FRONTIERS IN SYNAPTIC PLASTICITY: DENDRITIC SPINES, CIRCUITRIES AND BEHAVIOR

EDITED BY: Alberto A. Rasia-Filho, Rochelle S. Cohen and Oliver von Bohlen und Halbach

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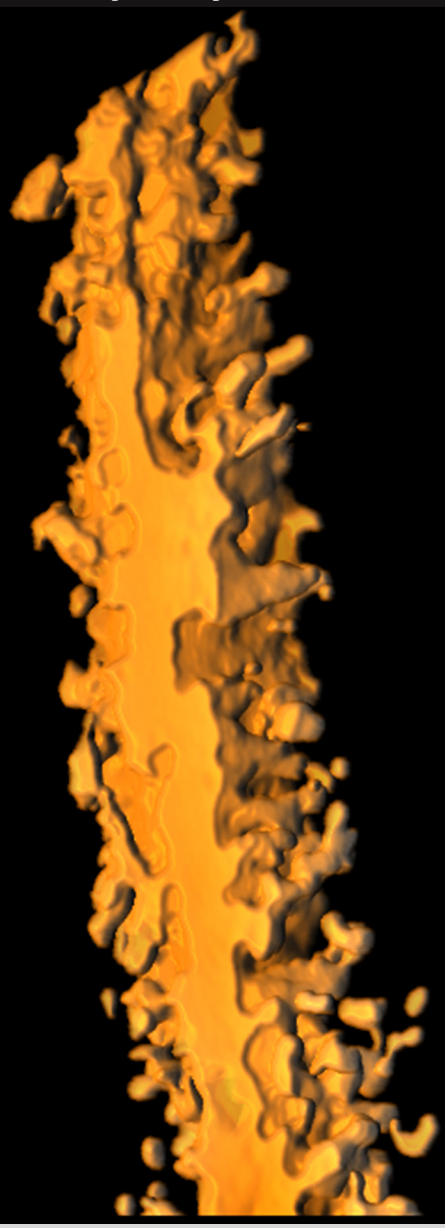

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FRONTIERS IN SYNAPTIC PLASTICITY: DENDRITIC SPINES, CIRCUITRIES AND BEHAVIOR

Topic Editors:
Alberto A. Rasia-Filho, Federal University of Health Sciences, Brazil
Rochelle S. Cohen, University of Illinois at Chicago, USA
Oliver von Bohlen und Halbach, Universitätsmedizin Greifswald, Germany

Reconstructed dendritic spines from a human hippocampal pyramidal neuron visualized by the Golgi method under light microscopy. Original magnification × 1300. ImageJ, Volume Viewer software (NIH, USA). Courtesy of R. Reberger, C.R. Jung, A. Dall´Oglio and A.A. Rasia-Filho.

The term "synaptic plasticity" is a broad concept, which is studied with a variety of experimental approaches. One focus is the impact of changes in synaptic, neuronal and glial morphology on brain circuitry and behavior. In this regard, unique animal models have been key to the study of affective and social behaviors and neurological and psychiatric diseases. However, there is a paucity of compilations directed toward the correlation of alterations in synaptic structure with various physiological and behavioral paradigms. This Frontiers Research Topic will, therefore, serve as an exciting forum for the exchange of novel hypotheses and data and an important resource and reference for investigators studying synaptic and brain plasticity, as well as those in related fields.

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Table of Contents

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EDITORIAL
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Editorial: Frontiers in Synaptic Plasticity: Dendritic Spines, Circuitries and Behavior

Alberto A. Rasia-Filho[1], Rochelle S. Cohen[2] and Oliver von Bohlen und Halbach[3]*

[1]Department of Basic Sciences/Physiology, Federal University of Health Sciences, Porto Alegre, Brazil, [2]Department of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, IL, USA, [3]Institute of Anatomy and Cell Biology, Universitätsmedizin Greifswald, Greifswald, Germany

Keywords: synaptic function, synaptic transmission, synaptic remodeling, dendritic spine function, neural circuits and behavior, sexual dimorphism, neuroimmune interactions, psychiatric disorders

The Editorial on the Research Topic

Frontiers in Synaptic Plasticity: Dendritic Spines, Circuitries and Behavior

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****Correspondence:***
Alberto A. Rasia-Filho
aarf@ufcspa.edu.br

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More than a century ago, in 1906, the Nobel Prize in Physiology or Medicine was awarded to Camillo Golgi and Santiago Ramón y Cajal "in recognition of their work on the structure of the nervous system." Using the Golgi technique, Cajal discovered and described dendritic spines, which, since then, have received considerable attention. Dendritic spines are the major targets of excitatory synapses within the brain. Their disparate morphologies appear to reflect cellular processes involved in neuronal and synaptic plasticity. Dendritic spines reach high levels of complexity in humans (1). Neuronal and synaptic plasticity are manifested by changes in structure (e.g., dendritic spine shape, size, density, and connectivity) and activity (e.g., long-term potentiation) leading to dynamic changes in circuitries for neuronal processing. Furthermore, some of these changes in the brain can translate into altered behavior and even can contribute to psychiatric disorders. Animal models have been key to the study of affective and social behaviors, as well as neurological and psychiatric disorders. They provide insight into mechanisms underlying basic to complex neural functions and disturbances in behavior. However, there is a paucity of compilations correlating alterations in synaptic structure with various physiological and behavioral paradigms. This Research Topic is a forum for the exchange of data and novel hypotheses about synaptic and brain plasticity. It comprises 10 articles with 3 original research articles, 3 reviews, 2 hypothesis and theory papers, 1 opinion, and 1 general commentary elaborated by 39 authors from various countries. These contributions present state-of-the art approaches to the study of dendritic spines, circuitries, and behavior from animal models, including rodents and primates, to humans. The research strategies used range from classic techniques to cutting-edge technologies, including imaging techniques, electrophysiology, and experimental-based hypothetical approaches.

Tønnesen and Nägerl provide up-to-date STED microscopy data on structure and function relationships of dendritic spines. These data include the spine head volume and local postsynaptic density associated with the neck diameter and its variable resistance. In conjunction, they modulate the spine electrical compartmentalization or the influence on dendritic voltage and synaptic plasticity. These data are crucial in evaluating the impact of spine geometry on neuronal function and dynamic synaptic processing and enduring changes in neural circuits.

Hansberg-Pastor et al. describe the broad and complex actions of estradiol and progesterone on the regulation of protein components of the cytoskeleton of neurons and astrocytes that ultimately affect cellular morphology, function, and connections, including dendritic spine growth. These properties correlate with region-specific features in the brain of females. This modulation begins early in

development and persists along the life span, notably during the estrous cycle, suggesting a continuous plastic transformation of dendritic spines, synapses, and neural networks.

