Author:
Cross, Christine

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Postnatal Development of Neural Networks in the Healthy and Premature Brain

Postnatal Development of Neural Networks in the Healthy and Premature Brain

Christine Mairi Cross

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Abstract

Late fetal and early neonatal life is a period of rapid neurological development, with dramatic structural and functional changes unfolding to create the complex neuronal networks of the mature brain. Disruptions to early life experience can have profound effects on the developing brain, with alterations to network formation that can have life-long impacts. A common adverse early life condition in humans is premature birth, which often results in neurodevelopmental deficits. This project develops a mouse model of prematurity to investigate the impact that being born early has on the development of the sensorimotor networks. It finds, using measures of behavioural development, and cellular and synaptic maturation of neurons that the developing brain is remarkably robust. If an animal can survive the initial dramatic changes of being born, the sensory networks of the brain continue to develop along their typical trajectory. This project further explores the impact of premature birth on sensory network development, with a neuroimaging experiment in human preterm infants. A somatosensory stimulation based functional magnetic resonance imaging paradigm is established to investigate evoked responses in the preterm infant brain. *In vivo* neuroimaging techniques offer valuable information about the functional development of neuronal networks. This project also utilises a newly established pan-cortical calcium imaging technique to investigate the postnatal development of cortical activity in mice. Recordings of both endogenously generated and sensory stimulated activity in healthy and sensory deprived conditions are made at high spatial and temporal resolution. It finds complex patterns of spontaneous activity across the cortex that have intracortical coordination, that are independent of sensory experience in the first postnatal week. Somatosensation is active from the first postnatal day and the developmental trajectory of evoked cortical activity is mediated by early life sensory experience. This thesis details an investigation into the development of the sensorimotor networks in both healthy and adverse early life environments, discovering both the vulnerability and robustness of their functional development.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ……………………………………………… DATE:……………………

To my parents, who tirelessly worked to give their children the opportunities to explore their passions. For my Mum, who's unceasing kindness and nurture gave me the bravery and resilience to take on the world. I wish you were here to see this. I hope I make you proud. And for my Dad, who nurtured my curiosity and pushed me to find the answers to my own questions. I do not believe I would be the scientist I am today without your encouragement and example. Thank you both for all you have done. This achievement is yours as well as mine.

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Abbreviations

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – analysis of variance

AP – action potential

BAPTA - 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

$CaCl_2$ – calcium chloride

CGA – corrected gestational age

CP – cerebral palsy

$CsMeSO_4$ - cesium methanesulfonate

DAB - Diaminobenzidine

DAPI - 4',6-diamidino-2-phenylindole

DMSO – dimethyl sulfoxide

EEG - electroencephalogram

EGTA - ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HI – hypoxic-ischemic

HRF – haemodynamic response function

IQR – interquartile range

IR – infrared

IVH - intraventricular haemorrhage

KCl – potassium chloride

$KMeSO_4$ - potassium methyl sulfate

MG-ATP - adenosine 5'-triphosphate magnesium salt

$MgSO_4$ – magnesium sulfate

NaCl – sodium chloride

NA-GTP - guanosine 5'-triphosphate **sodium** salt

$NaHCO_3$ – sodium bicarbonate

NMDA - N-methyl-D-asparate

PBS - phosphate buffered saline

PC - post-conception

P - postnatal

PTX – pertussis toxin

PVL - periventricular leukomalacia

QX314 - *N*-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium

RMP – resting membrane potential

ROI – region of interest

TTX - Tetrodotoxin

1 General Introduction

The brain is a complex structure that has evolved to effectively process the information that is received from the external environment. Major sensory inputs from the environment - visual, auditory, and tactile - all drive neural networks that are specialised to receive and process incoming sensory stimulation. These sensory pathways have a structure that is evolutionarily conserved across mammalian species, where sensory stimulation is detected at the periphery by sensory receptors and this information ascends to the cortex via subcortical structure such as the thalamus (Hodos and Butler, 1997; Kaas, 2008). Sensory information from the environment is often complex and multimodal, and processing of this information is required for an animal to navigate the world they live in. The brain does not come fully formed and capable of such processing abilities. Interpretation of incoming sensory signals must be learned and calibrated, a process that requires the organisation and cooperation of many cells (Kostović and Jovanov-Milosević, 2006). Disruptions to sensory experience early in life can have a profound effect on the development of functional neuronal circuits in the brain (Wiesel and Hubel, 1963). Critical periods of heightened sensitivity, which in normal circumstances are a key period of anatomical and physiological plasticity are times when developing neural circuits are particularly vulnerable to disruptions in sensory experience (Hensch, 2004).

1.1   Primary sensory networks

The basic structure of the neocortex is conserved across mammalian species. It is comprised of horizonal layers and is organised vertically into functional columns of 300-600μm in diameter (Mountcastle, 1997). Afferent neurons from the thalamus project into the cortex synapsing mainly into layer IV. There are connections between layers within cortical columns and corticocortical connections between cortical regions (Purves *et al.*, 2001a). The primary sensory regions of the cortex have organised structures which are important for faithful representation of incoming sensory information in the mature system (Gilbert and Wiesel, 1992; Diamond, Petersen and Harris, 1999; Carl C H Petersen, 2007; Tsukano *et al.*, 2017).

In rodents, tactile stimulation provided by whisker deflection is an important source of sensory information about the environment around them. Because of this it is a prominently

used model for investigating the development of sensory networks. Whisker tactile information ascends the pathways from the mechano-gated sensory neurons of the trigeminal nerve which initially synapse in the trigeminal nuclei of the brainstem. The topographical organisation of the whiskers is preserved in the brainstem in 'barelettes' (Veinante and Deschênes, 1999). From here information travels to the ventral posterior medial (VPM) nucleus of the thalamus, which is also topographically organised to preserve the whisker pattern, in 'barreloids' (Brecht and Sakmann, 2002). The final stage of this pathway is thalamocortical projections primarily into layer IV of the cortex which form discrete clusters known as 'barrels' (Woolsey and Van der Loos, 1970). This topographical organisation means that spatially adjacent whiskers are represented in adjacent regions throughout the sensory pathway, providing a faithful representation of whisker organisation in the cortex. The thalamocortical projections form excitatory connections with spiny stellate neurons (Fox, 1995). The cell bodies of these cortical neurons form a wall around the thalamocortical projection clusters, sending dendrites into the barrel centre to form synaptic connections (Benshalom and White, 1986; Feldmeyer, 2012). This topographical organisation through the sensory pathway allows sensory information from individual whiskers to precisely input into the cortex (Carl C H Petersen, 2007).

The other sensory systems, such as visual and auditory pathways have a similar periphery to central layout. Sensory information is received through the sensory organs - the retina and the cochlear - and information ascend the pathway relaying through subcortical regions and terminating in the primary sensory cortex (Malmierca, 2003; Seabrook *et al.*, 2017). As with the whisker related organisation of the somatosensory system the visual and auditory pathways have topographical organisation throughout (Dräger, 1975; Purves *et al.*, 2001c). This sensory pathways organisation, from peripheral sensory receptors to sensory cortex via subcortical relays is conserved across mammalian species including the human (Kaas, 2008).

1.2   Development of the sensory networks

1.2.1   Structural changes to the developing sensory networks

The highly organised sensory networks, which faithfully represent information from the external environment, develop during prenatal and early postnatal life. In humans the last

trimester of gestation is a period of rapid neurological development, with profound molecular, structural and function changes occurring within the brain (Kostović and Jovanov-Milosević, 2006). At 30 weeks gestation the brain is only 50% of its term-birth weight (Guihard-Costa and Larroche, 1990), with a 4-5 fold increase in the size of the cortex (Hüppi *et al.*, 1998; Kapellou *et al.*, 2006) and dramatic development of gyri (Dubois *et al.*, 2008) occurring in this final gestation period. During this period there is extensive migration of thalamocortical afferents (Allendoerfer and Shatz, 1994; Kostović and Judas, 2010; Krsnik *et al.*, 2017), which are the primary subcortical input into the cortical plate. Alongside the migration of thalamocortical fibres there is a rapid increase in the number of synapses in the cortex and dendritic arborisation of cortical neurons (Molliver, Kostović and van der Loos, 1973; Huttenlocher and Dabholkar, 1997).

Key neurodevelopmental processes are conserved across mammalian species but the specific timing of these developmental events can be different (Clancy *et al.*, 2007). Rodents are born in a relatively immature state compared to humans (Clancy, Darlington and Finlay, 2001). Many of the events described above, which occur during the third trimester in humans, happen postnatally in rodents. The majority of cortical cell migration in humans happens by the end of the second trimester (Kostović and Jovanov-Milosević, 2006), whereas in rodents this is still occurring during the early neonatal period (Fox, 1995; Inan and Crair, 2007). Excitatory cortical neurons are born in the ventricular zone (Gilmore and Herrup, 1997) and begin to migrate to the cortical plate between embryonic day (E) 13-17 in the mouse (Figure 1.1). Inhibitory cortical neurons are born in the subventricular zone and migrate to the cortical layer, during late embryonic and early postnatal life in rodents (Kirischuk, Luhmann and Kilb, 2014). The cortical plate differentiates into the 6 layers seen in the mature cortex in an inside to outside sequence (Angevine and Sidman, 1961). Layer VI excitatory neurons are born first and migrate to the pial surface, with more superficial layer neurons being born later and migrate through these lower layers of more mature cells. There are two transient neuron population that are born during this early development, Cajal-Retzius (CRNs) and subplate neurons (SPNs). CRNs migrate to the pial surface which will eventually become layer I (Kirischuk, Luhmann and Kilb, 2014) and SPNs are located below layer VI (Patrick O Kanold and Luhmann, 2010). These neuron populations both facilitate the migration and organisation of cortical neurons during layer formation

(Luhmann *et al.*, 2016a), along with radial glia (Barry, Pakan and McDermott, 2014). In rodents, layers V and VI are present at birth and cortical migration is complete by the end of the first postnatal week, where both excitatory and inhibitory neurons are present across the cortical layers. As in humans once neurons reach the cortical plate they undergo maturation, in the form of cellular morphology changes such as dendritic arborisation (Catalano, Robertson and Killackey, 1996; Baloch *et al.*, 2009), a dramatic increase in synaptic density (Ashby and Isaac, 2011) and changing molecular composition (Sanderson and Murphy, 1981). In rodents the thalamocortical projections begin to innervate late in gestation (embryonic day 18) and continue into the neonatal period (Molnár and Blakemore, 1995), occurring simultaneously with cortical layer migration. These thalamocortical projections are topographically organised before they reach their destination in layer IV of the cortex (Erzurumlu and Jhaveri, 1990; Auladell *et al.*, 2000), with further refinement of their organisation in the postnatal period (Martini *et al.*, 2018).
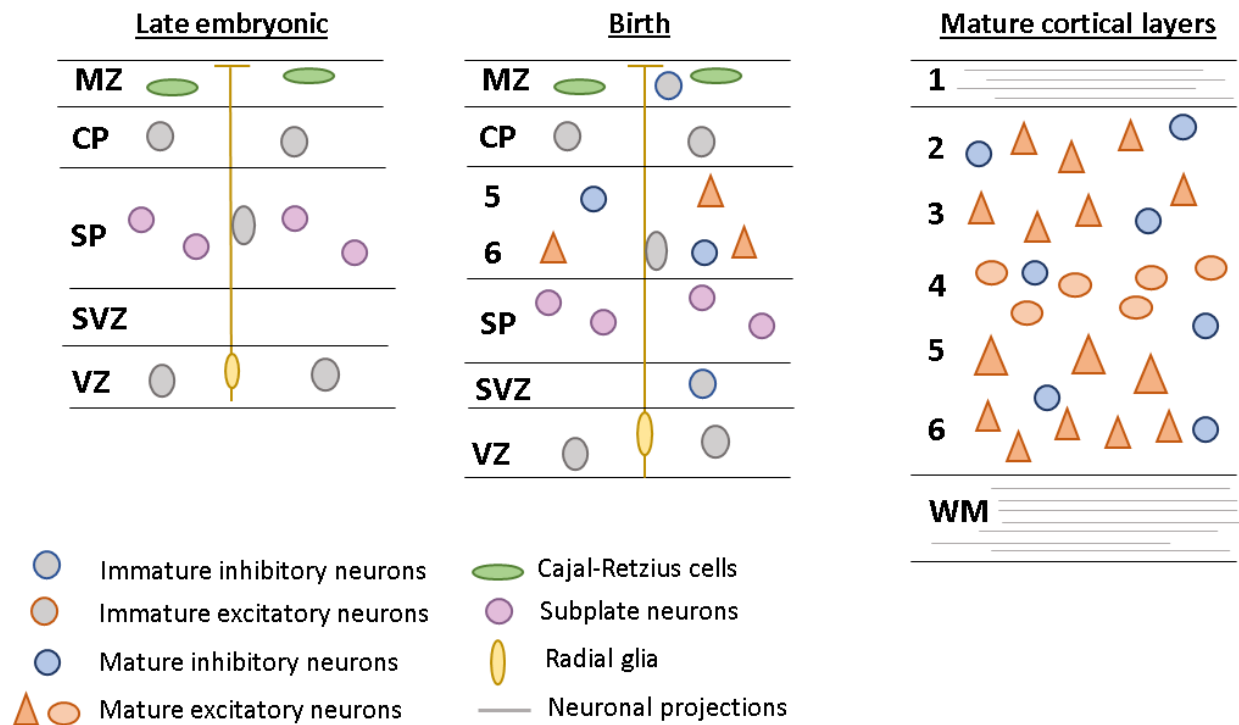
Figure 1.1 **Schematic of the late prenatal and postnatal cellular development of the rodent cortex.** *This is a simplified schematic of the major events occurring during cortical layer development during late embryonic and early postnatal life in the rodent. Cajal-Retizus cells and subplate neurons are transient early developing neuron populations that facilitate the development of the cortical layers. Excitatory cortical neurons are born in the ventricular zone (VZ) and migrate by radial glia to the cortical plate. Inhibitory neurons are born in both the subventricular zone (SVZ) and the marginal zone (MZ) and migrate tangentially. Once neurons reach their destination in the cortical plate the undergo maturation processes such as the formation of projections and synaptic connections.*
Modified from Luhmann et al., 2016.

1.2.2 Developmental behavioural changes

The different timings of structural development of the sensory systems in rodent and humans is observed in behavioural development as well. Rodents are born functionally blind and deaf with their eyes and ear canal closed, with active sensing in these networks does not beginning until the second postnatal week (Froemke and Jones, 2011; Jing Shen and Colonnese, 2016). The somatosensory network develops earlier, with withdrawal responses to tactile stimulation being found at embryonic day 13 (Schiffman and McHale, 1990) and cortical activity following tactile whisker stimulation present at birth (Mitrukhina *et al.*, 2015b). Tactile sensory experience continues to develop postnatally with active whisking also fully emerging in the second postnatal week (Landers and Philip Zeigler, 2006). This development in sensory perception ability allows for the development of more complex behaviours that integrate sensory experience (Arakawa and Erzurumlu, 2015a). During the first few postnatal weeks a variety of behaviours develop that allow pups to interact with their environment and interpret the world around them (Fox, 1965).

Active sensing in humans begins before birth. Responses to tactile stimulation are observed from as early as 8 weeks of gestation (Montagu, 1986) and responses to auditory stimulation are observed in-utero from 19 weeks gestation (Hepper and Shahidullah, 1994). Cortical activity in response to external stimulation is observed in preterm infants in all primary sensory networks (Majnemer, Rosenblatt and Riley, 1990; Eldredge and Salamy, 1996; Taylor, Boor and Ekert, 1996; Smit *et al.*, 2000; Chipaux *et al.*, 2013)(Kaminska *et al.*, 2017) indicating that active sensing in humans is present before term birth.

1.2.3 Activity-dependent neonatal brain development

The developmental changes in the immature brain are a multistage process. Initially genetically-driven molecular cues guide the formation of the embryonic brain, signalling migration and maturation of neurons (Yamamoto and López-Bendito, 2012). This results in the early parcellation of different brain regions, formation of gross network connections and activity in the brain begins. Initially this activity is intrinsically generated by neurons and

begins to refine the network connections (Blankenship and Feller, 2010). This leads to a rapid maturation of the neuronal networks, organising them so they are prepared to process information from the external environment. This leads to the final stage of the developmental process, where once the sensory pathways are established enough external sensory information generates activity in the network that further refines its organisation (Leighton and Lohmann, 2016). This pre-sensory maturation and organisation of the networks by spontaneously generated activity means that effective processing in the cortex occurs as soon as external sensory information starts (Zhang *et al.*, 2012; Ko *et al.*, 2013). These stages of development do not happen in discrete periods but are overlapping and influence each other. Spontaneously occurring activity is known to have a role in gene regulation (Buonanno and Fields, 1999) and molecular signalling during neuron migration in the developing brain (Ming *et al.*, 2001; Tang, Dent and Kalil, 2003; Hanson and Landmesser, 2004; Yamada *et al.*, 2010). Once active sensing begins, spontaneous activity does not disappear, although its patterns and origins transition to a more mature state around the period of active sensation (Colonnese *et al.*, 2010; Minlebaev *et al.*, 2011).

1.2.3.1   *Generation of spontaneous activity*

Early brain activity is vitally important for the formation of neural circuits (Spitzer, 2006; Kirkby *et al.*, 2013; Luhmann *et al.*, 2016a). Spontaneously generated activity in the sensory networks begins embryonically in rodents (Jones, Jones and Paggett, 2001; Yamamoto and López-Bendito, 2012; Maccione *et al.*, 2014) and continues into early postnatal life, increasing with development (Luján, Shigemoto and López-Bendito, 2005). Spontaneous activity has been defined as neuronal activity that occurs in the absence of external stimulation (Colonnese and Khazipov, 2012). Recordings of the immature cortex during restful behaviour have revealed spontaneously occurring activity, in both rodents and humans (Colonnese and Khazipov, 2012). This endogenously generated spontaneous activity has been reported throughout the developing sensory networks. In the visual and auditory networks coordinated waves of activity are generated in the periphery by the retina (Torborg and Feller, 2005) and the cochlear (Wang *et al.*, 2015). This activity propagates through the sensory pathways to the primary sensory cortex (Ackman, Burbridge and Crair, 2012; Babola *et al.*, 2018).

Investigations into the generation of spontaneous activity in the somatosensory network have found it to be not as clear cut as the visual and auditory systems. Some of the spontaneous activity observed in the somatosensory cortex is generated by twitch movements of the limbs and whiskers (Khazipov, Sirota, Leinekugel, Gregory L Holmes, *et al.*, 2004; Tiriac *et al.*, 2012; Akhmetshina *et al.*, 2016). These small fast movements, known as myoclonic twitches, occur during sleep and are generated by spontaneous activity bursts in the spinal cord (Khazipov, Sirota, Leinekugel, Gregory L Holmes, *et al.*, 2004). They are discrete movements, sometimes of individual joints and whiskers and others a combination of a few simultaneously (Tiriac *et al.*, 2012; Blumberg *et al.*, 2013). These movements result in activation of the somatosensory cortex in discrete areas related to the area of the body moved. Not all of the spontaneous activity observed in the somatosensory cortex is stimulated by movement. Mizuno *et al.*, 2018 found that 89% of activity in layer IV of the barrel cortex was not correlated with whisker movements, suggesting that most spontaneous activity observed in the barrel cortex in the neonate is endogenously driven. In addition silencing of the whisker pad results in a suppression but not totally elimination of barrel cortex spontaneous activity (J.-W. Yang *et al.*, 2009), meaning that some of this activity is generated in the central network such as the thalamus or cortex rather than the sensory periphery (Khazipov, Sirota, Leinekugel, Gregory L Holmes, *et al.*, 2004; Adelsberger, Garaschuk and Konnerth, 2005).

1.2.3.2   *Spatial pattern of spontaneous activity*

The endogenously generated activity in the sensory networks is coordinated, with non-random spatial and temporal patterns (Thivierge, 2009). These precise patterns of activity play an important role in the development of the sensory systems which require a highly organised structure to effectively process sensory information (Kirkby *et al.*, 2013). In the visual and auditory networks the endogenously generated activity propagates in waves, and these wave patterns are conserved throughout the pathways (Ackman, Burbridge and Crair, 2012; Babola *et al.*, 2018). In the somatosensory network activity is more discrete, with bursts of activity often confined to functional cortical columns (J.-W. Yang *et al.*, 2009; Mizuno *et al.*, 2018). These difference in patterns of activity are reflective of the continuous

retinotopic and tonotopic organisation of the visual and auditory networks and the more discrete topographical map of the somatosensory system (Carl C H Petersen, 2007; Rothschild, Nelken and Mizrahi, 2010; Garrett *et al.*, 2014). These coordinated patterns of activity are important for the development of organised functional networks, as alternations to the patterns of early spontaneous activity results in disrupted formation of the sensory pathways. For example, using genetic manipulations to disrupt the pattern of spontaneous activity in the visual pathway during the postnatal week results in imprecise mapping in the visual cortex (Cang *et al.*, 2005a), and suppression of spontaneous activity in the somatosensory cortex resulted in insufficient development of both thalamocortical (Yamada *et al.*, 2010) and callosal projections (C.-L. Wang *et al.*, 2007).

Rodents can move at birth and produce coordinated wakeful movements as well as myoclonic twitches (Tiriac, Del Rio-Bermudez and Blumberg, 2014). These wakeful movements result in large amounts of somatosensory stimulation which do not have the localised and organised spatial activity restrictions that spontaneous generated twitching does (Dooley and Blumberg, 2018). This spatially-unrestricted activity would be expected to disrupt the formation of the organised topographic map of the somatosensory cortex. It was discovered that this was prevented by a unique inhibitory gating mechanism in the subcortical areas of the pathway that prevents this tactile experience from reaching the immature cortex (Tiriac *et al.*, 2012; Tiriac and Blumberg, 2016; Dooley and Blumberg, 2018). This further emphasises the importance of patterned spontaneous activity in the developing sensory pathways, with tight control of activity being a characteristic of these systems.

This spontaneous activity is coordinated and optimised to organise and stabilise the initial topographical organisation of the incoming thalamocortical projections in the primary sensory cortical regions (Colonnese and Phillips, 2018). As well as migrations of thalamocortical projections during this early postnatal period interhemispheric connections are also forming. One of the major interhemispheric connections is through the corpus callosum. These projections are developing during the first postnatal week in rodents, crossing the midline at P3 and penetrating the grey matter of the opposite hemisphere by P6 (C.-L. Wang *et al.*, 2007). After entering the cortex they continue further refinement, with visual cortex connections reaching a mature arrangement at around P12, the time of eye

opening (Ivy and Killackey, 1981). The migration and organisation of these projections also requires spontaneous cortical activity (C.-L. Wang *et al.*, 2007; Tagawa, Mizuno and Hirano, 2008; Suárez *et al.*, 2014). It was discovered that that the symmetry of hemispheric activity was more important that absolute activity levels (Suárez *et al.*, 2014), suggesting that, as with development of thalamocortical connections, the organisation of activity is vitally important to the formation of callosal projections.

In the mature brain interhemispheric connections are important in the transferring and integrating of lateralised sensory inputs (Ebner and Myers, 1962; Shuler, Krupa and Nicolelis, 2001, 2002) and mediate the coordination of spontaneous activity between hemispheres (Mohajerani *et al.*, 2010). Even while these pathways are forming, during the first postnatal week, it has been found to have an important role in spontaneous activity patterns in the sensory cortex, with lesions of the callosal fibres during the first postnatal week resulting in increased activity bilaterally in the primary somatosensory cortex (Marcano-Reik and Blumberg, 2008).

1.2.3.3 *Temporal pattern of spontaneous activity*

As well as spatial organisation to the activity in the developing brain different temporal patterns have been discovered. Early network oscillations (ENOs) are present in the developing sensory cortex (Yang *et al.*, 2009; Koepsell *et al.*, 2010; Li *et al.*, 2017), including spindle bursts and gamma oscillations (Figure 1.2). Gamma oscillations are spatially restricted to functional cortical columns occurring in short bursts of around 100-300ms every 10-30s (Khazipov, Minlebaev and Valeeva, 2013). Spindle bursts have a longer duration of around 0.5-3s and occur spontaneously about every 10s, and synchronising activity across multiple columns at once (Yang *et al.*, 2016). These oscillation patterns have also been recorded in the developing thalamus (Minlebaev *et al.*, 2011; Yang *et al.*, 2013). In addition to these discrete activations of the sensory cortices, more wide spread propagation of spontaneous activity travelling across the cortex has been reported (Garaschuk *et al.*, 2000; Adelsberger, Garaschuk and Konnerth, 2005; Namiki *et al.*, 2013; Ackman, Zeng and Crair, 2014). Long lasting oscillations have been recorded in the developing cortex, that occur less frequently and have a larger spatial extent (Allène *et al.*, 2008; Yang *et al.*, 2009).

These different temporal patterns of activity are thought to play unique roles in neuronal network development. In the somatosensory cortex gamma oscillations are confined to individual barrels and are thought to facilitate to organisation of the cortical layers, whereas spindle bursts spread over several different barrels and play a role in the coordination of activity between them (Suchkov, Sharipzyanova and Minlebaev, 2018).
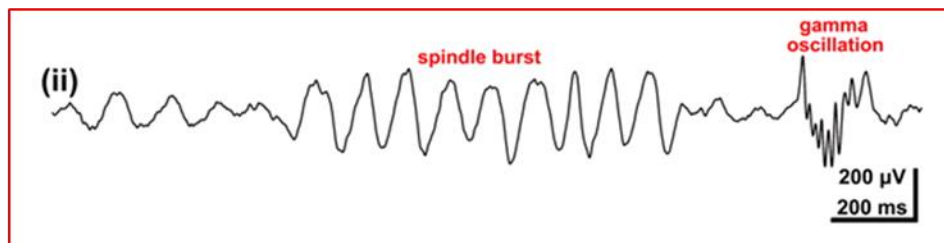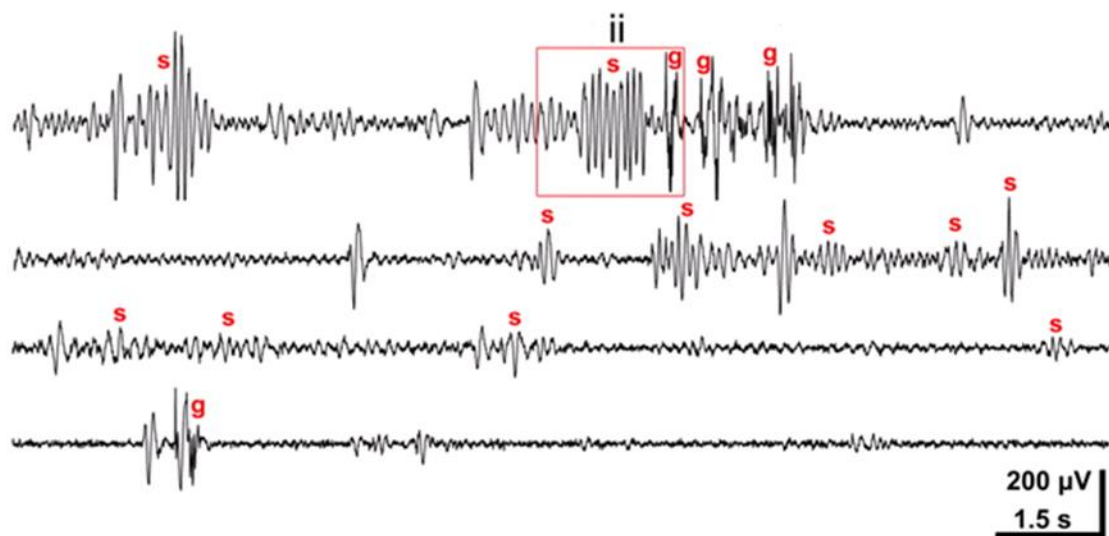
*Figure 1.2 **Examples of early network oscillations (ENOs) in the neonatal rodent cortex.***
In vivo spontaneous activity recorded by multielectrode array implanted in the rat somatosensory cortex during the first postnatal week. Showing examples of spindle bursts (S) and gamma oscillations (g). (ii) is an expanded timescale showing period from the red box.
Modified from (Yang et al., 2009).

1.2.3.4 *Activity in the immature human brain*

The temporal patterns of activity found in the immature brain result in discontinuous burst-like recordings (Dreyfus-Brisac and Larroche, 1971; M. André *et al.*, 2010). From EEG recordings in preterm infants this bursting pattern of activity in the immature brain has also been found in humans (Vanhatalo and Kaila, 2006a). Activity bursts that appear to be homologues for spindle bursts, called delta brushes, have been identified in the preterm brain, (Lamblin *et al.*, 1999; Vanhatalo *et al.*, 2002; M André *et al.*, 2010; Kaminska *et al.*, 2017). Furthermore, gamma oscillations have recently been discovered in the auditory cortex of preterm infants (Kaminska *et al.*, 2017). EEG recordings in preterm infants revealed that spontaneous activity is present at 24 weeks gestation, and continues through the third trimester becoming more temporally synchronised with development (Vanhatalo and Kaila, 2006b). This activity is particularly prominent over primary sensory cortical regions around 30 weeks, which is when thalamocortical projections are entering the cortex (Vanhatalo and Kaila, 2006b). As with the gross structural maturation of the developing brain, the transient, coordinated activity patterns in later fetal development in humans appears to parallel the development occurring in rodents during early postnatal life (Kostović and Jovanov-Milosević, 2006).

1.2.3.5 *Developmental transition to mature activity states*

Activity patterns found in the immature brain are grossly different to those occurring in adults (Cirelli and Tononi, 2015). The discontinuous activity that is characteristic of the developing brain (Figure 1.2) is not present in adults, where it has been replaced by a continuous oscillating brain state. The activity in the developing brain is not thought to just be a underdeveloped version of the adult, but appears to be specialised to facilitate the formation of organised sensory systems (Colonnese and Phillips, 2018). The immature network is arranged to amplify spontaneously generated activity, so even weak signals can reach the cortex – with this amplification process reducing around the emergence of active sensing (Colonnese *et al.*, 2010). The local neuron populations in the sensory cortices are highly synchronised in their activation early in development, but activity becomes more

sparse over the neonatal development period (Peyman Golshani *et al.*, 2009; Rochefort *et al.*, 2009). Dense firing patterns are poor at discriminating incoming sensory information (Smith *et al.*, 2015) but are primed for transmitting topographical information to the cortex with enough signal to induce plasticity and formation of the network (Colonnese and Phillips, 2018).

As the neuronal networks mature, they go from a pre-sensory state where this organisation and formation of networks is occurring to actively sensing and processing external information. There is a transitionary period where the immature activity patterns change, and mature ones begin to emerge. In rodents this transition occurs in the second postnatal week (Colonnese *et al.*, 2010). In the visual network responses to light stimulation begin around P10 and occur alongside spontaneous retinal wave activity, and then as full active vision emerges at P12 the background activity in the visual cortex becomes more continuous, with low amplitude slow-wave activity present between activity bursts (Colonnese *et al.*, 2010; J. Shen and Colonnese, 2016) (Figure 1.3).In the somatosensory cortex a similar transition occurs a couple of days before active whisking begins (Minlebaev *et al.*, 2011). The dense firing patterns of local neuron populations also transitions around this time, with firing becoming more sparse, as found in the mature cortex (Peyman Golshani *et al.*, 2009; Rochefort *et al.*, 2009). During dense firing patterns adjacent neurons fire simultaneously and with maturation less coordinated firing of local neurons is found (Figure 1.4). These dense firing patterns and thought to be suited to amplify incoming signals that may be weak in the immature network (Colonnese *et al.*, 2010), with sparse cellular firing thought to be more efficient at processing information (Olshausen and Field, 2004) and so more suited to the requirements of the mature sensory cortex. During this transition period spontaneous cortical activity goes from being driven by thalamocortical inputs to intracortical origins (Minlebaev *et al.*, 2011), which exhibit bistable network activity, resulting up and down states (Colonnese, 2014).

The transition from discontinuous to continuous activity patterns is also found in the developing human. Over the preterm period cortical activity recordings reveal an increase in frequency of activity resulting in shorter periods of quiescence. Around term (~40 weeks gestation) activity patterns stop bursting and become more continuous (Tolonen *et al.*, 2007), maturing to adult like patterns over the first few postnatal months (Vanhatalo and

Kaila, 2006b). Although active sensation develops earlier in humans than rodents and is present in preterm infants the cortex, is thought to still be poorly connected for sensory inputs and continues to develop during the third trimester (Pihko and Lauronen, 2004; Tolonen *et al.*, 2007; Fabrizi *et al.*, 2011). Cross cortical connections are also developing during this period (Kostović and Jovanov-Milošević, 2006) meaning this transitionary activity period in the human infant is occurring alongside the structural formation of neuronal networks as in rodents.
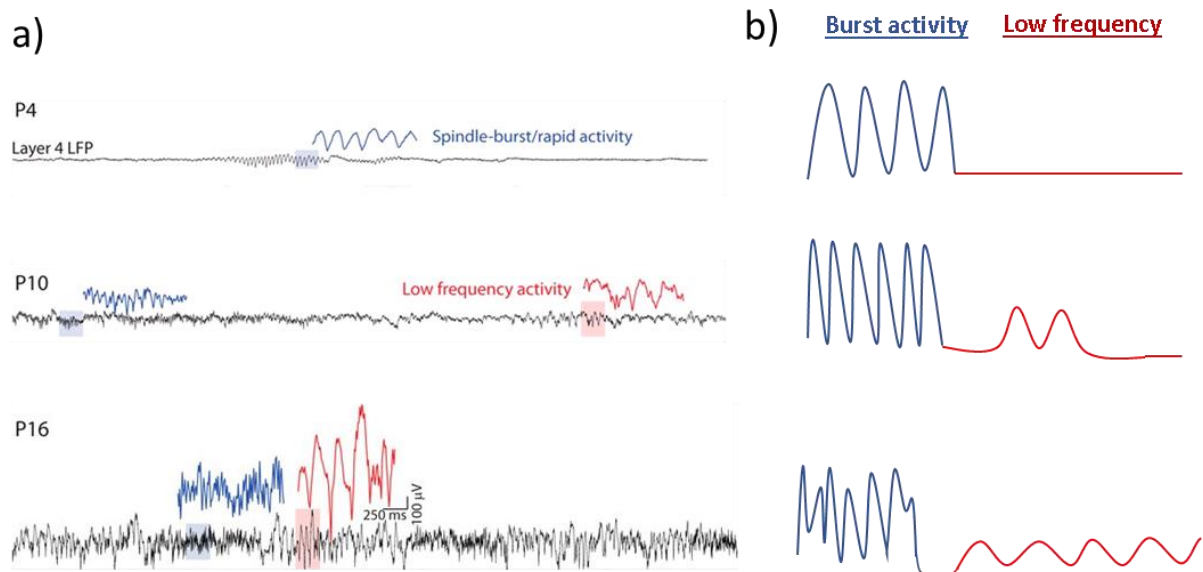
*Figure 1.3 **Development of discontinuous to continuous activity in the rodent neonatal cortex***
a) Example local field potential recordings from the neonatal rat visual cortex (Shen and Colonnese, 2016). With periods of rapid burst activity (and blue) and low frequency activity (red) highlighted.
b) A schematic of the activity patterns recorded at each age. At P4 activity is discontinuous, with rapid early network oscillations followed by network silence. By P10 there is some low frequency activity between bursts and by P16 activity is continuous with slow-wave activity interjected with rapid bursts of activity.
Figure modified from (Shen and Colonnese, 2016).
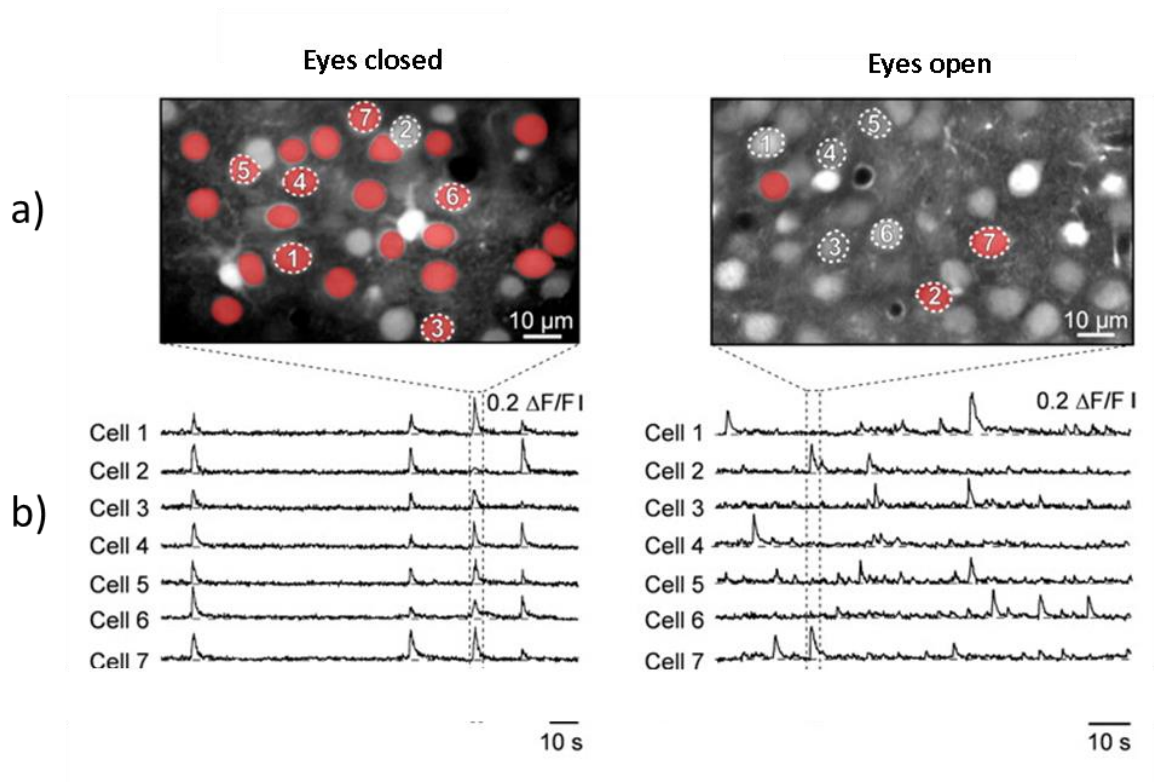
*Figure 1.4 D**ense to sparse spontaneous firing patterns of local neurons in the visual cortex***
a) Images from in vivo two-photon calcium imaging (OGB-1 dye) in layer 2/3 of the visual cortex, and
b) baseline corrected calcium transient wave forms of delineated neurons - showing activity patterns
from neuron population before and after eye opening.
Before opening these neurons usually fire simultaneously, but after their firing becomes more independent (sparse).
Modified from (Rochefort et al., 2009).

1.2.3.6   *Critical periods of development*

During development there are certain windows of time when neuronal network formation is particularly sensitive to sensory experience and a heightened level of plasticity is present; these time windows are known as critical periods (Berardi, Pizzorusso and Maffei, 2000; Feldman, 2009). These critical periods have been demonstrated in all the major sensory modalities, across a variety of species (Banks, Aslin and Letson, 1975; Fox, 1992; Issa *et al.*, 1999; Hensch, 2004; Barkat, Polley and Hensch, 2011). Critical periods are associated with dramatic changes in synaptic connectivity and function (Hensch, 2005; Feldman, 2009). This heightened plasticity, often in the form of long-term potentiation (LTP) facilitates the rapid formation of neuronal networks occurring during these early developmental periods. There is a critical period in the barrel cortex during the first postnatal week, where it has been demonstrated that LTP in thalamocortical afferents projecting into layer IV is at its peak at P3 and reduces around P8 (M. C. Crair and Malenka, 1995). It is at the end of this period that spontaneous activity is transitioning from immature to mature patterns (Minlebaev *et al.*, 2011), and shortly after this active whisking begins (Gordon and Stryker, 1996). The barrel cortex layer II/III critical period is later, in the second to third postnatal week (Stern, Maravall and Svoboda, 2001; Wen and Barth, 2011; Erzurumlu and Gaspar, 2012), along with a critical period for connectivity between these two layers (Maravall, Stern and Svoboda, 2004). In the rodent visual network, the initial postnatal days are a pre-sensory period where vision is not active. During this period spontaneous activity is vital for the initial formation of the retinotopic map in the visual cortex (Cang *et al.*, 2005b; Huberman, Speer and Chapman, 2006). Shortly after eye opening plasticity in the visual cortex increases and a critical period for ocular dominance occurs, which is a sensory experience dependent process (Gordon and Stryker, 1996; Hooks and Chen, 2007). It appears that the critical periods for sensory cortex development are multi levelled and different mechanisms mediate them.

One cellular mechanism known to play a role in critical periods of development is a phenomena known as silence synapses (Wu, Malinow and Cline, 1996). These are glutamatergic synapses that contain NMDA receptors (NMDAr) but not AMPAr. NMDAr

have a magnesium molecule blocking them at resting membrane potential and so are not readily activated (Nowak *et al.*, 1984). Activation of these synapses during the critical period results in plasticity where AMPAr are inserted into the post synaptic membrane and these synapses become active (Liao, Hessler and Malinow, 1995; Isaac *et al.*, 1997; Ashby and Isaac, 2011). The prolonged activity of spindle bursts occurring in the sensory cortices during development are thought to be ideal for activating NMDA only synapses and facilitating this unsilencing process (Colonnese and Phillips, 2018). This involvement of NMDAr in the critical periods of sensory development has been found across the sensory modalities - in the somatosensory (Schlaggar, Fox and O'Leary, 1993; M. C. Crair and Malenka, 1995; Iwasato *et al.*, 2000) visual (Erisir and Harris, 2003), auditory (Feldman, Brainard and Knudsen, 1996) and olfactory cortex (Poo and Isaacson, 2007). To form synaptic connections dendrite and axons must migrate close enough to one another. Spontaneous synaptic activity plays a role in activation of NMDAr receptors in immature neurons and blocking NMDAr receptors during this period results in a dramatic reduction in dendritic arbour complexity (Andreae and Burrone, 2014). Alterations to the function of NMDAr during this critical period has been found to disrupt the formation of the organised arrangement of the barrel cortex, both in the thalamocortical projections cortex (Iwasato *et al.*, 2000) and the within cortex columnar organisation (Fox *et al.*, 1996). Silent synapses are present during the critical period of thalamocortical maturation in the barrel cortex during the first postnatal week, but have disappear by P8/9, which coincides with the closure of the critical period and the loss of heightened LTP (Isaac *et al.*, 1997). The critical period of ocular dominance formation in the visual cortex has been found to be mediated by the function of NMDAr (Fagiolini *et al.*, 2003) and that the maturation of silent synapses can govern the duration of this period (Huang *et al.*, 2015).

This evidence indicates that activity during critical period of development is vitally important for the successful formation of sensory networks. During these critical periods the neuronal networks are primed for changes and this makes them particularly vulnerable to insult. Seminal work by Wiesel and Hubel in the 1960's discovered that deprivation of visual experience during the critical period of development disrupted the formation of the visual cortex structure and function (Wiesel and Hubel, 1963). Subsequently this has been shown across the sensory modalities (Hensch, 2004), and the impact of this altered sensory

experience has the most impact during these critical periods of development. In the somatosensory pathway, whisker damage at birth affected the development of the topographical organisation of the cortex but if this damage occurred at P5 development happened normally (Weller and Johnson, 1975). It is not just in controlled sensory deprivation that these altered early life experiences occur. Changes to cortical activity in the developing cortex have been found in a mouse model of autism (Fragile X syndrome) (Berzhanskaya *et al.*, 2016). In the pre-sensory period the visual cortex was hypo-excitable with reduced spontaneous firing, and although onset of active visual responses happened at the expected time, altered sensory-evoked functional activity in the mature cortex was found. Neonatal hypoxic-ischemic (HI) injury has also been found to reduce the plasticity during critical periods of development in both the visual (Failor *et al.*, 2010) and somatosensory cortices (Ranasinghe *et al.*, 2015). Neonatal HI injury also depressed spontaneous activity in the developing rodent cortex, and reduced spindle burst events (Ranasinghe *et al.*, 2015). Interestingly, EEG recordings in preterm infants have found a similar reduction in background activity, which has been used as a marker for neonatal brain injury (Wikström *et al.*, 2012; Ranasinghe *et al.*, 2015). In humans the rapid formation of sensory networks is occurring in late gestation (Kostović and Jovanov-Milosević, 2006), and a common clinical occurrence is premature birth, resulting in infants being outside of the uterus during this important developmental period (Harrison and Goldenberg, 2016). Many preterm infants develop sensory neurological deficients (Davis *et al.*, 2001; Suellen M. Walker *et al.*, 2009; Hellström, Smith and Dammann, 2013; Wickremasinghe *et al.*, 2013). These changes in neuronal network development in response to clinically relevant environmental changes makes the investigation of the mechanisms underlying them an area of great interest.

1.3 Project outline

Late fetal and early neonatal life is a period of rapid neurological development with many structural and functional changes unfolding (Gilmore and Herrup, 1997; Kostović and Jovanov-Milosević, 2006; Molnár *et al.*, 2006; Wang *et al.*, 2010; Erzurumlu and Gaspar, 2012). This period is primed for the formation and maturation of neuronal networks, which

are required in the mature animal for processing and interpreting information from the world around them. Neuronal activity has a vital role in this development, with disruptions to it in the maturing system having consequence for the functional connectivity of neuronal networks later in life (Spitzer, 2006; Blankenship and Feller, 2010; Kirkby *et al.*, 2013; Leighton and Lohmann, 2016; Luhmann *et al.*, 2016a). These early life disruptions can come from neonatal environmental changes, such as specific sensory deprivations or dramatic whole organism changes such as premature birth. These altered early life conditions can result in changes to neurodevelopmental trajectories on a number of scales, including; changes to synapse formation and cellular morphology (Burd *et al.*, 2010a; Chen, Bajnath and Brumberg, 2015) that can lead to altered neuronal network activity (Ranasinghe *et al.*, 2015; Sieben *et al.*, 2015), that may play a role in the altered behaviours found in animals who experience altered early life environments (Toso *et al.*, 2005; Arakawa and Erzurumlu, 2015a).

This project investigates functional development of the sensory networks in both human preterm infants and neonatal mice, in both healthy and adverse early life environments. The differential developmental time scales between these species (Clancy *et al.*, 2007) means that the early neonatal period of rodent development is informative of the preterm human brain. Neonatal mice in both health, premature and sensory deprived conditions are used to investigate the specific roles of early life experience on the development of sensory perception and functional network connectivity. A mouse model of prematurity is used to investigate developmental changes at a behaviour, cellular and synaptic level. Pan-cortical calcium imaging is utilised to investigate the functional activity of the developing mouse brain in both healthy and sensory deprived conditions, to explore the developmental trajectories of endogenously generated and sensory stimulated activity and the role that evoked sensory experience plays.

Naturally premature birth seems to be a uniquely human experience (Phillips, Abbot and Rokas, 2015a). Animals models of clinical pathologies offer a wealth of experimental exploration that is not possible in the human infant, but the ideal way to explore the impact of preterm birth on human neurodevelopment is with direct investigation of the human brain. This project further explores the impact of preterm birth on the development of the sensory networks with a neuroimaging study in preterm infants. It utilises the high spatial

resolution of functional magnetic resolution to explore the evoked activity in the central

network in response to somatosensory stimulation.

1.3.1 Thesis organisation

This thesis is divided into three studies that are presented in individual chapters.

Chapter 2 documents the development of a mouse model of premature birth. It investigates the neonatal development of the sensorimotor networks following this altered early life experience, using behavioural assays, electrophysiological investigation of cortical neuron functional maturation, and the structural development of thalamocortical projections in the somatosensory cortex. It seeks to investigate the impact that being born early has on the developing brain.

Chapter 3 documents the development of an *in-vivo* pan-cortical calcium imaging technique in neonatal mice. It investigates the development of cortical activity during the first 9 postnatal days, in both healthy and sensory deprived animal. Both spontaneously occurring and sensory stimulated activity is recorded, and the developmental trajectory of their spatiotemporal patterns are explored. Deprivation of early life somatosensory experience allows the investigation of which aspects of this functional development are experience-dependent.

Chapter 4 documents the development of a functional imaging paradigm in preterm human infants, to explore the development of the somatosensory network using evoked-sensory experience. Tactile stimulation was delivered during functional magnetic resonance image acquisition carried out on preterm infants at term equivalent age during natural sleep. The study both investigated the feasibility of this protocol for preterm infants and validates the sensory stimulation paradigm in a healthy adult population.

2 Mouse Model of Prematurity

2.1 Introduction

2.1.1 Clinical prematurity

Prematurity is a major global health problem (Harrison and Goldenberg, 2016). It is defined as births before 37 weeks of gestation and occurs in around 10% of pregnancies, and is associated with an increased risk of mortality and morbidity (Blencowe, Cousens, *et al.*, 2013). Medical advancements in recent decades means the rate of mortality has declined (Ananth *et al.*, 2005; Iams *et al.*, 2008; Saigal and Doyle, 2008), with infants as young as 22 week surviving (Ishii *et al.*, 2013; Rysavy *et al.*, 2015). While morbidity rates have also seen some decline (Wilson-Costello *et al.*, 2007; Hack and Costello, 2008; Moore *et al.*, 2012; Radesky, 2017), they are still a major problem, with many preterm infants developing neurological sequelae and life-long disabilities (Allen, 2008; Kidokoro *et al.*, 2014; Soleimani, Zaheri and Abdi, 2014; Dusing and Tripathi, 2015; Ortinau and Neil, 2015; Ream and Lehwald, 2018) - with estimates that around half of all childhood disabilities are an outcome of preterm birth (Glass *et al.*, 2015). There are sex differences in outcomes of preterm birth, with boys being born prematurely more often and having worse long-term outcomes (Zeitlin *et al.*, 2002; Ingemarsson, 2003; Kent *et al.*, 2012; Shim *et al.*, 2017; Lorente-Pozo *et al.*, 2018). Cerebral palsy (CP) is a group of disorders that affect movement and posture (Bax *et al.*, 2005) and is one of the major neurodevelopmental complications in preterm infants, as are other motor deficits (Allin *et al.*, 2006). Sensory processing changes are seen following preterm birth, with impairments to hearing (Davis *et al.*, 2001) and vision (Hellström, Smith and Dammann, 2013) being common, as well as changes in somatosensory processing (Bart *et al.*, 2011; Chorna *et al.*, 2014; Cabral *et al.*, 2016).

The severity of prematurity is subcategorised, into extremely preterm (<28 weeks gestation), very preterm (28-<32 weeks) and moderate to late preterm (32-<37 weeks) (Quinn *et al.*, 2016). The severity of outcomes is correlated with gestation length at birth, with infants born earlier generally having poorer outcomes (Himpens *et al.*, 2008; Stoll *et al.*, 2010; Sansavini *et al.*, 2011; Blencowe, Lee, *et al.*, 2013; Ishii *et al.*, 2013). However, even late preterm infants (Morse *et al.*, 2009; Osrin, 2010; Teune *et al.*, 2011; Dusing and Tripathi, 2015) and infants with mild presentation in infancy (Prempunpong *et al.*, 2018) are more likely to have adverse outcomes than full-term infants. This includes initial outcomes

such as increased mortality (Bulut, Gürsoy and Ovalı, 2016), risk of respiratory distress (Colin, McEvoy and Castile, 2010; Consortium on Safe Labor *et al.*, 2010; Natile *et al.*, 2014), rates of sepsis (Wang *et al.*, 2004; Melamed *et al.*, 2009) and occurrence intraventricular haemorrhage (Bastek *et al.*, 2008; Melamed *et al.*, 2009). Late preterm infants are also more likely to develop long-term developmental morbidities, including motor delays (Woythaler, McCormick and Smith, 2011; Odd *et al.*, 2013) and increased occurrence of cerebral palsy (Petrini *et al.*, 2009; Hirvonen *et al.*, 2014), and higher rates of asthma (Goyal, Fiks and Lorch, 2011; Liu *et al.*, 2014; Zhang *et al.*, 2018). Several studies have found that children born in the late-preterm period have poorer cognitive and academic performance (Chyi *et al.*, 2008; Morse *et al.*, 2009; Baron *et al.*, 2011; Chan *et al.*, 2016), and even some alterations in structural development of the brain have been found, with smaller tissue volumes during childhood being reported (Brumbaugh *et al.*, 2016).

The mechanisms behind these long-term developmental abnormalities is a complex issue, as prematurity is often accompanied with comorbidities which could contribute. It is thought that intrauterine infection is the cause of up to 40% of spontaneous preterm deliveries (Lamont, 2003; Bastek, Gómez and Elovitz, 2011; Agrawal and Hirsch, 2012; Galinsky *et al.*, 2013). This condition exposes the fetus to an adverse environment before being born early, and this has been shown to have implications for neurological development (Malaeb and Dammann, 2009; Burd, Balakrishnan and Kannan, 2012; Mwaniki *et al.*, 2012; Cordeiro, Tsimis and Burd, 2015; Hagberg *et al.*, 2015). Another common cause of preterm birth are placental pathologies (Morgan, 2016), which can compromise the supply of oxygen to the fetus and has also been implicated in long-term neurodevelopmental problems (Gagnon, 2003)

When an infant is born early many of its organ systems, such as the cardiovascular (Seri, 2001; Bennet *et al.*, 2012; Brew, Walker and Wong, 2014; Fyfe *et al.*, 2014; Wu, Azhibekov and Seri, 2016) and respiratory (Fraser, Walls and McGuire, 2004; Moss, 2006; Jones, 2009) systems, are underdeveloped and are not yet ready to sustain the infant outside the uterus. As a result, postnatal hypoxic and ischemic episodes are common in preterm infants and can have detrimental effects on the development of the brain (Logitharajah, Rutherford and Cowan, 2009; Scafidi *et al.*, 2009; Gopagondanahalli *et al.*, 2016). The immune system is also

underdeveloped meaning neonatal infection is more likely (Melville and Moss, 2013), which is linked to adverse neurodevelopmental outcomes (Mwaniki *et al.*, 2012).

The mechanisms underlying neurodevelopmental changes in preterm infants are not fully understood - due to the complex nature of the premature situation, the quickly changing structure of the brain during the preterm period, and the limited experimental exploration possible in human infants. Each preterm infant is unique in their early life experience and their long-term outcomes. It may be that different components of the preterm experience are contributing to the neurodevelopmental disruptions in different ways.

2.1.2   Animal models of prematurity

Animal models are a valuable experimental resource when trying to elucidate the mechanisms behind clinical neurodevelopmental injury such as in preterm birth. The timings of these key neurodevelopmental processes are different between species (Clancy *et al.*, 2007), with many of the events occurring in the third trimester of gestation in humans happening postnatally in rodents. Cortical neuron migration is mostly completed during fetal development in humans (Kostović and Jovanov-Milošević, 2006) but formation of cortical layers occurs postnatally in rodents (Kirischuk, Luhmann and Kilb, 2014) – with the peak in brain growth occurring during the final gestational weeks and in the second postnatal week for rodents (Dobbing and Sands, 1979). Once cortical neurons reach their destination they undergo maturation, such as axonal and dendritic growth (Semple *et al.*, 2013). Subcortical projections, such as thalamocortical neurons develop at a similar time, again being different in humans and rodents (Molnár and Blakemore, 1995; Kostović and Jovanov-Milošević, 2006). The formation of synapses spans a long period, starting embryonically and continuing into postnatal life, in both (Huttenlocher and Dabholkar, 1997; Li *et al.*, 2010; Pressler and Auvin, 2013; Semple *et al.*, 2013). There are also differences in key timings for the emergence of functional and behavioural aspects of brain development. Rodents are born blind and deaf and do not develop active sensing until the second postnatal week of life (Froemke and Jones, 2011; Jing Shen and Colonnese, 2016). Whereas cortical processing of visual and auditory stimulation is present in preterm human infants (Pryds, Greisen and Trojaborg, no date; Eldredge and Salamy, 1996), with continued

development of acuity during the postnatal period (Catford and Oliver, 1973; Litovsky, 2015).

Previous animal models of prematurity have considered these developmental timing differences and the common comorbidities in human preterm birth. Documented models include those based on: intrauterine infection and inflammation (Rousset *et al.*, 2008), intrauterine and neonatal hypoxic injuries (Brockmann *et al.*, 2013), and premature birth in isolation (Calmus *et al.*, 2011). A variety of species have been used including rats (Rousset *et al.*, 2006), mice (Toda *et al.*, 2013; McCarthy *et al.*, 2018), rabbits (Gorenberg *et al.*, 2005; Balakrishnan *et al.*, 2013), guinea pigs (Shaw *et al.*, 2016), sheep (Penning *et al.*, 1994; Back *et al.*, 2012), pigs (Sangild *et al.*, 2013; Andersen *et al.*, 2016)and non-human primates (Dieni *et al.*, 2004; Loeliger *et al.*, 2006; Verney *et al.*, 2010). These models result in a variety of neurodevelopmental changes on a structural, functional and behavioural level.

Impairments in the development of sensory motor behaviours during the neonatal period have been found in a variety of animal models. Intrauterine acute oxygen deprivation was found to impair lamb behaviour immediately following birth, including time to standing and to suckling (Castillo-Melendez *et al.*, 2013). Movement and suckling impairments were also found in P1 rabbits following intrauterine infection (Balakrishnan *et al.*, 2013), and prematurely born pigs took longer to stand and walk after birth compared to term controls (Andersen *et al.*, 2016). In rodents a variety of reflexive behaviours that integrate sensorimotor abilities develop during the first 3 postnatal weeks (Fox, 1965). The development of these behaviours have been found to be delayed following intrauterine infection in both mice (Chahboune *et al.*, 2009a) and rats (Toso *et al.*, 2005). Although neonatal hypoxic ischemic (HI) injury was not found to impair the development of these early behaviours (Brockmann *et al.*, 2013; Feather-Schussler and Ferguson, 2016) it does reduce locomotion and exploratory behaviour in adolescent rats (Girard *et al.*, 2009).

Gross morphological changes to the brain have been found in animal models of preterm comorbidities. Reductions in brain weights have been found following acute neonatal HI injury (Brockmann *et al.*, 2013), chronic neonatal hypoxia (Fagel *et al.*, 2006) and intrauterine infection (X. Wang *et al.*, 2007a; Rousset *et al.*, 2008). Further investigation found that these models of insult resulted in reductions in cortical volume, including neonatal HI injury in rodents (Failor *et al.*, 2010; Brockmann *et al.*, 2013) and intrauterine HI

injury (Dean *et al.*, 2013) and infection (Dean *et al.*, 2011) in sheep. Chronic neonatal hypoxia in mice resulted in a reduction in cortical volume at P11, which was found to be a result of reduced cortical neuron count (Fagel *et al.*, 2006). Interestingly, following the return to normoxic conditions at P11 this cortical deficit recovered, due to increased neurogenesis, demonstrating remarkable resilience of the developing brain to injury. In many studies no change in cortical neuron numbers were reported but changes in the morphology of cells were. Intrauterine infection was found to reduce the complexity of the dendritic arbour of cortical excitatory neurons and the number of spines on them in both rabbits (Balakrishnan *et al.*, 2013) and mice (Burd *et al.*, 2010a). A reduction in dendrites and spines was also found following intrauterine hypoxia in sheep (Dean *et al.*, 2013) and neonatal HI injury in rats (Ranasinghe *et al.*, 2015). Prematurely born rhesus monkeys, who underwent neonatal care for the first few weeks of life had altered cortical neuron synapse morphology but no change in the density (Bourgeois, Jastreboff and Rakic, 1989).

