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2. Alipio, J. B., **Haga, C.**, Fox, M. E., Arakawa, K., Balaji, R., Cramer, N., Lobo, M. K., & Keller, A. (2021). Perinatal fentanyl exposure leads to long-lasting impairments in somatosensory circuit function and behavior. *The Journal of Neuroscience*, JN-RM-2470-20. <https://doi.org/10.1523/JNEUROSCI.2470-20.2020>
3. Eley JG, **Haga CW**, Keller A, Lazenby EM, Raver C, Rusek A, Dilmanian FA, Krishnan S, Waddell J. Heavy Ion Minibeam Therapy: Side Effects in Normal Brain. *Cancers (Basel)*. 2021 Dec 9;13(24):6207. doi: 10.3390/cancers13246207. PMID: 34944825; PMCID: PMC8699126.

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2. **Haga CW**, Koenig JA, Keller A. Effects of nicotine on parvalbumin neurons in rat somatosensory cortex during the critical period. Society for Neuroscience, November 2022, San Diego, CA. Virtual poster.
3. Alipio JB, Li Y, **Haga CW**, Balaji R, Fox ME, El-Metwally D, Lobo MK, Picciotto MR, Keller A. Perinatal exposure to drugs of abuse causes permanent aberrations in somatosensory circuits. Gordon Research Conference on Thalamocortical Interaction, Feb 2020, Ventura, CA.

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Abstract

From Early Development to Adult Circuits: Nicotinic Modulation of Fast-Spiking Interneurons in the Somatosensory Cortex

Catherine W. Haga, Doctor of Philosophy, 2025

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Signaling at nicotinic acetylcholine receptors (nAChRs) is vital for normal development of cerebral cortical circuits. These developing circuits are also shaped by fast-spiking (FS) inhibitory neurons in somatosensory cortex, an important model of development in rodents. Long-term deficits in sensory and cognitive processing can result from insults to either FS neurons or cholinergic function. Nicotine, the psychoactive component of tobacco, has been used extensively as an agonist to elucidate the functional properties of these receptors in the cholinergic system. Early exposure to nicotine during development results in long-term deficits in attention and sensory processing. While nicotinic dysfunction in FS neurons is implicated in a number of psychiatric and neurodevelopmental disorders, FS neurons have not been shown to have nicotinic responses in adults. Here, we establish the early timeline of how FS neurons respond to nicotine presynaptically and postsynaptically and test the prediction that nicotinic responses in FS neurons are developmentally regulated. We explored presynaptic effects from the early postnatal period through adolescence and found that nicotine drives early increases in frequency and amplitude of spontaneous synaptic currents. The increases in frequency persisted through development, but there were no amplitude changes after the second postnatal week, revealing an early window of nicotinic responses. The presynaptic effects we discovered demonstrate a potential weakness to nicotinic disruptions, including prenatal nicotine exposure

and genetic mutations of nAChRs, specifically affecting FS neurons, which drive development of early cortical circuits. We also investigated somatodendritic effects of nicotine and found that FS neurons in primary somatosensory cortex (S1) lack these responses throughout development, as early as these neurons can be identified by their kinetic properties at P10. We ruled out desensitization as a mechanism for this finding, and RNAscope™ further revealed that FS neurons express abundant mRNA for multiple nAChR subunits beginning early in development. Finally, we found that *Lynx1*, a unique nicotinic modulator, is expressed in FS neurons in S1 from early in development and may act as a brake on nicotinic responses. The nicotinic mechanisms driving the development of FS neurons are understudied, despite the importance of both FS neurons and cholinergic signaling in cortical development. This work addresses this gap by examining nicotine's effects on FS neurons in the somatosensory cortex across different developmental stages, contributing to a better understanding of cholinergic regulation of these interneurons during cortical development.

From Early Development to Adult Circuits: Nicotinic Modulation of Fast-Spiking Interneurons
in the Somatosensory Cortex

by
Catherine W. Haga

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For my husband, Dan Haga, and my children Izabel, Grayson, Archer, & Russell.

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Abbreviations List

ACh	Acetylcholine
ACSF	Artificial cerebrospinal fluid
AHP	After-hyperpolarization
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	DL-2-amino-5-phosphonopentanoic acid
cDNA	Complementary deoxyribonucleic acid
CHRNA4	Gene coding for neuronal acetylcholine receptor subunit α 4
CHRNA7	Gene coding for neuronal acetylcholine receptor subunit α 7
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
DIC	Diffusion interference contrast
EPSC	Excitatory postsynaptic current
FS	Fast-spiking
FWHM	Full width at half max
GABA	γ -Aminobutyric acid
GPI	glycosylphosphatidylinositol
IgG	Immunoglobulin G
iPSC	Induced pluripotent stem cell
IPSC	Inhibitory postsynaptic current
L4	Cortical layer 4
Lynx1	Lymphocyte antigen 6 neurotoxin 1, or Lypd1
mAChR	Muscarinic acetylcholine receptor
mRNA	Messenger ribonucleic acid

mOsm/L	milliosmole per liter
nAChR	Nicotinic acetylcholine receptor
NBF	Neutral buffered formalin
NIH	National Institutes of Health
NIMH	National Institute of Mental Health
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
PLC	Phospholipase C
PNN	Perineuronal net
PV	Parvalbumin
RS	Regular-spiking
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S1	Primary somatosensory cortex
sEPSC	Spontaneous excitatory postsynaptic current
sIPSC	Spontaneous inhibitory postsynaptic current
sPSC	Spontaneous postsynaptic current
tPA	Tissue plasminogen activator

I. GENERAL INTRODUCTION

The development of cerebral cortical circuits is guided, in part, by the maturation of both fast-spiking, GABAergic, parvalbumin-expressing neurons (FS neurons) and the cholinergic system (Dwyer et al., 2008; Lo et al., 2017). Indeed, insults to either FS neurons or cholinergic function can result in cortical dysfunction and in deficits associated with a number of psychiatric disorders (Hijazi et al., 2023; Thippaiah et al., 2022; Lin et al., 2014). To better understand the roles of FS neurons and nAChRs in cortical development, here we establish the developmental timeline of FS neurons' responses to nicotine in the barrel field of primary somatosensory cortex, a well-established developmental model of cortical circuits and functions (Erzurumlu & Gaspar, 2012; Erzurumlu & Gaspar, 2020).

A. Early Cortical Development

The second postnatal week is a developmental turning point in rodents. The first weeks of life in rodents represent a somatosensory 'critical period', in which the cortical representation of topographic maps of vibrissa, or whiskers, in the brain ("barrels") are mapped and refined (Erzurumlu & Gaspar, 2012). Whisker trimming or plucking, a form of sensory deprivation, during or prior to this period have lifelong effects on somatosensory processing, and thus on an animal's perception (Erzurumlu & Gaspar, 2012; Lee et al., 2009). The vibrissa system represents a well-studied developmental model of sensory perception in a modality that is highly relevant to rodent behavior. The second postnatal week is a unique developmental milestone in

the somatosensory cortex (Lo et al., 2017). This period of brain development in rodents is the equivalent to the third trimester in human pregnancy (Clancy et al., 2007).

Development is also driven by FS, parvalbumin-expressing GABAergic neurons, a large class of inhibitory cortical neurons with characteristic fast-spiking properties (Hensch et al., 2005). FS neurons drive use-dependent plasticity and circuit development during critical periods (Choi et al., 2018; Rupert & Shea, 2022; Lee et al., 2012; Kaplan et al., 2016; Hensch et al., 2005).

During the second postnatal week in rodent S1, the biophysical and chemical properties of FS neurons mature (Itami et al., 2007). γ -Aminobutyric acid (GABA) switches polarity from excitatory to inhibitory as the chloride gradient changes (Liu et al., 2006), the window of experience-dependent plasticity closes (Lo et al., 2017), and bidirectional Hebbian plasticity emerges (Itami et al., 2012). FS neurons are crucial regulators of use-dependent plasticity during sensory critical periods (Choi et al., 2018), and are involved in many of these developmental changes. They may be particularly sensitive to developmental toxicants during this time period.

B. FS Neurons: Background

Inhibitory neurons make up approximately 10 to 20% of cortical neurons, and FS neurons constitute the largest group (40%) of these neurons (Llorca & Deogracias, 2022; Rudy et al., 2011). Most FS neurons are either basket or chandelier cells, which selectively innervate the somata and proximal dendrites (Micheva et al., 2021) or axon initial segments (Gallo et al., 2020) of other neurons, respectively. FS neurons can be identified by the distinct properties of their action potentials (Subkhankulova et al., 2010; Tateno et al., 2004; McCormick et al., 1985). These include a brief spike duration, high firing frequency, a large after-hyperpolarization (AHP), and little or no spike-frequency adaptation (McCormick et al., 1985; Faini et al., 2017).

FS neurons provide powerful inhibition to other neurons, regulate excitation-inhibition balance, drive cortical oscillations, and contribute to the highly precise spike-timing of pyramidal neurons (Faiti et al., 2017; Micheva et al., 2021; Rupert & Shea et al., 2022; Mannekote et al., 2022).

Networks of FS neurons drive synchronous neuronal activity that is critical for normal information processing (Sohal et al., 2009; Onorato et al., 2025). Synchronous inhibition of pyramidal neurons driven by FS neurons generates gamma oscillations correlated with cognitive tasks, including attention, working memory, and sensory processing (Carlen et al., 2012; Guo et al., 2023; Onorato et al., 2025). These oscillations are likely driven by glutamatergic neurotransmission mediated by NMDA receptors in PV neurons, as selective deletion of NMDA receptors from PV neurons causes deficits in gamma wave induction (Carlen et al., 2012). In a number of neuropsychiatric disorders, including schizophrenia, autism, and dementia, cognitive and behavioral deficits are associated with disrupted gamma oscillations driven by reduced function of networks of FS neurons (Thippaiah et al., 2022; Bueno-Junior et al., 2027; Carlen et al., 2012; Hijazi et al., 2023). Nicotine exposure in adult rodents potentiates gamma oscillations in prefrontal cortex, with a greater effect after repeated exposure (Bueno-Junior et al., 2017).

FS neurons also regulate use-dependent plasticity, particularly during development.

Use-dependent plasticity is a remarkable capacity of the brain to undergo structural and functional modifications in response to sensory input, environmental stimuli, and learning throughout life. These modifications may include changes to synaptic strength resulting from dendritic spine density, size, and shape, expression of neurotransmitter receptors, and cell excitability (Sadahiro et al., 2016; Morishita et al., 2010; Rupert & Shea, 2022). Use-dependent plasticity is also regulated by perineuronal nets (PNNs) (Fawcett et al., 2022). PNNs are a unique

component of the extracellular matrix that are expressed in close proximity to PV neurons (Miao et al., 2014) and serve as a brake on plasticity, though the mechanism of this brake is not fully understood (Faini et al., 2017, Hensch et al., 2005; Hijazi et al., 2023; Fawcett et al., 2022).

PNNs are thought to regulate number of dendritic spines, receptor motility, and maturation, size, and strength of synapses (Fawcett et al., 2022). PNNs are upregulated during the first postnatal weeks in cortex as multiple critical periods are closing and as use-dependent plasticity is reduced (Faini et al., 2017). FS neurons can rapidly mobilize use-dependent plasticity mechanisms by breaking down PNNs during early development (Sadahiro et al., 2016; Bukhari et al., 2015). This mechanism is greatly reduced in adulthood, as is use-dependent plasticity, but it can be restored through disruption of PNNs (Sadahiro et al., 2016; Bukhari et al., 2015). Removal of PNNs in adult animals is also associated with increased gamma oscillations during sensory tasks, a mechanism consistent with increased activation of PV neurons (Faini et al., 2017).

These mechanisms intersect in the etiology of a number of disorders associated with aberrant cognitive functioning. Neuronal hyperexcitability is a hallmark of mouse models of Alzheimer's disease (Hijazi et al., 2023). A proposed mechanism driven by early increases in soluble amyloid-beta protein implicates increased excitability of FS neurons coupled with an increase in PNNs around FS neurons (Hijazi et al, 2023). In later stages, FS neurons become hypoactive while pyramidal neurons remain hyperexcitable due to increasing amyloid-beta pathology and more amyloid plaques. This corresponds to reduced expression of Nav1.1 and Kv3 channels that are expressed in PV neurons and contribute to their fast-spiking kinetics (Hijazi et al, 2023; Du et al., 1996; Okaty et al., 2009). In human and rodent data, this phenotype includes reduced gamma oscillations and increased epileptic activity as part of the disease process (Hijazi et al,

2023). Enhancing GABA transmission via PV neurons restores some of the deficits associated with Alzheimer's disease models (Verret et al., 2012).

FS neurons mature relatively late in development. The first detectable immunoreactivity for parvalbumin is around postnatal day 9 (P9) in rodents (Alcántara et al., 1993; de Lecea et al., 1995), with expression becoming more easily detectable by P13 (de Lecea et al., 1995; Itami et al., 2007). FS neurons receive excitatory inputs from the thalamus and intracortical excitatory projections, as well as inhibitory projections from FS neurons and other GABAergic neurons, such as those expressing somatostatin (Lo et al., 2017). After migration of FS neurons to the cortex ends by the end of the first postnatal week, dendritic branching increases and synaptic connections mature as they continue to develop (Gallo et al., 2021; Tai et al., 2019). Many of these connections are selectively pruned in the third postnatal week, leading to a reduction in expression of parvalbumin protein in the cortex as part of the normal course of development (Gallo et al., 2021; Steinecke et al., 2017). FS neurons also develop their characteristic kinetic properties towards the end of the second postnatal week due to increasing expression of specialized ion channels, including Kv3 potassium channels (Du et al., 1996; Okaty et al., 2009). Developmental studies of inhibitory drive from FS neurons are complicated by the switch in GABA polarity from excitatory to inhibitory that also occurs during this time period (Liu et al., 2006; Ben-Ari et al., 1989).