Bittencourt et al. demonstrate that synapses and circuitries can undergo post-lesion reorganization, as is the case for mossy fiber sprouting and its debatable relationship with epileptogenesis. Here, ultrastructural data reveal the number and type of asymmetric contacts involving spine and shaft synapses and the likely restorative connectivity of the dentate gyrus molecular layer of rats with induced chronic seizures.

Vargas-Barroso et al. report cytological, whole-cell patch-clamp electrophysiological and connectional data indicating an anatomical and functional interaction between the accessory and the main olfactory bulb in rats. These findings are relevant for the animal's perception of complex chemosensory clues from the environment and the neural circuits implicated in the display of various social behaviors.

Calcagnotto addresses the role of interneurons in synaptic plasticity and the ways in which cellular replacement approaches can rescue defects in local circuit activity and synaptic plasticity. Strategies that alter interneuronal networks, including those that control inhibitory interneurons and the use of precursor cell grafts, may have the potential to restore synaptic plasticity and brain oscillations.

Zimmermann-Peruzatto et al. provide a comprehensive review of the relationship between vasopressin receptors and specific brain circuits. Alterations in vasopressinergic pathways may lead to changes in synaptic plasticity and parental and sexual behaviors.

de Sousa et al. link important data about the secretion and modulatory actions of hormones to developmental ages in young primates. They show that sex differences, cortisol levels, acute or chronic social isolation, and coping strategies are important for the development of neural circuitries and learning in male and female monkeys, an approach that can serve as a model for the study of emotional and behavioral disorders.

Gottfried et al. propose that neuroimmune responses are central to translating the effect of environmental risk factors and genetic and epigenetic changes to deficits in brain function and behavior in autism spectrum disorder (ASD). They present an immunological sequence of events leading to neuroinflammation, neuronal-gial responses, and brain connectivity dysfunction that may be involved in ASD pathogenesis.

Siniscalo highlights the impact of aberrations in neuroimmune responses in ASD, as proposed by Gottfried et al., citing the role of pro-inflammatory cytokines in disruption of the immunological interface between the peripheral immune system and central nervous system, leading to deleterious neuronal and behavioral consequences.

Pandey's group (Kyzar et al.) provides evidence for the effects of disturbances in epigenetic programming during adolescence due to repeated exposure to binge levels of alcohol. Alcohol exposure during adolescence leads to alterations in epigenetic, neurotrophic, and neuroimmune pathways in the brain, manifested by widespread and persistent changes in synaptic remodeling and neurogenesis in strategic brain areas. Rodent and human data link alcohol exposure, impaired dendritic spines, and neural circuitry to long-lasting behavioral consequences in the adult.

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AUTHOR CONTRIBUTIONS

AARF, RSC, and OvBuH are co-editors of this research topic.

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HYPOTHESIS AND THEORY
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Dendritic Spines as Tunable Regulators of Synaptic Signals

Jan Tønnesen[1,2] *and U. Valentin Nägerl*[1,2]*

[1] Interdisciplinary Institute for Neuroscience, University of Bordeaux, Bordeaux, France, [2] CNRS UMR 5297, Bordeaux, France

Neurons are perpetually receiving vast amounts of information in the form of synaptic input from surrounding cells. The majority of input occurs at thousands of dendritic spines, which mediate excitatory synaptic transmission in the brain, and is integrated by the dendritic and somatic compartments of the postsynaptic neuron. The functional role of dendritic spines in shaping biochemical and electrical signals transmitted via synapses has long been intensely studied. Yet, many basic questions remain unanswered, in particular regarding the impact of their nanoscale morphology on electrical signals. Here, we review our current understanding of the structure and function relationship of dendritic spines, focusing on the controversy of electrical compartmentalization and the potential role of spine structural changes in synaptic plasticity.

Keywords: synapses, synaptic plasticity, hippocampus, super-resolution fluorescence microscopy, dendritic spines

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Jon I. Arellano, Yale University School of Medicine, USA
Jochen Herms, German Center for Neurodegenerative Diseases (DZNE), Germany
Hjalmar Brismar, KTH Royal Institute of Technology, Sweden

***Correspondence:**
U. Valentin Nägerl
valentin.nagerl@u-bordeaux2.fr

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INTRODUCTION

Dendritic spines harbor glutamatergic synapses and mediate the vast majority of excitatory synaptic transmission in the mammalian brain. They represent fundamental computational units of information processing that underlie sensory perception, emotions, and motor behavior. Spine structural and functional plasticity is an important substrate of learning and memory (1), while spine dysfunction is linked to neuropsychiatric and neurodegenerative disorders of the brain, including autism (2) and Alzheimer's disease (3).

Ever since the discovery of dendritic spines by Ramon y Cajal more than a century ago, progress in understanding their anatomy and physiology has strongly depended on the development of new techniques to experimentally probe them (4).

Using the latest Golgi staining and light microscopy techniques of his days, Cajal hypothesized that spines harbor synapses and receive signals from other neurons (5). Still, it was not until 1959 that definitive proof for this idea was provided by the first electron microscopic (EM) images of synapse ultrastructure, revealing the presynaptic specialization, synaptic cleft, and postsynaptic density (PSD) (6).

Long before direct visualization of spine plasticity in live tissue became possible, EM provided the first hints of their dynamic nature, indicating that they change shape and size in response to repetitive synaptic stimulation (7). In parallel, theoretical studies formulated the idea that spines might compartmentalize biochemical and electrical signals, and thereby shape the functional properties of synapses (8–10).

The development of two-photon microscopy (11) opened up manifold opportunities to study synapses and their structure–function relationship deep inside live brain tissue with high temporal and spatial resolution (12). In addition to imaging the morphology of fluorescently labeled neurons, two-photon microscopy allows for targeted stimulation of single synapses

by photolysis of caged glutamate and other bioactive compounds (13) and measurements of molecular diffusion and enzymatic reactions using fluorescence recovery after photo-bleaching (FRAP) (14) and fluorescence lifetime imaging (FLIM) (15) in individual spines.

While being a powerful modality for imaging and stimulating neurons in living brain tissue, the spatial resolution of two-photon microscopy is limited by the diffraction of light to around 500 nm and, hence, falls short of resolving many important morphological details of neurons and glia cells. In particular spine necks, distal glial processes, and the shafts of axons have spatial dimensions of around 50–200 nm and, therefore, are not resolvable by two-photon microscopy (16).

