

Effects of Early-Life Stress on AMPA Receptors in the Auditory Cortex

by

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Abstract

Critical period (CP) plasticity in the auditory cortex (A1) has been known to be crucial for both functional brain development and cognitive function. Impaired A1 development during a CP for tonotopic mapping has been implicated in many neurological disorders of learning and memory, including Autism. Our recent results have shown a critical role for α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) in the auditory CP for tonotopic mapping. Here, we aim to determine if early-life stress (ELS) during rapid synaptic development affects the function of AMPARs required for normal CP plasticity. ELS was induced at P3-15 in a c-Fos based transgenic mouse model. Using whole-cell patch-clamp recordings, we recorded pyramidal cells in layer IV of A1 to measure AMPAR function and the maturation of glutamatergic synapses in P12-15 mice. We found that AMPAR functional maturation is highly correlated to the opening of A1 tonotopic CP plasticity during normal development. We further identified that ELS selectively activated a subpopulation of A1 pyramidal neurons as evidenced by selective activity-dependent green fluorescent protein tagging. Interestingly, while ELS did not cause significant changes in AMPAR function in overall randomly sampled neurons, ELS activated neurons showed enhancement of AMPAR function compared to non-activated neurons. These results provide a potential synaptic mechanism following exposure to a stressor during a CP of brain development and might identify novel strategies to modulate ELS-induced neurodevelopmental impacts.

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Abbreviations

A1	Primary auditory cortex
AC	Auditory cortex
ACSF	Artificial cerebral spinal fluid
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPA	AMPA receptor
AP	Action Potential
ASD	Autism Spectrum Disorders
ATP-Mg(Na₂)	Adenosine 5'-triphosphate magnesium salt
BSA	Bovine Serum Albumin
CA1	Region of the hippocampal circuit
CaCl₂	Calcium chloride
CORT	Corticosterone
CPs	Critical periods
DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELS	Early-life stress
EPSC	Excitatory post-synaptic current
eEPSC	Evoked EPSC
sEPSC	Spontaneous EPSC
GABA	γ -aminobutyric acid
GFP	Green fluorescent protein
GluA1,2,3,4	AMPA subunits
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic-pituitary-adrenal axis
KCl	Potassium chloride

LTP	Long-term potentiation
MgCl₂	Magnesium chloride
MgSO₄	Magnesium sulfate
mPFC	Medial pre-frontal cortex
MS	Maternal separation
NaCl	Sodium chloride
NaHCO₃	Sodium bicarbonate
NaH₂PO₄	Sodium phosphate monobasic
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NGS	Normal Goat Serum
PB	Phosphate buffer
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PND or P	Post-natal day
PTX	Picrotoxin
PV-cell	Parvalbumin-positive fast-spiking interneuron
QX-314 chloride	N-Ethylidocaine chloride
SHRP	Stress hypo-responsive period
TEA-Cl	Tetraethylammonium chloride

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Introduction

Throughout infancy and early childhood, the developing brain is defined by critical periods (CPs) of heightened synaptic plasticity, rapid learning, and synaptogenesis (Jeanmonod, Rice, & Van der Loos, 1981). These CPs tend to last a short amount of time and occur early in postnatal development, creating a “sensitive period” of increased susceptibility to alterations by experience (Jeanmonod et al., 1981). During these CPs the immature brain undergoes an intricate process of adapting itself to the surrounding environment, in turn, creating long-lasting functional connections (de Villers-Sidani, Chang, Bao, & Merzenich, 2007). As a result, the brain is increasingly susceptible to adverse experiences, such as early-life stress (ELS), during this period of time. There is growing evidence to indicate that negative experiences during CPs of development directly impact behaviour and increase the risk for psychiatric disease throughout the lifespan (Derks, Krugers, Hoogenraad, Joels, & Sarabdjitsingh, 2016; Pechtel & Pizzagalli, 2011). According to the National Comorbidity Survey (2005), childhood adverse experiences preceded nearly 32% of psychiatric disorders and 44% of disorders with childhood onset (Green et al., 2010). The current study aims to explore potential changes at the level of the synapse following ELS during a well-defined critical window of neural development.

Background and Rationale

Critical Period Plasticity

CPs have been defined as brief periods of postnatal heightened nervous system sensitivity (Simons & Land, 1987; Wiesel & Hubel, 1965). During this time the developing brain undergoes massive synaptic changes, both through eliminating unnecessary and reinforcing necessary synapses. They occur in a predictable sequence during the development of various modalities, including vision, language, and higher cognitive function (Hensch & Bilimoria, 2012). These series of CPs reflect dynamic changes in both excitatory and inhibitory circuits (Takesian, Bogart, Lichtman, & Hensch, 2018). Neural circuit refinement in early development is important for normal sensory processing that remains throughout life (Pan et al., 2017), and therefore, understanding the molecular mechanisms underlying the timing of these CPs is important (Lo, Sng, & Augustine, 2017).

Inhibitory Control. Early in postnatal life, the vast majority of neuronal connections are excitatory. It is believed that CPs begin once the developmental switch occurs in which inhibitory neurotransmission arises (Bavelier, Levi, Li, Dan, & Hensch, 2010; Hensch & Bilimoria, 2012). While CPs have been defined largely by the balance between inhibitory and excitatory circuits, much of the focus has been on the maturation of γ -aminobutyric acid (GABA)ergic inhibition. Specifically, how the rise of GABAergic inhibition is concurrent with the onset of CPs across brain regions (Kolb, Harker, & Gibb, 2017). As observed in early models of CP plasticity in the visual cortex, a disruption in GABA production is sufficient to prevent ocular dominance plasticity from occurring (Hensch et al., 1998; Wiesel & Hubel, 1965). Additionally, research has suggested that reductions in inhibition may delay the end of the CP, potentially extending the window of heightened plasticity (Kuhlman et al., 2013).

In particular, parvalbumin-positive (PV) fast-spiking interneurons have been implicated in the formation of CPs for sensory plasticity (Fagiolini et al., 2004; Huang et al., 1999). They develop in time for the onset of CPs (Chattopadhyaya et al., 2004; del Rio, de Lecea, Ferrer, & Soriano, 1994), acting as a key player in the temporal control of CPs (Hensch & Bilimoria, 2012; Katagiri, Fagiolini, & Hensch, 2007). As observed in ocular dominance and whisker deprivation work, PV-cells receive large sensory input in response to sensory manipulations during sensitive periods of development. They provide inhibitory control over local excitatory circuits and synchronize the activity of cortical pyramidal neurons (Lo et al., 2017). The sensory input typically strengthens connections between PV interneurons and pyramidal cells over the course of the CP (approx. first two weeks), with little effect for the rest of the postnatal period. However, detailed knowledge about the role of glutamatergic excitation in the regulation of CP plasticity in the immature brain is sparse.

Silent Synapses. A silent synapse is defined as “a synapse in which an excitatory postsynaptic current is absent at resting membrane potential but appears following depolarization” (Kerchner & Nicoll, 2008; Malenka & Nicoll, 1997). CPs are defined by a natural progression in the conversion of silent synapses from functionally “silent” to “unsilent”, or functionally mature (Hanse, Seth, & Riebe, 2013). Necessary activity is required to unsilence existing silent synapses, driving the development of various cortical networks (Durand, Kovalchuk, & Konnerth, 1996; Isaac, Crair, Nicoll, & Malenka, 1997; Wu, Malinow, & Cline, 1996), yet the molecular mechanisms underlying this process have been largely debated. The concept of “ineffective synapses”, later becoming known as silent synapses, was first proposed by Merrill & Wall (1972) after observing that selective presynaptic stimuli was not always effective in triggering postsynaptic firing in the dorsal horn of the spinal cord (Merrill & Wall,

1972). This was later confirmed by Kullmann (1994) who observed that the number of morphologically identified synapses and the number of functionally active synapses did not match (Atwood & Wojtowicz, 1999; Hanse et al., 2013; Kullmann, 1994). At this time, and for years to come, it remained elusive whether the lack of postsynaptic conduction was a result of presynaptic dysfunction or absent postsynaptic receptors (Kerchner, Li, & Zhuo, 1999; Malenka & Nicoll, 1997; Redman, 1990).

Typical mature glutamatergic synapses consist of three types of ionotropic receptors: α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors, and kainate receptors. Kinetically, AMPA receptors (AMPA receptors) mediate currents that are quickly activated and decay over milliseconds, while NMDA receptors (NMDARs) mediate currents that activate slower and decay over hundreds of milliseconds (Kleppe & Robinson, 1999). Previous research has indicated multiple explanations for the molecular mechanisms fundamental to silent synapses. First, it was suggested that these synapses are presynaptically silent, meaning they may release less glutamate than regular synapses or not release any glutamate at all. As proposed by Kullmann (1994) the silent synapse is composed of “incompetent presynaptic terminals and mature postsynaptic membranes”, indicating that while AMPARs are present they are not activated (Kullmann, 1994). This theory is rooted in the fact that NMDARs show a much higher affinity to glutamate than AMPARs (Patneau & Mayer, 1990), allowing for low levels of glutamate to elicit NMDAR-mediated responses without observing any AMPAR-mediated responses. Another related theory for unfunctional presynaptic synapses is the spillover of glutamate from neighbouring mature synapses, once again having minimal glutamate available to activate NMDARs only (Kullmann, Erdemli, & Asztely, 1996). It is suggested that following long-term potentiation (LTP) the release of neurotransmitters is

enhanced and becomes sufficient to activate the already existing surface receptors (AMPA receptors). This model has been refuted a number of times including a case in which exogenous glutamate was applied and only NMDARs were activated, not the AMPARs (Renger, Egles, & Liu, 2001). If the presynaptically silent model was true, it was expected that glutamate release would increase with unsilencing resulting in a greater amplitude in NMDAR-mediated currents, though this was not the case. It was observed in multiple cases that NMDAR-mediated current amplitude was unaltered after unsilencing (Durand et al., 1996; Hanse et al., 2013; Liao, Hessler, & Malinow, 1995; Montgomery, Pavlidis, & Madison, 2001; Rumpel, Hatt, & Gottmann, 1998; Ward et al., 2006), yet unsilencing was associated with a shift in the subunit composition of NMDARs (Bellone & Nicoll, 2007).

An alternative model for silent synapses focused on the functionality of synapses as a result of the density of postsynaptic receptors, namely NMDARs and AMPARs. In this case, immature synapses may contain either non-functional AMPARs or lack them completely. Growing evidence is in favour of this model, suggesting silent synapses lack functional AMPARs. Studies have shown that unsilencing was inhibited by blocking postsynaptic exocytosis (Ward et al., 2006) and increased selectively following the application of exogenous AMPA (Montgomery et al., 2001). Further evidence in support of this model has been obtained through studies using laser photolysis of caged glutamate onto single dendritic spines (Bagal, Kao, Tang, & Thompson, 2005; Busetto, Higley, & Sabatini, 2008; Harvey & Svoboda, 2007; Kerchner & Nicoll, 2008; M. Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). Additionally, there is morphological evidence for the lack of AMPARs in postsynaptic membranes of silent synapses (Gomperts, Rao, Craig, Malenka, & Nicoll, 1998; Nusser et al., 1998; Petralia et al.,

1999). With particular focus on this model it is clear that AMPARs play an integral role in silent synapses conversion.

A focus on AMPARs. AMPARs mediate the majority of excitatory transmission in the brain and are key in mediating synaptic strength and plasticity (Jurado, 2017). As synapses are being rapidly formed in the developing brain, these recently created glutamatergic synapses lack functional AMPARs and are classified as “AMPA silent” or simply silent synapses (Hanse et al., 2013). These NMDAR-only synapses lack activity at hyperpolarized potentials due to the voltage sensitive NMDAR’s Mg^{2+} block. Therefore, at the resting membrane potential, only AMPARs mediate excitatory currents (Kerchner & Nicoll, 2008; Malenka, Kauer, Perkel, & Nicoll, 1989). During the CP these synapses are either matured and stabilized or eliminated, which is achieved through the trafficking and integration of AMPARs on the synaptic surface with development, activity, and experience. The already existing surface NMDARs allow for calcium-dependent processes to take place and recruit AMPARs from intracellular or extracellular stores to the surface. In addition to the increase in surface AMPARs over early synaptic development, the receptor subunit composition is fundamental in the maturation of these synapses.