C. nAChRs in Development

Acetylcholine (ACh) is the endogenous neurotransmitter that acts on muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively), which are expressed widely in

neocortex (Dwyer et al., 2008; Fiedler et al., 1987). In the periphery, ACh acts directly as a neurotransmitter, primarily at neuromuscular junctions. In the brain, ACh tends to act as a bulk neuromodulator, increasing neurotransmitter release, and shaping neural circuits through volume transmission mediated by diverse and widespread receptor expression (Picciotto et al., 2012). Through this mechanism, nAChRs are critically important for enhancing attention, cognition, and sensory processing (Demars & Morishita, 2014; Gil & Metherate, 2019; Picciotto et al., 2012). mAChRs are G-protein-coupled receptors, and nAChRs are ligand-gated ion channels (Garduño et al., 2012; Picciotto et al., 2012). nAChRs can be heteromeric combinations of α and β subunits or homopentamers made of α subunits only (Metherate et al., 2004; Albuquerque et al., 2009). The $\alpha 4\beta 2$ heteromeric and $\alpha 7$ homomeric receptors are the most expressed nAChRs in mammalian cerebral cortex (Albuquerque et al., 2009; Nair & Liu, 2019; Millar & Gotti, 2009). These receptors play complementary roles in shaping early circuits. Both can be expressed presynaptically or postsynaptically (Picciotto et al., 2012; Tregellas & Wylie, 2019). Presynaptic nAChRs induce neurotransmitter release including GABA, glutamate, dopamine, serotonin, norepinephrine, and even acetylcholine itself (Picciotto et al., 2012; McGehee & Role, 1995). Postsynaptic nAChRs contribute to neuronal excitation and long-term potentiation (Picciotto et al., 2012; McGehee & Role, 1995; Kawai et al., 2007).

Homomeric $\alpha 7$ nAChRs gate Ca^{2+} and Na^{+} currents and have a high ratio of Ca^{2+} to Na^{+} permeability, contributing to their importance for regulation of plasticity and development (Dwyer et al., 2008; Tregellas & Wylie, 2019). They have relatively low affinity for ACh and nicotine, but selectively respond to endogenous choline (Albuquerque et al., 1998; Dwyer et al.,

2008). $\alpha 7$ nAChRs desensitize rapidly to nicotine at concentrations over 1 μM , but also recover rapidly (Dwyer et al., 2008).

Heteromeric $\alpha 4\beta 2$ nAChRs primarily gate Na^+ currents, leading to membrane depolarization and facilitation of excitatory inputs (Dwyer et al., 2008; Anderson et al., 2020). They are characterized by their high affinity for nicotine, and they desensitize more slowly than $\alpha 7$ nAChRs, but at much lower concentrations (0.1 μM) (Dwyer et al., 2008; Lambe et al., 2003). The broad kinetic properties of these receptors have some variation based on stoichiometry of assembled subunits and inclusion of other subunits with unique parameters (Gotti et al., 2006; Anderson et al., 2020).

Neuronal nAChRs play an important role in early circuit development. They can enhance sensory processing during the early postnatal period when the sensory cortex is undergoing rapid development (Gil & Metherate; 2019; Naeff et al., 1992). These receptors are upregulated during this time and their expression in the cerebral cortex peaks by the end of the second postnatal week (Fiedler et al., 1987; Naeff et al., 1992; Tribollet et al., 2004). This time period represents a critical period of development for the sensory cortex (Erzurumlu & Gaspar, 2012; Lo et al., 2017). Disruption during these periods has lifelong effects on the brain's ability to respond appropriately to sensory inputs and produce use-dependent plasticity. Nicotine has been used extensively as an agonist to elucidate the functional properties of nAChRs and the cholinergic system in the brain (Metherate et al., 2004; Dwyer et al., 2008).

D. Nicotinic modulation of FS Neurons

Nicotinic signaling in FS neurons is implicated in neuropsychiatric disorders that involve network dysfunction, including autism and schizophrenia (Lin et al., 2014; Deutsch & Burkett, 2020). Mutation or microdeletion of the *CHRNA7* gene coding for the $\alpha 7$ homomeric receptor is associated with human disorders including schizophrenia, intellectual disability, autism spectrum disorder, and epilepsy (Lin et al., 2014; Sharp et al., 2008; Tregellas & Wylie, 2019). Behavioral abnormalities in attention, working memory, and learning associated with these disorders have also been functionally linked to deletion of this receptor in rodent models (Lin et al., 2014; Deutsch & Burkett, 2020;). FS neurons are implicated in the mechanisms of these associations, as deletion of the $\alpha 7$ gene causes abnormal PV neuron development (Lin et al., 2014). Up to 70% of adults with schizophrenia are smokers, the highest of any neuropsychiatric disorder – likely due to $\alpha 7$ -mediated dysfunction in FS neurons (Tregellas & Wylie, 2018). Thus, nicotinic signaling in FS neurons is critical for normal cortical development.

There is considerable debate as to whether PV neurons express nAChRs. While some authors maintain that most PV neurons do not express nAChRs (Askew et al., 2019; Couey et al., 2007), co-expression of both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in PV neurons has been demonstrated in human temporal cortex (Krenz et al., 2001), and $\beta 2$ receptors have been identified in PV neurons in macaque visual cortex (Disney et al., 2007). However, FS neurons do not respond postsynaptically to nicotine, at least during late postnatal development through adulthood. This has been confirmed in auditory, visual, somatosensory, and prefrontal cortical areas (Couey et al., 2007; Gullledge et al., 2007; Askew et al., 2019). In contrast, whether FS neurons respond to

nicotine at earlier developmental ages—prior to postnatal day 15 (P15)—has not been established.

E. The Ly6 Family of Proteins: Endogenous Nicotinic Modulators

In the rodent brain, FS neurons express Lynx1, a member of the Ly6/uPAR/neurotoxin superfamily of proteins that directly binds nAChRs and reduces their signaling (Ibañez-Tallon et al., 2002; Miwa et al., 2021). Nicotinic signaling in cortex promotes neural plasticity, particularly during early development. Windows of plasticity in development close at prescribed time periods as the brain matures. This plasticity at the transcriptional level is largely controlled by increasing Lynx1 expression, rather than modulation of nAChR expression or desensitization (Anderson et al., 2020; Takesian et al., 2018). Lynx1 acts as a molecular brake on nicotinic signaling by orthosterically binding receptors and thus suppressing plasticity, memory, and cognition to mature status (Miwa et al., 2021; Morishita et al., 2010; Takesian et al., 2018).

In the rodent visual cortex, Lynx1 has been implicated in the monocular deprivation model of use-dependent plasticity. In this model of functional and structural plasticity, young animals are deprived of visual input from one eye-- resulting in a permanent loss of visual acuity, or amblyopia (Morishita et al., 2010). Amblyopia does not develop when animals undergo monocular deprivation in adulthood, as this is past the critical period of plasticity in visual cortex (Morishita et al., 2010). In mice lacking the *Lynx1* gene, this window of plasticity remains open well into adulthood, resulting in amblyopia following monocular deprivation at older ages. From this research and other studies, Lynx1 has been established as a brake on nicotinic signaling and

nicotinic-receptor-dependent plasticity in cortex (Morishita et al., 2010; Anderson et al., 2020; Takesian et al., 2018; Miwa et al., 2021). Lynx1 has also been considered as a promising molecular target for treating disorders affecting neuroplasticity, memory, and cognition, such as Alzheimer's disease and Fragile X Syndrome (Thomsen et al., 2016; Talvio et al., 2022).

One possible mechanism linking the role of Lynx1 and nAChRs to regulation of plasticity by PV neurons revolves around the control of perineuronal nets. PV neurons express tissue plasminogen activator (tPA), a protein that degrades PNNs and is linked to activation of plasticity in an activity-dependent manner (Lepine et al., 2022; Takesian et al., 2018). PV neurons lacking tPA are surrounded by PNNs that are denser, resulting in less plasticity (Lepine et al., 2022; Takesian et al., 2018). This mechanism of activating plasticity via tPA-driven breakdown of PNNs is mostly downregulated in adulthood but can be reactivated by removing the Lynx1 brake (Bukhari et al., 2015). In the ocular dominance plasticity model in visual cortex, knockdown of Lynx1 reopens the critical period window in adult animals (Bukhari et al., 2015). As Lynx1 acts by inhibiting nicotinic signaling, this plasticity mechanism likely relies on intact nicotinic signaling within PV neurons at young ages when the brain has the highest plasticity, but this has not been directly tested.

Importantly, all studies of Lynx1's control of plasticity have focused on auditory (Takesian et al., 2018) or visual cortex (Morishita et al., 2010; Bukhari et al., 2015; Sadahiro et al., 2016; Sajo et al., 2016). The timeline of Lynx1 expression and its role in the somatosensory cortex is not known. Lynx1 is expressed in PV neurons in adult rodents in the primary visual cortex (V1), but not somatostatin neurons (Demars et al., 2014). The increase in Lynx1 expression in V1 at the

end of the visual critical period (Morishita et al., 2010) may be a developmental feature in somatosensory cortex as well, but this yet to be explored.

Lynx1 may be an important cholinergic plasticity brake in multiple cortical areas, which presents treatment possibilities for perinatal nicotine exposure as well as other cognitive and developmental disorders that involve impaired cortical plasticity. In a model of Alzheimer's disease, mice with amyloid-beta and tau pathology have decreased Lynx1 levels in frontal cortex as they age (Thomsen et al., 2016). There is also evidence that Lynx1 and amyloid-beta compete for binding to nAChRs (Thomsen et al., 2016). Lynx1 may be protective against amyloid-beta pathology, and this mechanism may be mediated by PV neurons (Thomsen et al., 2016). Fragile X Syndrome (FXS) is a developmental disorder associated with abnormal neuroplasticity (Martin & Huntsman, 2012). In studies of induced pluripotent stem cell (iPSC) derived progenitors from control patients and patients with FXS, *lynx1* expression is significantly reduced (Talvio et al., 2022). Aberrant process growth in FXS iPSCs is associated with a tPA-dependent mechanism downstream of Lynx1 (Talvio et al., 2022).

F. Hypothesis and Predictions

Despite evidence that FS neurons express nAChRs, these neurons have been shown not to respond to nicotinic stimulation in adults. In contrast, whether FS neurons respond to nicotine at earlier developmental ages—prior to postnatal day 15 (P15)—has not been established. FS neuron activity is critically involved in cortical development, as are nAChRs. Here, we address this knowledge gap by studying the actions of nicotine on both presynaptic and postsynaptic responses of FS neurons across development. We also investigate the developmental expression

of Lynx1, an important modulator of nicotinic signaling and use-dependent plasticity, in S1 and in FS neurons.

We hypothesize that nicotinic responses in FS neurons in somatosensory cortex are developmentally regulated. We expand this hypothesis to posit that FS neurons have unique responses to nicotinic stimulation exclusively early in development, and that late responses are blocked by increasing expression of Lynx1 protein. We test several predictions that rise from these hypotheses in two experimental chapters of this Dissertation:

Chapter III:

Specific Aim 1: Nicotine increases excitatory inputs to FS neurons in S1 region of the somatosensory (barrel) cortex of rats from early ages.

- Prediction: Nicotine increases frequency and amplitude of sEPSCs in FS neurons in the early postnatal period through adolescence

Chapter IV:

Specific Aim 2: Nicotine has somatodendritic effects on FS neurons exclusively early in development

- Prediction 1: Nicotine induces somatodendritic responses in FS neurons in the second postnatal week
- Prediction 2: FS neurons lack somatodendritic nicotinic responses in late development through adolescence
- Prediction 3: FS neurons express mRNA for nAChR subunits beginning in the second postnatal week

- Prediction 4: Developmental increase in *lynx1* expression correlates with developmental loss of FS responses to nicotine

II. GENERAL METHODS & STATISTICS

A. *Animals*

All procedures adhered to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine. Male and female Long-Evans rats purchased from Charles River were bred in our temperature- and humidity-controlled vivarium. Animals were fed standard chow *ad libitum* and maintained on a 12-hour light/dark cycle. Rats were bred in monogamous pairs with gestational timing confirmed by the presence of sperm in vaginal lavage samples. Dams were single housed immediately before delivery until study endpoint. Litters were culled to 8-12 pups per dam. Preweanling offspring were used in most experiments. In some cases, pups were weaned at P21 and socially housed until study endpoint. Preweanling (P12) and adult (P100) male and female C57BL/6 mice were used for Lynx1 RNAscope™ experiments.

B. *Statistical Analyses*

Statistical tests were conducted using Graphpad Prism 10 software (Boston, MA). Sample sizes were determined *a priori* using G*Power software suite (Faul et al., 2007). Statistical significance level was set at $p < 0.05$. We individually tested for sex differences in each sample where powered and grouped animals according to age if none were found. Where relevant, figures depict sex of the animal for each associated data point. Parametric tests were used when assumptions of normality were met, otherwise, nonparametric tests were used.