For this reason, it has remained impossible to properly quantify the dynamics of these anatomical structures in live tissue, let alone to evaluate them relative to functional measurements. This is a major limitation for understanding the physiology of axons and spines, because their small size renders their functional properties particularly susceptible to minute morphological changes.

Given their conspicuous morphology, typically featuring a bulbous spine head attached to the dendrite via an elongated neck, spines are bound to be immensely important for synapse physiology and neural plasticity (**Figure 1**). Indeed, activity-dependent remodeling of spines, such as changes in spine turnover and spine head size, has been a consistent finding across cell types and brain regions under a wide range of (patho-) physiological experimental conditions *in vitro* and *in vivo* (1).

Notably, a recent study showed that newly acquired motor skills can be disrupted by light-induced shrinkage of those spines that were potentiated during motor learning (20). Two other recent studies reported on spine changes in the hippocampus *in vivo* (21, 22), which is the brain area most closely associated with learning and memory formation. The reported rates of spine turnover were very different between these studies, which highlights the methodological challenge of visualizing spines over time in deeper brain regions.

The invention of fluorescence super-resolution STED microscopy (23, 24), which was recognized by the Nobel Prize in 2014, has substantially facilitated synapse imaging (25, 26). STED microscopy is not limited by the diffraction of light and allows visualization of even the finest details of synaptic structures and their dynamics in living brain tissue (27, 28). Initially restricted to just a few microns, the depth penetration of STED has been significantly extended to tens of micrometers tissue depth. This is achieved either by the use of two-photon excitation (29, 30), or glycerol objectives that match the refractive index of brain tissue better than oil objectives, and which are equipped with a correction collar to reduce the spherical aberrations from the residual refractive index mismatch (31).

In this review, we summarize our current understanding of the structure–function relationship of dendritic spines, and highlight current controversies and open questions. We discuss the potential impact of nanoscale spine structural plasticity on the electrical function of synapses, by relating recent live cell structural and functional data to earlier theoretical predictions.

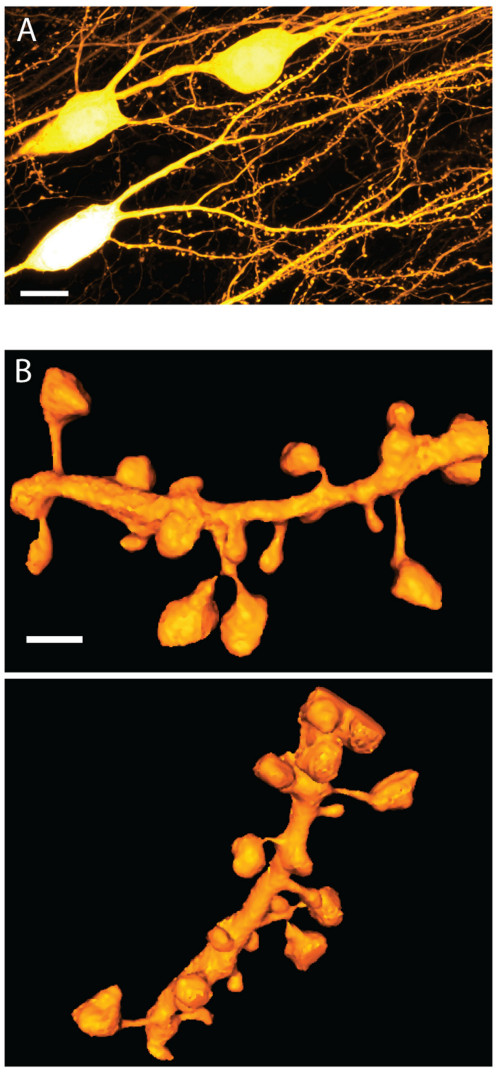

FIGURE 1 | Dendritic spine morphology. **(A)** STED image of basal dendrites on live CA1 pyramidal cells in organotypic hippocampal slice prepared from Thy1-YFP transgenic animals. The image is a maximum intensity projection over 10 µm and is subjected to a 1-pixel median filter. Scale bar is 10 µm. **(B)** Two rotated views of a surface rendered 3D STED image of live spines on a dendritic segment in organotypic hippocampal slice as above. The rendering was prepared using an ImageJ 3D viewer plugin (17). The scale bar is 1 µm. Images acquired as in Ref. (18), with the addition of 3D STED (19).

SPINE STRUCTURE AND FUNCTION

Spines stand out as unique neuro-anatomical specializations, and apart from their general head-and-neck design, no spine looks quite like any other (**Figure 1**). In fact, spine morphology is highly diverse, covering a broad distribution of shapes and sizes, which defies obvious categorization. Spine head volumes range from 0.01 to 1 μm^3, while spine necks measure between 50 and 500 nm in diameter and are roughly up to 3 µm in length (32–34). Moreover, these morphological parameters show little correlation with each other.

Despite of this morphological continuum, spines are commonly grouped into a small number of distinct categories, such as stubby, mushroom, thin, and filopodial, based on their appearance (35). While this categorization scheme may be practical for analysis purposes, it is a gross over-simplification, where the categorization results depend strongly on image quality, which vary between studies. Moreover, image projection artifacts and limited spatial resolution mask short spine necks, which leads to the false identification of stubby spines (18).

There are consistent differences in the spectrum of their morphology across different dendritic locations and laminar positions, cell types, brain areas, animal age, and disease states (36), while the density of spines on dendrites is also highly variable; aspiny interneurons lack spines altogether, while cerebellar Purkinje cells carry more than 200,000 spines.

The ubiquity of dendritic spines across the phylogenetic tree points to a highly specialized and fundamental role; however, the rhyme and reason behind their remarkable structure and diversity remains enigmatic. Over the last decade, extensive experimental studies using EM or two-photon imaging combined with glutamate uncaging and electrophysiological approaches have established several ground rules for the relationship between their structure and function.

First and foremost, there is a broad consensus that the size of the spine head scales with the size of the PSD (32, 34), and the amplitude of the excitatory postsynaptic current (EPSC) (37, 38).

Accordingly, the induction of synaptic long-term potentiation (LTP) leads to spine head enlargement that scales with the potentiation of the EPSC (39–41). This structural effect primarily occurs in smaller spines (40), and is saturable as repeated rounds of induction lose their effectiveness, much like LTP (42).

While synaptic potentiation and spine enlargement occur within seconds after the induction protocol, the increase in PSD size develops more slowly over tens of minutes (43), indicating that multiple, kinetically distinct processes underlie the molecular and morphological remodeling of synapses.