AMPARs are tetrameric heteromeric complexes, containing a combination of GluA1, GluA2, GluA3 and GluA4 receptor subunits. Each subunit type differs primarily in the C-terminal sequence (Song & Huganir, 2002). These receptor subunits are dynamic, with receptor composition being altered in response to excitatory-inhibitory regulation, forming different receptor subtypes (Luján, Shigemoto, & López-Bendito, 2005; Rakhade & Jensen, 2009). These receptors have been described as symmetric ‘dimer of dimers’ (Greger, Ziff, & Penn, 2007; Mayer, 2005), with most AMPARs containing the GluA2 subunit, making them calcium

impermeable. GluA2 induced calcium impermeability is determined by a posttranscriptional modification involving the switch from an uncharged amino acid (glutamine) to a charged amino acid (arginine) inside the ion channel pore, creating an unfavourable environment for calcium ions to pass through (Kim, Kim, Choi, Min, & Gwag, 2001). Some AMPARs do not contain the GluA2 subunit and are able to permeate calcium. These GluA2-lacking receptors cannot last long on the synaptic surface and are soon replaced by GluA2-containing receptors. The switch follows synaptic activity, believed to be induced by calcium influx. The switch to favour calcium impermeability is believed to protect from excitotoxicity of the cell (Kim et al., 2001). In addition, these GluA2-lacking receptors are sensitive to voltage-dependent block by polyamines (Rozov, Zakharova, Vazetdinova, & Valiullina-Rakhmatullina, 2018).

AMPA unsilencing involves the initial integration of GluA2-lacking AMPARs, calcium permeable receptors, into the synaptic surface. As this point calcium permeability is important for downstream signalling and gene transcription (Fortin et al., 2010; Henley & Wilkinson, 2016). This then leads to their replacement by GluA2-containing AMPARs, calcium impermeable receptors, through calcium dependent post-translational modifications of AMPAR subunits (Huganir & Nicoll, 2013; Kerchner & Nicoll, 2008; Petralia et al., 1999). Once GluA2-lacking AMPARs are inserted into the synaptic membrane surface they are no longer silent yet are not fully matured. Trafficking of AMPARs to the postsynaptic membrane can originate from either intracellular stores (Hayashi et al., 2000; Park, Penick, Edwards, Kauer, & Ehlers, 2004; Shi et al., 1999) or alternative extrasynaptic locations along the plasma membrane (perisynaptic stores) (Adesnik, Nicoll, & England, 2005). As observed in unsilenced primary auditory neurons (Eybalin, Caicedo, Renard, Ruel, & Puel, 2004), calcium influx through GluA2-lacking AMPARs drives their replacement by GluA2-containing receptors, and is observed within the

first few weeks following birth. It is suggested that these calcium permeable AMPARs play a key role in experience-dependent plasticity, thus their surface integration appears to coincide with synapse maturation following experience. In all, while the brain matures, the ratio of silent synapses is significantly reduced. As observed in the hippocampus and somatosensory cortex, the expression and function of the excitatory AMPARs is much higher during the CP than in adulthood. Although AMPARs are key in shaping neural networks and their expression coincides with CPs, it is unknown the exact role of AMPARs in CP plasticity in the auditory cortex (AC).

Plasticity in the Auditory Cortex

A variety of auditory functions perform optimally when learned early in life (i.e. musical experience, and language acquisition) (Penhune, 2011). The reason for this lies in the role of CPs. Experience-dependent plasticity during CPs has been well characterized in several sensory areas, such as the primary auditory cortex (A1) (Hensch, 2004; Hensch & Bilimoria, 2012; Kral, 2013). Sound representation in the cortex is very adaptive during a CP early in life (de Villers-Sidani et al., 2007; Insanally, Köver, Kim, & Bao, 2009; Zhang, Bao, & Merzenich, 2001). During a finite three-day CP for tonotopic plasticity in A1, from postnatal day (P) 12 to 15 (Barkat, Polley, & Hensch, 2011), passive tone exposure leads to an expanded representation of that tone subserved by altered auditory thalamocortical connectivity. Changes in mapping resulting from tone manipulations occur largely during this time window, with tone expansion observed to be much larger when presented earlier in development (Kral, 2013; Stanton & Harrison, 1996; Zhang et al., 2001).

This window of heightened cortical plasticity was determined through experiments in which rodent pups were exposed to a specific tone, inducing selective increases in cortical representation of that tone, leading to changes in tone perception (de Villers-Sidani et al., 2007; Insanally et al., 2009; Zhang et al., 2001). These animal models for prenatal development show underdeveloped tonotopic maps which are defined by spontaneous sounds (Jones, Leake, Snyder, Stakhovskaya, & Bonham, 2007; Lippe, 1994, 1995; White, Hutka, Williams, & Moreno, 2013). Following birth, tonotopic maps become more response specific through exposure to environmental sound stimuli, eventually leading to highly organized maps through progressive differentiation and refinement of tone-specific receptive fields (de Villers-Sidani et al., 2007; Zhang et al., 2001). These findings confirm that auditory experiences early in the stages of neurodevelopment are integral in the formation of functional AC development (Zhang et al., 2001).

In addition to sound stimuli, other forms of stimuli early in development can have lasting effects on tonotopic mapping in the AC. In particular, the CP for A1 has been shown to be altered by early life interventions. One such study from our lab (Sun et al., 2018) involving the introduction of early life seizures, indicated that when seizures occur in the context of a sensitive period of tonotopic plasticity, it results in aberrant synaptic connections and ultimately dysfunctions related to auditory functioning. The seizures appear to bypass the typical CP for auditory circuit development by diminishing the population of NMDA-only synapses (Sun et al., 2018). It is anticipated that this disruption to the CP may contribute to long-lasting deficits in auditory functioning as observed in disorders of learning and memory.

Disorders of Learning and Memory. Regulation of CPs through excitatory and inhibitory imbalance and other signalling pathways have implications in both normal and

abnormal neurodevelopment. Alterations of these predetermined temporal sequences (either through delay or acceleration) may underlie disorders of learning and memory. The CP in A1 is crucial for both functional brain development and cognitive function. Impaired auditory cortical development during the CP for tonotopic mapping has been implicated in many neurological disorders of learning and memory; including but not limited to Intellectual Disability and Autism Spectrum Disorders (ASD) (Barkat et al., 2011; LeBlanc & Fagiolini, 2011; Port et al., 2017). It has been suggested that aberrant AC development may underlie related language deficits associated with the aforementioned disorders (J. Matsuzaki et al., 2012; McKavanagh, Buckley, & Chance, 2015; Oram Cardy, Flagg, Roberts, Brian, & Roberts, 2005).

ASD related behaviours involve the integration of a variety of sensory systems (i.e. auditory, visual and somatosensory), directing communication, socialization, and learning. The AC in particular is implicated due to its critical role in language acquisition and learning implications in ASD. In a number of animal models of ASD, CP plasticity disruptions have been reported, varying depending on the etiology of ASD (LeBlanc & Fagiolini, 2011). Mouse models of ASD display impaired auditory cortical development, most notably during the CP for tonotopic mapping (Engineer et al., 2014; S. Rotschafer & Razak, 2013; S. E. Rotschafer, Marshak, & Cramer, 2015). Further rodent models of ASD have shown distorted primary auditory maps, with over representation of high frequencies, broad receptive fields and higher sound intensity thresholds compared to controls (Anomal et al., 2015). Questions remain regarding whether these disturbances result from aberrant neural circuitry formation or a delay in development. Genetic research has indicated a key role for excitatory postsynaptic structural plasticity in the development of neurodevelopmental disorders (Hall, Trent, Thomas, O'Donovan, & Owen, 2015; Hamdan et al., 2011; Kirov et al., 2012; Sanders et al., 2015). Many

genes key in creating and maintaining the inhibitory-excitatory balance have been implicated in genetic mouse models of ASD (LeBlanc & Fagiolini, 2011), commonly showing deficits in neocortical PV-cells (Gogolla et al., 2009).

ASD and intellectual disability have been thought to be associated with aberrant connectivity and immature synaptic networks resulting from early disruptions in sensory and social experiences (Hanse et al., 2013; LeBlanc & Fagiolini, 2011). A potential contributing factor to neurodevelopmental disorders has been proposed as the disrupted activity dependent selection of NMDA-only synapse conversion, through excessive, indiscriminate or deficient unsilencing. Deregulation in experience-dependent synaptic plasticity across brain regions has been observed in animal models of ASD, with impairments in sensory processing, learning related synaptic abnormalities, and developmental synapse pruning (Hutsler & Zhang, 2010). It has been hypothesized that ASD may result from disruptions in the expression and/or timing of CPs across various brain regions. Animals models of ASD in fact display fewer NMDA-only synapses in the developing hippocampus contributing to faulty connectivity (for review see Hanse et al., 2013). What has not been established is a link between early-postnatal stress and later development of such disorders. Uncovering the neural mechanisms driving ELS-induced social deficits is important for treatment strategies aimed at neurodevelopmental disorders associated with learning and social impairments, such as ASD (Rai et al., 2012).

Early-Life Stress

CPs typically end when plasticity halts, though emerging research indicates that early-life experience can interrupt the timing and progression of CPs. The developing brain is highly sensitive to experiences, despite its inherent resilience and flexibility, showing an increased

capacity for plasticity that may in turn influence lifelong behavioural outcomes (Kolb et al., 2017). The immature brain is subject to a variety of factors including but not limited to sensory and motor inputs, language, caregiver and peer interactions, drugs, diet, microbiome, immune system, and ELS. ELS is among the most studied of the aversive effects on brain development. Additionally, emerging evidence indicates that a large array of stressful experiences alter brain development, regardless of few seemingly impactful events (Kolb et al., 2017). With this considered, it is believed that chronic stress in early life could lead to impaired brain development rather than segmented acute stressors.

Stress on the adult brain is a factor in the induction of adaptive plasticity (Chocyk et al., 2013; McClelland, Korosi, Cope, Ivy, & Baram, 2011; Schmidt, 2011), while in the context of a sensitive period of brain maturation, stress may rather interfere with normal experience-induced plasticity and lead to maladaptive consequences (Bock & Braun, 2011; Chocyk et al., 2013; McClelland et al., 2011). Chronic stress induces neuronal and behavioural abnormalities, related to aberrant structural and functional synaptic plasticity (Christian, Miracle, Wellman, & Nakazawa, 2011; Yang et al., 2017). Diverse chronic stress leads to abnormal synaptic function of AMPAR mediated excitation in the synapses (Kallarackal et al., 2013; Makino & Malinow, 2009; Schmidt et al., 2010). This is supported by the observation that glucocorticoids show regulation of AMPAR trafficking, implementing the stress hormone in synaptic plasticity regulation (Hubert, Li, Rainnie, & Muly, 2014; Krugers, Hoogenraad, & Groc, 2010). To date, the problem remains that much of the literature indicates opposing findings. Generally, corticosteroids have been suggested to alter the diffusional properties of AMPARs (Groc, Choquet, & Chaouloff, 2008). Some work suggests that stress facilitates the insertion of AMPARs containing the GluA2 subunit into existing newly unsilenced synapses, in turn fully

maturing these synapses and inducing long-term potentiation following the stress exposure. In this case, there is an increase in GluA2-containing AMPARs without altering the number of AMPARs overall (Martin et al., 2009). Rather, other work has identified chronic stress induced disturbances in AMPAR dependent synaptic plasticity. As observed in the hippocampal CA1 region, there was reduced AMPAR expression following chronic stress (Kallarackal et al., 2013). In order to further explore the effects of ELS on AMPAR-dependent synapse conversion a well-defined window of CP sensory development may be investigated.

ELS and the Auditory Cortex. Similar findings have yet to be confirmed in the context of a well-established CP of synaptic plasticity for A1. Generally, research indicates a relationship between stress and auditory functioning. Many structures involved in auditory processing show sensitivity to stress, some studies indicating the AC as an additional target for stress-induced neuronal damage (Campeau, Dolan, Akil, & Watson, 2002; Ma, Li, Li, Wang, & Qin, 2017; Simoens et al., 2007). First, Simoens and colleagues (2007) indicate psychosocial stressor anticipation may reduce general and divergent sound processing, potentially indicating stress in the dysfunction of cortical memory-trace formation. Second, emotional stress has been associated with the occurrence of various auditory dysfunction (i.e. hyperacusis, tinnitus and vertigo) (Mazurek et al., 2010). The focus of stress neurobiology has been primarily focused on the limbic system and the medial prefrontal cortex (mPFC) (McEwen & Chattarji, 2004).