C. In vitro slice electrophysiology

We anesthetized preweanling and adolescent rats with ketamine (40-80mg/kg)/xylazine (5-10 mg/kg), removed their brains, and prepared 300- μ m coronal sections containing primary somatosensory cortex using a vibratome. Slices from preweanling rats up to P14 were prepared in ice cold normal artificial cerebrospinal fluid (ACSF) containing the following: 119 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 2.4 mM NaHCO₃, 12.5 mM glucose, 2 mM MgSO₄·7H₂O, and 2 mM CaCl₂·2H₂O. Slices from rats P15 and older were prepared in ice cold NMDG ACSF containing the following: 92 mM NMDG, 30mM sodium bicarbonate, 20mM HEPES, 25mM glucose, 5mM sodium ascorbate, 2mM thiourea, 1.25 mM monosodium phosphate, 2.5mM potassium chloride, 3mM sodium pyruvate, 10mM magnesium sulfate heptahydrate, 0.5mM calcium chloride dihydrate. Slices prepared in NMDG ACSF were allowed to recover in 35–37°C NMDG ACSF for 7 minutes immediately after slicing before being placed in room temperature normal ACSF. All ACSF solutions were adjusted to a pH of 7.4 using HCl, and osmolarity was adjusted to 305 \pm 5 mOsm/L. Solutions were saturated with carbogen (95% O₂ and 5% CO₂) throughout use.

We placed slices in a submersion chamber and continually perfused (2 ml/min) with normal ACSF. We obtained whole-cell patch-clamp recordings of spontaneous postsynaptic currents (sPSCs) and whole cell resistance measurements from S1 L4 neurons in voltage-clamp mode (-65 mV holding potential), through pipettes (4-6 Ω) containing: 120 mM potassium gluconate, 10mM potassium chloride, 10mM HEPES, 1mM magnesium chloride, 0.5mM EGTA, 2.5mM magnesium ATP, 0.2mM GTP-Tris, and 0.1% biocytin (Thermo Fisher Scientific), adjusted to pH 7.3 and 290 mOsm/L. Biocytin was included in the internal

solution to allow for reconstruction and further identification of recorded cells. In some sPSC experiments gabazine (1 μ M) was added to the ACSF to suppress the effects of inhibitory inputs on the recorded cells. In all current clamp experiments, gabazine (1 μ M), CNQX (20 μ M), and AP5 (50 μ M) were added to the ACSF to suppress synaptic activity and study post-synaptic effects of nicotine. Nicotine bitartrate dihydrate (Sigma-Aldrich, St. Louis, MO) was applied in slice electrophysiology experiments by washing in with the ACSF for 3-6 minutes (10 μ M) or directly applying near recorded neurons through a glass pipette (4-6 Ω), using a Picospritzer (20 to 30 μ M). Series resistance was monitored in all electrophysiological recordings by measuring the current evoked by a -5mV square pulse, and recordings were discarded if resistance changed by >20%. Recordings were made with the Axon Pclamp 11 Software Suite (Molecular Devices, Silicon Valley, CA) and analyzed with Easy Electrophysiology V2 software (Easy Electrophysiology Ltd, London, England).

D. Histology

After recordings, slices were fixed in 10% neutral buffered formalin (NBF) at room temperature for 24-48 hours after recordings and stored at 4° C in PBS until processing. Slices were placed in blocking solution containing PBS, 1% BSA, and 0.3% Triton for two hours, then incubated for 48-72 hours in blocking solution (1% BSA, 0.1% Triton) containing streptavidin-conjugated Alexa 488 (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) and parvalbumin polyclonal antibody (1:10,000; PA1-933; Thermo Fisher Scientific). Slices were then washed and incubated in a secondary antibody solution (Alexa Fluor 594 donkey anti-rabbit IgG, 1:500; Thermo Fisher Scientific) then mounted in aqueous media and imaged using confocal microscopy. Neurons were analyzed

for parvalbumin immunoreactivity, soma and dendritic morphology, and presence of dendritic spines to confirm phenotype as FS or RS.

III. Nicotine Drives Excitatory Inputs to FS neurons in S1 from Early Ages

A. INTRODUCTION

As FS neurons and nicotinic inputs to cortex are both important for cortical development, we sought to investigate how these inputs drive development of FS neurons' responses to nicotine. These neurons and receptors are vital for proper cortical circuit formation and plasticity during critical periods in sensory cortex. Understanding their interaction, particularly the excitatory inputs driven by nicotinic signaling onto FS cells, can provide fundamental insights into normal brain development, the mechanisms underlying critical period plasticity, and the origins of neurodevelopmental disorders, potentially paving the way for future therapeutic interventions. Determining how nicotinic inputs regulate excitation of FS neurons also may reveal a potential mechanism for the long-term effects of nicotinic insults early in development, such as through prenatal exposure via maternal smoking or vaping, or exposure to environmental agents that affect cholinergic transmission.

Nicotinic effects on inputs to FS neurons are largely unknown, and those that have been reported are region- and cortical layer-specific. Layer 6 FS neurons in prefrontal cortex from rats aged P15-39 showed a significant increase in frequency, but not amplitude, of sEPSCs in response to acetylcholine, in the presence of atropine to isolate nicotinic responses (Kassam et al., 2008). These responses are blocked by the AMPA receptor antagonist CNQX, suggesting a presynaptic site of action. This effect may be mediated by excitatory, $\alpha 4\beta 2\alpha 5$ nAChR-expressing projections to other neurons. $\alpha 4\beta 2\alpha 5$ nAChRs are suggested to have unique kinetic properties, and may be resistant to desensitization (Kassam et al., 2008; Brown et al., 2007). Also in medial prefrontal

cortex, frequency and amplitude of spontaneous EPSCs (sEPSCs) in FS neurons are substantially increased by bath application of 10 μ M nicotine in adult animals, with a similar non-desensitizing response pattern (Couey et al., 2007). To our knowledge, these studies represent the extent of the literature on nicotinic responses in FS neurons, and these have not been investigated in other cortical areas, including somatosensory cortex. These effects have also not been studied in the early postnatal period, when nAChRs reach their highest expression and maturing FS neurons are actively patterning cortical circuits. As nAChR expression and function is highly dependent on individual cortical region and cell type, we sought to address this gap in the field by developing a timeline of excitatory nicotinic responses in FS neurons in somatosensory cortex, beginning early in development.

We chose to focus on the effects of nicotine on excitatory inputs to FS neurons in layer 4 of somatosensory cortex. Layer 4 (L4) constitutes the main thalamic recipient layer in this area, and FS neurons in layer 4 receive fast and strong excitatory projections from the thalamus, contributing to their ability to precisely control the responses of pyramidal neurons (Schuman et al., 2019; Brecht et al., 2007; Swadlow et al., 2005). Thalamic projections to cortical pyramidal neurons express nAChRs and are stimulated by nicotine (Gil et al., 1997; Poorthuis et al., 2013; Lambe et al., 2003), but the effect of nicotine on excitatory projections to FS neurons in sensory cortex is not known. Understanding the effects of nicotine on these projections is important to develop a full picture of nicotinic modulation of early sensory cortical function.

In this study, we use nicotine (10 μ M) to study persistent effects on frequency and amplitude of sEPSCs recorded from FS neurons, beginning as early as they can be identified at P10, through

early adolescence. We also explore the ability of nicotine to evoke whole-cell currents from recordings, as a measure of postsynaptic responses, in FS neurons (see in Chapter 4). We develop a protocol to reliably identify these neurons early in the second postnatal week and confirm their identify by verifying parvalbumin expression and aspiny morphology in recorded neurons. Based on published nicotinic responses in other cortical areas (Kassam et al., 2008; Couey et al., 2007), we predict that nicotine will increase both frequency and amplitude of sEPSCs in FS neurons in somatosensory cortex beginning in the second postnatal week and persisting into adolescence, representing consistent nicotinic drive of excitatory responses in these neurons.

B. MATERIALS & METHODS

sEPSC Recordings

Slices were prepared and maintained as described in *General Methods*. We identified putative FS neurons in S1 L4 by their characteristic morphology as further described in Figure 3.1, Chapter 3 *Results*. We obtained whole-cell patch-clamp recordings from these neurons first in current-clamp mode to establish their kinetic parameters from intracellular current injections. Using a series of 1000 ms current pulses of increasing amplitude, we recorded and analyzed the firing pattern of each neuron for the first 500 ms of stimulation. We subsequently recorded sPSCs in voltage-clamp mode (-65mV holding potential). Where specified in *Results*, gabazine (1 μ M) was added to the external recording solution to block inhibitory inputs acting on GABA-A receptors and isolate excitatory inputs. However, even in cases where we did not block GABA-A signaling, most inhibitory currents driven by chloride would not be detectable due to a lack of

driving force for this ion. We calculated the reversal potential for chloride based on our solutions to be -64mV. We calculated the liquid junction potential (LJP) to be +16mV, but we did not correct for LJP. For recordings in voltage clamp mode, cells were held at -65mV-- effectively the reversal potential of chloride-- and thus we assume that recorded sPSCs are glutamatergic in nature and refer to them in *Results* as sEPSCs.

We recorded baseline sEPSCs from FS neurons for a minimum of 3 minutes before washing in ACSF containing nicotine (10 μ M) for 6 minutes. We compared frequency and amplitude of sEPSCs during 3 minutes of baseline and 3 minutes of nicotine application, following the initial 3-min wash-in period. Series resistance was monitored in all electrophysiological recordings by measuring the current evoked by a -5mV square pulse, and amplitude recordings were discarded if access resistance changed by >20%.

Statistical analyses

Statistical tests were conducted using Graphpad Prism 10 software (Boston, MA). Sample sizes were determined *a priori* using G*Power software suite (Faul et al., 2007). Statistical significance level was set at $p < 0.05$. Effects of nicotine on individual neurons as well as group effects were analyzed. Inter-event intervals (IEI) for each sEPSC were calculated, and frequency is reported as the instantaneous frequency of events, or the inverse of IEIs. For individual neurons, effects of nicotine on frequency and amplitude of events from each neuron were separately analyzed using nonparametric tests (Mann-Whitney), as they did not meet assumptions of normality, and results reported. For each neuron, we also calculated an average

frequency and amplitude at baseline and with nicotine and analyzed group effects with paired t-tests. All experiments used male and female animals and sex is indicated for individual data points where applicable. As these experiments did not have enough males and females to power for potential sex effects, we did not test for these effects specifically.

C. RESULTS

Identifying PV neurons

FS neurons can be identified by the distinct properties of their action potentials, evoked by intracellular current injections (Subkhankulova et al., 2010; Tateno et al., 2004; McCormick, 1985). These include a brief spike duration, high firing frequency, a large after-hyperpolarization (AHP) (Fig. 3.1B), and little or no spike frequency adaptation (Fig 3.1D). Fig 3.1D depicts a representative recording from an FS neuron from a P24 rat, demonstrating these characteristics. Fig. 3.1A depicts an FS neuron as viewed through diffusion interference contrast (DIC) during live-slice electrophysiology with characteristic morphology including oblong shape and absence of a pronounced apical dendrite. Whenever possible, we confirmed the identity of these neurons with *post hoc* parvalbumin immunohistochemistry and verification of absence of dendritic spines (Fig. 3.1G). The FS characteristics contrast with those of regular spiking (RS) neurons that have longer spike durations, small or no AHP, and whose spike trains accommodate (Fig. 3.1C, F). RS cortical neurons may include both excitatory neurons and inhibitory neurons that are not FS and do not express PV (Rudy et al., 2011; Porter et al., 1999).

It is more challenging to identify FS neurons in younger animals in which currents that drive the distinctive firing characteristics are still developing (Subkhankulova et al., 2010; Okaty et al., 2009), and in which significant PV expression begins only around P12 (Itami et al., 2007; Alcántara et al., 1993; De Lecea et al., 1995). To address this, we developed quantitative criteria to distinguish between FS and RS neurons. These criteria were obtained from recordings of 87 neurons from animals ranging in age from P7 to P26, and were validated by IHC in a subset of cells (Fig. 3.1C). Neurons were identified as FS if they had after-polarization (AHP) ≤ -10 mV, action potential width (at half max) ≤ 1.7 ms, and slope of inter-spike intervals (IEI slope, or spike adaptation index) $\leq 7.1 \times 10^{-4}$. Spike adaptation index is transformed by a scaling factor in 3.1C for simplicity of presentation. Neurons that did not meet these criteria were classified as regular spiking (RS) neurons. Fig. 3.1E-F depict the differences in action potential kinetics obtained from P12 neurons classified as FS and RS, respectively. The depicted P12 FS neuron (Fig. 3.1E) has an AHP of -17.9 mV, half-width of 1.5 ms, and IEI slope of 1.7×10^{-4} , while the RS neuron (Fig. 3.1F) has a longer half-width (1.8 ms) and more pronounced spike adaptation (IEI slope = 1.0×10^{-3}).

