $F_{(3, 15)}$ = 6.1, P=0.0062; post hoc Dunnetts P<0.05), which returned to baseline at 10 mins (Figure 4A and B). In addition Figure 3 shows that hindpaw incision had a more persistent effect on the response to subsequent noxious electrical stimuli. In animals with prior hindpaw incision, the mean peak amplitude of the electrical evoked potential was not significantly altered by step increase in isoflurane level, except at step 4 (RM ANOVA $F_{(4, 20)}$ = 3.7, P=0.0210; post hoc Dunnetts P<0.01 between baseline 1.5% and step 4), and even at the maximal inspired isoflurane concentration, the mean response remained at 67±16% of the peak amplitude evoked at the initial 1.5% isoflurane concentration (Figure 3D and E). This is further confirmed by the time frequency analysis (Figure 3F). At baseline 1.5% isoflurane and at step 1, the strong increase in energy at all frequencies in the first 500 ms post stimulus does not differ in animals with and without skin incision, but this is not so at higher levels of isoflurane. Thus, following hindpaw incision, the energy of cortical activity does not diminish with increasing inspired concentration of isoflurane. Comparison of oscillation energies with and without plantar hindpaw incision at Step 4, shown in Figure 3, shows that the presence of surgical injury increases the resistance of infant S1 cortex nociceptive activity to isoflurane (Figure 3C and Figure 3F, bottom panels).

To test whether this effect of skin incision was nociceptive specific, we compared the effects upon potentials evoked by low intensity innocuous A fiber stimulation versus those evoked by high intensity noxious C fiber stimulation in the same animal. Figure 5A and B show that, while in intact skin A fiber stimulation evokes distinct potentials which slowly diminish with increasing levels of isoflurane, following skin incision the A fiber evoked response is completely absent at all isoflurane levels (Fig. 5C and D). This is in marked contrast to the C fiber evoked potentials which persist unchanged following skin incision at all but the highest step level of isoflurane (Fig.5E and F).

Effects of isoflurane on spontaneous intracortical activity are influenced by postnatal age

We next examined the effect of postnatal age upon the relationship between primary somatosensory cortical spontaneous and evoked activity at increasing inspired isoflurane concentrations. To do this, the same experiment was performed at P14, P21 and P30 and the results compared with those obtained at P7.

Figure 2 shows the intracortical spontaneous activity in the primary somatosensory cortex of the rat, at the four postnatal ages following equilibration at 1.5% isoflurane and through subsequent step increases in inspired isoflurane concentration (*n*=7 animals at each age). In contrast to the lack of spontaneous activity at P7, Figure 2A shows that at P14, P21 and P30, an inspired isoflurane concentration of 1.5% induced burst-suppression activity in the somatosensory cortex, characterized by intermittent, highly synchronized neuronal discharges (bursts) separated by silent periods (suppression), as described elsewhere [29]. The burst-suppression activity disappears as inspired isoflurane concentration increases. Figure 2B shows the distribution of frequency components of spontaneous somatosensory cortical activity at each age. At P14-30, activity across all frequencies decreases steadily as the inspired isoflurane concentration increases, and there is no spontaneous activity and an iso-electric intracortical activity by step 4.

Noxious-evoked activity in the somatosensory cortex is more sensitive to increasing isoflurane concentration in older rats

We next compared the primary somatosensory (S1) cortical potentials evoked by noxious, C fiber intensity electrical stimuli in the contralateral hindpaw (10 stimuli/step/animal, n=7 animals) in P7, P14, P21 and P30 rats during increasing inspired isoflurane concentrations (Figure 6). Sample traces are shown in Figure 6A and the mean peak amplitude of the evoked potential is plotted in Figure

6B, as the percentage decrease with each step increase in isoflurane, normalized to the initial values recorded following equilibration at 1.5% isoflurane. The relative decrease in evoked potential peak amplitude with each step increase in isoflurane is more marked in older animals (P14, P21, P30) than at P7. At step 3, the mean peak amplitude has reduced to half in P7 animals (50 ± 9% of initial 1.5% isoflurane values), in contrast to P21 and 30 where it drops below 10% (P21: 6 ±1 %; P30: 4 ±1 %). At Step 4, no discernible evoked potentials were recorded from any P21 or P30 animals but were recorded from three out of seven P14 animals, and five out of seven P7 animals.

Figure 7 shows time frequency analysis of evoked activity at each age. The mean energy of cortical activity across different frequencies (1-100Hz) evoked in the 3 seconds following noxious electrical stimulation is shown at the four steps of increasing inspired isoflurane (10 stimuli/step/animal, n=7 animals for each age group). At all ages, the evoked response energy decreases with increasing inspired isoflurane. Whereas noxious evoked cortical activity is relatively resistant to increasing isoflurane concentration at P7, there is a gradual increase in the degree of suppression by isoflurane at older ages (Figure 7).

The effect of hindpaw incision upon cortical nociceptive activity at P30 differs from that at P7

As surgical incision had a profound effect upon the subsequent cortical response to noxious electrical stimulation at P7, we tested whether this also occurred at an older age. Figure 4 shows that there was no immediate effect of the plantar incision upon spontaneous activity during, and immediately after, the incision at P30 (Figure 4C and D). Figure 8 illustrates the effect of plantar incision upon nociceptive evoked potential at each anaesthetic step in P30 rats. Electrical evoked nociceptive potentials are progressively suppressed by increasing inspired isoflurane concentration, in the absence (Figure 8A and B) and presence (Figure 8C and D) of skin incision. The reduction in peak evoked potential amplitude produced by step increases in inspired isoflurane (Figure 8B) was not

altered following hindpaw incision (Figure 8D). There was a steady fall in mean evoked potential amplitude, expressed as a percentage change from baseline (1.5% isoflurane) in both non-incised (Step 1, 88±14; Step 2, 62±8; Step 3: 4±1; Step 4: 1.4±0.5, $F_{(4, 20)} = 49$, $P<0.0001$) and incised groups (Step 1, 62±9; Step 2, 50±19; Step 3: 3.5±1; Step 4: 0.13±0.1, $F_{(4, 20)} = 25$, $P<0.0001$) (Figure 8B and 8D). Time frequency analysis (Figure 8E and 8F) shows that skin incision at 1.5% isoflurane causes an increased energy and duration of evoked cortical oscillations at P30, not seen at P7, suggesting some underlying sensitization of cortical pain circuits. Although clear at an inspired isoflurane concentration of 1.5%, this increased cortical response is highly sensitive to subsequent increases in isoflurane, and the difference in response between P30 animals with (Figure 8F) and without (Figure 8E) hindpaw incision is largely lost at step 1, and evoked responses in both groups are significantly diminished at step 2 and 3 and effectively gone at step 4. These results differ significantly from those obtained at P7 (Figure 3), where cortical evoked potentials and oscillatory activity persisted and were resistant to increasing inspired isoflurane concentration.

Discussion

The primary aim of this study was to test the impact of increasing isoflurane concentration upon spontaneous and evoked nociceptive activity in the somatosensory cortex of infant rats undergoing hindpaw incision. We hypothesised that, in infant rats, noxious evoked brain activity is more resistant than spontaneous brain activity to isoflurane anesthesia. The results, obtained by recording intra cortical neuronal activity from layer 5-6 of the somatosensory cortex in postnatal day (P)7 rats during increasing inspired concentrations of isoflurane , support this hypothesis. The data show that isoflurane influences spontaneous activity and evoked activity in the infant rat somatosensory cortex quite differently.

At P7, all concentrations of inspired isoflurane (1.5-5%) silenced cortical neurons and suppressed spontaneous bursts and oscillations. This effect is consistent with a previous study in the neonatal somatosensory cortex, where cortical activity was completely suppressed in P7 animals by 1.5-2% isoflurane[16]. This is in contrast to the spontaneous cortical activity observed in awake or very lightly anesthetised animals at this age, which is characterized by intermittent bursts organized in oscillations in alpha-beta (spindle bursts) and gamma frequency bands (early gamma oscillations, EGOs)[16,30–33].

In contrast to the absence of spontaneous activity, noxious somatosensory evoked potentials and evoked oscillatory activity persisted, even at high inspired concentrations of isoflurane. This result highlights the differences in the neural mechanisms generating spontaneous activity and evoked activity in the immature somatosensory cortex. Activity in the developing brain changes with age and early cortical development is marked by unique patterns of activity as functional circuits mature[34,35]. Early oscillatory bursts are generated in thalamocortical circuits [32,36] but can also be triggered by sensory inputs and are likely to be generated by glutamatergic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptors while GABAergic

(gamma-aminobutyric acid releasing) interneurons compartmentalize the activated areas via surround inhibition[37,38]. The transition from predominantly nonspecific neuronal bursts to specific cortical sensory evoked potentials appears to be triggered by increasing sensory input [31] in the neonatal rodent somatosensory cortex [16]. A similar transition begins in the preterm human infant brain at around 35 weeks postconceptional age [26,39]. Thus the nociceptive evoked activity in the newborn infant cortex recorded at P7 is still relatively immature and may be less tightly coupled within specific networks, rendering it less susceptible to suppression by volatile anesthetics than at older ages.