Various studies have pointed toward a number of links between the auditory and stress systems: the limbic system, responsible in part for instinctive behaviour and emotions. The limbic system extends vast upstream and downstream connections to sensory systems. The amygdala and hippocampus being two major limbic regions that send and receive neuronal inputs/outputs to/from the central auditory system (LeDoux, 2007; Mohedano-Moriano et al.,

2007; Munoz-Lopez, Mohedano-Moriano, & Insausti, 2010; Sah, Faber, Lopez De Armentia, & Power, 2003; Winer, 2006; Winer & Lee, 2007) and connections modulate neuronal activity and plasticity (Kraus & Canlon, 2012; Marsh, Fuzessery, Grose, & Wenstrup, 2002; Weinberger, 2007). This includes projections sent directly from the A1 and medial geniculate body to the amygdala, outputs from the lateral amygdala to the auditory association areas (LeDoux, 2007; McDonald, 1998; Sah et al., 2003), and direct connections from the CA1 to auditory association and primary auditory cortices in rat models (Cenquizca & Swanson, 2007). In a review by Dagnino-Subiabre (2012) it was hypothesized that “the auditory system is affected in some neuropsychiatric disorders related to increases in amygdala activity” (Dagnino-Subiabre et al., 2012). Rat studies show activation of the amygdala through fear conditioning alters tonotopic map plasticity in the A1 (Froemke & Martins, 2011; Gao & Suga, 2000; Morris, Friston, & Dolan, 1998; Suga, 2008; Weinberger, 2007), with some studies indicating amygdala stimulation inhibits response in the A1 via GABA receptors (He, Chen, & Zhou, 2004). More recently, Ma and colleagues (2017) observed whether chronic stress impaired inhibitory gating in the AC. Their results indicated that inhibitory gating of the AC was significantly reduced in chronically stressed rats, confirming that their model of chronic restraint stress disrupted physiological functioning of the AC (Ma et al., 2017).

There remains little evidence regarding excitatory synaptic changes in the AC following exposure to stress, particularly during a CP in neurodevelopment. Considering the literature outlined above, it is clear that there is a need for comprehensive research on ELS induced changes during CPs, specifically in the context of a well-defined window of plasticity, as seen in the AC.

Maternal Separation as a Model of ELS. Maternal interaction is an important source of stimulation in early development. Mother-pup interactions have a great deal of control over neonatal development (Hofer, 1984; McCormick, Kehoe, & Kovacs, 1998; Suchecki, Rosenfeld, & Levine, 1993). In rodents a commonly used model for early postnatal stress is the maternal separation (MS) model. MS models vary widely in terms of separation duration, time variability, and compounding it with additional stressors such as early weaning (George, Bordner, Elwafi, & Simen, 2010; Tan, Ho, Song, Low, & Je, 2017), reduced nesting materials (Rice, Sandman, Lenjavi, & Baram, 2008), and/or social isolation (McCormick et al., 1998; Niwa, Matsumoto, Mouri, Ozaki, & Nabeshima, 2011). Short periods of maternal deprivation alter HPA axis functioning, potentially inducing positive effects, while longer periods (3 hours or more daily) lead to dysfunction in the stress response system. Long-lasting effects depend highly on the age of the pup and duration of separation, with more robust effects when manipulated earlier in development and for longer periods of time (de Kloet & Oitzl, 2003). The use of this model in rats has led to reliable findings of long-lasting behavioural consequences following a period of MS. Studies in mice have resulted in inconsistent findings for behavioural outcomes. Ultimately, MS paradigms are in need of further work to be validated in all rodent models (Tan et al., 2017).

ELS and the Stress Hypo-responsive Period. The stress response system differs greatly between neonatal animals and adults. During CPs early in development compensatory mechanisms protect highly active mechanisms of neuronal growth and myelination (Morgane, Mokler, & Galler, 2002). The period in which there is a reduction in HPA axis response is known as the stress hypo-responsive period (SHRP) (Rosenfeld, Wetmore, & Levine, 1992; Sapolsky & Meaney, 1986). It is considered a protective period in which limiting corticosterone (CORT) to low levels is required for normal growth and development of the central nervous

system (Nishi, Horii-Hayashi, Sasagawa, & Matsunaga, 2013). This period is observed in mice between P1-P12, displaying low basal levels of glucocorticoids and minimal responses to stressors that would typically induce enhanced stress response in adults (Rosenfeld et al., 1991). Though, studies suggest that during this period the stress system may respond specifically to developmentally important stressors, such as maternal interaction stressors (Molet, Maras, Avishai-Eliner, & Baram, 2014; Nishi et al., 2013). MS has been shown to be a sufficient inducer of a stress response, even during the SHRP. Meaney and colleagues (1999) suggest that since mother-pup interactions are important in maintaining this reduced HPA axis response period, through maternal licking, grooming, nursing etc. (Francis, Champagne, Liu, & Meaney, 1999; Levine, 1994), a disruption in such behaviours may remove the suppression of the stress response system, creating an environment for adversity during this intended period for protection.

Rationale for the Current Study

Region specific CPs in early life are characterized by elevated synaptogenesis and synaptic plasticity essential for normal development, where learning and “imprinting” occurs, and persists throughout the lifespan (de Villers-Sidani et al., 2007; Kral, 2013). It has been established as a particularly sensitive period in which environmental factors, such as ELS, may lead to lasting synaptic organizational consequences. Emerging research has indicated that: 1) Synaptic maturation during CPs can be observed through the conversion of silent synapses, evident primarily through a shift in AMPAR functioning, 2) Stress is related to changes in synaptic organization, 3) Dysfunction of the AC is correlated with disorders of learning and memory (i.e. ASD), and 4) Stress may induce disruptions in AC neuronal functioning. However, much of the research on stress and synaptic plasticity continues to be contradictory, while little research attempts to understand the impact of ELS on synaptic development of the immature brain. To date, the effects of ELS on synaptic development in the AC has never been explored. Therefore, in this study we aim to examine whether ELS will affect the development of excitatory synapses of A1 pyramidal neurons in the AC. We hypothesize that if ELS occurs in the context of rapid synaptic development, there is the potential for disruption of the normal synaptic development during the CP of development. The following experiments are designed to test this hypothesis.

Aim 1: To test whether ELS induces CORT responses and affects postnatal development in mouse pups.

My first set of experiments were designed to investigate the effects of ELS from P3-15 on CORT levels during the CP of AC development in mouse pups. Additionally, I evaluated the changes in body weight of mouse pups subjected to ELS compared to control pups.

Aim 2: To determine whether ELS affects intrinsic membrane properties of A1 pyramidal neurons in the AC.

Intrinsic membrane properties critically determine the neuronal excitability. Therefore, I examined whether ELS affects intrinsic membrane properties including resting membrane potential, input resistance, the action potential (AP) amplitude, AP threshold and duration, and input-output function using whole-cell current-clamp recordings in A1 layer IV pyramidal neurons in slices from P12-15 post ELS pups and control pups.

Aim 3: To investigate whether ELS affects the development of excitatory synapses in A1 pyramidal neurons in the AC.

Experiment 1: Can ELS affect AMPA receptor function in A1 layer IV pyramidal neurons during the CP of A1 development? To study this, I first analyzed the spontaneous AMPAR mediated excitatory postsynaptic currents (EPSCs) in A1 layer IV pyramidal neurons in slices from P12-15 post ELS pups and control pups. Then, I examined the amplitude of minimally evoked AMPAR EPSCs in A1 layer IV pyramidal neurons in slices from P12-15 post ELS pups and control pups.

Experiment 2: Can ELS affect NMDAR-only silent synapses in A1 layer IV pyramidal neurons during the CP of A1 development? To further elucidate how ELS affects maturation of these excitatory synapses, I examined the NMDAR-only silent synapses in A1 layer IV pyramidal neurons in slices from P12-15 post ELS pups and control pups using whole-cell voltage clamp recordings.

Aim 4: To identify what population of neurons are selectively activated by ELS and to determine whether these neurons display altered intrinsic properties and development of excitatory synapses in A1 pyramidal neurons in the AC.

To further explore the impacts of ELS on a subpopulation of A1 pyramidal neurons I used a c-Fos based transgenic mouse model with selective activity-dependent green fluorescent protein (GFP) tagging. I examined selective neuronal activation through immunohistochemistry, measuring intrinsic membrane properties, and measuring AMPAR-mediated sEPSCs in A1.

Our study has the potential to lead to the knowledge necessary to identify candidate molecular sites for modulating the neurodevelopmental impacts of ELS and will undoubtedly lead to subsequent research and potential targets for intervention.

Methods

Experimental Animals

Male and female P3-P18 c-Fos-GFP mouse pups were used for all experiments. All mice were maintained in a 12 h light/dark cycle schedule with a room temperature of 22°C. All mice had free access to food and water, and provided with nestlets. All animal care protocols and procedures (#109150) were approved by and in accordance with the guidelines of the Animal Care and Use Committee at Carleton University. Efforts were made to minimize animal suffering and the number of animals used.

Early-Life Stress Model: Maternal Separation with Social Isolation

Early-life stress was induced daily in a specific quiet room from P3 to P15 using a procedure of MS with social isolation adapted from protocols by Millstein et al., 2007 and Shin et al., 2018.

At the time of birth (P0) a litter was assigned to either the control or stress group. Regardless of whether assigned to the control or stress group, the litters were left undisturbed until P3 in which the sires are removed and housed separately, and pups counted.

The pups assigned to the stress group were subjected to daily MS from P3 to P15. Pups were removed from the home cage and placed in separate isolated chambers (12.5 cm x 14.4 cm) with fresh 1/8" bedding, excluding them from both dams and littermates (S. Shin et al., 2018), on a heating pad maintained at 32°C (Horii-Hayashi et al., 2013; Millstein & Holmes, 2007). This was done for a duration of 3 hours with an unpredictable stress schedule by randomizing the timing of separation (within the light period 8:00-20:00) (Tan et al., 2017). This makes it difficult for the dam and pups to anticipate the stressor which is believed to increase the effects

of the stressor (Enthoven, Oitzl, Koning, van der Mark, & de Kloet, 2008). Dams remained in their home cage during the entire period of separation. At the end of the separation period, pups were returned to their home cage and reunited with their respective dams. Litters assigned to the control group were left undisturbed until cage/food and water changes, and at P10 for genotyping. Pups from both groups were removed from P11-P15 for experiments.

Measuring CORT

Blood Collection. At the time of sacrifice, blood was collected from the trunk and pipetted into 1.5 mL tubes contained 10 μ L of EDTA on ice. The samples were then centrifuged for 20 mins at 2000g. Once adequately separated, approximately 30 μ L plasma was collected, divided and placed into two new 1.5mL tubes. Samples were then stored at -80°C prior to further analyses.

ELISA Analyses. Plasma CORT was subjected to an enzyme-linked immunosorbent assay (ELISA) using the Enzo Life Sciences CORT Elisa kit (Cat #ADI-900-097). It is a plate-based assay technique designed to detect and quantify a variety of substances including peptides, proteins, antibodies, and hormones (Willey, Sherwood, & Woolverton, 2008). It relies on highly specific antibody-antigen interactions, the antigen being immobilized on the plate, which complexes with an antibody. Following conjugation, a detection enzyme linked to the antibody produces colour (Willey et al., 2008). Relative intensities of colour determine the relative concentration of the substance of interest, in this case CORT. All samples were assayed in duplicate.

Auditory cortex slice preparation

The auditory cortex slices (300 μ m) were prepared from P7-18 pups as described previously (Sun et al., 2018). Briefly, mouse brains were quickly dissected from the skull and placed in the chilled oxygenated cutting solution containing (nM): 119 choline chloride, 5 KCl, 4 MgSO₄, 1.25 NaH₂PO₄, 0.8 CaCl₂, 26 NaHCO₃, 18 glucose, and 5 sucrose. The brain was then sectioned into 300 μ m coronal slices containing the AC using a vibrating microtome (Leica Microsystems VT1000S). Slices were allowed to recover in oxygenated artificial cerebral spinal fluid (ACSF) containing (mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 1.2 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 glucose, at 35°C for 30 minutes, then left at room temperature for at least 1h before electrophysiological recordings. For recordings, the slices were then transferred to a 2.5mL recording chamber placed in an upright Zeiss Axio Scope A1 microscope equipped with infrared and differential interference contrast imaging devices and perfused with oxygenated ACSF at room temperature (22-24°C).

Whole-cell patch clamp recording

Whole-cell patch clamp recordings were obtained from layer IV pyramidal neurons in the A1 from P7-P18 mice using an Axopatch 200B amplifier (Axon Instruments, Molecular Devices), as previously described (Sun, Kosaras, Klein, & Jensen, 2013; Sun et al., 2018; Zhou, Lippman, Sun, & Jensen, 2011). Within layer IV of A1, pyramidal neurons were identified initially based on morphology, a conic shaped soma, and further identified by observing intrinsic properties (i.e. AP firing frequency). Patch electrodes with a resistance of 3-10 M Ω were prepared from borosilicate glass capillaries with a Narishige micropipette puller (Model PC-100, Tokyo, Japan). Pipette intracellular solution for current clamp recordings contains (mM): 130 K-

Gluconate, 2 MgCl₂, 0.6 EGTA, 10 HEPES, 5 KCl, 2 ATP-Mg(Na₂), pH 7.3. For voltage clamp recordings, internal solution contains: 0.3 GTP-Na₃, 110 Cs-methanesulfate, 10 TEA-Cl, 4 NaCl, 2 MgCl₂, 10 EGTA, 10 HEPES, 4 ATP-Mg(Na₂), and 0.3 GTP-Na₃, with 5 QX-314 chloride and 7 Phosphocreatine-Na₂, pH 7.25. Spontaneous EPSCs (sEPSCs) were recorded at a -60mV holding potential to isolate AMPAR mediated currents from NMDAR mediated currents. Additionally, picrotoxin (PTX) (100 μM), a GABA_A blocker, was administered to further pharmacologically isolate AMPAR mediated currents. Signals were filtered at 2kHz, digitized at 20kHz by a Digidata 1320A interface, acquired by the pClamp 9.2 software, and analyzed with the Clampfit 10.7 program (Molecular Devices).