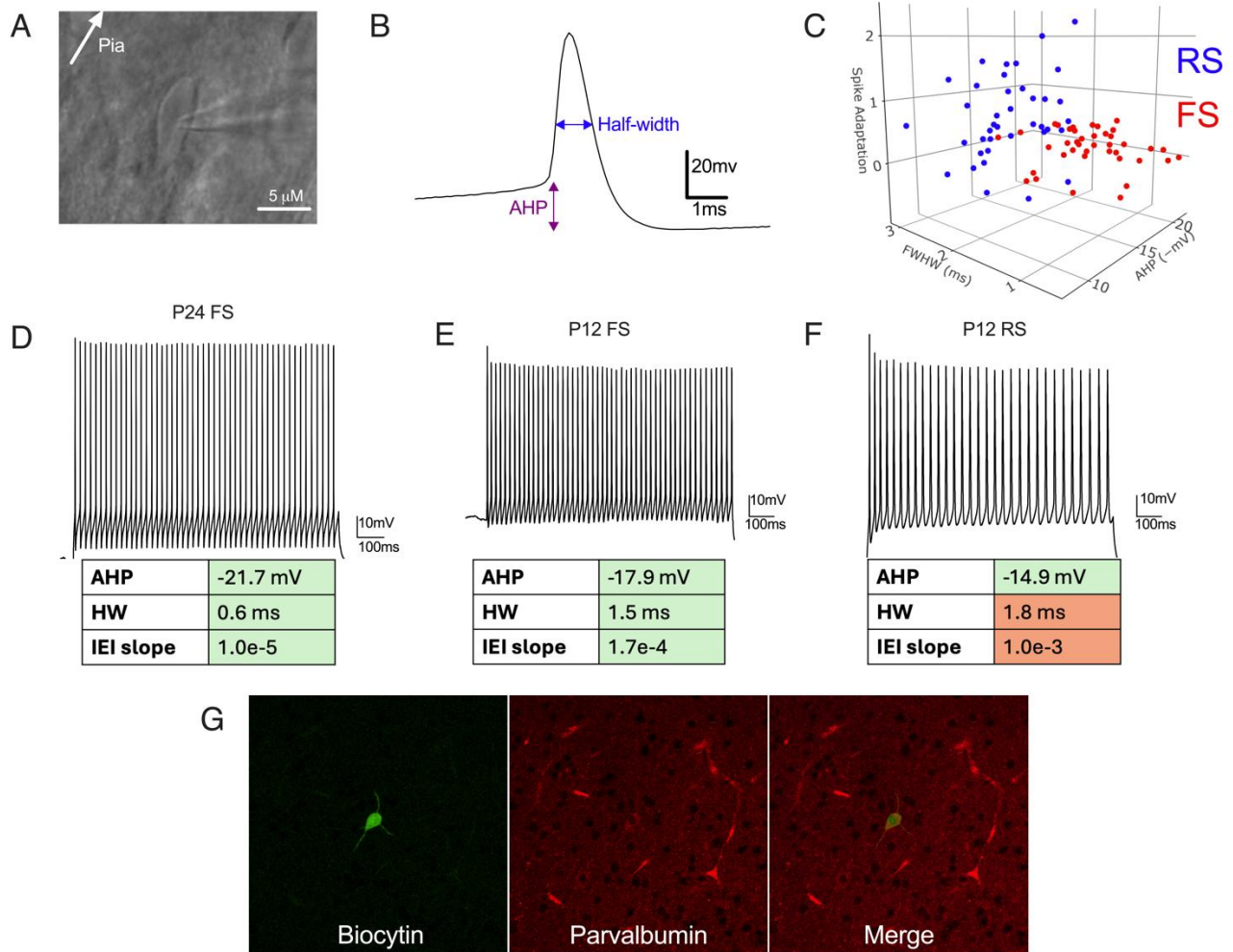


Fig. 3.1 Identification of PV neurons in early development

(A) Example of FS neuron soma morphology imaged using differential interference contrast during whole-cell patch clamp recording. (B) Example of a recorded action potential from a FS neuron. Spike half-width and AHP measurement locations are as indicated. (C) Distribution of kinetic parameters used to identify neurons as FS or RS (Spike adaptation index, FWHW = full width at half maximum, or half-width, and AHP). (D) Example trace of a FS neuron recorded from an adult rat. Additional example traces and kinetics from neurons from P12 rats classified as FS (E) or RS (F). (G) Example of biocytin-filled recorded neuron positive for parvalbumin IHC in a 300 μm section.

Nicotine increases excitatory synaptic inputs to FS neurons in S1 across development

nAChRs are expressed by inhibitory, GABAergic cortical neurons, and by the presynaptic, glutamatergic terminals that provide excitatory inputs to these neurons (Gil et al., 1997, Askew

et al., 2019; Demars & Morishita, 2014; Gil & Metherate, 2019). We predicted nAChR activation would increase synaptic drive onto FS neurons, beginning in early development through adolescence. We recorded sEPSCs in S1 layer 4 FS neurons from postnatal rats ($N = 19$ rats, $n = 1-2$ neurons per rat). We assessed the frequency and amplitude of events from 3-min recordings at baseline in ACSF and after a 3-min wash-in of $10 \mu\text{M}$ nicotine. Frequency and amplitude of events were increased in some neurons by application of nicotine. Fig. 3.2A depicts representative traces of sEPSCs before and after nicotine application recorded from a rat in the second postnatal week. A representative cumulative probability distribution curve for a neuron that responded to nicotine with an increased frequency of sEPSCs is shown in Fig. 3.2B, and for a neuron that responded to nicotine with increased amplitude of sEPSCs is shown in Fig. 3.2C. Nicotine produced significant rightward shifts in the cumulative probability distribution of each neuron depicted. In younger rats (P10-14), nicotine increased frequency of sEPSCs in 57% of neurons (Fig. 3.2D: $n = 14$ neurons, Mann-Whitney), and increased the amplitude in 25% of them (Fig. 3.2E: $n = 12$ neurons, Mann-Whitney). In 7% of recorded FS neurons, frequency and amplitude increased (data not shown: $n = 1$ neuron, Mann-Whitney).

In a subset of FS neurons from P10-14 rats, we included the GABA_A receptor antagonist, gabazine ($1 \mu\text{M}$) in the ACSF to suppress effects of inhibitory interneurons on the recorded cells. When gabazine was included in the recording solution, similar ratios of FS neurons responded to nicotine with an increased frequency (50%, Fig. 3.2F: $n = 6$ neurons) and amplitude (17%, Fig. 3.2G: $n = 6$ neurons). In older rats (P15-26), a similar proportion of neurons responded to nicotine with an increased frequency of sEPSCs with nicotine (Fig. 2.2H: $n = 6$ neurons, Mann-Whitney), but none of the neurons responded with an increase in sEPSC amplitude at these ages

(Fig 2.2I: $n = 6$ neurons, Mann-Whitney). Nicotine significantly increased mean frequency of sEPSCs for FS neurons from P10-14 animals (Fig. 3.2J, $p = 0.03$, paired t-test), but not amplitude (Fig. 3.2K). Gabazine blocked the effects of nicotine on the sEPSC frequency in group analysis (Fig. 3.2L) and had no effect on sEPSC amplitude (Fig. 3.2M). We found no group effects of nicotine on frequency or amplitude of sEPSCs in older animals (Fig. 3.2N-O). These data suggest that nicotine increases frequency of excitatory synaptic events in a majority of FS neurons, beginning in the second postnatal week and continuing into adolescence. Nicotine increased amplitude of sEPSCs in a smaller subset of these neurons. These effects are sufficient to drive group differences in frequency at these ages, but not in the presence of gabazine, nor in older animals.

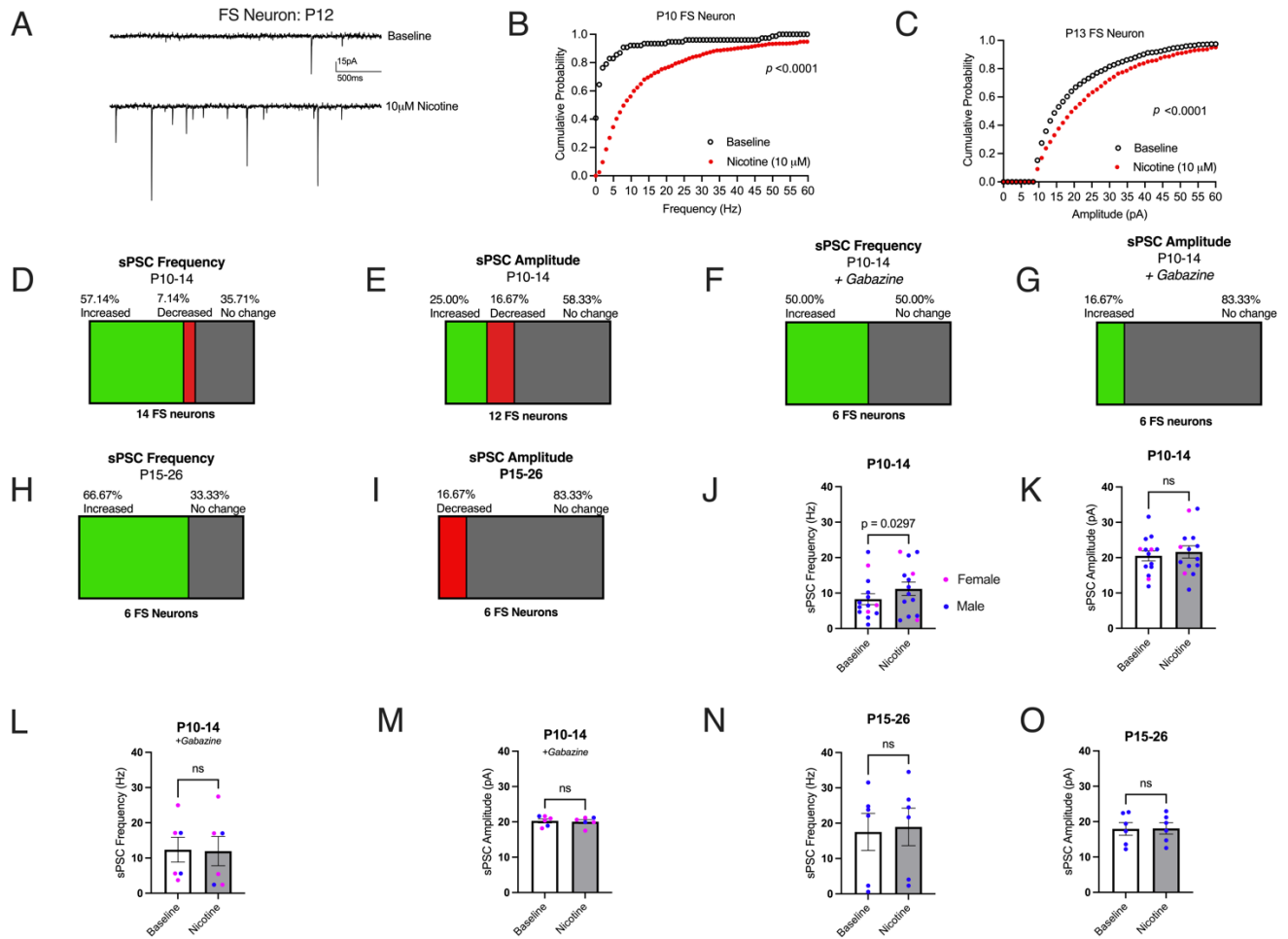


Fig. 3.2 Nicotine enhances excitatory inputs to FS neurons

(A) Example recording of sEPSCs from a P12 FS neuron that responded to nicotine by increasing frequency and amplitude of sEPSCs. (B) Example cumulative probability distribution of sEPSC frequency from a P10 FS neuron. (C) Example cumulative probability distribution of sEPSC amplitude from a P13 FS neuron. Ratios of FS neurons from P10-14 animals that significantly increased frequency (D) and amplitude (E) of sEPSCs with nicotine (Mann-Whitney tests). Ratio of FS neurons from P10-14 animals that significantly increased frequency (F) and amplitude (G) of sEPSCs with nicotine in the presence of gabazine (Mann-Whitney tests). Ratio of FS neurons from P15-26 animals that significantly increased frequency (H) or decreased amplitude (I) of sEPSCs with nicotine (Mann-Whitney tests). Individual means of sEPSC frequency (J) and amplitude (K) from P10-14 FS neurons before and after nicotine application (t-tests). Individual means of sEPSC frequency (L) and amplitude (M) from P10-14 FS neurons before and after nicotine application, with gabazine (t-tests). Individual means of sEPSC frequency (N) and amplitude (O) from P15-26 FS neurons before and after nicotine application (t-test).

IV. Nicotinic modulation of FS neurons in the postnatal and adolescent period

A. INTRODUCTION

In Chapter 3, we investigated presynaptic effects of nicotine on FS neurons in S1 and found that nicotine increases frequency of sEPSCs across development, and also increases amplitude in a small population of FS neurons, but only at young ages. In Chapter 4, we explore direct postsynaptic effects of nicotine on FS neurons, beginning in early development. In other cortical regions, these neurons do not respond to nicotine. In mouse prefrontal cortex, FS neurons are not depolarized by direct nicotine application (Couey et al., 2007). FS neurons in rat motor cortex do not respond to direct application of a nicotinic agonist (Porter et al., 1999). In rat visual, somatosensory, and prefrontal cortex, FS neurons are unresponsive to ACh directly applied in the presence of muscarinic blockers (Gulledge et al., 2007). Bath application of nicotine does not depolarize FS neurons in auditory cortex (Askew et al., 2019). In contrast, RS neurons across multiple cortical areas depolarize in response to nicotine, with either bath application (Askew et al., 2019) or direct puff application (Gulledge et al., 2007; Couey et al., 2007). When cortical neurons are further differentiated by cell type, vasoactive intestinal peptide-expressing (VIP) neurons have the most robust responses to nicotine, pyramidal neurons modestly depolarize, and somatostatin (SST) neurons do not respond to nicotine (Askew et al., 2019), though they are depolarized by other cholinergic agonists (Kawaguchi et al., 1997). The effects of nicotine on pyramidal and somatostatin neurons are suggested to result from an indirect mechanism mediated by VIP neurons (Askew et al., 2019; Porter et al., 1999).