Changes to cell morphology can result in changes to their function. Early brain activity recordings (EEG) in preterm infants have been found to be predictive of neurodevelopmental out comes in humans (Wikström *et al.*, 2012; Hüning *et al.*, 2018). Changes to cortical function have been found in several animal studies of moderate neonatal HI injury at P2. At the end of the first postnatal week a reduction in gamma oscillation in the prefrontal cortex was found (Brockmann *et al.*, 2013). During the first 2 postnatal weeks a reduction in background cortical activity was also found (Ranasinghe *et al.*, 2015) which was not found in the same model at 4 weeks old (Failor *et al.*, 2010), suggesting that this is a transient functional change occurring during a critical period of cortical development. It was also discovered that HI injury impaired plasticity during critical development periods in both the visual (Failor *et al.*, 2010) and somatosensory cortex (Ranasinghe *et al.*, 2015). Even if these activity changes are transient their presence during the critical periods of functional network development could result in long-term deficits in sensory function.

All the rodent models described here used comorbidities associated with prematurity during the early neonatal period to mimic insults in human preterm infants. Few rodent studies have investigated the long-term developmental outcomes following birth before term. One of the challenges of animal models of prematurity is survival. Spontaneously occurring

preterm birth is uncommon in species other than humans (Rubens *et al.*, 2014; Phillips, Abbot and Rokas, 2015b) and when premature birth is induced experimentally, long-term offspring survival is reduced (Loctin and Delost, 1983). However, a successful induction of prematurity with viable offspring in mice has been achieved and long-term investigation of neurodevelopment carried out (Toda *et al.*, 2013). This study finds an acceleration of thalamocortical development in the somatosensory pathway which is an interesting contrast to deficits in development found in many comorbidity models. The specific role of being born early in the premature infant in isolation of other pathophysiological changes is an interesting and relatively underexplored topic.

2.1.3   Aims

This study uses a similar approach to Toda *et al.*, 2013., using a progesterone antagonist to induce preterm birth, in isolation of other comorbidities. Toda et al., 2013 induce early birth in ICR mice by subcutaneous injection of 150µg of RU486 on embryonic day 17. This study uses the same drug quantity and delivery method to induce early birth in C57/BL6 mice. Due to the differing gestation lengths of different mouse strains (Murray *et al.*, 2010) the earliest possible induction for C57/BL6 mice will be trialled to maximise the degree of prematurity in this study.

This study asks the question of how being born early affects the development of the mammalian brain, with focus on the development of the sensorimotor networks which are in a particularly vulnerable developmental stage during the preterm period. This will help to tease apart the complex situation of prematurity and shed light on how the altered environment of being outside of the uterus before fetal development is complete affects the development of the brain. As previously discussed late preterm infants and those with mild clinical presentations can still develop long-term neurological alterations. By exploring an animal model of exclusive prematurity it may be possible to identify the mechanisms that alter neurological development in these situations. When a human clinical situation is as complex and varied as the preterm infant experience it is valuable to understand the contribution of different factors as this can allow a more individualised approach to each unique situation. There is relatively little evidence about the contribution of being born

early on the development of the mammalian brain and so this study will be a valuable

contribution to the existing body of work in the field of animal models of prematurity.

2.2 Methods

All procedures were carried out in accordance with UK Home Office guidelines set out in the Animals (Scientific Procedures) Act 1986.

2.2.1 Breeding

C57BL/6 adult mice (over 8 weeks old) were purchased from Harlan Laboratories (UK). They were reared in normal 12hr light/dark cycles, with *ad libitum* access to food and water. Timed breeding was carried out, where estrus states of females was assessed by vaginal cytology (Byers *et al.*, 2012), and if found at a receptive stage they were housed with a male for 1 hour (9-10am). Successful mating was confirmed by the presence of a vaginal plug (Behringer *et al.*, 2016). This time point was assigned post-conception (PC) day 0. All experiments were time matched to this timepoint. (timeline of experimental procedures is outlined in Figure 2.1)

2.2.2 Preterm induction

A drop in progesterone levels precedes parturition in rodents (Pasqualini, Kincl and Sumida, 1985). As such, artificially reducing progesterone signalling, by blocking the activation of progesterone receptors, can be used to induce preterm birth. RU489 (also known as mifepristone), a competitive progesterone receptor antagonist, has previously been used in mice for this purpose (Dudley *et al.*, 1996), and the dose used in this study is based on these findings.

A subcutaneous injection of RU486 (Bio-Techne Ltd, UK) (150μg dissolved in 100μl 2% DMSO (Sigma-Aldrich) in sesame oil (Sigma-Aldrich)) was administered on PC 17d + 9hrs to induce preterm birth. Vehicle control dams received a 100μl subcutaneous injection of sesame oil at the same time point. Dams were monitored by video surveillance (H.264 DVR - RF Concepts Ltd. UK) with an IR sensitive camera (RF Concepts Ltd, UK) from the time of injection to birth of her litter, to assess precise gestation length and monitor wellbeing. 6-12 hours after birth the number of pups were counted, dead pups removed, and live pups

weighed (TS120, OHAUS, Switzerland). Then mother and surviving pups were returned to the standard unit house, in a Tecniplast (UK) 1284 conventional cage. Pups were weighed daily, and an assessment of developmental stage was made based on the Jackson Laboratory's (USA) 'Appearance by Age' chart.

An additional cohort of natural births was collected. Time breeding was carried out and at PC17d + 9hrs dams were placed under video surveillance, for precise recording of gestation length. 6-12 hours after birth the number alive and dead of pups were counted, and living pups were weighed. Offspring from these litters were not further assessed.

In alternative animal models of prematurity sex differences in outcomes have been seen (Johnston and Hagberg, 2007; J.D.L. Bergeron *et al.*, 2013; Shaw *et al.*, 2016), so in all experiments male and female offspring were used, and sex was considered in statistical analysis. Sex was visually assessed by anogenital distance (Greenham and Greenham, 1977).
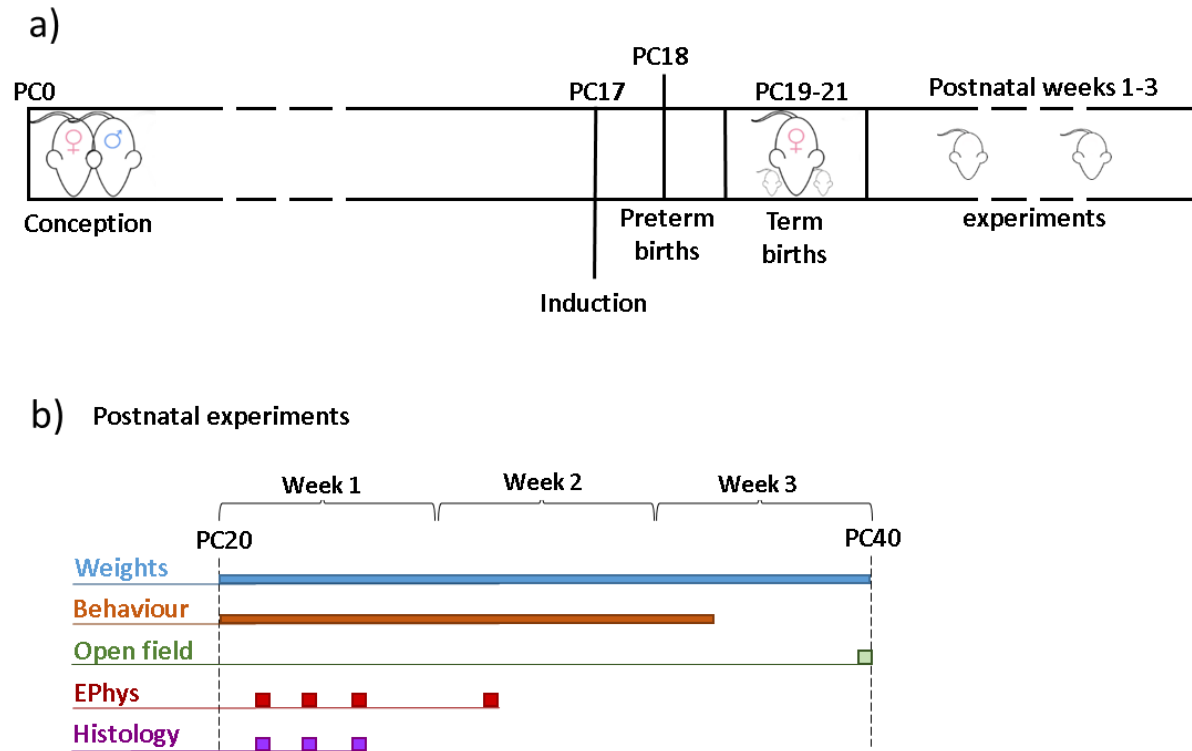
Figure 2.1 **Timeline of preterm mouse model experimental procedure**
a) Preterm birth was induced at post-conception day (PC) 17 resulting in births on PC18. Control animals received a vehicle at PC17 and term litters were born between PC19 and PC21.
b) Pups underwent experiments during the first 3 postnatal weeks (before weaning). Weights were taken daily from PC20 to PC40. In the same animals, developmental behaviours were recorded daily from PC20-35 and open field exploration investigated at PC40. In animals from other litters electrophysiological (EPhys) recordings were taken on PC22d, 23d, 24d and 28d, and brain fixed for histological investigation on PC22, 23 and 24. Both preterm and control groups were collected for all experimental protocols.

2.2.3   Electrophysiology

2.2.3.1   *Solutions*

Details of the solutions used for whole cell patch clamp solutions are below. Compounds were purchased from Sigma-Aldrich Ltd (UK), Fisher scientific UK Ltd (UK) and Hellobio (UK).

2.2.3.1.1   Artificial cerebrospinal fluid

Compound	Concentration (mM)
NaCl	119
KCl	2.5
Glucose	11
NaH_2OPO_4	1
$NaHCO_3$	26.5
$MgSO_4$	9/1.3*
$CaCl_2$	2.5

*Slicing/recording concentrations

Saturated in 95% oxygen and 5% carbon dioxide

2.2.3.1.2 Current clamp internal solution

Compound	Concentration (mM)
$KMeSO_4$	130
NaCl	8.5
HEPES	5
EGTA	0.5
Mg-ATP	4
Na-GTP	0.3

Adjusted to pH 7.25 using concentrated KOH, and osmolarity of 285 mOsM

Junction potential = 9mV

2.2.3.1.3 Voltage clamp internal solution

Compound	Concentration (mM)
$CsMeSO_4$	135
NaCl	8
HEPES	10
BAPTA	5
Mg-ATP	4
Na_GTP	0.3
QX314	5

Adjusted to pH 7.25 using concentrated CsOH, and osmolarity of 285 mOsM

Slice preparation

Brain slice experiments were carried out on PC 22-24d and 28d. Pups were killed by UK Home Office Schedule 1 procedure (cervical dislocation). Brains were rapidly dissected out and placed in ice cold slicing ACSF. Slices were collected to preserve long range thalamocortical projections (Agmon and Connors, 1991). The rostral portion of the brain was removed with a scalpel blade at a 45-55^0 angle (reducing angle with PC age), the right hemisphere was removed at a 90^0 angle to the first cut, and any remaining cerebellum was removed (Figure 2.2). The rostral cut surface of the brain was superglued into the vibratome cutting chamber (Leica VT1200 vibratome, Leica Microsystems, UK) and submerged in cold slicing ACSF, with the pial surface facing the blade. The top portion of the brain was discarded (1000μm at PC 22-24d and 1500μm at PC 28d) and 5 slices of 400μm were cut and collected in cold cutting ACSF. Slices were transferred into recording ACSF at room temperature and allowed to rest for at least 1 hour before experiments and were usually viable for 6-8 hours.
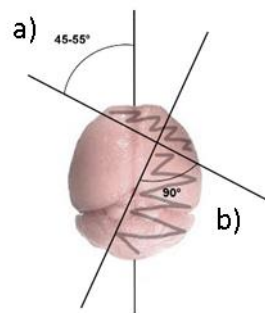
*Figure 2.2 **Orientation of gross brain dissection for slice preparation***
First the rostral portion of the brain was dissected away at 45-55^0 from the midline (a) and then the right hemisphere was dissected away at 90^0 to this initial cut (b).

2.2.3.3 *Whole cell recordings*

Whole cell patch clamp recordings were made in Stellate neurons in layer IV of the barrel cortex. Slices were placed in the recording chamber of a Zeiss Axioskop microscope (Carl Zeiss Ltd, UK) and held into place with a slice anchor (Harvard Apparatus Ltd). Temperature has been shown to be important in both synaptic transmission (Hardingham and Larkman, 1998) and intrinsic cellular states (Simkus and Stricker, 2002), so slices were perfused continuously with ACSF (using a MINIPULS 3 - Gilson U.K) kept at 32 ± 1^0C - using a single channel heat controller (TC-324B, Harvard Apparatus Ltd).

The barrel cortex was visualised at low power (Zeiss 2.5x) magnification using infrared differential interference contrast (DIC) optics (Figure 2.3a). Patching was performed under high power (Olympus 40x) magnification, and stellate neurons were identified by their characteristic small, spherical shape (Figure 2.3b), and later confirmed by their electrophysiological properties.
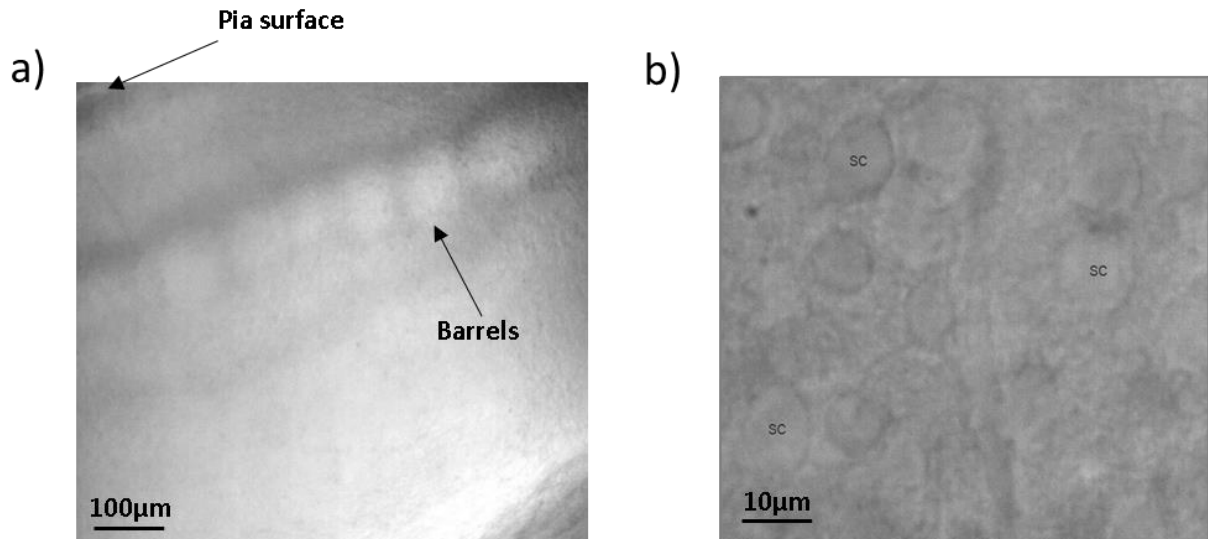
*Figure 2.3. **Visualisation for whole cell patch clamp experiments***
(a) 2.5x magnification image of the barrel cortex. (b) 40x magnification of barrel cortex, with spiny Stellate neurons (sc) identified.

Microelectrodes with a tip resistance of 3-6MΩ were made using borosilicate glass capillaries (Harvard Apparatus) pulled with a micropipette puller (Model P-87, Sutter Instrument Co.). Recordings were performed with a Multiclamp 700A amplifier (Molecular Devices, USA) and digitised with a Micro1401 data acquisition box (Cambridge Electronic Design Ltd, UK), and acquired using Signal (version 5 - Cambridge Electronic Design Ltd, UK). Signals were acquired at a 10kHz sampling rate, and were Bessel filtered at 4kHz. High quality recordings were confirmed by: a gigaohm seal achieved before breaking in and access resistance <25MΩ throughout the recording.

Whole cell current clamp recordings were made with patch electrodes filled with current clamp intracellular solution (section 2.2.3.1.2). Recordings were made at resting membrane potential (RMP) and -70mV holding potential (before junction potential correction), which is close to RMP in mature stellate neurons (Fleidervish, Binshtok and Gutnick, 1998). A protocol of an injected hyperpolarisation current step (-0.05V, 500ms), followed by 18 increasing (in amplitude) depolarising square wave pulses (0-0.36v in 0.02 steps, 500ms) was run on each cell. A separate depolarisation protocol, with fine increments (40 increasing ramp steps, with a change of 20mV over 100ms, start points from 0.01 to 0.21V) was used to determine the minimal current injection require to drive the membrane to threshold and fire an action potential (AP) (rheobase).

Voltage clamp recordings were made with patch electrodes filled with voltage clamp intracellular solution (section 2.2.3.1.3). 50µM picrotoxin (PTX) and 500nM tetrodotoxin (TTX) were added to the standard ACSF for these experiments, to block GABAA receptors and voltage-gated sodium channel current respectively. Spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded. AMPAr and NMDAr have differential likelihood of opening at different membrane potentials due to magnesium blocking NMDAr receptors at hyperpolarised membrane potentials (Nowak *et al.*, 1984). By recording at both -70 and +40mV holding potential AMPAr and NMDAr mediated activity was isolated. These were recorded in 2 second sweeps, with a 50ms, 5mV pulse at the start to each, with alternation between the holding potential occurring every 60 sweeps, for approximately 200 sweeps per holding potential per cell (minimum 130 sweeps).

2.2.3.4   *Analysis*

Fast spiking interneurons are also present in the barrels and have similar morphological characteristics when visualised in this experimental protocol. They however have distinct firing patterns in response to depolarisation steps (Gibson, Beierlein and Connors, 2005; Daw, Ashby and Isaac, 2007), making it possible to identify them post hoc and eliminate them from the analysis pool.

2.2.3.4.1   Current clamp recordings

Current clamp recordings were analysed with custom software in Matlab R2015a (Mathworks, UK). Junction potential (Gorman and Murphy, 1949) was calculated and included in the analysis to adjust membrane potential calculations to the actual value.

Passive membrane properties were measured from the hyperpolarisation step of the protocol (Figure 2.12a). Resting membrane potential (RMP) was calculated from the mean membrane voltage when no current injection was present at the start of the protocol. Input resistance (Ri) was calculated using Ohm's law ($V = I \times R$), taking V from the steady state membrane potential change driven by the hyperpolarisation step (Figure 2.12a). The membrane time constant (tau) and sag were measured by fitting an exponential to the

membrane charging curve, with Ri used to guide its final value. Tau was defined as the point where the membrane potential reached 63% of this final value and sag is the difference between the voltage change estimated by the exponential fit and the actual membrane charge curve.

An automated analysis script was used to detect all APs triggered by the depolarising current steps (peak voltage that occurred after a first derivative value of >30V/s). The first AP generated by each cell was used to analyse waveform properties. The first derivative of the waveform was calculated, and the maximal rise time was taken as the largest value (Figure 2.13d) and the threshold was designated as the point at which the rise time became greater than 11.25V/s (Figure 2.13b). The height of the waveform was the voltage change between the threshold and the peak of the waveform (Figure 2.13c), and the width was taken at half of the AP height (Figure 2.13e). As a measure of excitability, the total number of APs fired during the depolarisation protocol was calculated and normalised to the number of steps they were fired during (which step AP firing began varied between neurons).

2.2.3.4.2   Miniature excitatory post-synaptic currents

Voltage clamp recordings were analysed in Clampfit 10.5 (Molecular Devices, LLC). Templates of averaged event shape were created for both +40mV and -70mV recordings, based on two randomly selected data files. They were then tested on 10 additional random recordings, and manually checked to confirm they did not produce false positives or negatives. Then all traces were analysed with the software's automated template search, which marks events that match the custom templates. This gave the number of events in the recording, and the amplitude (the change from baseline to peak) can be calculated for each event.

2.2.4 Histology

Brains were collected at PC 22-24d and 28d. Pups were killed by UK Home Office Schedule 1 procedure (cervical dislocation). Brains were dissected out and placed in 4% paraformaldehyde for 48hrs at 4°C. They were then transferred into PBS and stored at 4°C.

2.2.4.1 *Cytochrome oxidase staining*

Whole brains were encased in low melting point agarose (2-3% in distilled water) block to provide stability while sectioning. Coronal sections were cut through the barrel cortex at 50µm thickness (Leica VT1200 vibratome, Leica Microsystems, UK), and placed in PBS in a 24 well plate (Greiner Bio-One, UK). Sections were washed a further two times in PBS for 5 minutes, and then incubated in 1ml of Cytochrome stain (see below) for 36 hours in a 37^{0}C water bath. Sections were then rinsed twice in 0.1M Tris buffer (pH 8) (Sigma-Aldrich, UK), mounted on slides (Leica Microsystems, UK), left to air dry overnight, then cleared in Histoclear for 10 minutes (Sigma-Aldrich, UK) and then cover slipped (VWR, UK) using DPX (Sigma-Aldrich).

Cytochrome Oxidase Stain

DAB	3mg
Cytochrome C	1.5mg
Sucrose	225mg
0.1M phosphate buffer	5ml

All compounds were purchased from Sigma-Aldrich (UK).

2.2.4.2 *Analysis*

Optical density of the cytochrome oxidase staining was measured to quantify barrel structural development. The coronal brain section where the hippocampus was first visible

was identified and the 1st and 6th section from this point were analysed. Images were captured on a contrast microscope (DM IRB – Leica Microsystems, UK) at 2.5x magnification, with a Nikon colour microscope camera (Nikon UK Limited), as 1600X1200 pixel TIFF files.

Images were analysed using Fiji (ImageJ). Images were rotated so barrels were parallel to the top of the image, then inverted and smoothed using a mean filter at a five-pixel radius. A narrow rectangle was drawn across the 3 barrels and 2 septums (Figure 2.11d) and the pixel density plotted. In young animals the barrels are not visible (Figure 2.11c). The location of the ROI was determined using the pia surface and the hippocampus as landmarks and sections from older animals where the barrels were visible. The value at the peaks (barrels) and troughs (septums) of the plot were used in Equation 1, along with the mean pixel value of the whole slice (T) to give a ratio of barrel to septum optical density (BFI-CO) [adapted from (Toda *et al.*, 2013)].

Equation 1. BFI-CO = ((Barrel 1 + Barrel 2 + Barrel 3) / 3) – ((Septum 1 + Septum 2)/2) / T

The width of the cortex was measured on the section where the hippocampus was first visible - from the pia surface to the corpus callosum at the outer edge of the hippocampus (
Figure 2.4).
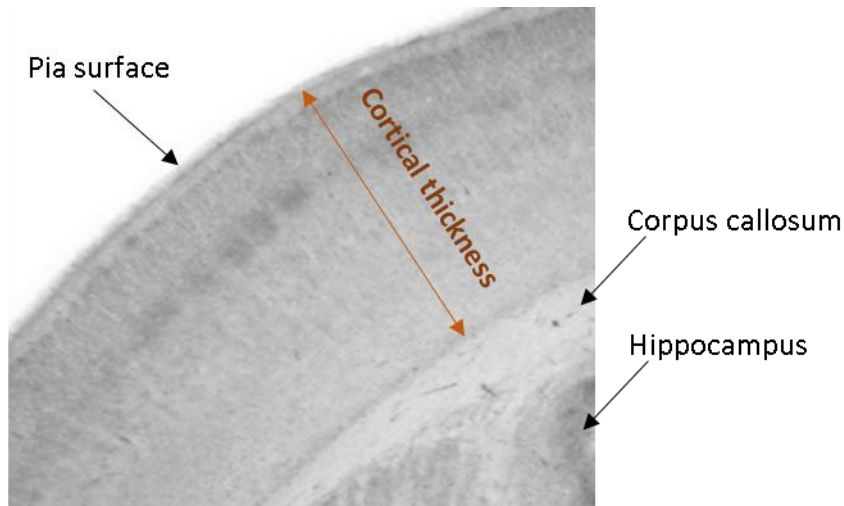
*Figure 2.4 **Measuring cortical thickness**.*
The location cortical thickness measurements were taken

2.2.5 Behaviour

A series of behavioural tests - similar to those reported in (Fox, 1965; Arakawa and Erzurumlu, 2015a; Feather-Schussler and Ferguson, 2016) - which are designed to assess motor and sensory development were carried out between PC 22-35d. The cohort includes 20 pups in each group, from 6-7 litters, with 10-14 subjects being assessed at each time point, with balanced male and female participation. Testing was carried out at the same time each day, and light and noise levels were kept consistent. Subjects went through all behavioural assays in a set order and were then placed back in their home cage.

2.2.5.1 *Righting reflex*

Pups were placed in a circular 20cm Pyrex staining dish on their back. The time taken to roll over and place all four paws on the surface was recorded. If this time exceeded 30 seconds the pup was righted by the experimenter - to conform to the specifications of the Home Office PPL 30/3108 – and was recorded as 30s. This was repeated twice each day.

2.2.5.2 *Cliff avoidance*

Pups were placed with their head and forepaws over the edge of a 10cm high custom-made cliff drop (a flat surface with a 90^0 angle edge). The time taken to back away and turn 45^0 from the edge was recorded. Again, PPL specifications allowed up to 30s to complete the task and if pups failed or fell off the apparatus a time of 30s was recorded. This was repeated twice each day.

2.2.5.3 *Negative geotaxis*

Pups were placed head down on a custom made 45^0 slope, which was covered in fine mesh to allow some traction. Time taken to turn 180^0 and face up the slope was recorded. Again, 30s was allowed and if pups failed or fell off the apparatus a time of 30 seconds was recorded. This was repeated twice each day.

2.2.5.4   Grasping reflex

A thin steel rod (galvanised steel, 1mm diameter) was placed under the palmar surface of the forepaws and the response recorded. This behaviour was graded by the following system: No grasping = 0 points, bent digits = 1 point and full grasping = 2 points. If grasping was present the rod was slowly raised until the pup was stretched out with hind paws remaining on the surface. Whether the pup remained grasping was recorded (1 point for success). This was repeated for right and left paw twice each day.

2.2.5.5   Whisking behaviour

Pups where observed for 60 seconds to detect the presence of whisking behaviour. This behaviour was graded by the following system: No whisker movement = 0 points, whisker twitching = 1 point and full whisking = 2 points. There were two 60s observation periods each day.

2.2.5.6   Whisker stimulation

Pups were presented with manual whisker stimulation using a thin steel rod (galvanised steel, 1mm diameter) for 30 seconds at a 1Hz frequency across the entire whisker field - with care taken not to touch the face. Head movements in response to stimulation were counted. This stimulation was presented twice per day on left and right whisker fields.

2.2.5.7   Tactile stimulation

Pups were presented with a tactile stimulation using a small plastic rod (3mm diameter), which was used to manually touch 7 points on their body (head, back, tail, forepaws and hind paws), 5 times each, in rotation. Twitching or withdrawal responses to each stimulation were counted.

2.2.5.8 *Open field*

At PC 40d (weaning age) pups were presented with an open field experiment. They were placed in a clear plastic 30x50x25cm (width x length x height) arena and left alone in the room to freely move for 6 minutes, whilst being recorded (Quickcam - Logitech, Switzerland). Subject movement was tracked using an open source Matlab toolbox (Autotyping, University of Pennsylvania). The first 60 seconds were discarded, allowing time for the experimenter to leave the room and for the mouse to acclimatise from being handled. The subsequent 5 minutes of open field movement were quantified, giving values for distance moved, average speed and a ratio of time spent in the outer 5cm and inner region of the arena (thigmotaxic behaviour).

2.2.6 Statistical analysis

Statistical analysis was performed using R version 3.5.0 (The R Project).

Data was checked for normality of distribution using both a Shapiro-Wilk's test and visual assessment using a histogram and quartile-quartile plot. If either group failed normality testing non-parametric tests were used, typically a Mann-Whitney U test, with two tailed t-tests and ANOVAs being used for normally distributed data.

Most data are presented as boxplots, where mean, 25th and 75th quartiles are shown in the main box, and whiskers representing the largest values, with observations more than 1.5 times the IQR shown as outlier points.

Longitudinal behavioural data had individual subjects repeatedly tested across time. The data did not meet requirements for a repeated measure two-way ANOVA as there were missing values, the distributions were not normal and there was not homogeneity of variance (Liu, Cripe and Kim, 2010). Instead mixed-model regression analysis was carried out on this data. Another data assumption that influences model choice is the linearity, which the data was assessed for. All data sets appeared to be non-linear so generalised additive mixed models (GAMM) were used, with the package MGCV (Wood, 2006). This uses smooth spline fitting to model non-linear relationships. Age and group were accounted for as fixed independent variables and animal ID as a random factor in all models, and other

random variables (sex, litter, litter size, gestation length of controls, repeated trial) were considered and dropped in a stepwise process if they did not significantly contribute to the model fit. Different distribution and types of data (e.g. continuous or count) were accounted for in the model using different family options as is standard with generalised linear modelling. Model fit was checked by assessment of the residuals for normality and heteroscedasticity, and by how well predicted values correlated to raw data. Final model assessment was made by adjusted R-squared (R-sq.) values, indicating the goodness of it as a proportion of the variance in the data that was explained by the model.

Sample sizes were calculated a priori, using G*Power (version 3.0.10) – with two tails, a power of 0.90 and an α error of 0.05. Effect sizes were determined for each experimental section individually based on previous experimental findings. For electrophysiological experiments unpublished data of normal electrical development of Stellate neurons in mice during the first 2 postnatal weeks was used (collected in Dr Ashby's laboratory, University of Bristol). Histology effect size was determined from previous published experiments investigating the anatomical development of barrels following early delivery (Toda *et al.*, 2013), and behavioural experiment effect size from previous published data on the developmental trajectory during the early neonatal period and in impact of altered sensory experience (Arakawa and Erzurumlu, 2015b).

2.3 Results

All data in this section are presented as post-conception (PC) days. PC0d is given to the hour that pregnancy was confirmed with the presence of a vaginal plug (Behringer *et al.*, 2016), and all experimental time points are matched to this hour.

2.3.1 A mouse model of premature birth

Clinical preterm birth is a complicated situation of comorbidities. Previous rodent models of prematurity have investigated the effects of one or more comorbidities, but little information is available about the effects of preterm birth alone. In this study the effects of being born before term gestation are investigated with a mouse model of prematurity. This model uses a progesterone receptor antagonist to induce premature birth in isolation of the comorbidities, and offspring development during the first few postnatal weeks is investigated.

First the success of inducing premature birth with this model needs to be confirmed Premature birth was induced by a single administration of the progesterone receptor antagonist RU486 by subcutaneous injection into pregnant dams at PC 17d + 9hrs. RU486 administration triggered parturition within the next 18 hours, resulting in significantly shorter gestation lengths compared with animals who received a vehicle only injection at the same time point (Figure 2.5a) ($p < 0.001$ - Mann-Whitney U test) [n: preterm = 35; vehicle = 21, litters]. The average control gestation length is 19.5d±17.5hr and in preterm induction it is 18d±1.3hrs, meaning preterm birth in this model is 92.5% of average gestation. This gestation length is in line with the clinical definition of prematurity in humans, which is live births occurring before 37 weeks gestation (92.5% of 40 weeks full term gestation) (Gibson and McKeown, 1951).

Clinically preterm birth is associated with increased mortality (Manuck *et al.*, 2016), even in late preterm infants (Jain, 2007). The survival fraction of litters was calculated as the percentage of living pups against the total litter size. In this model of preterm birth there was a reduction in pup survival at birth compared to control litters (Figure 2.5b) ($p < 0.001$ - Mann-Whitney U test) [n: preterm = 32; vehicle = 21, litters]. Further mortality was

observed in preterm pups over the first 24 hours, with survival dropping from 54% at birth to 45% by P1. No pup deaths were observed in the control group over the first 24 hours. PC18d is the earliest preterm birth that resulted in live offspring for longitudinal assessment with this protocol. On 5 occasions preterm birth occurred before PC18d (17d+21 hrs ± 2.4 hrs) and in all cases no pups survived beyond 2 days old. As such this protocol is on the cusp of prematurity that can be investigated in mice with no additional support for survival given. Clinically a differential effect of preterm birth is seen in male and female offspring (Ingemarsson, 2003; Johnston and Hagberg, 2007; Shim *et al.*, 2017), so sex was considered in this study. There was no significant difference between the ratio of live male and female pups in preterm or control litters (p = 0.7524 – Fishers Exact test) [n: preterm – 20/7, control – 20/7, (pups/litters)].

Surviving pups were weighed on the day of birth. Animals from preterm litters were significantly smaller than those from control litters (Figure 2.5c) (p <0.001 – unpaired, t-test) [n: preterm = 23; vehicle = 14, litters].

The control group for this study received vehicle injections at the same time point as preterm inductions were given. This is to control for the potential stress that receiving an injection induces in the mother. To see whether vehicle control injections affected pregnancy a cohort of natural births were compared to the vehicle control group.

Gestation lengths in vehicle control pregnancies were not different to untreated ones (Figure 2.5a) (p = 0.714 - Mann Whitney U) [n: natural = 8; vehicle = 21]. This suggest that the vehicle injections used as a control in this protocol do not induce premature labour. There was also no adverse effects on pup survival (Figure 2.5b) (p =0.4847 - Mann-Whitney U test) [n: natural = 7; vehicle = 21], and birth weights were the same between vehicle injected and natural litters (Figure 2.5c) (p =0.432 – unpaired, t-test) [n: natural = 7; vehicle = 14]. Natural pregnancy litters were not investigated further.
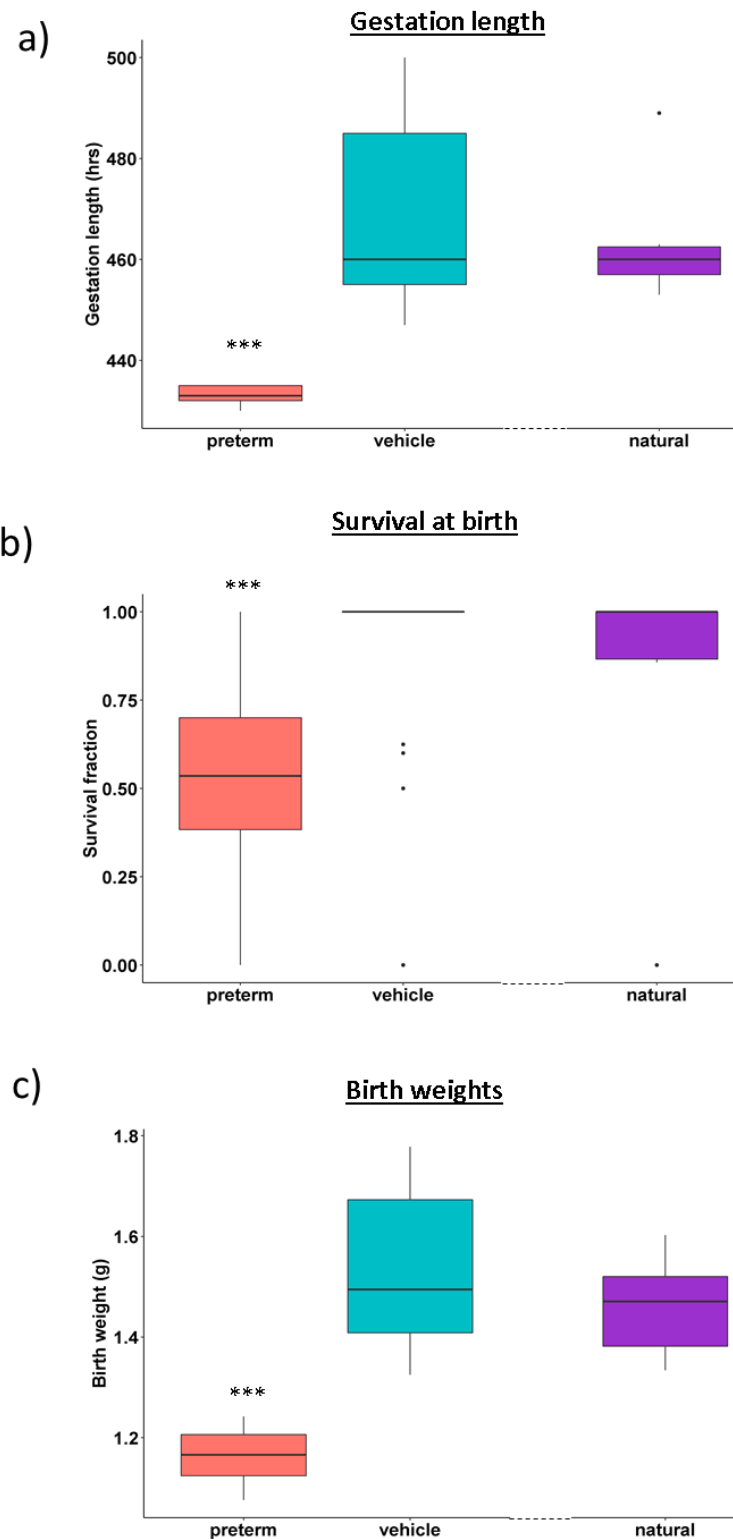
*Figure 2.5 **Initial outcomes of mouse model of prematurity***
Injection with RU486 at post conception day 17 resulted in shorter gestation lengths (a) (p < 0.001),
with reduced pup survival at birth (b) (p < 0.001) and lower birth weights (c) (P < 0.001) compared to
vehicle injected control births. Vehicle injection does not affect the gestation length (a), pup survival
(b), or birth weight (c) when compared to natural untreated pregnancies.
n# (litters) - Gestation length [n: preterm = 35; vehicle = 21; natural = 8], Survival [n: preterm = 32;
vehicle = 21; natural = 7], Birth weight [n: preterm = 23; vehicle = 14; natural = 7]

In control pregnancy cohorts there were a wide range of gestation lengths - between PC18d + 18hrs and PC20d + 21 hrs. This difference in gestation length correlated with litter size, with larger litters being born earlier (Figure 2.6a) (r = 0.6559, p < 0.001 – linear regression), which agrees with previous reports (Biggers *et al.*, 1963). The pups born from larger litters are also born smaller (Figure 2.6c) (r = 0.573, p<0.001), which has been reported in a variety of species (Werner and Griebeler, 2011). This relationship with litter size and gestation length is not evident in the preterm cohort (Figure 2.6b), as birth is artificially induced and there is little variance in gestation length (Figure 2.5a). There is however still a significant relationship between litter size and birth weight (Figure 2.6d) (r = 0.3, p < 0.01), suggesting that pups in large litters are smaller from before their natural birth date.

Gestation length vs litter size

a)

b)

Birth weight vs litter size

c)

d)
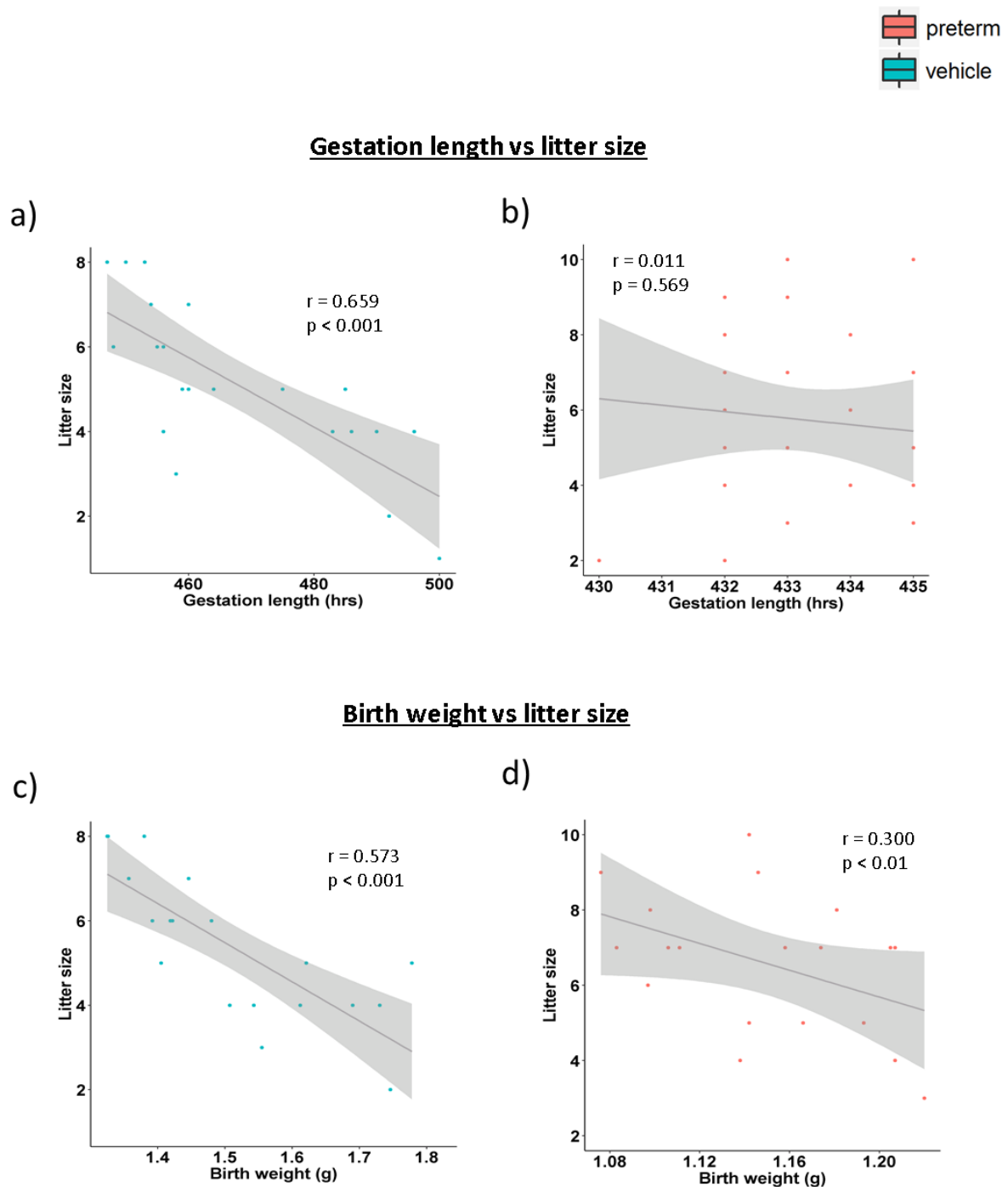
*Figure 2.6 **Correlations between gestation length, litter size and pup birth weights***
In control pregnancies there is a negative correlation between litter size and gestation length (a) and average litter birth weight (c), with larger litters being born earlier (r = 0.659, p < 0.001) and the pups being smaller (r = 0.573, p < 0.001) – shown as raw data points, with linear regression line + standard error. There is no correlation seen between litter size and gestation length in preterm pups (b) but there is a significant negative correlation between birth and litter size (d) (r = 0.3, p < 0.01)
n# (litters) - Gestation length vs litter size [n: preterm = 21; vehicle = 21], Birth weight vs litter size [n: preterm = 19; vehicle = 18]

As preterm pups are born on an earlier gestational day, and so have had less development time it is not surprising that preterm pups are smaller at birth. To assess whether prematurity affected continued growth, pups were weighed daily the first three postnatal weeks and weights of preterm pups were compared to control pups at matched post-conception ages (Figure 2.7a) (GAMM, R-sq. = 0.973, n: preterm = 20, vehicle = 19, pups). When weight is compared based on post-conception day the growth curve of preterm animals is not significantly different to control animals. Significant random factors included in the model where individual animal ID and litter ID. These are variables that were found to improve the fit of the model, but were not factors of interest in the study, and so were included as random rather than fixed effects. This means that there was both individual animal and whole litter differences that resulted in some variability in weight. Growth curves of male and female animals were not significantly different.

As well as weight there are phenotypical markers of development that can be used to assess pup development (see Methods 2.2.2). The post-conception age that these developmental milestones were reached in preterm pups were compared to controls (Figure 2.7b) GAMM, R-sq. = 0.979, n: preterm = 20, vehicle = 19, pups). Developmental milestones were reached at the same PC day in preterm and control animals, with no data variance being explained by individual animal or litter ID suggesting less inter-animal variation than with weights. One key marker of neonatal development is eye opening, which occurs in mice at around 2 weeks after birth. In this cohort the day of eye opening was not different between preterm and control animals, happening on average (mean) at PC34d (Figure 2.7c) (p = 0.467 - Mann-Whitney U test) [n: preterm = 17; vehicle = 20, pups].

Gestation length in control pregnancies varied by up to 2 days (PC18d + 18hrs and PC20d + 21 hrs), however developmental milestones were reached based on post-conception age not postnatal, with later born pups appearing more developed at birth. This is also seen in the regression model where gestation length was not found to significantly improve the model fit. This suggests that being born early and therefore undergoing the late fetal development outside of the uterus does not affect the gross physiological development of mouse pups.

Growth curves

Weights

a)

Anatomical markers

b)

Day of eye opening

c)
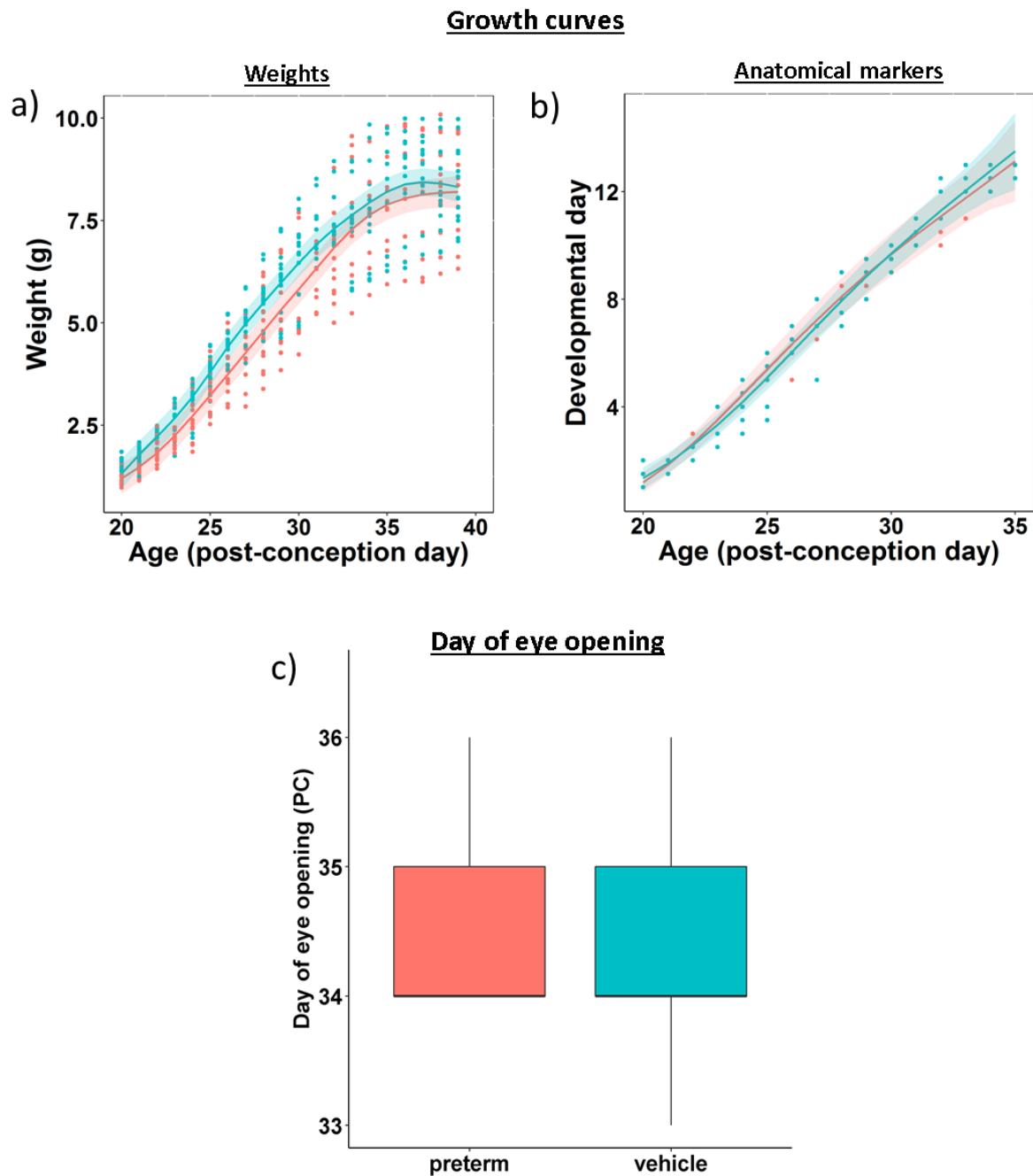
*Figure 2.7 **Pup growth and developmental milestones***

Pup weight (a) increases with age, as does phenotypic development (b) but neither have different developmental trajectories in preterm compared to control animals. Data presented as raw data points, with GAM model predicted mean and 95% CI error bars.

Day of eye opening (c) was not different between preterm and control animals.

n# (pups) - [n: preterm = 20; vehicle = 19]

2.3.2 Behaviour

During the first 2 postnatal weeks mice undergo a rapid period of development, where behaviours required for survival outside of the nest emerge (Fox, 1965). Many of these behaviours require sensorimotor coordination, and they develop at varied rates according to their complexity. Other animal models of prematurity have shown differences in early life behavioural development (Toso *et al.*, 2005; Chau *et al.*, 2009; Feather-Schussler and Ferguson, 2016). In this study these behaviours were assessed daily from PC22d (beginning of the first postnatal week) until PC35d (early in the third postnatal week).

2.3.2.1 *Righting reflex*

Righting reflex is the ability for an animal to turn over from supine position and place all four paws on the ground. This is a skill that involves the integration of sensory information and motor abilities. At PC22d many animals cannot achieve this task in the 30 seconds time limit, with a large variability in times in those who can. Task performance improved with age in a non-linear way, until PC31d where all pups could perform this task successfully every time. The development of this behaviour was not significantly different in preterm compared with control animals (Figure 2.8a) (GAMM, R-sq. = 0.58).

2.3.2.2 *Cliff aversion*

When presented with a cliff edge, mice will back away from it to avoid the risk of falling from heights. This is a behaviour that integrates both sensory perception, motor abilities and anxiety responses. At PC22d pups could not perform this task (Figure 2.8b), with them often failing to support themselves on the apparatus and falling from the cliff edge (failures marked as 30s). This behaviour emerges during the first postnatal week, and by PC34d all pups could complete this task every time. Being born prematurely had no apparent effect on the development of cliff avoidance behaviour (Figure 2.8b) (GAMM, R.sq. = 0.772).

2.3.2.3 *Geotaxis*

Another situation mice will avoid is having their head lower than their body (negative geotaxis). Like cliff aversion this behaviour integrates sensory and motor skills, as well as levels of anxiety the animal perceived from the situation. At PC22d pups could not complete the task, and again often lack the motor skills to even stay on the apparatus (failures marked as 30s). The emergence of success with this task is slower and more gradual than cliff aversion (Figure 2.8c), but mastery of this behaviour does occur before PC35d. The developmental trajectory of this behaviour is not significantly different in preterm compared to control animals (GAMM, R.sq. 0.764).

2.3.2.4 *Grasping behaviour*

Grasping behaviour is seen when a small object is presented at the paw. The performance at this task was given a score between 0 (no grasping) and 3 (able to sustain weight from grip). This behaviour is rarely seen at PC22d, but increased grasping and supporting of weight occurred over the following week (Figure 2.8d). The frequency of this behaviour began to fall again around PC30d, which has been previously reported (Arakawa and Erzurumlu, 2015b). Rather than the ability to perform this task decreasing during this period, it appears that the animal's interest in the task reduces with age. This pattern of behaviour emergence and then decreasing interest occurs at the same ages in preterm and control animals (GAMM, -sq. = 0.526).
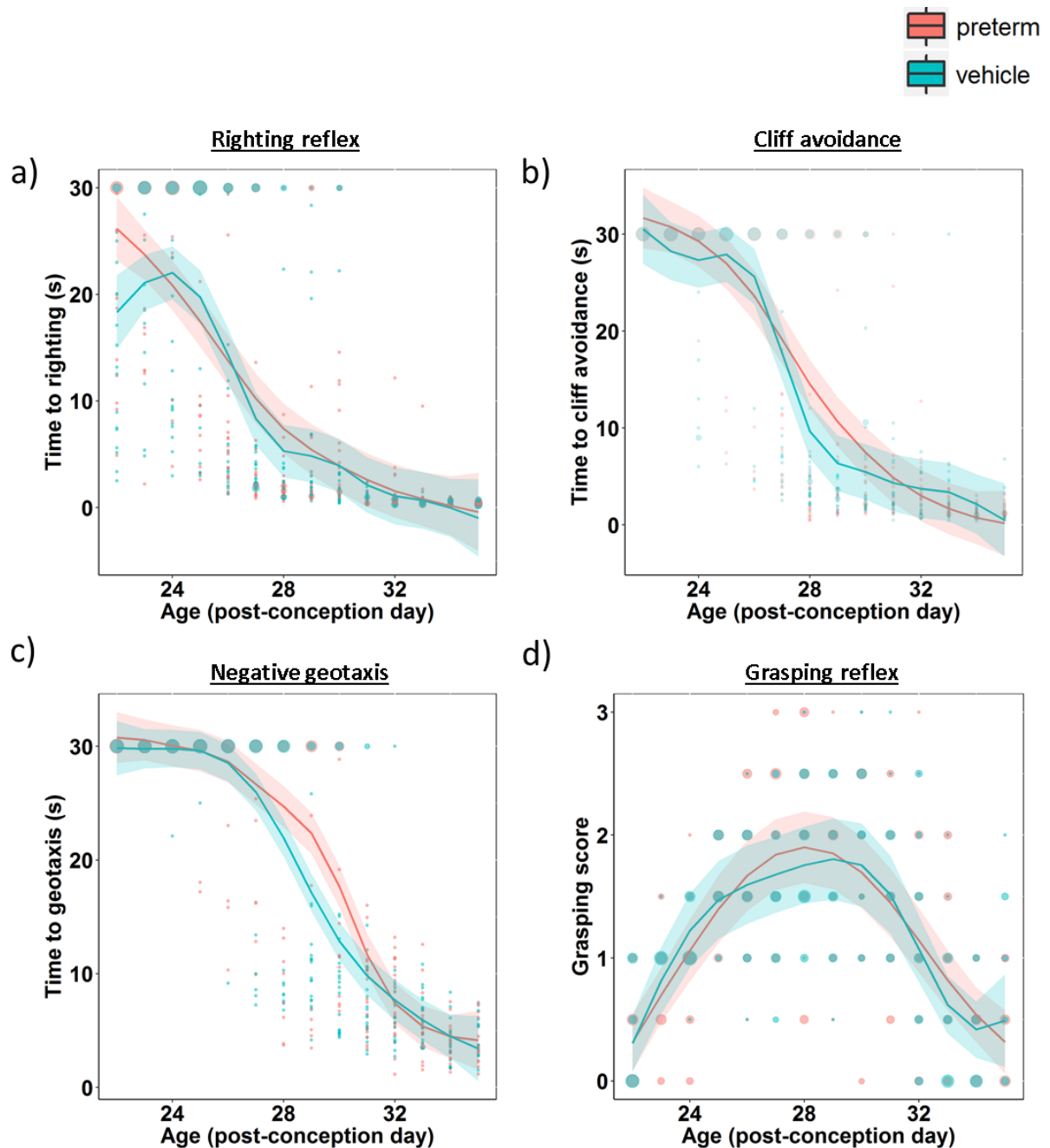
Figure 2.8 **Sensorimotor behaviour development**

The ability to right from a supine position (a) emerges during the first postnatal week, from PC22d until mastery in the second week at around PC31d. The ability to avoid a cliff edge is a behaviour that emerges slowly during the first postnatal week (b), as is the ability to correct a negative geotaxic position (c). The ability to grasp an object placed at the paw also improves during the first postnatal week, then declines as their attention for the task decreases (d).

The developmental trajectory of these sensorimotor behaviours is not significantly different in preterm compared to control animals.

Data is presented as individual data points (where size indicates number of observations) and the line is the GAM model's predicted fit with 95% confidence interval error bars.

2.3.2.5 *Whisking behaviour*

Rodents primarily use their whiskers to navigate the environment (Sofroniew and Svoboda, 2015). They use bilateral sweeping movements of the vibrissae to contact objects, which inform them of their surroundings. This behaviour is not present at birth, developing over the first 2 postnatal weeks. Whisking behaviour was scored, including whisker twitching that occurs before full whisking develops. At PC22d no active whisker movements were seen (Figure 2.9a), but they begin to emerge the next day increasing until full whisking is seen in all observations at PC34d. There is a lot of variability in the data, as only a 30 second window of observation was given each day, meaning that if whisker movements are present but infrequent they may be missed. The development of whisking behaviour is not significantly different in preterm pups when compared to controls (GAMM, R-sq. = 0.665).

2.3.2.6 *Whisker stimulation*

As well as active whisking to explore, passive deflection of the whiskers supplies mice with information about their environment. When presented with a 1hz deflection of the whiskers mice move their heads towards the stimulation, and the number of head movements in a 30s period were counted. The responsiveness to whisker stimulation increases with age (Figure 2.9b). There is no significant difference in the responsiveness to whisker stimulation between preterm and control animals (GAMM, R.sq. = 0.621). Individual animals show a significantly different responsiveness to stimulation, which was incorporated into the model and helps explain some of the data variance.