AMPA mediated spontaneous responses

AMPA-mediated sEPSCs were recorded at a -60mV holding potential. sEPSCs were recorded over a period of 10 minutes and analyzed further using Clampfit 10.2. sEPSC events were detected automatically using Clampfit 10.2, and frequency and amplitude histograms were constructed using this program. The threshold for detection of sEPSC events was set at 5-6pA, depending on the noise level. All events were confirmed visually on the basis of the rise and decay times. The cumulative distributions of the sEPSCs were constructed from at least 10 min of recording from each cell, using a bin width of 1 pA for amplitude.

Measuring silent synapses using failure rates

Silent synapses are quantified through the use of “failure rates”, the proportion of trials where no synaptic response is detected, at depolarized and hyperpolarized potentials. Minimally evoked EPSCs (eEPSCs) were elicited by stimulating L6 of the A1 under voltage clamp mode in

P12-P15 mice. The stimulation location in L6 was determined when consistent recordings at a single stimulation intensity were established. Postsynaptic responses were first evoked around threshold stimulus using minimal stimulation intensity. Then, stimulation intensity was gradually increased to elicit detectable synaptic responses with about 50% failure rate to consecutive 20-40 trials of the same stimulus (Zhou et al., 2011). To examine the postsynaptic silent synapses, cells were first held at -60mV, and then stimulated with the same intensity at +40mV holding potential. The fraction of silent synapses was calculated using the established formula: % of silent synapses = $(1 - \ln(F_{-60}) / \ln(F_{+40})) \times 100\%$.

Intrinsic Membrane Properties

To examine the membrane properties of A1 pyramidal neurons, 500 ms rectangular hyper- and depolarizing pulses (-200 to 200pA, 20pA/step) were used to evoke membrane voltage responses in the current clamp mode.

To determine the input resistance, membrane potential changes evoked by current injections (0 to -80 pA) were measured between the baseline potential and the peak hyperpolarization to plot current–voltage (I–V) curves. The slope of the curve was calculated to estimate the input resistance of the cell membrane. AP amplitude is measured by the difference between the peak voltage of the AP and initial membrane potential at which the membrane potential started to rise rapidly (inflection point). The AP threshold was determined by the membrane potential at which only one or two spikes are generated. The threshold current for firing is defined as the lowest level of current injection required to elicit at least one AP. Duration of the AP was measured at its half amplitude.

Immunohistochemistry

Perfusion. Mice were anesthetized with sodium pentobarbital (200mg/Kg, Euthasol®, Virbac USA) and perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Whole brains were extracted from the perfused animals and left in 4% PFA overnight at 4°C. The following day the tissue was washed in 10% sucrose in PB solution for 4 hours then another 4 hours or overnight, followed by a final wash in 30% sucrose in PB + azide solution at 4°C until tissue sectioning.

Tissue Sectioning. Perfused brain tissue was sectioned at 40µm. The tissue was flash frozen in a cryostat using Fisherbrand Super Friendly Freeze-it (Fisher cat # 23022524) prior to cutting. Floating sections were placed in 0.1 M PB + azide and stored at 4°C until staining.

GFP Staining. Sections were first rinsed 2 x 5 mins in 10mM Phosphate buffered saline (PBS). The slices were then blocked for 1 hour in 5% Normal Goat Serum (NGS, Sigma cat# G9023) and 0.3% Triton-X in PBS. The blocker was removed, and the slices incubated in GFP primary antibody (chicken IgY; 1:5000; Aves Labs Inc.) overnight at room temperature in a primary dilution solution (2% BSA in PBS, and blocker). The following day the slices were rinsed 3 x 5 minutes in PBS. Next, the slices were incubated in anti-GFP fluorescent secondary Alexa Fluor 488 (anti-chicken IgG; 1:1000; Invitrogen Alexa Fluor series) dissolved in the secondary dilution solution (NGS, 10% Triton-x, BSA, and PBS) for 2-3 hours. Once again, the slices were rinsed 3 x 5 minutes in PBS followed by mounting onto positively charged slides (Thermo Fisher cat# 12-550-15) and coverslip (Thermo Fisher cat# 12-545M) with Fluoromount containing DAPI (4',6-diamidino-2-phenylindole; Thermo Fisher # 00-4959-52). Slices were left to air dry overnight and sealed with nail polish the next day. They were finally stored at -20°C until imaging.

Imaging and Quantification. The AC anatomical location was determined using the Allen mouse Brain Reference atlas (2008). All images were captured using the ZEISS AX10 microscope with the digital camera HAMAMATSU C10600 attached (Zeiss Canada, ON). The percentage of GFP⁺ cells was quantified using NIH ImageJ software. The final value per animal was averaged over 3 tissue sections. In order to calculate % activation the whole image was counted for GFP⁺ cells versus DAPI stained cells (stain for nuclei of all cells).

Statistical Analysis

Graph Pad's Prism (Version 8) was used for all statistical analyses. The data is given as mean \pm S.E.M. All data was first tested for normality using the Shapiro-Wilk normality test. When normally distributed, statistical significance differences are established as $p < 0.05$ using two-way ANOVA with post hoc Tukey's procedure or a two-tailed student's unpaired t test. Non-normally distributed data were analyzed using a Mann-Whitney rank test. Two-way ANOVAs with sex or PND and treatment group as factors were performed for animal weight and plasma CORT analyses. Two-tailed student's unpaired t tests or Mann-Whitney rank tests were performed for electrophysiological data. Additionally, two-way ANOVAs with sex and treatment group as factors were performed for intrinsic and excitatory data of randomly sampled neurons. No significant factor or interaction effects were observed when considering sex for intrinsic and excitatory data of randomly sampled neurons.

Results

ELS does not impact macro physiological measures

Our first goal was to examine the impact of chronic ELS on hormonal stress reactivity and postnatal development of mouse pups. To determine whether chronic ELS induces CORT responses and affects postnatal development in mouse pups, plasma CORT (pg/mL) was collected and analyzed. Additionally, animal weights (g) were recorded at the time of sacrifice in P12-15 ELS and control mice.

Animal Weight. A two-tailed student's unpaired t-test was completed to determine the effects of ELS on animal weight. There were no significant differences in weight between control (6.694 ± 0.290 g, $n=16$) and ELS animals (6.432 ± 0.195 g, $n=22$, $p=0.4404$). A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to observe the influence of sex on weight changes. When considering sex (females $n=18$, males $n=20$), all factor effects were determined to be insignificant ($F(1,34)=0.1150$, $p=0.7366$, $F(1,34)=0.5776$, $p=0.4525$). Additionally, there were no significant interactions between factors ($F(1,34)=0.1378$, $p=0.7128$). A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to observe the influence of PND on weight changes. Similar insignificant outcomes for interaction ($F(3,30)=1.203$, $p=0.3257$) and stress related effects factor effects ($F(1,30)=0.02705$, $p=0.8705$) were observed when considering PND (P12 $n=10$, P13 $n=13$, P14 $n=8$, P15 $n=7$). The age factor was observed to have a significant effect ($F(3,30)=3.605$, $p=0.0246$) on weight as developmentally expected. Post hoc tests revealed a significant change in weight from P12 ELS mice vs. P15 ELS mice (95% DI -4.190 to -0.06763 , $p=0.0390$).

Plasma CORT. A Mann-Whitney test was completed to determine the effects of ELS on plasma CORT levels. There was a significant difference between groups in plasma CORT levels with increased CORT levels observed in control (49198 ± 4723 pg/mL, $n=16$) animals over ELS animals (36091 ± 2964 pg/mL, $n=19$, $p=0.0373$). A two-way ANOVA followed by a Tukey's multiple comparisons test was performed to observe the influence of sex on CORT changes. When considering sex (females $n=18$, males $n=17$), there was no significant effect ($F(1,31)=0.5703$, $p=0.4559$) or interaction ($F(1,31)=2.479$, $p=0.1255$). The effect of ELS remained significant ($F(1,31)=5.539$, $p=0.0251$). Post hoc tests revealed that this effect was only observed in male mice, with control mice displaying heightened stress reactivity over ELS mice (95% DI 431.8 to 41862, $p=0.0440$). A two-way ANOVA followed by a Tukey's multiple comparisons test was performed to observe the influence of PND on CORT changes. When considering PND (P12 $n=10$, P13 $n=12$, P14 $n=7$, P15 $n=6$), stress related factor effects were determined to be insignificant ($F(1,27)=2.432$, $p=0.1305$), while age showed significant effects ($F(3,27)=14.90$, $p<0.0001$). Additionally, there was no significant interaction ($F(3,27)=1.139$, $p=0.3510$) between age and stress effects. Post hoc tests revealed no significant changes at each age but over the span of the CP, with significant changes in CORT between P12 and P15 mice. Although our model of ELS did not induce significant changes in weight and CORT reactivity, we sought to examine the effects of chronic ELS at a microscopic level, the neuron. The average intra-assay coefficient of variability was determined to be 9.901%, meeting the suggested cut off of 10% (Enzo Life Sciences).

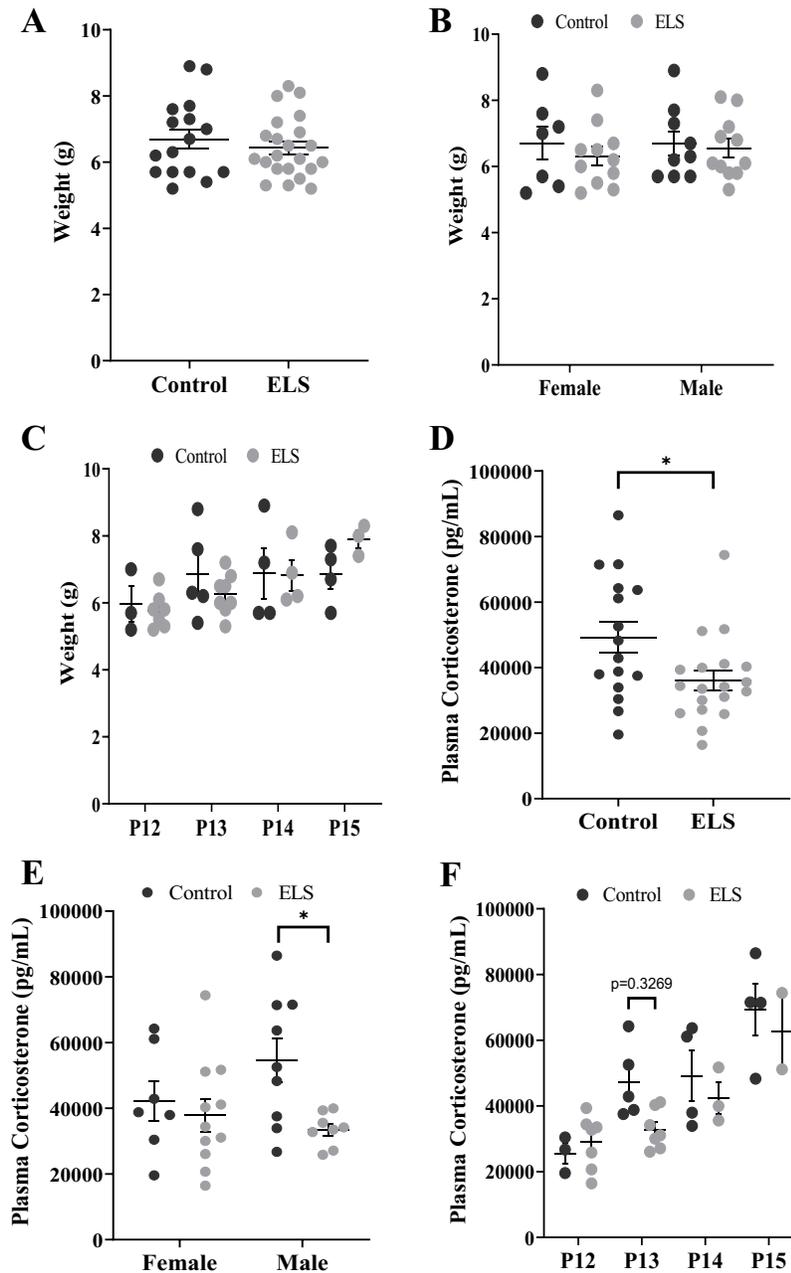


Figure 1. Macro physiological measures are not significantly altered by ELS. (A-C) Weights of mouse pups in control and ELS groups show no significant differences, even when sex and PND are considered. (D) Plasma CORT levels (pg/mL) for control vs. ELS groups show control animals with a significantly increased and more variable stress response. (E) When comparisons are made considering sex, females show no significant difference in CORT levels between groups whereas males show a significant difference with the control group showing a greater stress response. (F) Comparisons considering PND show no significant stress dependent changes in CORT levels. Data are expressed as mean \pm SEM. *p < 0.05.