A further finding here is that the relative insensitivity of noxious evoked activity to isoflurane was enhanced following plantar hindpaw incision. This well-established model of surgical incision pain[21], adapted for younger rat pups [40,41], rendered infant rat nociceptive evoked cortical potentials and oscillatory network activity totally resistant to the maximum inspired isoflurane tested (5%). The mechanism for this is not clear but may reflect widespread depolarization of central neurons by the incoming nociceptive barrage. It is notable also that the incision itself increased cortical activity in young animals, consistent with the reported spike activity and sensitization of receptive fields in infant dorsal horn cells following hindpaw incision[42]. Importantly, the enhanced isoflurane resistance following skin incision at P7 was selective for C fiber nociceptive evoked potentials. In contrast, A fiber innocuous evoked potentials were completely abolished following skin incision at all isoflurane levels, reflecting the different sensitivities of immature A and C fibre evoked activity to skin incision[43].

A secondary outcome of this study was the significant age-dependence of the isoflurane effects upon spontaneous and noxious-evoked activity in the somatosensory cortex. At P14 and older ages, isoflurane 1.5% produced a typical pattern of burst suppression with high-amplitude low-frequency activity, and a progressive decrease in spontaneous activity with step increases in inspired isoflurane concentration, as reported elsewhere[29,44,45]. This contrasted with the suppression of spontaneous

spindle bursts and gamma oscillations even at the initial inspired concentration of 1.5% isoflurane at P7. More important was the greater sensitivity of the noxious evoked potentials to isoflurane in older animals. As reported in adult rats[9] noxious evoked potentials at P14-30 decreased markedly with increasing concentrations of inspired isoflurane and at P21 and P30 were completely absent at 5% isoflurane. Differences were especially clear using time frequency analysis, which reveals the energy and power of oscillating signals in the cortex and thus the changes within different frequency bands that are linked to specific sensory and motor functions[25]. This form of analysis has been used during isoflurane anesthesia in adult rats to characterize concentration-dependent changes in both low (30-50Hz) and high (70-140Hz) frequency gamma power in different brain regions [44].

A further secondary outcome was the failure of surgical incision to affect noxious evoked potential sensitivity to isoflurane at the older age of P30. Unlike the P7 rat, surgical incision itself at P30 caused no immediate cortical activity, but did have a sensitizing effect upon noxious cortical evoked activity at 1.5% isoflurane. This is consistent with previous reports of increased C fibre input to the adult rat somatosensory cortex in a UV-B irradiation model of hyperalgesia[8]. However, in contrast to P7 rats, the reduction of evoked potentials and oscillatory activity by isoflurane were not altered by surgical incision in rats at P30. These data suggest that the powerful effect at P7 is not due to the effects of central sensitization, but a different, as yet unknown effect.

Our aim in this study was not to compare 'hypnotic potency' at the different ages, but rather to evaluate within age-group changes in cortical activity during isoflurane anesthesia. Equipotent concentrations of volatile anesthetics have been traditionally based upon the minimum alveolar concentration (MAC) that prevents movement to a standardized noxious stimuli. However, these values do not reflect hypnotic potency in the brain[46]. The MAC of isoflurane is higher in P7-P9 rodents,[47,48] as spinal reflex excitability is greater at this age, but a clear stimulus-response relationship that is sensitive to injury, analgesia and inspired volatile agent concentration is evident across all ages

[22,49]. While isoflurane actions in the spinal cord may reduce ascending somatosensory information and indirectly alter cortical activity[50], the level of cortical-evoked activity cannot be inferred from the presence or absence of a visible reflex response. Here, hindpaw incision (but not electrical stimulation) produced a brief visible reflex response in P7 but not older animals, consistent with the reported greater MAC, but in older animals noxious stimuli produced clear cortical evoked responses at 1.5% isoflurane, despite a lack of reflex response. The age dependence of cortical evoked response sensitivity to isoflurane may have been influenced by alterations in physiological parameters. Heart rate was monitored throughout the experiments and even in the more prolonged protocols including hindpaw incision, decreases were only seen at the highest level of inspired isoflurane, and to the same degree (approximately 20% reduction) in P7 and P30 groups. Animals were mechanically ventilated in isoflurane and oxygen with age-adjusted settings, and although we cannot confirm that partial pressures of carbon dioxide (pCO2) were the same across the different ages, changes with increasing isoflurane concentration would not have been influenced by respiratory parameters.

These rat data obtained have considerable implications for clinical practice. The timing and sequence of key events in brain development are exceptionally similar across mammals and a recent neuroinformatic analysis shows that sensorimotor events in the cortex of the P7 rat translate to 1-2 months in the human [51]. This study clearly demonstrates that spontaneous intracortical network activity is more effectively silenced by isoflurane in the neonatal brain, but that isoflurane has less effect upon cortical activity evoked by peripheral noxious sensory inputs. As the developing central nervous system is vulnerable to changes in neural activity, maintaining appropriate anesthesia in infants is likely to require avoidance of both excessive reductions in cortical activity that may enhance neuronal apoptosis and excessive increases in activity due to uncontrolled noxious inputs[1–4]. Anesthesia-induced developmental neurotoxicity has been clearly demonstrated in neonatal animals[3], and the associated long-lasting cognitive impairment has raised concern for young children undergoing

anesthesia[4]. The data here highlight the complexity of using intracortical activity parameters to define or measure the level of anesthesia required to produce 'hypnosis' in neonates and infants. The persistence of noxious sensory-evoked responses despite increasing isoflurane concentration in young rats emphasizes the critical need to provide analgesia in neonates and infants. Surgery and tissue injury in neonatal rodents can produce long-term changes in sensory processing,[52] but this can be modified by morphine[53,54] or local anesthetic blockade[40,55]. Increased understanding of age- and anesthesia-dependent changes in intracortical activity may improve algorithms for evaluating depth of anesthesia in neonates and infants, and comparative studies of the ability of different types and doses of analgesic to minimize noxious evoked responses are likely to improve acute clinical care and long-term neurodevelopmental outcome following neonatal surgery.

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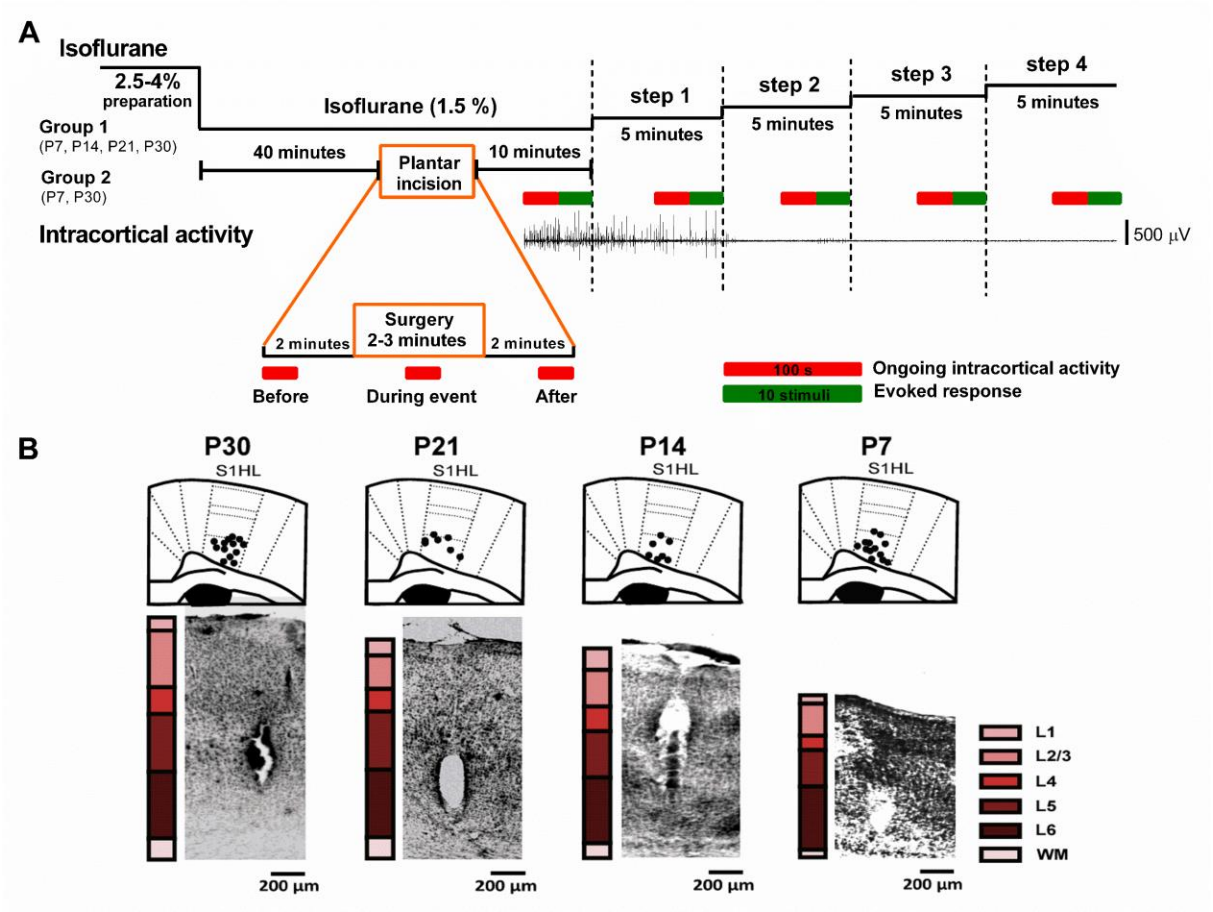