In addition to modifications of existing spines, spines can grow *de novo* in response to a variety of triggers, including LTP-inducing electrical stimulation, two-photon glutamate uncaging, or altered sensory experience (44–47), leading to the formation of new functional synapses (48, 49).

Conversely, electrical induction of long-term depression (LTD) leads to shrinkage of the spine head and increased spine loss (45, 50), which can also be induced by glutamate uncaging (51, 52) and optogenetic stimulation (53).

Taken together, these studies support the view that during synaptic plasticity spine heads undergo size changes followed by remodeling of the PSD to accommodate a higher or lower number of receptors, depending on whether LTP or LTD is induced. According to this view, spines serve primarily as placeholders for the PSD and changes in postsynaptic strength are mediated by modulating the efficacy or number of synaptic receptors, e.g. Ref. (54).

Due to lack of spatial resolution, structural plasticity studies have traditionally been limited to reporting changes in spine numbers or spine head size, neglecting the spine neck, despite its potentially critical biophysical role as pointed out early on, as in Ref. (10, 55).

BIOCHEMICAL COMPARTMENTALIZATION IN SPINES

There is ample evidence that dendritic spines can spatially constrict the diffusion of second messenger molecules. Biochemical compartmentalization is thought to allow neurons to independently regulate each of their thousands of synapses, endowing the brain with an enormous information processing capacity.

The first experimental evidence for compartmentalized signaling came from calcium imaging studies showing that presynaptic stimulation can elicit calcium transients that are confined to single spines (56, 57). In addition, compartmentalized activation of a variety of signaling molecules, including second messengers and enzymes, has been demonstrated in spines after plasticity-inducing synaptic stimulation (58). Quantitative analyses of diffusion between spine and dendrite based on FRAP experiments demonstrate that diffusion rates vary widely between different spines, ranging from tens to hundreds of milliseconds for small fluorescent molecules (14, 59).

Interestingly, the diffusional coupling between spine and dendrite is reduced following repetitive stimulation of individual spines by two-photon glutamate uncaging (60), indicating that the degree of biochemical compartmentalization is subject to activity-dependent regulation.

These studies clearly established that spines form diffusionally isolated micro-compartments, even though the underlying biophysical mechanism remained unclear for a long time. While a correlation between FRAP time constant and spine neck length was observed (14, 59), additional intracellular factors, such as a meshwork of actin filaments or the spine apparatus (61, 62), are likely also to contribute to the diffusion barrier. Interestingly, micrometer-scale synaptic signaling domains exist even without spines in smooth dendrites of neocortical interneurons, suggesting that compartmentalization can be achieved in non-morphological ways (63).

Combining FRAP experiments with super-resolution imaging allows for direct comparisons of molecular diffusion and nanoscale morphology in identified spines (**Figure 2**). Through this approach, we recently found that more than half of the measured variation in FRAP time constants across spines can be accounted for by spine morphology (18). While it is clear that the diffusional properties of spines are strongly shaped by spine morphology, there is still considerable variation that may be explained by other factors, such as organelles or cytoskeletal structures in the spine head and neck.

By first approximation, the diffusional FRAP time constant τ depends on spine morphology as follows (14, 64):

$$\tau = \frac{V \times L}{A \times D} \quad (1)$$

where V is the volume of the spine head, L the length, A the cross-sectional area of the spine neck, and D the diffusion coefficient of the fluorophore.

This simple formula shows that changes in τ can be realized in different ways: τ will increase if the spine head enlarges or if

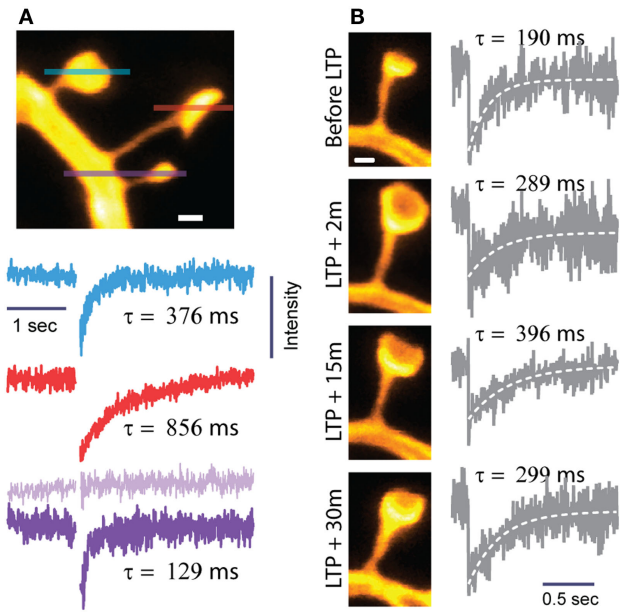

FIGURE 2 | Biochemical compartmentalization of dendritic spines. Spine morphology defines the spine as a biochemical compartment. **(A)** Neighboring spines often differ widely in shape and size and, hence, compartmentalize diffusive signals in a very different way. The lines through the spines show where FRAP was recorded by line scanning. The FRAP traces and their diffusional time constants (τ) correspond to the individual spines according to the color code and sequence (top to bottom). Spines with thin, long necks and large heads experience slower diffusional recovery and, hence, higher τ values. Modified from Ref. (18). **(B)** Induction of LTP by glutamate uncaging triggers structural changes in spine heads and necks, which have opposite effects on compartmentalization, so that τ changes less than predicted by looking at either neck or head dynamics alone [modified from Ref. (18)]. Scale bars are 0.5 µm.

the neck becomes longer or thinner. Parallel changes in head-and-neck morphology may be additive or cancel each other out regarding their effects on overall compartmentalization. For instance, τ will stay more or less constant if the spine head becomes larger and the spine neck widens at the same time.

These distinctions are pertinent given that the induction of LTP not only enlarges spine heads (40) but also leads to shorter and wider spine necks, so that τ changes less than what would be predicted if only one parameter were to change (**Figure 2**) (18).

While τ remains largely unaltered after LTP, the biophysical environment of the synapse and the compartmental properties of the spine are certainly affected, as the increase in spine head size will effectively lower the concentration of molecules released into the enlarged spine volume, and more permissive spine necks will facilitate the exchange of material (molecules, vesicles, organelles) between the spine head and parent dendrite.

Finally, it is worth mentioning that spine morphology is likely to influence other diffusion-dependent processes, including the spread of chloride in dendritic shafts, which impacts short-term plasticity of GABAA receptor signaling and inhibitory drive (65) and the mobility and trafficking of synaptic receptors and synaptic scaffold proteins within nano-domains that have been recently reported (66, 67).