ELS does not significantly alter intrinsic properties of randomly selected layer IV pyramidal neurons in A1

Prior to exploring the effects of ELS on AMPAR activity we sought to determine if intrinsic membrane properties of layer IV pyramidal neurons in A1 were altered. Whole-cell current clamp recordings were performed and resting membrane potential (mV), input resistance ($M\Omega$), AP amplitude (mV), AP threshold (mV), and AP duration (ms) were obtained for control and ELS groups. As all measurements of intrinsic properties displayed a normal distribution, multiple two-tailed student's unpaired t-tests were completed.

Resting membrane potential was found not to be significantly different between control (-67.97 ± 1.50 mV, n=10 cells) and ELS animals (-68.43 ± 1.52 mV, n=6 cells, $p=0.8417$). Input resistance, measured as the linear relationship between current and resulting voltage change, was found not to be significant between control (407.4 ± 38.87 $M\Omega$, n=10 cells) and ELS animals (453.8 ± 71.90 $M\Omega$, n=6 cells, $p=0.5424$). AP amplitude, measured as the difference in voltage between the point of AP threshold and AP peak, showed no significant difference between control (82.47 ± 4.79 mV, n=10 cells) and ELS animals (81.98 ± 6.73 mV, n=6 cells $p=0.9533$). AP threshold, measured as the membrane potential in which the first AP is generated, showed no significant difference between control (-36.77 ± 1.21 mV, n=10 cells) and ELS animals (-36.60 ± 1.74 mV, n=6 cells, $p=0.9373$). AP duration, measured as the time of the AP half-width, showed longer AP duration in ELS animals (4.522 ± 0.438 ms, n=6 cells) over control animals (3.575 ± 0.225 ms, n=10 cells), but failed to reach significance ($p=0.0506$).

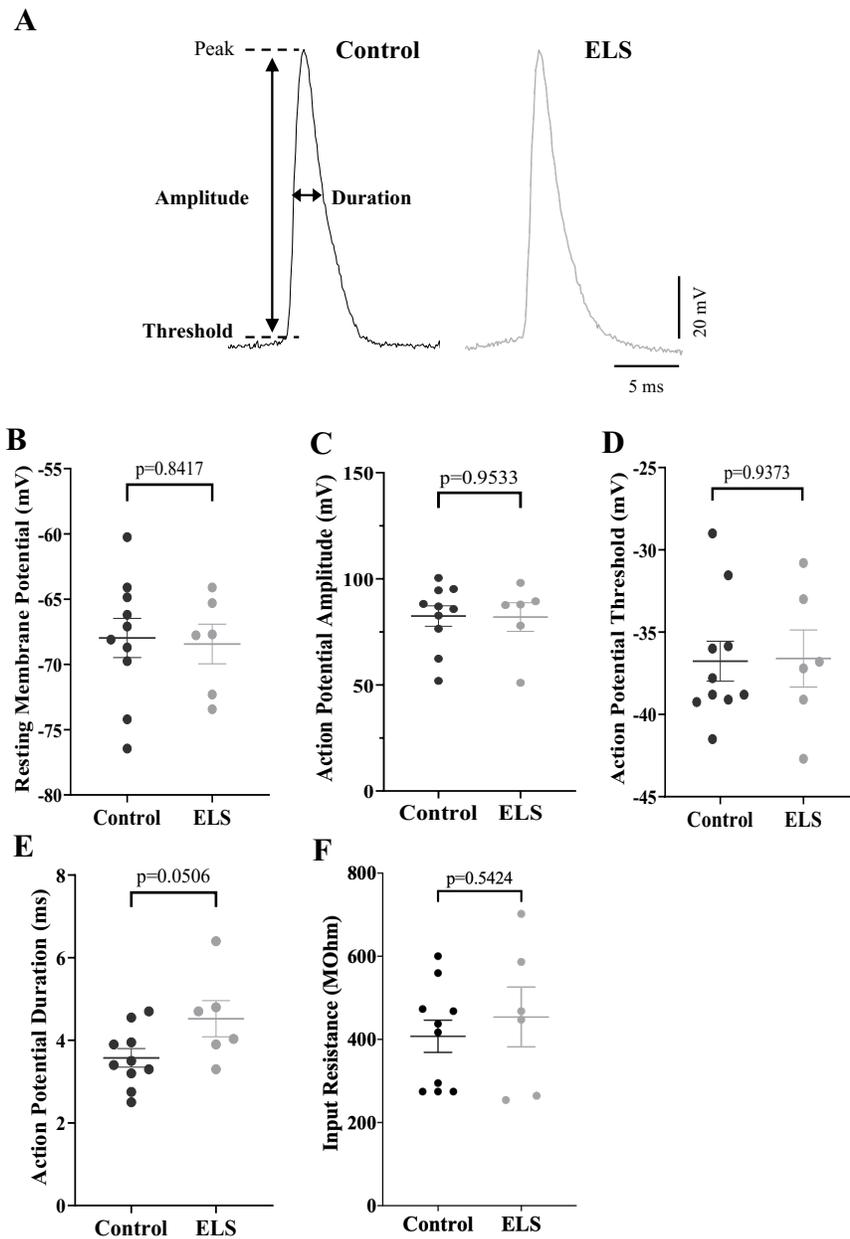


Figure 2. Intrinsic properties of layer IV A1 pyramidal neurons are not altered following exposure to ELS. (A) Representative AP trace from a control and ELS recordings with indicated intrinsic measures. (B-F) Resting membrane potential (mV), action potential amplitude (mV), threshold (mV) and duration (ms), and input resistance (MΩ) of layer IV A1 pyramidal neurons are not significantly different between control and ELS groups. Data are expressed as mean \pm SEM. n.s. $p > 0.05$.

The auditory cortex displays a developmental increase in AMPAR-mediated function across a CP of plasticity

Prior to exploring AMPAR functioning following an early life insult, ELS, we first aimed to establish a typical development course for AMPAR-mediated development. We established the developmental maturation of AMPAR-mediated sEPSCs across a CP for auditory tonotopic plasticity. Whole-cell voltage clamp recordings were performed at a -60mV holding potential to isolate spontaneous AMPAR-mediated currents. AMPAR-mediated sEPSC amplitude (pA) and frequency (Hz) were examined for differences across the CP of A1 development (prior to the CP P7-11, during the CP P12-15, and following the CP P16-18).

AMPA sEPSC amplitude data for the P12-15 group did not display a normal distribution and therefore multiple Mann Whitney Rank tests were performed. For comparisons between P7-11 and P16-18 groups a two-tailed student's unpaired t-test was completed. We observed a significant change in AMPAR amplitude between P7-11 (13.92 ± 0.85 pA, n=17 cells), P12-15 (17.82 ± 1.37 pA, n=17 cells, p=0.0043), and P16-18 (18.61 ± 1.31 pA, n=15 cells, p=0.0046). We did not observe any difference between P12-15 (17.82 ± 1.37 pA, n=17 cells) and P16-18 (18.61 ± 1.31 pA, n=15 cells, p=0.7658) groups. Frequency did not display a normal distribution and therefore multiple Mann Whitney Rank tests were performed. We observed a significant change in AMPAR frequency between P7-11 (0.1395 ± 0.01045 Hz, n=17 cells), P12-15 (0.3056 ± 0.05651 Hz, n=17 cells, p=0.0038), and P16-18 (0.2148 ± 0.03220 Hz, n=15 cells, p=0.0177). Once again, we did not observe a significant change between P12-15 (0.3056 ± 0.05651 Hz, n=17 cells) and P16-18 (0.2148 ± 0.03220 Hz, n=15 cells, p=0.2948) groups.

The data presented is consistent with literature pertaining to CP specific development of AMPAR-mediated currents, displaying a significant increase in AMPAR-mediated activity during the CP with a less significant increase in activity following the closure of the CP. Once we established this normal development of AMPAR-mediated activity we sought to explore whether it could be disrupted by chronic ELS in the form of maternal separation.

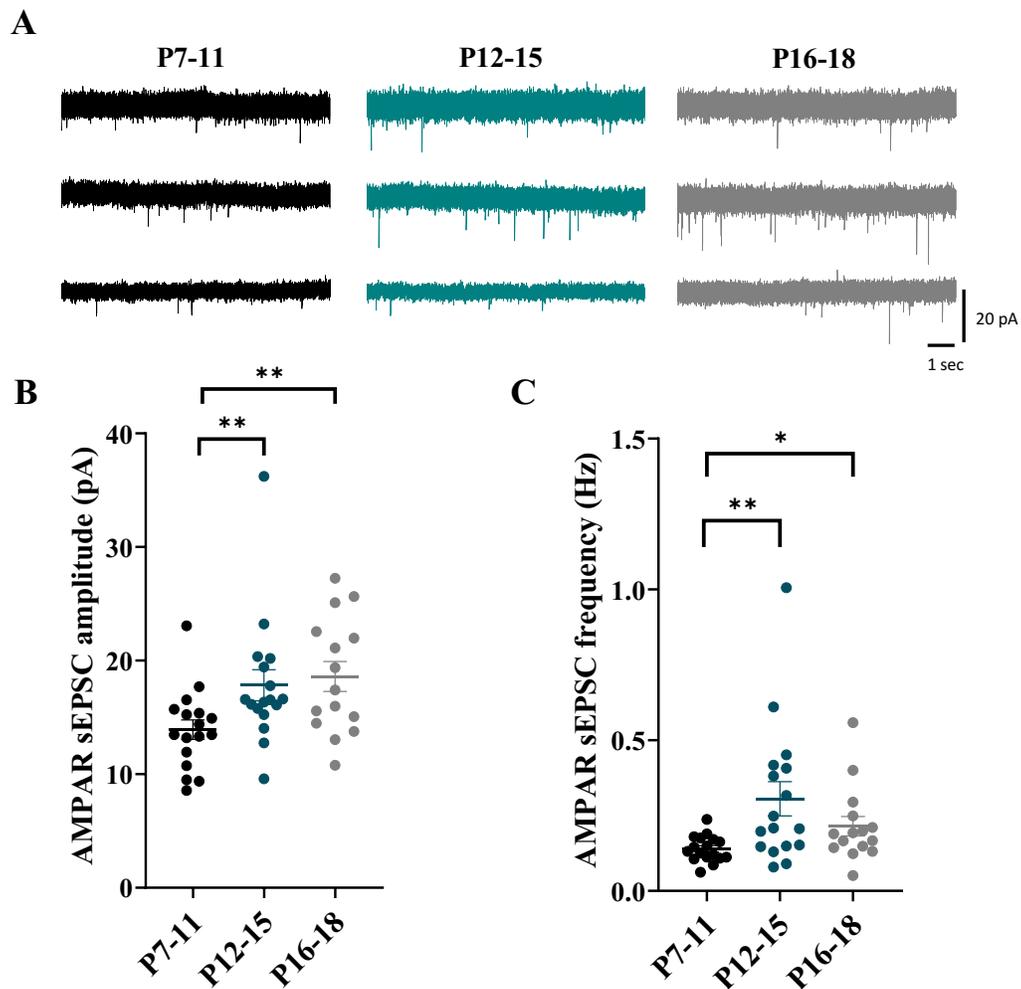


Figure 3. Developmental maturation of AMPAR-mediated sEPSCs in layer IV pyramidal neurons in A1. (A) Sample recordings of AMPAR sEPSCs in layer IV pyramidal neurons from P7-11 auditory coronal slices (left), P12-15 brain slices (middle), and P16-18 brain slices (right). (B-C) Amplitude (B) and frequency (C) data show AMPAR sEPSC events with larger amplitudes in layer IV pyramidal neurons during and following the CP, while sEPSC frequency also displays an increase during and following the CP in comparison to prior the CP. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

ELS does not significantly alter AMPAR function of randomly selected layer IV pyramidal neurons in A1

ELS effects on AMPAR sEPSCs. To determine the effects of chronic ELS on AMPAR-mediated sEPSCs of layer IV pyramidal neurons in A1, whole-cell voltage clamp recordings were performed at a -60mV holding potential to isolate spontaneous AMPAR-mediated currents. AMPAR-mediated sEPSC amplitude, frequency, rise and decay times were examined in A1 layer IV pyramidal neurons from both control and ELS animals.