Frequency and amplitude of spontaneous inhibitory currents (sIPSCs) in cortical pyramidal neurons are enhanced by bath nicotine application, acting at multiple types of nAChRs (Couey et al., 2007; Askew et al., 2019). FS neurons have been excluded from the mechanism for these effects (Couey et al., 2007; Askew et al., 2019; Kawaguchi et al., 1997). To our knowledge, nicotinic effects in FS neurons have not been studied earlier than P15 in rodents.

As a result of these studies, some authors have proposed that FS neurons do not possess nAChRs (Couey et al., 2007; Askew et al., 2019). However, co-expression of both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in PV neurons has been established in human temporal cortex (Krenz et al., 2001), and $\alpha 7$ receptors have been identified in PV neurons in macaque visual cortex (Disney et al., 2007). To address this deficiency in understanding of nicotinic modulation of FS neurons, here we study the direct, postsynaptic effects of nicotine across development. We further investigate these developmental changes herein by quantifying whole cell currents in FS neurons in response to bath nicotine, beginning early in development. We also explore direct measures of neuronal excitability in response to nicotine application. We predict that FS neurons will demonstrate nicotinic responses in the second postnatal week but will lack responses in the third postnatal week and beyond. We further seek to clarify whether FS neurons express nAChRs, which ones, and when these receptors appear during development.

FS neurons may possess multiple types of nAChRs, but apparently lack nicotinic responses in adults. Understanding whether these receptors are non-functional, modulate FS neuron activity through indirect mechanisms, or if there are specific developmental or contextual factors that influence their expression and function is crucial for a comprehensive understanding of

cholinergic modulation in cortical circuits. FS neurons express *Lynx1*, an endogenous modulator of nicotinic function, with structural similarity to snake venom toxin (Miwa et al., 1999; Anderson et al., 2020; Demars & Morishita, 2014; Morishita et al., 2010). *Lynx1* is proposed to act as a plasticity inhibitor by shutting down signaling at nAChRs (Miwa et al., 1999; Morishita et al., 2010). While it has been found in multiple cortical neuron types, it is overwhelmingly expressed in FS neurons in regions where it has been studied (Sadahiro et al., 2016; Morishita et al., 2010). Up to 90% of PV-expressing neurons in visual cortex in adult rodents express *Lynx1* (Demars & Morishita, 2014). In contrast, 5HT3AR+ neurons, which include VIP neurons, do not show detectable levels of *Lynx1* or *Lypd6*, another prominent member of the Ly6 family of proteins (Demars & Morishita, 2014). While a direct link between *Lynx1* and nicotinic function in FS neurons has not been found, the involvement of *Lynx1*, nicotinic signaling, and FS neurons in plasticity mechanisms in early development suggests a direct interaction. As *Lynx1* is developmentally regulated in other cortical areas, it may increase in somatosensory cortex during the second postnatal week in order to decrease nicotinic responses and regulate plasticity.

In this study, we use various methods to apply nicotine while recording postsynaptic responses in FS neurons in somatosensory cortex, from as early as the second postnatal week through early adolescence. We establish the developmental timeline of nicotinic responses in these neurons, as well as their expression of $\alpha 4^*$ -containing and $\alpha 7$ nAChRs during the early postnatal weeks. We predict that FS neurons evoke nicotinic currents exclusively during the second postnatal week but lack these responses during later development. We expect to find expression of nAChRs from early in development through adolescence. We further predict an increase in *Lynx1*

expression in S1 during the second postnatal week, and expression in PV neurons during this important time period.

B. MATERIALS & METHODS

Whole cell currents

Slices were prepared and maintained as described in *General Methods*, and FS and RS neurons were identified as described in Chapter 3. We recorded neuronal resistance, an indirect measure of ionic currents, by measuring the voltage response during the steady-state period of intracellular current injections, before and after bath nicotine application for 3 minutes (10 μ M).

Current clamp recordings

We measured resting membrane potential and rheobase of FS neurons from P10-13 rats in current clamp configuration, with the inclusion of NMDA and AMPA receptor antagonists (AP5, 50 μ M & CNQX, 20 μ M) and the GABA_A agonist gabazine (1 μ M) in the ACSF. These measurements were repeated after 3 minutes of bath nicotine application (10 μ M). Rheobase was calculated from 500ms current steps in increments of 10-20pA until cells reached action potential threshold. Rheobase was further refined for each neuron in 500ms current steps in increments of 2pA until a more precise threshold was reached.

For puff recordings, the puff pipette was attached to a Sutter Instruments micromanipulator for positioning and pressure ejection of nicotine solution controlled with a Picospritzer. Prior to recording, the puff pipette was lowered into a region of the brain outside of the cortex and the air

pressure adjusted (~ 5 to 10 PSI) so that the pressure wave extended at least 50 μm from the tip of the pipette while causing minimal mechanical disturbance to the cells at this range. A putative FS neuron was identified, and the puff pipette placed ~ 50 μm away, but aimed directly at, the cell. A second pipette was used to obtain whole cell recordings and the cell was phenotyped by injecting square current pulses in current clamp mode as described in Chapter 3.

We checked for responses to nicotine puff application by comparing the area under the curve (AUC) of each recording (current clamp mode) 3 seconds prior to each puff application (baseline) and 1 second after the onset of puff application. The AUCs for the first 3 to 5 consecutive applications in each cell were compared with using a paired t-test with $p < 0.05$ indicating a significant response to nicotine. For cells that showed a significant response to nicotine, we report the peak change in membrane potential. Cells that did not respond to nicotine were assigned a value of 0 mV.

RNAscope™

In situ mRNA expression levels were determined using RNAscope™ Multiplex Fluorescent V2 Assays (ACDbio, Newark, CA). Animals were deeply anesthetized by intraperitoneal injection of ketamine/xylazine and transcardially perfused with ice-cold PBS followed by 10% neutral buffered formalin (NBF). Brains were extracted, fixed overnight in 10% NBF, cryoprotected in a sucrose solution, frozen, and sectioned using a cryostat (12 μM). Ready-to-use reagents from the RNAscope™ Multiplex Fluorescent V2 Assay Kit were used with Rn-Chrna4, Rn-Chrna7, and Rn-Pvalb probes (ACDbio) to process sections from P12 and P19 male and female rats to detect mRNA for nAChR subunits and parvalbumin. Mm-Lynx1 and Mm-Pvalb probes (ACDbio) were

used to process sections from P12 and P100 male and female mice. Sections were mounted in aqueous media and imaged using confocal microscopy. *In situ* mRNA expression levels were quantified using Imaris 10 software (Oxford Instruments, Abingdon, United Kingdom).

RT-qPCR

Male and female rats were euthanized at P0, P4, P7, P14, and P21 ($n = 6-8/\text{age}$). Brains were extracted and dissected in ice-cold PBS to collect 14-gauge tissue punches from 1mm sections of S1 and V1. Two brains were pooled for each region from P0 and P4 animals due to tissue volume. RNA was isolated from cortical tissue punches with Trizol reagent (Invitrogen) and the MicroElute Total RNA Kit (Omega; Cat# R6831) with a DNase step (Qiagen, Germantown, MD; Cat# 79254). RNA volume was measured on a Nanodrop (Thermo Scientific) and 1000ng of mRNA from each sample was used to synthesize complementary DNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA; Cat# 1708891). This cDNA was diluted to a concentration of 2 ng/ μL , which was used to measure relative mRNA expression of *Lynx1* by age via quantitative PCR with PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA; Cat# 95072). Primer sets used were as follows (F, R; 5'-3'): *Lynx1* ACCACTCGAACTTACTTCACC, ATCGTACACGGTCTCAAAGC; β -*Actin* TCATGTTTGAGACCTTCAACAC, GTCTTTGCGGATGTCCACG; *GAPDH* CCCACTCTTCCACCTTCGATG, TCCACCACCCTGTTGCTGTAG. All samples were run in duplicate, and samples with a difference in CT value greater than 0.6 were excluded. Relative quantification of mRNA expression was performed using the $2^{-\Delta\Delta C_t}$ method with standard protocols, using *Gapdh* and P21 values in each respective cortical region to normalize expression.

C. RESULTS

Nicotine does not produce whole-cell currents in FS neurons in S1

We hypothesized that nicotine may have postsynaptic effects on FS neurons at young ages. We calculated normalized neuronal resistance changes, an indirect measure of whole-cell currents, after applying nicotine (10 μ M) for 3 min. An example I-V curve used to produce these data is shown in Fig. 4.1A. Nicotine application did not decrease whole-cell resistance in FS neurons at any age, from P10-26 (Fig. 4.1B, $n = 20$ neurons, one-sample t-test), P10-14 (Fig. 4.1D, $n = 14$ neurons, one-sample t-test), or P15-26 (Fig. 4.1F, $n = 4$ neurons, one-sample t-test). In a subset of FS neurons from young (Fig. 4.1C, $n = 9$ neurons, one-sample t-test) and older (Fig. 4.1G, $n = 3$ neurons, one-sample t-test) animals, we recorded whole-cell resistance changes with nicotine in the presence of gabazine (1 μ M) and similarly found no effect. In RS neurons, nicotine also did not decrease normalized whole-cell resistance (Fig. 4.1I, $n = 31$ neurons, one-sample t-test) recorded in the absence of any synaptic blockers. However, in the presence of gabazine, nicotine modestly decreased whole-cell resistance (Fig. 4.1J, one-sample t-test, $p < 0.05$).

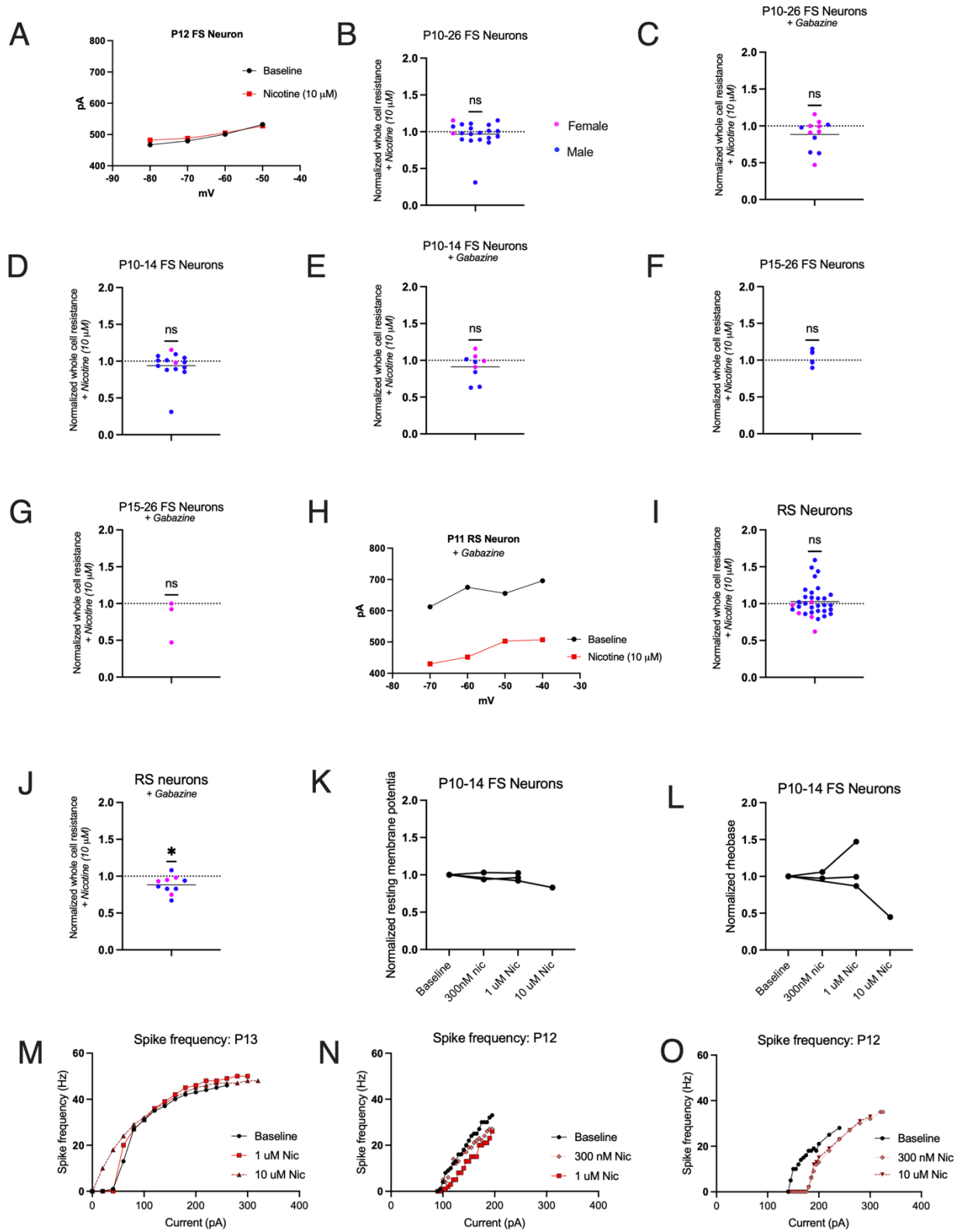


Fig. 4.1 Somatodendritic responses of FS and RS neurons in S1

(A) Example I-V curve of a P12 FS neuron before and after application of nicotine, with no significant change in input resistance. Normalized whole cell resistance of all FS neurons (B, $n = 20$), FS neurons with gabazine (C, $n = 12$), FS neurons from P10-14 rats (D, $n = 14$), FS neurons from P10-14 rats with gabazine (E, $n = 9$), FS neurons from P15-26 rats (F, $n = 4$), and FS neurons from P15-26 rats with gabazine (G, $n = 3$). (H) Example I-V curve from a P11 RS neuron before and after application of nicotine. Normalized whole cell resistance of all RS neurons (I, $n = 31$) and RS neurons with gabazine (J, $n = 10$, $p < 0.05$). Normalized resting potential (K) and rheobase (L) of $n = 3$ FS neurons with bath application of nicotine at listed concentrations, recorded with synaptic blockers. Spike frequency with bath nicotine application with synaptic blockers from 3 rats, P12-13 (M – O).