Figure 1. (A) Diagram showing the experimental timelines and recording sites. Animals were initially anesthetized with isoflurane 2.5-4% in oxygen for surgical preparation and electrode insertion, and then maintained at 1.5% for 40 minutes to allow equilibration. The electrocardiogram (ECG) was monitored throughout. Group 1: On postnatal day (P) 7, 14, 21 or 30, continuous intracortical spontaneous activity in the right somatosensory cortex was recorded for 100s, followed by evoked responses to electrical stimulation of the left hindpaw (10 stimuli of 3.2 mA for 500ms at 10s intervals). Recordings were repeated at 5-minute intervals following step increases in inspired isoflurane concentration: step 1 (2%), step 2 (3%) step 3 (4%) and step 4 (5%). Group 2: In P7 and P30 animals, spontaneous activity was recorded 2 minutes before, during and 2 minutes following left plantar hindpaw incision. Ten minutes following incision, spontaneous and electrical activity were recorded

during 1.5% and step increases in inspired isoflurane concentration. **(B)** Recording sites in layer 5/6 of the rat somatosensory cortex. Schematic representation of the location site of intracortical recording in the area of primary somatosensory representing the hindpaw (S1HL) in rats aged postnatal day (P) 7, P14, 21 and 30 (P7=12; P14=6; P21=6; P30=12). The locations were determined by post-hoc histological analysis and recording sites (filled circles) were reconstructed using a standard stereotaxic atlas (Paxinos and Watson 1998). L1, layer 1; L2/3, layer 2 and 3; L4, layer 4; L5, layer 5; L6, layer 6; WM, white matter.

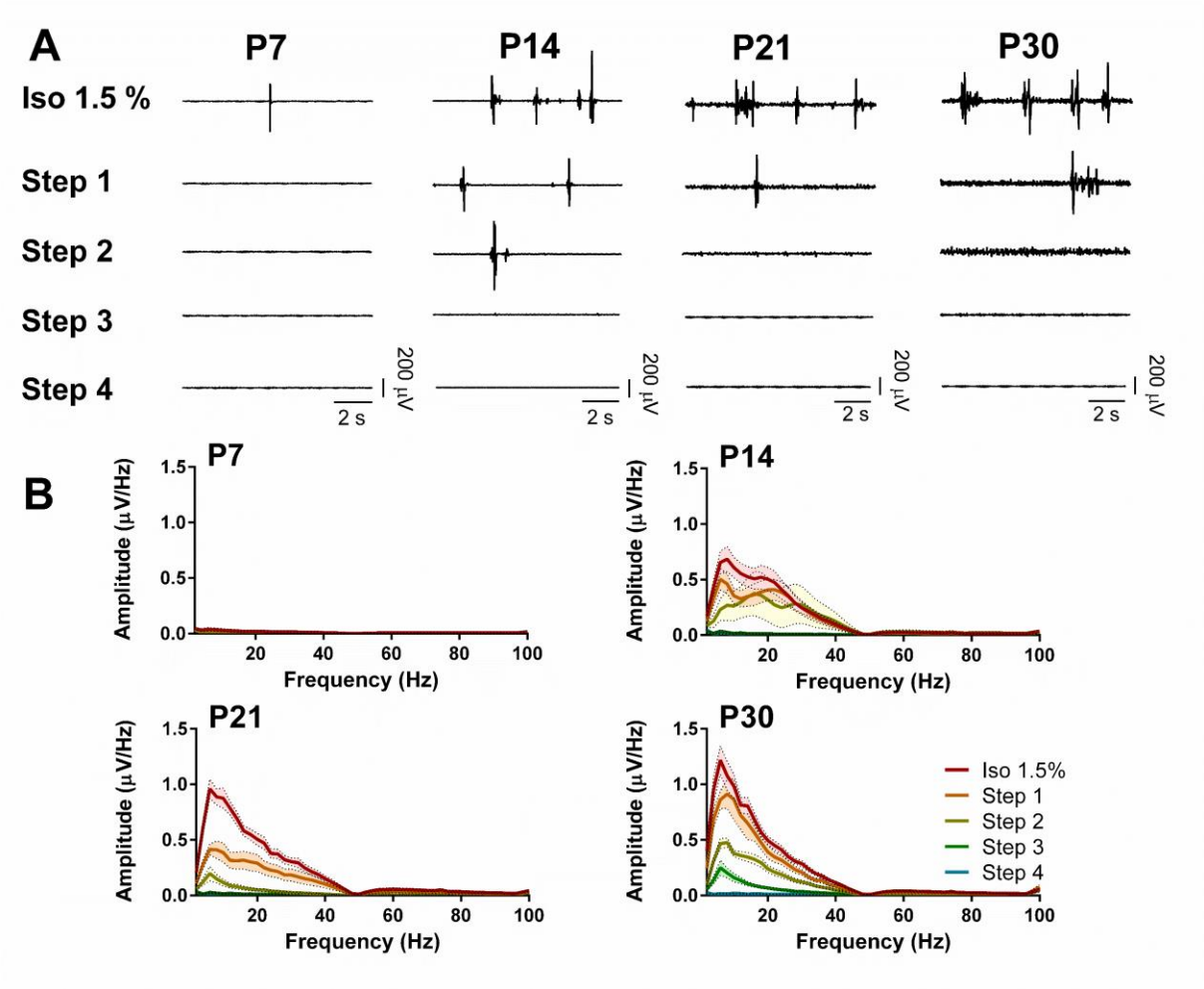


Figure 2. The effect of postnatal age and inspired isoflurane concentration upon ongoing spontaneous somatosensory intracortical activity. **(A)** Typical 10s traces of spontaneous activity at 1.5% inspired isoflurane (iso 1.5%) and during step increases every 5 min in inspired isoflurane (step

1: 2%; step 2: 3%; step 3: 4%; step 4: 5%) in rats aged postnatal day (P) 7, P14, 21 and 30. **(B)** Spectral properties of intracortical activity (frequency range: 1-100 Hz) at each age and inspired isoflurane concentration. Spectral analysis (Fast Fourier Transformation, frequency range: 1-100 Hz) was performed on 100s epochs. Mean ± SEM (n=7 animals per age).