ELECTRICAL COMPARTMENTALIZATION OF DENDRITIC SPINES

In contrast to biochemical compartmentalization, the case for electrical compartmentalization remains highly controversial, primarily due to technical limitations in measuring electrical signals directly in the spine, which forces experimenters to infer them by indirect means.

Several early studies based on cable theory (55, 68) and FRAP experiments (14) indicated that spines cannot modify synaptic signals appreciably. Subsequent experimental work based on Ca^{2+} imaging, two-photon glutamate uncaging, electrophysiology, and mathematical modeling has pointed to the contrary, indicating that spines are sufficiently electrically isolated to impact synaptic potentials and their dendritic integration (69–71). More recently, the pendulum has swung back, with studies based on voltage-sensitive dye imaging (72) and super-resolution STED microscopy (73), arguing that the spine neck has no effect on synaptic signals in the dendrite or soma. The lack of consensus effectively leaves open the basic question of the impact of spine morphology on the electrical signaling of synapses (**Figure 3**).

Modeling Voltage Transfer in Dendritic Spines

To gain insights into how spine morphology may influence synaptic signaling, we will consider an equivalent electrical circuit, which models the electrical phenomena in the postsynaptic neuron at steady state (**Figure 4**) (55). The model does not take into consideration the membrane capacitance and active conductances other than the ligand-gated synaptic conductance. Therefore, the synaptic current is modeled to flow without capacitive losses or active amplification from the spine head to the dendrite.

We point out already here that the spine neck will simultaneously have differential effects on the voltage in the spine head and the dendrite, and that the effects in the spine head are more pronounced in absolute voltages (**Figure 4**). However, only the effects manifested on the dendritic side will matter for dendritic integration and action potential firing.

When an excitatory synapse is stimulated, glutamate receptors (primarily of the AMPA type, but also NMDA) open, causing a net inward ionic current. The synaptic current (I_{syn}) scales with the synaptic conductance (g_{syn}) and driving force:

$$I_{syn} = g_{syn} \times (V_{spine} - E_{syn}) \quad (2)$$

where V_{spine} is the voltage in the spine head, E_{syn} is the reversal potential of the synaptic conductance (around 0 mV for glutamate receptors), and the term $V_{spine} - E_{syn}$ denotes the driving force (around 70 mV).

The amplitude of the excitatory postsynaptic potential (EPSP) in the spine head ($\Delta V_{spine} = V_{spine} - V_{rest}$) can be described by the following equation:

$$\Delta V_{spine} = \frac{g_{syn} \times (R_{neck} + R_{dendrite}) \times (E_{syn} - V_{rest})}{1 + g_{syn} \times (R_{neck} + R_{dendrite})} \quad (3)$$

where V_{rest} is the resting membrane potential (typically around −70 mV), R_{neck} the electrical resistance of the spine neck,

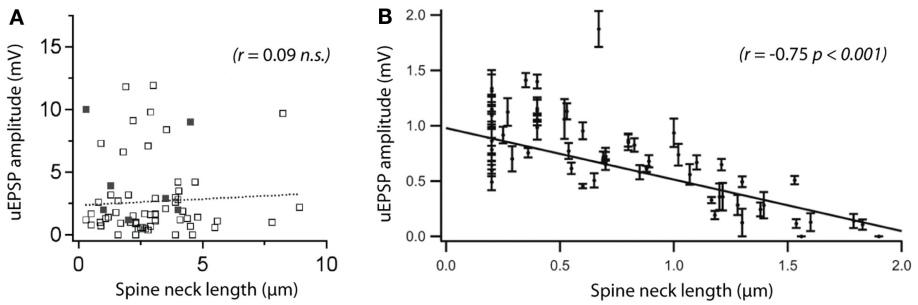

FIGURE 3 | Are spines capable of compartmentalizing electrical signals? There is no consensus on the role of the spine neck in electrical signaling, and conflicting results have been reported. **(A)** A recent two-photon microscopy study comparing spine morphology with uncaging (u)EPSP amplitude did not see a correlation between somatic uEPSPs and neck length. The solid dots represent spontaneous synaptic activity (evaluated by calcium imaging). Reprinted from Bywalez et al. (74), with permission from Elsevier. **(B)** Using a similar experimental approach, a previous study reported a strong correlation between the same parameters. The discrepancy between the two studies adds to an ongoing controversy about the importance of the spine neck in electrical compartmentalization of synapses. Modified with permission from Ref. (69) Copyright (2006) National Academy of Sciences, USA.

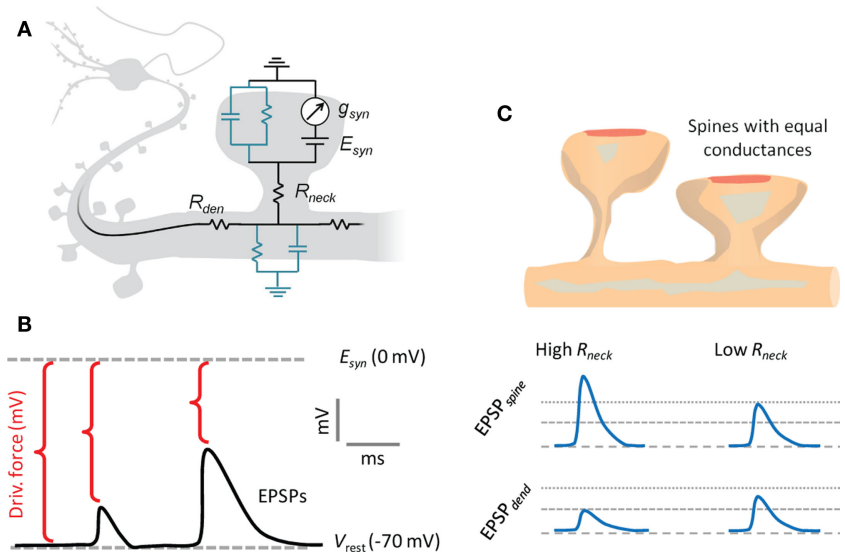

FIGURE 4 | Electrical compartmentalization of dendritic spines. **(A)** In the spine electrical circuit diagram, a variable current enters through the synaptic receptors, scaling with their conductance, g_{syn}, and with the electrical driving force, which is the difference between resting membrane potential and the reversal potential of the conductance, E_{syn}. The membrane resistance is so high that current will not escape, and it will instead pass first the neck resistance, R_{neck}, and then the dendritic input resistance, $R_{dendrite}$, on the way to the soma. The EPSP that the synaptic current generates along the way is defined by Ohm's law and follows voltage divider law. **(B)** As the synaptic current scales with driving force, the depolarizing EPSPs produced by the current will have a self-dampening effect as they approach the glutamate receptor reversal potential, E_{syn}. **(C)** A thin and long spine neck will have a high R_{neck}, which will locally boost the EPSP in the spine head. This in turn causes a loss of driving force, so that less current will flow over the synaptic conductance. While the EPSP in the spine head sees both the boosting and the loss of driving force, the corresponding EPSP in the dendrite only experiences the loss of driving force. Conversely, a spine with a low R_{neck} will see less boosting of the spine head EPSP and less current attenuation, so the spine and dendritic EPSPs are more similar. Beyond the illustrated passive effects of morphology, the boosted spine head EPSP may locally recruit voltage-gated conductances on the spine, which may in turn increase or decrease the synaptic current.

and R_{dendrite} the dendritic input resistance at the location of the spine (**Figure 5**).