AMPA-mediated sEPSC amplitude and rise and decay time data displayed a normal distribution. Therefore, two-tailed student's unpaired t-tests were completed. We found that AMPAR-mediated sEPSC amplitude, rise time and decay time were not significantly altered by ELS (amplitude: control 10.57 ± 0.40 pA, n=19 cells, ELS 11.61 ± 0.59 pA, n=17 cells, p=0.1480; rise time: control 1.998 ± 0.0988 ms, n=19 cells, ELS 1.659 ± 0.1624 ms, n=17 cells, p=0.0773; decay time: control 5.125 ± 0.344 ms, n=19 cells, ELS 5.263 ± 0.484 ms, n=17 cells, p=0.8143). AMPAR-mediated sEPSC frequency data was not normally distributed, and therefore a Mann-Whitney rank test was completed. We found that AMPAR-mediated sEPSC frequency was not significantly altered by chronic ELS (control 0.9927 ± 0.1097 Hz, n=19 cells, ELS 0.9731 ± 0.1270 Hz, n=17 cells, p=0.9252).

ELS effects on NMDAR-only silent synapses. Next, we sought to determine whether ELS impacts the conversion of silent synapses during the CP. Whole cell voltage clamp recordings in layer IV of A1 were performed with stimulation in layer VI of A1. Minimally evoked AMPAR and NMDAR-mediated eEPSCs were obtained by holding a cell at a -60mV holding potential and gradually increasing stimulation intensity until the cell responds approximately 50% of the time, achieving what we consider a 50% failure rate. Once this is

established the cell was then held at +40mV with the same stimulation intensity to evoke eEPSCs mediated by both AMPARs and NMDARs. The difference between the failure rate at -60mV and +40mV holding potentials provides enough information to establish an estimate for the percentage of silent synapses (NMDAR-only synapses).

All measures displayed a normal distribution and either paired or unpaired two-tailed student's t-tests were completed. Control neurons (n=7 cells) displayed failure rates at -60mV ($63.63 \pm 7.90\%$) and +40mV ($35.00 \pm 10.91\%$, $p=0.0010$) that were significantly different indicating a higher ratio of silent synapses. ELS neurons (n=5 cells) displayed failure rates at -60mV ($37.51 \pm 5.65\%$) and +40mV ($26.00 \pm 11.34\%$, $p=0.1989$) that were not significantly different indicating a potentially lower ratio of silent synapses. The failure rate difference for control ($28.62 \pm 4.83\%$, n=7 cells) and ELS ($11.49 \pm 7.47\%$, n=5 cells, $p=0.0710$) did not reach significance. Ultimately, the fraction of silent synapses did not significantly differ between control ($66.03 \pm 8.86\%$, n=7 cells) and ELS ($38.25 \pm 13.77\%$, n=5 cells, $p=0.1051$) mice during a CP of AC tonotopic mapping plasticity.

During early postnatal development neuronal networks at baseline are known to be heterogenous both spatially and temporally (Baroni & Mazzoni, 2014; Keller et al., 2010). Therefore, we decided to go a step further by observing whether a specific population of neurons became activated (positive for GFP expression) following chronic ELS, and whether these neurons displayed changes in AMPAR functioning compared to neurons not selectively activated.

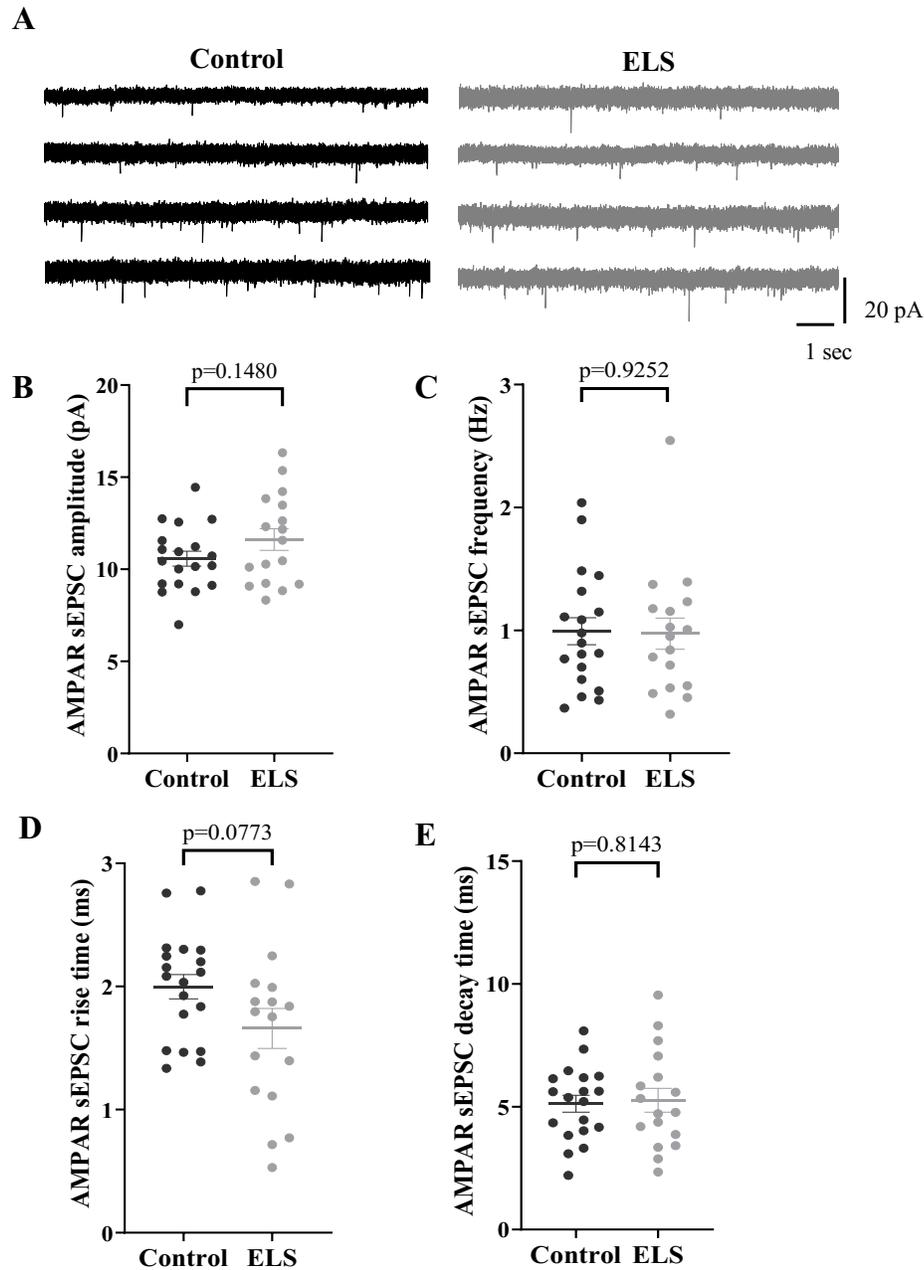


Figure 4. No observable changes in AMPAR-mediated sEPSCs during a CP in layer IV pyramidal neurons in A1 following ELS. (A) Sample recordings of AMPAR sEPSCs in layer IV pyramidal neurons from control (left) and ELS (right) brain slices. (B-C) Amplitude (B) and frequency (C) data show no changes in AMPAR sEPSC events between control and ELS groups. Data are expressed as mean \pm SEM. n.s. $p > 0.05$.

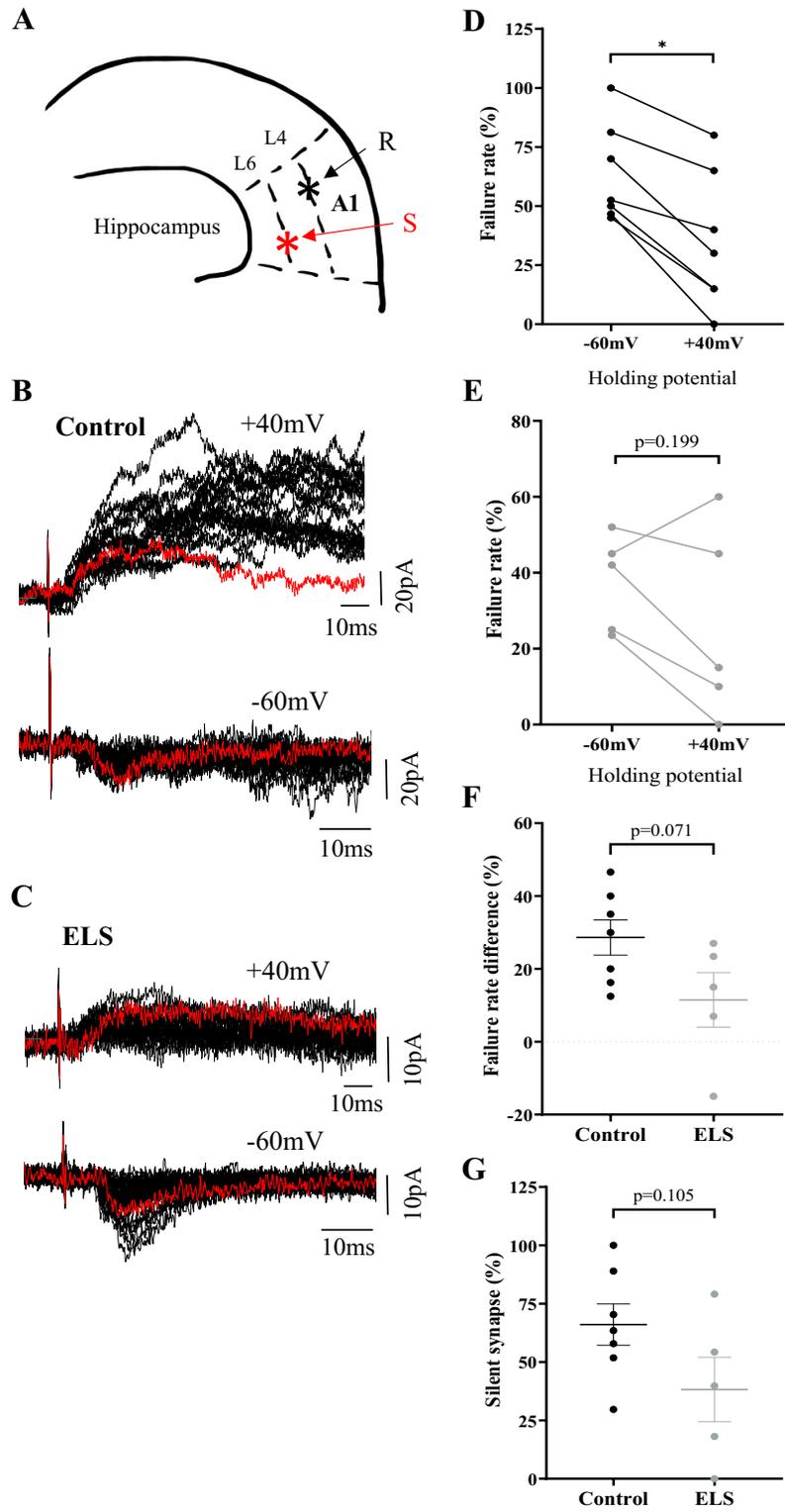


Figure 5. NMDAR-only silent synapses in A1 layer IV pyramidal neurons are not significantly altered by ELS. (A) Schematic of stimulation and recording sites in coronal A1 slices. (B-C) Sample minimal layer VI-evoked eEPSCs at +40mV (upper traces) and -60mV (bottom traces) from control (B) and ELS mice (C). (D-F) Failure rates at -60mV and +40mV holding potentials from control (D) and ELS (E) pyramidal neurons in layer IV. (G) Fraction of calculated silent synapses shows decrease in layer IV pyramidal neurons following ELS. (mean \pm SEM, * p <0.05).

ELS Selectively Activated Neurons display increased AMPAR-mediated activity

Immunohistochemistry. First, to identify whether a selective population of AC neurons are activated by chronic ELS, immunohistochemistry was performed using c-fos based transgenic mice with GFP antibody as an exogenous marker. A two-tailed student's unpaired t-test was performed on the data. There was a significant difference in the percent of neuronal activation via GFP tagging between control (10.75 ± 2.06 %, $n=4$) and ELS animals (36.00 ± 1.00 %, $n=2$, $p=0.0013$) in the A1 region. These findings encouraged further exploration into the electrophysiological properties of ELS activated neurons versus neurons in the AC that were not activated.