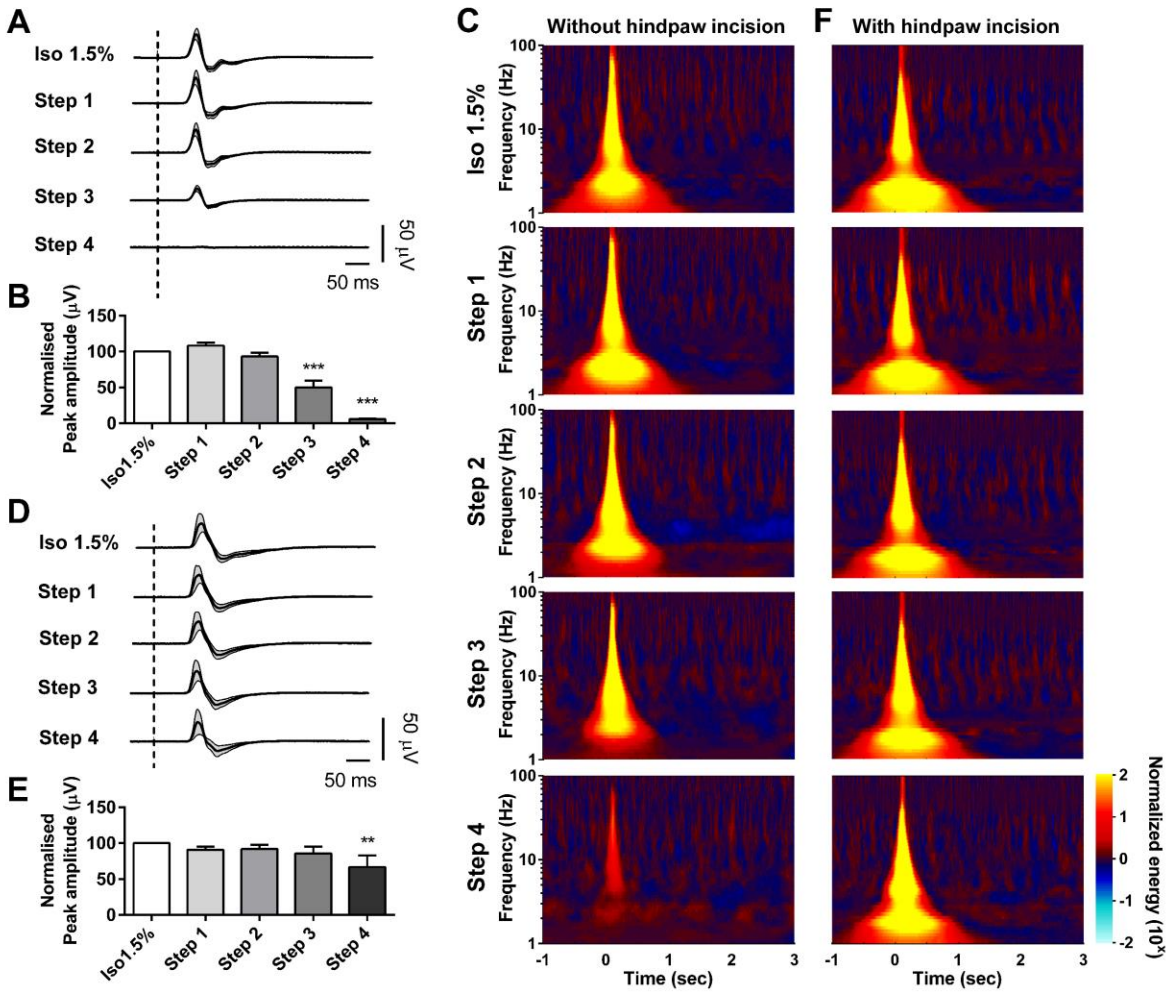


Figure 3. Effect of inspired isoflurane concentration on noxious-evoked activity in the primary somatosensory cortex at postnatal day 7 (A) Typical recordings of right somatosensory-evoked potentials (SEPs) following electrical stimulation of the left hindpaw (10 x 3.2 mA, 500 µs stimuli applied at the time indicated by the dotted line) during increasing inspired isoflurane (Iso) concentrations. Grand average evoked potentials (filled lines) ± SEM (grey area) from 10 stimuli per

animal (n=7 animals). **(B)** Bar chart showing the peak evoked potential amplitude at each isoflurane step, normalised to initial recordings during 1.5% isoflurane (RM ANOVA $F_{(4, 24)}$ =78, P < 0.0001; Dunnett's post hoc test, *** P<0.001, compared to activity during inspired isoflurane 1.5%). **(C)** Time-frequency decomposition of the evoked somatosensory cortical neural activity shown in A. The time-frequency energy changes, time-locked to each stimulus, are presented as a group median. Results are displayed as increases and decreases in energy relative to a baseline period of 1 sec prior to stimulation. Energy values between 0 and -2 correspond to energy decreases, while values between 0 and 2 correspond to energy increases. n=7 animals for each age group. **(D)** Evoked potentials as in A, but 10 minutes after plantar skin incision, (10 stimuli per animal in n=6 animals). **(E)** Bar chart showing the peak evoked potential amplitude after hindpaw incision at steps of increasing of inspired isoflurane concentration, normalised to recordings during 1.5% isoflurane (RM ANOVA $F_{(4, 20)}$ =24, P < 0.0001; Dunnett's post hoc test **P<0.01, compared to activity recorded during 1.5% isoflurane. **(F)** Time-frequency decomposition of the evoked somatosensory cortical neural activity shown in D, 10 minutes after plantar skin incision.

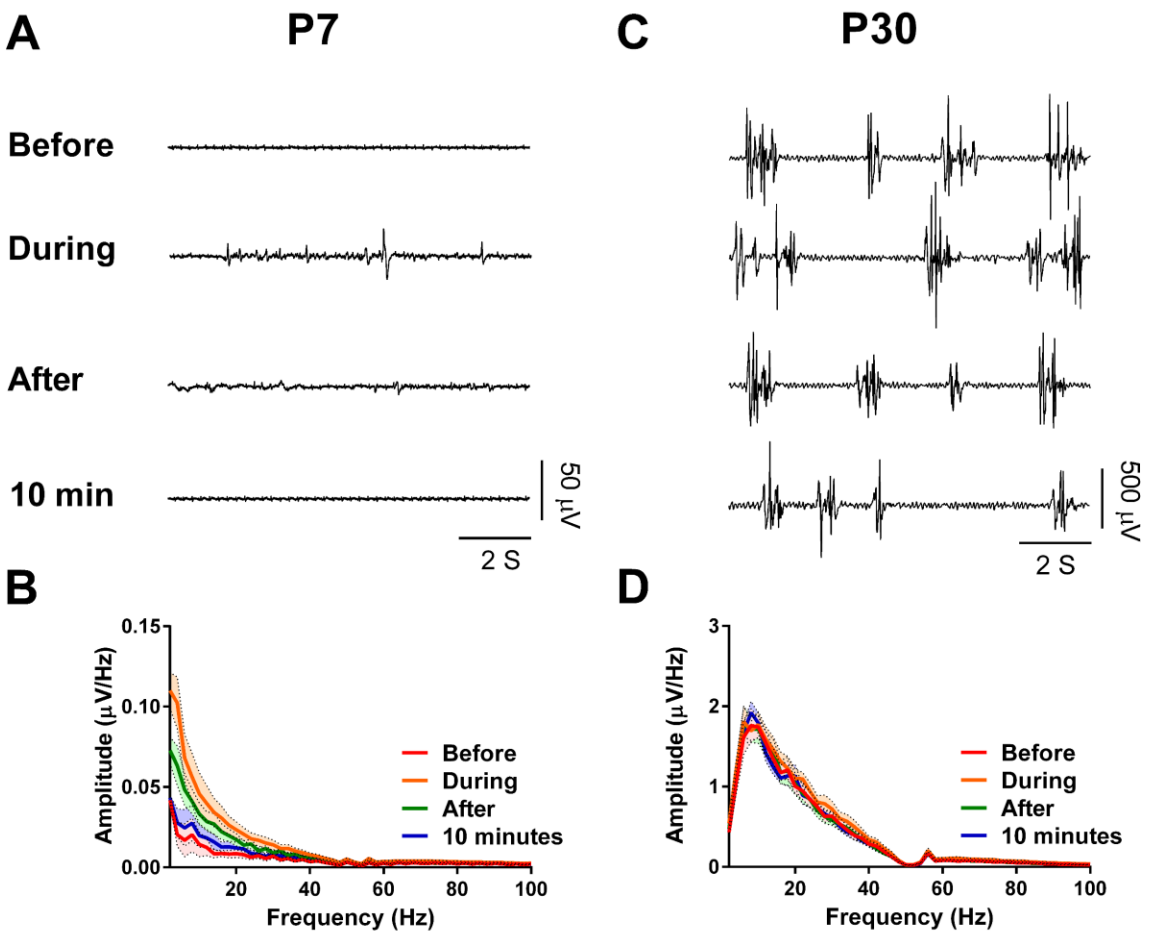


Figure 4. The effect of skin incision upon spontaneous activity in the somatosensory cortex in postnatal day (P) 7 and P30 rats. **(A)** Typical 10s intracortical traces from P7 rats sampled from the 2 min interval before, during and after plantar skin incision and again 10 minutes later. **(B)** Spectral analysis (Fast Fourier Transformation) from 100s epochs quantified changes across the frequency range 1-100 Hz. The graph represents the amplitude (µV/Hz) across the frequency range 1-100 Hz. Data=mean± SEM (n=6 animals). A significant main effect of hindpaw incision (before and during incision) was observed in the increased delta band activity (0.02±0.01 µV/Hz to 0.10±0.02 µV/Hz; RM ANOVA $F_{(3, 15)}$ = 13, P=0.0002; post hoc Dunnetts P<0.001), and beta band activity (0.01±0.00 µV/Hz to 0.02±0.01 µV/Hz, RM ANOVA, $F_{(3, 15)}$ = 6, P=0.0062; post hoc Dunnetts P<0.05). This had recovered by

10 mins post-surgery. **(C)** Typical 10s intracortical traces from P30 sampled from the 2 min interval before, during and after plantar skin incision and again 10 minutes later. **(D)** Spectral analysis in P30 rats. In contrast to P7 rats, the frequency distribution of the mean spontaneous activity at P30 (n=6 animals at each age) in a 100s epoch before, during and after incision under 1.5 % isoflurane anaesthesia was unchanged (δ band from 1.05±0.14 µV/Hz to 1.22±0.12 µV/Hz, RM ANOVA $F_{(3, 15)}$ = 0.8, P=0.4994, non-significant; β band from 0.01±0.00 µV/Hz to 0.02±0.01 µV/Hz, RM ANOVA $F_{(3, 15)}$ = 0.7, P=0.5688, non-significant.