It is instructive to consider the two limiting cases of Eq. 3, where both g_{syn} and R_{neck} are either very small or very large, respectively. More precisely, if $g_{\mathrm{syn}} \times (R_{\mathrm{neck}} + R_{\mathrm{dendrite}}) \ll 1$, the expression for $\Delta V_{\mathrm{spine}}$ simplifies to:

$$\Delta V_{\mathrm{spine}} = g_{\mathrm{syn}} \times (R_{\mathrm{neck}} + R_{\mathrm{dendrite}}) \times (E_{\mathrm{syn}} - V_{\mathrm{rest}}) \quad (4)$$

In this regime, the deflection in spine head voltage is a small fraction of V_{rest} and depends linearly on g_{syn}, and the sum of the electrical resistances, R_{neck} and R_{dendrite}. The spine effectively acts as a current source, meaning that the synaptic current is independent of the downstream electrical resistance.

In the opposite limiting case, when the synaptic conductance and synapse input resistance are very large, i.e., if $g_{\mathrm{syn}} \times (R_{\mathrm{neck}} + R_{\mathrm{dendrite}}) \gg 1$, the expression is reduced to:

$$\Delta V_{\mathrm{spine}} = (E_{\mathrm{syn}} - V_{\mathrm{rest}}) \approx 70\ \mathrm{mV} \quad (5)$$

In this regime, the spine head voltage approaches the reversal potential of the synaptic conductance (0 mV) and, thus, becomes

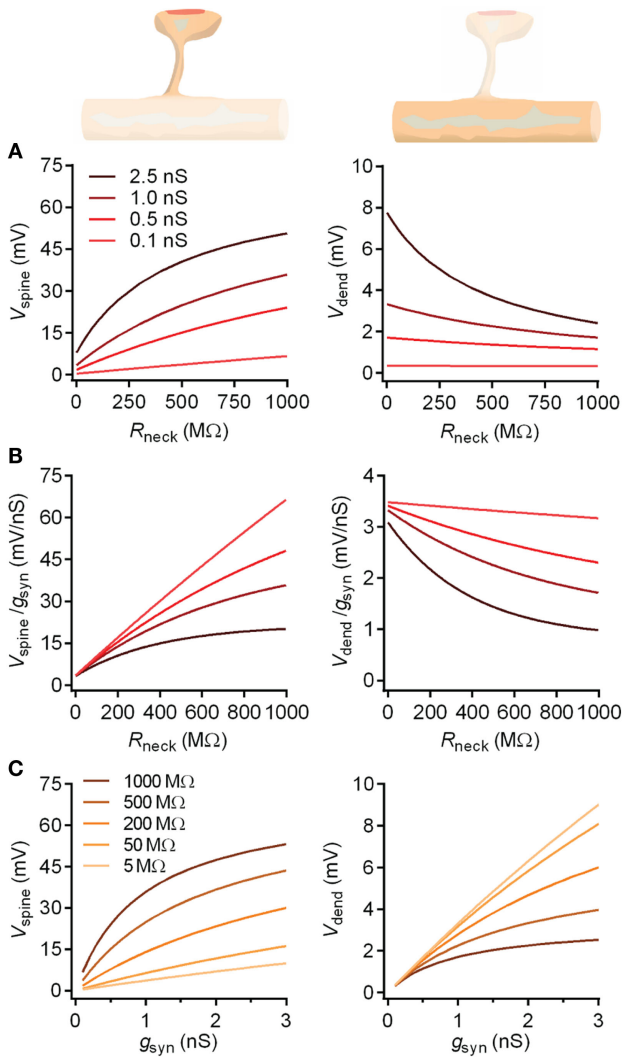

FIGURE 5 | The impact of the spine neck resistance on EPSPs in spine heads and dendrites. The spine neck resistance has opposite effects on EPSPs in the spine head and in the dendrite. **(A)** For a given conductance, the neck will boost the spine head voltage, which in turn will reduce driving force and saturate the boosting effect. By contrast, in the dendrite, the neck no longer boosts the voltage, and only the reduced driving force is manifested as a decreasing voltage with increasing R_{neck}. Both effects are more pronounced for synapses with higher conductances, as these produce higher voltages and stronger reductions in driving force. **(B)** This simultaneous boosting and saturation effect of the neck is manifested as a reduced voltage per conductance (synaptic gain) as a function of R_{neck}, which is more pronounced for stronger synapses. Again, the dendrite sees only the saturation effect, while the spine voltage is also boosted. **(C)** Conversely, for a fixed R_{neck} value, increasing the synaptic conductance will boost both the spine and dendritic voltages, with an accordingly stronger saturation effect if R_{neck} is higher. From the different R_{neck} values plotted, it is evident that while R_{neck} boosts the spine voltage, it simultaneously attenuates the dendritic voltage.

independent of any other parameter, including R_{neck}. In contrast to the former case, the spine now acts like a constant voltage source, effectively clamping the voltage to 0 mV in the spine head.

After entering the spine head, the synaptic current passes through the spine neck into the dendrite, spreading mostly to the somatic region, from where it exits the cell. Along the way, the current causes local changes in membrane voltage, leading to the EPSP in the soma, which can be measured electrophysiologically.

From the spine head to the dendrite, the voltage drops according to the voltage divider law, yielding a voltage signal ($\Delta V_{dendrite}$) at the adjacent dendritic location:

$$\Delta V_{dendrite} = \frac{R_{dendrite}}{R_{neck} + R_{dendrite}} \Delta V_{spine} \quad (6)$$

which, given Eq. 3, can be expressed as (**Figure 5**):

$$\Delta V_{dendrite} = \frac{g_{syn} \times R_{dendrite} \times (E_{syn} - V_{rest})}{1 + g_{syn} \times (R_{neck} + R_{dendrite})} \quad (7)$$

Also here, it is interesting to consider the two limiting cases for Eq. 7. In the case of $g_{syn} \times (R_{neck} + R_{dendrite}) \ll 1$, the voltage deflection in the dendrite becomes:

$$\Delta V_{dendrite} = g_{syn} \times R_{dendrite} \times (E_{syn} - V_{rest}) \quad (8)$$

which is similar to Eq. 4, except now the voltage only depends on $R_{dendrite}$. An important implication is that any changes in R_{neck} will be inconsequential for the dendritic voltage as long as the limiting case applies.