2.3.2.7 *Tactile stimulation*

Tactile information is also received through the rest of the rodent body. Like with whisker deflection, touching the body elicits a motor response, in the form of twitching and withdrawal of body parts away from the stimulation point. At the beginning of the first postnatal week few responses are seen, and over the next 2 postnatal weeks the pups become significantly more responsive to tactile stimulations (Figure 2.9c). Preterm animals were more responsive to tactile stimulation across all ages compared to control (R.sq. =

0.482; p < 0.001), with individual variability accounting for a significant amount of variance in the data. This could indicate increased sensory sensitivity, heightened anxiety behaviours, or differing motor reflex responses in preterm animals.
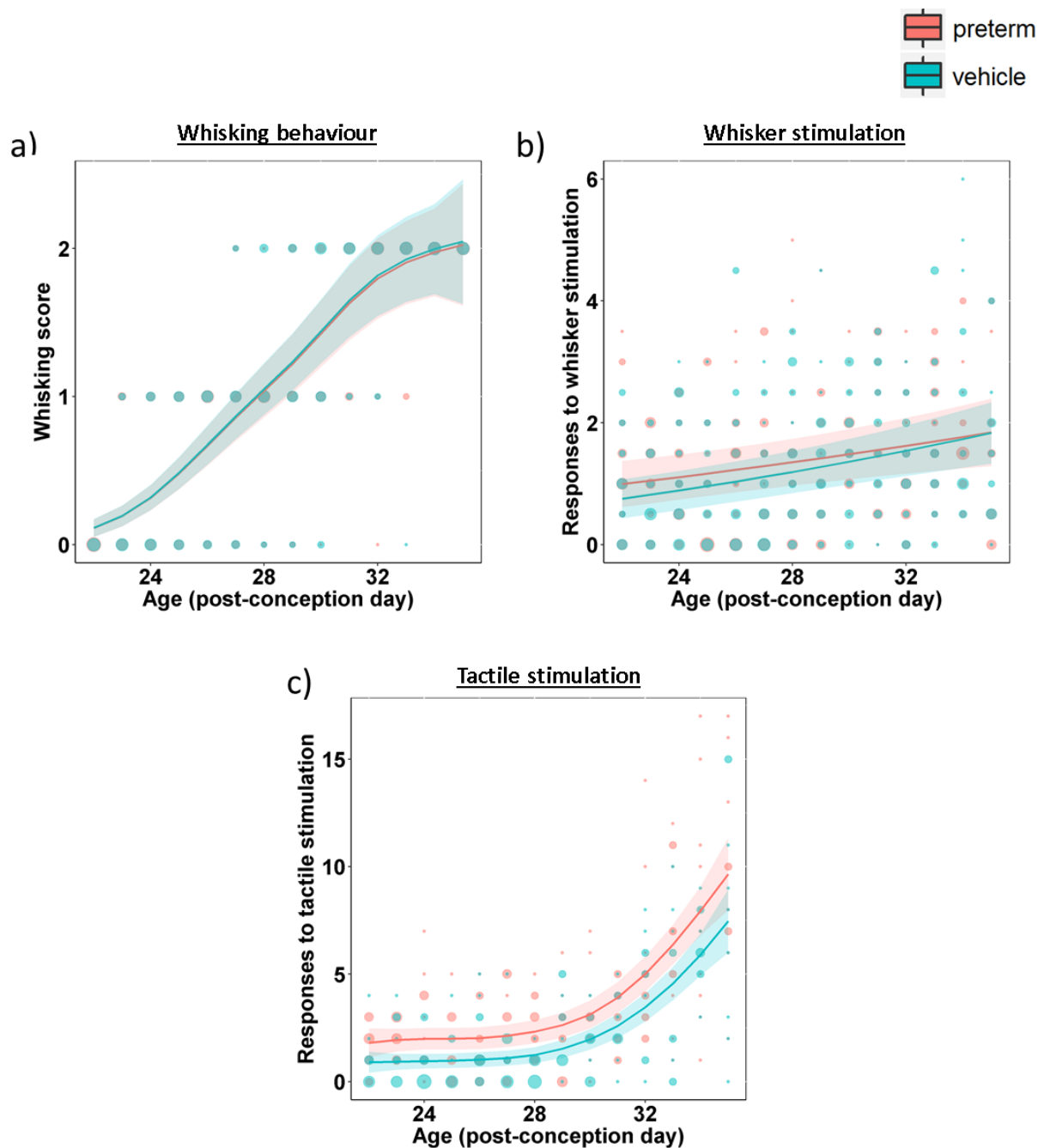
*Figure 2.9 **Sensory behaviour development***

Whisking behaviour (a) (graded, 0 = none, 1 = twitching, 2 = full whisking) develops with age, with full whisking being present by the end of the second postnatal week. The developmental trajectory of this skill is not different between preterm and control animals.

When whiskers are stimulated animals move their head in response (b), and the responsiveness increases with age, and does not differ between preterm and controls.

Animals also move in response to tactile stimulation on the body (c), with the likelihood of response increasing with age. Preterm animals are significantly more responsive to tactile body touches than controls across all ages (p < 0.001).

Data presented as individual data points (where size indicates number of observations) and the line is the model's predicted fit with 95% confidence interval error bars.

2.3.2.8 *Open field*

Open field exploration experiments are often used in adult rodents to measure locomotion, exploratory and anxiety-like behaviour (Seibenhener and Wooten, 2015). At PC40d - which is weaning age - this cohort of animals was given five minutes of open-field exploration. The animal's movement was tracked with automated detection software - see Figure 2.10aii for an example path of exploration. Both the distance travelled (Figure 2.10b) and the speed of travel (Figure 2.10c) did not differ between the preterm and control animals, suggesting motor and exploratory behaviour is unaffected by premature birth. The time spent close to the walls compared with the inner segment is known as the thigmotaxic ratio and is used as a measure of level of anxiety in rodents (Simon, Dupuis and Costentin, 1994). Preterm animals were significantly less likely to spend time exploring the inner region of the arena than control (Figure 2.10d) (p = 0.0413 – Mann-Whitney U test) [n: preterm = 17; vehicle = 18], potentially suggesting an elevated level of anxiety in preterm animals.
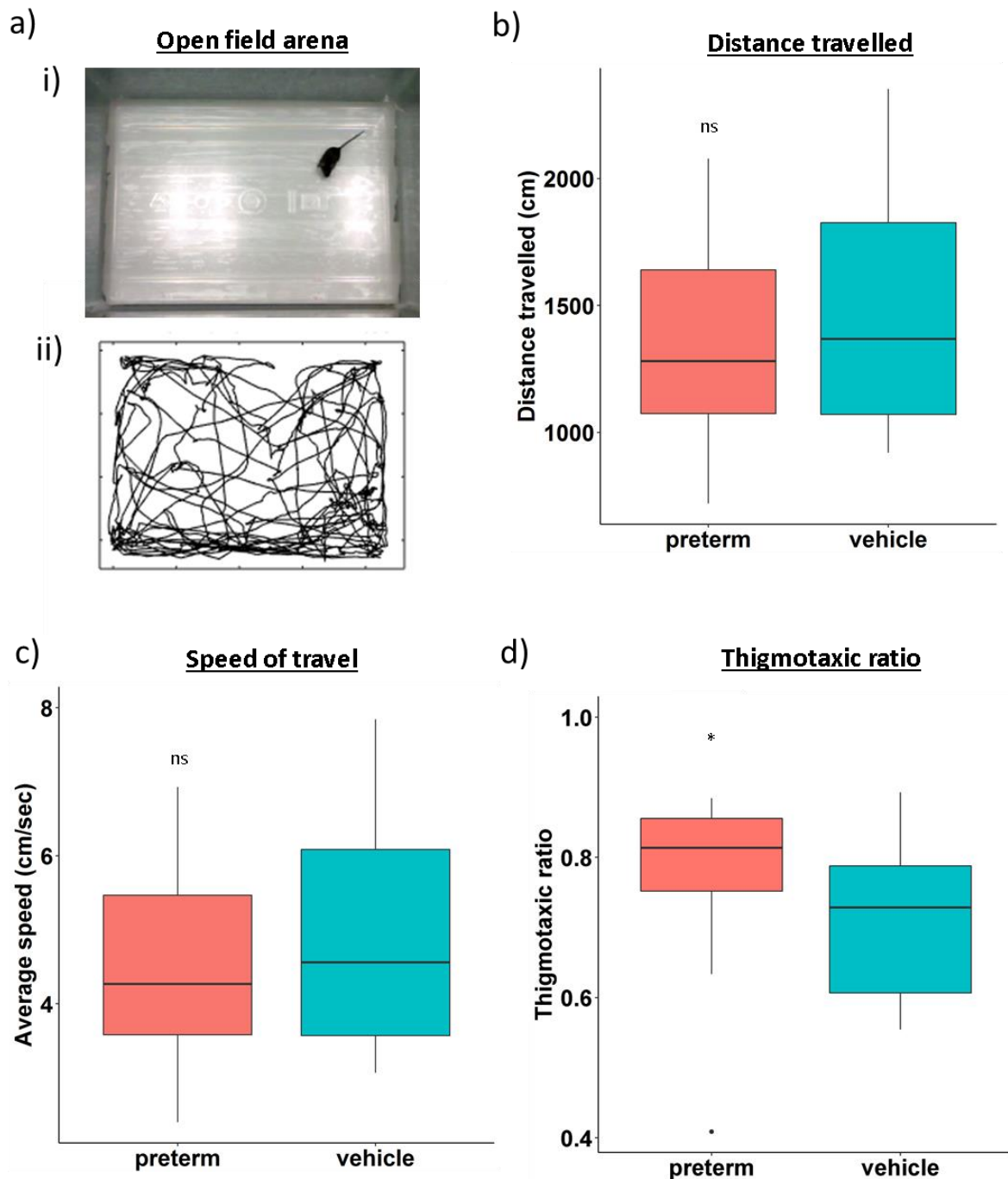
*Figure 2.10 **Open field exploration***

Pups were given 5 minutes of locomotion in a novel arena at PC40d.

(a)(i) Photograph of open field arena with subject in, and (ii) illustration of tracked movement over 5 min.

The distance travelled (b) and the speed of movement (c) did not significantly differ between preterm and control animals. The tendency to stay near the wall of the arena (thigmotaxis) (d) was different between groups, with preterm pups spending significantly more time near the wall (p = 0.0413).

n# (pups) - [n: preterm = 17; vehicle = 18]

2.3.3 Histology of barrel cortex

The rodent brain grows rapidly during the first postnatal week with new cells being born and migrating to their destination. In the cortex, the characteristic layers of the mature brain are formed sequentially in the first postnatal week (Semple *et al.*, 2013). This process results in an increase in brain weight (Figure 2.11a) (F = 40.21, p < 0.01 – two-way ANOVA) [n: preterm – PC22d = 13, PC23d = 8, PC24d = 15; vehicle – PC22d = 12, PC23d = 8, PC24d = 15] and in thickening of the cortex (Figure 2.11b) (F = 44.238, p < 0.001 – two-way ANOVA) [n: preterm – PC22d = 5, PC23d = 6, PC24d = 6; vehicle – PC22d = 6, PC23d = 6, PC24d = 7]. However, the rate of growth of the brains of preterm animals, as measured by weight and by cortical thickness, was not significantly different to controls.

The critical period for barrel development is during the first 7 days of postnatal life (Erzurumlu and Gaspar, 2012). During this period thalamocortical axons migrate into layer IV of the cortex and form synapses onto neurons in the barrels (Carl C H Petersen, 2007). It is this distribution of presynaptic terminals that gives rise to the characteristic barrel structure seen. To assess barrel development, staining of the mitochondrial enzyme cytochrome oxidase was used to visualise the terminal ends of these thalamocortical neurons (Land and Simons, 1985). Examples of this staining are shown in Figure 2.11c. The density of staining in the barrels compared to the septum was used as a measure of barrel development (Figure 2.11d). At PC22d and 23d there is little staining in the barrels. By PC24d this has increased (Figure 2.11d) (F = 12.592, p<0.01 – two-way ANOVA) [n: preterm – PC22d = 5, PC23d = 6, PC24d = 6; vehicle – PC22d = 6, PC23d = 6, PC24d = 7]. This rate of increase in thalamocortical terminal in the barrels was not significantly different in preterm animals compared to controls.
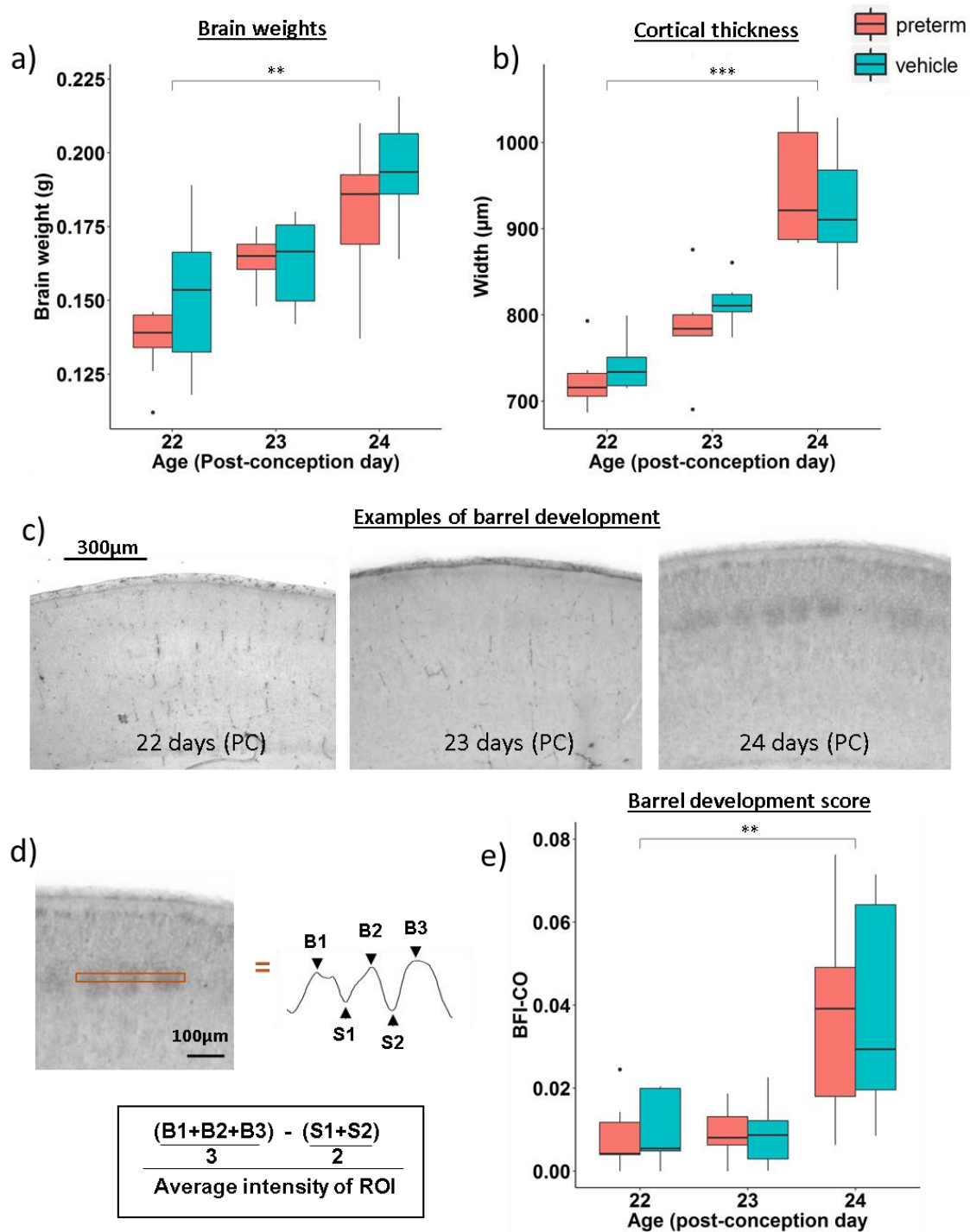
Figure 2.11 **Anatomical formation of the barrel cortex**

Brain weight (a) increases with age (p < 0.01), as does cortical thickness (b) (p < 0.001), with no significant difference between preterm and control animals.

(c) Example images of barrel cortex in cytochrome oxidase stained coronal slices.

BFI-CO is measured from comparing the staining intensity of barrels and septums (d) as a proxy of barrel maturation. This increases with age (e) (p <0.01), with no significant difference between preterm and control animals.

n# (pups) - [n: preterm – PC22d = 13, PC23d = 8, PC24d = 15; vehicle – PC22d = 12, PC23d = 8, PC24d = 15], - Thickness & BFI-CO [n: preterm – PC22d = 5, PC23d = 6, PC24d = 6; vehicle – PC22d = 6, PC23d = 6, PC24d = 7].

2.3.4 Intrinsic cellular properties of layer IV Stellate neurons

Most neurons in the barrels within layer IV of the mouse cortex are stellate cells (Simons and Woolsey, 1984), and are the primary recipients of thalamocortical synaptic input (Inan and Crair, 2007). These cortical neurons and thalamocortical projections migrate during the late embryonic and early postnatal period (Kirischuk, Luhmann and Kilb, 2014). Stellate neurons are born embryonically but their electrical properties continue to mature during the neonatal period (Callaway and Borrell, 2011) - coinciding with preterm birth in this mouse model. Alterations in the activity input into neurons during this developmental period have been shown to affect the formation of the dendritic arbour (Wong and Ghosh, 2002; Andreae and Burrone, 2015), and such morphological changes in turn affect the electrical activity of the neuron (Callaway and Borrell, 2011). Preterm animals in this cohort are in an altered sensory environment during what should be the late fetal stages of development and these changes may impact the activity input into the sensory cortical neurons. In addition microstructural changes have been found in human preterm infants that suggest delayed development of dendritic arborisation and synapse formation (Ball, Srinivasan, *et al.*, 2013). To assess whether premature birth in this mouse model has affected sensory cortical neuron development in similar ways, the intrinsic electrical properties of stellate neurons were measured.

In this study whole cell current clamp recordings from Stellate cells in layer IV of the barrel cortex, at both resting membrane potential and -79mV holding potential were made to investigate both passive membrane and firing properties. Recordings were made on PC22d, 23d, 24d and 28d, covering the first into the beginning of the second postnatal week. All n numbers in this section are presented as individual cells, which came from 4-5 animals per group at each time point.

2.3.4.1 *Passive membrane properties*

At PC22d Stellate cells were relatively depolarised, with the vehicle group at a mean RMP of -45.4±6.6mV and preterm group at -50.3±4.4mV. RMP became progressively hyperpolarised with age (Figure 2.12b (RMP)) (F = 13.778, p<0.001 – two-way ANONA), towards more adult-like RMP (Fleidervish, Binshtok and Gutnick, 1998). There was no significant difference in

RMP in cells from preterm animals compared to controls. At all ages and in both groups holding at around -79mV was achieved (Figure 2.12b (-79)).

Input resistance of a neuron is a measure of how easily current can pass across its membrane. Measured using a hyperpolarisation step (Figure 2.12a), input resistance of the developing Stellate cells progressively decreased between PC22d and 28d (Figure 2.12c) at both -79mV holding (F = 17.411, p<0.001 – two-way ANOVA) and RMP (F = 13.778, p < 0.001). This changing input resistance was not significantly different in preterm animals compared to controls.

Other measures taken from the hyperpolarisation step include the membrane time constant of decay (tau) and the time-dependent rectification (sag). The membrane time constant, which is a measure of the time the membrane takes to change to a potential, does not change between PC22d and 28d (Figure 2.12d).

Sag is the difference in the hyperpolarisation potential compared to what would be expected from a single exponential fit from the initial tau. Sag is mediated by hyperpolarisation-activated cation currents (I_h) mediated by hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels, that rectify the hyperpolarisation of the membrane towards resting membrane potential (Kaupp and Seifert, 2001). This current plays a role in regulating action potential firing (Pape, 1996), and has been found in mature somatosensory neurons (Momin *et al.*, 2008; Breton and Stuart, 2009). In layer IV Stellate cells of developing mice little sag is present at either RMP or -79mV holding (Figure 2.12e) and there is no significant change between PC22d and 28d. This suggests that there is not much I_h in these immature neurons, which agrees with previous data from Dr Ashby's group (Murray, 2016). The contribution of I_h is not significantly different between preterm and control groups.
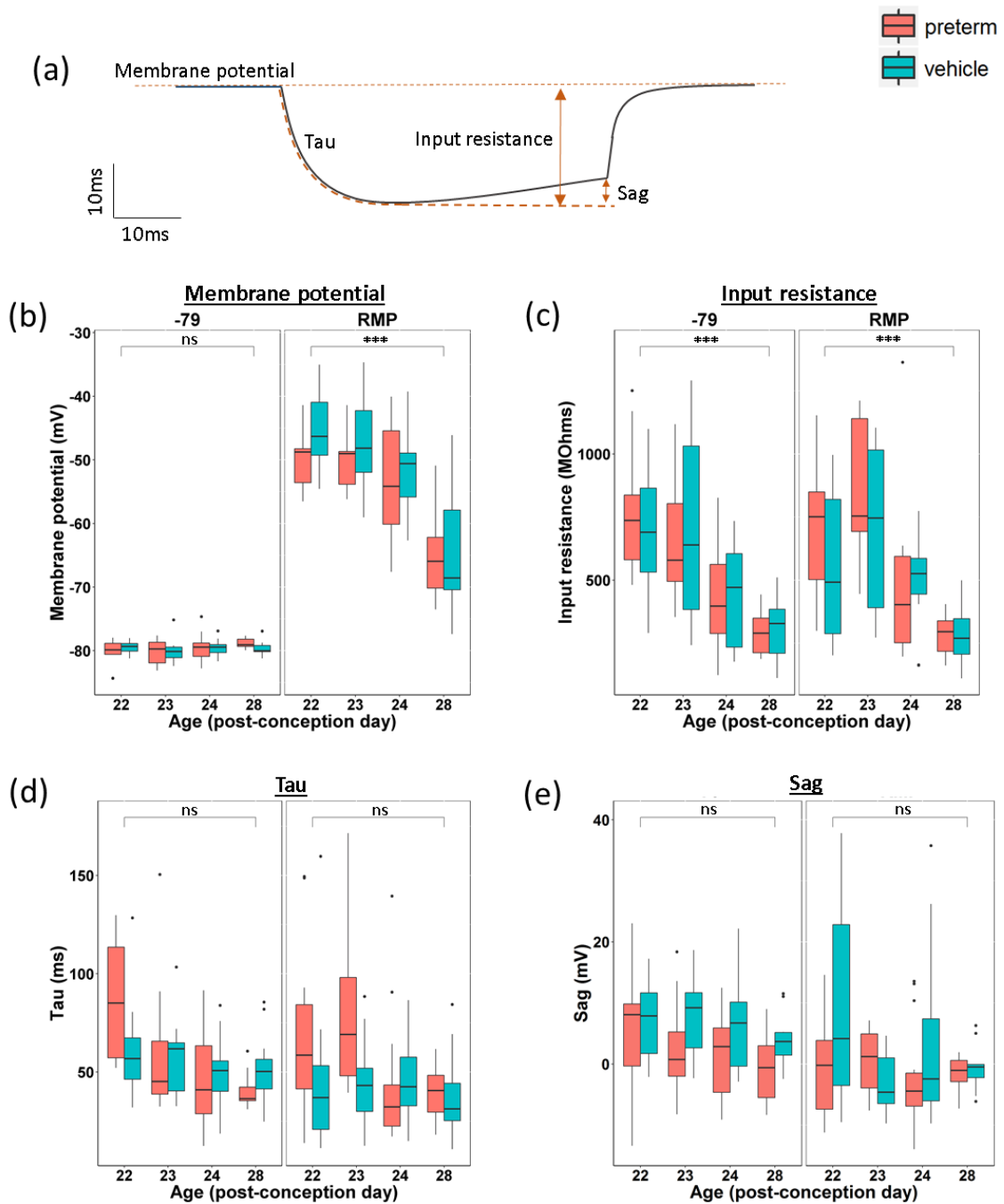
*Figure 2.12 **Passive membrane property development in layer IV Stellate cells.***
(a) Measures of passive membrane properties from the hyperpolarisation step.
The resting membrane potential (RMP) (b) of Stellate cells decreases with age (F = 13.778, p <0.001) and holding potential was not different between groups or ages. The input resistance of cells decreased with age at -79mV holding (F = 17.411, p <0.001) and RMP (F = 13.778, p <0.001). The tau(d) and sag (e) do not change with age. There is no significant difference in these passive membrane properties in preterm compared to control animals.

n# (cells) -79mV holding [n: preterm – PC22d = 10, 23d = 10, 24d = 18, 28d = 9; vehicle – 22d = 11, 23d = 9, 24d = 12, 28d = 11]. RMP holding [n: preterm – PC22d = 11, 23d = 8, 24d = 14, 28d = 8; vehicle – 22d = 8, 23d = 9, 24d = 11, 28d = 13].

2.3.4.2 *Action potential waveforms*

Alongside developmental changes in passive electrical properties the characteristic of action potential (AP) firing also matures postnatally. AP waveform shape is important for effective synaptic transmission, and is dependent on the ion channels present in the membrane (Bean, 2007). As such, any effect of preterm birth on subsequent development of AP dynamics was assessed in layer IV stellate neurons between PC22d and 28d. As the neuron matures the AP waveform changes, examples from each experimental age are shown in Figure 2.13a. The AP waveform appears to become larger and faster with age. To quantify these changes the first AP fired during a stepped current injection protocol was used to measure AP properties.

Action potentials occur when a neuron's membrane potential reaches a threshold that triggers a cascade of ion channels opening (Bender and Trussell, 2012). The threshold membrane potential at which APs fire decreases with age at RMP (Figure 2.13b) (F = 6.735, p<0.001 – two-way ANOVA), but is constant over ages when the cells is held at -79mV. There is no significant difference in threshold in preterm compared to control animals at either membrane potential. The height of the AP is measured as the change in membrane potential from threshold to peak (Figure 2.13c). The AP height increases with age at both RMP (F = 19.48, p <0.001 – two-way ANOVA) and -79mV holding (F = 14.237, p < 0.01), and this developmental change is not different in preterm compared to control animals. Post-hoc test (TukeyHSD) finds that the significant change in AP threshold and height occurs at PC28d, with no significant difference between PC22d and 24d. This is indicative of a transition in neuron firing properties from the first to second postnatal week, which is known to be a transition point out of the critical period in the barrel cortex (Isaac *et al.*, 1997).

To calculate how quickly this full AP height was reached the first derivative of the waveform was calculated, and the maximum rate of change measured (Figure 2.13d). There was a significant increase in the maximum rate of rise with age at both RMP (F = 124.26, P <0.01 – two-way ANOVA) and -79mV holding (F = 25.308, P < 0.01), with post-hoc testing find that the significant change occurs again at PC28d. This increasing rate of rise of the AP waveform will influence the duration of the event. This can be seen in the width (full-width at half maximum height) of the AP, which decreases with age (Figure 2.13e) at both RMP (F =

19.48, p <0.001 – two-way ANOVA) and -79mV holding (F = 9.901, P < 0.01). Again, the developmental increase in AP rate of rise was not significantly different in preterm animals compared to controls.
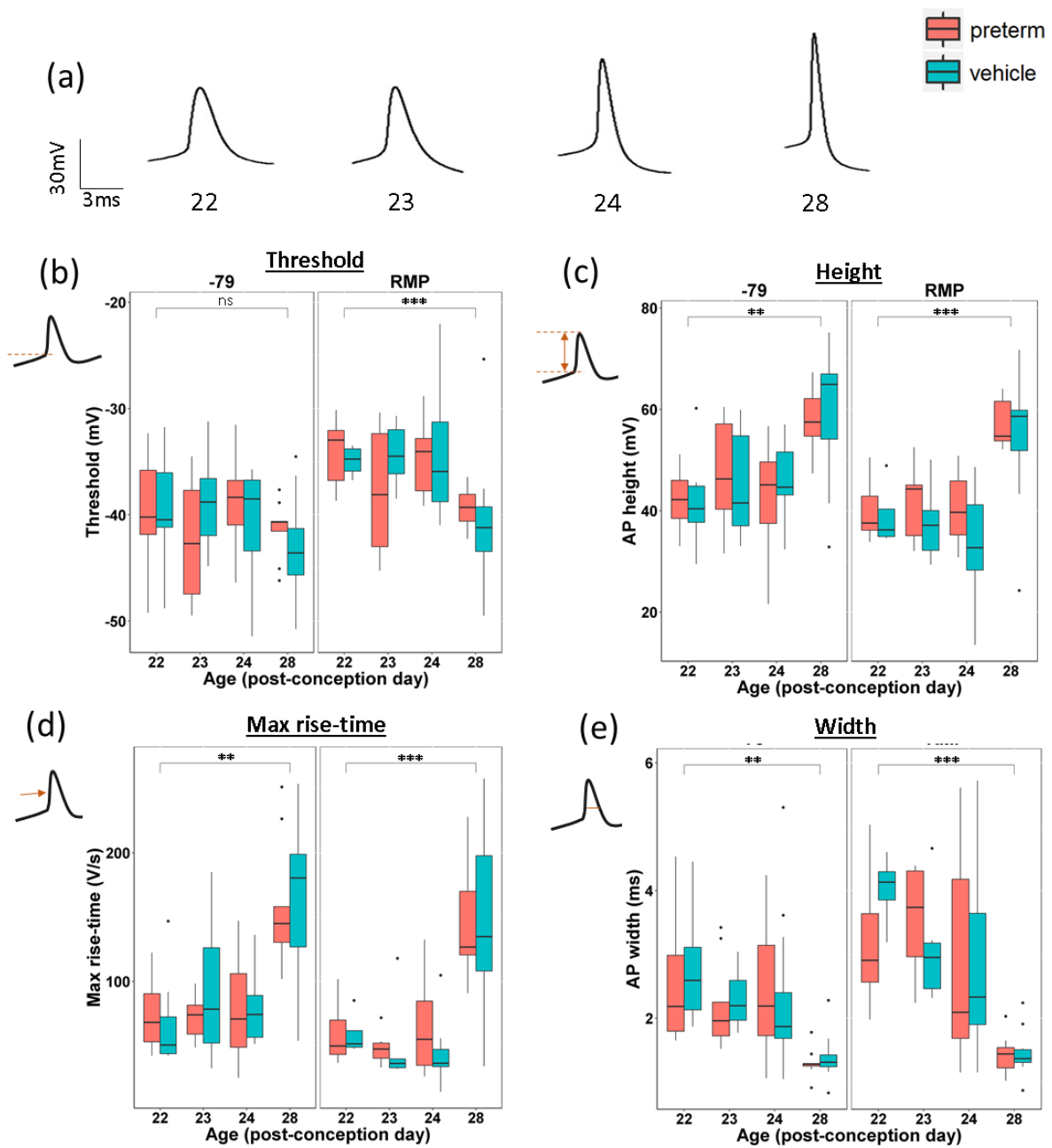
Figure 2.13 **Action potential waveform development in layer IV Stellate cells**
(a) Shows example action potential wave forms from the ages measured.
The threshold that AP fire (b) decreases with age at RMP (F = 6.735, p <0.001) but not at -79 holding.
Action potential (AP) height (c) increase with age (at: -79 - F = 14.237, p < 0.01; RMP, F = 19.48, p < 0.001), as does the maximum rise-time (e) (at: -79 - F = 25.308, p < 0.01; RMP, F = 124.263, p < 0.001), and the width (e) decreases (at: -79 - F = 9.901, p < 0.01; RMP, F = 12.644, p < 0.001). There is no significant difference in action potential waveform parameters between preterm and control animals.

n# (cells) - -79mV holding [n: preterm – PC22d = 10, 23d = 10, 24d = 18, 28d = 9; vehicle – 22d = 9, 23d = 8, 24d = 12, 28d = 11], RMP holding [n: preterm – PC22d = 8, 23d = 6, 24d = 11, 28d = 9; vehicle – 22d = 4, 23d = 6, 24d = 8, 28d = 12].

2.3.4.3 Firing properties

Rheobase is the minimum current required to drive the neuron's membrane potential to AP threshold. The rheobase of layer IV Stellate cells progressively increases between PC22d and 28d (Figure 2.14b) at both RMP (F = 4.374, p < 0.01 – two-way ANOVA) and when held at -79mV (F = 3.9484, P < 0.05), but was not significantly altered in preterm compared to control animals at any age or either membrane potential.

Neurons do not always just fire a single AP. The protocol used for this study was a series of 18, 500ms current step injections of increasing size (0-0.36V), which often resulted in a train of APs being fired (examples in Figure 2.14a). As well as the individual AP waveforms changing with developmental age, there were changes to the train of AP events. Once cells reach their rheobase and begin to fire APs the number of APs fired during each depolarisation step increased with age, at both RMP (Figure 2.14c) (F = 11.286, p < 0.001 – two-way ANOVA) and -79mV holding (F = 5.22, p <0.01). This increasing firing rate was not significantly different between preterm and control animals. There was little difference in firing rate between P22d and 24d (first postnatal week) at either holding potential, but with the increase in number occurring at PC28d (second postnatal week). So, although it takes a larger current injection for P28d neurons to fire APs once they reach this threshold they are more excitable and fire in longer trains.
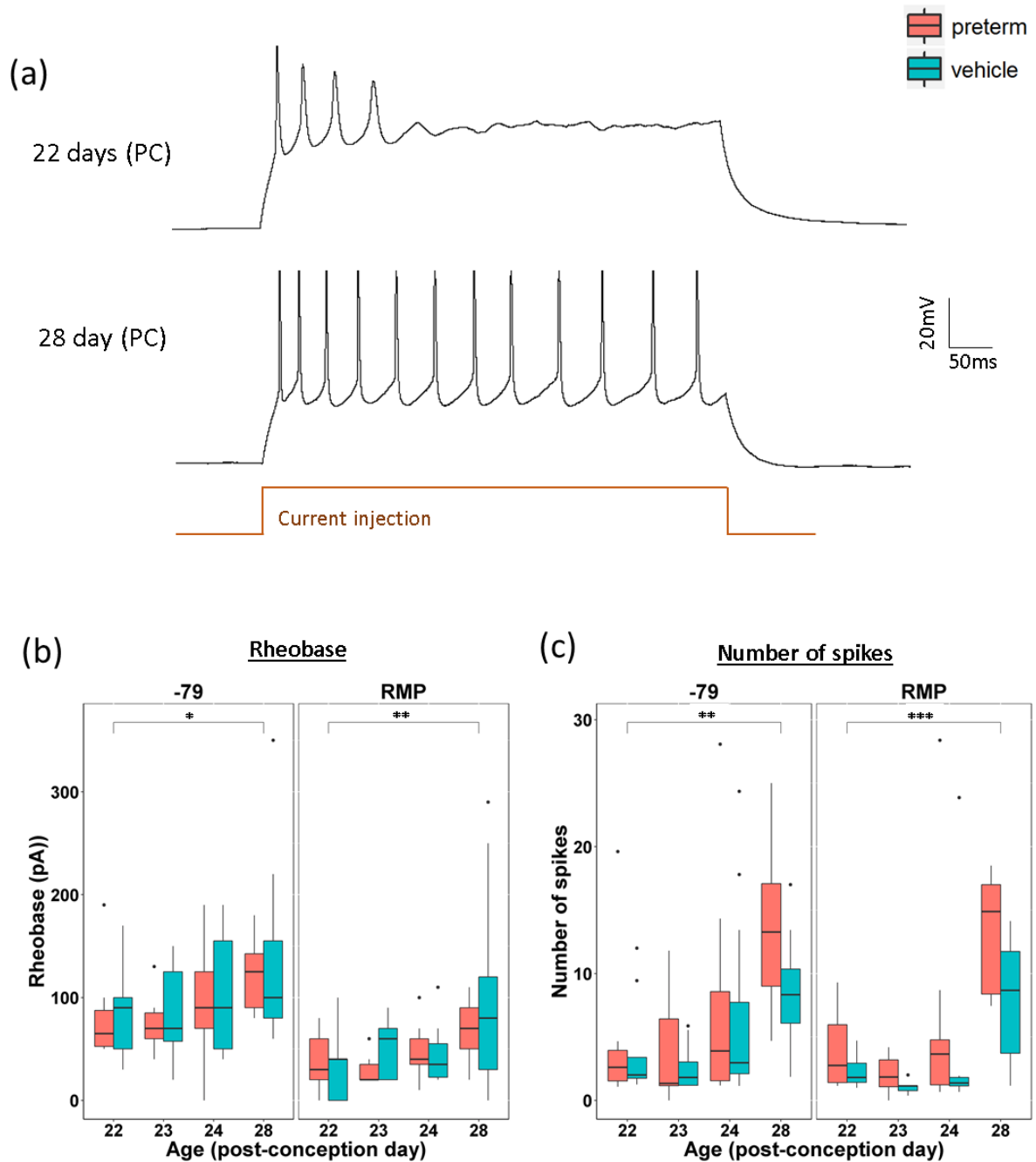
*Figure 2.14 **Action potential firing property development in layer IV Stellate cells.***
(a) Example of action potential trains following current injection at the developmental ages recorded.
The current required to drive the cell past threshold (rheobase) (b) increased with age at both -79mV
holding potential (F = 3.948, p < 0.05; RMP – F = 4.374, p < 0.01).
The number of action potentials fired per current injection sweep once past rheobase (c) also increased
with age (at: -79 – F = 5.222, p < 0.01; RMP – F = 11.286, P <0.001). There is no significant difference
in the likelihood of firing APs in preterm compared to control animals.
n# (cells)- -79mV holding [n: preterm – PC22d = 10, 23d = 11, 24d = 18, 28d = 9; vehicle – 22d = 11, 23d
= 9, 24d = 12, 28d = 11], RMP holding [n: preterm – PC22d = 11, 23d = 8, 24d = 14, 28d = 9; vehicle –
22d = 10, 23d = 10, 24d = 12, 28d = 12].

2.3.4.4 *Miniature excitatory postsynaptic currents*

As well as release of neurotransmitter into the synaptic cleft during AP propagation there is spontaneous release that occurs independent of incoming signal (Kavalali, 2015). Neurotransmitter is stored in vesicles in the pre-synaptic terminal, each containing a standard amount. These are sometimes spontaneously released, and this neurotransmitter present in the synaptic cleft results in a small potential change in the postsynaptic neuron (Katz and Miledi, 1963), that can be measured in whole cell voltage clamp. When this neurotransmitter release is excitatory (e.g. glutamate) these are known as a miniature excitatory post-synaptic current (Simkus and Stricker, 2002), and when it is inhibitory (e.g. GABA) they are miniature inhibitory post-synaptic current (Takahashi *et al.*, 2006). When initially discovered it was thought that these events were random 'noise' in the neuronal system, however evidence has emerged that support the idea that these events have a specialised and vital role in neuronal network connectivity, including in the developing brain (Andreae and Burrone, 2018). Spontaneous synaptic activity in the immature brain has been found to facilitate the arborisation of dendrites (Andreae and Burrone, 2015) and the formation of spines (Bouwman *et al.*, 2004), both of which are important developmental stage in the formation of functional neuronal networks. This spontaneous release can provide information about the synaptic property of the local neural network, and changes in these events' properties could indicate disruptions in the development of network connectivity.

Layer IV stellate cells are excitatory glutamatergic neurons, that express AMPA and NMDA receptors (Fröhlich and Fröhlich, 2016). These receptor types have different likelihoods of opening at different membrane potentials (Ascher, Bregestovski and Nowak, 1988). This is a result of NMDAr being blocked by a Mg+ ion at negative membrane potentials, which is removed during depolarisation (Nowak *et al.*, 1984). Experimentally this means that events mediated by each receptor type can be isolated by holding the cell at different potentials. At -70mV holding potential it is predominantly AMPA receptors that are activated, and at +40mV it is largely NMDA receptor mediated events that are recorded. Examples of AMPAr and NMDAr mediate events can be seen in Figure 2.15a. Both the frequency and size of these events supply information about the structural and functional composition of the synapses.

The frequency of both AMPAr (Figure 2.15b) and NMDAr (Figure 2.15c) mediated mEPSCs significantly increase between PC22d and 28d (AMPA – F = 31.753, p < 0.001, NMDA – F = 44.958, p < 0.001 – two-way ANOVA), with post-hoc Tukey testing finding this significant increase occurs at PC28d, but not in the first postnatal week. This is a similar developmental time-scale to the intrinsic electrical properties of these neurons (Figure 2.13), with a dramatic transition from first to second postnatal week. There is no significant difference in the frequency of mEPSCs in preterm animals when compared to controls. There is relatively small change between PC22d and 24d (first postnatal week), with a large increase in both AMPAr and NMDAr mediated events seen at PC28d (second postnatal week). The ratio of AMPAr to NMDAr mediated events gives information about the relative number of synapses containing each type of receptor. Only data for PC24d and 28d are presented as at PC22d and 23d there were too few events recorded to give a reliable estimate of ratios. Between PC24d and 28d there is an increase in the AMPA:NMDA event frequency ratio (Figure 2.15d) (F = 9.246, p < 0.01 – two-way ANOVA). This agrees with previous work (M. Crair and Malenka, 1995) which showed an increase in the relative number of AMPAr containing synapses with maturation. Insertion of AMPAr into previously NMDAr only synapses is a process known as unsilencing and is known to be occurring during this critical period of barrel development (Isaac *et al.*, 1997; Ashby and Isaac, 2011). The AMPAr to NMDAr frequency ratio was not different in preterm animals compared to controls, suggesting that maturation of synapse number and unsilencing of silent synapses in not influenced by prematurity.
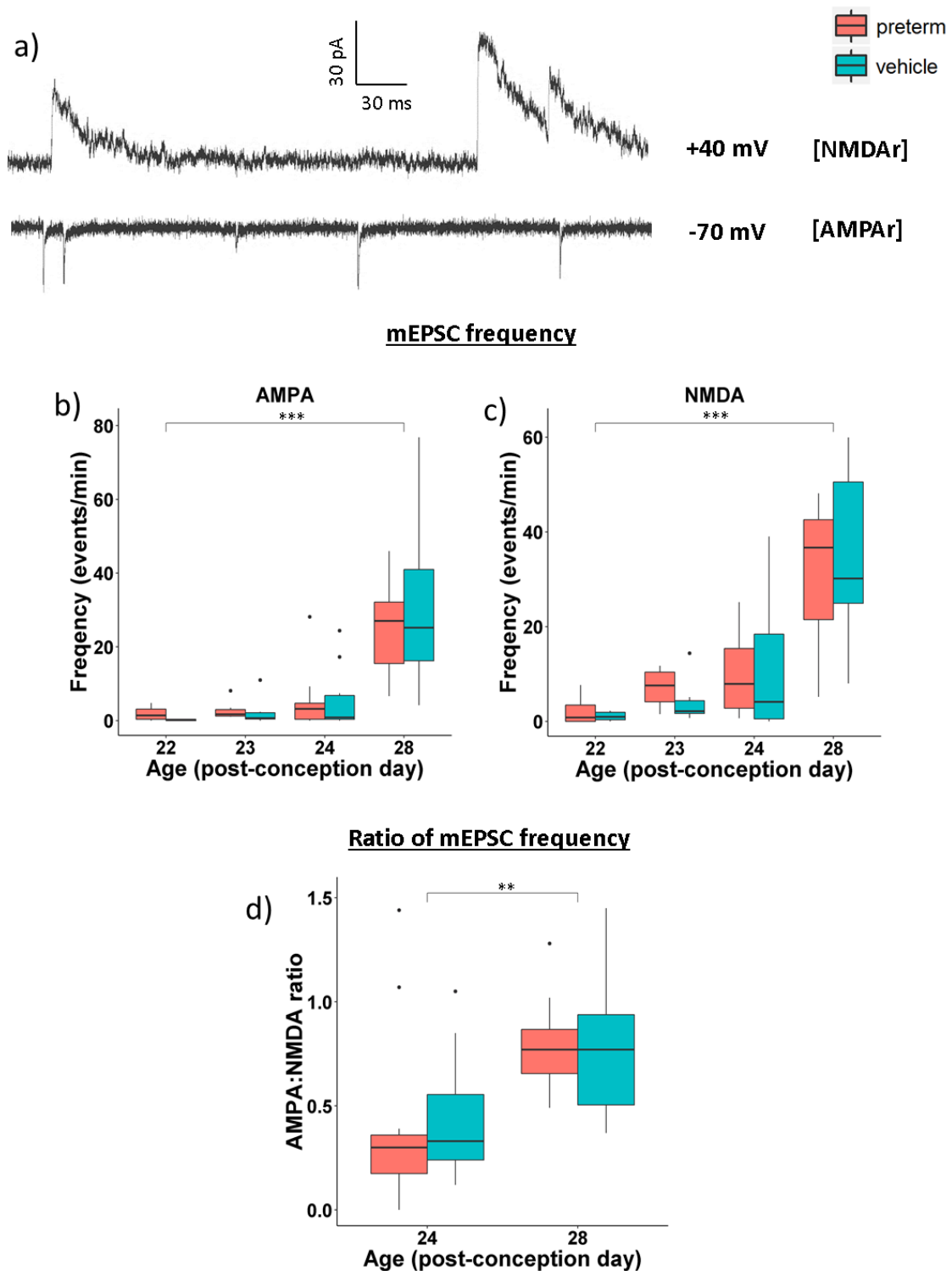
*Figure 2.15 **Frequency of miniature excitatory postsynaptic currents in layer IV Stellate cells.***
(a) Example recordings of spontaneous mEPSCs mediated by AMPAr (-70mV holding) and NMDAr (+40mV holding) The frequency of mEPSCs increases with age from both (b) AMPA receptor (F = 31.753, p < 0.001) and (c) NMDA receptor (F = 44.958, p < 0.001) mediated events. The ratio of AMPAr and NMDAr mediated events (d) changes with age (F = 9.246, p < 0.01). The frequency of EPSCs is not significantly different in preterm compared to control animals.
n# (cells) [n: preterm – PC22d = 8, 23d = 8, 24d = 14, 28d = 16; vehicle – 22d = 7, 23d = 7, 24d = 18, 28d = 15].

The amplitudes of mEPSCs is an indicator of the number of receptors in the post-synaptic density (Han and Stevens, 2009). mEPSC amplitudes are only considered for PC24d and 28d as there were not enough events at 22d and 23d to get a robust estimate of mean and distribution of events. Between PC24d and 28d the amplitude of AMPAr mediated events increases (Figure 2.16a) (F = 7.395, p < 0.01 – two-way ANOVA) but there is no change in NMDAr mediated events (Figure 2.16b). This is more evidence to support the theory that AMPAr are being inserted into the post-synaptic density with maturation. This maturation process is not significantly different in preterm animals compared to controls. These are the averaged amplitudes of each cell, but there is a range of amplitude seen for each. This is because each event is due to the activation of a different synapse, each of which contain different numbers of receptors. To measure the distribution of events as well as the average, the cumulative frequency of mEPSC amplitudes were compared at each age for cells from preterm and control animals. There is no significant difference in the distribution of amplitudes between preterm and control animals from either AMPAr (Figure 2.16c) or NMDAr (Figure 2.16d) mediated events, again suggesting that early birth does not have a profound effect on synaptic receptor maturation.

mEPSC amplitude

a) **AMPA**

b) **NMDA**

mEPSC amplitude distribution

c)

d)
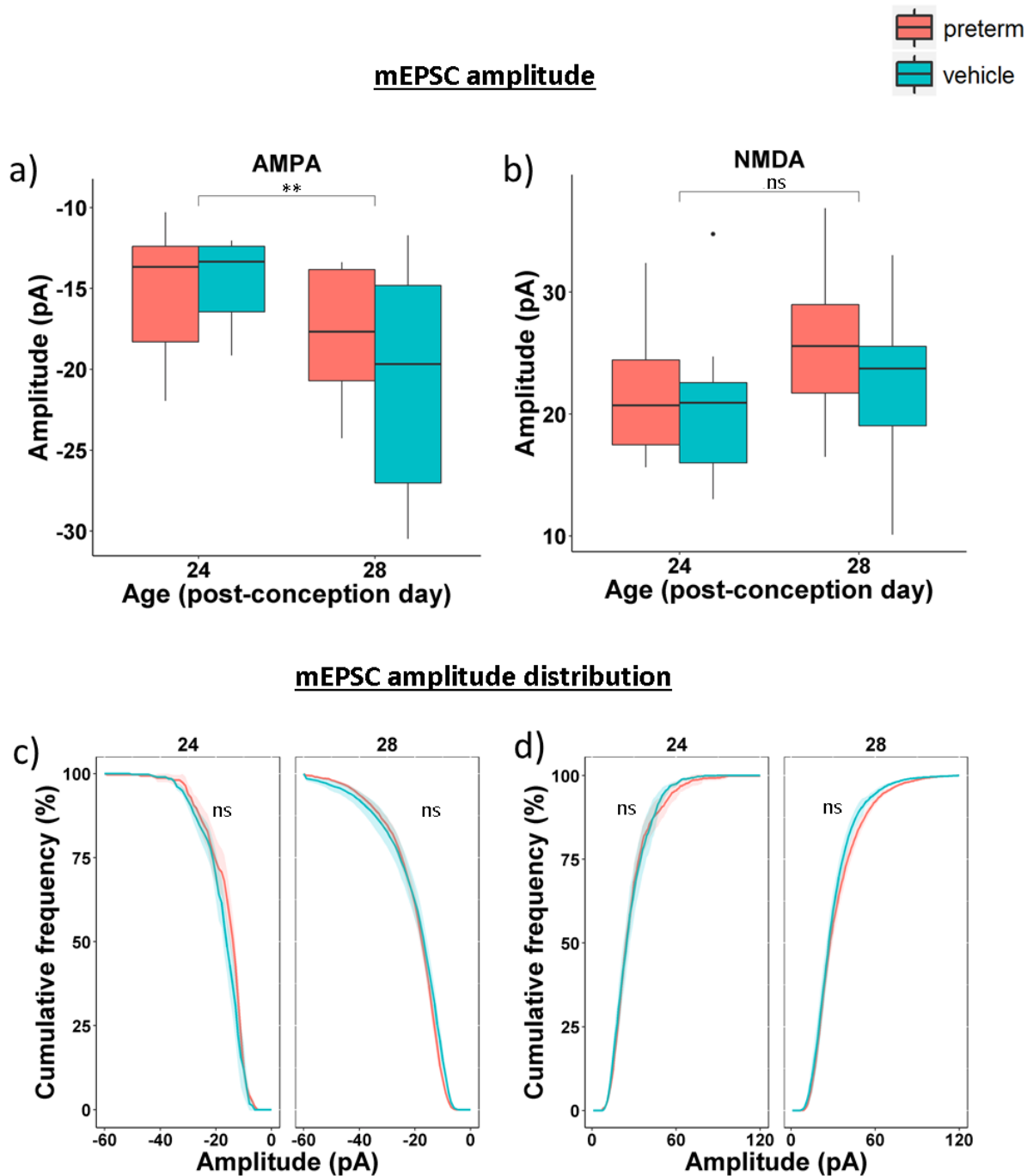
*Figure 2.16 **Size of miniature excitatory postsynaptic currents in layer IV Stellate cells.***
The average amplitude of AMPAr mediated events (a) increases with age (F = 7.395, P <0.01, but the average amplitude of NMDAr events remains the same. Neither the average amplitudes or the cumulative frequency of amplitudes at both AMPAr (c) and NMDAr (d) events are different between preterm and control animals.
n# (cells) [n: preterm –24d = 14, 28d = 16; vehicle –24d = 18, 28d = 15].

2.4 Discussion

2.4.1 Model of prematurity

Human prematurity is a complex health problem, where being born early is usually accompanied by a variety of comorbidities. Previous studies in humans and animals have looked at the outcomes of prematurity with these accompanying conditions, but few have looked at the effects of being born early in isolation. This study uses an induction method that mimics the physiological decrease in progesterone receptor activity responsible for triggering labour in mice (Sugimoto *et al.*, 1997). In contrast to premature birth triggered by other models (e.g. intra-uterine infection (Hirsch, Saotome and Hirsh, 1995)). this results in preterm birth with no apparent accompanying health concerns. Having characterised this model, it is used to investigate the influence of being born early on neurological postnatal development and further tease apart the individual components of the clinical situation. It combines behavioural assays, histological assessment, and electrophysiological investigation of cellular and synaptic properties of neurons to investigate the effects of preterm birth on the development of the sensorimotor network.

This model of prematurity successfully induced preterm birth (Figure 2.5b) on PC18d, which is 92.5% of term gestation. This was the earliest gestation possible for long-term offspring survival, with birth before PC18d resulting in 0% survival beyond 2 days old. Previous studies have shown that with spontaneous preterm birth in mice occurring before PC18d resulted in no offspring survival (Calmus *et al.*, 2011) and mouse model using caesarean section found no survival before PC19 days (Loctin and Delost, 1983). This difference in the day that survival is achieved is possibly due to the difference in the average term gestation lengths between mouse strains (Murray *et al.*, 2010), which differs up to 42hrs. Because of this difference it is important to consider the day of preterm birth relative to term gestation. Strain difference in gestation may not be the only factor contributing as *Loctin and Delost* (1983) used CD-1 mice which have a similar gestation length to the C57BL/6 used in this study (Giknis, Charles and Clifford, 2007). The other major difference between these studies is the method of birth, caesarean section versus vaginal delivery. In the vaginal delivery in this study mothers were observed tending to the pups as soon as they were born which may have helped stimulate survival. It is also possible that the changes in progesterone signalling

induced with RU486 administration stimulated the normal parturition signalling cascade (Ratajczak and Muglia, 2008), resulting in a more usual birth experience that could have increased pup survival. Whatever the source of these differences in day of survival it emphasises that there is a delicate balance to achieve viable preterm offspring in mice, and this study appears to have found the cusp of when it is possible in C57BL/6 mice.

Previous animal models have looked at offspring development following preterm birth at around 90% of gestation (Sangild *et al.*, 2013; Toda *et al.*, 2013; Caminita *et al.*, 2015; Andersen *et al.*, 2016; Shaw *et al.*, 2016). In humans, birth at 90% of gestation is classed as late prematurity (Gibson and McKeown, 1947), however the differential developmental times between humans and rodents need to be considered. Rodents are born in a relatively immature state compared to humans (Clancy, Darlington and Finlay, 2001) and the neurodevelopmental stage of a term born rodent is comparable to a third trimester human fetus (Kostović and Jovanov-Milosević, 2006). As a result, inducing birth a couple of days early in mice results in them being in an altered developmental environment around the same time as more severally preterm human infants.

Even with birth at 92.5% of gestation survival rate of offspring born prematurely in this model were reduced (Figure 2.5c). This reduced survival in preterm birth is seen in humans (Manuck *et al.*, 2016), as well as in other mammals both naturally (Wildman *et al.*, 2011) and in experimental models (Loctin and Delost, 1983). As well as lower survival at birth there was an increase in neonatal deaths in the first postnatal day in preterm litters. It is not only the brain that is underdeveloped in preterm offspring, but also other organ systems such as cardiovascular (Seri, 2001; Bennet *et al.*, 2012; Brew, Walker and Wong, 2014; Fyfe *et al.*, 2014; Wu, Azhibekov and Seri, 2016) and respiratory (Fraser, Walls and McGuire, 2004; Moss, 2006; Jones, 2009). Clinical interventions are taken to artificially maintain these systems and aid maturation. Without any interventions in this model it is likely that the reduced survival of offspring is due to failure of one or more of these other organ systems. Lung maturation in mice is occurring during this late fetal period (McCarthy *et al.*, 2018) and following preterm birth mice have been found to lack sufficient lung maturation (Loctin and Delost, 1983), and also have trouble feeding during the first few postnatal days. This could explain the mortality rate at birth and the subsequent neonatal death seen in this cohort.

A measure of fetal maturation is skin barrier function, which develops by late gestation in humans (Evans and Rutter, 1986) and also in rodents (Hardman *et al.*, 1998). In C57BL/6 mice it has been shown that the epidermis is not fully developed at PC18d, but is by 19d, which is evidence that the preterm mice in this study are in an immature state. This underdeveloped state can also be seen in the reduced birth weights of preterm offspring in this model (Figure 2.5c), which is observed both clinically and in other experimental models of prematurity (Andersen *et al.*, 2016; Shaw *et al.*, 2016).

Although they are born smaller, premature offspring gain weight at the same rate as term born animals, so their growth curve over the first few postnatal weeks is not different to control animals (Figure 2.7a). In a previous progesterone antagonist induced preterm model, guinea pigs were found to have reduced birth weights but by 28 days old there was no weight difference compared to controls (Shaw *et al.*, 2016). Another marker of overall postnatal development is reaching physical milestones such as fur development and eye opening. Preterm offspring in this model reached these gross physical milestones (Figure 2.7b&c), at the same post-conception age as control animals. This suggests that the emergence of these characteristics is genetically determined, and that preterm birth does not disrupt their development. In contrast a rodent intrauterine infection found a delay in eye opening (Toso *et al.*, 2005).

2.4.2 Behaviour

During the first few postnatal weeks mice develop a range of reflexive behaviours, that involve integration of several neural systems and are required for their independent survival outside the nest (Fox, 1965). Previous animal models of prematurity have investigated the development of these sensorimotor behaviour in offspring. Intrauterine infection was found to delay development of righting reflex, cliff avoidance and geotaxis (Toso *et al.*, 2005; Dada *et al.*, 2014), whereas neonatal hypoxic-ischemic (HI) injury did not affect the development of these behaviours (Brockmann *et al.*, 2013; Feather-Schussler and Ferguson, 2016), but did show deficits in ambulation and grasping (Feather-Schussler and Ferguson, 2016). Preterm pigs (delivered by caesarean section) were found to have delayed onset of early motor behaviours (Andersen *et al.*, 2016). In this study preterm offspring developed behaviours

that involved sensorimotor integration at the same rate as control animals (Figure 2.8). Some of these behavioural tests were limited to 30 second attempts, to conform with Home Office licence requirements. At the youngest time-points animals were not able to complete cliff avoidance or negative geotaxis experiments in the time allocated and few were able to complete righting reflex tests. It is possible that with a longer test period these young animals would have eventually been able to complete the task, resulting in a more gradual development of these behaviours being observed, and possible revealing more subtle changes to the preterm animals in these early stages.

Longer term motor behaviour deficits have also been seen in HI injury and infection based preterm animal models and in preterm pigs (Chahboune *et al.*, 2009b; J D L Bergeron *et al.*, 2013; Andersen *et al.*, 2016; Shaw *et al.*, 2016). A common outcome of prematurity in humans is motor deficits, particularly cerebral palsy (Pascal *et al.*, 2018) and so long-term motor deficits are an important behaviour to investigate in animal models of prematurity. In this study, when given an open field exploratory task at 3 weeks old preterm offspring showed no deficits in locomotive behaviour (Figure 2.10b&c).

Preterm infants are also at higher risk of developing sensory processing deficits (Suellen M Walker *et al.*, 2009; Wickremasinghe *et al.*, 2013). In rodents the primary sensory input from the environment comes though the whiskers and the development of active whisking emerges during the early postnatal period (Landers and Philip Zeigler, 2006). To assess whether premature birth affected the emergence of whisking whisker movements were observed daily during the first 3 postnatal weeks (Figure 2.9a). This fine resolution measure of this behaviour found no deficits in preterm animals. Sensory perception is also experience throughout the rest of the body, and when presented with body stimulation pups sometimes respond with movement (Figure 2.9c). Preterm pups were more responsive to body stimulation throughout the developmental period which is a possible indicator of increased sensitivity to stimulation or an increased level of anxiety. In addition, when presented with the open field exploration task at weaning preterm animals were less likely to enter the central portion of the area (Figure 2.10d), which is a characteristic associated with anxiety (Simon, Dupuis and Costentin, 1994). Similar results have been previously observed in other preterm animal models, with increased salivary cortisol levels following open-field exploration in prematurely born guinea pigs (Shaw *et al.*, 2016), and decreased

exploration of the central area of an open-arena found in adult mice following chronic neonatal hypoxia (Weiss *et al.*, 2004). Increased anxiety has been reported in humans following premature birth (Johnson and Marlow, 2011). Anxiety in humans is a complex mood disorder and many factors that occur in the preterm period could influence the development of these behaviours, such as exposure to painful procedures as part of neonatal care (Barker and Rutter, 1995). Early life pain has been shown to increase anxiety behaviour in both humans (Hall and Anand, 2005) and rodents (Victoria *et al.*, 2013). Painful experience however is not something that was part of this animal model of prematurity. Changes in non-painful tactile experience during the neonatal period has also been found to alter anxiety responses in adult mice, with whisker deprivation resulting in hyperactive neuronal activation in the amygdala following stressful experience (Soumiya *et al.*, 2016). Being born early alters the sensory experience of preterm offspring. Whether this change in environment impacts the development of anxiety behaviour in the way chronic sensory deprivation does is unknown, but it is a possible mechanism behind the altered anxiety-like behaviours in preterm pups in this study.

Maternal stress during gestation has been found to induce heightened anxiety-like behaviour in offspring (Zagron and Weinstock, 2006; Iturra-Mena *et al.*, 2018). Maternal stress from the experimental procedure (handling and injection) were accounted for by delivering a vehicle injection to the control group at the same time-point. However, it is possible that the induction of preterm labour increased stress levels in the dam which could have impacted the offspring in the 18hrs between induction and birth. Another possible source of alerted early life experience could have been changes to maternal care. In rodents poor maternal care during the postnatal has been found to increase anxiety-like behaviour in offspring (Caldji *et al.*, 1998). Maternal care was not quantified in this study, and although no major neglect was observed, and pups grew along the same trajectory as controls (Figure 2.7a) - indicating sufficient care - it is possible that changes in maternal behaviour occurred and this impacted the development of anxiety behaviour in preterm offspring.