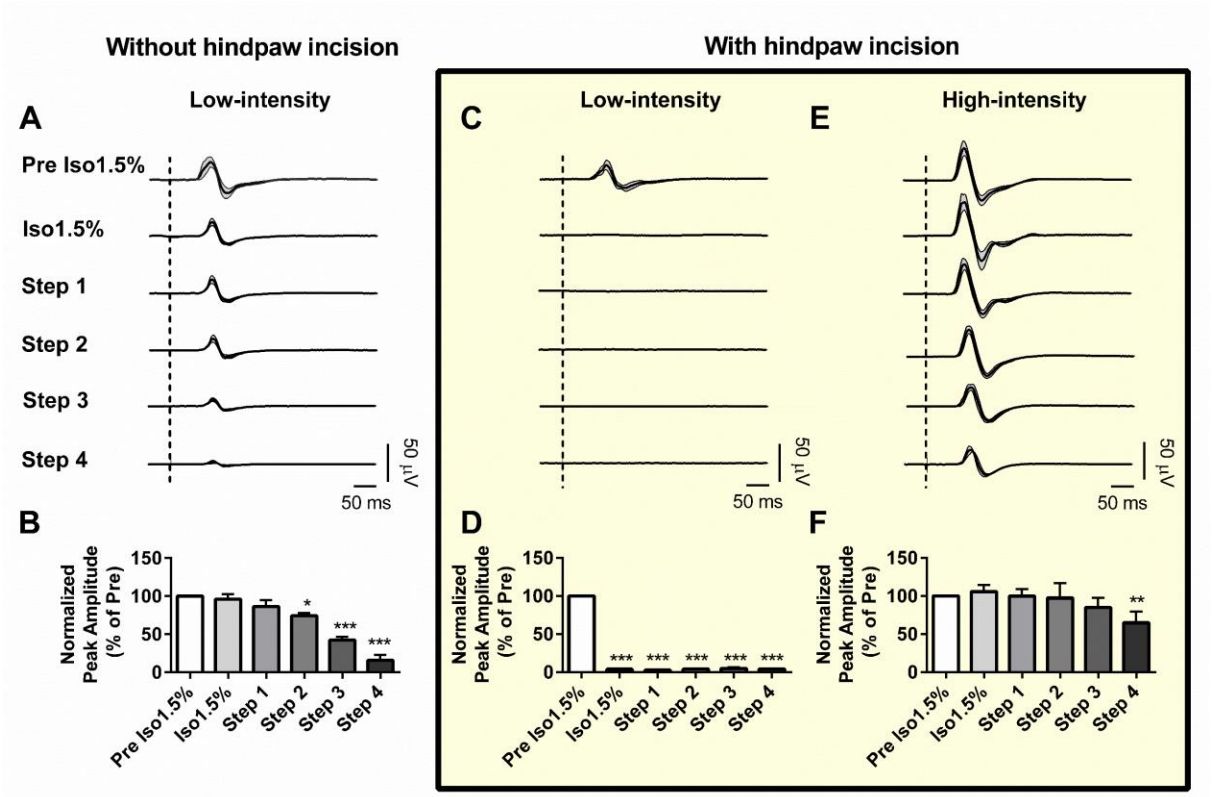


Figure 5. Effect of isoflurane and hindpaw incision on non-noxious, A fiber (0.32 mA, 50 µs) and noxious, C fiber (3.2 mA, 500 µs) evoked potentials in the primary somatosensory cortex at postnatal day (P) 7. (A) Typical recordings of right somatosensory-evoked potentials (SEPs) following low intensity A fiber electrical stimulation of the left hindpaw (10 x 0.32 mA, 50 µs stimuli) applied at the time indicated by the dotted line) during increasing inspired isoflurane concentrations. Grand average

evoked potentials (filled lines) ± SEM (grey area) from 10 stimuli per animal (n=5 animals). **(B)** Bar chart showing the peak evoked potential amplitude at each isoflurane (Iso) step, normalised to initial recordings during 1.5% isoflurane (Pre Iso 1.5%) (RM ANOVA $F_{(5, 20)}$ =40, P < 0.0001; Dunnett's post hoc test, *P<0.05; *** P<0.001, compared to activity during Pre Iso 1.5%. **(C)** and **(E)** show typical recordings of P7 right somatosensory-evoked potentials (SEPs in response to **(C)** low-intensity, A fiber (C, 5 x 0.32 mA, 50 µs stimuli) and **(E)** high-intensity, C fiber (5 x 3.2 mA, 500 µs) electrical stimulation of the left hindpaw following hindpaw incision at increasing inspired isoflurane steps. Traces represent grand average evoked potentials (filled lines) ± SEM (grey area) from 5 stimuli (5 x low-intensity, and 5 x high-intensity) per animal (n=5 animals). The dotted line indicates the time of electrical stimulation. **(D)** and **(F)** show bar charts of the peak evoked potential amplitude at each isoflurane step with low-intensity electrical stimulation **(D)** and high-intensity electrical stimulation **(F).** The data were normalised to initial recordings during 1.5% isoflurane (Pre Iso 1.5%) (For low intensity stimulation: RM ANOVA $F_{(5, 20)}$ =1673, P < 0.0001; for high intensity stimulation: RM ANOVA $F_{(5, 20)}$ =6, P =0.0011, Dunnett's post hoc test, *P<0.05; **P<0.01; *** P<0.001, compared to activity during Pre Iso 1.5%).