By contrast, in the case of $g_{syn} \times (R_{neck} + R_{dendrite}) \gg 1$, the dendritic EPSP is as follows:

$$\Delta V_{dendrite} = \frac{R_{dendrite}}{R_{neck} + R_{dendrite}} \times (E_{syn} - V_{rest}) \quad (9)$$

which means that R_{neck} and $R_{dendrite}$ determine the EPSP amplitude in the dendrite and soma, and that changes in spine neck dimensions can directly affect this.

Electrical Resistance of the Spine Neck

The key parameters to consider in this discussion are g_{syn}, R_{neck}, and $R_{dendrite}$, because they determine the amplitude of the current entering the synapse and the resultant voltages in the spine head and dendrite. While g_{syn} and $R_{dendrite}$ can be reasonably well determined by patch-clamp recordings, measuring R_{neck} is much more difficult, because of the inaccessibility of the spine head for electrophysiological recordings. However, this important biophysical parameter can be estimated in several indirect ways, all of which have specific advantages and caveats.

(1) The spine neck can be modeled as a passive ohmic resistor, which is defined by its cross-sectional area (A), length (L), and cytoplasmic electrical resistivity (ρ). R_{neck} can be then be calculated by the formula (9, 10):

$$R_{neck} = \frac{\rho \times L}{A} \quad (10)$$

Given sufficiently resolved images of dendritic spines, this morphology-based estimate is straightforward. However, it ignores the intracellular constituents of the spine neck, such as the spine apparatus or other organelles, which are likely to affect the electrical resistance of the spine neck.

Based on spine morphology obtained from EM images, and assuming a value of 100 Ωcm for ρ, spine neck resistances were estimated to range between 1 and 400 MΩ for CA1 pyramidal neurons (32). We recently reported a similar range for live spines, between 2 and 600 MΩ, based on STED microscopy in brain slices (18).

(2) R_{neck} can be also estimated by FRAP experiments (**Figure 2**). After bleaching a substantial fraction of small diffusible fluorophores inside the spine head (which is equivalent to a concentration jump), the time constant of fluorescence recovery (τ) is related to R_{neck}, ρ, the spine head volume V, and the diffusion coefficient D according to the formula (14):

$$R_{neck} = \frac{\tau \times \rho \times D}{V} \quad (11)$$

This method has the advantage that it is sensitive to contributions from intracellular factors, and does not require any knowledge of spine neck morphology, only the volume of the spine head that is easier to estimate.

Using this strategy, more variable ranges have been reported for R_{neck}, between 4 and 150 MΩ (14), up to 1 GΩ (60), and between 5 MΩ and 1.2 GΩ (18).

(3) R_{neck} has been estimated based on a combination of calcium imaging and modeling, where voltage-dependent calcium channels are used as a sensor of the voltage deflection in the spine head. However, the calcium fluorescence signal depends on the voltage in a highly non-linear way, which makes quantitative measurements challenging.

Based on this method the reported range is between 400 and 800 MΩ (71) and up to 1.2 GΩ (70). These values are generally higher and show less variation than the estimates based on morphology and FRAP. However, the discrepancies might reflect measurement biases, where spines with high neck resistances produce larger and, hence, more detectable calcium transients than spines with lower neck resistances.

(4) Finally, voltage imaging in dendritic spines is emerging as a new method, which may, in principle, provide a direct measure of R_{neck}. While holding great promise, optical detection of sub-threshold voltage deflections in spatial microcompartments still poses considerable challenges concerning signal sensitivity, accuracy, and calibration.

A recent study based on voltage-sensitive dye imaging in spines on thin basal dendrites of cortical pyramidal neurons provided an estimate of R_{neck} around 27 MΩ (72), contrasting sharply with previous higher estimates from calcium imaging (70, 71), although still falling within the low-end range of the FRAP and morphological estimates (14, 18, 32).

It is obvious that there is substantial disagreement in the literature on the mean value and variability of R_{neck}, and it remains unclear to what extent these discrepancies reflect physiological (brain area, cell type, etc.) or methodological (accuracy, experimental preparation, temperature, etc.) differences.

However, given the available evidence, it seems likely that R_{neck} varies widely, ranging from a few mega ohms to at least several hundred mega ohms. This variability implies that electrical compartmentalization of spines is also highly variable. Assuming a value of 50 MΩ for $R_{dendrite}$, the spine head voltage may be similar or more than ten times larger than the dendritic EPSP, depending on the value of R_{neck}.

REGULATION OF SYNAPTIC STRENGTH THROUGH STRUCTURAL PLASTICITY

It is a long-standing question whether spine structural plasticity represents a mechanism to tune synaptic strength. While the basic idea was conceived decades ago (8, 75, 76), it has laid largely dormant after being dismissed on theoretical grounds (9) and given the technical difficulties to explore it experimentally.

While it is clear that spine head enlargement or shrinkage is associated with functional changes, structural plasticity has essentially been viewed as a mere space issue: changes in spine head size reflect a dynamic capacity to accommodate a higher or lower number of synaptic receptors or scaffolding proteins. Hence, changes in synaptic strength are usually attributed to mechanisms that converge on modifying the conductance level of the synapse, through changes in presynaptic release probability, the clearance of glutamate from the synaptic cleft, or the number and biophysical properties of synaptic receptors. From this conductance-centric perspective, structural plasticity plays a permissive role for functional plasticity, but in and of themselves structural changes do not have direct effects on synaptic transmission.

More than 30 years ago, pioneering work based on EM provided the first indirect evidence for spine neck plasticity (7). But being limited to fixed preparations EM could not provide a smoking gun, and this work was ignored until 20 years later, when two-photon microscopy was able to provide time-lapse evidence for neck changes in live spines.

However, the scarce published results have been conflicting; on the one hand, neuronal activity was shown to slow down diffusion across the spine neck (60) and, on the other hand, it was shown to drive spine neck shortening (77, 78), which should rather facilitate diffusion.