Given the cognitive deficits found in preterm infants, even those classified as late-preterm (Chyi *et al.*, 2008; Morse *et al.*, 2009; Baron *et al.*, 2011; Chan *et al.*, 2016) it would be interesting in future to investigate cognitive development in this mouse model. This would require extended investigation into adulthood and the use of rodent behavioural assays

such as memory tasks (Brooks *et al.*, 2005; Rodriguiz and Wetsel, 2006; Zeleznikow-Johnston *et al.*, 2018).

2.4.3   Anatomical brain development

Clinical changes in brain growth are seen in preterm infants, including reduced brain volumes and cortical thickness (Inder *et al.*, 1999; Kesler *et al.*, 2004; Inder *et al.*, 2005a; Pierson *et al.*, 2007; Srinivasan *et al.*, 2007). Similar changes have also been seen in previous animal models of preterm comorbidities, including intrauterine infection (X. Wang *et al.*, 2007b), and intrauterine and neonatal HI injury (Fagel *et al.*, 2006; Brockmann *et al.*, 2013). However a previous study using a similar protocol to this one found no changes to cortical thickness in the early post-natal period (Toda *et al.*, 2013) which is replicated here with no change in the increase in brain weight (Figure 2.11a) and cortical thickening (Figure 2.11b) in the early neonatal period following preterm birth.

In rodents thalamocortical projections go through a coordinated development during the first postnatal week (Fox, 1995; Inan and Crair, 2007). Using a similar method of induction of preterm birth (Toda *et al.*, 2013) found that the migration of thalamocortical projections into the barrel cortex was stimulated by birth and so accelerated in preterm induction. This is a transient difference appearing at around PC23d, with the developmental stages at PC24d appearing the same as controls. In contrast in this study there was no apparent difference in barrel development in preterm and term born animals (Figure 2.11e). This difference in outcome could be due to the different strain of mice, owing to the fact that natural gestation in different strains has been shown to vary (Murray *et al.*, 2010) and that genetics has been shown to be an influencing factor in the effects of prematurity (Bezold *et al.*, 2013). In addition to altered gestation lengths, the ICR mouse strain used by Toda *et al.* have litters of an average size of 11.5 offspring (Shin *et al.*, 2017) whereas the C57BL/6 strain used in this study have an average litter size of 8 (Murray *et al.*, 2010). Another difference is that C57BL/6 mice are an inbred strain whereas ICR are outbred, meaning they would have more genetic variability between animals (Festing, 1976). It has previously been found that differences in body weight affect the development of the barrel cortex during this early postnatal period, with larger animals developing them earlier in typical

developmental conditions (Hoerder-Suabedissen, Paulsen and Molnár, 2008). Toda *et al*. reported no difference in body weight during the first postnatal week following premature birth - which was also found in this study (Figure 2.7a) – but barrel formation was still accelerated suggesting a change in developmental mechanisms compared to typical developmental conditions. The developmental differences between these strains that may impact the outcomes of prematurity are not known, but these findings suggest that subtle genetic differences within species need consideration when developing an animal model of prematurity.

2.4.4   Cellular properties

During early postnatal life cortical neurons undergo a maturation process which results in changes to the passive membrane properties and active function of the cells (McCormick and Prince, 1987; Zhou and Hablitz, 1996; Maravall, Stern and Svoboda, 2004; Zhang, 2004; Etherington and Williams, 2011). These result from changes in cellular morphology and composition, such as dendritic arborisation (Callaway and Borrell, 2011), increased number of synapses, and changes in the number and type of ion channels in the membrane (Hamill and Huguenard, 1991).

Previous studies using preterm models, including those caused by HI injury (Dean *et al.*, 2013; Ranasinghe *et al.*, 2015), intrauterine inflammation (Burd *et al.*, 2010b) and prenatal stress (Gutiérrez-Rojas, Pascual and Bustamante, 2013), have shown changes to cortical development. Altered microstructure in the cortex have been seen in preterm infants (Vinall *et al.*, 2013) which is an indicator of cell morphological changes. However, induction with RU486 was previously shown to not change the morphological development of neurons (Burd *et al.*, 2010b).

Changes to cell morphology affects the functional output of neurons (Connors and Regehr, 1996; Mainen and Sejnowski, 1996), and early cortical activity is known to be important for the formation of cortical networks (Colonnese *et al.*, 2010; Yang *et al.*, 2013). Together this means that these structural changes could alter neural activity which could disrupt the formation of neuronal networks. Activity changes have been seen in animal models of prematurity, with impaired maturation of EEG signals following intrauterine infection in

sheep (Keogh *et al.*, 2012) and changes in excitatory neural activity across the cortex following neonatal HI injury (Failor *et al.*, 2010; Brockmann *et al.*, 2013; Ranasinghe *et al.*, 2015).

Early postnatal developmental changes to passive membrane and firing properties of layer IV excitatory neurons have previously been reported in rats (Valiullina *et al.*, 2016). These results were mirrored in this study in mice, decreasing RMP (Figure 2.12b) and input resistance (Figure 2.12c) with developmental age, with APs becoming larger (Figure 2.13c) and faster (Figure 2.13d&e), with a lower threshold (Figure 2.13b) and neurons firing more AP as they mature (Figure 2.14c). These developmental changes are not effected in the premature cohort, suggesting once again that this maturation process is set at conception and is not disrupted by the experience of preterm birth.

2.4.5   Synaptic properties

The preterm period coincides with an explosion of synaptogenesis (Molliver, Kostović and van der Loos, 1973; Huttenlocher and Dabholkar, 1997) and in rodents this process is occurring during the early postnatal period (M. C. Crair and Malenka, 1995; Ashby and Isaac, 2011). Delayed synaptogenesis has been reported in preterm infants (Sarnat and Flores-Sarnat, 2016), and synaptic changes in animal prematurity (Bourgeois, Jastreboff and Rakic, 1989), with early life insults associated with clinical prematurity, such as hypoxia and inflammation also affecting synaptic formation in the developing brain (Dean *et al.*, 2013; Kuypers *et al.*, 2013; McClendon *et al.*, 2014). In contrast following prematurity in primates no delays in synaptogenesis in the cortex were seen (Bourgeois, Jastreboff and Rakic, 1989).

Synapse formation and refinement is an experience-dependent process, and alterations to early life sensory experience disrupts this process (M. C. Crair and Malenka, 1995; Ashby and Isaac, 2011; Dunn *et al.*, 2013). Being born prematurely alters the environment of the developing neonate and this change to early life sensory stimulation could have experience-dependent effects on the formation of neuronal network connections. To assess whether prematurity impacts the development of synaptic inputs to cortical cells, this study investigated the spontaneous release of glutamate at single synapses, measured as mEPSCs (Katz and Miledi, 1963). During the early neonatal period there is an increase in the

frequency of these events (Figure 2.15b&c), which has previously been seen in other cell types in the developing cortex (Desai *et al.*, 2002; Simkus and Stricker, 2002; Yuan *et al.*, 2016). This increase may be from increasing numbers of synapses and/or from an increased likelihood of release as the existing synapses mature. As well as an overall increase in both AMPAr and NMDAr mediated events there is a change in the ratio of their activation, with a higher proportion of AMPAr events occurring at older timepoints (Figure 2.15d). This differential change is also seen in the increase in size of AMPAr mediated events but not NMDAr (Figure 2.16a&b). These are likely due to a developmental process known as synaptic unsilencing (M. Crair and Malenka, 1995; Isaac *et al.*, 1997; Ashby and Isaac, 2011). Early in development many synapses do not contain AMPAr making them functionally silent (Wu, Malinow and Cline, 1996), as at hyperpolarised resting membrane potentials NMDAr are blocked with magnesium and so cannot be activated (Nowak *et al.*, 1984). This allows for large amounts of synaptic plasticity in the form of LTP to occur, and with AMPAr inserted into the post-synaptic density, resulting in active synapses. This is most prominent during the critical period of barrel development in the first postnatal week (M. Crair and Malenka, 1995). This process has been shown to be effected in some developmental disorders (Hanse, Seth and Riebe, 2013), and a reductions in AMPAr density has been seen following neonatal HI injury (Ranasinghe *et al.*, 2015). In this model there are no deficits in this process in preterm animals suggesting that early birth does not disrupt the critical neonatal synaptic development of the barrel cortex.

It is the development of excitatory cortical neurons that have been investigated here, but it is important to briefly consider inhibitory network development - as in the mature network these opposite activities have highly interconnected function. Even more importantly, in the early neonatal brain the inhibitory neurotransmitter $GABA_A R$ has an excitatory action on the cortical network (Ben-Ari, 2002). As a result the E/I balance that is crucial to efficient processing in the mature brain (Zhou and Yu, 2018) is not yet present. Instead recurrent depolarisation potentials are prevalent in the immature brain, with GABA playing a vital role in their generation (Ben-Ari, 2002). As a result, GABA also plays a role in the synaptogenesis explosion seen in the early neonatal brain, with its depolarising action being enough to remove the magnesium blocker of NMDAr (Nowak *et al.*, 1984) and stimulate the previously described LTP during this critical period (M. Crair and Malenka, 1995).

These lack of alteration to the developmental trajectory of excitatory synapses in the barrel cortex seen in this investigation suggests that the altered early sensory experience of being born prematurely does not impact this maturation process. The first few postnatal days is a critical window where sensory deprivation has the most impact on synapse formation in the developing barrel cortex, which coincides with a peak in plasticity at P4 (M. C. Crair and Malenka, 1995). The unchanged synapse formation in preterm animals may be because the altered sensory experience occurs before this window. If the neuronal network is not yet primed for experience-dependent developmental processes the intrinsic development may continue unaffected (Rubenstein, 2000; Yamamoto and López-Bendito, 2012) and normal development continues. Previous studies have investigated the affect that early life deprivation of sensory experience has on developing synaptic connectivity, but the effects of enhanced sensory experience are unknown. The preterm period in this model may not be a deprivation of experience but an addition of stimulation that would not have been present in the intra-uterine environment, and this may not influence the development of the sensory pathways. Alternatively, the alteration in sensory experience may not have been that profound in preterm mouse pups. They were reared under normal conditions by their mother following birth and all aberrant sensory experience, such as handling were received by the control animals as well. Mice are born relatively immaturely and so in the early postnatal days spend their time nested under their mother. This environment may be similar enough to the intra-uterine experience to not impact experience-dependent neuronal development.

2.4.6   Conclusion

Prematurity is a complex clinical situation and teasing apart the factors that contribute to neurological damage in humans is difficult. This study provided one of the first investigations into the long-term structural and functional development of the mouse brain following premature birth. It successfully delivered prematurely born mice and offers insight into the effects of being born early in isolation of other pathologies. It finds that in mice born early - through induction by a progesterone antagonist - the brain appears to be relatively robust and develops along its expected trajectory.

It is important to acknowledge that the degree of prematurity in this model compared to the human situation is relatively mild, and it may be that the degree of prematurity is too small to impact the developing neuronal networks. However, the time point that these animals are born does occur at a neurodevelopmental stage that parallels a more severely preterm human brain. These findings could suggest that it is not being born early per se that is disrupting the neural developmental processes in the immature brain but other factors that often accompany the preterm situation. Previous large animal models of premature birth have used supportive intervention to aid neonatal survival, such as prenatal steroid administration for lung maturation (Shaw *et al.*, 2016) and postnatal nutritional and respiratory support (Andersen *et al.*, 2016). So, although no other pathologies were experimentally introduced these procedures and possible side-effects could have been enough to explain the neurodevelopmental deficits found. The offspring in this study did exhibit behavioural changes that suggest heightened levels of anxiety. The neuronal mechanism of this were not investigated and further investigation would be needed to confirm the source of these behavioural changes. A possible source of this increased anxiety in preterm animals is a deficit in maternal care, which has been previously shown to increase anxiety in offspring (Caldji *et al.*, 1998). This would support the idea that alterations to early life experience are the factors driving altered neurodevelopment in the preterm situation. It also emphasises the difficulty in teasing apart the contributing factors of the premature condition, even in controlled animal models.

Some limitations to this study need to be considered. The control group received a vehicle injection at the same time point as preterm RU486 injections to control for the stress of the procedure. However, this did not control for the effects of RU486 itself on the fetus. It is known that in humans RU486 crosses the placenta and enters the fetal circulation, although changes in circulating steroid concentrations were not found (Hill *et al.*, 1990). The placental transfer of this drug in mice is unknown. An additional control group could be used to account for the potential effects of RU486 directly on offspring development. This group would receive the same dose of the drug as the preterm group but timed to induce birth at normal term gestation. This was considered in the original study design, but the variability of natural gestation length in C57/BL6 mice posed a problem. The expected gestation of any pregnancy was not known and so induction at the exact time was not possible. With a

variability of 2 days (Figure 2.5) the degree of prematurity received by an induced term control group would be uncertain and could impact the development of the offspring. A possible solution going forward would be to investigate other mouse strains to find one that has less variability in natural gestation length.

Another limitation is the lack of blinding. This was not possible as all the experimental procedures, including induction of preterm birth were carried out by a single researcher (*Christine Cross*). To reduce the impact of this on the results all data was blinded post collection and automated data analysis was carried out using custom written software. However, it was not possible to add control measures for some of the behavioural assays, as subjective measurements had to be recorded in real-time. This included the assessment of developmental day (Figure 2.7b) and twitch responses to tactile stimulation (Figure 2.9). In future experiments improvements would be made by having separate researchers induce birth and carry out further investigations on the offspring.

The development of a mouse model of preterm birth also opens up the opportunities to combine it with transgenic mouse lines, which offer physiologically relevant genetic manipulations and novel experimental tools (Kumar *et al.*, 2009). The experiments presented in Chapter 3 would be an example of the use of transgenic mice for new investigations in this preterm model. It would allow an investigation into the functional changes to cortical network activity following premature birth, such as shifts in developmental trajectories or longer-term deficits. This would complement functional neuroimaging studies in human preterm infants (see Table 1 in Chapter 4 for details).

This model of preterm birth is a good foundation for investigating the complex clinical situation of prematurity, in a small animal widely used across the research community. Further expansion on this study, as described above could further explore the effects that being born early has on the neurodevelopment of mice and help uncover more subtle alterations not measured in this current study.

3 Pan-cortical Calcium Imaging of Postnatal Development

3.1 Introduction

In the healthy developing brain endogenously and exogenously generated neural activity is present, and both play an important role in the formation of neuronal networks, with disruptions to activity during the development of these networks have long term consequences (Zhang *et al.*, 2011). Because of this vital and vulnerable activity period investigation into the developmental trajectories of activity in the developing networks is of great interest. When investigating the activity dynamics of neuronal networks, the optimum method is to record from the intact system, in a naturalistic environment. To try to achieve this optimum situation this study develops a technique to investigate the development of spontaneous and stimulated activity across the entire cortex of awake neonatal mice.

Activity in the developing networks in vivo has previously been investigated using a number of different techniques. Electrical activity of neurons has been directly recorded, in both humans and rodents. Non-invasive recordings using scalp electrodes are routinely used in human infants, which capture cortical activity with high temporal resolution (Vanhatalo and Kaila, 2006a). However, the spatial resolution of this technique is not as well defined, with a summation of activity from multiple locations being captured by each electrode. Electrical activity in the rodent cortex can be collected in a more invasive manner. Electrodes can be implanted into the brain and more localised electrical signals can be collected, giving a more refined spatial resolution to the data (Yang *et al.*, 2009). However, the spatial resolution of these recordings is still restricted due to the limited number of electrodes it is possible to implant simultaneously in the rodent brain. The developing rodent brain is small and the insertion of electrode into the cortical tissue causes damage, both limiting the number of electrodes that can be implanted in the young animal (Zayachkivsky *et al.*, 2013)

To overcome this spatial information issue another option for recording activity in the cortex is to visualise neuronal activity by imaging. Direct visualisation of neuronal electrical activity is not possible in the naïve brain with current technologies, but proxies of activity can be. Active neurons have an increased metabolic demand that results in an increased consumption of metabolites, changing the composition of the blood flowing in active regions (Cipolla, 2009). These changes in the blood content can be visualised in a number of ways. This still doesn't provide high spatial resolution but does increase the information

regarding where the activity is occurring. A commonly used technique in both animals and humans is functional magnetic resonance imaging (fMRI). This technique measures changes in blood oxygenation, taking advantage of the different magnetic properties of oxygenated and deoxygenated blood (Ogawa *et al.*, 1990). fMRI can be captured either during rest (Lee, Smyser and Shimony, 2013; Gorges *et al.*, 2017) or in response to stimulation (DeYoe and Raut, 2014; Boussida, Traoré and Durif, 2017). These measurements can be taken throughout the brain and 3D data can be resolved (Glover, 2011). This signal is collected at the spatial resolution of voxels, in the order of millimetres in size, providing information about areas of blood flow changes that cover regions of the brain. From fMRI data spatial localisation of responses to single digit stimulation have been identified (Kim *et al.*, 2014a; Choi *et al.*, 2016) but is far from cellular resolution.

The temporal resolution of fMRI technique is much slower than the previously described electrical measurements. The rate of image acquisition is relatively slow (Boubela *et al.*, 2014) – typically 1-3 seconds - and even with technological improvements that could improve acquisition times the actual changes in blood oxygen levels are much slower than neuronal activity (Buxton, Wong and Frank, 1998). Another issue with this technique in the developing brain is that the link between blood oxygen changes and neuronal activity is not well understood. It is not only neurons that are developing in the brain but also the cerebral vasculature (Wang *et al.*, 1992). It has been found that the link between neuronal activity and blood oxygen in the immature brain are not the same as adults and change over the neonatal period in mice (Kozberg *et al.*, 2016). Another restriction of this technique is that to collect good quality data the subject must stay very still. In animals this is done thorough anaesthesia, which in young rodents has been found to suppress the blood oxygen changes (Colonnese et al 2008).

Changes in blood oxygenation in animals can also be measured by intrinsic optical imaging (IOI) (Frostig *et al.*, 1995; Zepeda, Arias and Sengpiel, 2004). Rather than measuring the magnetic properties of oxygenated and deoxygenated blood, IOI utilises the difference in their reflectance of visible light (Frostig *et al.*, 2009). IOI provides similar spatial information to fMRI, with changes in blood flow supplying regions of the brain identifiable, such as cortical activity in response individual whisker stimulation (Tsytsarev *et al.*, 2010). This technique is more invasive, requiring the exposure of the cortical surface, and can only be

taken at the surface rather than throughout the brain volume (Zepeda, Arias and Sengpiel, 2004). It can however be captured in awake animals, including neonatal rodents (Sintsov *et al.*, 2017), overcoming the issue of anaesthesia activity suppression. However, the signal to noise is low with this technique, with averaging of many trials being required to give confident result meaning it is not an optimal technique for recording resting-state activity. Also, as with fMRI this method captures changes in the blood rather than direct neuronal changes, and so does not overcome the issue of neuron to vascular coupling in the immature brain being unclear.

Techniques for visualising neuronal activity more directly have been developed. These techniques require the addition of a biosensor into the neurons that can be imaged using microscope technologies. One of these types of markers are calcium indicators. These are engineered molecules that are capable of binding to calcium ions which results in a change in their fluorescent properties (Adams, 2010). When neurons are active there is an increase in free intracellular calcium ions (Baker, Hodgkin and Ridgway, 1971; Verkhratsky and Shmigol, 1996), meaning that the change in fluorescence of calcium indicators when bound to these calcium ions is directly related to neuronal activity. Importantly this rise in intracellular calcium in active neurons is present in the immature brain (Rosenberg and Spitzer, 2011). These molecules are available as chemical (Paredes *et al.*, 2008) and genetically encoded calcium indicators (GECIs) (Tian, Hires and Looger, 2012). Chemical indicators are exogenously added to the brain and taken up by cells in a non-specific manner (Paredes *et al.*, 2008), whereas GECIs can be specifically expressed in certain cell lines using transgenic breeding strategies or recombinant gene delivery (e.g. viral infection or electroporation) (Palmer and Tsien, 2006). An advantage of transgenic expression of GECIs is that they do not have to be invasively introduced into the brain of each animal. The use of transgenic mice means that GECIs can be expressed in a specific cell type through endogenous protein production from the beginning of development. The most commonly used GECIs are the GCaMP family of molecules (Akerboom *et al.*, 2012; Chen *et al.*, 2013). The more recent versions of these molecules are bright enough to visualise through the adult mouse skull (Silasi *et al.*, 2016). Being able to image through the intact skull makes using these indicators relative less invasive compared to the removal of the skull and replacement with cranial windows (Holtmaat *et al.*, 2012).

Because of this direct relationship to neuronal activity rather than blood oxygen changes data can be collected at a higher temporal resolution than fMRI or IOI. However the kinetics of GECIs are slower than the electrical activity of neurons, with the fastest available at the time of this study, GCaMP6f having a rise time of approximately 50ms (Chen *et al.*, 2013). This indicator is also sensitive enough to detect the calcium rise associated with individual action potentials (Chen *et al.*, 2013).

In vivo calcium imaging is a powerful tool for investigating the functional circuitry of the brain (Russell, 2011). It can be implemented at a variety of spatial scales, from single cell resolution (T. H. Kim *et al.*, 2016) to simultaneous whole cortex activity (Mohajerani *et al.*, 2010). Macroscopic widefield imaging is becoming an increasingly popular technique, that allows the recording of the combined activity of thousands of neurons across the neuronal network simultaneously, and in combination with modern GECIs which are bright enough to visualise over large scales with single photon microscopy it is becoming a valuable method for investigating *in vivo* neuronal activity across large areas of the. Wide field imaging can be used to capture activity from across the cortex in awake animals, which has been demonstrated in both adult cortex (Ackman, Zeng and Crair, 2014; Vanni and Murphy, 2014; Xiao *et al.*, 2017) and neonatal mice (Ackman, Zeng and Crair, 2014; Kozberg *et al.*, 2016). The signal being captured during widefield single photon calcium imaging is the summation of activity from of many neurons, from different layers of the cortex (Ratzlaff and Grinvald, 1991). Because of this large spatial scale temporal limitations of the GECIs are not as much of an issue as data offers valuable insight into the population activity but discrete kinetics of single neuron activity cannot be resolved spatially.

The functional development of individual regions of the cortex have been extensively investigated (Khazipov and Luhmann, 2006; Froemke and Jones, 2011; Kilb, Kirischuk and Luhmann, 2011; Erzurumlu and Gaspar, 2012; J. Shen and Colonnese, 2016), but the development of global cortical activity is relatively unexplored. No single region of the neuronal network is developing in isolation, and a synchronisation between different areas is required for the successful formation of a functional mature brain. The use of wide-field calcium imaging in the neonatal mouse offers an opportunity to explore the activity across the cortex in the resting state and during external sensory stimulation, and to track the changes over development.

3.1.1 Aims

This study develops a pan-cortical calcium imaging technique, using wide-field single-photon microscopy and the genetically encoded calcium indicator GCaMP6f expressed in cortical excitatory neurons. This technique is used to explore the cortical network development in awake neonatal mice with high spatial and temporal resolution. Both sensory stimulated and resting-state recording were captured and analysed to investigate the dynamics of cortical activity across early neonatal development.

3.2 Methods

 All procedures were carried out in accordance with UK Home Office guidelines set out in the Animals (Scientific Procedures) Act 1986.

3.2.1 Breeding

Mice expressing GCaMP6f in excitatory cortical neurons were generated by crossing two transgenic lines: Emx1-IRES-cre, expressing cre, which expresses recombinase under the Emx1 promotor, and the reporter line Ai95D, which carry a floxed copy of the GCaMP6f gene under the CAG promotor in the ROSA26 locus (The Jackson Laboratory, USA). Animals were housed in breeding groups of 1-2 females to 1 male, in 12 hr light/dark cycles, with *ad libitum* access to food and water. These two transgenic lines were crossed to produce *Emx1-IRES-cre:loxP-GCaMP6f-loxP (Ai95D)* offspring, who would express GCaMP6f in excitatory cortical neurons (Gorski *et al.*, 2002; Kummer *et al.*, 2012). Pregnant dams were checked every morning before 10am and postnatal day 0 (P0) was assigned on the day a new litter was found. Expression of GCaMP6f was confirmed by using a flashlight and goggles set (Nightsea, USA) to visualise GCaMP6f fluorescence in the brain during the first 2 postnatal days without any skin removal.

In a subset of animals PCR was used to verify genotype and check it matched the expression seen through fluorescent visualisation. 1mm tail tip samples were collected in accordance with Home Office regulations before P10. PCR-based detection of the two transgenes was completed by the departmental technical support team. The primers used were (The Jackson Laboratory, USA):

Emx1-IRES-cre:
Mutant
oIMR1084: 5'-GCGGTCTGGCAGTAAAAACTATC-3'
oIMR1085: 5'-GTGAAACAGCATTGCTGTCACTT-3'
Wild-type
oIMR4170: 5'-AAGGTGTGGTTCCAGAATCG-3'
oIMR4171: 5'-CTCTCCACCAGAAGGCTGAG-3'
Ai95D:
Mutant
19908: 5' -ACGAGTCG ATCTCCCTTTG- 3'
Wild-type
olMR9020: 5' - AAGGGAGCTGCAGTGGAGTA- 3'

3.2.2   Head-fixation surgical procedure

Mice aged P1-9 were prepared for mesoscale optical imaging by surgical procedure to expose the skull and implant a head-fixation device. Pups were placed on a heat-mat (Harvard Apparatus, UK), covered in a surgical drape, set at 38°C to maintain body temperature throughout the experiment. They were briefly (~7 min) anaesthetised using inhalation of isoflurane (ISO) in medical oxygen. First placed in an induction box for 1 minute at 5% ISO to suppress movement, and then placed on a facemask at 3% ISO for the rest of the procedure. Neonatal rodents are more complicated to anesthetise than adult animals (Research, 2003) but successful induction with inhalation of ISO in neonatal rodents has been reported (Gotoh, Matsumoto and Imamura, 2004; Loepke *et al.*, 2006; Huss *et al.*, 2016). Anaesthesia depth was monitored, aiming for a loss of pinch reflex and a steady respiratory rate (Research, 2003). Local analgesia was given by subcutaneous injection of xylocaine (0.02ml 2% with adrenaline – Astrazeneca, UK) under the scalp. The scalp was removed to expose the skull surface using curved micro dissection scissors (World Precision Instruments, USA), and the periosteum cleared using cotton swabs. The skull was covered in a thin layer of clear dental cement (C&B super bond kit - Prestige Dental UK, Bradford, UK) and 4-40 ¼" stainless steel set screw (Thorlabs Inc. USA) was affixed over the cerebellum (Figure 3.1a). Anaesthesia was then turned off, and pups were given a 30-minute recovery period, where they were nested in cotton swabs and covered in bubble wrap on the heatmap, breathing air.

3.2.3   Wide field calcium imaging protocol

Following recovery pups were attached to head fixation device, similar to the setup in (Silasi *et al.*, 2016), which gave head stability in adult mice. All components were purchased from Thorlabs (USA). An articulated ball and socket mount (TRB1/M) was attached to an aluminium optical breadboard using two optical posts attached through 90⁰ with an adjustable bracket (SWC). The set screw fixed to the pup's skull was attached to the ball mount and secured in place with a ¼" plastic hex nut (RS Components Ltd, UK) (Figure 3.1a). The articulated head-mount and the adjustable post design means the pup's head could be adjusted into a comfortable position. A piezoelectric wafer (Piezo Systems INC, USA) was

placed under the pup's torso (Figure 3.1a) to record body movements. The piezoelectric wafer was connected to a Micro1401 data acquisition box (Cambridge Electronic Design Ltd, UK), and the analogue output from the device was recorded in Signal (version 5 - Cambridge Electronic Design Ltd, UK). A small piece of plastic bubble wrap was secured over the pups back to keep their dorsal side warm. This setup was all attached to the breadboard and so could be moved into position for imaging without disrupting the animal.

Images were captured using a tandem lens fluorescence macroscope, composed of 2 SLR camera lenses (50mm, 1.4f - Sigma Imaging, UK) positioned front to front, and a Q Imaging (Canada) optiMOS sCMOS camera, with a blue light emitting diode (LED) (ThorLabs, USA) attached to the side of the macroscope column, directed at a filter cube (bandpass filter for illumination light 473nm and emitted light long pass 500nm) (Figure 3.1b). The brain was positioned in the centre of the LED beam and the z-plane focus was set manually to be just below the skull surface (Figure 3.1c). Images were captured using Micromanager (https://micro-manager.org) at 12-bit 960x540 pixels TIFF images, at 50Hz (20ms frame rate) frame rate. The camera was controlled by a TTL pulse that triggered each frame, which was sent from Signal via the Micro1401 data acquisition box.

3.2.4   Sensory stimulation

Whiskers were stimulated with a custom paddle that was designed to deflect multiple whiskers at a time. It was a 15x8mm plastic paddle secured to a 9V micro servo motor (SG92R – Tower Pro) (Figure 3.2a). The device was powered and controlled through an Arduino Uno board, which was connected to the Micro1401 data acquisition box and was commanded through Signal. Having the stimulation device commanded through the same software as the camera meant that exact timings of stimulation in relation to acquired images was known. The stimulation paddle was positioned caudal to the right whisker field, so it was not touching the face or the whiskers, and at an angle that could contact the whiskers as it arched (Figure 3.2b). It moved in a caudal to rostral direction for 30ms per stimulation – which is enough time for the paddle to move across the entire whisker field to the snout - and then returned to the rest position. There was a 500ms rest period before the

stimulation and a 10s inter-stimulus interval (ISI) between stimulations, with 20 stimulations per recording block.

Single whisker stimulation was delivered using a piezoelectric bending actuator (Piezo Systems INC, USA) with a small clear pipette tip attached (Figure 3.2c). This device was powered by an isolated voltage simulator (DS2A – Digitimer Ltd, UK) and controlled again from Signal software via the Micro1401 data acquisition box to once again be time locked to the camera TTL pulse. Whiskers D1, E1 and delta on the right side were visualised under a binocular microscope (S6E, Leica Microsystems, UK) and trimmed to ~10mm using micro dissection scissors (World Precision Instruments, USA). A whisker was then threaded into the pipette tip, to ~2mm from snout. A 100ms 75V pulse was delivered to the piezoelectric bending actuator which resulted in a 150μm deflection at the end of the device. There was a 500ms rest period before the simulation and a 10s ISI between stimulations and 20 repeats per block.

Auditory stimulation was delivered using an Arduino passive buzzer module (HESAI, China), powered by the Arduino Uno board, and controlled from Signal software via the Micro1401 data acquisition box. The device was positioned 10mm from the pup's right ear and a 30ms auditory stimulation at 10kHz was delivered with a 500ms rest period before the stimulation, a 10s ISI, and 20 repeats. This auditory stimulation was then immediately repeated at 20kHz.
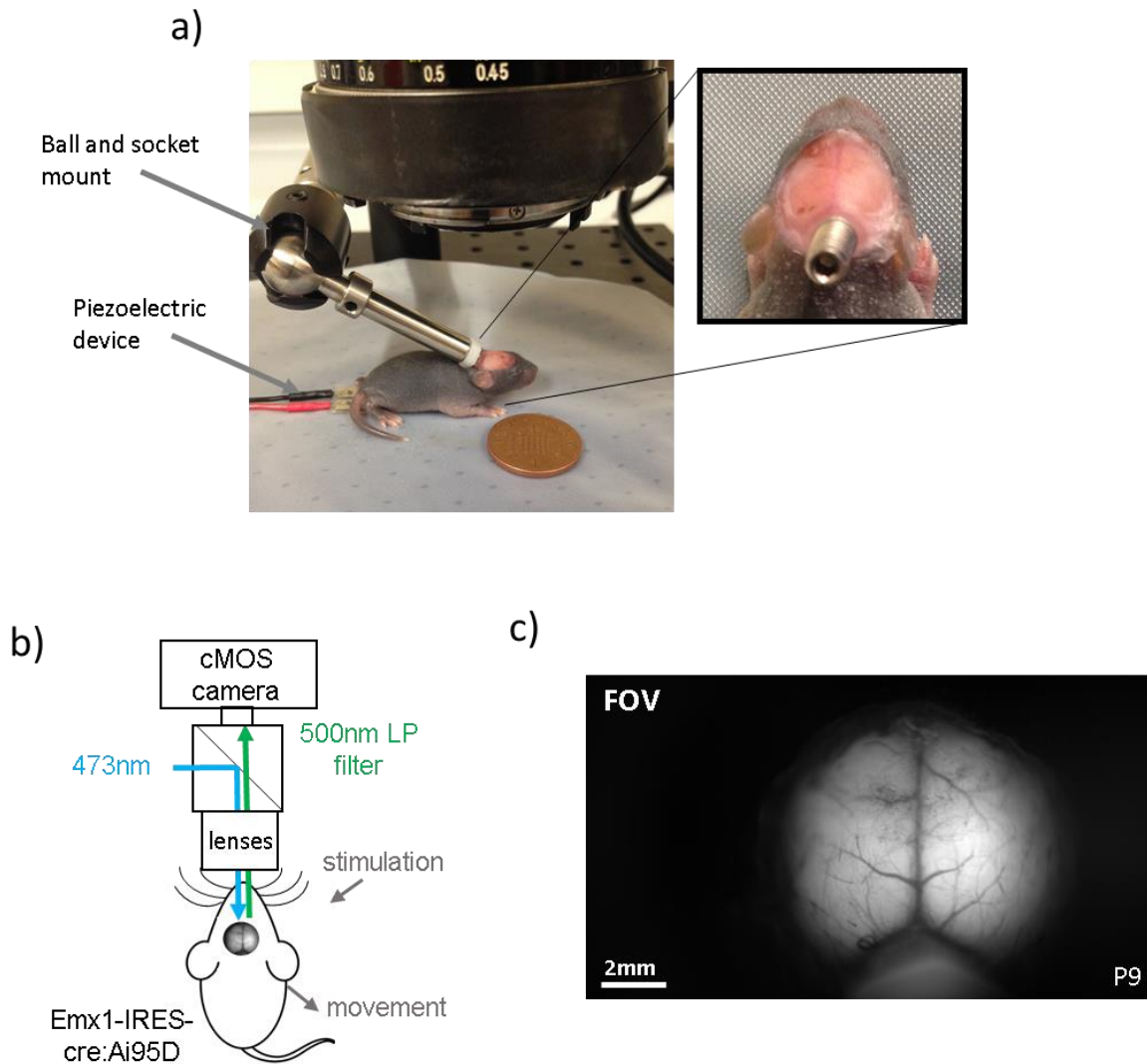
Figure 3.1 **Macroscope wide-field calcium imaging setup**

a) Head-fixation setup. Animal is placed on top of a heat-mat set to 38^0C, with a piezoelectric device under its torso for capturing movement. The animal's head is attached to the ball and socket mount head-fix using a set screw attached with dental cement (posterior to lambda – see zoom in).

b) Illustration of the macroscope setup. 473nm blue LED illumination focused through a back-back tandem macroscope lens onto cortical surface (of GCaMP6f expressing animal), with emitted light filtered through a 500nm long-pass filter and captured by a cMOS camera at 50Hz. Movement is captured by the piezoelectric device under the animal's torso, and external sensory stimulation is delivered (both tactile and auditory).

c) Example field of view captured by macroscope (example P9 animals)
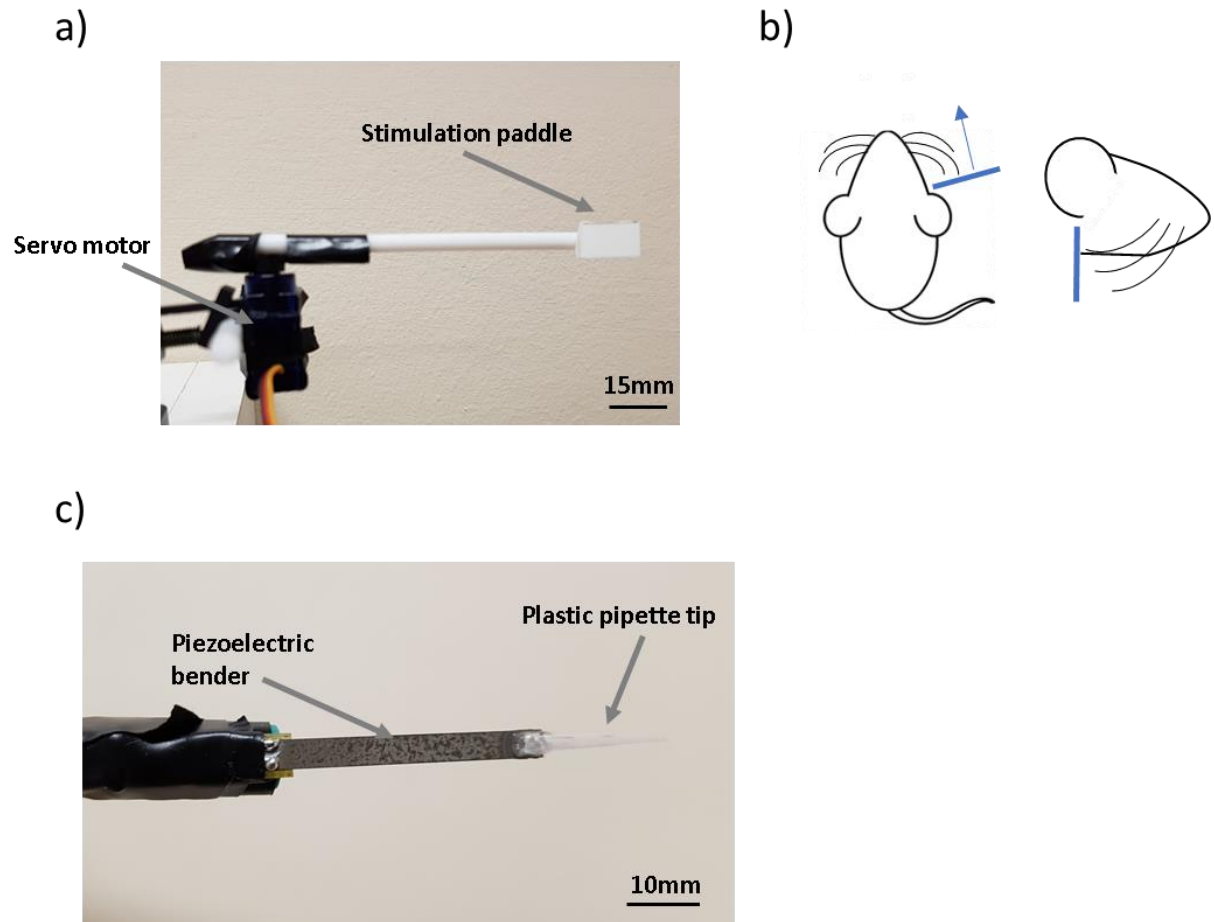
Figure 3.2 **Whisker stimulation setup**
   a) *Whisker array stimulator, consisting of a plastic paddle that displaces the whiskers and a servo motor to drive movement.*
   b) *Position of whisker array paddle for stimulation is represented by the blue line, with the rostral to caudal direction of travel indicated by the blue arrow.*
   c) *Single whisker stimulator, consisting of a piezoelectric bender with a plastic tip for threading the whisker into.*

3.2.5 Whisker trimming

For whisker trimming experiments, unilateral trimming of the left whiskers was carried out daily from the day of birth. Animals were restrained by their neck scruff and held up to a light to visualise the whiskers clearly and trimmed down to the surface of the whisker pad with curved micro dissection scissors (World Precision Instruments, USA). Control animals were restrained in the same way and had the left whiskers gently ruffled with the dissection scissors as a sham stimulation experience. All pups in the litters were weighed at the same time as the trimming protocol. Trimmed and control pups were mixed within litters. Animals in this protocol were imaged at P3 and P7 using the same imaging protocol as above.

3.2.6 Order and timing of imaging protocols

10,000 frames (200s) of continuous images were captured for each block of both resting-state and stimulated recordings. The order and timing of the blocks was kept consistent across subjects (Figure 3.3). In the initial cohort (P1-9 ontogenetic recordings) a resting-state recording, followed by whisker field stimulation and then auditory stimulation were repeated 3 times at 30-minute intervals. In the whisker trimmed cohort a resting-state recording, followed by two whisker field stimulation, followed by single whisker stimulation of D1, E1 and delta repeated twice for each and then finally a second resting-state recording 1hr after the first.
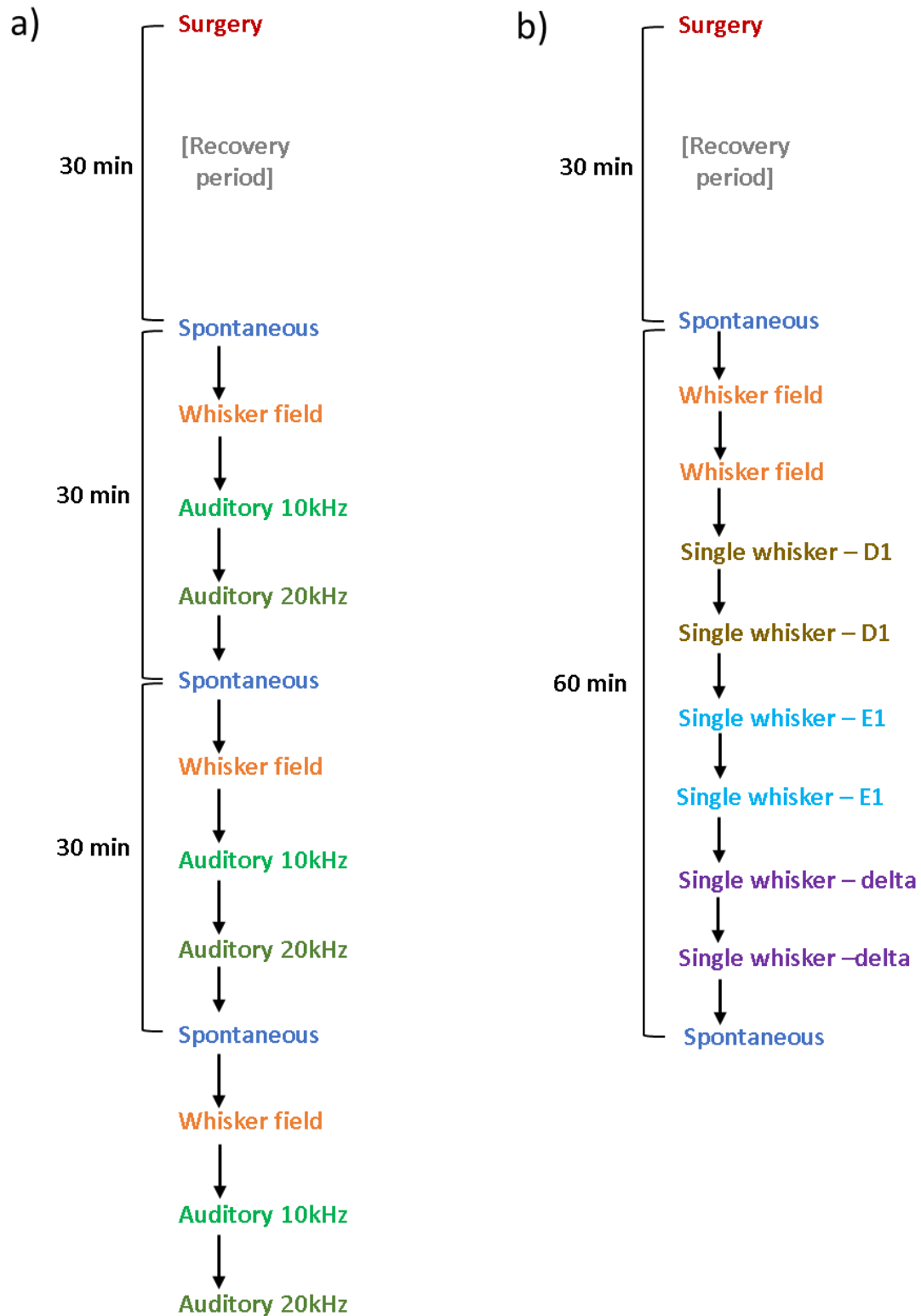
Figure 3.3 ***Order and timing of imaging protocol***
a) Ontogenetic cohort experimental timings. 30 minutes post-surgery recovery followed by 3 blocks of spontaneous recording, followed by whisker and auditory stimulation protocols.
b) Whisker trimmed cohort experimental timings. 30 minutes post-surgery recovery followed a spontaneous recording, followed by whisker field and single whisker stimulation.

3.2.7 Peripheral silencing

To silence the whisker pad 30µl of 2% xylocaine (with adrenaline – AstraZeneca, UK) was injected subcutaneously into the right whisker pad. Immediately a 7 min resting-state recording was captured, followed by a block of whisker stimulation to the right whiskers.

3.2.8 Histology

To verify the GCaMP expression pattern, brains were removed from a subset of P7 animals and drop-fixed in 4% paraformaldehyde made in Dulbecco's PBS (Sigma-Aldrich Ltd, UK) for 48hrs at 4^0C and then stored at 4^0C in Dulbecco's PBS. Whole brains were encased in low melting point agarose (2-3% in distilled water) block to provide stability while sectioning. Coronal sections were cut through the whole brain at 50µm thickness (Leica VT1200 vibratome, Leica Microsystems, UK), and mounted directly onto slides (Leica Microsystems, UK). Once dry they were cover slips were added using Vectashield with DAPI mounting medium (Vector Laboratories LTD, UK). Slices were visualised using a fluorescence microscope (DM IRB Leica Microsystems, UK) and captured on a Leica DFC 450C camera at 5x magnification as tile scanned 696x520 8-bit .lif files.

3.2.9 Analysis

Imaging data were imported and analysed using custom designed software in MATLAB (Mathworks, MA, USA). All code used for analysis can be found at https://github.com/cx749/calcium-imaging.

Image timeseries (TIFF stacks) were imported and underwent a bilinear transformation to reduce the spatial resolution of the image by half (from 960x540 to 480x270). This was done to improve computational efficiency during the first pass of analysis over the whole cortex.

3.2.9.1 *Movement data*

Voltage generated by the piezo wafer placed under the animal's torso was collected at a frequency of 1khz (20 times faster than the imaging). Changes in piezo output voltage occurs whenever it undergoes deflection caused by movement of the body on top of it. Electrical interference from the heat mat introduced high frequency noise to the movement trace, which was suppressed using a 20x averaging temporal filter centred on each image frame. This downsampled the movement data to 50hx, matching the image acquisition while preserving the movement related amplitude changes but reducing the electric noise. Matlab's 'Envelope' function was used to assign a signal envelope over the positive values of the averaged trace to account for the bidirectionality of the signal. A threshold at which the movement trace amplitude changed from the baseline was assigned, and whether the signal at each point exceeded this threshold was calculated, resulting in a binary table of movement or no movement for each imaging frame.

3.2.9.2 *Spontaneous activity analysis*

3.2.9.2.1 Pre-processing

Regions of interest (ROIs) were manually delineated for each animal. These regions included the boundary of the exposed cortex, the left and right hemisphere individually and individual regions of the sensory (B - barrel cortex, FL - front limb, HL - hind limb, V - primary visual and A – auditory), motor (M1 - primary motor, M2 - secondary motor) and sensory

association cortices (RS - retrosplenial, PPC - posterior parietal). For individual regions the coordinates of a pixel in the centre of each region was assigned to represent that area's activity. The position of these regions was determined using a variety of resources - the atlas of the developing cortex defined by Ackman, Zeng and Crair, 2014, the Allen Institute's adult mouse atlas, the location of the barrel cortex identified by whisker stimulation experiments in this study and characteristic spontaneously occurring activity such as retinal waves identified in resting-state recordings.

For the multipixel ROIs the average pixel intensity, and for the individual regions the intensity of the assigned pixel was calculated for each frame. This raw timeseries of regional fluorescence activity was then baseline corrected to account for any variability in baseline fluorescence across cortical regions and different animals. The baseline value (f) was calculated by averaging the lowest 5% of pixel values in the timeseries. This is one of several methods previously used in calcium imaging analysis (Keemink *et al.*, 2018). The raw timeseries values were corrected by subtracting the baseline (df) and then dividing by it (df/f) and multiplying by 100 to give a percentage change (df/f%). Baseline corrected images were temporally filtered at <1Hz using a Butterworth filter (Del Giorno *et al.*, 2012). The variance in the filtered baseline was calculated for a 2s rest period in all traces and the average for all traces across all ages was calculated as 0.42 ±0.09%.

3.2.9.2.2   Frequency of spontaneous activity

Periods of activity were identified using an automated function that selected peaks with an amplitude exceeding twice the baseline variance (i.e. 1%). The locations of these peaks were compared to the binary log of movement, and if movement had occurred 500ms before or after the peak of activity it was assigned as activity related to animal movement. For each trace the frames related to animal movement were excluded, the peaks per minute were then calculated for the remainder of the trace.

3.2.9.2.3 Correlation of spontaneous activity

To calculate the correlation of activity in different regions the pre-processed traces were compared by Pearson's correlation and a coefficient value was calculated. To calculate activity peaks that occurred in regions simultaneously the location of peaks in one timeseries were identified and if a peak in the other region's timeseries occurred within 200ms before or after it was assigned as a match, and the percentage of matching peaks out of the total activity peaks in the timeseries was calculated as a percentage of coordinated activity.

3.2.9.3 *Stimulated activity analysis*

For stimulated recordings a region of activation was calculated. Each stimulation had a 500ms period preceding them and the average fluorescence value of this was used as the baseline for each individual stimulation. First the binary movement log for the recording was consulted and if movement occurred in the 1s following stimulation this sweep was removed from further analysis. If the animal was still during the stimulation period the baseline value was calculated for all pixels, and then a df/f% for each pixel in the 1s following a stimulation was calculated and mean averaged. This was repeated for all still stimulation sweeps and each pixel location was averaged producing a single activation map. From this averaged map the maximum change in fluorescence was identified and then the continuous area surrounding this pixel that exceeded 50% of its value was delineated and assigned as the activation ROI. This average df/f% for the 1s following stimulation was visualised using a heatmap and the activation ROI border was marked on it, allowing the location of the ROI to be manually assessed. For whisker stimulation this ROI was consistently in the contralateral cortex in an area that aligns with the barrel cortex. In auditory stimulation there was no consistent ROI location identified and so no further quantification of response was carried out.

3.2.9.3.1 Amplitude of whisker stimulation response

The amplitude of fluorescence change following simulation was calculated from the maximal response pixel in the heatmap. The pixel intensity at this location was extracted for each 10s stimulation period. The first 500ms were used as the baseline (f) and the 9.5s following the stimulation was baseline corrected (df/f%). The peak amplitude was calculated as the maximum value in the 1s period following stimulation. The average amplitude peak was calculated for each animal by averaging all 10s stimulation periods and calculating the peak amplitude following stimulation.

3.2.9.3.2 Area of whisker stimulation response

The spatial extent of the whisker stimulation response was calculated from the activation ROI. The pixel intensity of this was calculated as a percentage of the entire expose cortical surface. This was to account for the growth of the cortex during the postnatal period investigated.

3.2.10 Statistics

Statistical analysis was performed using R version 3.5.0 (The R Project).

Data were checked for normality of distribution using both a Shapiro-Wilk's test and visual assessment using a histogram and a quartile-quartile plot. If either group failed normality testing non-parametric tests were used. Most data are presented as boxplots, where mean, 25th and 75th quartiles are shown in the main box, and whiskers representing the largest values, with observations more than 1.5 time the IQR shown as outlier points.

3.3 Results

3.3.1 Validation of transgenic breeding strategy

In order to measure neural activity across the developing cortex, a transgenic strategy was implemented in which the calcium indicator GCaMP6f was specifically expressed in excitatory cortical neurons. GCamp6f was used because at the time of experimentation it was the fast GECI available, giving the closest temporal resolution to neural activity possible with genetically expressed indicators. It had also been previously been shown that this fast version of GCAMP6 was still bright enough to visualises through the intact skull in mice (Kozberg *et al.*, 2016; Xiao *et al.*, 2017). To achieve this, *Emx1-IRES-cre* mice, in which the IRES-cre gene is inserted into the locus of the Emx1 homeobox gene (Gorski *et al.*, 2002), was crossed with the Ai95D mouse line that has a floxed-STOP cassette ahead of the GCaMP6f gene driven by the CAG promoter knocked in to the ROSA26 locus (Ding *et al.*, 2014). The *Emx1-IRES-cre* mouse is a commonly used and well characterised transgenic line, that drives expression of cre recombinase in ~88% of neurons in the neocortex (Gorski *et al.*, 2002; Kummer *et al.*, 2012). In animals carrying both transgenes (Emx1-Ai95D), the cre activity should recombine the floxed STOP cassette, permitting expression of GCaMP6f in cortical neurons. Expression of GCaMP6f was initially verified using goggles to visualise fluorescence in the newborn offspring of the transgenic parents. Unexpectedly, some offspring appeared to be expressing GCaMP6f across their bodies (including brain, skin and muscle), suggesting off-target expression. To assess expression patterns the brains of these animals and those that appeared to express only in the brain (as would be expected of the Emx1-Ai95D cross) were fixed and coronally sectioned. Clear differences in expression patterns were observed. Animals that had shown fluorescence in only the brain in vivo (Cortical expressing - Figure 3.4a) had expression of GCaMP6f in the cortex and hippocampus but not in the deep grey matter, which agrees with previous publications documenting expression of cre in the Emx1-IRES-cre line (Gorski *et al.*, 2002; Kummer *et al.*, 2012). However, animals that had shown fluorescence across the body (Body expressing – Figure 3.4b) had expression also in the deep grey matter. In addition, bodies of these animals were dissected after termination and visualised with the goggles. GCaMP6f expression was seen in other organ systems as well (including the liver and hearts).

Germline recombination has been previously reported in cre transgenic lines (Steinmetz *et al.*, 2017), which lead to the theory that males carrying both transgenes may have the recombined Ai95D allele in some sperm. Therefore, breeding strategy was modified so singly transgenic males (with either Emx1-IRES-cre or Ai95D) were crossed with females carrying any combination of the genes. Following this modification, no more offspring with aberrant expression were born. To validate the transgene expression from this new breeding strategy a subset of litters had their DNA from tail-tips tested with PCR. This confirmed that animals expressing GCaMP6f in their cortex carried both Emx1-IRES-cre and Ai95D. This confirmed that visualisation of fluorescence using goggles in the newborn animals accurately identified transgene expression and was used throughout the rest of the study.
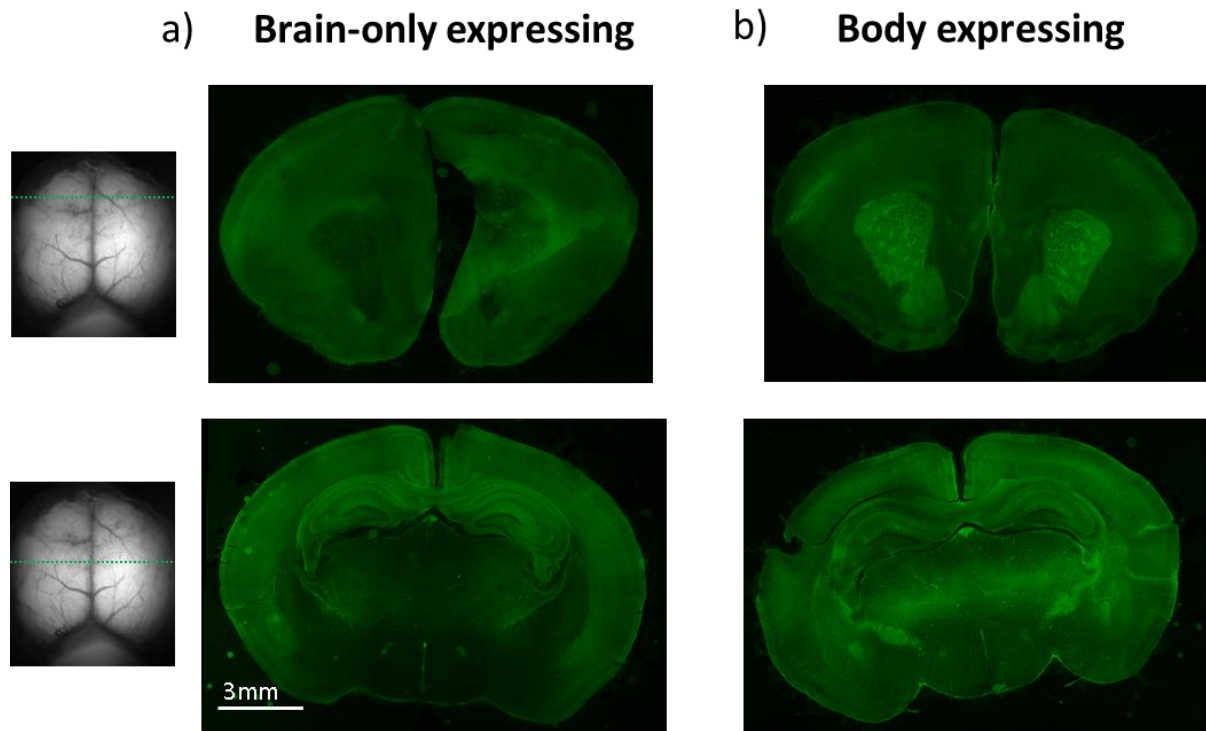
*Figure 3.4 **Expression of GCaMP6f in the brain of both on and off target transgene expression***
Coronal sections of the brain showing the expression of GCaMP6f from a P7 animal that appear to have on target cortical expression in vivo (a) and one that had off target expression across the rest of the body (b). Clear differences in expression can be seen, with a) showing cortical and hippocampal expression as expected from Emx1-Ai95D cross and b) showing additional deep grey matter expression which would not be expected from this cross.

3.3.2   Validation of movement detection

In this study, pan-cortical neuronal activity was imaged using awake neonatal Emx1-Ai95D mice using a tandem lens fluorescence macroscope. Animals were head-fixed for imaging but with free movement of the body allowed. Periods of body movement during image acquisition were recorded with a piezoelectric device placed under the animal (Figure 3.1a). When bent, such as when the animal moved, the piezoelectric device outputs a voltage, the amplitude of which reflected the size of the deflection. To identify periods of movement for use in the automated analysis protocol a threshold voltage was assigned for each recording and when the piezo recording exceeded this threshold, this point was classified as periods of movement (Figure 3.5a).