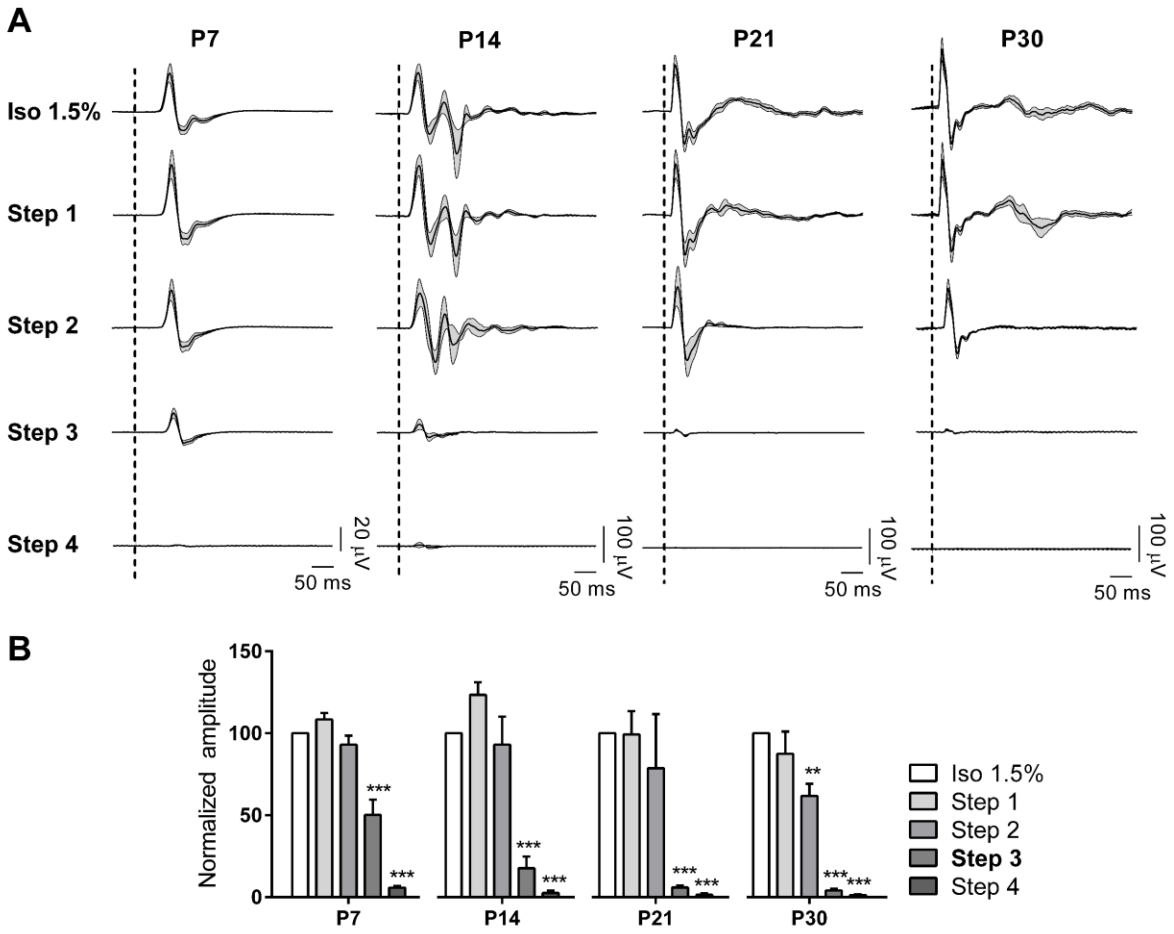


Figure 6. The effect of postnatal age upon somatosensory evoked potentials evoked by C fiber electrical hindpaw skin stimulation. (A) Typical recordings of right somatosensory-evoked potentials (SEPs) following electrical stimulation of the left hindpaw (10 x 3.2 mA, 500 µs stimuli applied at the time indicated by the dotted line). Recordings were performed at 1.5% inspired isoflurane (Iso 1.5%) and during 5-minutely step increases (step 1: 2%; step 2: 3%; step 3: 4%; step 4: 5%) in rats aged postnatal day (P) 7, P14, 21 and 30. Traces represent grand average evoked potentials (filled lines) ± SEM (grey area) from 10 stimuli per animal (n=7 animals). Note the different y-axis scale at P7 compared to older ages. **(B)** Comparison of the evoked potential peak amplitude at each isoflurane step, normalised to initial values obtained during 1.5% isoflurane. (RM ANOVA P7 $F_{(4, 24)}$ =78, P < 0.0001;

P14: $F_{(4, 24)}$ =37, P < 0.0001; P21: $F_{(4, 24)}$ =17, P=0.0008; P30: F(4, 24) =49, P < 0.0001. Dunnett's post hoc multiple comparisons test **P<0.01, and *** P<0.001, compared to evoke peak amplitude during inspired isoflurane 1.5%)

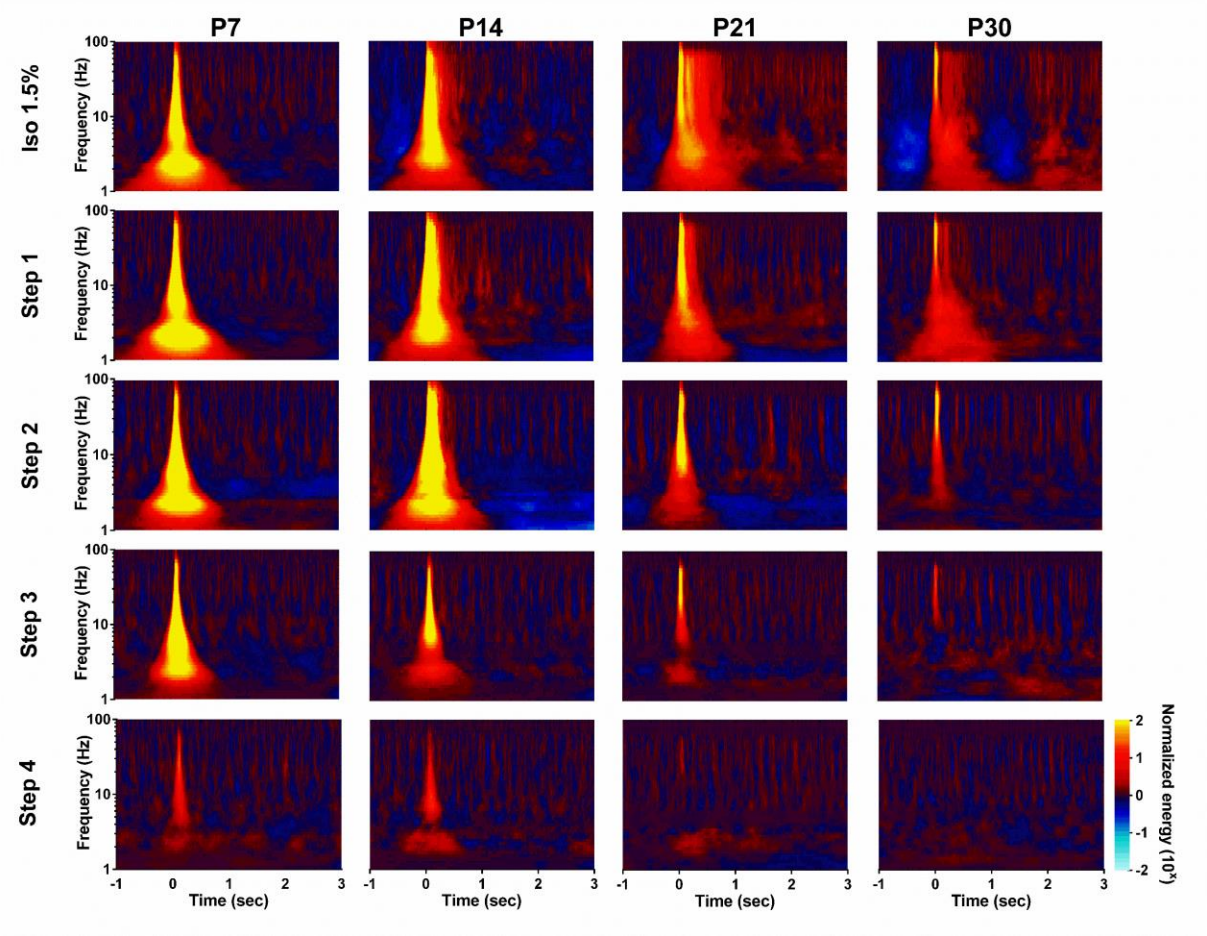


Figure 7. The effect of postnatal age upon cortical oscillatory activity following C fiber electrical hindpaw skin stimulation. Time-frequency decomposition of the evoked somatosensory cortical neural activity at each isoflurane step at postnatal day (P) 7, 14, 21 and 30. The time-frequency energy changes, time-locked to each C fiber stimulus, are presented as a group median (10 stimuli per animal, n=7 animals). Results are displayed as increases and decreases of energy changes relative to a baseline period of 1 sec prior to stimulation. Energy values between 0 and -2 correspond to energy decreases, while values between 0 and 2 correspond to energy increases (n=7 animals for each age group).