To investigate direct somatodendritic effects of nicotine on FS neurons at young ages, we recorded a subset of neurons from P10-13 rats in current-clamp in the presence of NMDA and AMPA receptor antagonists (AP5, 50 μ M & CNQX, 20 μ M) and gabazine (1 μ M). Nicotine application (300nM – 10 μ M) had no effect on resting membrane potential (Fig. 4.1K) or rheobase (Fig. 4.1L). Nicotine application also did not increase the spike frequency of these neurons (Fig. 4M-O).

Absence of nicotinic responses in FS neurons is not attributable to receptor desensitization

The sustained effects of nicotine bath application (10 μ M, 3 to 6 minute application) on inputs to FS neurons (Fig. 3.2) may represent a non-desensitizing nicotinic mechanism (Askew et. al., 2019). Our finding that bath-applied nicotine did not produce postsynaptic responses in FS neurons (Fig. 4.1) suggests that rapid receptor desensitization may preclude observation of postsynaptic nicotinic effects on these cells. To test this, we focally applied puffs of nicotine (20 to 30 μ M) using a Picospritzer, while recording in current clamp in the presence of synaptic blockers (Fig. 4.2A-B). Nicotine did not depolarize any FS neuron in either young or older pups (Fig 4.2C, E, $N = 6$ animals, $n = 9$ neurons). In contrast, nicotine depolarized one third of RS neurons by an average of 4.6 mV (Fig. 4.2D-E, $N = 10$ animals, $n = 18$ neurons), in both younger

and older animals. This result is consistent with previous findings that nicotine induces small depolarizations in some RS neurons in sensory cortical areas (Askew et al., 2019), though previous literature has not demonstrated this effect in the presence of synaptic blockers. As this brief nicotine application method reduces the likelihood of receptor desensitization, this suggests that FS neurons lack postsynaptic responses to nicotine. These results demonstrate that FS neurons do not have postsynaptic responses to nicotine applied via bath or directly to the soma, as early as these neurons can be reliably identified through adolescence.

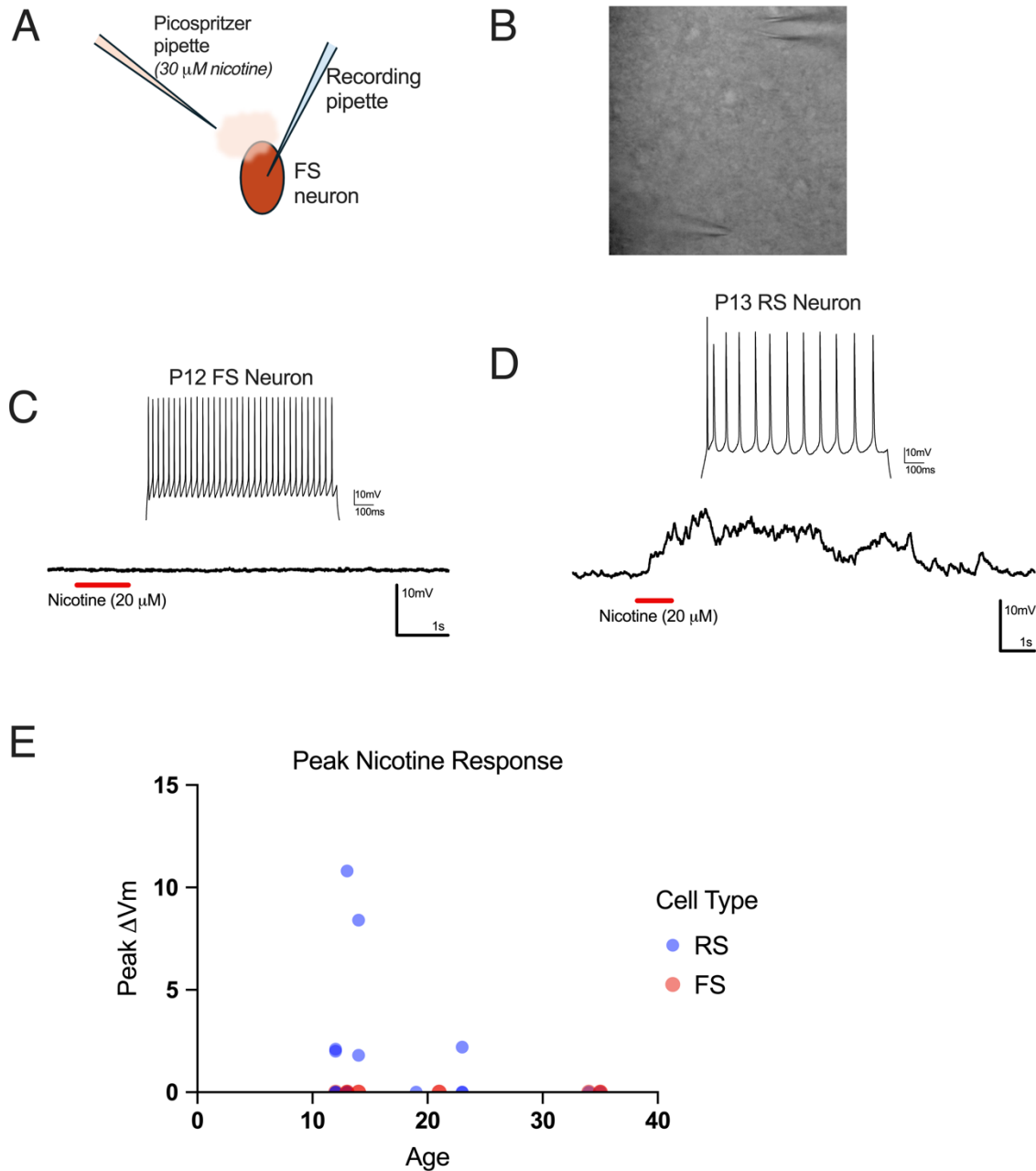


Fig. 4.2 FS & RS responses to direct nicotine application

(A) Schematic for nicotine puff recordings. (B) Image of puff recording with recording pipette (*top*) and puff pipette (*bottom*). (C) Representative recording from a FS neuron with direct nicotine application. (D) Representative recording from a RS neuron that depolarized in response to direct nicotine application. (E) Summary of voltage changes from RS and FS neurons with direct nicotine application at various ages.

FS neurons express two common nicotinic receptor subunits

The $\alpha 4\beta 2$ heteromeric and $\alpha 7$ homomeric nAChRs are the most commonly expressed nicotinic receptors in mammalian cortex (Nair & Liu, 2019; Millar & Gotti, 2009). Fig. 4.2A-B depicts images of sections through the barrel cortex of P12 rats, demonstrating localization of mRNA for PV and for *CHRNA4* and *CHRNA7*, the genes coding for the $\alpha 4$ and $\alpha 7$ nicotinic subunits, respectively. At P12, 64.2% of PV⁺ neurons expressed *CHRNA4*, and 75.1% expressed *CHRNA7* (Fig. 4.3C-D, $N = 2$ rats, $n = 2$ sections per rat). More than half of PV⁺ neurons at P12 co-expressed mRNA that encode $\alpha 4$ and $\alpha 7$ nAChR subunits (Fig 4.3E). At P19, 59.7% of PV⁺ neurons expressed $\alpha 4$ nAChR-encoding mRNA, 80.0% expressed $\alpha 7$ -nAChR-encoding mRNA, and 45.4% co-expressed both mRNAs (Fig. 4.3C-D, $N = 2$ rats, $n = 2$ sections per rat). These expression levels did not significantly differ between P12 and P19. These data indicate that PV neurons express mRNAs that encode $\alpha 4$ and $\alpha 7$ nAChR subunits from early in development, near the time point when these neurons develop characteristic fast-spiking properties.

Lynx1 expression increases early in development in S1

The expression of mRNAs that encode nAChR subunits by PV neurons, including in younger animals, contrasts with the lack of responses of these cells to nicotine. One mechanism that may prevent activation of nAChRs is the expression of Lynx1 protein. Lynx1 is a nAChR modulator co-expressed with nAChRs in the brain. It is expressed in PV neurons in adult animals in other cortical areas (Miwa et al., 1999; Demars et al., 2014). Therefore, we tested the hypothesis that Lynx1 is expressed in the developing S1 cortex and compared this expression to that in the visual cortex, which matures later in development (Morishita et al., 2010; Fagiolini et al., 1994).

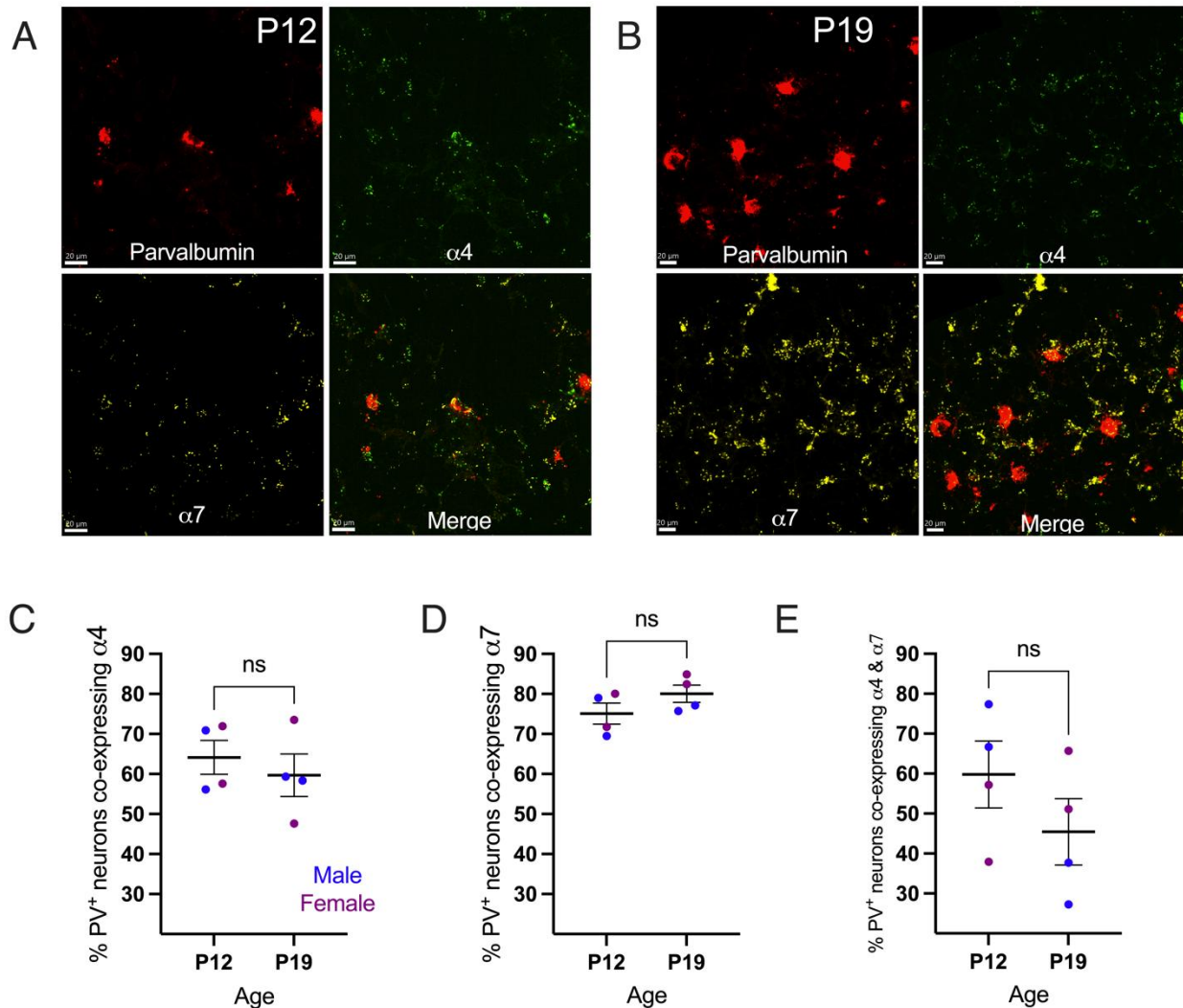


Fig. 4.3 PV neurons express mRNAs that encode multiple nAChR subunits in S1: $\alpha 4^*$ and $\alpha 7$

RNA scope™ images from S1 in a P12 (A) and P19 (B) rat showing co-expression of $\alpha 4^*$ and $\alpha 7$ mRNA in neurons also expressing parvalbumin. (C), Co-expression levels of $\alpha 4^*$ mRNA in PV⁺ neurons from P12 and P19 rats ($N = 2$ rats/age, $n = 2$ sections/rat). (D), Co-expression levels of $\alpha 7$ mRNA in PV⁺ neurons in P12 and P19 rats ($N = 2$ rats/age, $n = 4$ sections/rat). (E), Co-expression levels of $\alpha 4^*$ and $\alpha 7$ mRNA in PV⁺ neurons in P12 and P19 rats ($N = 2$ rats/age, $n = 4$ sections/rat).