To validate whether this method captured all periods of movement reliably some sessions of simultaneous piezo and video recording of the animal's body were acquired. From this recording a timeseries of the average change in pixel intensity was extracted to give a profile of body movement visually recorded. Movement observed in the video recordings was compared with the piezo recording and were found to be well correlated (Figure 3.5b). In addition, the video was manually assessed for movement types, and this revealed that the piezoelectric recording was able to capture individual limb movements as well as gross body movements (Figure 3.5b). These results are strong evidence that the recording of movement with a piezoelectric device under the animal's body is a reliable method and hence it was used throughout this study.
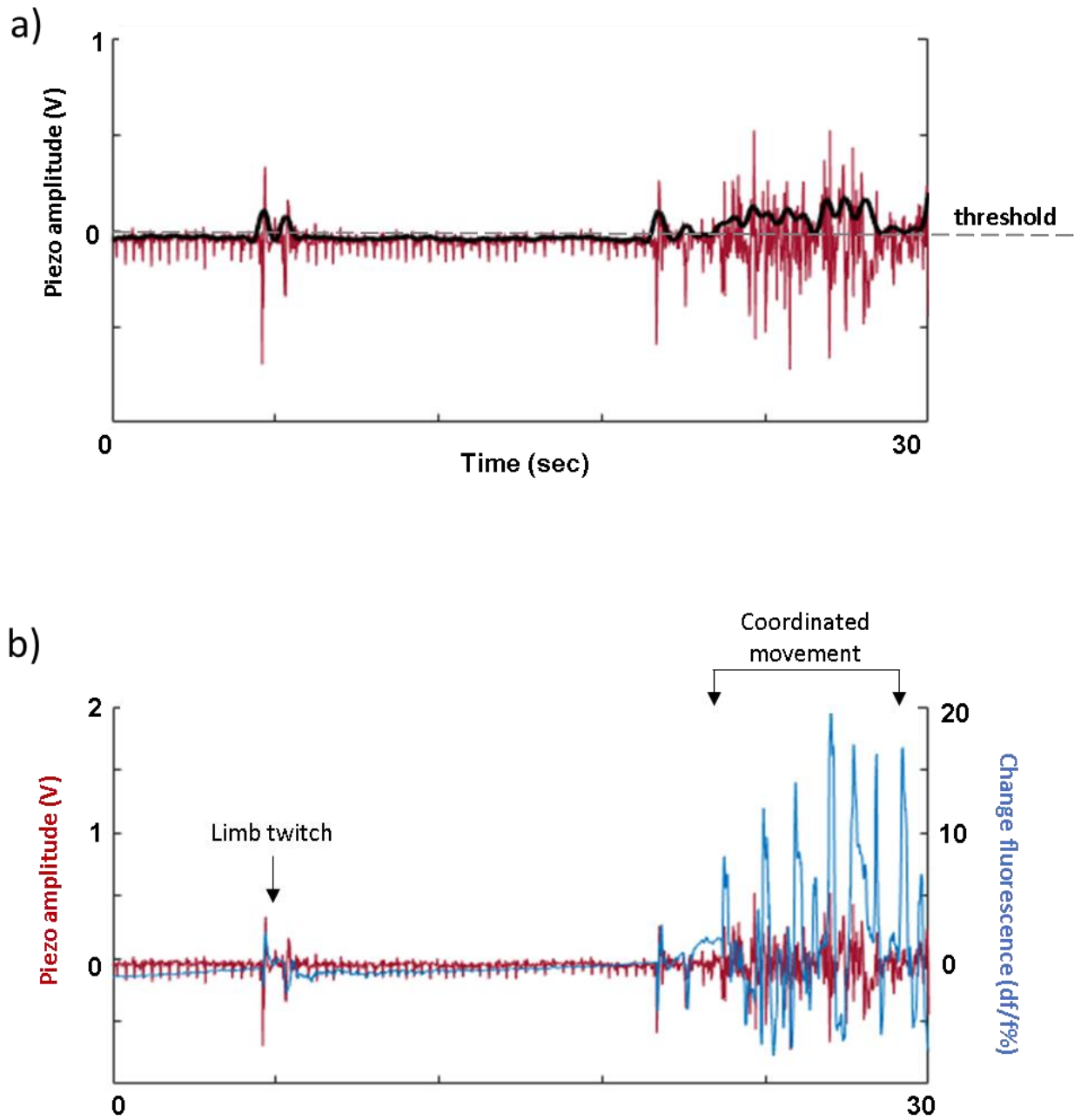
Figure 3.5 ***Validation of movement monitoring***
  a) *Example of a piezoelectric movement recording, with an automated envelope delineated over positive values (black), and the assigned threshold of movement values (grey).*
  b) *Example of piezoelectric movement recording and simultaneous video recording of pup's body (blue). Marked are visually detected periods of individual limb movement and coordinated body movement.*

Animals in these experiments showed typical neonatal rodent behaviour, with cycles of movement and rest periods (Daszuta and Gambarelli, 1985). In neonatal mice it has been reported that these behavioural changes correlate with awake/sleep cycles, where neonatal mice are asleep when they are at rest (Durand *et al.*, 2005; Balbir *et al.*, 2008). Traditionally sleep-wake cycles in experimental mammals have been measured using electromyogram (EMG) of the nuclear muscle as it was found to be well correlated to behavioural states (Pivik, Sircar and Braun, 1981; Schwartz and Kilduff, 2015). However due to the invasive nature of these recordings alternative methods of assessment in neonatal rodents were investigated. They revealed that in young animals, body movements were well correlated to the sleep-wake cycle. This study has recorded the body movements of mice during image acquisition (Figure 3.5) and so by identifying periods of movement and quiescence it is possible to have a proxy for these animals' sleep/wake cycles to compare to cortical activity.

In the developing brain movement has been shown to lead to tactile stimulation resulting in neural activity in the somatosensory cortex being generated (Khazipov *et al.*, 2004; Tiriac *et al.*, 2012; Akhmetshina *et al.*, 2016), but not in the motor cortex (Dooley and Blumberg, 2018). Activity during periods of movement were preliminarily investigated and a characteristic spatial pattern of activation was observed at all ages recorded (Figure 3.6a). From P1 to P9 activation of the body and limb somatosensory cortex was found while no activation was detected in the motor cortex.

Body movement also resulted in instability in the image capture due to movement of the brain, under the head-fixed skull. Due to the multidimensional nature of the movement, with the images coming in and out of focus in the z plane, image alignment was not possible. Because of the characteristic cortical activation patterns during movement and the contamination of the imaging plane periods of movement were separated from periods of quiescence. Movement periods were not further investigated, and periods of rest were used for the rest of the remainder of the presented data. It has previously been seen that spontaneous calcium wave activity in the developing mouse brain mostly occurs during this restful sleep-like behavioural state (Adelsberger, Garaschuk and Konnerth, 2005).

Neonatal rodents are born with limited motor skills, which develop during the first few postnatal weeks (Brust, Schindler and Lewejohann, 2015). The neonatal period investigated in this study is during this rapid acquisition of motor skills and so it is possible the movement

behaviour could be changing. Because the periods of movement are eliminated from the analysis any changes to the amount of movement could affect what is being assessed. The proportion of time spent moving during both resting-state (Figure 3.6b) and sensory stimulation recordings (Figure 3.6c) was calculated. There was no change in the amount of time spent moving with age in either condition. This ratio of movement to rest is similar to a previous report of neonatal mouse awake, head-fixed in vivo recordings (Adelsberger, Garaschuk and Konnerth, 2005).
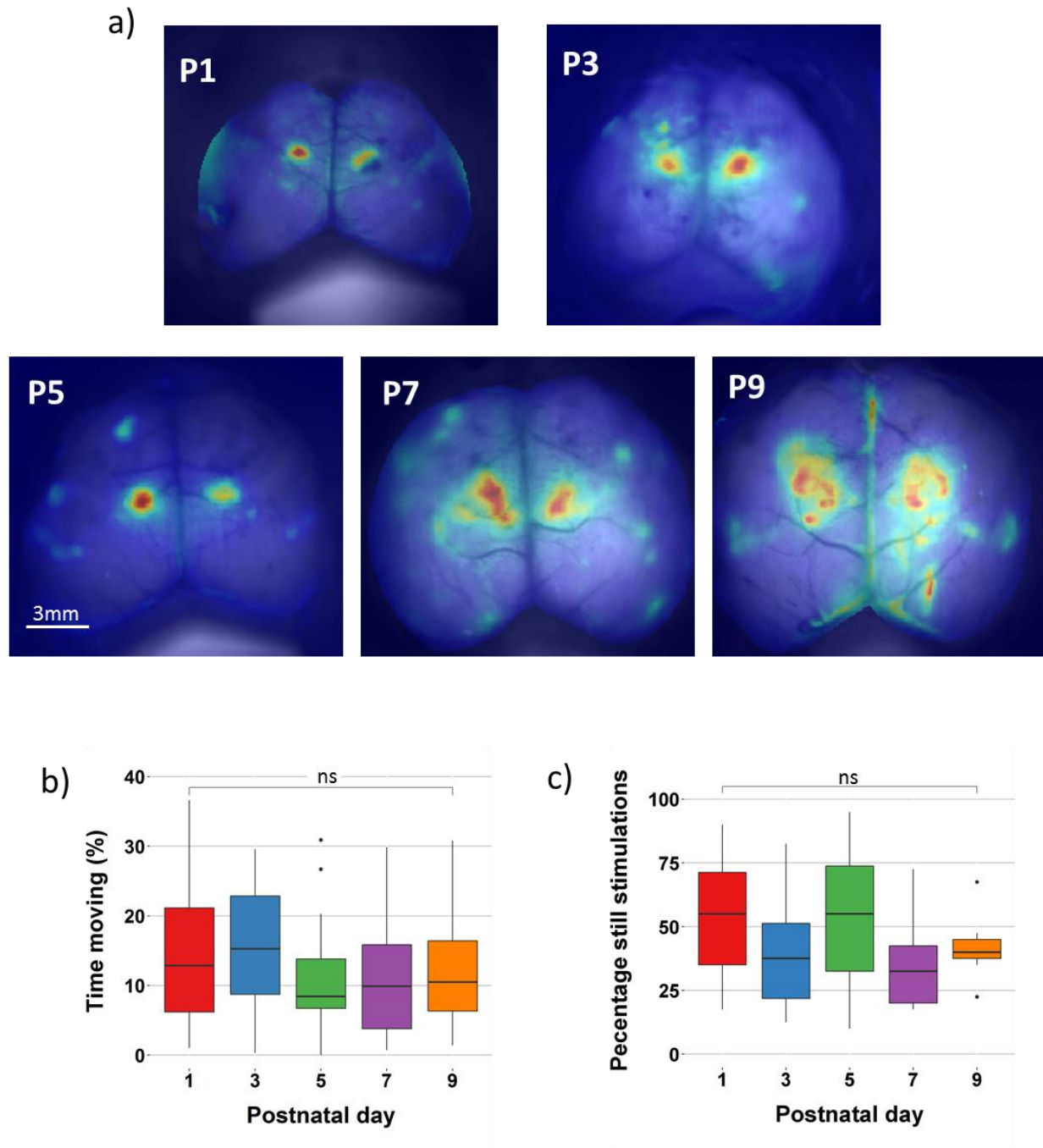
Figure 3.6 **Characterisation of movement**
a) Example of cortical activity in the 1s following a bout of active movement at all postnatal ages investigated. A characteristic activation of somatosensory cortex is seen at all ages.
The percentage of time spent moving during resting-state recordings (b) or during whisker stimulations (c) does not change with postnatal age (one-way ANOVA).

n# (pups) - [n: P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

3.3.3   Resting-state pan-cortical calcium imaging

The occurrence of spontaneous activity – no external stimulation required – in the developing neonatal cortex is well documented and is an important developmental stage occurring in the pre-sensory brain (Colonnese and Khazipov, 2012). This developmental stage has been extensively investigated in individual regions of the cortex, particularly the sensory areas but there is little research into how this activity is interacting across these cortical areas. In this study several minutes of pan-cortical calcium imaging were captured in unanaesthetised, resting neonatal mice, from P1 to P9.

Spontaneous activity was present from P1 and had complex spatiotemporal dynamics, occurring as both propagating waves and discrete regions of activity. Figure 3.8a is a time projection map of 10s of activity, showing that within this relatively small period, activity is present across the entire cortex. Initial exploration of activity identified discreet bursts confined to sensory regions, including retinal waves (Ackman, Burbridge and Crair, 2012) and activity in individual barrels of the primary somatosensory cortex (Mizuno *et al.*, 2018) (Figure 3.7a). These discrete activity bursts occurred both bi- and uni-laterally, sometimes in isolation (Figure 3.7b) and with other active regions simultaneously (Figure 3.7c), and sometimes activations of areas that are members of established networks in the mature brain were seen (Figure 3.7d).

a) — Retinal wave, Barrel activation

b)

c)

d) — Motor cortex, Somatosensory cortex, Activation of somatomotor network
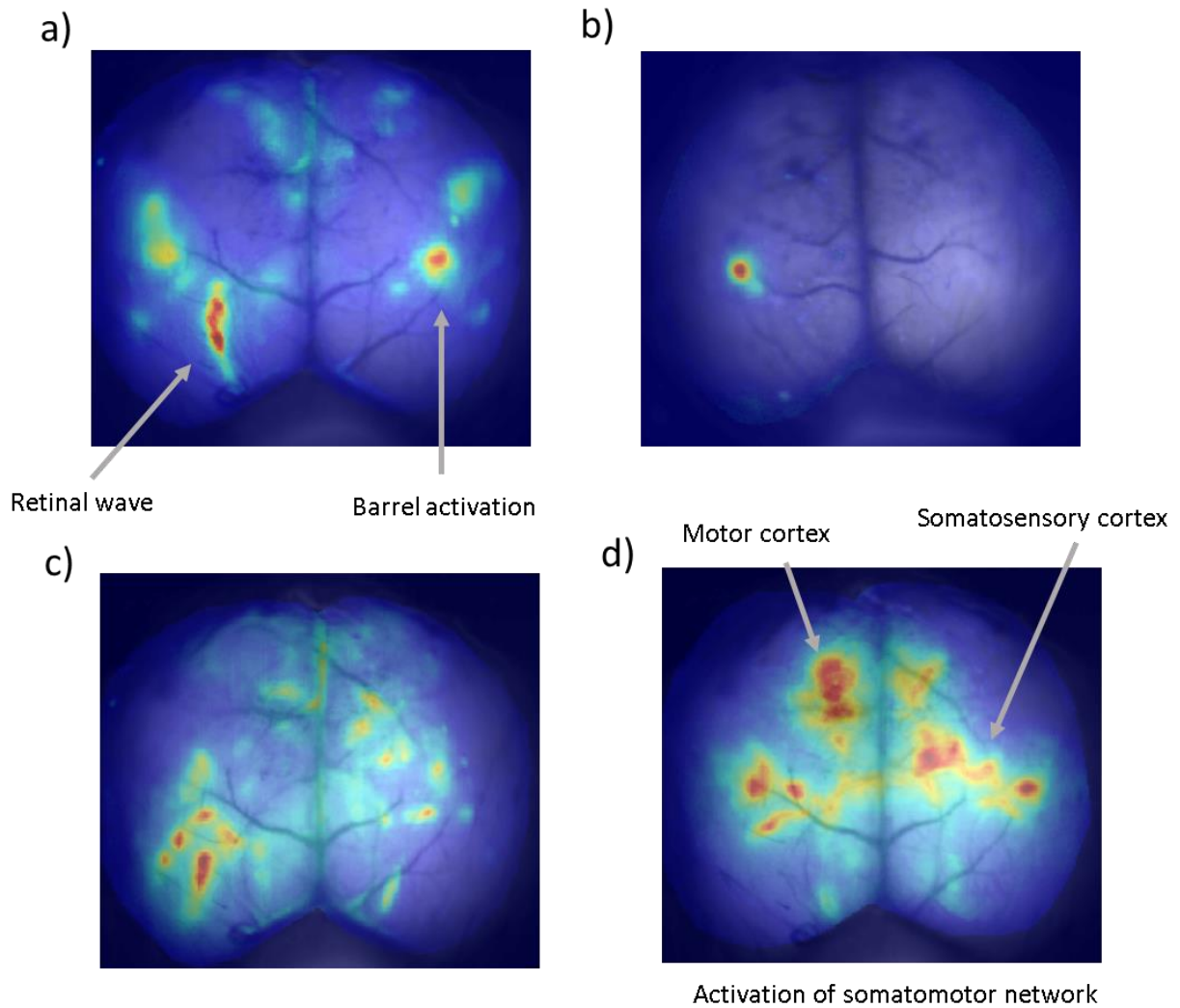
Figure 3.7 **Examples of spontaneous pan-cortical activity**
Spontaneous activity occurring in the developing cortex during periods of rest are varied. Here are examples of 1s of cortical activity. Regional events such as retinal waves and barrel bursts are seen (a). Activity sometimes occurs in a single region (b) and others a large proportion of the cortex is active simultaneously (c). Sometimes this large spread of cortical activity is coordinated amongst associated regions, such as the somatomotor network (d).

3.3.4   Pre-processing resting-state recordings

Due to this complex spatiotemporal nature of activity, the cortex was parcellated into distinct anatomical regions that allowed for the temporal properties of each to be analysed and compared. The brain is rapidly growing during the early postnatal period (Figure 3.9a) and it is likely that the boundaries of regions are changing with it. Unlike the adult mouse, there is no established atlas of the developing neonatal brain. For this study, regions of interest were defined manually for each animal using a selection of resources to identify their location. The locations of the primary sensory regions, sensory association areas and motor cortex were defined based on anatomical and functional criteria (see methods 3.2.9.2.1)(Figure 3.8b). A single pixel in the centre of each ROI was chosen and the activity of this was used as a measure of that region (Figure 3.8b).

To investigate the temporal properties of activity in these regions timeseries of fluorescence changes in these regions were calculated. These traces reveal periods of quiescence and bursts of activity can be seen, similar to the discontinuous electrical activity seen in both rodents (Colonnese and Khazipov, 2012) and humans (Lamblin *et al.*, 1999; Vanhatalo *et al.*, 2002; Kaminska *et al.*, 2017). These timeseries data were baseline corrected by normalising to the lowest5% of fluorescence intensities across the timeseries (df/f%) (Figure 3.8c) and then lowpass filtered at 1Hz (Figure 3.8d). Baseline correction aims to eliminate the effect of variability in different baseline fluorescence between animals due to variation in expression levels and brain/skull anatomy. Another common method used to calculate the baseline is the mean of the entire timeseries (Vanni *et al.*, 2017). This was not used initially due to the possibility that the amount of activity occurring would be changing with developmental age, and therefore the resulting in differing baseline values. To test the influence the chosen baseline method had on the results a post hoc Pearson's correlation coefficient was used. The comparison was made between timeseries baseline corrected using mean values versus the lowest 5% baseline traces. The average correlation coefficient across all ages was 0.996±0.003, meaning that there was little difference between these two methods of pre-processing. Temporal filtering at low frequencies has been previously used in calcium imaging studies, and calcium waves in both mature (Vanni and Murphy, 2014; Vanni *et al.*, 2017) and developing (Adelsberger, Garaschuk and Konnerth, 2005) brains have been shown to occur at low frequencies. In these data this temporal filtering process preserves

the overall shape of the trace, with periods of quiescence and bursts of activity being identifiable. In the raw trace a high frequency oscillation of around 8-10Hz was present (Figure 3.8c). This frequency is similar to the heart rate of neonatal mice (Zehendner *et al.*, 2013), and heartbeat frequency oscillations in GCaMP signals acquired at higher frequencies has previously been reported (Vanni and Murphy, 2014). The presence of this heart beat correlated oscillation in the GCaMP6f fluorescence signal is likely due to the autofluorescence of a mitochondrial flavoproteins (Masters and Chance, 1999), which are known to be a potential contaminant when imaging fluorescence in the 500–600 nm emission range. However, it has been shown to be a relatively small contributor in GCaMP imaging (Díez-García, Akemann and Knöpfel, 2007) particularly in young animals (Kozberg *et al.*, 2016), which can be seen in this data set where the high frequency oscillations have a small amplitude compared to GCaMP6f activity (Figure 3.8c).
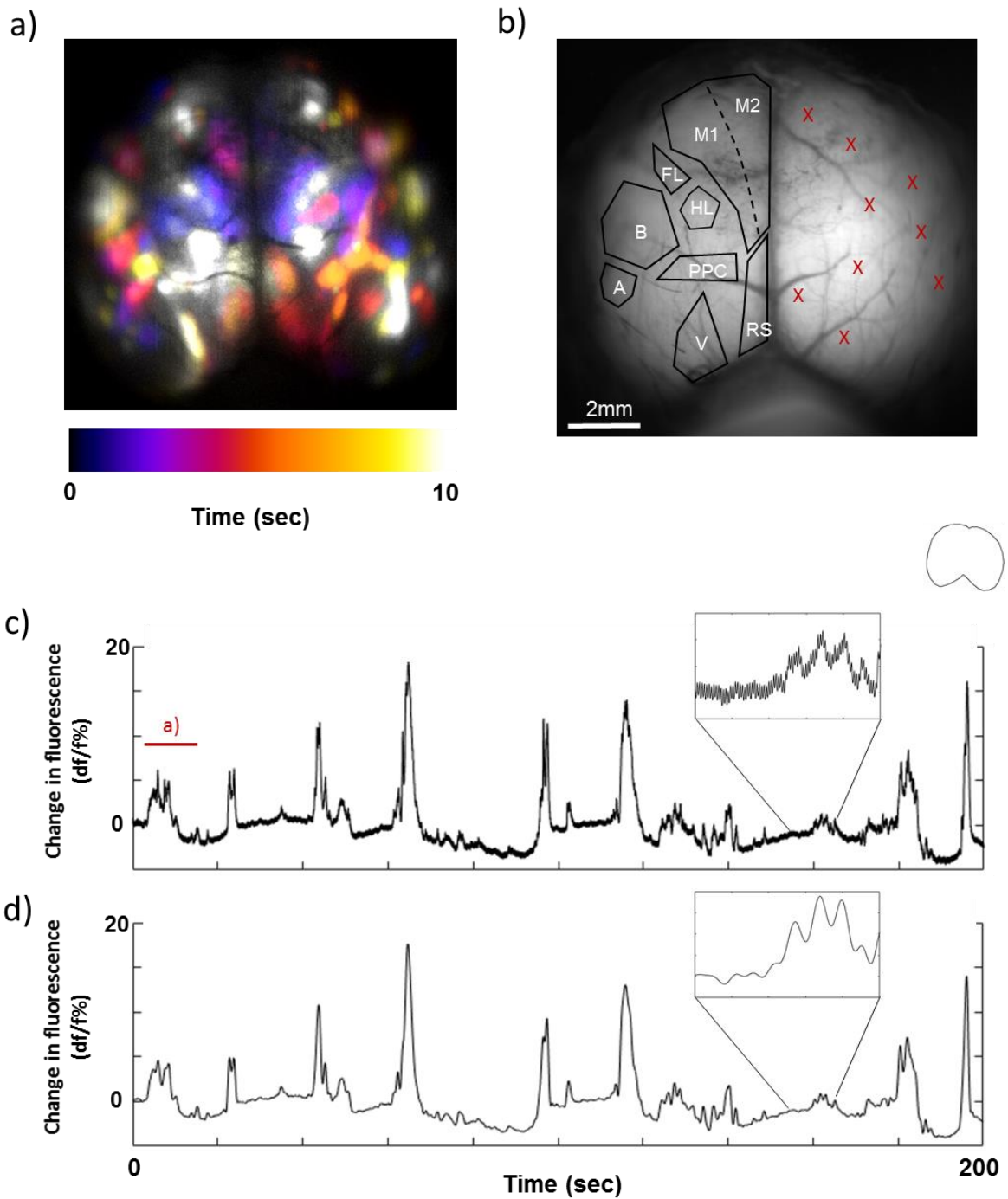
*Figure 3.8 **Pre-processing of resting-state activity***
a) Time projection map of 10s of spontaneous cortical activity.
b) example of location of cortical regions of interest in left and the regional pixel locations on the right
hemisphere (B - barrel, FL - front limb, HL - hind limb, M1 - primary motor, M2 - secondary motor, V -
primary visual, RS - retrosplenial, PPC - posterior parietal, A – auditory).
c) Example of a raw timeseries of spontaneous cortical activity, with the period of (a) marked. d) Is the
same example temporally filtered at <1Hz.

3.3.5 Stability of GCaMP6f expression across postnatal age

The neonatal ages investigated in this study (P1 and P9) are a period of rapid brain growth, which can be seen clearly from the size of the cortical surface imaged (Figure 3.9a). It is not only the size of the brain, but also the population of cells present in the cortex and their stage maturation that are changing. Emx1 is present in cortical excitatory neurons when they are born embryonically (Chan *et al.*, 2001), which means that the expression of GCaMP6f should be stably expressed in all postnatal age used in this study. Another developmental change that is occurring is the thickening of the skull. The more recent generations of GCaMP molecules are bright enough to visualise through the adult mouse skulls (Silasi *et al.*, 2016) however, changing bone thickness could affect the amount of fluorescence signal that reached the camera. Measures of both baseline and peak activity were calculated to confirm that the fluorescence signal being captured was consistent across postnatal age.

Periods of baseline quiescence were identified in resting-state recordings as the lowest 5% of pixel values in the timeseries. These were averaged across the whole cortical surface and compared between postnatal ages. This raw baseline fluorescence did not significantly change between P1 and P9 (Figure 3.9b). In these same resting-state recordings the maximum change in fluorescence from baseline was calculated and compared, and there was no significant difference across postnatal age (Figure 3.9c). These measures suggest that the signal being recorded in this study is consistent across ages.
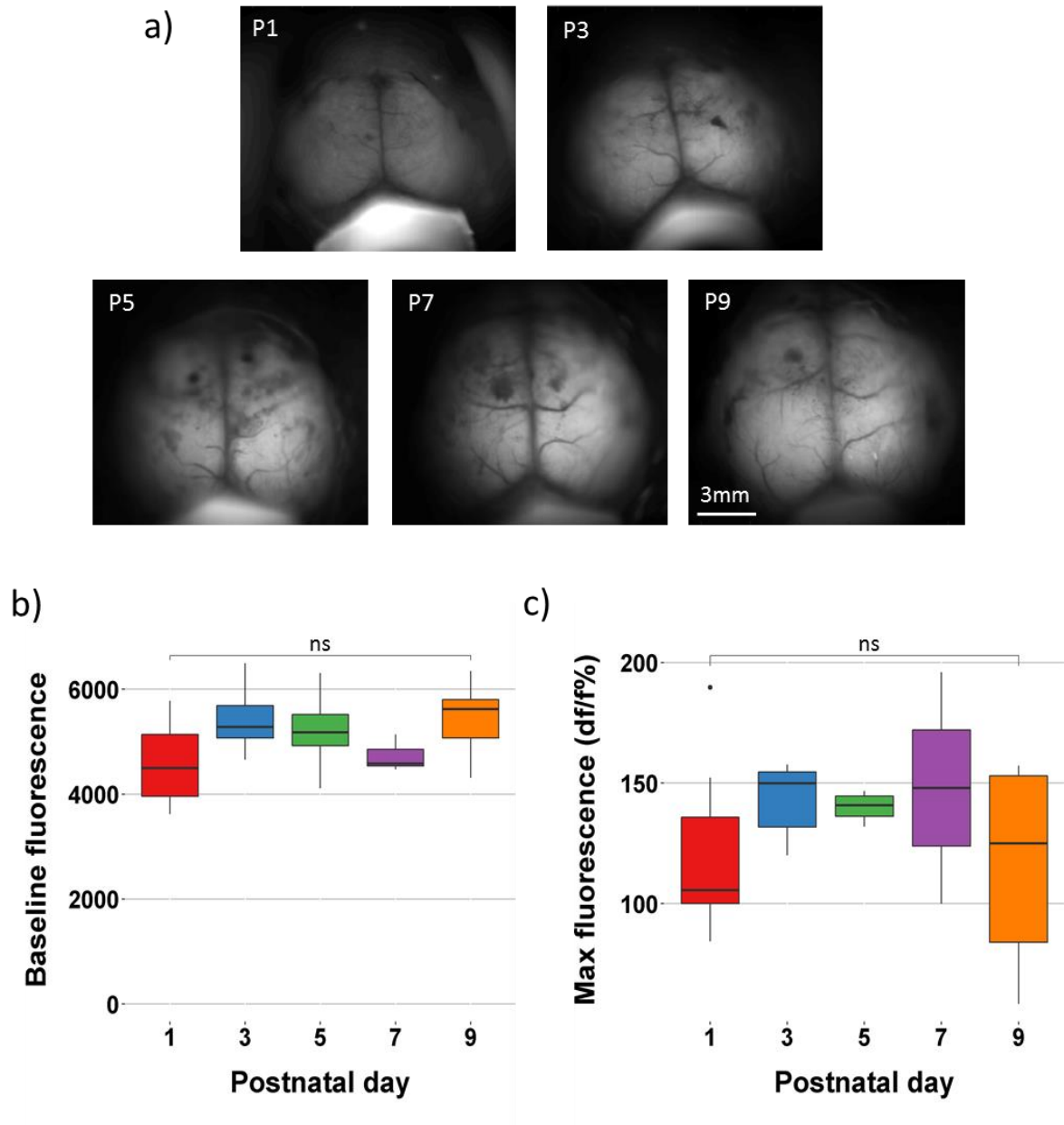
*Figure 3.9 **Stability of GCaMP6f expression across postnatal age***
The cortex increases in size during the postnatal period investigated in this study (a) (examples from each age).
The average lowest 5% of fluorescence values in resting-state recordings does not change with postnatal age (b). The maximum fluorescence change from baseline during spontaneous activity also does not change with postnatal age (c).

n# (pups) - [n: P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

3.3.6   Impact of anaesthesia on cortical activity

It has been shown that spontaneous activity across the cortex is suppressed with the administration of isoflurane anaesthesia in neonatal mice, with recovery after cessation (Adelsberger, Garaschuk and Konnerth, 2005; Hanganu, Ben-Ari and Khazipov, 2006; Ackman, Burbridge and Crair, 2012; Siegel *et al.*, 2012). As a result, a period of recovery time following anaesthesia is required before imaging to capture an accurate representation of neural activity in the developing brain. The length of time required for the full recovery of activity after anaesthesia is not known, but this is likely dependent on anaesthesia used, dose and duration. In these experiments, animals underwent approximately 7 minutes of isoflurane anaesthesia to allow skull exposure and preparation of the head-fix. To test the effect of the specific dose and duration of anaesthesia used during this head-fixation surgery, pan-cortical recordings were captured immediately following the cessation of anaesthetic in a subset of animals. The averaged timeseries of activity across the cortex was calculated (Figure 3.10a). Immediately following the cessation of anaesthesia there is no spontaneously occurring activity present in the cortex (Figure 3.10b). After approximately 2 minutes activity begins to return, with activity present across the cortex before 6 minutes post-anaesthesia (Figure 3.10b).

This quick recovery of activity informed the design of these experiments, with a 30-minute post-surgery recovery period left before the imaging protocol. To further validate this design, spontaneous imaging sessions were repeated 3 times at 30-minute intervals, and the frequency of global cortical activity in each session compared (Figure 3.10c). At P1, P3 and P5 there was a significant increase in frequency across these sessions (F = 5.25, p < 0.05 – two-way repeated measures ANOVA), and with post-hoc pair-wise comparisons this difference was found to be between the first (30-minitues post-anaesthesia) and second (60-minutes post-anaesthesia) recording session. This change in activity over session was not evident at P7 or P9. Because of this difference in the younger ages, the first recording session from all ages was excluded from the spontaneous activity analysis.

Figure 3.10 **Impact of anaesthesia on cortical activity**
Isoflurane anaesthesia supresses spontaneous activity in the neonatal cortex. a) is an example trace of cortex activity following 7 min of anaesthesia. Cortical activity immediately following the cessation is suppressed (b1). By 5 min post anaesthesia activity across the cortex has returned (b2).
Resting-state recordings were acquired at 30 min intervals (session 1-3). The frequency of the activity in these recordings significantly increases across the experimental sessions in P1-5 animals (c), but not at P7-9.

n# (pups) - [n: P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

3.3.7 Epileptiform events in GCaMP6 expressing mice

In 2017 Steinmetz *et al* identified the presence of epileptiform events in the adult cortex of multiple GCaMP6-expressing transgenic mouse lines. No epileptiform events were found in the Emx1-Ai95D cross, but to confirm this was the case in the cohort used in this study a subset of spontaneous recordings across developmental ages were analysed using the same methods as Steinmetz *et al*. Small ROIs in the frontal, somatosensory and visual cortices were defined (Figure 3.11a) and a df/f% timeseries was calculated (Figure 3.11b). From these raw traces the peaks of activity were identified using Matlab's (Mathworks, USA) 'findpeaks' function, and their height and full-width at half height were calculated and the relationship between them analysed. The original report categorises epileptiform events as large amplitudes, brief duration calcium elevations that form a distinct population. No events of this description were identified in any of the traces analysed, with a single continuous population of events found in all (Figure 3.11c).
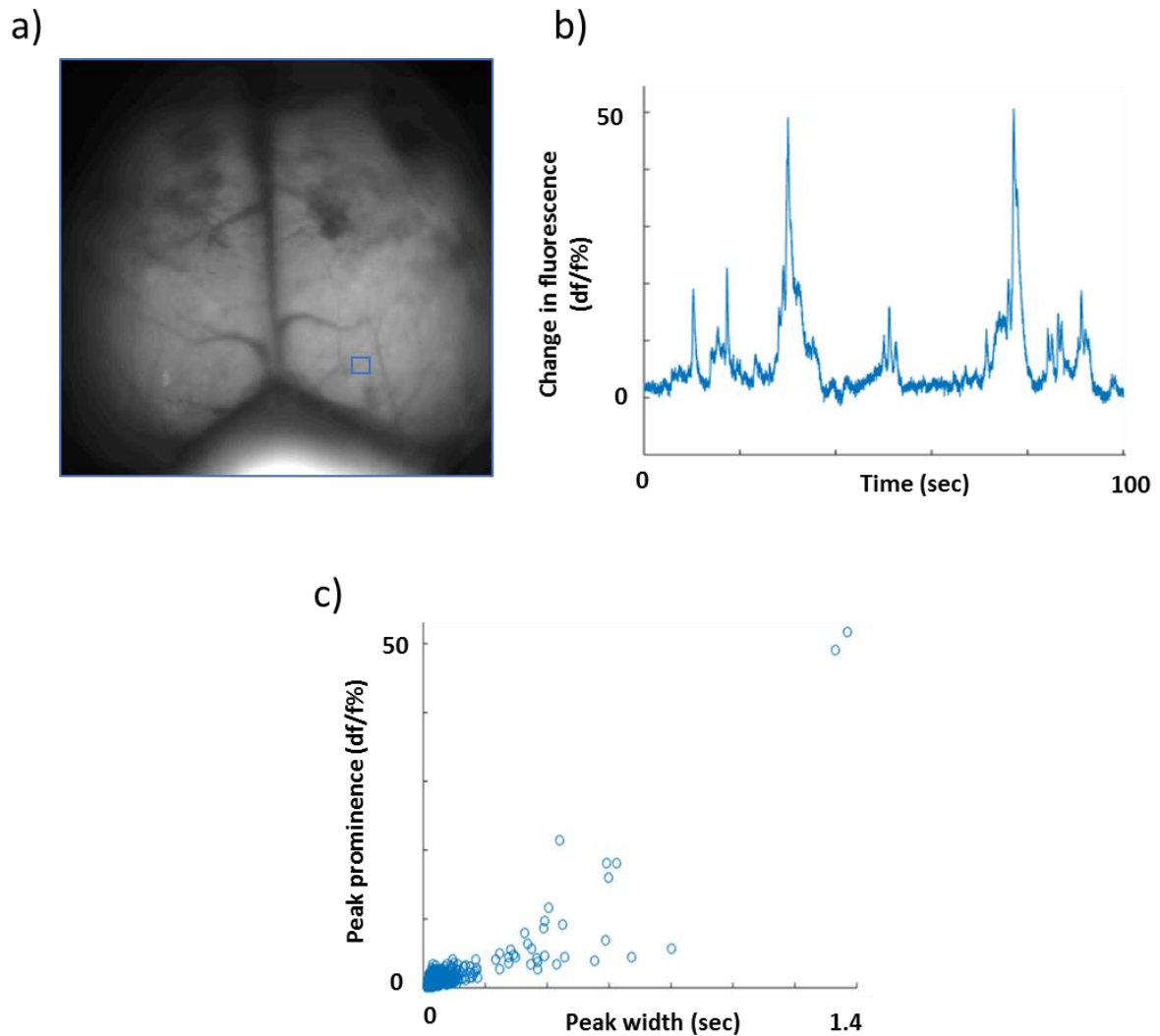
*Figure 3.11 **Identification of epileptiform events in GCaMP6 expressing mice***
The presence of epileptiform events in the developing cortex was investigated. a) Is an example of a selected ROI. b) Averaged timeseries of the ROI in (a) baseline corrected. The peaks of activity were identified and the peak prominence and the width at half-max was calculated (c). No examples of large prominence and small width which are characteristic of epileptiform events were identified in any of the recordings analysed.

3.3.8 Frequency of spontaneous activity

From pre-processed timeseries of resting-state recordings periods of activity were identified using an automated function, as follows (Figure 3.12a). The variance in signal during a 1s period of quiescence was calculated for all rest-state recordings and averaged across the population to give a value of change that was considered noise. To be defined as an event, its prominence had to exceed twice this noise level, which was 1% change in fluorescence from baseline. Events occurring during periods of movement were discarded and the frequency of activity during rest was calculated. The frequency of activity bursts across the cortex increases with postnatal age (Figure 3.12c) ($F = 17.41$, $p < 0.001$ – one-way ANOVA), and the frequency of activity in the right and left hemispheres were similar at all ages (Figure 3.12d). This activity was also investigated in individual regions, and an increase in frequency of spontaneous events was found in all cortical regions (Figure 3.13a)[a]. When the activity in each region is normalised to the global activity there is not a significant change with age (Figure 3.13b), suggesting that even though external sensory perception comes online at different ages in different modalities the spontaneous activity is developing in a more uniform, pan-cortical way.

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[a] (LB – $F = 7.302$, $p < 0.001$, RB – $F = 5.728$, $p < 0.01$, LFL – $F = 7.682$, $p < 0.001$, RFL – $F = 5.75$, $p < 0.01$, LHL – $F = 9.081$, $p < 0.001$, RHL – $F = 10.09$, $p < 0.001$, LM1 – $F = 7.233$, $p < 0.001$, RM1 – $F = 7.05$, $p < 0.001$, LM2 – $F = 10.71$, $p < 0.001$, RM2 – $F = 16.2$, $p < 0.001$, LV – $F = 16.78$, $p < 0.001$, RV – $F = 20.38$, $p < 0.001$, LRS – $F = 13.2$, $p < 0.001$, RRS – $F = 12.97$, $p < 0.001$, LPPC – $F = 24.8$, $p < 0.001$, RPPC – $F = 19.49$, $p < 0.001$, LA – $F = 16.88$, $p < 0.001$, RA – $F = 12.54$, $p < 0.001$ - one-way ANOVA).
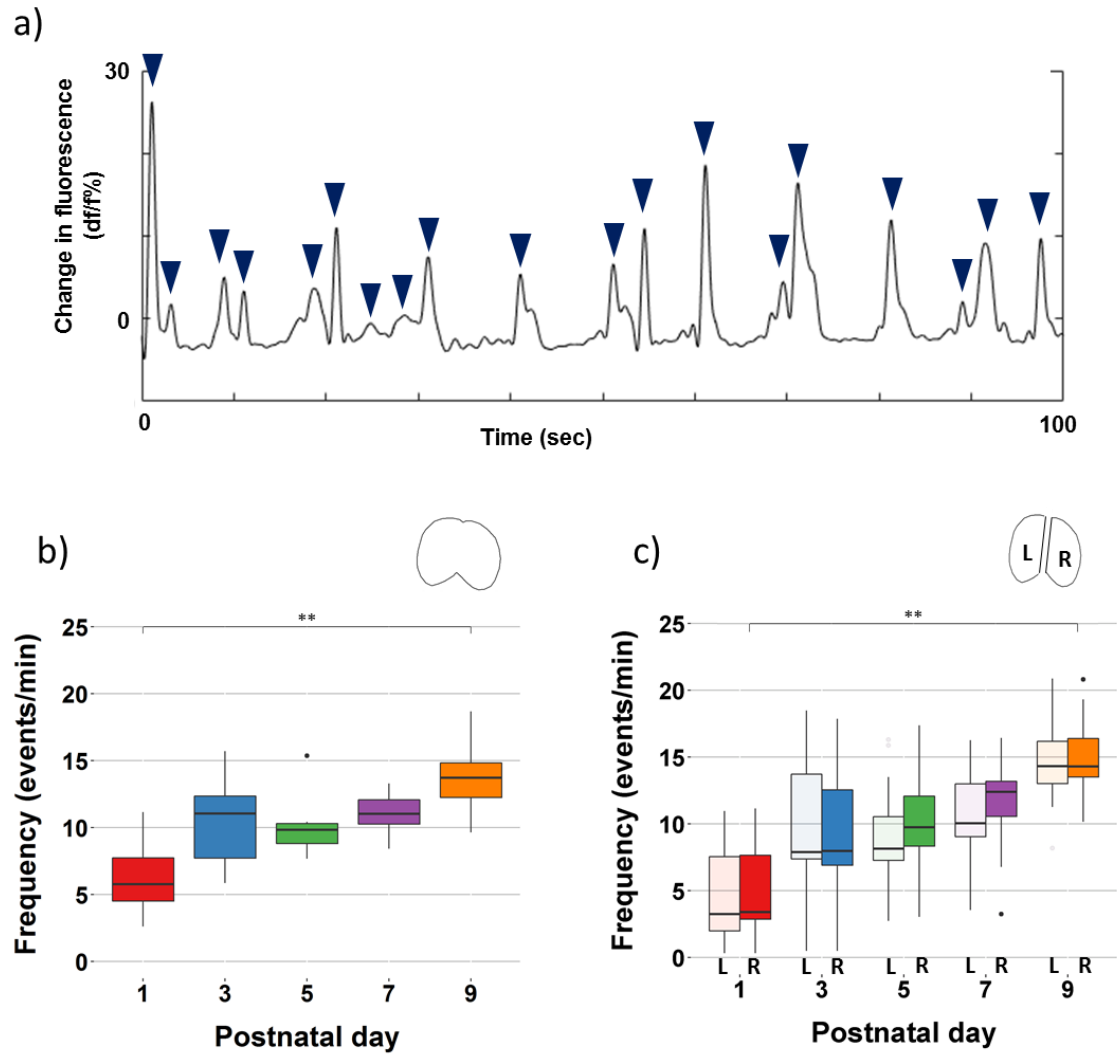
Figure 3.12 *Global frequency of spontaneous cortical activity*
a) An example pre-processed resting-state timeseries with peaks of activity above 1% prominence identified. The frequency of these activity bursts in the cortex significantly increases with postnatal age (b) (p <0.01) and this increase occurs at the same rate in both right and left hemisphere (c).

n# (pups) - [n: P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]
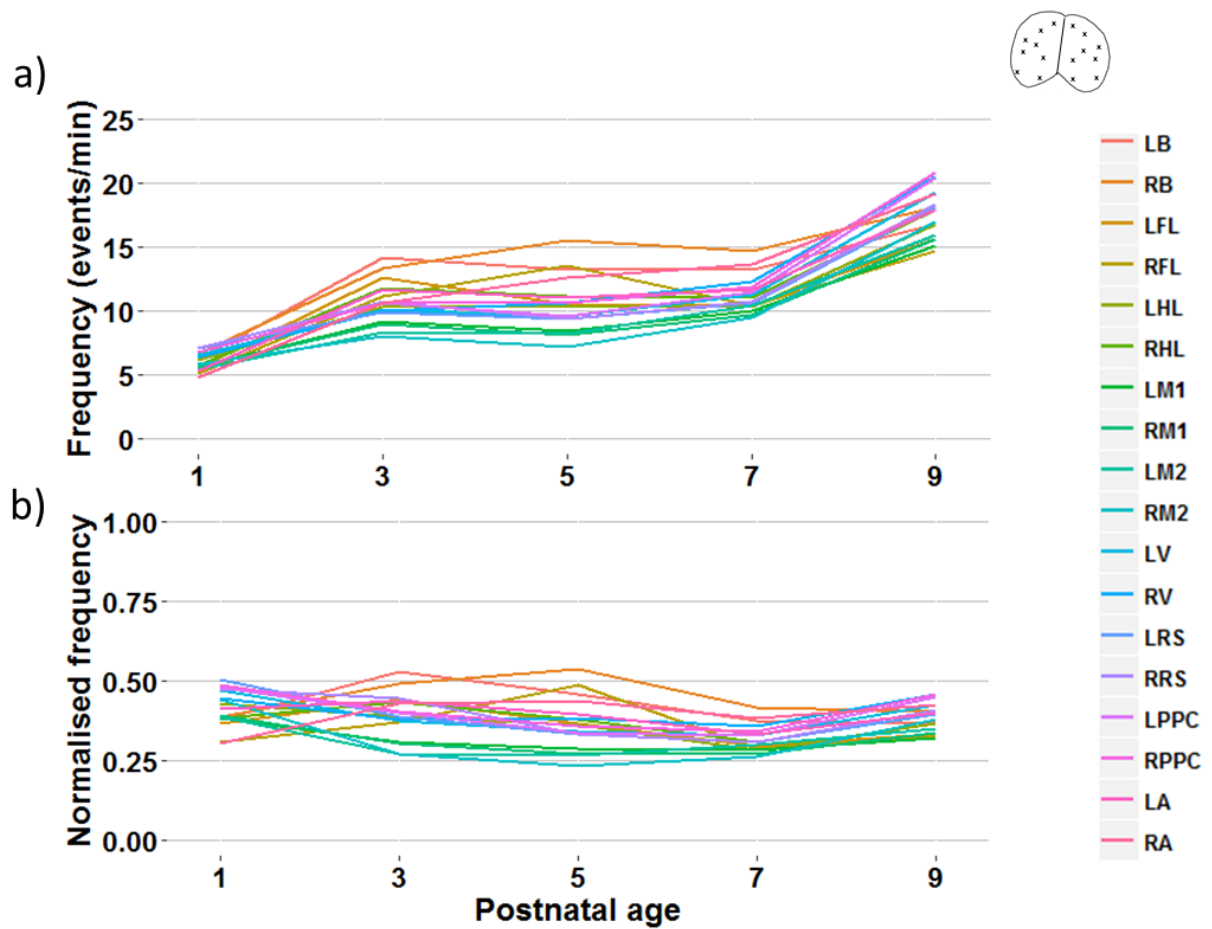
*Figure 3.13 **Regional frequency of spontaneous cortical activity***
The frequency of spontaneous activity increases with postnatal age in all cortical regions (a) (p <
0.001). When normalised to global event frequency there is no change with age (b).

n# (pups) - [n; P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

3.3.9   Coordination of spontaneous activity

The frequency of spontaneous events across cortical regions gives us some indication of the spatial properties of this endogenously generated activity. To further explore the coordination of this activity across regions, coinciding activity in different regions are often used as a measure of functional connection (van den Heuvel and Hulshoff Pol, 2010). This functional connection between regions could arise from common thalamocortical inputs or from corticocortical connections between cortical regions. Both of these pathways are maturing during the postnatal period investigated in this study and so the coordination of activity between cortical regions is possibly changing (Erzurumlu and Gaspar, 2012). It is known that the local coordination of activity is changing during this developmental period (Golshani *et al.*, 2009; Mizuno *et al.*, 2018), little is known about the global cortical functional activity.

A commonly used method of comparing the activity patterns between regions in multiple modalities (including, fMRI, EEG and calcium imaging) is Pearson's pairwise correlation (Zhang, Tian and Zhen, 2007; Mohajerani *et al.*, 2013; Rojas *et al.*, 2018), which generates a coefficient value indicating how similar the two timeseries are (Figure 3.14a). When global activity in the right and left hemispheres were compared, a decreased correlation coefficient was seen between P1 and P9 (Figure 3.14a) ($F = 23.12$, $p < 0.001$ – one-way ANOVA). The interhemispheric correlations of individual cortical regions also decreased with postnatal age (Figure 3.14b)[b].

Correlation coefficient calculations compare all the activity from a region, including the periods of quiescence. As the timeseries trace is relatively flat during this period they are counted as periods of correlated activity. Since event frequency changes dramatically over development (Figure 3.13), correlation coefficients may reflect coordination of varying amounts of quiet and active periods at different ages. Therefore, to specifically measure periods of coordinated activity, a second method of inter-region comparison was used. Specifically, the percentage of spontaneous events in the right and left hemisphere occurring within 200ms of each other were calculated. As with correlation coefficient, the percentage of coordinated activity between the hemispheres also decreased with postnatal age (Figure 3.14c) ($F = 18.07$, $p < 0.001$ – one-way ANOVA). When explored at a regional

level, most cortical areas had decreased interhemispheric coordinated activity, except for secondary motor (M2), auditory (A) and retrosplenial (RS) cortex (Figure 3.14d)[c].

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[b](B – F = 12.72, p < 0.001, FL – F = 8.6, p < 0.001, HL – F = 12.32, p < 0.001, M1 – F = 7.896, p < 0.001 , M2 – F = 7.42, p < 0.001, V – F = 9.515, p < 0.001, RS – F = 8.285, p < 0.001, PPC – F = 3.298, p < 0.05, A – F = 2.818, p < 0.05 – one-way ANOVA)

[c](B – F = 4.465, p < 0.01, FL – F = 814.52, p < 0.001, HL – F = 14.52, p < 0.001, M1 – F = 7.798, p < 0.001 , M2 – ns, V – F = 16.78, p < 0.001, RS - ns, PPC – F = 8.707, p < 0.001, A - ns – one-way ANOVA).
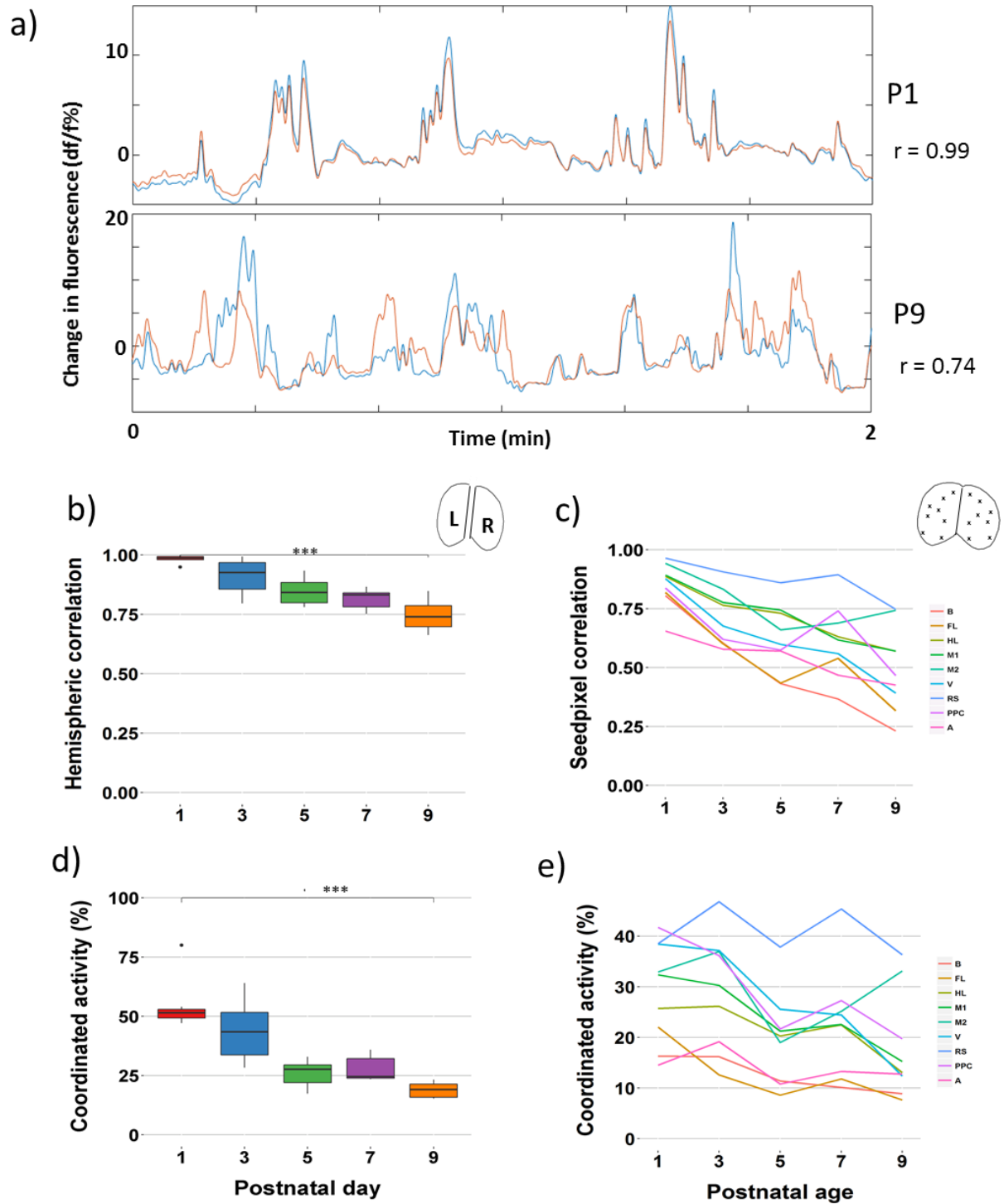
*Figure 3.14 **Hemispheric coordination of spontaneous activity***
a) Examples of right (blue) and left (orange) hemisphere timeseries at P1 and P9, and their Pearson's correlation coefficient r values. The correlation of hemispheric activity decreases with postnatal age (b) (p<0.001). This decrease in hemispheric correlation is seen across all regions (c). There is also a decrease in the percentage of activity peaks within 200ms of each other In the right and left hemisphere (d) across cortical regions investigated (e).
n# (pups) - [n; P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

Within this complex dynamic pan-cortical activity, functional networks could be emerging. These networks are not just intercortical connections, but also connections between regions within the hemisphere. In the mature cortex networks are found between somatosensory and motor regions, as well as areas of the medial-parietal cortex (Zingg *et al.*, 2014) with coordinated resting-state activity found between them (Mohajerani *et al.*, 2013; Vanni *et al.*, 2017). The emergence of these networks has been found early in postnatal development (Ackman, Zeng and Crair, 2014).

To investigate the functional connections of the data in this study Pearson's pairwise correlations were calculated between all cortical areas within and across hemispheres. These data were organised into correlation matrices with associated regions were adjacent (somatosensory, motor, and auditory cortices and medial-parietal region). This revealed organisation in spontaneous activity as early as P1 (Figure 3.15) with higher correlation coefficients within areas of the sensorimotor and the medial-parietal cortices meaning their activity is more coordinated than with other regions. These functional networks are present through until P9, with a decrease across all regions expect RS seen, but with more correlated networks remaining the most correlated at P9. In contrast in the adult mouse cortex slow-wave activity during sleep is correlated across the hemispheres with a coefficient of around 0.5, with RS cortex still being the most highly correlation region (Mohajerani *et al.*, 2010). These neonatal findings agree with previous findings of pan-cortical functional networks in the developing brain (Ackman, Zeng and Crair, 2014) The study presented here has a finer break down of postnatal age and shows that these network developments are gradually occurring.
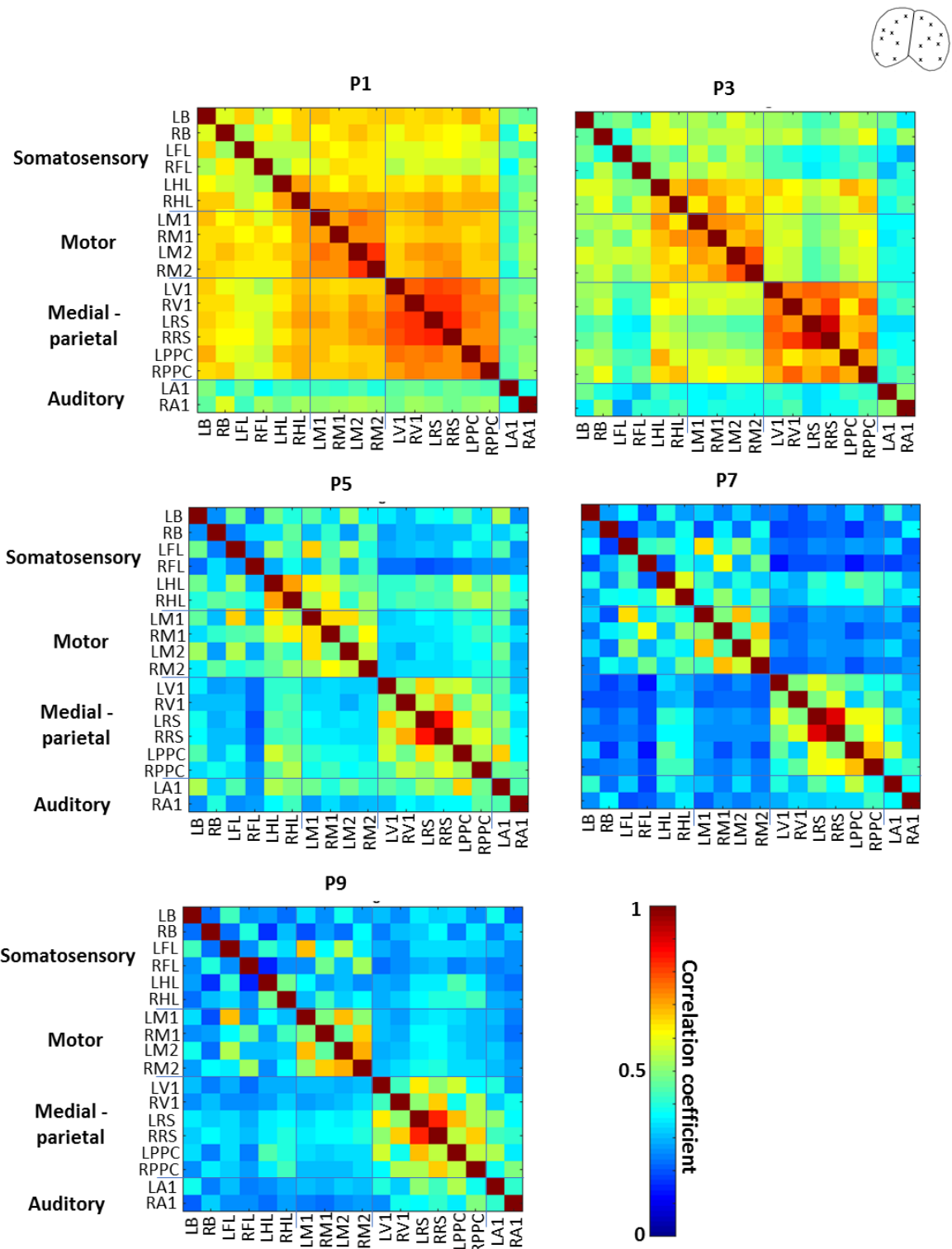
Figure 3.15 **Cortical network coordination of spontaneous activity**
Matrices of average correlation between cortical region resting-state activity from P1 to P9. (B - barrel cortex, FL - front limb, HL - hind limb, M1 - primary motor, M2 - secondary motor, V - primary visual, RS - retrosplenial, PPC - posterior parietal, A – auditory).
n# (pups) - [n; P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

3.3.10 Sensory stimulation

The endogenously generated activity so far described is known to be vital for the successful development of neural networks (Colonnese and Khazipov, 2012). This activity facilitates the organisation of the sensory cortices that allows accurate representations of incoming sensory stimulation to reach the cortex (Blankenship and Feller, 2010). Mice are born in a relatively immature state and not all sensory pathways are mature enough to communicate external stimulation at birth, but as was seen in the previous section endogenously generated activity is present in the cortex from P1. Responses to visual or auditory stimuli don't occur until the second postnatal week (Froemke and Jones, 2011; Jing Shen and Colonnese, 2016) whereas the somatosensory network matures earlier and cortical responses to tactile stimulation are present from birth (Mitrukhina *et al.*, 2015b). Once the sensory pathways do become active the incoming information facilitates further refinement of the organisation of the network to ensure a faithful representation of the external world can be processed by the animal. As with spontaneously occurring activity this is a progressive developmental process that is changing during the early postnatal weeks (Jamann, Jordan and Engelhardt, 2018).