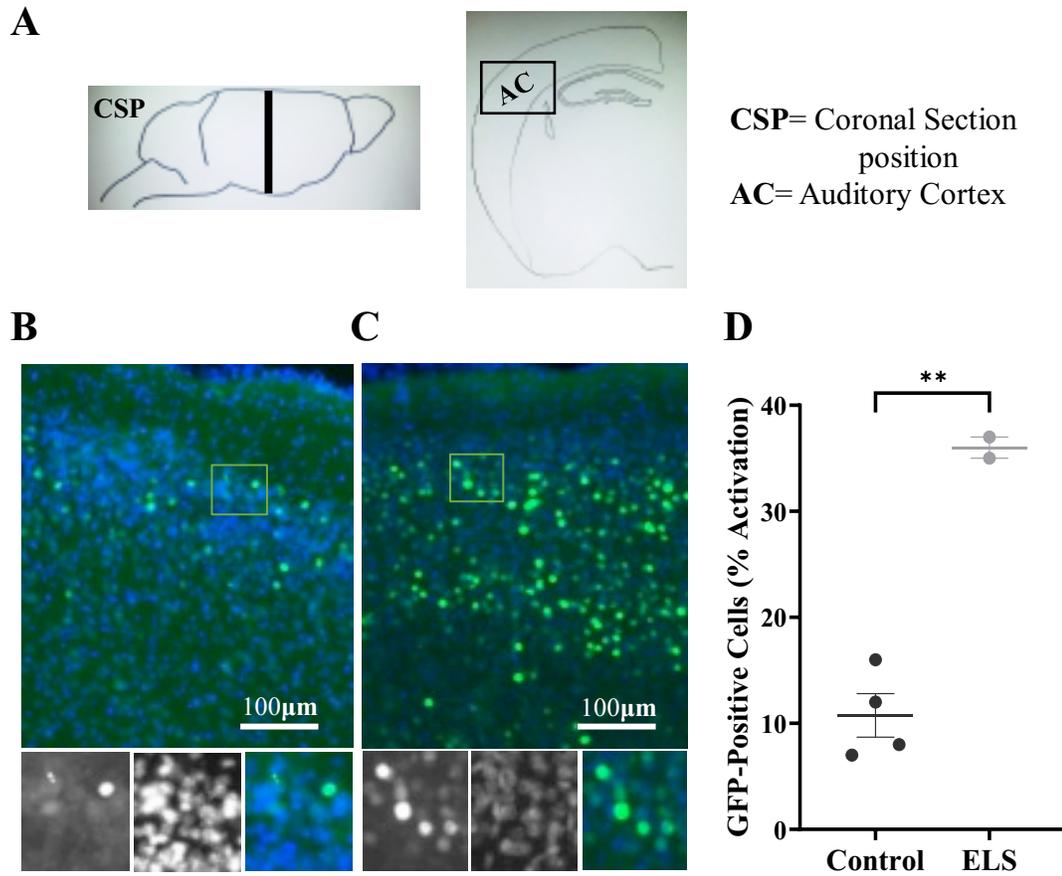


Figure 6. Chronic ELS induces selective neuronal activation in A1. (A) Schematic of the coronal slices containing the A1 region. (B) 20x (large) and 40x (small) images of GFP⁺ cells in A1 of control animals. (C) 20x (large) and 40x (small) images of GFP⁺ cells in A1 of ELS animals. (D) Mice following chronic ELS showed a significant increase in the number of GFP⁺ neurons in A1 compared to controls. Data are expressed as mean \pm SEM. ** $p < 0.01$.

AMPA function. After observing the subpopulation of layer IV pyramidal neurons in A1 activated by ELS we sought out to perform additional electrophysiological experiments targeting GFP⁺ (activated) and GFP⁻ (inactivated) neurons. By observing endogenous GFP tagging via our c-fos based transgenic mouse model, ELS sensitive and insensitive neurons were recorded. Whole-cell voltage clamp recordings were performed at a -60mV holding potential to isolate spontaneous AMPAR-mediated currents. AMPAR-mediated sEPSC amplitude, frequency, rise and decay times were examined in both GFP⁺ and GFP⁻ neurons.

Recordings on GFP^{+/-} neurons were performed on a different electrophysiological rig set up with epi-fluorescence, resulting in differing noise levels from previous data. We decided to present the following data as normalized to the mean values of GFP⁻ results. As a result, all values are first presented as the original value followed by a percentage of the mean GFP⁻ value. Amplitude, rise time, and decay time displayed a normal distribution and therefore two-tailed student's unpaired t-tests were performed on these data. Frequency was not normally distributed, and a Mann-Whitney rank test was performed. GFP⁺ neurons (25.37 ± 1.69 pA, 148.10 ± 9.86 %, n=6 cells) displayed a significant increase in AMPAR-mediated sEPSC amplitude over GFP⁻ neurons (17.12 ± 1.74 pA, 100.00 ± 10.18 %, n=5 cells, p=0.0082). Frequency measures did not show a significant difference between GFP⁻ (0.2146 ± 0.1562 Hz, 100.00 ± 72.77 %, n=5 cells) and GFP⁺ (0.5470 ± 0.1533 Hz, 254.50 ± 71.42 %, n=6 cells, p=0.1255) cells. Additionally, rise time for GFP⁻ (2.837 ± 0.737 ms, 100.00 ± 25.98 %, n=5 cells) and GFP⁺ (2.157 ± 0.141 ms, 76.01 ± 4.96 %, n=6 cells, p=0.3454) neurons and decay time for GFP⁻ (6.619 ± 0.585 ms, 100.00 ± 8.84 %, n=5 cells) and GFP⁺ (6.168 ± 0.618 ms, 93.19 ± 9.33 %, n=6 cells, p=0.6142) neurons showed no significant changes.

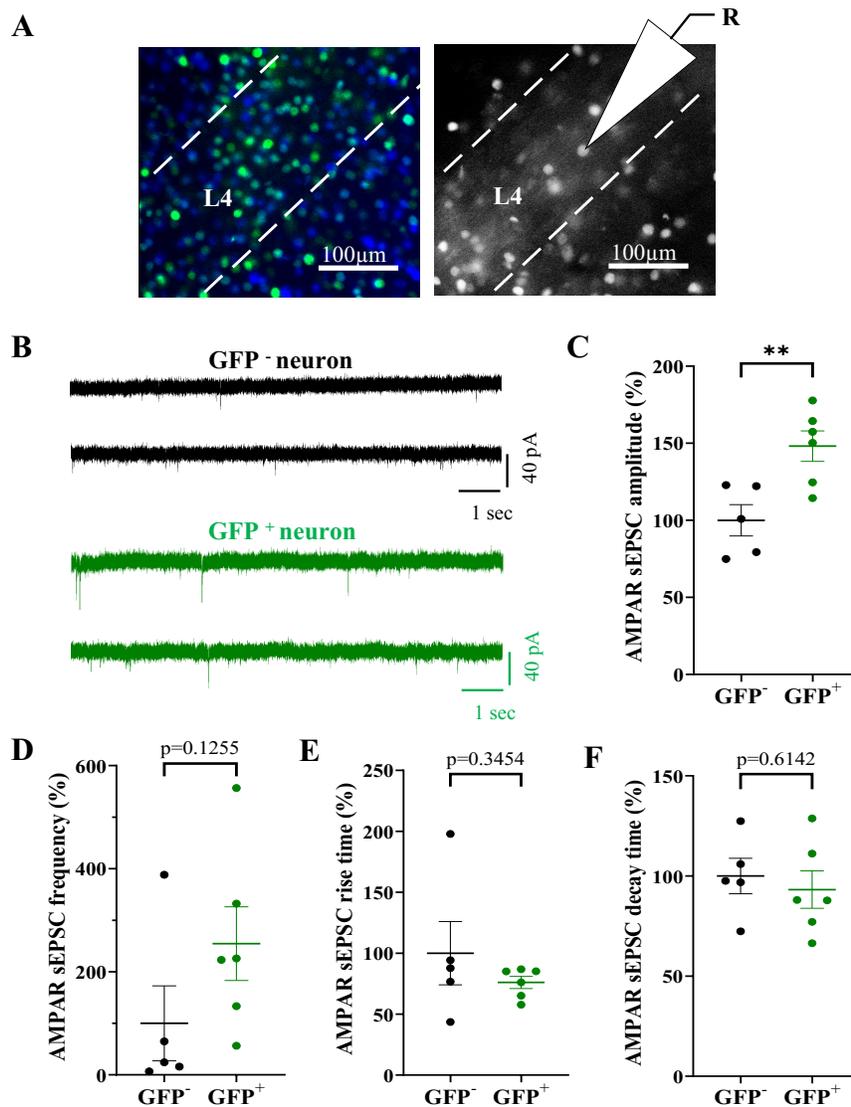


Figure 7. Chronic ELS increases AMPAR function in layer IV of GFP⁺ neurons in A1. (A) 20x images of endogenous GFP⁺ cells in layer IV of A1 from 300 μ m recording slices via imaging microscope (right) and electrophysiology microscope (left). (B) Representative spontaneous traces for GFP⁻ and GFP⁺ neurons. (C) Whole-cell voltage clamp recordings in layer IV pyramidal neurons in A1 from mice with chronic ELS show a significant increase in the amplitude of AMPAR-mediated sEPSCs in GFP⁺ neurons compared to GFP⁻ neurons. (D-F) No significant differences in frequency, rise and decay time of AMPAR-mediated sEPSCs were observed between GFP⁺ and GFP⁻ neurons in A1 following chronic ELS. Data are expressed as normalized mean (% of GFP⁻ mean) \pm SEM. ** $p < 0.01$.

Intrinsic properties. Selectively activated and non-activated neurons were further compared based on their intrinsic membrane properties. To determine the effects of chronic ELS on intrinsic membrane properties of GFP⁺ and GFP⁻ layer IV pyramidal neurons in A1 whole-cell current clamp recordings were performed and resting membrane potential (mV), input resistance (M Ω), the AP amplitude (mV), AP threshold (mV), and AP duration (ms) were obtained. Once again, data was normalized to the mean values of GFP⁻ results. Resting membrane potential, AP threshold, AP duration, and input resistance displayed a normal distribution and therefore two-tailed student's unpaired t-tests were performed on these data. AP amplitude was not normally distributed, and a Mann-Whitney rank test was performed. All normalized intrinsic measures did not display a significant change between neuronal subpopulations, resting membrane potential (GFP⁻ -67.57 ± 2.60 mV, 100.00 ± 3.83 %, n=3 cells, GFP⁺ -73.11 ± 4.50 mV, 108.20 ± 6.65 %, n=3 cells, p=0.3460), AP amplitude (GFP⁻ 54.15 ± 4.52 mV, 100.00 ± 8.36 %, n=3 cells, GFP⁺ 74.69 ± 6.44 mV, 137.90 ± 11.89 %, n=3 cells, p=0.1000), AP threshold (GFP⁻ -37.70 ± 2.10 mV, 100.00 ± 6.64 %, n=3 cells, GFP⁺ -41.70 ± 1.16 mV, 115.2 ± 3.19 %, n=3 cells, p=0.1089), AP duration (GFP⁻ 5.967 ± 0.982 ms, 100.00 ± 16.46 %, n=3 cells, GFP⁺ 5.000 ± 1.242 ms, 83.80 ± 20.82 %, n=3 cells, p=0.5746), and input resistance (GFP⁻ $0.0003972 \pm 4.638 \times 10^{-5}$ M Ω , 99.31 ± 11.59 %, n=3 cells, GFP⁺ $0.0002957 \pm 7.095 \times 10^{-5}$ M Ω , 73.92 ± 17.74 %, n=3 cells, p=0.2970). Although data did not reach significance, a trend in many of the intrinsic measures indicates further potential changes between GFP⁺ and GFP⁻ layer IV pyramidal neurons in A1 in addition to those observed in AMPAR-mediated sEPSCs.