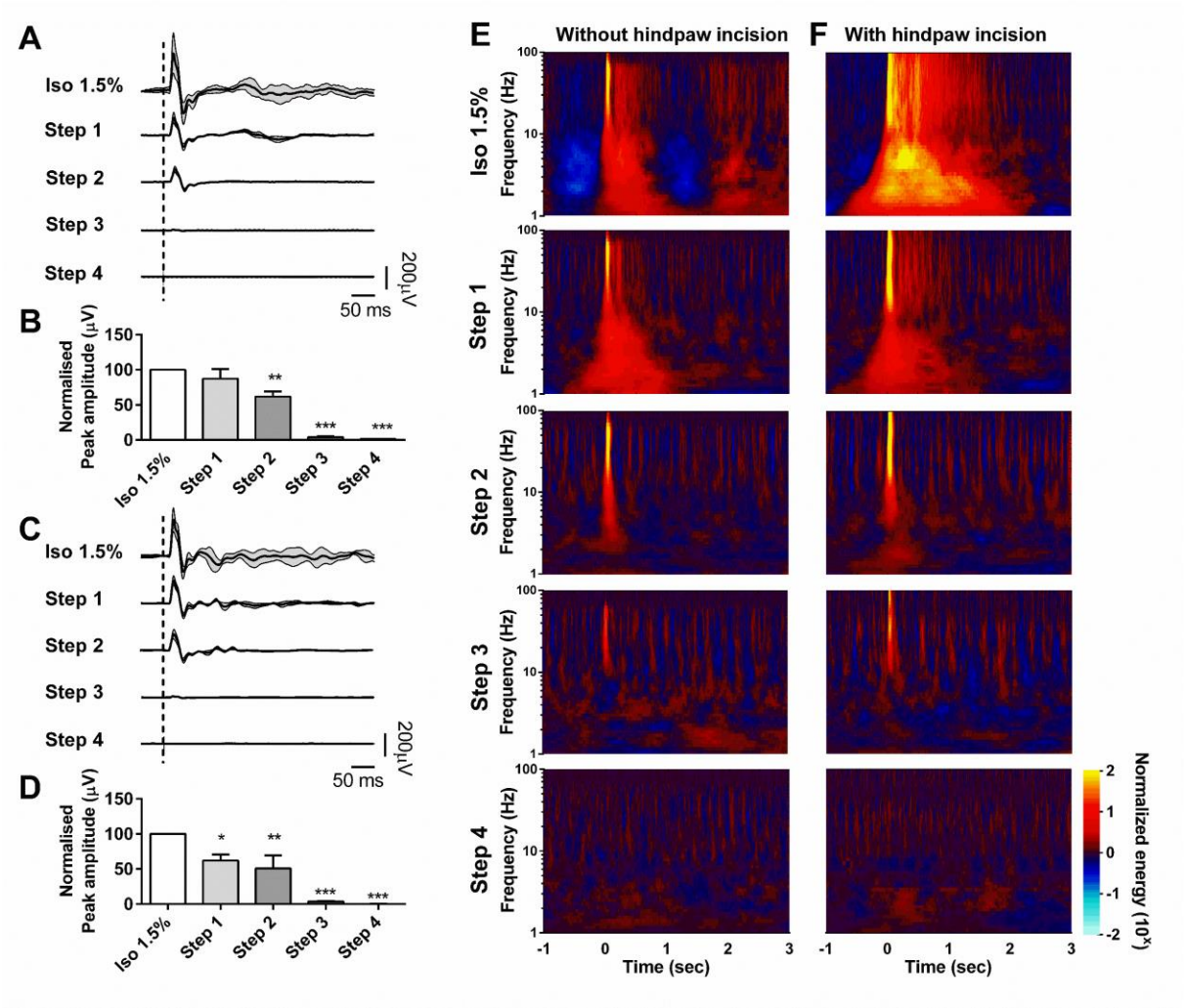
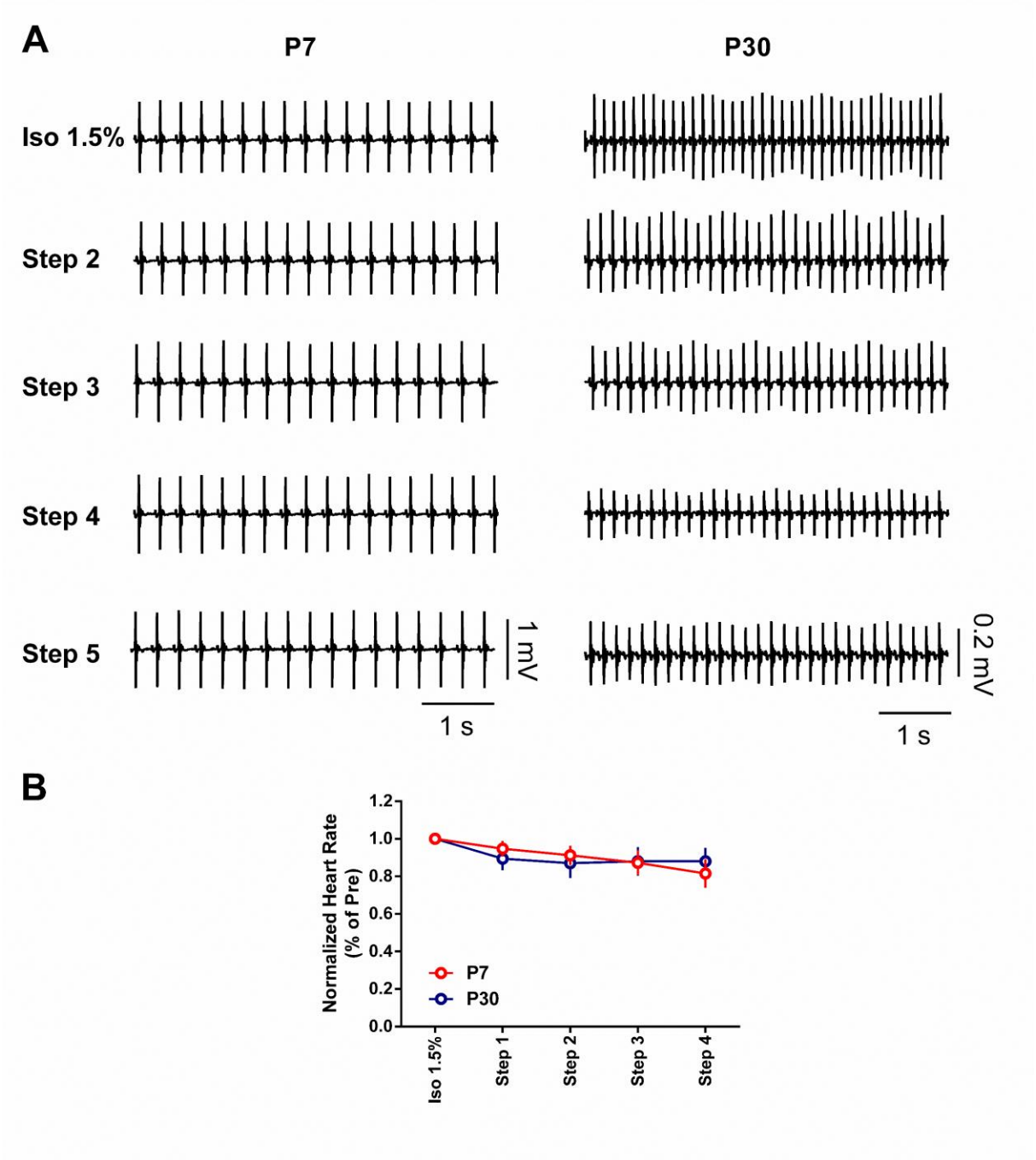


Figure 8 Effect of isoflurane and hindpaw incision on noxious-evoked activity in primary somatosensory cortex at postnatal day (P) 30. (A) Typical recordings of P30 right somatosensory-evoked potentials (SEPs) following electrical stimulation of the left hindpaw (10 x 3.2 mA, 500 µs stimuli applied at the time indicated by the dotted line) during increasing inspired isoflurane (Iso) concentrations. Grand average evoked potentials (filled lines) ± SEM (grey area) from 10 stimuli per animal (n=7 animals). **(B)** Bar chart showing the peak evoked potential amplitude at each isoflurane step, normalised to initial recordings during 1.5% isoflurane (RM ANOVA $F_{(4, 24)}$ =49, P < 0.0001; Dunnett's post hoc test, **P<0.01, and *** P<0.001, compared to peak evoked potential amplitude during 1.5% isoflurane). **(C)** P30 evoked potentials as in A, but 10 minutes after plantar skin incision,

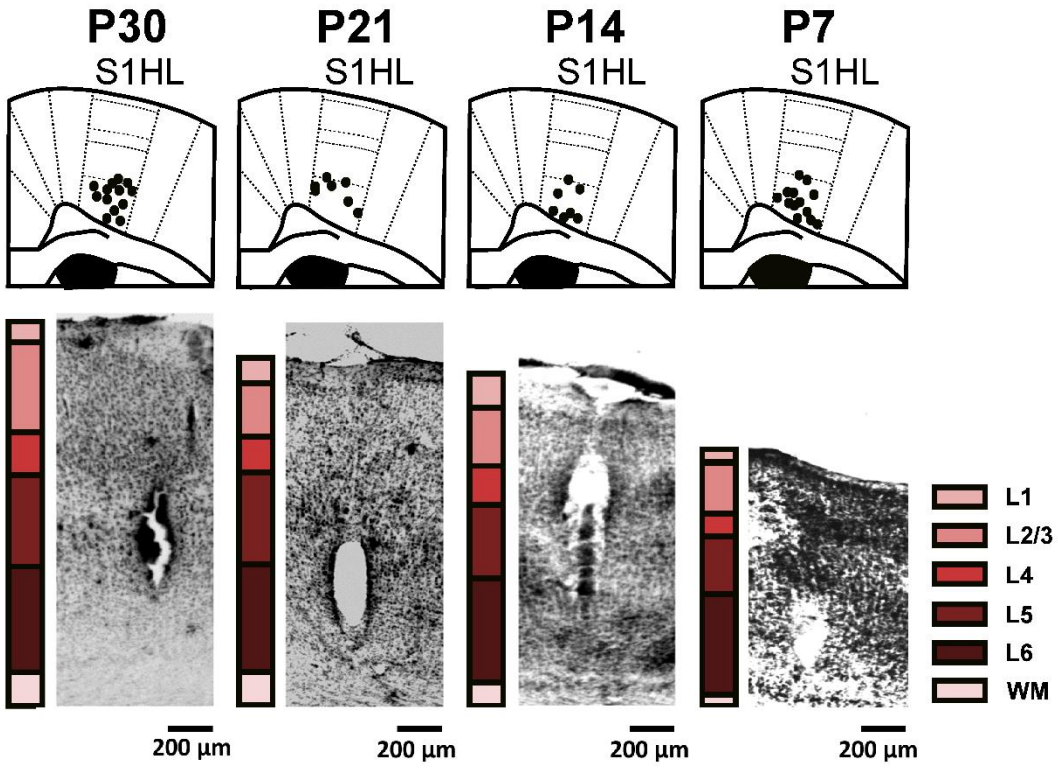
(10 stimuli per animal in n=6 animals). **(D)** Bar chart showing the peak evoked potential amplitude after hindpaw incision at steps of increasing of inspired isoflurane concentration, normalised to recordings during 1.5% isoflurane (RM ANOVA $F_{(4, 20)}$ =24, P < 0.0001; Dunnett's post hoc test, *P<0.05, **P<0.01, *** P<0.001, compared to activity recorded during 1.5% isoflurane. **(E)** Time-frequency decomposition of the evoked somatosensory cortical neural activity shown in A. The time-frequency energy changes, time-locked to each stimulus, are presented as a group median. Results are displayed as increases and decreases in energy relative to a baseline period of 1 sec prior to stimulation. Energy values between 0 and -2 correspond to energy decreases, while values between 0 and 2 correspond to energy increases. n=7 animals for each age group. **(F)** Time-frequency decomposition of the evoked somatosensory cortical neural activity shown in C, 10 minutes after plantar skin incision.