In this study tactile stimulation was delivered during imaging with a single 30ms deflection to a portion of the right whiskers in caudal to rostral motion. This stimulation resulted in a neural response in the contralateral barrel cortex, but no other cortical areas at any postnatal ages investigated (Figure 3.16a). The average fluorescence from these regions of activation was measured for each stimulation and an average response trace for each animal calculated (Figure 3.16b). The peak amplitude of cortical response to whisker stimulation did not significantly change between P1 and P9 (Figure 3.16c). The area of activation was calculated as a proportion of the whole exposed cortical surface, and this significantly increased with age (Figure 3.16d) (F = 10.49, p < 0.001 – one-way ANOVA).
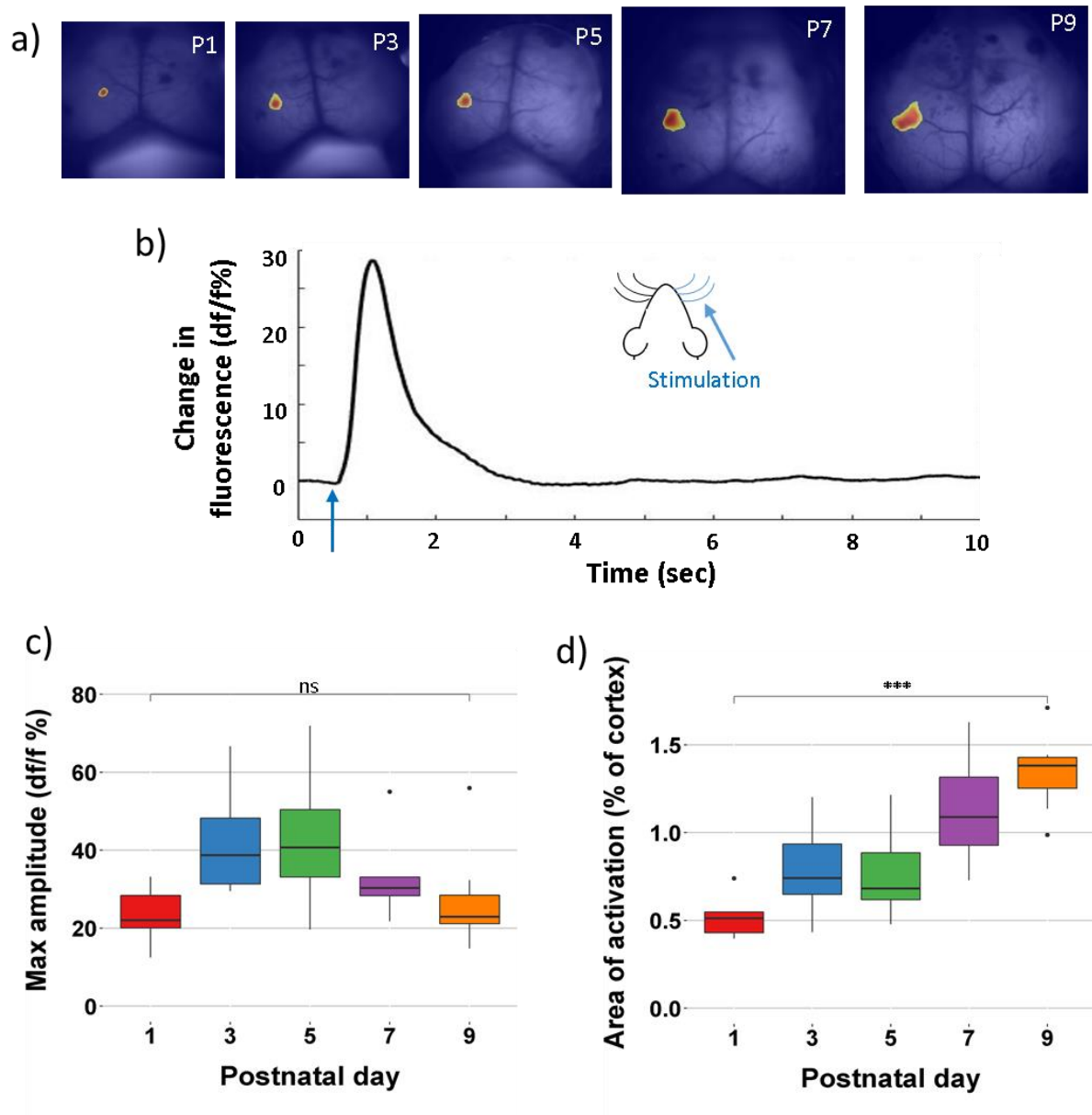
Figure 3.16 **Cortical responses to whisker field stimulation**
a) Examples of individual animals averaged cortical response to right whisker field stimulation from P1-9, and an individual animal example of the averaged timeseries response in the area of activation. The amplitude of the response to whisker field stimulation does not change with age (c), but the area of activation increased with postnatal age (d) (p<0.001).
n# (pups) - [n: P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

These data are averaged from 3 blocks of 20 stimulations for each animal. The amplitude of individual responses to repeated stimulation varied within individual animals (Figure 3.17a). Repeated stimulation can lead to receptor adaption and reduced signal (O'Mahony, 1986). The potential for this occurring was minimised by delivery only a single deflection at a time and leaving a 10s inter stimulus interval to allow recovery time. However, to confirm that desensitisation to stimulation was not occurring the amplitude variability was checked for a systematic decrease across the blocks. Between P3 and P9 there was not a significant decrease in response amplitude across stimulation trials (Figure 3.17b), however there was a decrease in cortical response with repeats at P1 ($F = 42.32$, $p < 0.001$ – one-way ANOVA).

A large variability in results can influence the averaged waveform and potential mask changes that are occurring. To investigate whether there was a change in variability that wasn't a run-down across repeated stimulations the overall variability of responses was calculated as a coefficient of variation (cv = (standard deviation/mean)*100). The variability of the amplitude of response to whisker stimulation did not change with postnatal age (Figure 3.17c).
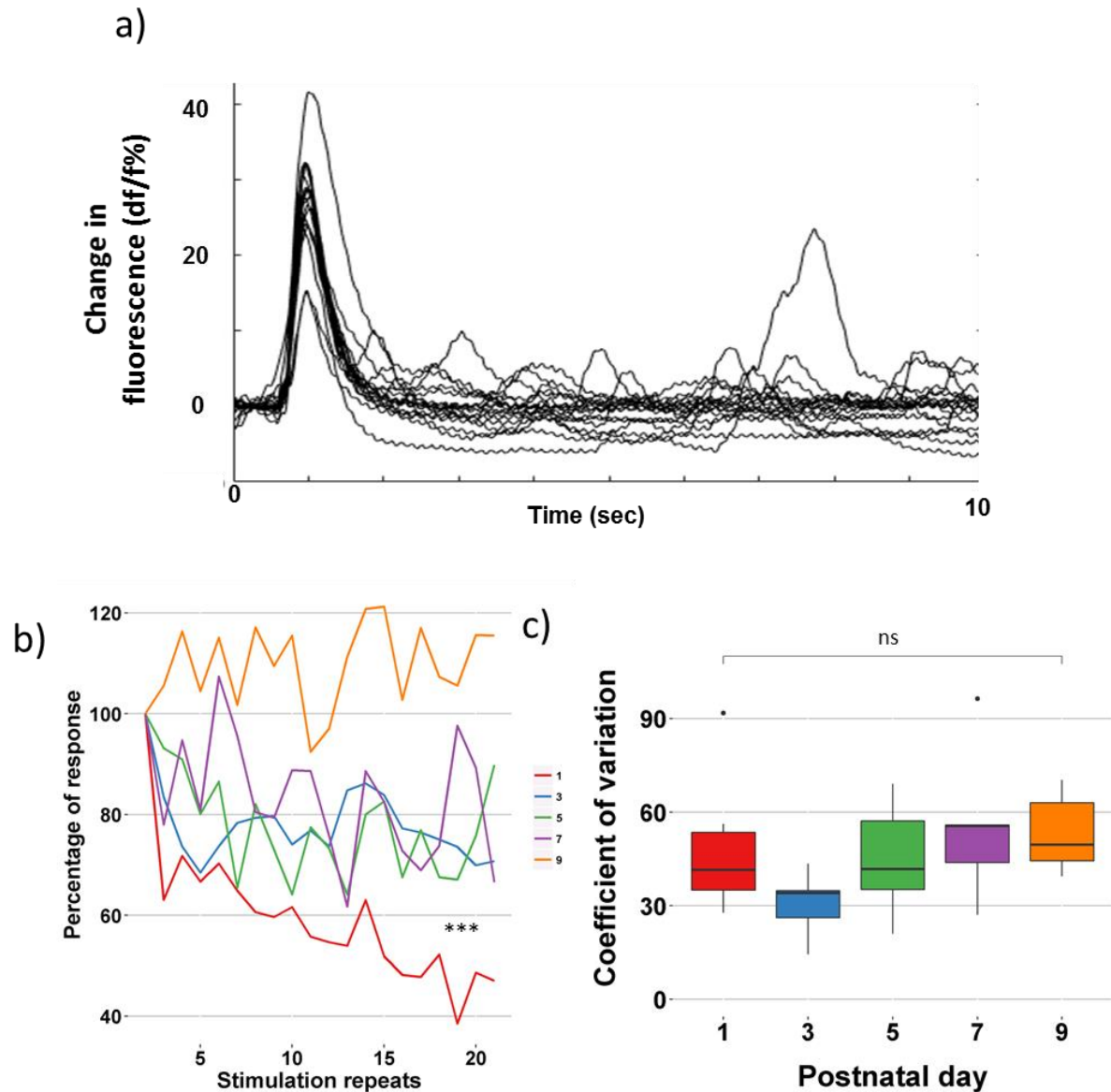
*Figure 3.17 **Variability in whisker stimulation responses***
a) Example of all 20 responses from a stimulation block, showing variability in individual trials
When compared with the first stimulation repeats did not significantly change across trials at P3 to P9
(b) but they significantly decreased at P1 (p < 0.001). The coefficient of variation of responses did not
change with postnatal age (c).
n# (pups) - [n: P1 = 7, P3 = 6, P5 = 6, P7 = 6, P9 = 7]

Although it has been shown that the primary auditory cortex does not respond to external sensory stimulation until P11 (Froemke and Jones, 2011) it is not known if there are any other areas of the cortex active earlier than this. There are cross-modal thalamocortical connections between primary sensory cortical areas and there is the possibility that activation of other areas of the cortex could occur before the primary pathway is established (Henschke *et al.*, 2015, 2017). To test this, animals were also presented with a 30ms auditory stimulation (separately at both 10kHz and 20kHz), again with a 10s ISI. These frequencies were chosen because it has previously been shown that when hearing onset emerges it is initially at high frequencies that the cortex is responsive (de Villers-Sidani *et al.*, 2007; Froemke and Jones, 2011). Unlike whisker stimulation, neither 10kHz or 20kHz auditory stimulation elicited a stereotypical response anywhere in the cortex (Figure 3.18).
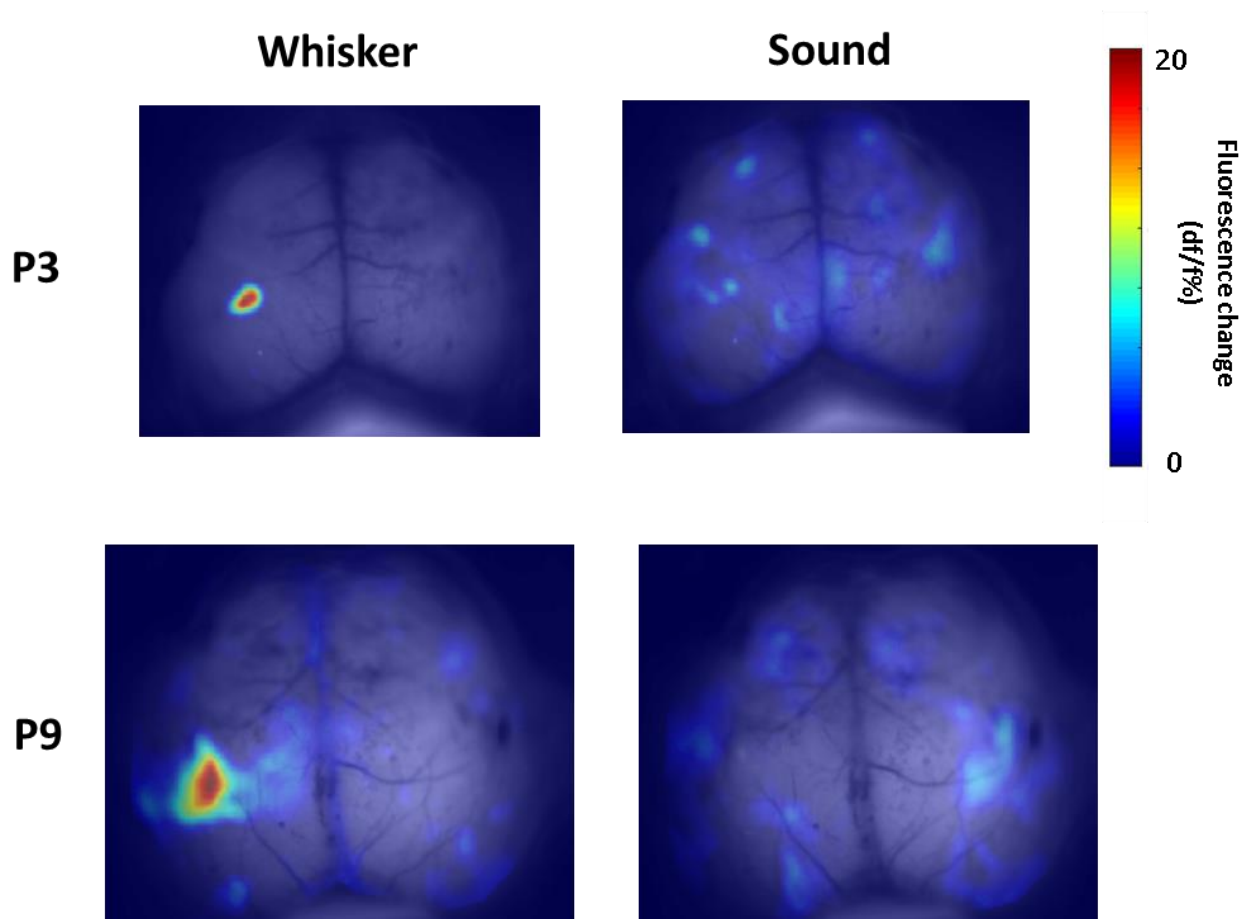
*Figure 3.18 **Comparison of somatosensory and auditory stimulation***
Examples at P3 and P9 of cortical responses to whisker and auditory stimulation. Whisker field stimulation results in a distinct activation area in the contralateral barrel cortex, whereas auditory stimulation does not stimulate activity in any cortical region.

3.3.11 Generation of spontaneous cortical activity

Early in development spontaneously occurring activity in the primary sensory cortices has been found to be driven by activity in sensory organ receptor neurons (Khazipov, Sirota, Leinekugel, Gregory L Holmes, *et al.*, 2004; Torborg and Feller, 2005; Wang *et al.*, 2015). Silencing of these peripheral regions has been found previously to alter the frequency of spontaneous activity in the somatosensory cortex. Yang *et al.*, 2009 injected lidocaine into the whisker pad whilst recorded electrical activity across the cortical layers of the barrel cortex, and found that this functional deafferentation supressed but did not eliminate (mean ~50% reduction) spindle burst and gamma oscillation activity. Mizuno *et al.*, 2018 also used lidocaine for acute peripheral silencing and found that the spontaneous calcium transients in layer IV excitatory neurons was almost eliminated. Both studies found no change to spontaneous activity frequency in the ipsilateral barrel cortex. This peripheral silencing has not been done in combination with pan-cortical activity acquisition before. The macroscopic wide-field imaging technique used in this study records the combined activity of many neurons from across the cortical layers and across regions simultaneously. In a subset of P7 animals in this study lidocaine was injected subcutaneously into the right whisker pad to silence the sensory neurons. To confirm the success of these injections whisker stimulation was delivered to the right whisker field. Before the lidocaine injection whisker stimulation resulted in activity in the contralateral barrel region of the cortex and following silencing of the periphery this response was abolished (Figure 3.19a&b). The frequency of spontaneous activity in the cortex was calculated before and after lidocaine injection. Activity in the corresponding barrel cortex was reduced, but not eliminated following lidocaine injection (p <0.05 – paired t-test) (Figure 3.19c). It should be noted that with only 3 samples this is a low powered statistical comparison which may be improved by addition of further data. In the barrel cortex ipsilateral to the injection there was no reduction in spontaneous activity (Figure 3.19d). There was also no reduction in the visual (Figure 3.19e) or retrosplenial (Figure 3.19f) cortex.
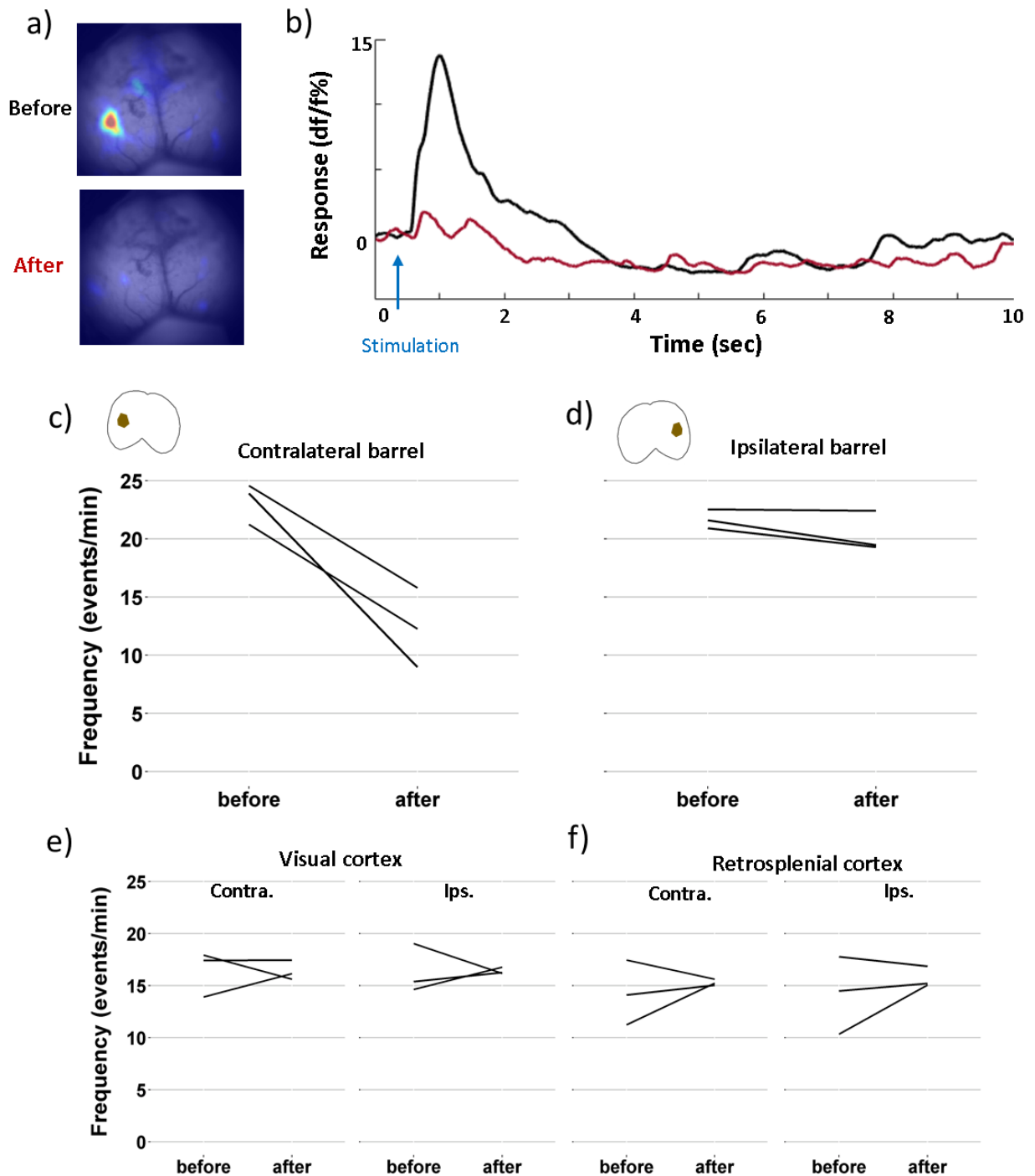
*Figure 3.19 **Generation of spontaneous cortical activity***
Lidocaine was injected into the right whisker pad. This supressed the cortical response to whisker stimulation. a) Example of cortical activity following right whisker and b) timeseries of barrel cortex activity stimulation before and after lidocaine.
The frequency of spontaneous activity in the contralateral barrel cortex decreased following lidocaine injection (c), but not in the ipsilateral barrel cortex (d), or in the visual (e) or retrosplenial (f) cortex in either hemisphere.

3.4 Introduction to neonatal sensory deprivation

Cortical activity during neonatal development is vitally important for the successful formation of mature neuronal networks (Luhmann *et al.*, 2016a). It has long been known that disruptions to this early activity affects the maturation of developing sensory networks resulting in dysfunction in the adult brain (Wiesel and Hubel, 1963; Fox, 1992). A lot of the early work on sensory deprivation was in the visual system, across a variety of mammalian species (Hubel and Wiesel, 1970; Le Vay, Wiesel and Hubel, 1980; Fagiolini *et al.*, 1994). These studies found abnormal development of the visual cortex that persisted into adulthood. These alterations include a decrease in the number of synapses (Turlejski and Kossut, 1985), reduction in the number of visually responsive cortical neurons (Hyvarinen, Hyvarinen and Linnankoski, 1981) and aberrant connections resulting in unrefined receptive fields (Callaway and Katz, 1991). More recent work has found functional activity changes such as alterations to endogenously generated cortical activity in the visual cortex (Colonnese and Khazipov, 2010) and to the functional connectivity of the cortical network (Kraft et al., 2017). It is evident that the visual network is an interesting area to explore for the effects of sensory deprivation. However, in rodents somatosensation is the dominant sense and processing of this incoming tactile stimulation is required for the animal to navigate the environment. It has been found that like the visual system alterations to whisker tactile experience in neonatal life has consequences for the development of the somatosensory network and the ability to interpret sensory information (Erzurumlu, 2010).

It has long been known that whisker damage in the early neonatal period affects the structural organisation of the barrel cortex (Weller and Johnson, 1975). Damage or removal of the whiskers means that sensory stimulated activity is not entering the developing somatosensory network. Deprivation of this whisker related sensory experience during the first few postnatal days affects the formation of the barrel cortex, but after P5 this is no longer seen (Weller and Johnson, 1975). This is because the critical period of heightened plasticity in the barrel cortex is during the first few postnatal days (M. C. Crair and Malenka, 1995) and this is the period that the thalamocortical projections are migrating to their location in layer IV of the cortex (Erzurumlu and Gaspar, 2012). Removal of the entire whisker field in early neonatal life through trimming has been found to have macroscopic

effects on the barrel cortex (Sieben *et al.*, 2015; Moreno-Juan *et al.*, 2017), whereas sensory deprivation through cauterisation of the whisker follicle have been found to disrupt the structural formation of the barrels (Kaas, Merzenich and Killackey, 1983; Suárez *et al.*, 2014). The difference in these findings is likely due to the degree of damage to the sensory neurons in the whisker pad, where trimming of the whiskers leaves them undamaged whereas cauterisation is likely causing damage to them. It is not only sensory driven activity that is important for the formation of the barrel cortex but also spontaneous generated activity (Yamada *et al.*, 2010). A large amount of this spontaneous activity is generated in the periphery (Mizuno *et al.*, 2018) and damage to the sensory neurons will disrupt this as well. This study is interested in sensory deprivation with the preservation of sensory neuron function and so will focus on sensory deprivation through whisker trimming.

Direct measures of activity in the somatosensory network following whisker deprivation have also been investigated. Changes in both spontaneously generated and sensory stimulated activity in the somatosensory cortex have been found following neonatal whisker deprivation. Increases in cortical neuron responses to whisker stimulation have been found to both bilateral (Sieben *et al.*, 2015) and unilateral (Simons and Land, 1987; Shoykhet, Land and Simons, 2005) neonatal whisker trimming. This increased responsiveness is thought to be a result of an altered excitatory inhibitory (E/I) balance, where inhibitory connectivity is decreased and excitatory is increased (Zhang *et al.*, 2013) resulting in an overall more excitable network. This increased excitability has also been seen in rest conditions with an increase in spontaneous activity in layer IV of the mature barrel cortex (Shoykhet, Land and Simons, 2005; Zhang *et al.*, 2013). As well as a disruption to the E/I balance an increased number of dendritic projections and synapses on excitatory neurons were found following whisker deprivation (Lee *et al.*, 2009; Chen, Bajnath and Brumberg, 2015; Simons, Carvell and Kyriazi, 2015). This increase in synapses may result in over-connectivity of the local excitatory neuronal circuits resulting in increased activity. Part of the developmental formation of cortical networks is an overproduction of dendritic processes that are later eliminated (Innocenti and Price, 2005), which is an activity dependent process (Segal and Andersen, 2000; Cline and Haas, 2008). As whisker trimming results in a decrease in sensory stimulated activity in the developing cortex this increased connectivity may result from a failure in this development pruning process. Indeed, it has been found that the rate of spine

elimination is reduced following whisker trimming (Zuo *et al.*, 2005). Another possible mechanism for an increased connectivity and excitability in the network following sensory deprivation is homeostatic plasticity (Turrigiano, 2012). This is a well-documented phenomena in both the mature and developing (Turrigiano and Nelson, 2004; Tien and Kerschensteiner, 2018) brain. An alteration in firing rates is compensated for - over a time scale of days (Li *et al.*, 2014; Glazewski, Greenhill and Fox, 2017) - to return the overall network activity back to its previous level. The major mechanisms for this are synaptic scaling (Turrigiano, 2008) and alterations in inhibitory tone (Li *et al.*, 2014). In sensory deprivation the reduction in input from the periphery could lead to an increase in synaptic connectivity and a decrease in inhibition to increase the overall cortical network activity. This mechanism could explain the alterations in connectivity and E/I balance previous reported following whisker trimming. As the timescale of these homeostatic mechanisms is days after the altered activity it is possible that these changes can be observed in the postnatal period during the initial period of sensory deprivation.

Somatosensory deprivation has also been found to affect the development of interhemispheric connections between the right and left barrel cortex. Unilateral whisker trimming disrupted the formation of callosal projection migration between the primary somatosensory cortices (Suárez *et al.*, 2014). Interestingly bilateral whisker trimming did not appear to affect the development of these interhemispheric pathways (Suárez *et al.*, 2014) suggesting that the balance of sensory stimulated activity in the developing network is more important than the absolute activity. It is not just altered connectivity between barrel cortices that is observed following whisker trimming. There is also increased spine density in the ipsilateral barrel following unilateral whisker trimming, which may be a result of the disrupted callosal projections previously described (Vees *et al.*, 1998). Changes to other sensory networks have also been found. Alterations to connections between sensory regions, in both intracortical and cross-modal thalamocortical connection have been found following early whisker deprivation (Nicolelis, Chapin and Lin, 1991; Sieben *et al.*, 2015; Henschke *et al.*, 2017). In addition to altered connectivity other sensory regions have been found to adapt, in what appears to be a compensatory way, such as olfactory hypersensitivity following whisker deprivation (Ye *et al.*, 2012). This compensatory adaption of other sensory modalities is seen in humans with chronic sensory deprivation such as blind

and deafness (Finney, Fine and Dobkins, 2001; Lewis, Saenz and Fine, 2010; Voss and Zatorre, 2012).

It is not just the amount of neuronal activity but also the coordination of firing amongst the neuronal population that is affected by neonatal sensory deprivation. The synchrony of activity between different cortical regions has been shown to be reduced following whisker deprivation (Sieben *et al.*, 2015). Detrimental effects on coordinated activity is also observed at a local level. Bilateral whisker trimming from birth resulted in degradation of the neuronal ensemble function, with a loss of coordinated spontaneous activity between pairs of neurons in the mature barrel cortex (Ghoshal *et al.*, 2009). Interestingly unilateral whisker trimming from birth did not result in this loss of neuronal ensemble coordination (Ghoshal *et al.*, 2014). Cortical populations of neurons in the sensory cortices have been previously shown to start highly synchronised and go through a development desynchronisation during the first few postnatal weeks as connections are refined (Golshani *et al.*, 2009). This process has been investigated following unilateral whisker trimming and it was found that this desynchronisation process in layer II/III of the developing cortex was not effected (Golshani *et al.*, 2009). Bilateral deprivation appears to disrupt correlated activity in the local neuron populations, whereas unilateral trimming has a greater impact on wider cortical connectivity (Suárez *et al.*, 2014).

Another factor that appears to influence the effects of sensory deprivation found in the cortex is the age of the animals used in the study. In adolescent mice an increase in dendritic arborisation and spine number in layer IV Spiny Stellate neurons was discovered (Lee *et al.*, 2009), but by adulthood there was a deficient in dendritic complexity and reduced spine numbers in the same cell types (Chu, Yen and Lee, 2013). It is hypothesised that the aberrant connections in adolescence are not functionally refined and eventually are lost through plasticity resulting in the decreased connectivity in adults (Chu, Yen and Lee, 2013). The development of neuronal networks is a complex series of structural and functional changes, that are precisely timed. The timing of deprivation is important, but the affects this have appear to transition through development. Few studies have investigated changes during the developmental period itself, and so little is known about the activity changes during this critical period. Investigations into these activity changes during this critical period of development could help elucidate the mechanisms behind the sensory

network alterations found in the mature brain. This study investigates the effect of unilateral whisker trimming from birth on the development of both spontaneous and sensory stimulated activity across the cortex during the first postnatal week. Unilateral whisker trimming was chosen because previous evidence has shown that asymmetrical deprivation disrupts the development of long range cortical connectivity in adulthood (Suárez *et al.*, 2014). This study aims to investigate whether there is evidence of these changes during early the postnatal period when these cortical networks are forming. Additionally, due to the immaturity of interhemispheric connections in the early postnatal brain (Wang *et al.*, 2007) unilateral deprivation may affect only the contralateral cortex, where the majority of the whisker pathway projections innervate. The ipsilateral hemisphere is therefore an interesting internal control for these experiments which would not be possible with bilateral trimming. Unilateral trimming also allows whisker stimulation of the naïve side to be carried out, allowing some investigation into the impact of sensory deprivation on the development of stimulated responses as well as spontaneous activity.

3.5 Sensory deprivation results

Pan-cortical activity was captured during resting state and sensory stimulation, at P3 and P7, in animals that had been unilaterally whisker trimmed from birth and sham controls who were litter mates that had been handled in the same way, but with their whiskers left intact.

3.5.1 Morphology and motor behaviour

Somatosensory input is important during the early neonatal period in rodents for suckling and huddling behaviours. Lesions to the infraorbital nerve, chronically removing all somatosensory input from the snout in the first postnatal week results in an inability to suckle, that is fatal (Hofer, Fisher and Shair, 1981). Just removing the sensory input of the whiskers by bilateral trimming has been found to acutely increase the latency in nipple attachment in 4 day old pups (Sullivan *et al.*, 2003). As well as feeding, neonatal rodents require huddling in a nest environment to stay warm and conserve energy (Alberts, 1978). Deficits in huddling behaviour have also been found in neonatal rodents following chronic whisker deprivation (Arakawa and Erzurumlu, 2015a). Reductions in nutrients intake and poor energy conservation from these behavioural deficient could result in a reduction in growth. Unilateral whisker trimming for the first postnatal week did not result in a change in body weight of pups (Figure 3.20a), suggesting that they were receiving adequate nutrition and care to thrive as well as sham control litter mates. In addition, brains were weighted after imaging studies at P3 and P7, and no difference was found in whisker trimmed animals compared to controls (Figure 3.20b).

Whisker trimming is known to affect the development of early life reflexive sensorimotor behaviours (Arakawa and Erzurumlu, 2015a). The time spent moving during resting-state imaging protocols (Figure 3.20c), and the percentage of whisker stimulations pups moved in response to were not different in whisker trimmed pups (Figure 3.20d), suggesting that there was no deficient in gross movement behaviour or altered sensitivity to the experimental setup. As with the previous cohort analysis was only done on imaging periods where the animals were resting, and so the same time spent moving in whisker trimmed

animals compared to controls also means the amount of data being analyses between groups is not significantly different.
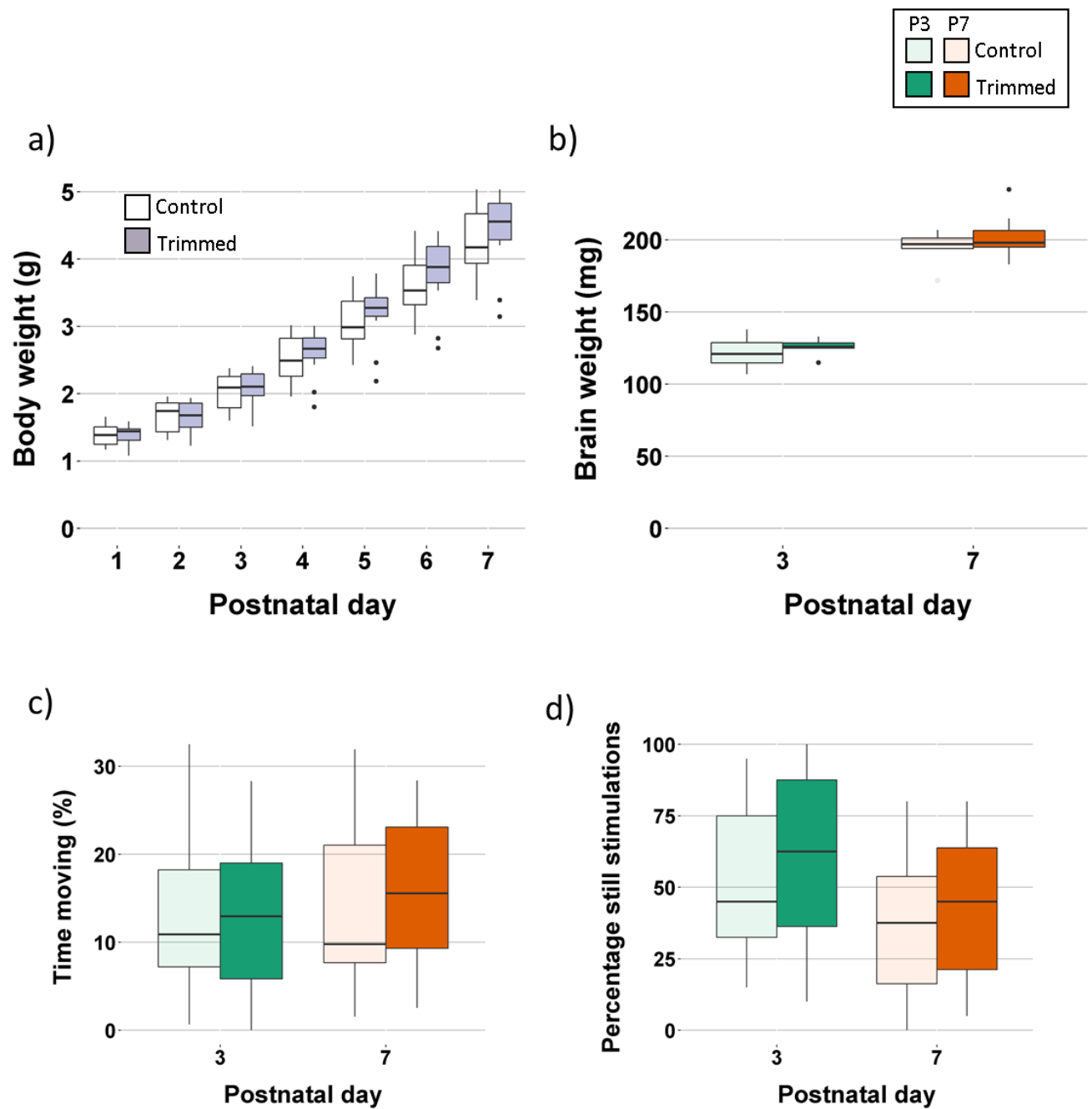
*Figure 3.20 **Changes to morphology and behaviour after sensory deprivation***
Sensory deprivation by unilateral whisker trimming did not affect the body weight of pups during the first postnatal week (a) or brain weights at P3 and P7 (b). There was also no change in the amount of movement during resting-state (c) or whisker stimulation (d) experiments.

3.5.2 Frequency of spontaneous activity

As with the previous cohort the frequency of spontaneous occurring activity across the cortex increases with postnatal age (F = 14.203, p <0.001 – two-way ANOVA (Figure 3.21a). This global frequency of spontaneous activity was not affected by whisker trimming at either age. The contralateral barrel cortex is the primary area connected to the whisker and so is an area of interest following unilateral whisker stimulation. It has previously been shown that whisker trimming results in an increased spontaneous firing in individual neurons in this area (Shoykhet, Land and Simons, 2005). However, the average spontaneous neuronal activity in the right barrel cortex (contralateral to trimming) was not significantly different than control animals (Figure 3.21b), with both groups having an increased frequency of events between P3 and P7 (F = 9.848, p <0.01 – two-way ANOVA).

Cross-modal modulation has been seen from uni-sensory deprivation and so the spontaneously occurring activity across the cortical regions was investigated (Sieben *et al.*, 2015; Henschke *et al.*, 2017). Pixels in the centre of the major cortical regions were selected as in the previous cohort (Figure 3.8b) and spontaneous events in these regions identified across the timeseries. There is again an increase in frequency of activity between P3 and P7 in all regions, with no significant difference between trimmed and control animals (Figure 3.21c).
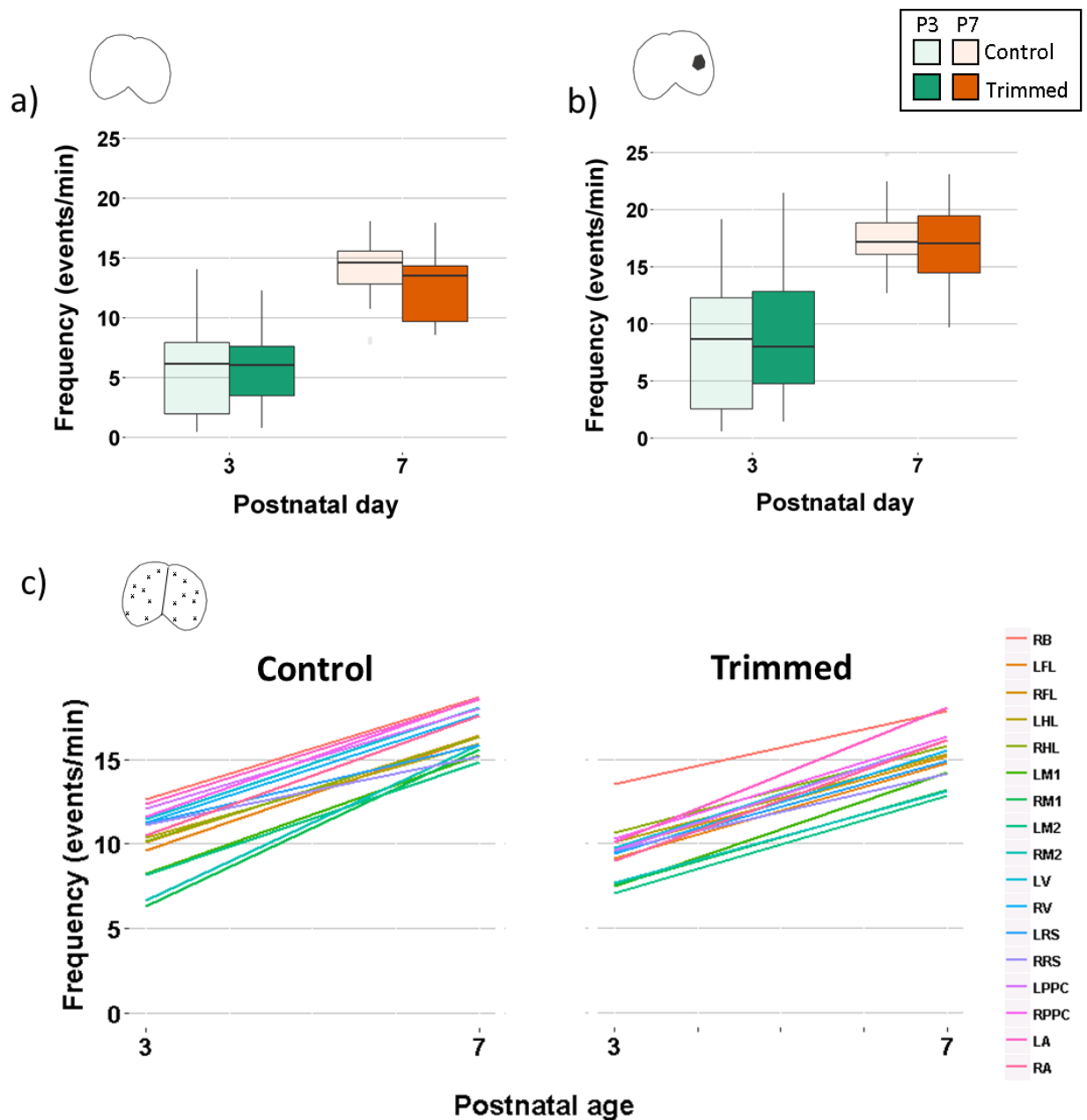
*Figure 3.21 **Frequency of spontaneous cortical activity after sensory deprivation***
The frequency of cortical spontaneous activity is not significantly different between trimmed and control animals, with both groups increasing in frequency between P3 and P7 (a). The frequency of activity in the barrel cortex contralateral to trimming is not significantly different between groups and again increase with postnatal age (b). This increase in activity with age is seen across cortical regions and is not different between trimmed and control animals (c)
[n: P3-trimmed = 7, P3-not = 5, P7-trimmed = 7, P7-not = 7]

3.5.3 Coordination of spontaneous activity

Intra-hemispheric connectivity is developing during this period of sensory deprivation (Erzurumlu and Gaspar, 2012). The spontaneous activity in the developing cortex is coordinated to give patterned activation which plays an important role in the organisation of the developing sensory networks (Kirkby *et al.*, 2013). Disruption to early sensory input has been shown to affect the coordination of local networks of neurons (Ashby and Isaac, 2011) as well as the correlation of interhemispheric activity between right and left sensory regions (C.-L. Wang *et al.*, 2007). The inter- and intra-hemispheric coordination of resting-state activity was investigated as in the previous cohort.

Whisker related sensory deprivation means that the barrel cortex is of particular interest. The Pearson's correlation coefficient between the average activity of the right and left barrel cortex was not significantly different in trimmed animals compared to controls at either P3 or P7 (Figure 3.22a). As with the previous cohort the correlation of activity significantly decreased with postnatal age (F = 28.956, P < 0.001 - two-way ANOVA). Coordinated activity bursts were also investigated, as previously described. There was no difference in the percentage of peaks in the right and left barrel cortex that occurred within 200ms of each other in trimmed animal compared to controls (Figure 3.22b), and in both groups this coordinated activity decreased with postnatal age (F = 5.281, P < 0.05 – two-way ANOVA). Because of the cross-modal connections in the developing brain sensory deprivation to one region could have effects in other sensory areas. The interhemispheric activity of all cortical regions previously defined were compared by Pearson's correlation and there was no significant different between trimmed and control animals (Figure 3.22c).

As has previously been discussed cortico-cortical connections are also within hemispheric regions as well as across. The functional connectivity between all defined cortical regions was calculated by correlation coefficient and collated in a matrix, with associated regions adjacent (somatosensory, motor, medial-parietal and auditory cortices) (Figure 3.23). The pattern of functional connectivity is similar to the previous cohort (Figure 3.15), and between trimmed and control animals. Unilateral whisker trimming does not appear to disrupt the spontaneous functional connectivity of the cortex during the first postnatal week of development.
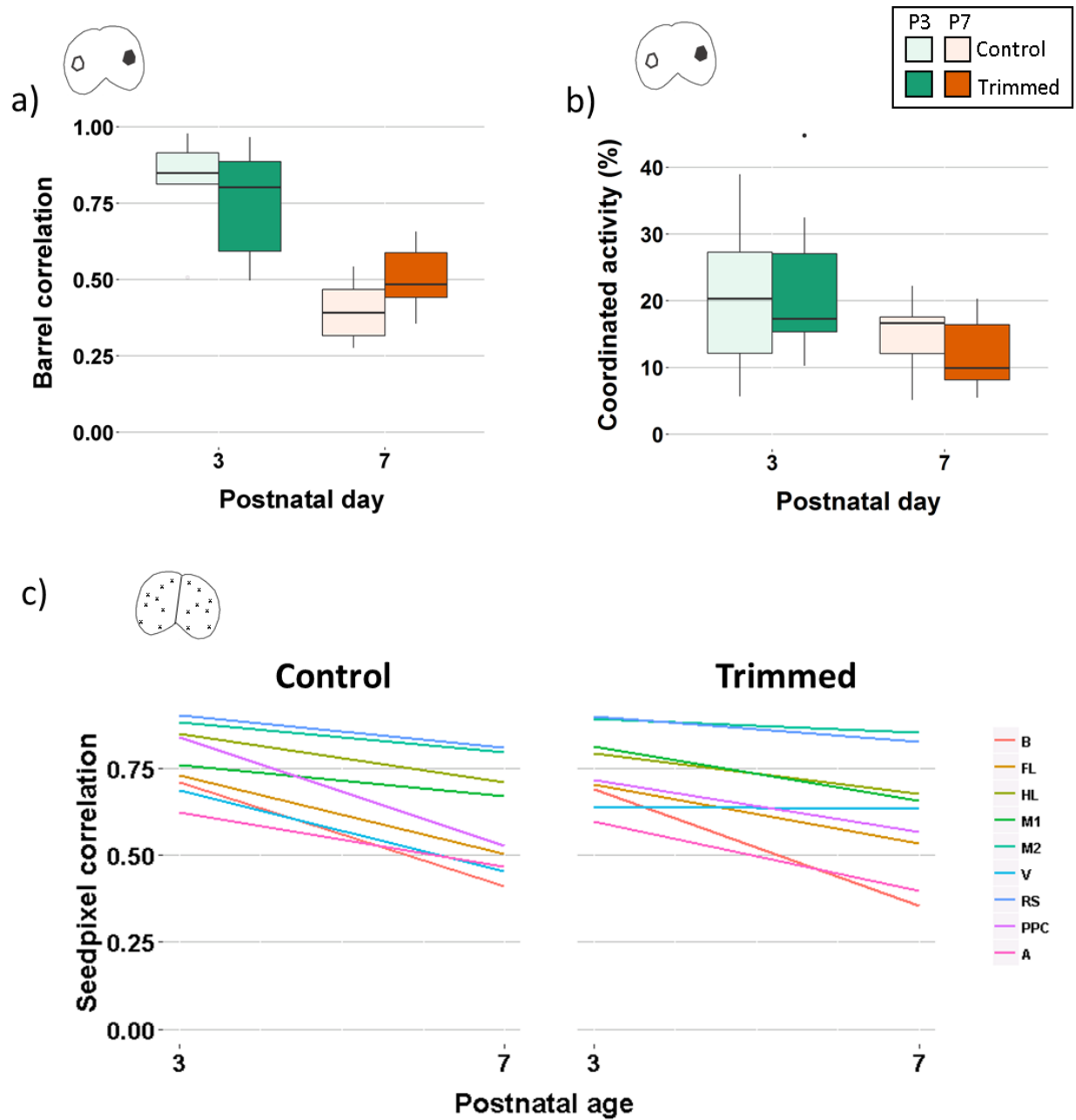
*Figure 3.22 **Coordination of spontaneous cortical activity after sensory deprivation***
There is no difference in the correlation of right and left barrel spontaneous activity in trimmed versus control animals at either P3 or P7, or in the percentage of coordinated activity bursts (b). The correlation of activity decreases across the cortex, with no difference between trimmed and control animals (c).
[n: P3-trimmed = 7, P3-not = 5, P7-trimmed = 7, P7-not = 7]
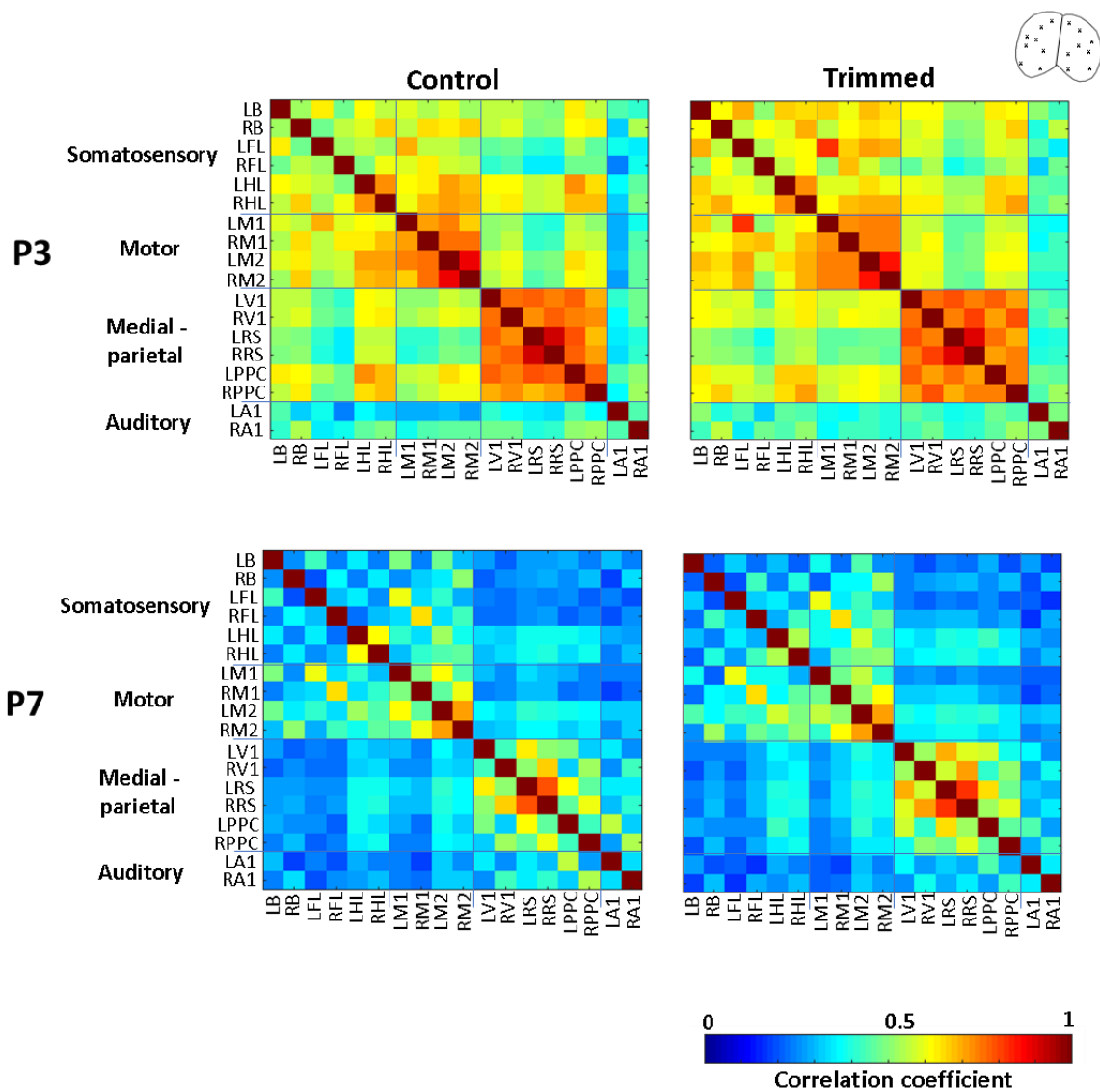
Figure 3.23 **Cortical network coordination of spontaneous activity after sensory deprivation**
The matrices of average correlation between cortical region resting-state activity at P3 and P7 appears to be the same in trimmed and control animals.
(B - barrel, FL - front limb, HL - hind limb, M1 - primary motor, M2 - secondary motor, V - primary visual, RS - retrosplenial, PPC - posterior parietal, A – auditory). [n: P3-trimmed = 7, P3-not = 5, P7-trimmed = 7, P7-not = 7]

3.5.4   Sensory stimulation

Whiskers were trimmed on the left side from the day of birth until the day of experiments in these animals and so stimulation of the trimmed whisker was not possible as no regrowth period occurred. To investigate whether deprivation of tactile sensation in the left whisker field affected the sensory process in the opposite side, spared whiskers on the right side were stimulated. As with the previous cohort stimulation of the right whisker field with a single 30s rostral to caudal deflection results in activation of the contralateral barrel cortex (Figure 3.24a). The amplitude of this response does not significantly change between P3 and P7, or between trimmed or control animals (Figure 3.24b). The size of the response area increased with age in control animals, as was observed in the previous cohort ($p < 0.05$ - Bonferroni corrected pair-wise comparison), whereas there was not a significant increase between P3 and P7 in the trimmed cohort. This means that at P7 the area of activation is significantly smaller after sensory deprivation when compared to controls ($p<0.05$ - Bonferroni corrected pair-wise comparison) (Figure 3.24c).

Across the postnatal ages investigated in this study the animals' whiskers are growing and changing orientation to the face. The stimulation of multiple whiskers was done with a small plastic paddle and was positioned by eye to catch as many whiskers as possible without touching the face, or the whiskers when in rest position. Because of this design and the changing whisker anatomy between ages it is possible that the stimulation was not consistent. Therefore, this cohort also received stimulation of individual whiskers (D1, E1 and delta) as a more controlled stimulation. A single 100ms whisker deflection resulted in activation of the contralateral barrel cortex at all ages investigated (Figure 3.25a), in distinct locations that agreed with the topographical conservation of axon projections from the whiskers to barrel cortex (Figure 3.25b). The average amplitude of the response to single whisker stimulation significantly increased between P3 and P7 ($F = 71.933$, $p < 0.001$ – two-way repeated measures ANOVA) but there was no difference between trimmed and control animals (Figure 3.25c). The area of activation also increases with postnatal age ($F = 13.843$, $p < 0.01$ – two-way repeated measures ANOVA), again with no significant difference between groups (Figure 3.25d).
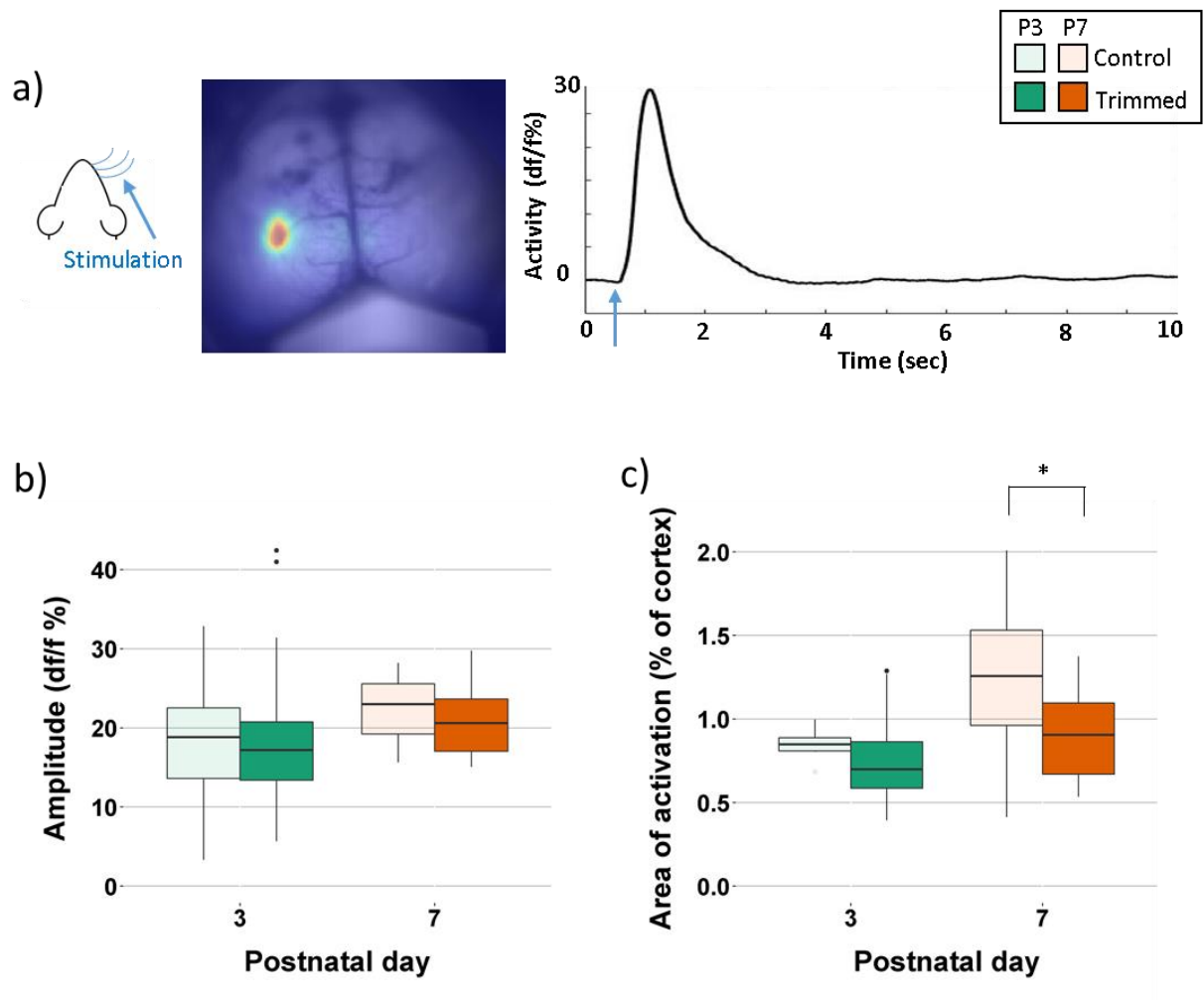
*Figure 3.24 **Cortical responses to whisker field stimulation after sensory deprivation***
a) An example of an individual animal's averaged cortical response to right whisker field stimulation, and an individual animal example of the averaged timeseries response in the area of activation.
The amplitude of the response to whisker field stimulation does not change with age or between trimmed and control animals (b). However, the area of activation is significantly smaller in trimmed animals at P7 (c) (p<0.05).
[n: P3-trimmed = 7, P3-not = 5, P7-trimmed = 7, P7-not = 7]
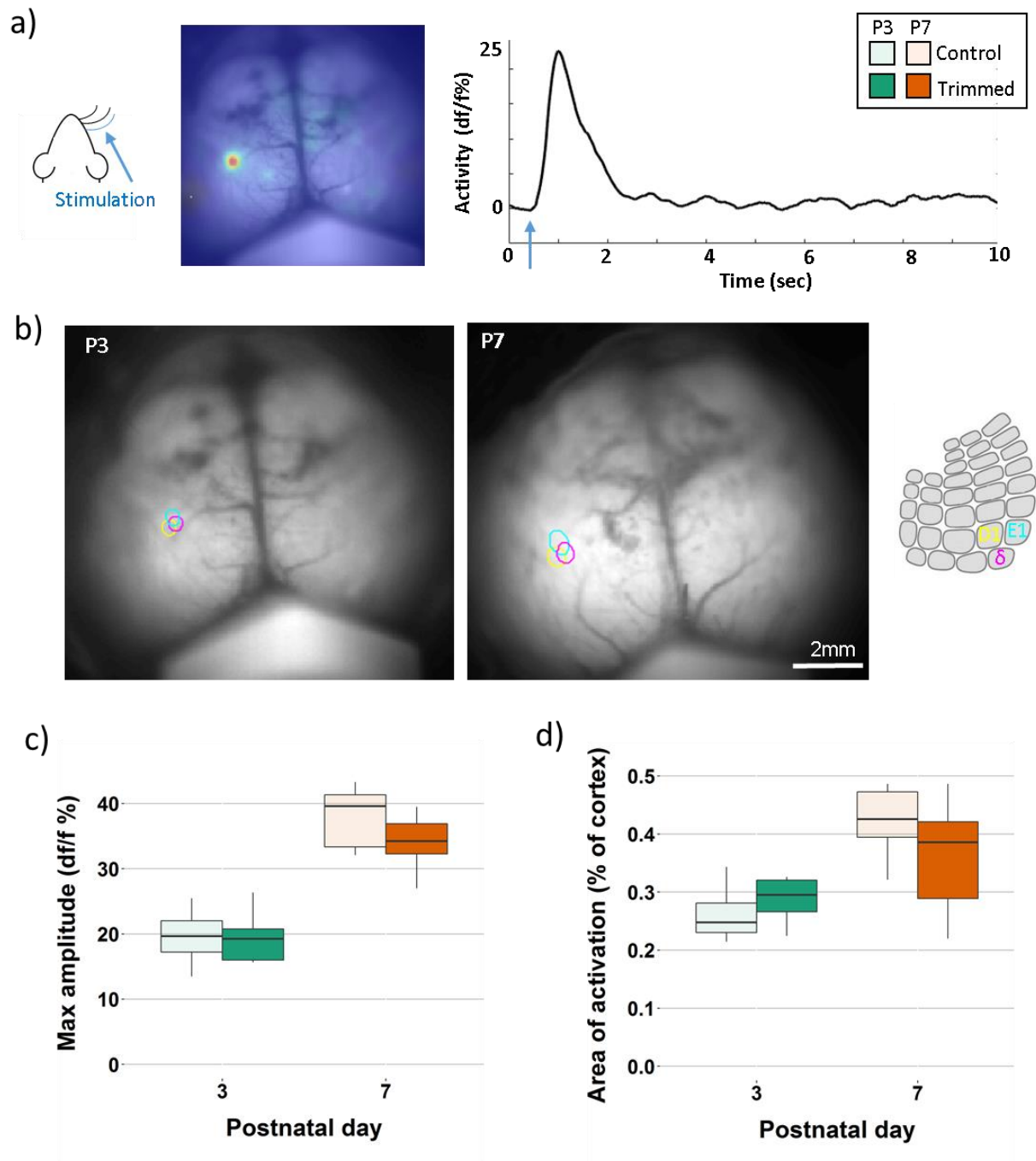
Figure 3.25 **Cortical responses to single whisker stimulation after sensory deprivation**

a) Example of an individual animals averaged cortical response to a single whisker stimulation, and an individual animal example of the averaged timeseries response in the area of activation. (b) Example outlines of areas of activation to D1, E1 and delta whisker stimulation, at P3 and P7. Areas correspond to the know barrel cortex topographical layout.
The amplitude of the response to single whisker stimulation increases with postnatal age, at the same rate in trimmed and control animals (c) (p<0.001). The area of activation also significantly increases with age (p<0.01) and is not different in trimmed compared to control animals (d).
[n: P3-trimmed = 5, P3-not = 4, P7-trimmed = 7, P7-not = 7]

3.6 Discussion

The work in this chapter is one of the first accounts of pan-cortical calcium imaging in neonatal mice. This study developed a reliable imaging setup that allows for collection of data during the first two postnatal weeks, in healthy and sensory deprived circumstances. Using this new technique, cortical activity was recorded with high spatial and temporal resolution, in both stimulated and resting-state conditions. From these data the developmental trajectory of activity across the cortex was investigated between P1 and P9, characterising the spatial and temporal properties, and investigating how sensory deprivation altered them. As this was a new technique a series of validation measures were carried out to ensure that the data being collected were being interpreted correctly.