We used RT-qPCR to quantify Lynx1 mRNA levels in rat S1 and V1 at different developmental ages. Lynx1 mRNA was not detectable in neonates (P0), underwent a gradual increase in the first postnatal week (P7), and greatly increased in the second postnatal week (Fig. 4.4A, $n = 4-8$

animals at each age). In primary visual cortex, *Lynx1* mRNA expression profile was similar, as the greatest increase in mRNA expression occurred during the second postnatal week (Fig. 4.4B, $n = 6-8$ animals at each age).

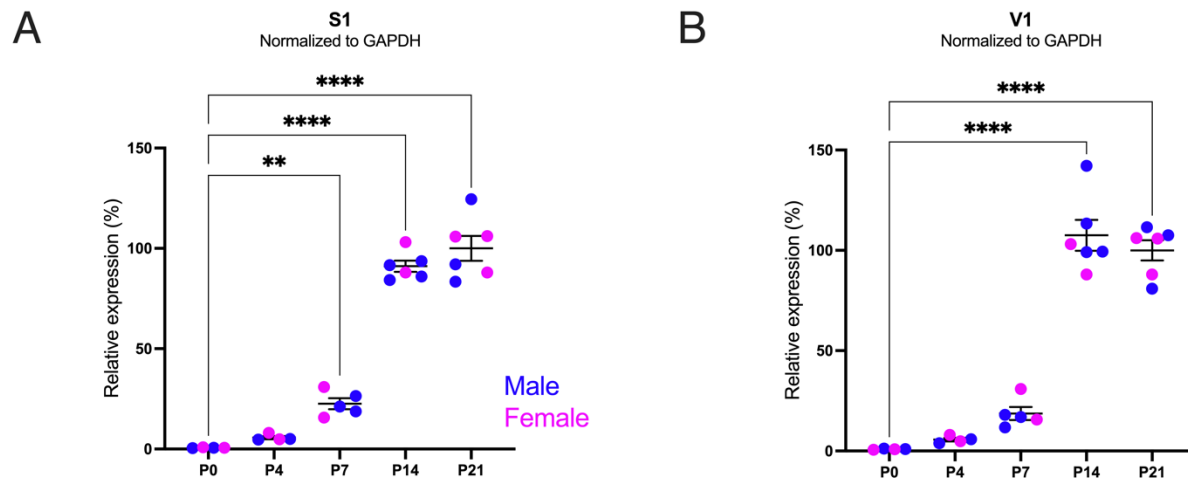


Fig. 4.4 *Lynx1* expression increases in S1 and V1 in the second postnatal week
Relative mRNA expression levels of *Lynx1* in S1 (A) and V1 (B).

We examined expression of *Lynx1* in PV neurons in mouse S1 during the second postnatal week (P12, Fig. 4.5A) and during adulthood (P100, Fig. 4.5B). At P12, 96.4% of PV+ neurons expressed *Lynx1*, and at P100, 83.5% expressed *Lynx1* (data not shown, $N = 2$ animals per time point), confirming that PV neurons express mRNA for a protein that can act as a brake on nicotinic receptor function from as early as they can be reliably identified and recorded using electrophysiology. *Lynx1* mRNA remains high in PV neurons well into adulthood.

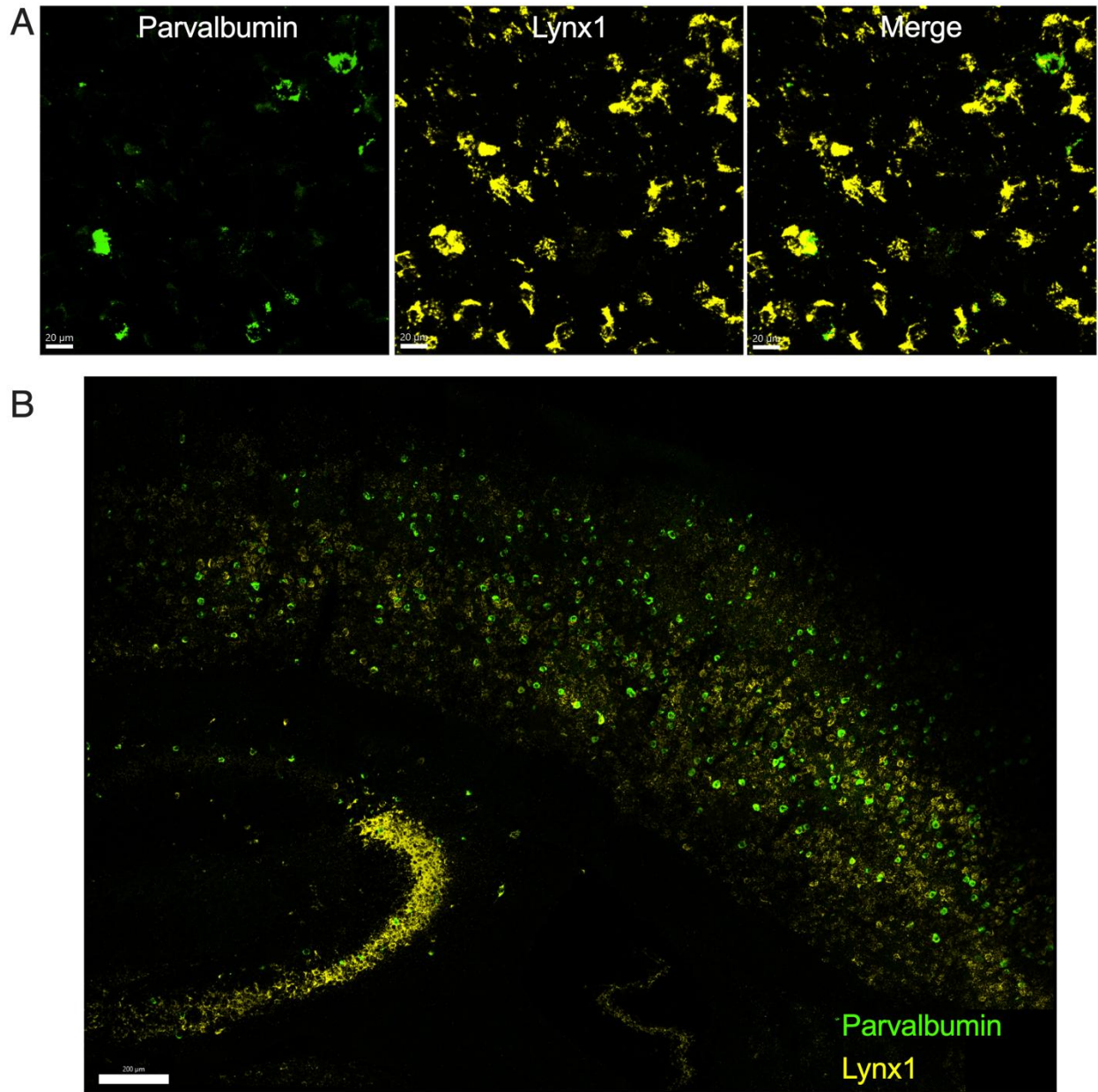


Fig. 4.5 Lynx1-encoding mRNA is expressed in PV neurons in mice early in development through adulthood. RNAscope™ images from S1 in a P12 (A) and P100 (B) mouse showing co-expression of *lynx1* and $\alpha 7$ mRNA in neurons also expressing parvalbumin. Characteristic *lynx1* expression pattern in hippocampus is shown in (B).

D. DISCUSSION

We report that FS neurons in S1 lack somatodendritic nicotinic responses from the second postnatal week through adolescence. We rule out desensitization as a mechanism for this lack of responsiveness, and further confirm that most FS neurons express multiple mRNAs that encode multiple nAChRs from as early as P12, and the receptor mRNA expression levels remain largely unchanged in these neurons across development. We quantified expression levels of Lynx1-encoding mRNA, as Lynx1 reportedly acts a negative modulator of nicotinic signaling, in S1 and V1 from birth through weaning, and identified early changes that have not been previously demonstrated in sensory cortex. We found the largest increase in *Lynx1* mRNA expression to occur during the second postnatal week, corresponding to the earliest time period that FS neurons can be identified and parvalbumin expression is detectable. Finally, we confirm that PV+ neurons express *Lynx1* mRNA as early as P12 through adulthood. Together, these findings lend support to the hypothesis that Lynx1 may block nicotinic responses in FS neurons from early ages through adolescence, though further studies will be needed to confirm this mechanism.

V. GENERAL DISCUSSION

A. Overall Summary of Hypothesis and Predictions

We hypothesized that nicotinic responses in FS neurons in somatosensory cortex are developmentally regulated. In Chapter 3, we established that nicotine enhances excitatory inputs to FS neurons as early as the second postnatal week in rats, a period corresponding to late human gestation (Clancy et al., 2007). There may be developmental changes earlier, but the difficulty of identifying FS neurons before P10 precludes this analysis. We revealed an early window during the second postnatal week where amplitude of these currents in a subset of FS neurons is increased by signaling at nAChRs. In Chapter 4, upon investigation of somatodendritic responses of FS neurons to nicotine, we found no evidence of direct effects of nicotine on the somatodendritic compartment of FS neurons at any time during development. We established a developmental timeline of expression of two important nAChR subtypes and found that they are both expressed in the majority of FS neurons from at least as early as postnatal day 12. Finally, we revealed a large developmental increase in expression of the mRNA that encodes Lynx1, a prototoxin that serves as a brake on nicotinic signaling (Morishita et al., 2010), in S1 and confirmed it is expressed in FS neurons in the second postnatal week. These findings demonstrate that neurons receive inputs from glutamatergic axons that are sensitive to modulation by nAChR activation early in development. They also suggest that, if functional nAChRs are expressed in the somatodendritic compartment of FS neurons, the activity of the receptors may be reduced or blocked by Lynx1 protein expressed by FS neurons.

B. Nicotinic currents in FS neurons in S1

Previous research has demonstrated that nicotine or ACh acting at nAChRs increase frequency of sEPSCs in cortical FS neurons (Kassam et al., 2008; Couey et al., 2007). The source of these inputs has not been conclusively identified, but intrinsic collateral axons from corticothalamic neurons are a likely component (Kassam et al., 2008; West et al., 2006; Kim et al., 2014; Heath et al., 2010). The earliest age this has been examined is P15 in rodents, though most studies have focused on FS neurons in adult animals. In this study, we successfully identified FS neurons at younger ages and explored the effects of nicotine on them during this early window in development. We found that nicotine increases the frequency and amplitude of sEPSCs in a population of FS neurons from animals age P14 and younger. These effects on sEPSCs in FS neurons in young animals are a novel finding that has not been reported in the literature, as nicotinic effects have not been studied at these ages.

In older animals, from P15 to P26, a period encompassing late development and early adolescence, we continued to see nicotine driving increased frequency of sEPSCs, but the amplitude effects were not evident after this time point in any recorded FS neurons. This reveals a unique early window of nicotinic effects on FS neurons in development. We also found that blocking the effects of signaling from intermediate inhibitory neurons using gabazine in the recording solution suppressed most of the effects of nicotine on FS neurons.

Including gabazine in the bath to block GABA-A receptors for sEPSC recordings reduced the group effect of nicotine on frequency in young animals but did not change the ratio of individual neurons that significantly responded with an increase in frequency. At the holding potential used

for these recordings (-65mV), most inhibitory currents would not be detectable, and we were primarily measuring excitatory currents. Nicotine may partially be acting on inhibitory neurons to drive increases in sEPSC frequency in FS neurons at young ages. As these recordings occurred before GABA currents switch from excitatory to inhibitory, a developmental milestone that occurs by the end of the second postnatal week, the increased frequency from nicotine application at early ages may be directly driven by excitatory GABA signaling. We have also considered an indirect mechanism involving disinhibition via VIP interneurons, as has been proposed in other studies (Askew, et al., 2019; Faini et al., 2017). These findings demonstrate significant neuromodulatory control of FS neurons via nAChRs beginning very early in development, possibly through other inhibitory neurons.

Neuromodulation via nAChRs is tightly developmentally regulated. In particular, expression of $\alpha 7$ receptors increases significantly during the second postnatal week in the cortex in rodents (Dwyer et al., 2008; Broide et al., 1995). This time corresponds to a period of developmentally regulated plasticity in somatosensory cortex (Aramakis et al., 2000; Tribollet et al., 2004). $\alpha 7$ receptors are thought to contribute to early patterning of sensory cortex by facilitating excitatory signaling. These effects on FS neurons may be driven in part by $\alpha 7$ receptors. However, $\alpha 7$ receptors, and most other subtypes of nAChRs are prone to desensitization. $\alpha 7$ receptors respond to relatively high concentrations of nicotine, but desensitize very rapidly (Aramakis et al., 2000; Deutsch & Burket, 2020). $\alpha 4\beta 2$ receptors respond to much lower concentrations of nicotine and also desensitize, but more slowly (Dwyer et al., 2008; Lambe et al., 2003). In this study, we demonstrated increases in frequency and amplitude of sEPSCs in response to a relatively high concentration of nicotine (10 μ M). The effects were resistant to desensitization, persisting for upwards of 10 minutes in some recordings. This reveals how sensitive synaptic inputs to FS

neurons are to nicotinic modulation, beginning from early in development. This presents a unique vulnerability to exogenous nicotine exposure, such as from maternal smoking or vaping. These effects may be driven by a less well-characterized nicotinic receptor, such as the $\alpha 4\beta 2\alpha 5$ receptor expressed in layer 6 cortical neurons that exhibits highly resilient nicotinic responses (Venkatesan et al., 2023; Bailey et al., 2014; Heath et al., 2010). This receptor is implicated in many of the long-term changes resulting from nicotine exposure during prenatal or early postnatal development (Heath et al., 2010), and the *CHRNA5* gene coding for the $\alpha 5$ nAChR subunit is linked to both nicotine addiction and schizophrenia (Hong et al., 2012).