Supplementary Figures



Supplemental Digital Content 1: The effect of isoflurane on the heart rate

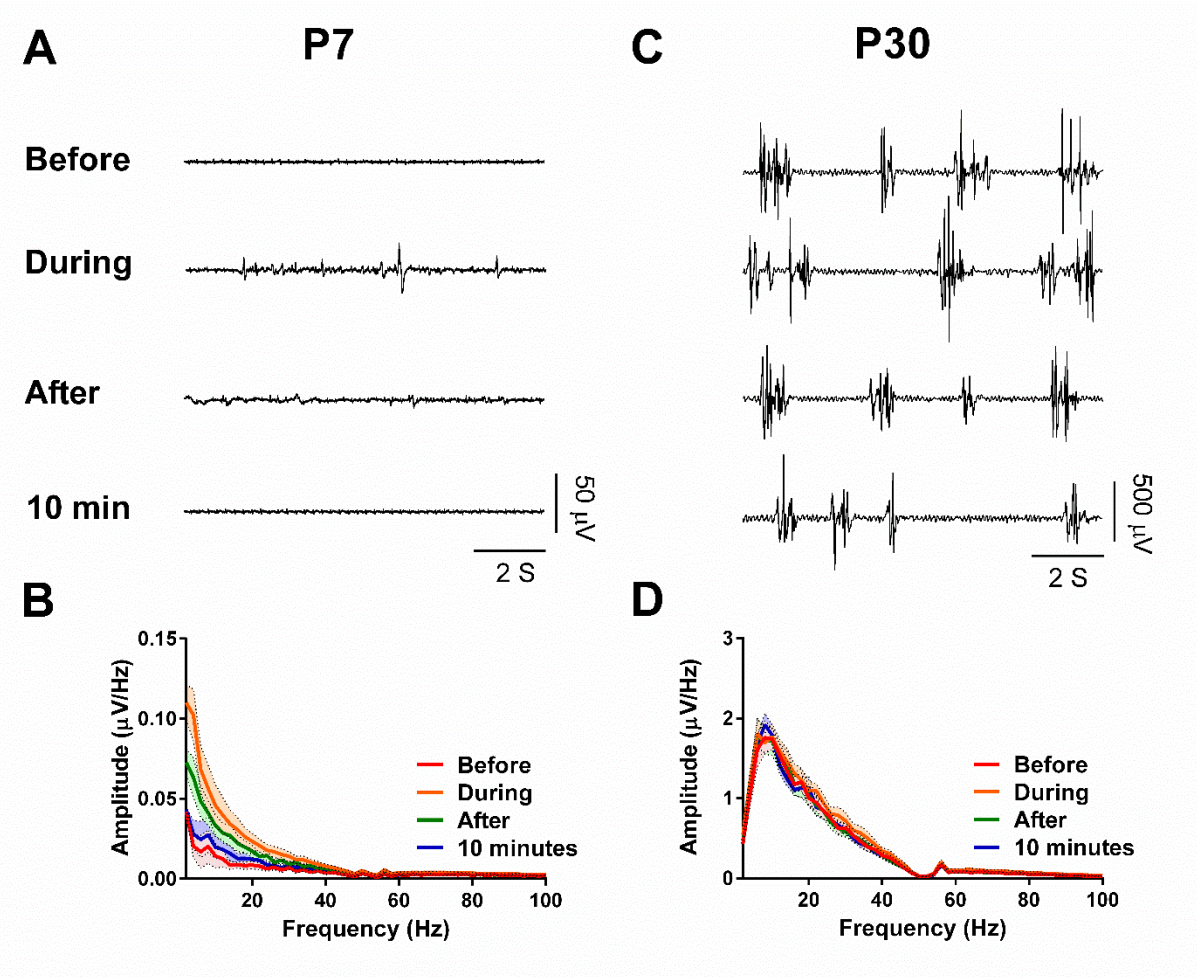
(A) Example of recordings of electrocardiogram (ECG) at 1.5% inspired isoflurane (iso 1.5%) and during step increases (step 1: 2%; step 2: 3%; step 3: 4%; step 4: 5%) in rats aged postnatal day (P) 7and 30. (B) The effect of step increasing of isoflurane on the heart rate in different age of group (n=6 in P7; n=6 in P30). The normalized heart rates (normalized to Iso 1.5%) were plotted against the step increase in isoflurane. The heart rate slightly decreases as isoflurane increases. However, there is no significant difference between ages. Step 1: t 0.05(2),10=1.762, P= 0.1086 ; Step2: t 0.05(2),10=1.072, P=0.3091; step 3: t 0.05(2),10=0.1818, P=0.8594; Step 4: t 0.05(2),10=1.530, P=0.1571, Student's T test).



Supplemental Digital Content 2: Recording sites in layer 5/6 of the rat somatosensory cortex

Schematic representation of the location of intracacortical recording sites in the primary somatosensory in rats aged postnatal day (P) 7, P14, 21 and 30 (P7=12; P14=6; P21=6; P30=12). The locations were determined by post-hoc histological analysis. The distribution of all recording sites

(filled circles) was reconstructed onto maps of the primary somatosensory cortex corresponding to hindpaw (S1HL) in a standard stereotaxic atlas [56](Paxinos and Watson 1998). L1, layer 1; L2/3, layer 2 and 3; L4, layer 4; L5, layer 5; L6, layer 6; WM, white matter.



Supplemental Digital Content 3: The effect of skin incision upon spontaneous activity in the somatosensory cortex in P7 and P30 rats

A. Typical 10s intracortical traces from P7 rats sampled from the 2 min interval before, during and after plantar skin incision and again 10 minutes later. **B**. Spectral analysis (Fast Fourier Transformation) from 100s epochs quantified changes across the frequency range 1-100 Hz (Fig. 2G). The graph represents the amplitude (V/Hz) across the frequency range 1-100 Hz. Spectral analysis (Fast Fourier Transformation, frequency range: 1-100 Hz) was performed from 100s epochs. Data=mean± SEM (n=6

animals). The significant main effect of hindpaw incision (before and during incision) was observed in the increased delta band activity (0.02±0.01 μV/Hz to 0.10±0.02 μV/Hz; RM ANOVA $F_{(3, 15)}$ = 13.42, P=0.0002; post hoc Dunnetts P<0.001), and beta band activity (0.01±0.00 μV/Hz to 0.02±0.01 μV/Hz, RM ANOVA, $F_{(3, 15)}$ = 6.136, P=0.0062; post hoc Dunnetts P<0.05) This had recovered by 10 mins post-surgery. **C.** Typical 10s intracortical traces from P30 sampled from the 2 min interval before, during and after plantar skin incision and again 10 minutes later. **D.** Spectral analysis in P30 rats. In contrast to P7 rats, the frequency distribution of the mean spontaneous activity at P30 (n=6 animals at each age) in a 100s epoch before, during and after incision under 1.5 % isoflurane anesthesia was unchanged (δ band from 1.05±0.14 μV/Hz to 1.22±0.12 μV/Hz, RM ANOVA $F_{(3, 15)}$ = 0.8270, P=0.4994, non-significant; β band from 0.01±0.00 μV/Hz to 0.02±0.01 μV/Hz, RM ANOVA $F_{(3, 15)}$ = 0.6960, P=0.5688, non-significant.