3.6.1 Origin of the calcium signal in macroscopic wide-field imaging

The fluorescence emission from many neurons is being captured with the macroscopic wide-field calcium imaging used for this study. In the adult mouse brain the fluorescent signal capture with macroscopic calcium imaging is well correlated with action potentials in cortex and sub-cortical regions (Xiao *et al.*, 2017). The transgenic mice, Emx1-Ai95D have GCaMP6f expressed in the excitatory neurons across the cortical layers (Chan *et al.*, 2001; Kummer *et al.*, 2012). In the mature cortex, layer I has few excitatory cell bodies, however during the first 2 postnatal weeks a transient population of a excitatory neurons called Cajal-Retzius cells are found there (Ma *et al.*, 2014), which have been shown to also express Emx-1 (Gorski *et al.*, 2002). Calcium signal is not just captured from the neuron soma, but also dendritic and axonal projections (Grienberger and Konnerth, 2012; Siegel and Lohmann, 2013; Broussard *et al.*, 2018). Somas from specific cell types can be found in distinct layers but their projections often cross into other cortical areas (Divac and Edward White, 1989).

The focal depth of this macroscope setup is ~200μm (Ratzlaff and Grinvald, 1991), and the fluorescence signal captured should primarily be from this volume. This original study used the macroscope setup to capture intrinsic optical imaging in the monkey striate cortex and compare the visualisation of the ocular dominance columns at the surface and 200μm below. They found that the characteristic patterning of this cortical region was resolved at

both depths providing evidence that emitted light from deeper cortical layers can be capture by this imaging setup. In the neonatal ages used in this study the focal depth of ~200µm covers from cortical layers I-IV (Figure 2.11c) - although the superficial layers will dominate this signal due to light scatter. As the emitted fluorescence from lower layers passes through the more superficial ones the light is scattered by the inhomogeneities in the tissue. This results in less of the signal penetrating the surface and being captured by the macroscope (Boustany, Boppart and Backman, 2010). In addition, this scattering also impacts the light that does reach the cortical surface - appearing as blurred edges – which is an important consideration when analysing this data. However, this technique is not aiming to resolve down to cellular level but is a summation of activity from thousands of cells that fall within a pixel of the image capture. The calcium signal detected will be a combination of activity from cell bodies and projections of excitatory neurons in these layers.

During the neonatal period cortical neurons are still migrating to their final location and are undergoing structural and functional maturation (Isaac and Feldmeyer, 2009). This growth and maturation mean that the type and properties of the excitatory neurons present in the imaging volume may be changing over the period imaged in this study. This could influence the origin of the fluorescence signal being detected with this wide-field calcium imaging technique and should be considered when interpreting these data. At the earliest age imaged - P1 – layers II/III and IV are not thought to be present (Luhmann *et al.*, 2016a) (Figure 1.1), and so in these animals the fluorescence signal recorded is from neurons in the cortical plate that will eventually become these superficial layers, and from Cajal-Retzius cells in layer I (Ma *et al.*, 2014). Over the first postnatal week the neurons continue to mature and separate into their distinct layers, with migration complete by the end of the first week (Luhmann *et al.*, 2016a). During the second postnatal week Cajal-Retzius neurons begin to die (Chowdhury *et al.*, 2010) and their contribution to the signal will be reduced in the P9 imaging experiments from this study. Cortical neurons will also be undergoing maturation processes such as dendritic arborisation and so their contribution to the signal will be increasing (Catalano, Robertson and Killackey, 1996; Baloch *et al.*, 2009). The exact contribution of these different cell types at each age is not known from these experimental results. A possible way to test this would be to use a genetic cross with GCaMP and a cell specific promotor, such as Thy-1 that localised to layer II/III (Dana *et al.*, 2014).

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3.6.2 Movement

Motor activity is observed in rodents from birth (Clarac, Brocard and Vinay, 2004). There have been two types of movement characterised in the developing rodent, myoclonic twitches that occur during sleep and coordinated active movement that occurs during wakefulness (Karlsson and Blumberg, 2002; Tiriac, Del Rio-Bermudez and Blumberg, 2014). Myoclonic twitches are small, precise and fast, occurring predominately in the limbs and whiskers (Blumberg *et al.*, 2013). In this study movement was measured using a piezoelectric device under the animal's torso, which captured any movement that could displace it. This method of movement detection was chosen as it is non-invasive, unlike EMG recordings of limbs (Blumberg *et al.*, 2013), and can capture movement across all limbs and the body from one device unlike photo diode measurements. Cross referencing this data with video recordings found that single limb movements were collected using this method of monitoring. However, as myoclonic twitches are even smaller, sometimes occurring at a single joint or whiskers, it is likely that they were not enough to displace the piezoelectric device and be recorded.

The neonatal motor network does not have the same connectivity as the mature brain. The motor cortex is not active during bouts of awake movement in the early neonate, with responses to awake active movements not emerging until at around P11 (Dooley and Blumberg, 2018). Movement is thought to be generated in subcortical regions during this immature period (Clarac, Brocard and Vinay, 2004; Tiriac *et al.*, 2012). Movement does result in somatosensory stimulation of the body and activation of the primary somatosensory cortex is seen in the immature network (McVea, Mohajerani and Murphy, 2012; Ackman, Zeng and Crair, 2014; An, Kilb and Luhmann, 2014). In agreement with these previous discoveries, this study finds that bouts of awake movement results in activation of the body and limb somatosensory cortex but not the motor cortex from P1 through to P9 (Figure 3.6a). Movement related activity in the immature cortex has a complex and characteristic profile, and as such should be considered separately to activity occurring during rest. Movement related activity was excluded from the analysis of resting-state and sensory stimulated so that the focus of this study is on the cortical activity during rest in the early neonatal brain.

Spontaneous activity in the motor cortex was observed during periods of rest however (Figure 3.13a). Activity in the immature motor cortex has previously been reported to occur during myoclonic twitches both before and after the movement (An, Kilb and Luhmann, 2014; Dooley and Blumberg, 2018). It has also been found that ~35% of activity in the motor cortex was not correlated with any detectable movement (An, Kilb and Luhmann, 2014). This activity uncorrelated with any movement is thought have another source, which may be intracortical or from subcortical areas of the pathway such as the thalamus. It is likely that the motor cortex activity found in this study is also generated by a combination of these sources.

Spontaneous activity in the immature somatosensory cortex during restful behaviour is thought to also be generated by a combination of myoclonic twitching, and endogenously generated activity from the peripheral sensory organs, the thalamus and cortex (Yang *et al.*, 2009; Mizuno *et al.*, 2018). Unlike wakeful movements twitches are thought to have a similar role to peripherally generated spontaneous activity bursts in that they give precise topographically organised activation of the ascending pathways which is vital for the initial formation of cortical organisation (Blumberg *et al.*, 2015). The likely inclusion of these twitch movements in this data set, due to not being able to identify them is therefore not a problem as it appears that their role is more closely related to spontaneous activity than active movement.

In future experiments, monitoring of finer movements would help to further classify behavioural states. A possible method would be to use high-speed image acquisition of the limbs and whiskers to capture twitches as well as active movement (Nashaat *et al.*, 2017)

3.6.3   Anaesthesia recovery

Because anaesthesia suppresses spontaneous activity in the immature brain (Adelsberger, Garaschuk and Konnerth, 2005; Hanganu, Ben-Ari and Khazipov, 2006; Ackman, Burbridge and Crair, 2012; Siegel *et al.*, 2012), this study was carried out on awake neonatal mice. Anaesthesia (inhaled isoflurane) was required for the implantation of a head-fixation device for these experiments and so there was a period of recovery following surgery before imaging. Spontaneous activity quickly returned (Figure 3.10a) and active body movement

returned during this recovery period. However, even though spontaneous activity was present in the first recording the frequency of activity in the younger animals (P1-5) continued to increase over the experimental session (Figure 3.10c). This could be a continued effect of anaesthesia, and although the total suppression of spontaneous activity ended quickly after anaesthesia withdrawal, there may not have been total recovery in the recovery period allowed. Another possibility is that the animal's arousal state is changing during the experimental protocol and this is impacting the frequency of activity occurring in the cortex. However, the amount of movement does not change across sessions or with age (Figure 3.6b&c), and an increase in spontaneous activity frequency was not found at P7 or P9. The frequency calculated is also only during periods of rest, which as previously discussed are well correlated to sleep. The exact source of this increase in spontaneous activity across the experiment are unclear. In future experiments a further investigation of this would be required to elucidate the reason and adjust the protocol to account for it. A longer recovery period following surgery would help identify if anaesthesia alone was the source. The increase in spontaneous activity is over the first 30 min of the experiment and so an addition 30 min recovery period could be provided. Initially a short period was given as the viability of the pups away from their mothers at such young ages was not known. However, all animals appeared healthy and active following the experiments, with milk spot still present - suggesting sustained sustenance was not an issue -and so a longer experimental protocol would be possible.

3.6.4   Defining cortical areas

The complex spatiotemporal patterns of the spontaneous activity observed in the developing brain (Figure 3.8a) required parcellation into defined spatial or temporal sections for quantification. The temporal properties of this pan-cortical activity are not known and so spatial parcellation was first explored. Defining the boundaries of cortical regions in the developing brain is challenging. During the postnatal period the brain is growing, and the structure is changing daily (Figure 3.9a) and no systematic mapping of these changes has been carried out. This means there are no standardised coordinates for the developing brain as there are for the adult mouse brain (Allen mouse brain project). There are transgenic mouse lines available that target expression of reporters in the

terminals of the thalamocortical projections. These have previously been used in the developing mouse to locate the position of the primary sensory regions, as there is a high density of thalamocortical terminals in these regions (Ackman, Zeng and Crair, 2014; Mizuno et al., 2014). There has however been no publication of this data across the neonatal developmental period and so the challenge of defining region boundaries across the different ages studies here was not eliminated. Because there is no established data for the structural layout of the developing cortex this study used a combination of resources to manually define them across the postnatal ages used. This included the location of cortical activation during whisker stimulation (Figure 3.16a) which gave an accurate location of the barrel cortex for individual animals, acting a reference point for all other ROIs. Rather than whole regions of interest a point in the centre of the region, where the location was more certain were used to represent these cortical areas (Figure 3.8b). Because of the implementation of these limitations there is confidence that the data analysed is from the locations stated. A possible future experiment would be to use the thalamocortical targeted transgenic mouse lines to create a standardised atlas of the mouse brain during the first few postnatal weeks. This could allow full regions of interest in the developing cortex to be defined and investigated. The TCA-RFP mouse (Tg5), developed by the Iwasato Laboratory (Mizuno et al., 2018) is one such mouse line. Red fluorescent protein (Miyawaki, Shcherbakova and Verkhusha, 2012) is expressed in the terminal ends of the thalamocortical projections of these mice and these neurons can be visualised through fluorescence widefield imaging – similar to setup used in this study. The primary sensory cortical regions are innervated by numerous thalamocortical fibres and as a result the location of these regions can be identified in this mouse line (Figure 3.26). These mice could be imaged across the postnatal period and from these images a map of the major cortical regions at each age could be created. With these delineations a more confidence spatial parcellation of the data from this study could be made.

Defining the structural boundaries of cortical regions facilitates analysis of pan-cortical imaging as it reduces the dimensions for the complex spatiotemporal properties of the data. However in the adult it has been found that the functional boundaries seen through wide

field imaging techniques do not exactly align to the structural boundaries defined (Vanni *et al.*, 2017). It would be interesting to see if this was true for the developing cortex as well.
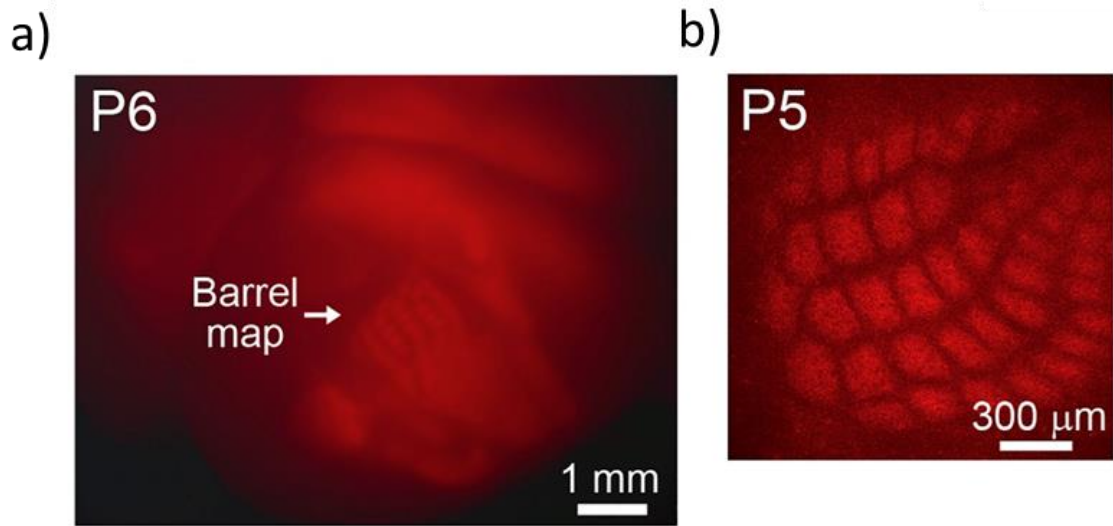
Figure 3.26 **Example expression of the TCA-RFP transgenic mouse line**
a) Visualisation of the Right hemisphere of a P6 TCA-RFP mouse, where the barrel map is visible.
b) A tangential slice of the barrel cortex in the TCA-RFP mouse at P5, imaged by confocal microscopy.
Showing clearly the thalamocortical terminal ends, creating the characteristic barrel pattern of this
region.

Image adapted from (Mizuno et al., 2018)

3.6.5   Spontaneous activity

Using these defined regions of interest, the temporal properties of cortical activity were investigated and compared across developmental ages. Investigation of spontaneously occurring neuronal activity is a valuable opportunity in the developing brain as some of the sensory pathways are not receptive to external stimulation at birth. It also comes with unique challenges as there are no known stimulus-related time-points to synchronise to, and this study has found that pan-cortical spontaneous activity in the neonatal brain has varied and complex spatiotemporal dynamics resulting in multidimensional data to quantify over development stages.

Spontaneous activity was found across the cortex from P1 (Figure 3.12). Early neonatal cortical activity has previously been reported (Khazipov, Sirota, Leinekugel, Gregory L Holmes, *et al.*, 2004; Ackman, Zeng and Crair, 2014), and in some areas of the cortex spontaneous activity is known to begin in the embryo (Moreno-Juan *et al.*, 2017). The presence of spontaneous cortical activity continues throughout the neonatal period assessed (until P9), with the frequency of activity bursts increasing with age (Figure 3.12). An increase in pan-cortical activity during the first 2 postnatal weeks has previously been reported (Ackman, Zeng and Crair, 2014), as well as a continued increase into adulthood (Kozberg *et al.*, 2016). An increased firing frequency of neurons at a local population level have been reported in the barrel (Golshani *et al.*, 2009) and visual (Rochefort *et al.*, 2009) cortices during neonatal development as well. This increasing frequency of cortical spontaneous activity agrees with electrical recordings of cortical activity in rodents and humans, where discontinuous bursting activity becomes more frequent over neonatal development and eventually become continuous in the mature brain (Colonnese *et al.*, 2010).

There are a variety of drivers of spontaneous cortical activity, which have been found to differ depending on the sensory modality. Activity in the immature motor and somatosensory cortex during rest is generated in both the periphery and centrally (Yang *et al.*, 2009; Mizuno *et al.*, 2018). In the visual and auditory systems spontaneous activity in the network has been found to predominately come from endogenously generated activity in

the peripheral sensory organs (Torborg and Feller, 2005; Wang *et al.*, 2015). It is not just in primary sensory regions of the cortex that spontaneous activity was found, but also association areas (Figure 3.13a). Less is known about the early neonatal development of these areas, but in the mature network they play an important role in integrating and processing sensory information (Purves *et al.*, 2001b). These connections between primary sensory and association regions may mean that the observed spontaneous activity is underpinned by intracortical connections between these regions.

During the neonatal period investigated in this study thalamocortical fibres are migrating to the cortex and forming functional synaptic connections at a rapid rate (Molnár and Blakemore, 1995). These connections are part of the ascending sensory pathways connecting the peripheral sensory organs with the central network. The bursting nature of the immature neuronal networks is thought to be linked to poor connectivity resulting a slow accumulation of depolarisation in the circuit before firing occurs. As connectivity increases with developmental age these periods of depolarisation would become shorter and the frequency of bursting increase. As well as increasing connectivity in the ascending sensory pathways during this neonatal period intracortical connections are developing, both locally (Ashby and Isaac, 2011) and between hemispheres (Wang *et al.*, 2007). This further increases the excitatory inputs into the cortical networks which could result in increased firing.

It is not just excitatory neurons that are maturing and forming connections in the postnatal network. Inhibitory neurons are also developing in the cortex during this early neonatal period (Kelsom and Lu, 2013; Lim *et al.*, 2018), and their effect on the overall network activity should be considered. During the early postnatal period it has been documented that they have a depolarising effect, and so rather than being inhibitory they actually contribute to the excitation of the network (Ben-Ari, 2002) - with a transition to inhibitory actions of GABA occurring during the second postnatal week. If this is the case, then the developing inhibitory network could be contributing to the spontaneous activity recorded from excitatory neurons in this study. However, more recent *in vivo* studies have found that GABA does have its traditional inhibitory effect even during the first postnatal week (Kirmse *et al.*, 2015; Valeeva *et al.*, 2016; Che *et al.*, 2018). As the nature of GABA during the early neonatal period is unclear it is difficult to know its contribution to the activity found in this

study. The cortical network is a complex system that is rapidly developing during this early postnatal period, and further investigation is required to dissect each cell populations contribution to this overall state of endogenously generated activity. A possible experiment to do this would be to rerun this experimental protocol with mice that have GCaMP crossed with an inhibitory neuron specific promotor, such as GAD67 (Rasmussen *et al.*, 2007; Dougherty, Sawchuk and Hochman, 2009). This would give a comparison to the activity of the excitatory network, and knowing both populations developmental trajectory could shed light on how they interact during this early neonatal period.

As well as an overall increase in connectivity and excitability in the central network another possibility could be that more spontaneous activity is being generated in the periphery. Across this early neonatal period there is an increase in frequency of the endogenous generated activity in the retina (Ackman, Burbridge and Crair, 2012) and the cochlea (Tritsch and Bergles, 2010). Some activity in the somatosensory cortex is generated by whisker twitches and activity of the whiskers is known to be developing during this early neonatal period (Tiriac *et al.*, 2012). An increase in the frequency of whisker twitching could contribute to the increased activity found in the barrel cortex.

To further investigate the origin of spontaneous activity in the somatosensory network the periphery was acutely silenced by injection of local anaesthesia into the whisker pad. Drive from the periphery was inhibited by local anaesthesia and spontaneous activity was dramatically suppressed, although not abolished in the contralateral barrel cortex (Figure 3.19c). Similar results have previously been found with local field potential recordings from across the cortical layers during the first postnatal week (Yang *et al.*, 2009). In contrast, spontaneous activity in layer IV excitatory neurons are almost entirely supressed during peripheral silencing (Mizuno *et al.*, 2018). These results suggest that spontaneous activity in layer IV in the neonatal brain is driven by peripheral input, but that activity in other cortical layers have another origin.

The advantage of pan-cortical imaging is that activity in the entire cortex can be captured simultaneously. This allowed the effect of acute localised peripheral silencing on other cortical regions to be investigated. No change in the frequency of activity was observed in any other area (Figure 3.19). This suggests that the peripherally generated spontaneous activity propagates to the primary sensory cortex but not in other regions, and that a

change in activity frequency in one region does not affect the activity in other areas of the cortex.

Region-of-interest-based methods used to detect activity only assess the temporal aspect of the calcium signal and identifies activity as a change in fluorescence that exceeds the baseline. However, events were not spatially homogenous. Some events have been observed to be discrete bursts of activity confined to a local region. These include characteristic patterns of retinal wave activity in the primary visual cortex (Figure 3.7a) that are known to be generated in the periphery (Ackman, Burbridge and Crair, 2012). Also observed are activations of discrete barrels (Figure 3.7b) which have similar spatial characteristics to single whisker stimulation (Figure 3.25a) and to the spontaneous bursts in layer IV previously reported (Mizuno *et al.*, 2018). This would suggest that these bursts of activity are stimulated by activation of the ascending sensory pathways. Other times, activity appears to travel across wider areas of the cortex as propagating waves between anatomical regions, which may be a result of intracortical connections. The varied and complex nature of these different activity patterns makes classification and separation of them difficult. A previous report of pan-cortical activity in the developing brain found that the propagation of spontaneous activity changed across neonatal development (Ackman, Zeng and Crair, 2014) with propagation going from patterned activity in discrete areas, such as retinal waves and bursts in individual barrels, to propagation across the entire cortical surface. This change in spatial properties of spontaneous activity across development must be considered when considering the change in frequency found in this study. Not only may it be an increase in the frequency of the same types of activity but also an introduction of new activity types such as intracortical travelling waves. To explore the spatial relationship of spontaneous activity between cortical regions investigation of coordination of activity was carried out.

3.6.6   Coordination of activity

Correlation of activity in regions during resting-state is widely used as a measure of functional connectivity (Shen, 2015; Wright *et al.*, 2017). Coordinated cortical activity could come from mutual subcortical connections (Henschke *et al.*, 2017) or from intracortical

connections between the regions (Mohajerani *et al.*, 2010). Interhemispheric activity at P1 is highly correlated, with an average coefficient of 0.987±0.01 (Figure 3.14b). This correlation coefficient gradually drops over development, to an average of 0.758±0.08. In the adult mouse cortex slow-wave activity during sleep is correlated across the hemispheres with a coefficient of around 0.5 (Mohajerani *et al.*, 2010). In adults this activity is mostly coordinated via interhemispheric callosal connections (Mohajerani *et al.*, 2010), although lesioning of the callosal fibres reduced but did not eliminate the correlated activity, suggesting some subcortical connections are involved, such as thalamocortical. In the mature brain it was found that the retrosplenial cortex had the highest interhemispheric correlation (Mohajerani *et al.*, 2010), which was also true for the immature brain (Figure 3.14c).

Callosal projections are not present at birth but migrate and innervate the opposite hemisphere by the end of the first postnatal week (C.-L. Wang *et al.*, 2007). If these interhemispheric projections are not present in the immature brain another mechanism for coordination must be present, meaning it is possibly dominated by subcortical coordination at very young ages. In the first few postnatal days even thalamocortical projections have not completed innervating the cortex (Erzurumlu and Gaspar, 2012). Transient subplate connections are however present from before birth and persist in the first postnatal week (López-Bendito and Molnár, 2003; Patrick O. Kanold and Luhmann, 2010). These projections have been found to have both contralateral and ipsilateral connections (Hoerder-Suabedissen and Molnár, 2012). This common subcortical connection at the beginning of the first postnatal week could explain the high correlation between spontaneous activity in the right and left hemisphere.

Over the first postnatal week subplate connections are replaced by thalamocortical projections. Spontaneous activity in the immature brain is dominated by incoming signal form the peripheral, via thalamocortical projections, which switches to intracortical generation in the mature brain (Minlebaev *et al.*, 2011). The thalamus principally connected to the cortex in a topographically organised manner, with discrete nuclei for each sensory modality. However, it is thought that there are also cross-modal projections, with connections going to sensory modalities (Tyll, Budinger and Noesselt, 2011; Henschke *et al.*, 2015). As subcortical connections dominate the transmission of spontaneous activity into

the immature cortex these cross-modal subcortical connections could be involved in the interhemispheric correlation found in young animals. These changing structural and functional aspects of the hemispheric connectivity during this developmental period suggests that the communication methods between cortical areas are transitioning, and that correlated activity at P1 may not be mediated by the same structures as at P9. This interhemispheric decorrelation appears to be a transition towards a more mature activity state.

As well as interhemispheric correlation of activity there is also coordination between intra-hemispheric regions (Figure 3.15). As with the interhemispheric activity at P1 there is high correlation coefficient values between most cortical regions. This overall high correlation gradually disappears with development, with some intracortical correlated activity remaining. These patterns of functional connectivity are similar to those previously reported during early postnatal development (Ackman, Zeng and Crair, 2014).

3.6.7   Sensory stimulation

It is not just spontaneous activity that is present in the developing brain. Responsiveness to sensory stimulation is emerging and plays an important role in sensory network development. Deflection of the whisker is known to stimulate activity in the primary somatosensory cortex of neonatal rodents in a topographically organised pattern (Khazipov, Sirota, Leinekugel, Gregory L. Holmes, *et al.*, 2004), from the day of birth (Mitrukhina *et al.*, 2015b). In this study deflection of the right whiskers results in activation of the contralateral barrel cortex from P1 to P9 (Figure 3.16a). Thalamocortical (TC) projects are present in the upper cortical layers from P0 (Agmon *et al.*, 1995), with continue development during the early postnatal days, with barrels emerging around P4 (Erzurumlu and Gaspar, 2012). As well as TC projections subplate neurons transiently project and form functional synapses in the cortical plate (López-Bendito and Molnár, 2003; Patrick O. Kanold and Luhmann, 2010) and are functionally organised to topographically represent the whiskers from birth (Yang *et al.*, 2013). Thalamocortical projections migrate during the first postnatal week and by P4 they have innervated layer IV to form visible barrel structures (Erzurumlu and Gaspar, 2012) and the subplate connections disappear by the end of the first postnatal week (Higashi *et al.*,

2005). This study finds no change in the amplitude of activation suggesting the transition between neuron populations does not disrupt the functional input into the cortex.

It appears that the whisker sensory pathway is primed to process external sensory information from as early as P1 and that the strength of cortical response does not change over the next week of development. The fluorescence signal captured with this wide-field calcium imaging is the summation of activity of multiple neurons in the cortex. Given the nature of the signal being measure the consistence in amplitude of cortical response to whisker stimulation could be either that the same population of neurons is activated to the same degree or that the balance of individual signal to the total number of neurons active is balanced through development. Neuron populations within individual barrels are known to be highly coactive during spontaneous activity in the early neonatal period, with this correlation decreasing with neonatal development (Golshani *et al.*, 2009; Mizuno *et al.*, 2018).

The amplitude of the cortical response to whisker stimulation does not change with age but the relative area of activation increases significantly across postnatal age (Figure 3.16d). The barrel cortex is in a critical period of development during the neonatal period investigated in this study (M. C. Crair and Malenka, 1995). At P1 the layers of the cortex are not full formed and the characteristic barrel pattern of later IV is not (Erzurumlu and Gaspar, 2012). As thalamocortical projections migrate during the first postnatal week and form synapses in layer IV the barrel cortex begins to form. This process of neural migration is happening across the cortex and it overall size is increasing (Figure 3.9a). This increase in activation area relative to the total surface area may be caused by a disproportionate growth of the barrel cortex compared to the rest of the cortex. In the mature brain the barrel cortex covers around 13% of the cortical surface and accounts for 69% of the somatosensory cortex (Lee and Erzurumlu, 2005), but the growth trajectory of the immature brain is not known.

This increase in area of activation could also relate to variability in the delivery of sensory stimulation. The whisker field stimulation was stimulated using a plastic paddle manual placed caudal to the whiskers at an angle that would contact as many whiskers as possible on movement. The length and position relative to the face of the whiskers is changing during this neonatal period and while collecting the data a concern arose that as the

whiskers grew, more of them were being deflected by this stimulation method. To address this possibility, deflection of individual whiskers was carried out in the second cohort to deliver a precise stimulation location. Single whisker deflection also resulted in an increased area of activation with postnatal age (Figure 3.25d). This increase in single whisker stimulation supports the hypothesis that it is a relative increase in individual barrel size that is the reason the for increased area of activation to stimulation with age, as the location of a single whisker is conserved through the ascending pathway to a single barrel in the cortex (Carl C.H. Petersen, 2007).

Only the contralateral barrel cortex was activated with either multiple or single whisker deflection. In the mature brain whisker stimulation also stimulates activity in the motor cortex, at a slight delay to barrel cortex activation (Ferezou *et al.*, 2007; Vanni and Murphy, 2014). This suggests that in the mature brain there are somatomotor intracortical connections that are activated during sensory perception and these are not present in the first 9 postnatal days. This means that although cortical processing of tactile whisker information is present from P1 there are still developmental changes occurring to the network during the early postnatal period.

Repeated sensory stimulation can result in a desensitisation of response (Kheradpezhouh, Adibi and Arabzadeh, 2017). In this study rundown of stimulation response over repeats was found at P1 to about 40% of the first over 20 stimulations, but not in older animals (Figure 3.17b). Adaption to repeated whisker stimulation is seen in the mature sensory network, however only at much higher stimulation frequencies than were given in this study (Chung, Li and Nelson, 2002; Martin-Cortecero and Nuñez, 2014). A 10s ISI was left between each single 30ms deflection was delivered to avoid this kind of adaption. The fact that a rundown of response was not seen in older animals also suggests that this is not what is occurring in P1 animals. A possible cause is the immaturity of neurons in the sensory pathway meaning they are more susceptible to vesicle depletion as their replenishment is slow, which has previously been described in immature cortical neurons (Feldmeyer and Radnikow, 2009).

Unlike the somatosensory network that is responsive to external stimulation from birth, the auditory pathway does not reach the maturity required to process sensory information until the middle of the second postnatal week (de Villers-Sidani *et al.*, 2007; Froemke and Jones,

2011). In agreement with these previous findings this study found no cortical response to auditory stimulation between P1 and P9 (Figure 3.18).

3.6.8 Sensory deprivation

Using this newly established technique the effects of neonatal sensory deprivation on pan-cortical neural activity in the developing brain were investigated. Sensory experience is an important part of the development of neuronal networks and deprivation results in deficits in their formation, in the primary sensory region associated with the deprivation (Wiesel and Hubel, 1963; Fox, 1992) and in other cross-modal regions (Sieben *et al.*, 2015; Henschke *et al.*, 2017). Recordings of cortical activity at high spatial and temporal resolution gives a unique opportunity to investigate the effects of sensory deprivation across the network simultaneously.

The first postnatal week is a critical period of development in the whisker somatosensory pathway and early neonatal whisker deprivation has been shown to have life-long effects on the development of this network (Fox, 1992). Most studies have investigated the effects of early life sensory deprivation on the mature brain, and so recording this pan-cortical activity during the critical period of barrel cortex development can give unique insight into the developmental effects of early life whisker trimming. Spontaneous and sensory stimulated activity are both important for barrel cortex development and so in this study changes to both these activity types during the critical period of barrel development were investigated. By investigating this critical period, it may be possible to identify the mechanisms of these long-term alterations to the network, and even transient changes that are later not identifiable but that have implications for long-term network function.

3.6.8.1 *Spontaneous cortical activity following neonatal whisker trimming*

Resting-state recording of cortical activity found that unilateral whisker deprivation during the first postnatal week did not alter the frequency of spontaneous activity in any cortical region, including the contralateral barrel cortex (Figure 3.21). Previously it was found that neonatal unilateral whisker trimming followed by a period of whisker regrowth resulted in

an increase in spontaneous firing in layer IV excitatory neurons in the adult barrel cortex (Shoykhet, Land and Simons, 2005). A recent study however, found that at P6 following whisker plucking from birth there was no change in spontaneous firing frequency in the contralateral barrel cortex (Che *et al.*, 2018). Another recent study found that lesioning the infraorbital nerve at P4 did not affect the spontaneous firing in layer IV of the contralateral barrel cortex a day later (Mizuno *et al.*, 2018). These results suggest that the effects of sensory deprivation change over development. It could be that during the early neonatal period the spontaneous activity in the cortex is being entirely endogenously generated and not sensitive to external sensory input. A transition in the pattern of spontaneous activity in the barrel cortex is found around on the onset of whisking at the end of the second postnatal week (Colonnese *et al.*, 2010). This could be the time that external sensory inputs begin to impact the spontaneous cortical activity. Whisker trimming from birth has been found to reduce the strength of thalamocortical inputs into the barrel cortex at the end of the first postnatal week (Crocker-Buque *et al.*, 2015), so external sensory input is required for early developmental of thalamocortical connectivity. Interestingly, an abundance of thalamocortical connections are found in the mature brain following neonatal whisker trimming (Wimmer *et al.*, 2010; Oberlaender, Ramirez and Bruno, 2012), again suggesting a developmental transition in effects of sensory deprivation.

Deprivation of sensory input during the early neonatal period may result in a deficient in thalamocortical connectivity during the critical period of development (M. Crair and Malenka, 1995) but then later in development increased connections may be made by aberrant processes. The increased excitability found in the mature cortex after sensory deprivation is thought to come from alterations to the inhibitory circuits state (Zhang *et al.*, 2013) and an increased connectivity due to disrupted refinement pruning during development (Zuo *et al.*, 2005; Simons, Carvell and Kyriazi, 2015). The inhibitory, like the excitatory circuits are immature and developing in the barrel cortex during the first postnatal week (Erzurumlu and Gaspar, 2012). It has been found that in the immature cortex GABAergic synapses are excitatory during the first postnatal week before shifting to their inhibitory role in the second postnatal week (Ben-Ari, 2002). It has also been found that inhibitory circuits can also be hyperpolarising even in the first postnatal week, but are still undergoing development (Kirmse *et al.*, 2015; Valeeva *et al.*, 2016; Che *et al.*, 2018). If

the changes in excitability in the mature cortex after sensory deprivation are a result of imbalance between excitatory and inhibitory activity, the immaturity of the inhibitory network during the first postnatal weeks could explain why there is no increase in spontaneous activity found.

The first postnatal week is the critical period for layer IV of the barrel cortex but critical development processes in other layers occur at different times. The critical period for receptive field development in layer II/III is during the second and third postnatal week (Maravall, Stern and Svoboda, 2004; Wen and Barth, 2011; Erzurumlu and Gaspar, 2012), which include the development of local inhibitory circuits (Lo, Sng and Augustine, 2017). The activity captured with pan-cortical imaging is a summation of fluorescence signal from multiple layers of the cortex. The differential timings of the cortical layer development could mean that effects in the summed signal captured would not show an effect until all layers have also gone through their critical period of maturation. In this study it is likely that spontaneous activity in the transient layer I Cajal-Retzius neurons are being detected (Gorski *et al.*, 2002), which disappear is the second postnatal week. Differential effects of whisker trimming have previously been observed between different layers II/III and IV in the mature barrel cortex (Lee, Land and Simons, 2007).

Whisker trimming does not damage the sensory neurons and so intrinsically generated activity, in the sensory organs themselves or from myoclonic twitching) may still be occurring in the deprived sensory pathway. Active whisking has not yet emerged in the first postnatal week (Landers and Philip Zeigler, 2006) and during resting-state recordings in this study no stimulation was supplied to the whiskers, meaning activity observed during these recordings is spontaneously driven. It appears that a lack of actual whiskers does not disrupt this endogenously generated activity in the immature network. It has previously been found that unilateral cauterisation disrupts the formation of interhemispheric callosal projections, as did direct disruption of spontaneous activity in the barrel cortex (Suárez *et al.*, 2014). In this study the correlation of activity between inter- and intra-hemispheric regions is not affected by whisker trimming (Figure 3.23). Cross cortical connections are immature in the first postnatal week (Wise and Jones, 1978; Ivy and Killackey, 1981), and the coordinated cortical firing present during this time is likely driven by subcortical connections. Since spontaneous activity is still present in the sensory pathway this may be driving the

coordination of activity in this early neonatal period. The development of interhemispheric connections during the first postnatal week is dependent on activity (C.-L. Wang *et al.*, 2007; Suárez *et al.*, 2014) and although the input from whiskers is deprived in this study the spontaneously generated activity from the sensory organs (Yang *et al.*, 2009; Mizuno *et al.*, 2018) has not been disrupted. The activity that is endogenously generated by neurons in the pathway may also be enough to guide the migration.

3.6.8.2   *Whisker stimulation responses*

Neonatal whisker trimming, followed by regrowth of whisker in adulthood increases the responsiveness in the deprived barrel cortex to whisker stimulation, in the adult rodent (Lee, Land and Simons, 2007; Simons, Carvell and Kyriazi, 2015). The thalamic input was found to be unaffected, but the thalamocortical connectivity was increased (Simons, Carvell and Kyriazi, 2015). It was also found that excitatory neurons in the deprived barrel cortex also had less refined receptive fields, responding more to adjacent whisker stimulation than controls due to a weaker inhibition (Shoykhet, Land and Simons, 2005). This increased thalamocortical connectivity and altered E/I balance appears to be a similar mechanism for the increased spontaneous activity in the mature cortex (Shoykhet, Land and Simons, 2005).

In this study it was not possible to regrow the whiskers to investigate their responses to stimulation as animals were investigated at such a young age. However, the use of unilateral whisker trimming meant that the non-deprived side could be stimulated. Stimulation of the intact whisker field resulted in activation of the contralateral cortex in control and trimmed animals with both single (Figure 3.25) and multiple whisker deflections (Figure 3.25), and the amplitude of the cortical response was not affected by sensory deprivation. Stimulation of non-deprived whiskers following unilateral whisker stimulation has not been performed in adult rodents and so the effect on the preserved barrel cortex is not known. It may be that there is no change to the ipsilateral pathway as the sensory inputs from the whiskers are bilaterally segregated. It was found that unilateral whisker trimming resulted a reduction in thalamocortical connectivity in the deprived hemisphere, but not in the spared at the end of the first postnatal week (Crocker-Buque *et al.*, 2015). However, sensory experience is a whole organism experience and it would also be possible to find

compensation from the preserved pathway to compensate for the loss of incoming sensory experience.

Although the amplitude of the cortical response to whisker stimulation is not affected by whisker trimming, the area of activation is decreased at P7 (Figure 3.24c). The area of cortical activation with single whisker stimulation was not significantly different at P7 but there is a wider spread of smaller values compared to control (Figure 3.25d). As previously discussed the increase in size of activation area with postnatal age is likely due to the increasing size of the barrels as they mature, so it is possible that the decreased activation area following whisker trimming is due to a reduced increase in the structural size to the barrels. Previously it was found that the width of the barrels in the deprived hemisphere following unilateral whisker trimming were not different to the spared at the end of the first postnatal week (Crocker-Buque *et al.*, 2015), but this was not compared to non-deprived animal development. The brain weights of the whisker trimmed animals were not different that control (Figure 3.20b) so any changes in barrel structure are not accompanied by a gross morphological deficit.

This smaller area of activation could also be a reduced functional receptive field. The response of barrels at birth is imprecise but over the first few postnatal days barrel receptive fields segregate (Mitrukhina *et al.*, 2015a). In adults it was found that barrels in the deprived cortex had increased receptive fields, resulting in poorer discrimination between whisker stimulation (Shoykhet, Land and Simons, 2005). Although the sensory input into the spared barrel cortex is not altered, it could be that the receptive field of the spared side becomes more refined to compensate for the loss of input from the deprived side. It is also possible that behavioural changes occurred to compensate for the lack of whisker sensory input on the left side. Somatosensory input is known to be important for suckling a huddling behaviour in neonatal rodents (Sullivan *et al.*, 2003; Arakawa and Erzurumlu, 2015a) and it could be that the spared whiskers were more predominately used by the pups. This could result in an increased sensory input into this side, which may have resulted in an altered refinement of the neuronal circuits. Another possible source of compensation is subcortical interhemispheric connectivity. Coordinated spontaneous activity between barrel cortices before interhemispheric connections have developed early in the first postnatal week suggest mutual subcortical connections. Changes to the

barreloids in the thalamus have been found on the spared side in adults following unilateral whisker trimming (Simons and Land, 1994). Also, unilateral infraorbital nerve lesions in neonatal mice did not affect the spontaneous activity in the barrel cortex and it was hypothesised that this was due to coordinated subcortical generation of activity (Mizuno *et al.*, 2018).

3.6.9   Conclusion

This is one of the first accounts of pan-cortical activity during neonatal development. It characterises the developmental trajectory of cortex-wide spontaneous activity and sensory evoked responses, revealing a dynamically changing functional network during the first 9 postnatal days. Spontaneous activity in the immature cortex is non-random, with both temporal and spatial coordination that is developing during this early neonatal period. This activity becomes more frequent over time and the functional connections appear to become more refined. This increasing frequency of spontaneous activity parallels findings of electrical recordings from animals and humans (Colonnese *et al.*, 2010), and finds that the increase occurs simultaneously across the cortex regions. There is a dynamic spatiotemporal nature to this activity and the wealth of data produced by this pan-cortical imaging offers an opportunity to continue to explore these dynamics and tease apart these complex intracortical functional relationships. Tactile stimulation evokes cortical activity from the beginning of the first postnatal week, and this study demonstrates reliable single whisker stimulation during the first postnatal week when the whiskers are still developing. These evoked responses reveal developmental trajectories of cortical activity occurring alongside barrel growth and maturation (Erzurumlu and Gaspar, 2012). With both spontaneous and evoked activity present in the cortex during this early developmental period it would be interesting to explore the interactions between them in future work. The motor cortex has a transient mechanism that modulates the evoked activity in the cortex in early the neonatal period, that is believed to allow spontaneous activity to dominate the developmental processes occurring (Dooley and Blumberg, 2018), but little is known about cortex wide interactions between these two activity types. Again, the wealth of data collected in this study allows for further analysis into these questions to be carried out. A possible analysis to explore this interaction acutely would be to look at spontaneously occurring activity during

the 10 second inter-stimulus interval (3.2.4) and compare it to the resting-state recordings. Whether there are modifications to ongoing network activity immediately following stimulation could be investigated, and whether this was localised to the area of stimulated activation or a global phenomenon.

Sensory deprivation by whisker trimming from birth does not alter the frequency of spontaneous activity in the cortex during the first postnatal week, complimenting a recent study that found no change to spontaneous activity in the deprive barrel cortex at P6 (Che *et al.*, 2018). This suggests that this early spontaneous cortical activity is not an activity depended process but is endogenously driven at these ages (Luhmann *et al.*, 2016b). Although spontaneously occurring activity across the cortex was unaffected, the sensory evoked activity was changed. Cortical responses to stimulation in the spared barrel cortex spread over a smaller area of the cortex, suggest a structural or functional alteration in the non-deprived pathway during the first postnatal week. This may be an underdevelopment of barrel growth or could be an altered refinement of function receptive fields. It has previously been found that the thalamocortical connectivity in the spared barrel cortex at P7 is unaffected (Crocker-Buque *et al.*, 2015), and so a functional change in receptive fields is thought the more likely reason for these results.

This pan-cortical calcium imaging is a newly established, minimally invasive technique that can capture the functional activity of the pan-cortical network in the developing mouse. This imaging technique has similarities to functional magnetic resonance imaging, that is routinely used in humans, offering a valuable tool for use in investigating the alterations to function development in animal models of early life insults, such as prematurity.

4 Functional MRI in Preterm Infants

4.1 Introduction

4.1.1 Sensory network development in the preterm period

The third trimester of fetal development in humans is a vulnerable time in neuronal development (Kostović and Jovanov-Milosević, 2006) and with more than 10% of infants globally are born prematurely (Goldenberg et al., 2008) the investigation into neuronal network development in these infants is of great interest. White mater injury is a common and well-studied pathology associated with premature birth (Khwaja and Volpe, 2008; Volpe, 2009), that has been found to be associated with neuron loss and grey matter deficits (Pierson *et al.*, 2007; Ligam *et al.*, 2009). These neuronal deficits can result in abnormal cortical development in prematurely born infants (Inder *et al.*, 2005b; Kapellou *et al.*, 2006). Even in the absence of severe white matter injury microstructural alterations in cortical (Ball, Srinivasan, *et al.*, 2013; Bouyssi-Kobar *et al.*, 2018) and subcortical (Boardman *et al.*, 2006; Srinivasan *et al.*, 2007) grey matter – including the thalamus - have been found in preterm infants. Diffusion tensor MRI (DTI) is a non-invasive neuroimaging technique for measuring water diffusion in tissue (Alexander *et al.*, 2007). Measurements from this technique have been used to estimate the development of the cytoarchitecture in developing brain (Dobbing and Sands, 1973; McKinstry *et al.*, 2002; Jespersen *et al.*, 2012). It has been found using these methods that preterm infants have significantly higher fractional anisotropic and mean diffusivity across the cortex (including sensorimotor areas) compared to term infants, suggesting that they have reduced dendritic arborisation and synaptic density at term equivalent age (Ball, Srinivasan, *et al.*, 2013; Bouyssi-Kobar *et al.*, 2018). Thalamocortical connectivity develops during a critical window of vulnerability during the preterm period (Molliver, Kostović and van der Loos, 1973; Allendoerfer and Shatz, 1994). Impaired thalamocortical connectivity has been found in preterm infants (Ball, Boardman, *et al.*, 2013; Toulmin *et al.*, 2015) and these changes were found to help predict cognitive outcomes at 2 years of age (Ball *et al.*, 2015). Thalamocortical connections are an important component of the sensory pathways and alterations in their connectivity has implications for sensory processing. Indeed, alterations to sensory processing are observed in children born prematurely (Slater *et al.*, 2010; Bart *et al.*, 2011; Wickremasinghe *et al.*, 2013; Chorna *et al.*, 2014; Cabral *et al.*, 2016).

The sensorimotor network emerges early in fetal development. Structural connections in the sensorimotor pathways have been revealed through post-mortem studies from the second trimester (Molliver, Kostović and van der Loos, 1973; Kostovic and Rakic, 1990), with continue development of cortical connectivity seen in preterm infants during the equivalent of the third trimester (Ball, Srinivasan, *et al.*, 2013). Functionally, fetal movement in response to vibration stimulation have been recorded from 21 weeks gestation (Marx and Nagy, 2015) and cortical activation to somatosensory stimuli can be reliably recorded in the preterm infant cortex by 25 weeks gestational age (Hrbek, Karlberg and Olsson, 1973). These somatosensory evoked potentials (SEPs) then continue to develop during the preterm into neonatal period (Pihko and Lauronen, 2004; Tolonen *et al.*, 2007; Fabrizi *et al.*, 2011). The latencies of SEPs induced by median nerve stimulation decreased rapidly between 27 and 40 weeks gestational age in preterm neonates with normal neurological examinations, which is believed to reflect the development of the normal fetus (Karniski *et al.*, 1992; Taylor, Boor and Ekert, 1996b). This continued maturation has also been observed using functional MRI, where activation of the cortex to sensorimotor stimulation is present from 31 weeks gestation (not investigated earlier in this study). The patterns of activation continue developing until term equivalent age, when activation is more similar to the mature network (Allievi *et al.*, 2016). This continued development makes these pathways vulnerable to the altered and potentially pathological environment of the preterm period.

The preterm period is not just a vulnerable time for neurobiological development but also an altered sensory environment. Preterm infants are often hospitalised, which is an environment that is very different to the intrauterine environment they would normally be in. This environment often comes with altered exposure to sensory experience, such as bright lights and loud noise levels (White-Traut *et al.*, 1994) and painful stimulation (Machado *et al.*, 2017). These changes in sensory experience are thought to play a role in the sensory processing issues found in preterm infants as they mature (White-Traut *et al.*, 1994; Lickliter, 2011; Cabral *et al.*, 2016). Research in animals has found that the development of the sensory pathways is an experience-dependent process and that changes to early life experience have long-term impacts on sensory network connectivity (Fox, 1992). Changes in neuronal network function in preterm infants could give insight into the effects that altered environmental conditions during early life have on the developing connections in the human brain.

4.1.2 Functional magnetic resonance scanning

Techniques for measuring neuronal activity in human infants are more limited than in animals, due to the requirement of non-invasive procedures. fMRI is a neuroimaging technique that can provide visualisation of neuronal activity (Glover, 2011), and is widely used in both adults and infants (Raschle *et al.*, 2012; Smyser and Neil, 2015). Human neuroimaging is non-invasive, using measurements of endogenous molecules through the skull. fMRI detects metabolic changes in tissue, utilising the varied magnetic properties of oxygenated and deoxygenated haemoglobin, producing a blood oxygenation level dependent (BOLD) contrast (Ogawa *et al.*, 1990). It has been found that when neuron populations in the brain are active there is an increased supply of oxygen to the local area (Buxton, Wong and Frank, 1998), meaning BOLD changes are a proxy for neuronal activity. The most common form of fMRI used is echo planar imaging (EPI), which rapidly collects two-dimensional slice images of the brain sequentially that can then be constructed into three-dimensional whole brain volumes (Mansfield, 1977). These changes can be detected during both resting-state spontaneous activity (Lee, Smyser and Shimony, 2013) and in response to external stimulation (Golland *et al.*, 2007).

fMRI is a valuable technique in human neurological research, with advancements being made all the time. However, it does come with some limitations. First the changes in blood oxygen are slow compared to neuronal activity meaning the temporal resolution is slower than measurements of electrical activity such as EEG (Buxton, Wong and Frank, 1998). Also, by using changes in blood oxygenation as a measure of neuronal activity there is an assumption made about the relationship between neuronal activity and vascular changes, known as neurovascular coupling (Phillips *et al.*, 2016). Mechanisms of neurovascular coupling in the healthy adult brain have been identified (Girouard and Iadecola, 2006), and although not fully understood the relationship between neuronal activity and adjustments to oxygen supply are believed to be spatially and temporally localised enough to be a reliable measure (Kim *et al.*, 2004; Lecrux and Hamel, 2011). However, this relationship between neuronal activity and vascular changes can differ in disease (D'Esposito, Deouell and Gazzaley, 2003; Girouard and Iadecola, 2006) and during brain development (Harris, Reynell and Attwell, 2011; Kozberg and Hillman, 2016). This means that using this technique

to investigate the role of prematurity in development has an added confounding factor. However, a recent study using simultaneous fMRI and EEG recordings demonstrated a clear association between neuronal activity and BOLD signal during resting-state recordings in preterm infants (Arichi *et al.*, 2017). Further to this, fMRI has been used to successfully record BOLD responses to sensory stimulation in preterm infants in a number of studies (Erberich *et al.*, 2006; Heep *et al.*, 2009; T. Arichi *et al.*, 2010; Dall'Orso *et al.*, 2018). Another issue that can arise with fMRI is that to capture a clear image the participant must remain still. To achieve this in infants many studies have used sedation (Fransson *et al.*, 2007a; Heep *et al.*, 2009; Arichi, Fagiolo, Varela, Melendez-Calderon, Allievi, Merchant, Tusor, Serena J Counsell, *et al.*, 2012). However, there is some evidence that sedation may reduce the BOLD response in infants (Williams *et al.*, 2015), and in light of these studies using natural sleep during fMRI in infants have been carried out.(Smith-Collins *et al.*, 2015).

Despite these caveats, fMRI is an excellent technique for the visualisation of activity across the entire brain in a non-invasive way and provides higher spatial information than EEG. Further to this, it has successfully been used in preterm infants during both sensory stimulation (Heep *et al.*, 2009; Allievi *et al.*, 2016) and resting-state recordings (Fransson *et al.*, 2007a; Smith-Collins *et al.*, 2015). Previous investigation into sensory stimulation based fMRI in preterm infants have combined motor and somatosensory stimulation together (Erberich *et al.*, 2003; Heep *et al.*, 2009; T. Arichi *et al.*, 2010; Allievi *et al.*, 2016). Motor dysfunction is a common outcome of prematurity, with many preterm infants developing cerebral palsy (Han *et al.*, 2002; Larroque *et al.*, 2008) making it a keen area of research. However, with somatosensory processing also being affected in prematurity the development of this network alone is also of interest. Somatosensory evoked potentials using EEG are widely used in preterm infants (Taylor, Boor and Ekert, 1996a; Smit *et al.*, 2000b; Tombini *et al.*, 2009), but this technique does not provide accurate spatial information of where neuronal activity is being generated. The higher spatial information across the whole brain provided by fMRI offers an opportunity to explore the location of neuronal activation in response to somatosensory stimulation and investigate the development of these pathways in preterm infants.

Stimulation type	Subjects	Sedation?	BOLD activation	References
Passive unilateral sensorimotor – wrist movement	8 -Born 24-30 -Scanned 38-39	Chloralhydrate	5/8 – bilateral primary SMC	(Heep *et al.*, 2009)
Passive unilateral sensorimotor – hand movement	19 -Born 24+4-36 -Scanned 39-44	Chloralhydrate	10/18 - Contralateral SSC & 8/18 bilateral SSC	(T Arichi *et al.*, 2010)
Passive unilateral sensorimotor – hand movement	15 -Born 26+3-41 -Scanned 38+1-44	Chloralhydrate	15/15 - Contralateral SSC	(Arichi, Fagiolo, Varela, Melendez-Calderon, Allievi, Merchant, Tusor, Serena J. Counsell, *et al.*, 2012)
Passive bilateral sensorimotor – hand movement	42 -Born preterm -Scanned 38-49	Chloralhydrate	18/42 contralateral SSC 14/42 ipsilateral SSC and 14/42 bilateral SSC	(Erberich *et al.*, 2006)
Passive unilateral sensorimotor – wrist movement	15 -Born 29+6-39+5 -Scanned 37+6 – 43+2	Chloralhydrate	15/15 - Bilateral SMC	(Allievi *et al.*, 2016)
Passive unilateral sensorimotor – elbow movement	13 -Born 22+6 – 28 -Scanned 37+6-42+6)	Chloralhydrate	8/13- contralateral SMC	(Scheef *et al.*, 2017)

Resting-state (6 min 34s recording)	24 -Born preterm -Scanned 39+4-43+3	Chloralhydrate	Adult like networks – default mode, frontoparietal, executive control, sensory and SMC	(Doria *et al.*, 2010)
Resting-state (10 min recording)	12 -Born 24+4-27+5 -Scanned 39+1-44+2	Chloralhydrate	5 functional networks - occipital cortex, SMC, temporal-auditory, prefrontal, and partial-cerebella	(Fransson *et al.*, 2007b)
Resting-state (6 min 24s recording)	105 -Born 23+6-34+5 -Scanned 39-48	Chloralhydrate	Ex-preterm infants show alteration in adult-like functional networks compared to healthy-term infants	(Ball *et al.*, 2016)

Table 1 ***Summary of key stimulated and resting-state fMRI findings in ex-preterm infants***
Including type of stimulation, Subjects used (number, Gestation range and birth and scanning), sedation used during fMRI scanning, and significant BOLD activation found (SMC – sensorimotor cortex, SSC – somatosensory cortex

4.1.3   Study rationale

This study expands on the previous literature in sensorimotor stimulated fMRI in preterm infants. It uses a pure somatosensory stimulation in infants born before 32 weeks gestation at term equivalent age, during natural sleep fMRI acquisition. A type of pure somatosensory stimulation is vibration, also known as vibrotactile stimulation when perceived through touch. Vibrotactile stimulation has been used previously for fMRI in healthy adults in a number of studies, evoking a BOLD response in the central somatosensory network (Harrington, Wright and Downs, 2000; Golaszewski *et al.*, 2002; Chakravarty, Rosa-Neto, *et al.*, 2009; Kim *et al.*, 2014a; Choi *et al.*, 2016). Vibration responses have been found in third trimester foetuses *in utero* (Leader *et al.*, 1984), and vibrotactile stimulation is used to prevent apnea in preterm infants, as it is detected and produces a startle response (Cramer *et al.*, 2018). The detection of vibrotactile stimulation already in the preterm period and its stimulation of the somatosensory network makes it a good candidate for somatosensory stimulation in this study.