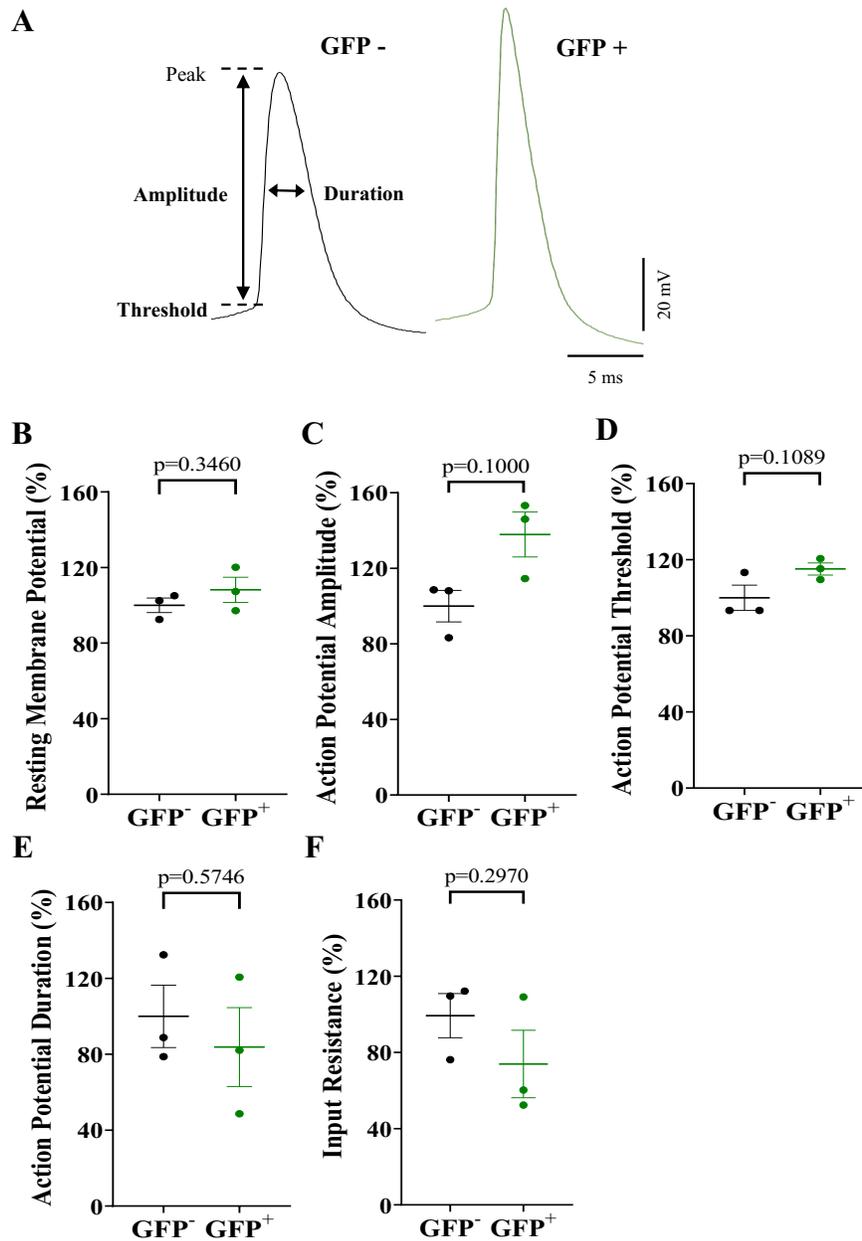


Figure 8. Chronic ELS does not significantly alter intrinsic properties of layer IV of GFP⁺ neurons in A1. (A) Representative AP trace from a GFP⁻ (left) and GFP⁺ (right) recording with indicated intrinsic measures. (B-F) Resting membrane potential, AP amplitude, threshold and duration, and input resistance of layer IV A1 pyramidal neurons are not significantly different between GFP⁻ and GFP⁺ neurons. Data are expressed as normalized mean (% of GFP⁻ mean) \pm SEM. n.s. $p>0.05$.

Discussion

The neonatal brain is particularly sensitive to experiences as a result of a series of CPs that define development. A key feature of this neural circuit refinement early in postnatal life is the developmental conversion of silent to functionally mature synapses. This process involves the trafficking of AMPARs into NMDAR-only synapses (Hanse et al., 2013), maturing and stabilizing these synapses during a CP of plasticity. As a result, the analysis of AMPAR-mediated synaptic functioning was of particular interest for this study.

CP plasticity in the AC has been known to be crucial for both functional brain development and cognitive function. Impaired A1 development during a CP for tonotopic mapping has been implicated in various neurological disorders of learning and memory, including ASD (Barkat et al., 2011; Port et al., 2017). Neurodevelopmental disorders, such as ASD, have been thought to be as a result of aberrant synaptic selectivity leading to immature synaptic networks. It is believed that early sensory and social experiences may contribute to this faulty connectivity, with particular interest in adverse experiences.

Stress in the context of a sensitive period of brain maturation may interfere with normal experience-induced plasticity and lead to maladaptive consequences (Bock & Braun, 2011; Chocyk et al., 2013; McClelland et al., 2011), including aberrant synaptic function of AMPAR-mediated excitation (Kallarackal et al., 2013; Makino & Malinow, 2009; Schmidt et al., 2010). What remains elusive is whether insults early in life such as ELS may directly alter AMPAR functioning in the context of a well-defined CP for tonotopic plasticity as modeled in A1.

The current study integrates knowledge related to ELS and aberrant excitatory synaptic activity during a CP of development in the AC. It was hypothesized that an insult such as ELS

occurring during a CP of rapid synaptic development may disrupt normal synaptic development, potentially leading to long term consequences. In all, this study suggests that ELS does have significant effects at the level of the synapse, and it is yet to be determined whether these changes will lead to long lasting aberrant synaptic connections and consequences. The main aims of the study were as follows: (1) To examine whether our model of ELS could induce CORT responses as well as disrupt normal early postnatal development, (2) To determine whether randomly sampled layer IV pyramidal neurons display altered intrinsic properties and AMPAR-mediated activity, (3) Further, to investigate what population of neurons are sensitive to chronic ELS and whether they display altered intrinsic properties and AMPAR function in A1. This study aimed to bridge a gap in the literature regarding early experience-dependent plasticity and the importance of AMPARs during this critical window, as well as, the effects of ELS in an uncommonly studied region, the AC.

Effects of ELS on Macro Physiological Measures

We induced ELS through the use of a MS model in P3-P15 transgenic c-Fos GFP mouse pups similar to the regime performed by Millstein et al., 2007 with the addition of social isolation as performed previously by Shin et al., 2018. Our results indicate that our model of ELS was insufficient to induce significant negative macro physiological changes in the mouse pups.

Weight of the mice did not significantly differ between control and chronic ELS groups. Based on previous knowledge and direct observation, we believe that mouse pup weight was not significantly altered by ELS because after returning the pups to their dam, the dam attempted to compensate for the separation by increasing maternal behaviours, including nursing (Mehta & Schmauss, 2011; S. Y. Shin, Han, Woo, Jang, & Min, 2016). Shin et al. (2016) displayed similar

findings following a MS protocol, observing no significant change in weight gain from P1 to 22 following exposure to repeated MS. We anticipate that the length of daily separation was not enough to induce significant weight changes as previously observed by Enthoven et al. 2008, where weight was altered following extended periods of separation of 8 hours from P3-5. It could also be argued that ELS induced weight changes would be more robust at a more sensitive stage in development.

CORT levels were largely heightened and variable in control animals that experienced handling and separation from their dam for the first time. Previous findings indicate that with repeated exposure to MS baseline CORT reactivity decreases over a 3-day period (Enthoven et al., 2008), and baseline (prior to separation) CORT levels are lower in repeated separation animals than controls (Horii-Hayashi et al., 2013) while acute CORT reactivity is heightened in comparison to mice without previous separation experience (Enthoven et al., 2008). Ultimately, we are unable to make conclusions regarding CORT reactivity in these animals at this time without “handled” controls (McCormick et al., 1998) (i.e. animals that were briefly handled daily without separation, to remove the effect of novelty exposure to handling as observed in our control group).

Effects of ELS on Randomly Selected Neurons

With little observable changes in macro physiological measures following ELS we sought to explore potential changes at the level of the synapse. First, we observed a significant increase in AMPAR-mediated current amplitude and frequency during and following a finite CP for plasticity in A1 from P12-15, supporting the importance of AMPARs during the course of a CP for synaptic plasticity. This data confirmed previous studies suggesting that over the course

of a CP AMPAR transmission is greatly increased relative to NMDAR transmission (Durand et al., 1996; Kerchner & Nicoll, 2008). With these observations considered we sought to examine possible alterations in intrinsic properties and AMPAR-mediated activity following ELS.

We found no significant changes in intrinsic properties including resting membrane potential, input resistance and various AP measures in layer IV pyramidal neurons in A1. These findings were consistent with those found by Herpfer and colleagues (2012). They observed no changes in multiple AP measures and input resistance in CA1 pyramidal neurons following exposure to MS from P1-14 (Herpfer et al., 2012). Shifting focus to our primary interest, AMPAR-mediated changes, previous work indicates that stress may influence the expression and functioning of AMPARs. With the focus on the hippocampus in mature male rats, Kallarackal et al. (2013) observed selective decreases in AMPAR number and function at specific synapses within the CA1 region following exposure to chronic unpredictable stress. With other research indicating alternative results (Martin et al., 2009), it remains largely unclear how widely these findings vary across other brain regions, models of stress, and periods of development. Here, we observed no significant change in AMPAR functioning as well as no significance as to whether silent synapses are being prematurely converted to its final state. The population of pyramidal neurons recorded for these measures included an unknown ratio of ELS selectively activated neurons and those not activated, which we believed to cause our results to show no significant trends toward ELS induced intrinsic and AMPAR-mediated changes. We next decided to identify a population of ELS sensitive neurons and explore the changes observed selectively in these neurons.

Effects of ELS on Selectively Targeted GFP Activated Neurons

Shin et al. (2016) have described the hippocampus as being heterogeneous with different cell populations displaying vast differences in vulnerability to neuropathological conditions. We aimed to show that this is the case in A1, with selective populations of neurons showing increased sensitivity to ELS. In order to do this, we utilized a widely used marker for neuronal activation, the immediate early gene c-Fos (Appleyard, 2009). Previous work has shown enhanced c-Fos activation in a number of brain regions following MS models of ELS (Horii-Hayashi et al., 2013; Nishi et al., 2013), with none taking interest in exploring auditory cortical regions. Through the use of our transgenic c-Fos-GFP mouse model, we were able to observe a significant population of neurons with increased neuronal activity in A1 following ELS compared to controls.

Following this observation, we further explored whether GFP positive or negative neurons displayed differing intrinsic and AMPAR-mediated spontaneous properties. Here, we observed that stress selectively targeted neurons show an increase in AMPAR sEPSC amplitude with a trend toward an increase in frequency as well. Additionally, we observed slight trends toward an increase in intrinsic measures such as resting membrane potential, AP amplitude and threshold in ELS activated neurons. These findings appear to oppose work discussed above (Kallarackal et al., 2013), however we cannot make direct comparisons as different brain regions, developmental time points, methods of chronic stress, and selective neuronal populations were considered. To our knowledge, no work has been done to selectively observe electrophysiological measures in experience-dependent activated neurons compared with neurons that were not selectively activated, particularly in A1 following ELS. With the

emergence of these results, there remains many unknowns that should be further explored with subsequent research.

Limitations and Future Investigations

While the experimental design of this study was sufficient to address our main research interests, limitations were experienced which may have confined our results. We observed a significant effect of the housing location on our animals when dams were transported to an alternative facility to carry out our stress protocols. AMPAR-mediated currents displayed significant changes in amplitude based on housing location, making comparisons between baseline and experimental data not possible. Related to access to space for experiments, time constraints disallowed for sufficient data before, during and following the CP to be collected for experimental animals. This additional data would have been very interesting in better understanding if ELS induced effects may have interacted with the addition of acoustic experience. It should be noted that none of the animals that were used for stress experiments had experienced the opening of the ear canal prior to sacrifice. Moreover, stimulation data for GFP positive and negative cells would have served to better understand the potential silent synapse changes occurring in the stress sensitive neuron population.

Further work is encouraged to address ELS induced changes over the span of the CP, particularly extending into the period following the CP. Perhaps changes are more robust following the closure of the CP, with the opening of the ear canal and the AC being fully functional. In addition, data regarding silent synapse ratios in GFP positive and negative neurons is also of particular interest going forward. With this data we may be able to better determine

whether changes following ELS have the potential to be long-lasting and lead to learning and memory dysfunction phenotypes.

Another avenue of interest would be to further explore genetic variations between stress sensitive and insensitive neurons as well as the AMPARs present in either neuronal subpopulation. As previously indicated by Schmidt et al. (2010), it is believed that individual variations in stress vulnerability may be linked to genetic variations in AMPAR subunits. With these discoveries we may eventually be able to genetically manipulate AMPAR composition to either increase or decrease vulnerability to stress induced effects opening up the possibility for specific therapeutic targets to be identified.

Conclusion

In summary, our results suggest that ELS can directly alter excitatory synaptic functioning during a CP of plasticity in the AC. It appears that while synaptic effects cannot be observed among randomly sampled population of layer IV pyramidal neurons in A1, they are evident when isolating selectively activated subpopulations of neurons in A1, which show significant increases in AMPAR function compared to non-activated neurons. At this time, we are unable to determine whether these changes will lead to lasting aberrant synaptic plasticity following the opening of the ear canal. Additional work is needed to further understand the role of these stress activated neurons in the functionality of the AC further in development. These ELS-sensitive neurons may have critical roles in ELS-induced long-term cognitive consequences including learning and memory impairments and increased sensitivity to stress and represent novel cellular targets for emerging therapeutic strategies.

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