C. FS neurons are not postsynaptically regulated by nicotine

We explored somatodendritic mechanisms of nicotinic regulation in FS neurons in Chapter 4. Using a variety of techniques, we did not find a direct effect of nicotine on FS neurons in either younger or older animals. Nicotine did not increase whole-cell currents in FS neurons at any age investigated, and gabazine did not have any effect on these recordings. Nicotine similarly did not increase whole-cell currents in RS neurons, though we noted a modest increase in the presence of gabazine, suggesting that direct nicotinic effects in RS neurons may be masked by intermediary nicotinic effects on interneurons. Current-clamp recordings from a subset of FS neurons from P10 to P13 demonstrated no effect of nicotine on measures of neuronal excitability, including resting membrane potential, rheobase, or action potential frequency, in the presence of synaptic blockers.

We further considered that observation of potential agonistic effects of nicotine on somatodendritic nAChRs that might be expressed by FS neurons could be obscured by rapid receptor desensitization in response to bath application of nicotine. A focal application technique in the

presence of synaptic blockers failed to depolarize any FS neuron at any age recorded, though we did note depolarization of RS neurons in both younger and older animals, indicating that nicotine application with this method was efficacious. The results in older animals are consistent with previous literature demonstrating that nicotinic agonists do not depolarize FS neurons (Couey et al., 2007; Porter et al., 1999; Gullledge et al., 2007), and we further confirmed that this holds true in the second postnatal week as well. Our finding that amplitude of sEPSCs in some FS neurons is increased by nicotine exclusively in early weeks is therefore likely driven by a presynaptic mechanism. A presynaptic nicotinic mechanism driving increased amplitude in cortical sEPSCs during a specific period in development has not been previously shown in any neuron type to our knowledge.

D. nAChRs in FS neurons

We focused on the expression mRNAs that encode the $\alpha 4$ and $\alpha 7$ nAChR subunits, the most commonly expressed in mammalian cortex (Nair & Liu, 2019; Millar & Gotti, 2009). Both heteromeric $\alpha 4\beta 2$ and homomeric $\alpha 7$ nAChRs are activated by ACh and nicotine, among other agonists. Heteromeric $\alpha 4\beta 2$ nAChRs gate Na^+ currents and tend to be expressed by GABAergic neurons (Gotti et al., 2006; Mansvelder et al., 2002), though they are also found on glutamatergic terminals (Lambe et al., 2003) and other neurons (Garduno et al., 2012). Homomeric $\alpha 7$ nAChRs also gate Na^+ but are much more highly permeable to Ca^{2+} (Deutsch et al., 2020). They are often expressed on glutamatergic terminals (Mansvelder et al., 2002). Both receptors are developmentally regulated (Naeff et al., 1992). Distribution of $\alpha 7$ nAChRs in somatosensory cortex in early postnatal weeks is dependent on thalamocortical innervation, a hallmark of the importance of this receptor in enhancing early sensory inputs (Broide et al., 1996). $\alpha 7$ nAChRs in

the context of PV neurons have been studied, as dysfunctional expression of $\alpha 7$ nAChRs is associated with abnormal development of PV neurons (Lin et al., 2014). This phenotype is linked to disorders including autism spectrum disorder, schizophrenia, and epilepsy (Lin et al., 2014; Sun et al., 2021). Despite this, a clear mechanism for how $\alpha 7$ nAChRs affect development of PV neurons has not been established.

Using fluorescent in situ hybridization, we established that quite early in development, nearly 2/3 of PV+ neurons in rat S1 express the gene coding for $\alpha 4$ nAChR subunits, and 3/4 of them express the gene coding for $\alpha 7$ nAChR subunits. We found that more than half express mRNAs encoding both receptor subunits, during a time period we have confirmed that nicotine does not directly stimulate FS neurons. This reveals a heterogeneity in expression of nAChR subunits that may correspond to different PV neuron subtypes, such as basket and chandelier cells. Our analysis also revealed very few PV+ neurons that expressed neither mRNA-encoding receptor subunit. We have demonstrated that the majority of PV neurons express both of the dominant nAChR subunits in rat S1 beginning very early in development. Despite known early upregulation of these mRNAs in broader cortex, the expression profile in PV neurons changed little between P12 and P19 in our analysis. The existence of these nAChR subunits in PV neurons, their early emergence in development, and relatively stable expression contrasts with the complete lack of responses of these neurons to nicotinic stimulation.

Whether nAChRs in PV neurons are expressed somatically or terminally has not been established, as the role of nAChRs in PV neurons is not well studied. Nicotine enhances frequency and amplitude of sIPSCs in cortical pyramidal neurons, possibly through effects on 5HT3AR+ neurons

(Couey et al., 2007; Askew et al., 2019; Takesian et al., 2018). Further studies may reveal whether $\alpha 4\beta 2^*$ and $\alpha 7$ receptors play a role in transmitter release at the terminals of FS neurons.

E. Lynx1 Emerges as a Candidate for Blocking Nicotinic Responses in FS Neurons

We considered possible mechanisms for FS neurons' lack of responsiveness to nicotine, despite abundant receptor expression. Lynx1 protein expression has been established in FS neurons in rodents, but its role in these neurons is unclear. Lynx1 is part of the large Ly6/uPAR/neurotoxin superfamily whose members are often expressed alongside nAChRs as accessory proteins (Miwa et al., 2021). Lynx1 is transported to the cellular membrane and binds to nAChRs in a similar manner to toxins in snake venom (Miwa et al., 1999).

The specific role of Lynx1 in FS neurons is left largely unexplored. Part of this is due to the difficulty of manipulating Lynx1 for experimental study. Much of the research into the actions of this protein is centered around gene knockout experiments, which present issues with abnormal development, compensatory mechanisms, and deficient maternal care in rodents (Anderson, 2020). The protein has been isolated and purified in water-soluble form for exogenous use in some experiments (Shenkarev et al., 2020; Venkatesan et al., 2022), but this strategy may not recapitulate how Lynx1 behaves *in vivo*, as a GPI-linked modulator that interacts directly with nAChRs within cell membranes (Ibañez-Tallon et al., 2002). Lynx1 has also been suggested to physically interfere with the assembly and function of nAChRs, changing their stoichiometry and functional properties (Anderson et al., 2020; Ibañez-Tallon et al., 2002)-- effects that cannot be replicated with exogenous application. Other studies have attempted to cleave off Ly6 family

proteins using a PLC-activator compound and measure altered nicotinic currents with results that are difficult to interpret due to lack of specificity of this technique (Venkatesan et al., 2022).

We first wanted to establish a timeline of expression of the *Lynx1* gene in PV neurons in somatosensory cortex. With fluorescent *in situ* hybridization, we found that *Lynx1* mRNA is present in nearly all of PV neurons (96.4%) in mouse S1 as early as P12, close to the earliest age that we are able to identify FS neurons using electrophysiology and immunohistochemistry. Further, we confirm that *Lynx1* persists in these neurons well into adulthood.

Somatosensory cortex develops earlier than either visual or auditory cortex (Erzurumlu & Gaspar, 2012), the only sensory cortical areas where *Lynx1* has been functionally studied. We confirmed that FS neurons express this important negative modulator of nicotinic currents at this early timepoint in development, in which we also confirmed that FS neurons do not respond to nicotinic stimulation. At earlier ages, when the somatosensory cortex is undergoing rapid development, putative FS neurons may be postsynaptically regulated by nicotinic currents when *Lynx1* expression is lower or absent.

The overall timeline of *Lynx1* expression in sensory cortex is important to understanding its function. In somatosensory cortex, the role of *Lynx1* has not been studied. In auditory cortex, *Lynx1* expression nearly doubles between P11 and P20, the same time period in which the critical period for auditory plasticity closes, and this closure can be delayed by *Lynx1* knockout (Takesian et al., 2018; Anderson et al., 2020). In visual cortex, a modest increase in *Lynx1* mRNA expression between P28 and P60 correlates with the closure of the visual critical period for ocular dominance

plasticity, and *Lynx1* knockout restores plasticity in adults (Morishita et al., 2010). This converging evidence implicates *Lynx1* in the closure of critical periods across sensory cortex, with a different developmental profile in each (Anderson et al., 2020).

In Chapter 4, we mapped the course of expression of *Lynx1* in S1 beginning at birth through P21, in male and female rats. *Lynx1* was not detectable in neonates, increased modestly in the first postnatal week, and had a very large increase in the second postnatal week. This is consistent with closure of the S1 critical period of use-dependent plasticity and *Lynx1* may be driving this closure as in other cortical areas. Our recordings of FS neurons in the second postnatal week in S1 occurred during this window of rapidly increasing *Lynx1* expression.

F. Future Directions

Earlier effects of Lynx1

We identified FS neurons as early as P10, in the second postnatal week, and found robust *Lynx1* mRNA expression during this time period. There may be *Lynx1*-associated changes in nicotinic responses in FS neurons even earlier in development that we are unable to capture due to the difficulty in identifying these neurons during this time period. Future research may capitalize on alternative strategies, including transgenic lines for other markers of putative PV neurons. However, current markers are not exclusive to PV neurons and may include other interneurons with the same lineage. Combining this strategy with genetic or optogenetic manipulation of nAChRs or *Lynx1* in PV neurons could be a powerful tool to reveal mechanisms of nicotinic signaling in early circuits patterned by FS neurons. Using more refined models to control the activity of *Lynx1* may also allow for further study of the mechanism through which *Lynx1* may

control plasticity via its activity in PV neurons, and present a more direct treatment target for disorders associated with aberrant use-dependent plasticity.

In utero nicotine exposure

Despite a successful public health campaign in past decades, developmental nicotine exposure remains a pressing issue. In the United States, 5.4% of mothers report smoking cigarettes during pregnancy (Kipling et al., 2024). Fetuses are also exposed to nicotine via smokeless tobacco, hookahs, nicotine replacement products, and e-cigarettes. Developmental nicotine exposure results in permanent effects on nAChR signaling in the brain (Dwyer et al., 2008; Baumann & Koch, 2017). On a structural level, cortical pyramidal neurons demonstrate increased dendritic branching and excitability (Bailey et al., 2014). Functionally, pyramidal neurons have reduced nicotinic currents in adults following prenatal exposure from conception to weaning (Bailey et al., 2014). This phenotype is thought to result from permanent desensitization and downregulation of nAChRs (Bailey et al., 2024; Heath et al., 2010). Children exposed to nicotine *in utero* demonstrate cognitive impairments, reduced ability to discriminate sensory information, deficits in auditory processing, and attention deficits, among other issues (Aramakis et al., 2000; Dwyer et al., 2009; Gorionova & Mansvelder, 2012).

Few studies on developmental nicotine exposure have examined effects on inhibition in the brain, and to our knowledge, none have looked at the effects on PV neurons specifically. Because these neurons are important for early circuits, sensory processing, and plasticity, unique effects on them would be important to study. Effects on Lynx1 expression in the brain as a result of developmental nicotine exposure also have not been studied. In nonhuman primates, Lynx1 expression is

increased in lung tissue after prenatal exposure to nicotine (Sekhon et al., 2005). *Lynx1* may be a mechanism through which developmental nicotine exposure has long-term effects on the brain, and future research should investigate its regulation as a result of this exposure. This is particularly relevant in the context of PV neurons, as nearly 100% of cortical PV neurons express *Lynx1* (Morishita et al., 2010).

G. Overall Conclusion

The studies presented herein reveal that FS neurons have presynaptic nicotinic drive beginning very early in development but lack somatodendritic responses despite expressing mRNAs that encode nAChR subunits. The presynaptic effects we discovered demonstrate a potential target to nicotinic disruptions, including those resulting from prenatal nicotine exposure and genetic mutations of nAChRs, specifically affecting FS neurons, which drive development of early cortical circuits.

We confirmed the lack of somatodendritic responses in FS neurons to nicotine from as early in development as the neurons can be reliably identified, and further discovered that these neurons express mRNAs encoding multiple nAChR subunits from very early through adulthood. We identified *Lynx1* expression in PV+ neurons in S1 from P12 through adulthood and mapped the course of *Lynx1* expression in S1 from birth through early adolescence. *Lynx1* is an important modulator of nicotinic responses and possibly blocks somatodendritic nicotinic responses in these neurons. Its role and mechanism in FS neurons during the first two weeks in development remains to be explored. Nicotinic dysfunction in FS neurons is associated with numerous neuropsychological and neurological conditions, but nicotinic mechanisms driving development

of FS neurons are understudied. This work adds to the body of research on nicotinic regulation of these neurons in development.

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