When investigating a patient population during fMRI it is important to know if changes in BOLD response are due to clinical factors or a problem with the methodology (Beisteiner, 2017). A control comparison is required to validate the paradigm design and represent a typical BOLD response. For a study with preterm infants an ideal control comparison would be a population of healthy term infant. However, this was not possible for this study, due to financial and ethical restrictions. Instead, a healthy adult population was used to validate whether the paradigm design produced a reliable BOLD activation of the somatosensory network. This mature, healthy activation pattern can then be used as a benchmark of network activity that the preterm infant data can be compared to for interpretation. Further to this a comparison within the preterm infant population can be made and fMRI findings can be statistically compared to individual clinical outcomes. In addition, previous studies have found cortical activation to sensorimotor stimulation in preterm infants (Table 1) and a maturation of this activation across the preterm period until term equivalent has been shown (Allievi *et al.*, 2016). A combination of a known healthy, mature activation pattern to this specific paradigm established through the adult validation study proposed, knowledge of the pattern of activity in similar stimulation types in preterm infants across a variety of

ages, and individual infant participants known clinical outcomes will be used to build a picture of somatosensory network development in preterm infants at term equivalent age.

The paradigm used is vibrotactile stimulation delivered the wrist in a block design, with 5 repeats. Two different lengths of stimulation and rest, and two different strengths of stimulation are used. This paradigm was selected to meet the requirements of the ex-preterm infant population and to investigate parameters that had unclear optimisation from previous literature. It was designed based on fMRI design theory (Amaro and Barker, 2006) and previous studies that successfully used vibrotactile stimulation to evoke BOLD responses in healthy adults (Harrington and Hunter Downs III, 2001; Golaszewski *et al.*, 2002; Siedentopf *et al.*, 2008; Chakravarty, Broadbent, *et al.*, 2009; Choi *et al.*, 2016). These resources presented a variety of potential design options that would be suitable, and the final designs were chosen based on their suitability for use in preterm infants.

A block design was chosen over single event stimulation, as the sensitivity to stimulation in the infants was not known. This increases the period of stimulation received, increasing the chance of activity in the somatosensory network being activated. Block designs with a total on/off cycle of 20-30s in duration have been found to be optimal in neonates (Cusack *et al.*, 2015), which is the lower end of recommended duration in adult studies (Carter *et al.*, 2008). Prolonged exposure to vibrotactile stimulation can result in desensitisation to the stimuli (Bensmaïa *et al.*, 2005). Different lengths of stimulation were trialled in the adult study (15TR and 5TR) to assess the balance between having a long enough stimulation to elicit a BOLD activation but not so long as to desensitise the receptors. Another measure taken to minimise the chances of desensitisation was that each block of stimulation had a rapid on/off (200-800ms intervals) within it, which is an approach previously used in vibrotactile fMRI studies in adults (Sanchez-Panchuelo *et al.*, 2012).

The number of repeated blocks used was limited by the length of time the infant could be in the MRI scanner and how long they would remain asleep. This project was added onto an MRI research scanning protocol that was already approximately hour long. The radiofrequency power delivered during MRI acquisition is transformed into heat within the participant's tissue (Bottomley and Andrew, 1978; Shellock, 2000). The energy absorbed per kg of body weight, the specific absorption rate, has a safety limit which may not be exceeded when scanning (Z. Wang *et al.*, 2007). This is not normally a problem in adult

participants as these effects are small, however, the small size of the infants means that this heating effect is significant and limits the scanning period they can undergo.

The wrist was chosen as the site of stimulation based on the small size of the infant and the swaddling technique used for scanning. It was determined to be the location that would give the secure attachment without irritating the infant and disrupting their sleep. The foot was considered as a stimulation site, however it was not chosen as the primary somatosensory cortex the foot representation is close to the midline of the brain (Akselrod *et al.*, 2017), and there were concerns that could make assessment of lateralisation of BOLD response in the infant brain difficult.

The frequency of 50Hz was chosen because it is mediated by both Pacinian and Meissner's corpuscles (Golaszewski *et al.*, 2002), and by covering both receptor types the chance of activation in the infants was hoped to be increased. The strength of the stimulation was trialled at two different levels in the adults. The strength of the stimulation was custom tested on each infant (see section 4.3.2) and the adult paradigm values were assigned within the ranges tolerated by the infants.

This study is the first report of delivery of vibrotactile stimulation to the wrist during fMRI acquisition. Although the paradigms used in this study have similarities to other adult vibrotactile stimulation fMRI studies – using 50Hz stimulation, delivered in a block design - the exact combination has not been previously used. Previous studies have reported a variety of different activation patterns in response to subtly different stimulation paradigms and so the changes used in this study need to be tested to reveal what sort of somatosensory network activation is produced in the healthy, mature brain. As previously stated, this will give a benchmark for expected somatosensory network activity in response to this stimulation and can be used to compare with fMRI findings in the ex-preterm infant population.

This chapter presents a feasibility study of the use of a vibrotactile stimulation fMRI protocol in preterm infants -born before 32 weeks gestation - at term equivalent age, during natural sleep, as well as a study validating the reliability of this paradigm in a population of healthy adult volunteers. The aim is to develop a protocol that can be used to investigate the functional development of the somatosensory network in the preterm infant, expanding on

previous work into sensorimotor function (Table 1), proving new evidence of the lesser explored area of somatosensory processing development in this patient population.

4.2 Methods

4.2.1 Adult validation of somatosensory stimulation fMRI paradigm

4.2.1.1 *Participants*

Eleven healthy adult volunteers (6 female, age range 24-42, mean age 30) were recruited. They were all tested as right handed based on Edinburgh handedness inventory (Oldfield, 1971). All participants gave written consent, and the experimental procedures were approved by the University of Bristol ethics committee. MRI acquisition was carried out with the guidance of the Clinical Research and Imaging Centre's (CRIC) lead radiographer, Aileen Wilson.

4.2.1.2 *Stimulation and paradigms*

Vibrotactile stimulation was delivered to the wrists using a custom non-magnetic vibrotactile stimulation device (Dancer Design, UK). The device is a piezoelectric bender, encased in a plastic housing, with a non-ferrous metal disk protruding (Figure 4.1ai). The stimulation disk was positioned on the distal wrist (Figure 4.1aii) of both hands, secured firmly but so was not uncomfortable, using surgical micropore tape (3M PLC, UK).

The stimulation device was controlled with custom Matlab (Maths works Inc., USA) code, which commanded a data acquisition board (National Instruments Corporation Ltd, UK) to deliver a 50hz sinusoidal wave voltage output to the device, resulting in two-way movement of the piezoelectric bender.

Three experimental paradigms were investigated. All were delivered in a block design, with a randomised order of unilateral right, left and bilateral stimulation repeated 5 times. During each stimulation period the device switched on and off at 200-800ms intervals, to give a 'chirping' pattern. This was done to try and minimise attenuation. Each block of stimulation was time locked to the incoming TTL pulse of the MRI scanner, which was relayed to the control room PC via a Lumina LP-400 response pad (Cendrus, USA).

For protocol A stimulation was delivered for 10 whole brain volume acquisitions (TR) with 15TR of rest between – with a 908ms TR time - with an amplitude of 5 (Figure 4.1bi).

Protocol B was 5TR of stimulation with 20TR between at an amplitude of 5 (Figure 4.1bii). Protocol C was a 10TR of stimulation with 15TR of rest, this time at an amplitude of 3 (Figure 4.1biii). Increasing the amplitude (from 3 to 5) results in a larger displacement of the piezo electric bender, delivering a stronger stimulation to the participant. Both these amplitudes (3 and 5) were suprathreshold for perception but not painful. Successful detection of stimulation was confirmed with each participant.

Protocol A was delivered to all participants, and half also received protocol B (5 subjects) and the other half C (5 subjects). The order of protocols was randomly assigned and between each protocol participants left the scanner and had a 15-minute break.
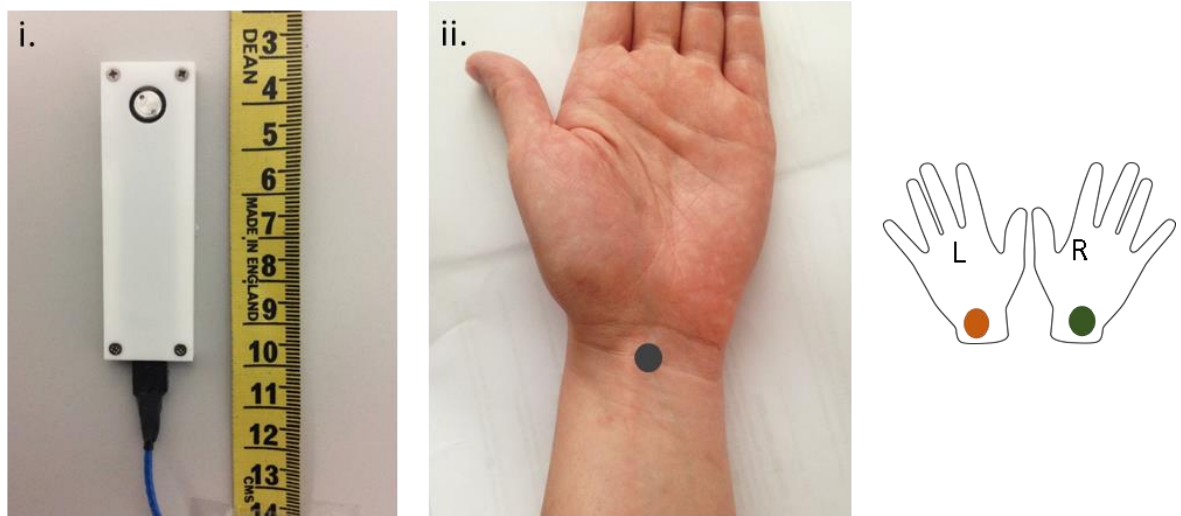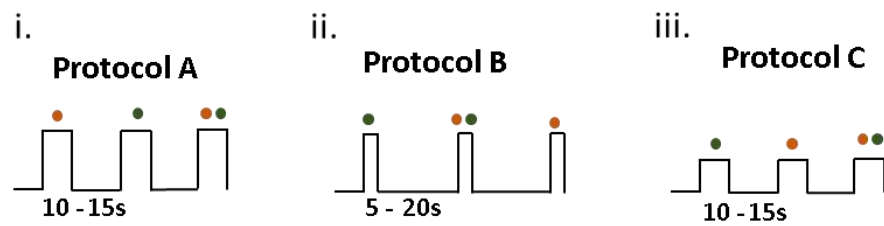
Figure 4.1 **Vibrotactile stimulation device and paradigm**
a) i. Custom design vibrotactile stimulation device. ii. Location the stimulation disk was positioned on the adult wrist (both right and left)
b) The three paradigm designs used in the adult validation study.

4.2.1.3 *Functional MRI data acquisition*

All scans were performed on a 3T Siemens Magnetom Skyra MRI scanner with a 32 channel adult head coil (Siemens, Germany), at the University of Bristol CRIC imaging centre. Ear protection was worn by all participants. Participants were instructed to lie relaxed in the scanner, with their eyes closed and arms by their side with the vibrotactile device facing up. To minimise head movement participants heads were stabilised using foam pads.

Functional MRI data were collected using the sequence reported in *Smith-Collins et al. 2015* (Smith-Collins *et al.*, 2015), with an increased field of view (FOV) to accommodate the full adult brains (multiband fast echo-planar imaging (EPI) sequence, repetition time (TR) 906ms, echo time (TE) 30ms, Flip angle 60^0, FOV 192 x 192, 30 slices 3 x 3 x 3 mm^3 voxel size, acceleration factor 3, interleaved, phase encoding direction A >> P, 400 volumes acquired for each experimental protocol). A high-resolution T1-weighted structural image of the whole brain was acquired for all participants (3D MPRAGE, TR 2300ms, TE 4.2ms, Flip angle 9^0, 192 slice, 1 x 1 x 1 mm^3 voxel size).

4.2.1.4 *Pre-processing*

fMRI images were pre-processed using Matlab (Mathworks Inc., USA) and the statistical parametric mapping package SPM8 (Wellcome Trust Centre for neuroimaging UCL, London; http://www.fil.ion.ucl.ac.uk/spm/). The first 10 functional images were discarded to ensure signal stability in analysed data. The remaining 390 images were realigned to the first scan to correct for any head movement during the session, and movement parameters detailing how much movement had occurred were produced. The adjusted images were then co-registered to the T1-weighted structural image. They were then normalised to standard space using SPM's standard EPI adult template. Then finally smoothed with a Gaussian kernel of 6mm full-width half-maximum (FWHM) to account for any small-scale variation in individual subjects. Successful spatial alignment of fMRI images to the template was visually checked in all data sets.

Statistical analysis of the fMRI data was performed using two methods, both of which have previously been used with somatosensory stimulated fMRI paradigms. First is a general linear model (Friston *et al.*, 1994) and then using multivariant pattern analysis (Haxby, 2012).

4.2.1.5.1 General linear model

Pre-processed functional data were analysed using a general linear model (with SPM8) - which is an established method of fMRI analysis (Friston *et al.*, 1995) – to estimate regions that showed changes in BOLD during stimulation. The experimental block design was used as an exploratory variable in the model, and the onset and duration of each block entered to millisecond precision. The response measured during fMRI is not a precise on/off as blood oxygenation changes are slow (Buxton, Wong and Frank, 1998). To account for this the exploratory variables were convolved with a canonical haemodynamic response function (HRF). The movement parameters obtained during realignment were included in the model as random variables, to be accounted for in model estimation but were not of interest. An additional regressor was added to temporally high-pass filter the data at 1/128hz, removing low frequency noise associated with fMRI signals Contrasts were made between rest and both right and left stimulation, using univariant t-tests on a voxel by voxel basis. Data was explored on a single subject level and two different statistical thresholds were considered, both when regional activation surpassed p < 0.001 uncorrected and when p < 0.05 family-wise corrected error (FWE). Correcting for multiple comparisons with FWE is more conservative and ensures robust results, whereas the uncorrected approach is more likely to reveal small activations. This study was to validate that the stimulation paradigm could robustly activate the somatosensory network, and although robust FWE corrected activation would be the optimum, smaller activations would also be of interest when taking the paradigm forward into infant studies. Areas of activation were labelled using the Talairach Atlas (talairach.org).

4.2.1.5.2 Multivariant pattern analysis

Another method that has been used with vibrotactile stimulation fMRI paradigms in healthy adults is MVPA (Sanchez-Panchuelo *et al.*, 2012; Kim *et al.*, 2014a). Instead of using a statistical model to estimate voxels of activation during a stimulation machine learning is used to classify the activity patterns of the brain during a stimulation condition, and then predict the condition of novel data. Unlike GLM where each voxel is considered individually, MVPA considers all voxels together and builds a classification model of the whole brain activity during a stimulation type.

For these data the toolbox PRoNTo (Schrouff *et al.*, 2013), which is compatible with SPM 8 was used. A binary support vector machine was used (Burges, 1998), and classifications run on right versus left side stimulation on individual subject data. Pre-processed fMRI data and the block design timings are input, so which scans correspond to which stimulation condition are labelled. A leave one block out cross validation method was used, where the classification is trained on the 4 stimulation blocks and then tested on the 5th. The classifications of the test blocks are compared to the true stimulation label. This process is repeated on all blocks and the percentage of accuracy can be calculated and compared against change (50% in a binary comparison).

4.2.2 Feasibility study for somatosensory stimulated fMRI in preterm infants

4.2.2.1 *Participants*

Participants were recruited from the Neonatal Intensive Care Unit at St Michaels Hill hospital, Bristol. Four preterm infants (two male) born at <32 weeks gestation underwent MRI acquisition at term corrected ages (38-41 weeks gestation). The exclusion criteria for the study included congenital abnormalities, known genetic conditions or required surgical interventions during the preterm period. No infants were receiving additional support at the time of scanning. All parents gave informed written consent, and the experimental procedures were approved by the NHS research authority ethics committee. Neonatal scanning was carried out with the assistance of a neonatologist, Dr Adam Smith-Collins and the CRIC's lead radiographer, Aileen Wilson.

Infants received the same vibrotactile stimulation as the adult volunteers. The stimulation device (Figure 4.1ai) was placed on the wrist and was secured using a neonatal arm splint. Their arms where placed in a comfortable position and the infants were swaddled in a blanket to keep them secure, minimise movement and facilitate sleep.

The same 50hz sinusoidal wave vibration was delivered to the device, in a 10TR of stimulation with 15TR of rest block design, with the same intermittent 'chirping' during stimulation. The block design was unilateral stimulation to right and left in a random presentation repeated 5 times. The amplitudes used were assessed for each infant individually before the experimental procedure. A test stimulation was delivered to both wrists and the amplitude gradually increased from 0 until an increase in heart rate was seen, without waking. This was used as an indication the infant had experienced the sensation and amplitude was set at this point for the experimental protocol.

4.2.2.3   *Functional MRI data acquisition*

Scanning was performed during natural sleep, with no sedation. Infants were fed prior to scanning and settled in the preparation room. Continual ECG and blood saturation were acquired using MRI compatible monitoring equipment (Invivio Expression, USA) with the Biopac MP150 system and AcqKnowledge software (Biopac Systems, USA). Using this data infants were continuously monitored by a neonatologist, for periods of oxygen desaturation or distress. Ear protection was used in all infants, with mouldable putty in the external ear canal (Affinis, Switzerland) and external ear covers place over ('minimuffs', Naurus, USA). Movement was reduced by placing participants in a custom made vacuum moulding cushion, secured around their body and head.

As with the adult study all scans were performed on a 3T Siemens Magnetom Skyra MRI scanner with a 32 channel adult head coil (Siemens, Germany) at the CRIC, and fMRI data was collected using the sequence reported in *Smith-Collins et al. 2015* (TR 906ms, TE 30ms, Flip angle 60^0, FOV 160 x 160, 30 slices 2.5 x 2.5 x 2.5 mm^3, Acceleration factor 3,

interleaved, phase encoding direction A >> P, 400 volumes acquired). All infants had high resolution T2-weighted structural images acquired according to the institution's clinical neonatal MRI protocol.

4.2.2.4 *Pre-processing*

Functional MRI images were pre-processed using SPM8 (Wellcome Trust Centre for neuroimaging UCL, London; http://www.fil.ion.ucl.ac.uk/spm/). The first 10 functional images were discarded to ensure signal stability in analysed data. The infant's head is not always in a central position and to standardise for any rotation the remaining functional images were coregistered to an infant brain template (Shi *et al.*, 2011). Then the open source Matlab toolbox ArtRepair (Centre for Interdisciplinary Brain Science Research, Stanford University) was used to remove any non-brain tissue from the images. To correct for any small head movements that occurred during the image series functional images were realigned and unwarped so the positions matched throughout the whole series. The structural T-2 weighted image was then also coregistered to the infant template, so that its orientation matched the adjusted functional scans. Functional images were then co-registered to the T2-weighted structural image for further alignment. Functional scans were then smoothed with a Gaussian kernel of 4mm FWHM. Successful spatial alignment of fMRI images to the template was manually checked in all data sets.

4.2.2.5 *Analysis*

Infant fMRI data was analysed using GLMs as described for the adult study.

4.3 Results

The aim of this study was to assess whether peripheral vibrotactile stimulation during fMRI acquisition was feasible in non-sedated preterm neonates at term equivalent age, and to validate the paradigm by assessing whether it produced a robust activation of the somatosensory network in healthy adult volunteers.

4.3.1 Healthy adult paradigm validation

11 healthy adult volunteers were presented with a vibrotactile stimulation to their left and right wrist in a block design paradigm during fMRI acquisition. This study was designed to assess whether this paradigm reliably activated the somatosensory network. This is an important step in designing a protocol for preterm infants as there needs to be a known response in the healthy mature brain that differences in the infant population can be compared to. Activation of the somatosensory network in response to vibrotactile stimulation was investigated on an individual subject level.

4.3.1.1 *Univariant GLM analysis*

The presence of significant clusters of activity in the key anatomical areas of the somatosensory network (Jones and Powell, 1970; Venkatesan *et al.*, 2014) were identified on a single subject level for all 3 protocols, for both right and left wrist stimulation. The area of most interest is the contralateral post-central gyrus (S1), which is the primary sensory area of tactile stimulation with projections from the periphery through subcortical relays to the cortex. In protocol A, with right wrist stimulation, 7 out of 11 participants had significant activity in the contralateral S1, and 5/11 had activation of the ipsilateral S1 region (Figure 4.2a). Between 1 and 6 participants also show activity in other areas of the somatosensory network (Figure 4.2a). Figure 4.2b shows example activity clusters from a participant that showed contralateral S1 activity (i) and one that didn't (ii) - both overlaid onto the participants T1-weighted structural scan. With left wrist stimulation 3 out of the 11 participants had activation of the contralateral S1, and 3 of the ipsilateral S1, with 1-4 showing activity in other areas of the somatosensory network (Figure 4.2c). Figure 4.2d

shows examples of successful activation of the contralateral S1 (i) and a participant with no activation (ii). Although the participant in Figure 4.2dii does not have S1 activation, they do show bilateral activity in the superior temporal gyrus (S2).

Protocol B and C show similar results to protocol A, with only some participants having activation of contralateral S1 and other areas of the somatosensory network. Protocol B resulted in 2/5 participants showing activation of S1 with right side stimulation and 3/5 with left. In protocol C it was only 2/5 with right side and 1/5 with left wrist stimulation that result in activation of S1 (Figure 4.3). Cortical activation was seen in all participants to some stimulation, but no one showed robust somatosensory network activation to all stimulation presentation.
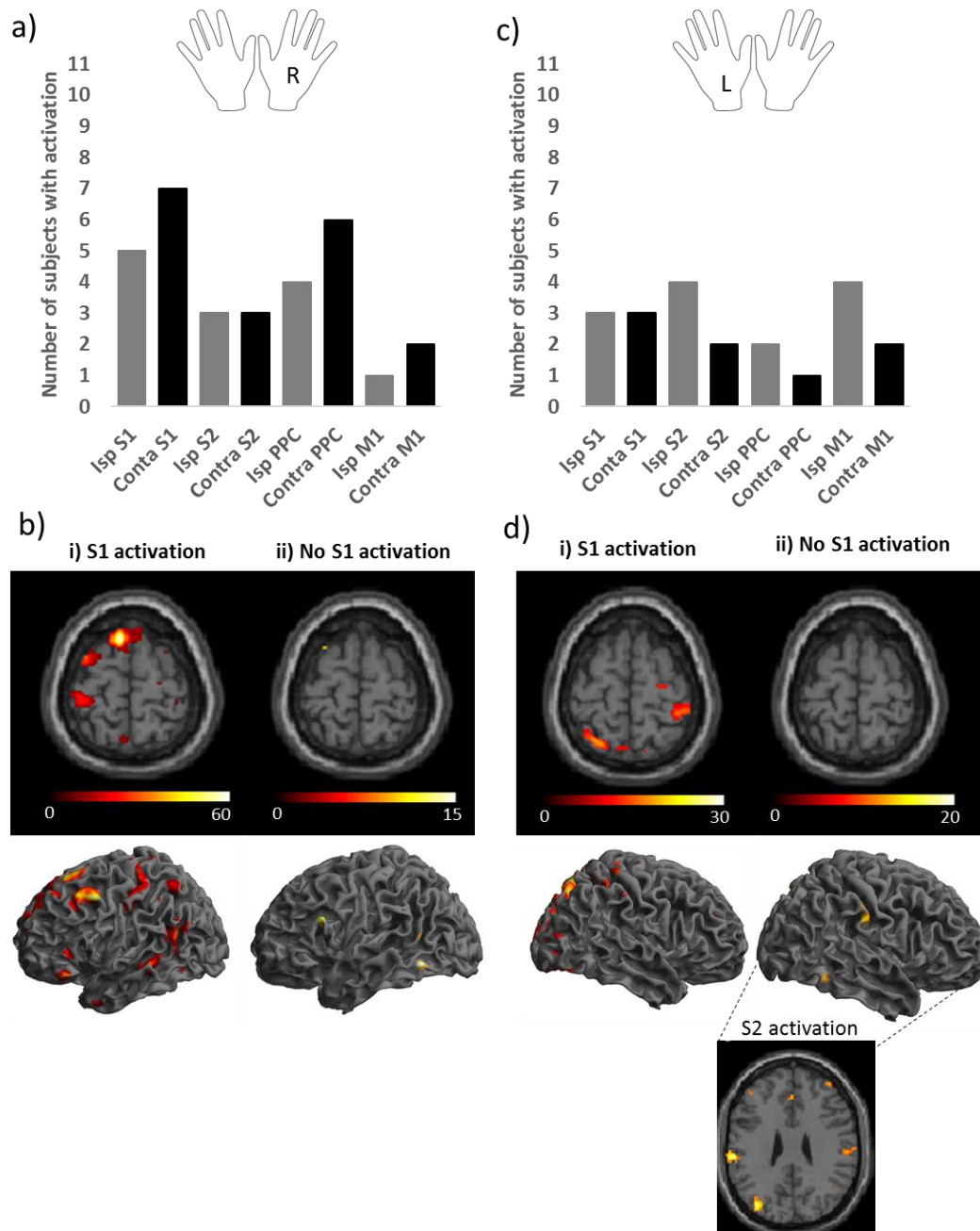
*Figure 4.2 **Activation patterns of protocol A***

  *a) The number of participants that showed significant activation in the areas of the
       somatosensory network when presented with right wrist vibrotactile stimulation.*

  *b) Example activation clusters from right side stimulation, overlaid on participants T1-weighted
       anatomical scan of i) S1 responder and ii) non-responder.*

  *c) Number of participants that showed somatosensory network activation in response to left
       side stimulation.*

  *d) Examples of activation clusters from left side stimulation of i) S1 activity and ii) no S1 activity,
       but bilateral activation of S2.*

  Colour scale is T-scores at uncorrected p < 0.001
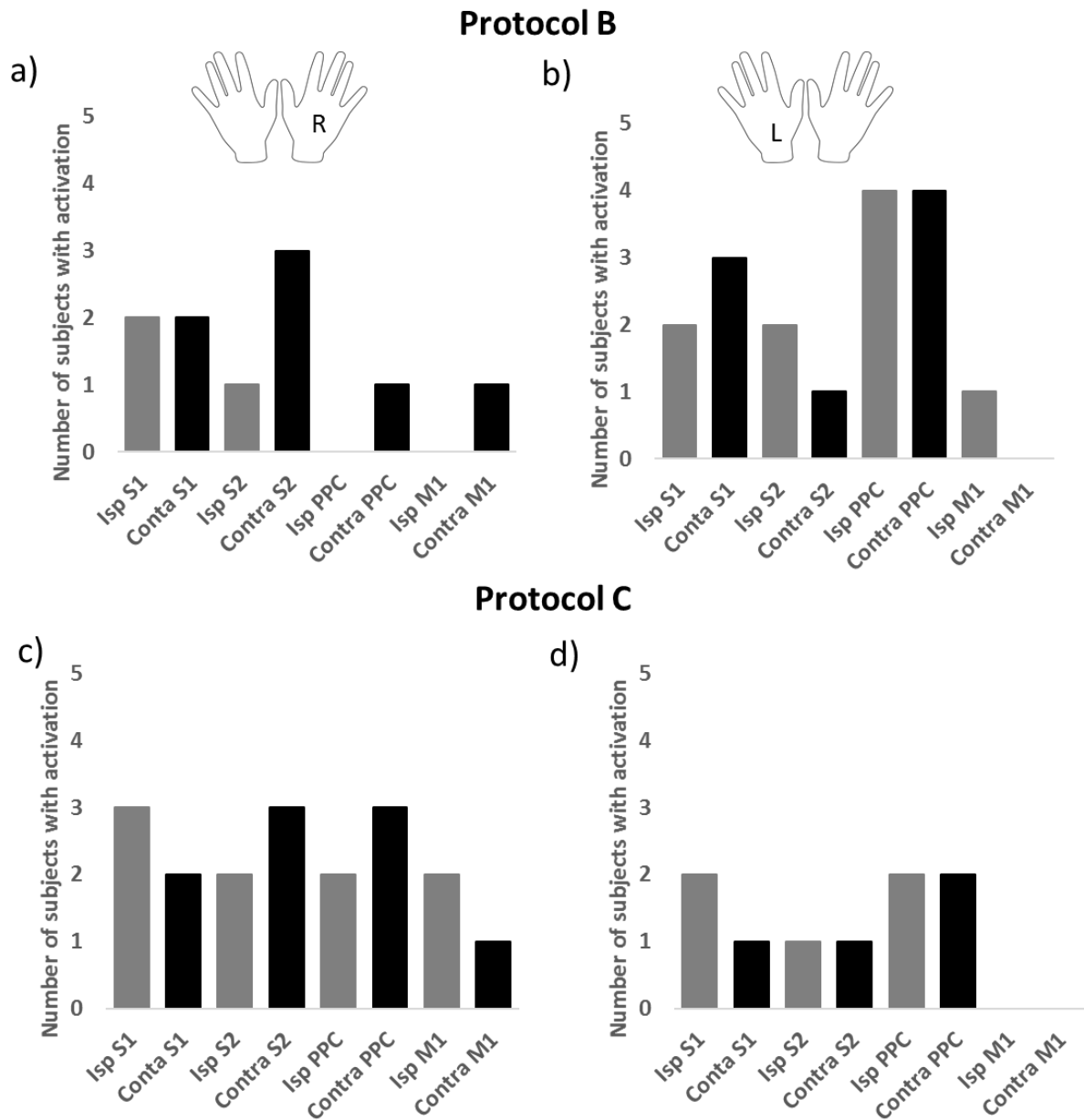
Figure 4.3 **Activation patterns of protocol B and C**
The number of participants that showed significant activation in the areas of the somatosensory network when presented with protocol B right wrist (a), and left wrist (b), and protocol C right wrist (c) and left wrist (d).

Significant changes in BOLD signal in the somatosensory network in response to vibrotactile stimulation have previously been reported in healthy adult volunteers (Golaszewski *et al.*, 2002; Siedentopf *et al.*, 2008; Chakravarty, Rosa-Neto, *et al.*, 2009; Sanchez-Panchuelo *et al.*, 2012; Kim *et al.*, 2014a). These studies use a variety of paradigm designs and locations of stimulation. Varied activation patterns in secondary and association areas were recorded, but all found activation of contralateral S1. Some possible differences between this study and previous reports, that could be addressed in analysis were adaption to the stimulation throughout the stimulation block and a non-canonical HRF.

With prolonged somatosensory stimulation it is possible that adaption could have occurred and a cortical response diminishing or disappearing over the block. If this had occurred including the whole stimulation period in the GLM could mask an initial activation that occurred immediately after the onset of stimulation. To address possible desensitisation a new GLM was run on the fMRI data, with the same parameters as before except that only the first 2s of the stimulation block were considered and the rest excluded from the analysis. This new analysis resulted in the same mixed results as the GLM on the full stimulation blocks. Some participants showed activation of areas of the somatosensory network but not all.

The on/off 'chirping' during blocks was implemented to reduce potential adaption. This non-continuous stimulation would have resulted in a non-continuous activation of the somatosensory network. The change is blood oxygenation measured by fMRI is a response to neuronal activation. This non-continuous activation may have resulted in a non-standard haemodynamic waveform, such as stacking of canonical waveforms on top of one another as another activation begins before the previous has returned to baseline. To investigate this another GLM was run this time with a Fourier set HRF. This makes less assumptions about the shape of the wave form than the canonical HRF (Gitelman *et al.*, 2003; Lindquist *et al.*, 2009) initially used and so may be more suitable for this data set. This new analysis again resulted in a mixture of somatosensory activation areas in participants, with not all individuals showing activation of the contralateral S1.

Multivariant pattern analysis

GLM is not the only method of fMRI analysis used for sensory stimulation paradigms. Kim *et al.*, 2014 found that multivariant pattern analysis gave more robust discrimination between individual finger vibrotactile stimulation than a unilateral GLM approach. Rather than look for significant changes in BOLD signal on a voxel level it uses supervised machine learning to try and classify a pattern of activity across the whole brain that is representative of the type of stimulation delivered. These classifications are tested on blind experimental data to see how accurate they are. With a two-way comparison (left vs right) chance level is 50% meaning a reliable classifier must be significantly above this to be reliable for analysis.

As with univariant GLM analysis not all subject's data produced an above chance decoding accuracy with MVPA. With protocol A the average decoding accuracy was 45.5±6.4%, with 4/11 participants having results above chance (Figure 4.4a). With protocol B 3/5 participants had decoding over chances, with a group average of 51.5±8.6% (Figure 4.4b) and with protocol C 3/5 participants had values over chance, with an average of 50.7±8.4% (Figure 4.4c).
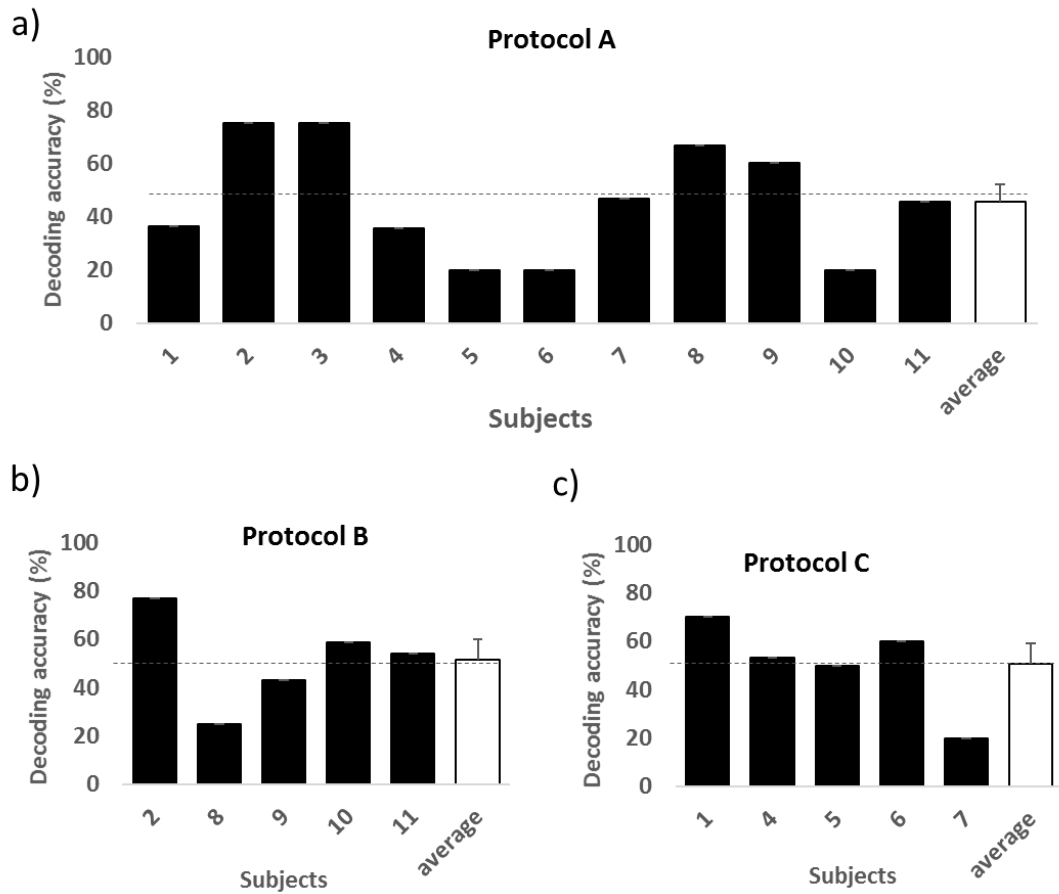
Figure 4.4 **Multivariant pattern analysis decoding accuracies**
Decoding accuracies for each participant from protocol A (a), B (b) and C (c). Chance level marked by the dashed line (50%)

Alongside validating the stimulation paradigm in healthy adults, the feasibility of delivering vibrotactile stimulation to non-sedated preterm infants during fMRI acquisition was tested. A neonatology research team at the University of Bristol have established an fMRI acquisition method for use in non-sedated neonates (Smith-Collins *et al.*, 2015) but delivery of stimulation during it had not been previously carried out. 4 preterm infants were included in this feasibility study, details in Table 2.

Subject ID	Sex	Gestation length (weeks)	Birth Weight (g)	CGA at MRI (weeks)	Days in ITU	Proven Sepsis	Clinical notes from MRI
M209	F	26.7	784	38	8	-	Mild prominence of trigones. Otherwise normal
M212	F	27.4	955	37.4	13	-	Mild, non-cystic PVL with some WM loss
M213	M	27.4	1132	37.4	27	-	Normal
M219	M	26.4	943	38.6	38	+	Bilateral IVH

*Table 2 **Description of infant study population**
 [CGA = corrected gestational age; PVL = periventricular leukomalacia; WM = white matter; IVH = intraventricular haemorrhage]*

Initial concerns about waking the infants with stimulation were addressed by customising the strength of stimulation for each of them. The heart rate of the infant was monitored as a stimulation was presented to an individual wrist staring at zero intensity and increasing in strength. The stimulation intensity at which an increase in heart rate was observed was an indicator of detection of the stimulation (without waking) and this strength was then used for the experiment. The strength of stimulation varied between infants. M209 received a strength of 2 (lower than the adult study tested), M212 and M213 received a strength of 4, and M219 a strength of 5, which are all in the range tested in adults. All infants slept through the scanning paradigm, and the functional image series was suitable for analysis.

Analysis was carried out as with the adult study, using GLM to find significant BOLD changes on a voxel by voxel basis. Significant clusters of activation (uncorrected p < 0.001) were overlaid on participants T2-weighted anatomical image and areas of activation were visually assessed for location. Possible activation of the primary somatosensory region in some conditions were found in this preterm infant population. M209 had significant activation of both left and right post-central gyrus when stimulated on the right wrist (Figure 4.5a), but no somatosensory network activity with left or bilateral stimulation. M212 had activity in the right primary somatosensory region when stimulated on the right wrist and on the left when stimulated bilaterally (Figure 4.5a) but showed no activation anywhere when stimulated on the left side. M213 had bilateral post-central gyrus activity when stimulated on the left wrist, and on the right side with left wrist stimulation, but no somatosensory network activity with right side stimulation (Figure 4.6a). M219 had right primary somatosensory region activation with all 3 stimulation types (Figure 4.6b).

This was an initial assessment to validate that the data acquired from these infants was of high enough quality to analyse. Further exploration of the data was not carried out once the adult validation study found this stimulation paradigm did not produce robust somatosensory network activations.
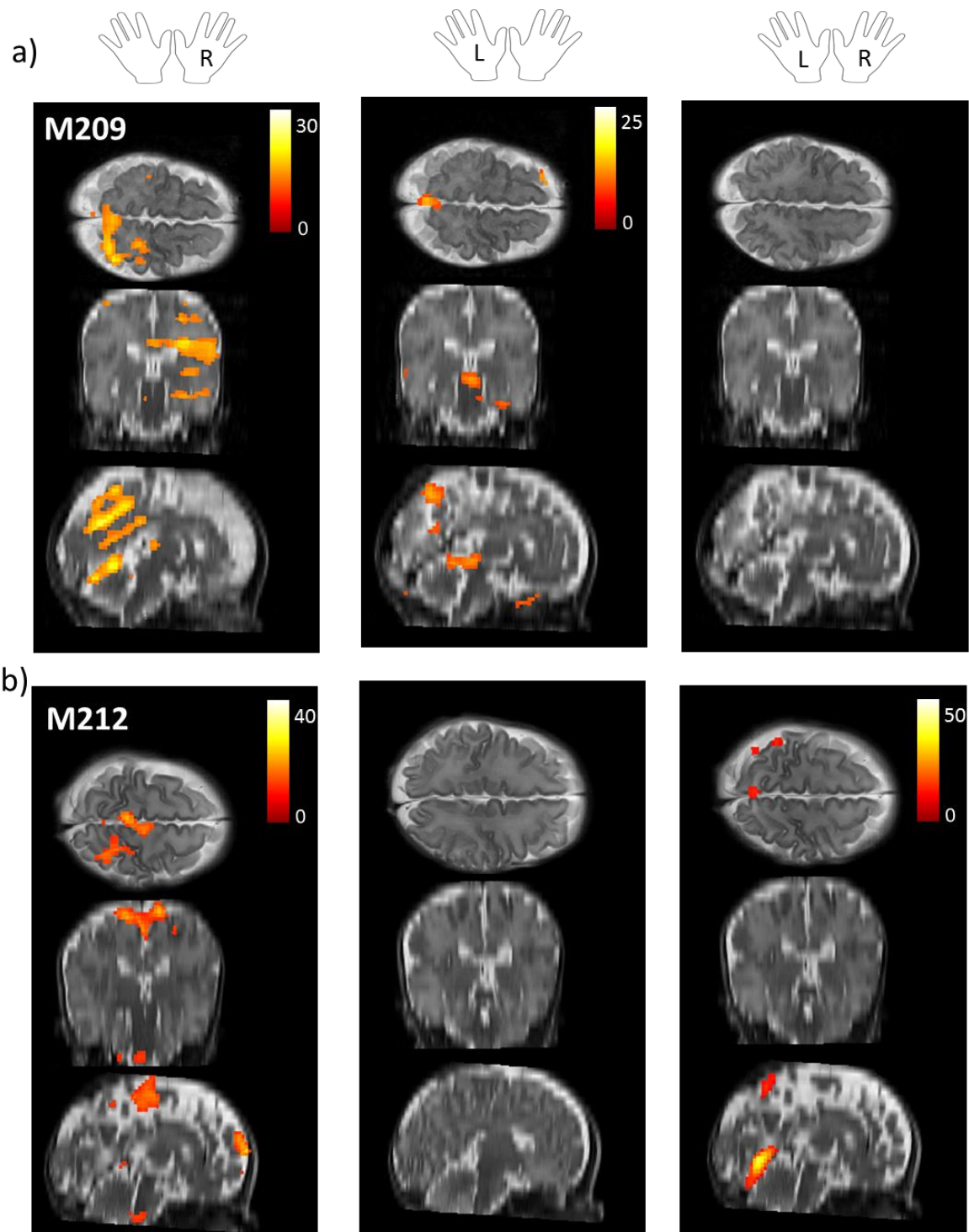
Figure 4.5 **Activation patterns of M209 and M212**
Significant clusters of activation for each stimulation for infant M209 (a) and M212 (b) overlaid on individual T2-weighted anatomical scan. Colour scale is T-scores at uncorrected p < 0.001
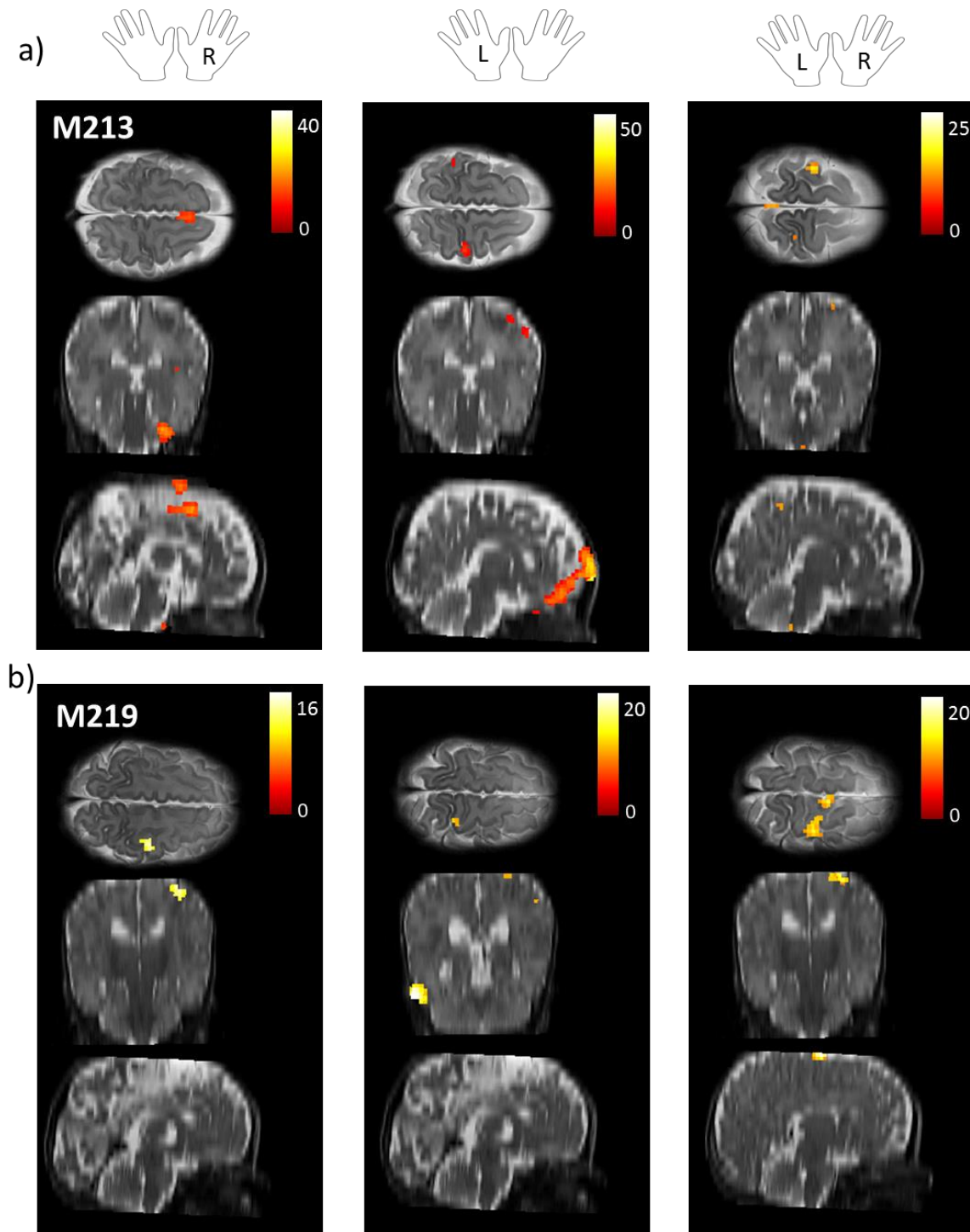
Figure 4.6 **Activation patterns of M213 and M219**
Significant clusters of activation for each stimulation for infant M213 (a) and M219 (b) overlaid on individual T2-weighted anatomical scan. Colour scale is T-scores at uncorrected p < 0.001

4.4 Discussion

This study presents for the first time the delivery of an MR compatible automated vibrotactile stimulation during fMRI acquisition in preterm infants. The aim of this study was to develop a somatosensory stimulation paradigm that could be used in preterm infants during natural sleep to explore evoked functional activity in the sensory pathway. To test the protocol design two steps were taken, to confirm that stable fMRI images could be acquired in preterm infants and that the stimulation paradigm used activated the somatosensory network in healthy adults.

4.4.1 Healthy adult validation study

The stimulation paradigm designed for use in preterm infants was delivered to 11 healthy adult volunteers. The aim of this validation study was to find a stimulation paradigm that produced reliable BOLD changes in the somatosensory network in all participants. Without a reliable activation in healthy adults, it would not be possible to make conclusions about a lack of activity in preterm neonates as changes in BOLD response could be due to methodological problems. The parameters that could be optimal for this vibrotactile stimulation were not known so 3 different protocols were tested, with adjustments to strength and duration of stimulation (see method 4.2.1.2).

The 3 paradigm designs used on the healthy adult volunteer population did not produce a reliable BOLD response in the somatosensory network. Previous studies using vibrotactile stimulation have reported robust BOLD activations in the primary somatosensory cortex, as well as a variety of other activation patterns with paradigm and subject variations. There are several variations in paradigm design that could explain the difference in result in this study compared to previous findings.

4.4.1.1 *Attention*

In the infant study participants were scanned during natural sleep. This meant that no attention to the stimulation could be accounted for. To try and create a similar

environment, adult participants were asked to lie quietly in the scanner with their eyes closed – again with no account for attention to stimulation given. In a debrief after the scanning sessions all the participants in this study reported being 'relaxed' or 'sleepy' during the experiments.

 It has been previously reported that attention during tactile stimulation increases somatosensory network activity (Johansen-Berg *et al.*, 2000; Meador *et al.*, 2002; Young *et al.*, 2004; Sanchez-Panchuelo *et al.*, 2012). Several studies using vibrotactile stimulation during fMRI in health adults have used tasks to maintain attention (Chakravarty, Rosa-Neto, *et al.*, 2009; Nordmark, Pruszynski and Johansson, 2012; Kim *et al.*, 2014b; Choi *et al.*, 2016). However, it has also been found the S1 activation evoked by fingertip vibrotactile stimulation was not different if the sensation was attended to or not (Burton, Sinclair and McLaren, 2008). Other studies have also reported robust S1 activity to vibrotactile stimulation when the participant was instructed to just lay quietly in the scanner with no further instruction on attending to the stimulation (Golaszewski *et al.*, 2002; Siedentopf *et al.*, 2008).

4.4.1.2   *Block design*

A general rule of fMRI stimulation paradigms is that block designs are optimised for detecting activity and event related designs are better for measuring precise amplitude and timing of response (Glover, 2011). In previous adult vibrotactile studies both block design (Golaszewski *et al.*, 2002; Chakravarty, Rosa-Neto, *et al.*, 2009; Sanchez-Panchuelo *et al.*, 2012; Choi *et al.*, 2016; J. Kim *et al.*, 2016; Sanchez Panchuelo *et al.*, 2018), and single event stimulation paradigms have been used (Golaszewski *et al.*, 2006; Burton, Sinclair and McLaren, 2008; Siedentopf *et al.*, 2008), all finding activation of S1 in response to stimulation in participants. Given that single event stimulation could reliably activate the central somatosensory network it is unlikely that the block used for this study were too short.

Alternatively, they could have been too long resulting in desensitisation (Bensmaïa *et al.*, 2005). The time course of desensitisation in Meissner and Pacinian corpuscle receptors have been reported as 13.2s and 28.1s, and the recovery of them as 26.6s and 29.6 (Leung *et al.*,

2005). All protocols tested in this study have blocks of stimulation shorter than these timings (see section 4.2.1.2). Previous studies have also reported BOLD responses following blocks of 30s (Choi *et al.*, 2016) and 40s (Golaszewski *et al.*, 2002) of stimulation. To investigate within block adaption analysis was carried out on the first 2s of stimulation alone, treating the paradigm more as a single event stimulation. This did not result in BOLD response patterns in the somatosensory network of all the participants either. Desensitisation was a factor considered when designing the protocol and an on/off jitter was assigned to the blocks. This approach has previously been taken with vibrotactile stimulation, and produced reliable BOLD responses (Sanchez-Panchuelo *et al.*, 2012; Sanchez Panchuelo *et al.*, 2018).

The number of times the blocks of stimulation were repeated in this study was limited by the amount of time the infants could spend in the scanner. The number of repeats chosen (5 for each side) is within the range previously used for adult vibrotactile stimulation, and studies using fewer repeats have produced robust activation patterns (Golaszewski *et al.*, 2002; Kim *et al.*, 2014a). Chakravarty, Rosa-Neto, *et al.*, 2009 specifically aimed to develop a paradigm that kept the acquisition time short for use clinically in adults. They delivered a 40-50hz vibrotactile stimulation to the whole hand in a 15s stimulation followed by 15s rest block design, repeated 4 times, and found that BOLD activation of S1 in all participants was present from the first block of stimulation, with stronger responses seen over repeated blocks. This suggests that it is possible to obtain robust activation with minimal repetition of stimulation.

4.4.1.3 *Location of stimulation*

A popular location for vibrotactile stimulation in adults is the finger-tips (Briggs *et al.*, 2004; Burton, Sinclair and McLaren, 2008; Nordmark, Pruszynski and Johansson, 2012; Kim *et al.*, 2014a; Choi *et al.*, 2016; J. Kim *et al.*, 2016; Sanchez Panchuelo *et al.*, 2018), and the palm of the hand (Chakravarty, Rosa-Neto, *et al.*, 2009) and the sole of the foot (Golaszewski *et al.*, 2002; Siedentopf *et al.*, 2008) have also been used - all produced robust activation of somatosensory cortex. The use of the wrist has not previously been reported. The area stimulated is in the location of the median nerve, which has been used as an area of

stimulation during fMRI with direct nerve stimulation using electrodes (Manganotti *et al.*, 2009; Xue *et al.*, 2009; Ai *et al.*, 2013). However, it may be that the stimulation delivered in these experiments was not strong enough to reliably activate the median nerve as electrical stimulation.

4.4.1.4 *Frequency and strength of stimulation*

Previous studies have used a variety of stimulation frequencies. in the flutter/Meissner corpuscle range (under 50Hz) (Sanchez Panchuelo *et al.*, 2018) , vibration/Pacinian corpuscle (over 50Hz) (Siedentopf *et al.*, 2008; Kim *et al.*, 2014a; Choi *et al.*, 2016) and at 50Hz (Golaszewski *et al.*, 2002; Chakravarty, Rosa-Neto, *et al.*, 2009). Some have also tested a range of frequencies to investigate differential activations (Briggs *et al.*, 2004; Nordmark, Pruszynski and Johansson, 2012; J. Kim *et al.*, 2016). All these previous studies have reported robust S1 activation with these ranges of frequencies.

The strength of stimulation used in different studies is difficult to compare because the stimulation devices and methods of attachment to participants are different. In this study adult participants were asked to confirm that they could perceive the stimulation. A study using Von Frey filament tactile stimulation found that stimulation near perception threshold did not produce BOLD responses in the contralateral S1 for healthy adult volunteers, and that even at a suprathreshold strength only 5/12 participants had S1 activation, with all participant showing activation once the strength of the stimulus was painful (Ramirez Garzón, Pasaye and Barrios, 2014). Although this study does not use vibrotactile stimulation it suggests that the strength of stimulation plays a role in the central network BOLD activation. In addition, increasing strengths of vibrotactile stimulation to the fingertip (at 20Hz) resulted in increased BOLD activation of contralateral S1 in healthy adults (Nelson *et al.*, 2004). In contrast, another study, comparing two different amplitudes of 100Hz vibrotactile stimulation to the foot found that a smaller amplitude resulted in a stronger activation of contralateral S1 (Siedentopf *et al.*, 2008). This differing result make it unclear exactly how strength of vibrotactile stimulation affects the activation of the somatosensory network, but importantly all the stimulation strengths in these 2 previous studies resulted in contralateral S1 activation in all participants (Nelson *et al.*, 2004; Siedentopf *et al.*, 2008).

The strength stimulation in this study was limited because a minimal stimulation strength for use in infants was desired. It may be that the strength of the stimulation was too small and that a large amplitude would have resulted in more reliable activation of the somatosensory network.

One other previous study using vibrotactile stimulation fMRI in healthy adults did not did not find activation of S1 in all participants (Harrington and Hunter Downs III, 2001). This study delivered vibrotactile stimulation through a device held between the thumb and index finger, a strength perceivable by participants at both 35Hz and 150Hz frequency. Participants were asked to lay with their eyes open, and the stimulation was delivered in a block design of 19.2s (with 35s rest between) with 5 repeats. Using standard GLM analysis 7/8 participants were found to have S1 activation with 35Hz stimulation and 4/8 with 150Hz. This paradigm design is similar to the ones used in this study, and many of the components of both studies overlap with protocols that resulted in BOLD activation in S1 in all participants.

The exact reason that the paradigms used in this study did not produce activation of S1 in all healthy adult participants tested cannot be known for sure, but there are several factors that are candidates. Although the exact paradigm used here has not previously been studied, many of the parameters are the same as other studies. There are some possible adjustments that could be made to the paradigm design to see if more reliable activation patterns could be produced. The location of stimulation was chosen as it was a suitable location for attaching the stimulation probes in the preterm infants. Another location that could be suitable for attachment of the probes is the sole of the infant's foot. Robust cortical BOLD activation has been found in adults with foot stimulation and so this may be a more suitable stimulation site for future experiments (Golaszewski *et al.*, 2002; Siedentopf *et al.*, 2008). Another option would be to increase the stimulation strength to the wrist. If the sensitivity of the area is less than that of the hands and feet, which have previously used in adult studies, a stronger stimulation may produce more cortical activation. The strength was initially kept low as there was concerns of waking the infants, but all 4 infants tested in the feasibility study slept well through the protocol and so an increase stimulation strength may be possible.

The area of the wrist stimulated is over the location of the median nerve, and electrical stimulation of this nerve is often used to induce SEPs in infants (Taylor, Boor and Ekert, 1996b; Pihko and Lauronen, 2004; Nevalainen *et al.*, 2014). This method of stimulation could be another alternative to use in this fMRI study in future iterations. As well as being known to stimulate a cortical response in preterm infants, electrical stimulation of the median nerve has been shown to stimulate reliable BOLD responses in healthy adults (Xue *et al.*, 2009) making it a good candidate for an ex-preterm infant fMRI study. However, this type of stimulation is moving away from pure somatosensory as the median nerve is the major peripheral nerve of the arm and transmits information for pain and motor function as well as tactile. I believe the stimulation details are the likely candidate for the failure of reliable somatosensory network activation, as the paradigm block design timings and lengths have been used previously in healthy adults with different stimulation locations and successfully produced robust cortical activation. Going forward testing these different stimulation alterations (location, strength and type) would be necessary to produce reliable adult validation of the paradigm. Establishing a reliable paradigm would then allow a full cohort of ex-preterm infants to be collected and their somatosensory network function to a pure somatosensory stimulation could be investigated.

4.4.2   Infant feasibility study

This study demonstrates that it is possible to deliver vibrotactile stimulation to non-sedated preterm infants during fMRI acquisition. Infants were placed in the MRI scanner during natural sleep and stimulation was delivered to their wrists. All 4 infants tested continued to sleep throughout the protocol and stable fMRI images were obtained (Figure 4.5 & Figure 4.6). Positive BOLD activation was found in all infants, including activity in areas of the somatosensory network. Although the stimulation paradigm used requires some adjustments to produce reliable network activation in a healthy population, this feasibility study shows that stimulation fMRI can be successfully acquired in an ex-preterm infant population. These results provide useful information that could be used in combination with an altered paradigm design to investigate the development of the somatosensory pathways in preterm infants.

Previous work has been done to investigate sensorimotor activation in sedated ex-preterm infants (Table 1) and this study would expand upon this body of work by investigating the response to somatosensory stimulation in non-sedated ex-preterm infants. It is thought that sedation may impact the BOLD response in infants (Williams *et al.*, 2015) and so now that a method to scan non-sedated infants during sleep has been established it will be valuable to collect data in this more natural state. Somatosensory and motor processing is highly interconnected and both networks are developing during the preterm period (Kostović and Jovanov-Milosević, 2006). This future experiment would further expand on the existing work into the development of the sensorimotor network by looking at the responses to somatosensory activity in isolation of motor function. Motor dysfunction is a well-known outcome of preterm birth (Bax *et al.*, 2005; Allin *et al.*, 2006; Goyen, Lui and Hummell, 2011), and although somatosensory processing changes have been found in children following preterm birth (Bart *et al.*, 2011; Chorna *et al.*, 2014; Cabral *et al.*, 2016) it is a relatively understudied area. This future study using somatosensory stimulation during fMRI would increase our understanding of the development of somatosensory processing during the preterm period. Going forward, further follow-up investigation of somatosensory function in infants scanned could be used to correlate cortical functional activity observed during fMRI in the neonatal period with long-term developmental outcomes.

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