Using super-resolution STED microscopy in combination with two-photon glutamate uncaging and patch-clamp electrophysiology, we obtained direct evidence that spine necks become shorter and wider after the induction of LTP, while the spine head is enlarged and the synaptic conductance increased (**Figure 3**) (18). Based on the morphological estimate of spine neck resistance (Eq. 10), these structural changes amount to a major reduction (on average by 50%) in R_{neck}.

In light of our discussions above, if the synapse operates in the current source regime, a change in R_{neck} will only affect the voltage in the spine head, whereas if it acts as a voltage source, it will only influence the dendritic EPSP. In reality, most synapses are likely to occupy a middle ground between these two extreme regimes, so that spine neck plasticity might simultaneously influence synaptic signals in the spine and dendrite.

Hence, a reduction in R_{neck} is likely to have at the same time differential effects on the EPSP on either side of the spine neck, lowering it in the spine head, while elevating it in the dendrite. Conversely, an increase in R_{neck} will boost the voltage in the spine head and lower it in the dendrite (**Figure 5**). The actual magnitude of the effects will depend on the relative sizes of the parameters g_{syn}, R_{neck}, and $R_{dendrite}$, according to the formulas above (Eqs. 3 and 7).

Counterintuitive at first sight, the local drop in spine head EPSP is actually facilitating LTP, because it reduces the negative feedback on the synaptic current resulting from a loss of driving force, which occurs as the spine head voltage approaches the synaptic reversal potential and effectively saturates. This negative feedback between spine EPSPs and driving force may under normal conditions be quite pronounced in spines with long and thin necks, which have high R_{neck} values. Thus, reducing R_{neck} may be a physiological mechanism during LTP, whereby the investment of increasing synaptic receptor numbers (i.e., synaptic conductance) is protected by counteracting voltage saturation in the spine head.

Beyond the immediate effects on EPSPs, large changes in R_{neck} might effectively shift the operating regime of the synapse, acting more like a voltage or current source. Such a major "parametric" change would modify the voltage transformation of the synapse and may, thus, affect dendritic integration and the computational performance of the neuron.

Secondary Effects on Active Conductances by Structural Plasticity

By influencing the spine head EPSP, changes in R_{neck} might strongly affect the activation of voltage-gated ion channels in the spine head, such as voltage-sensitive calcium and sodium channels, which in turn shape the EPSP (74, 79). Likewise, the voltage-dependent block of the NMDA receptor by extracellular magnesium will be directly affected by changes in the spine head EPSP.

At present, it is hard to make quantitative predictions on how electrical signaling at the synapse will be affected by these highly non-linear and dynamic interactions. Modeling can provide some intuitive insights; however, the results of numerical simulations will depend steeply on the model parameters for the active and passive properties of the synapse, many of which are still poorly known.

OUTLOOK

Ever since the discovery of dendritic spines by Ramon y Cajal, generations of neuroscientists have peeled away layers of their secrets. Yet, a comprehensive understanding of their structure–function relationship remains elusive, and continues to pose one of the great challenges in neuroscience.

The development of powerful optical microscopy techniques, such as super-resolution microscopy, two-photon glutamate uncaging, and voltage-sensitive dye imaging, is making it increasingly possible to measure key biophysical parameters with sufficient sensitivity and spatial and temporal resolution under a variety of physiologically relevant experimental conditions. Together with computer simulations, these new techniques will transform our understanding of the role of spines for synaptic function, neural computation, and ultimately behavior.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Sex Hormones Regulate Cytoskeletal Proteins Involved in Brain Plasticity

*Valeria Hansberg-Pastor[1], Aliesha González-Arenas[2], Ana Gabriela Piña-Medina[3] and Ignacio Camacho-Arroyo[3]**

[1] Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico, [2] Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico, [3] Unidad de Investigación en Reproducción Humana, Instituto Nacional de Perinatología-Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico

In the brain of female mammals, including humans, a number of physiological and behavioral changes occur as a result of sex hormone exposure. Estradiol and progesterone regulate several brain functions, including learning and memory. Sex hormones contribute to shape the central nervous system by modulating the formation and turnover of the interconnections between neurons as well as controlling the function of glial cells. The dynamics of neuron and glial cells morphology depends on the cytoskeleton and its associated proteins. Cytoskeletal proteins are necessary to form neuronal dendrites and dendritic spines, as well as to regulate the diverse functions in astrocytes. The expression pattern of proteins, such as actin, microtubule-associated protein 2, Tau, and glial fibrillary acidic protein, changes in a tissue-specific manner in the brain, particularly when variations in sex hormone levels occur during the estrous or menstrual cycles or pregnancy. Here, we review the changes in structure and organization of neurons and glial cells that require the participation of cytoskeletal proteins whose expression and activity are regulated by estradiol and progesterone.

Keywords: sex hormones, estradiol, progesterone, brain, glial cells, plasticity

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****Correspondence:***
Ignacio Camacho-Arroyo
camachoarroyo@gmail.com

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INTRODUCTION

Sex steroid hormones are known to play an important role during development and adulthood, regulating different functions and features of the central nervous system (CNS), such as brain differentiation, reproductive behavior, learning, and memory as well as neuroprotection. Structural plasticity is highly involved in the functional adaptation of the CNS in response to different environmental and physiological stimuli, including changes in hormone levels. In particular, female sex hormones can modify the size, morphology, synaptic density, and function of neuronal cells as well as the morphology of glial cells in sex steroid-responsive structures of the CNS (1). These changes are due to modifications in the neuronal and glial cytoskeleton where intracellular signals converge to regulate the direction and speed of outgrowth of different cell structures. Actin filaments, microtubules (MTs), and intermediate filaments, as well as the proteins associated with them, play a major role in synapse and dendritic spine formation (2). Neuronal projections are not only dependent on neuronal activity but also reliant on glial cells. The glia has an essential role in regulating the activity of CNS, where a mutual communication between glial cells and neurons exists. The activity and modifications in glial cell morphology also affect the formation and maintenance of synaptic contacts (3, 4). In this review, we will focus on the effects of female sex hormones on the

expression and regulation of cytoskeletal proteins, contributing to the remodeling of the adult brain.

SEX HORMONES AND THE BRAIN

Female sex hormones are known to have a wide range of effects in the brain regulating not only reproductive processes but also cognitive functions. Estradiol (E2) and progesterone (P4) are cholesterol-derived hormones that, given their lipophilic structure, can easily cross the blood–brain barrier and interact with their specific receptors in different target cells of the brain. These hormones are also synthesized inside the brain. P4 and E2 levels have been detected in different brain areas such as hypothalamus and hippocampus with concentration differences between female and male animals (5–7), and their synthesis in neurons and glial cells have been demonstrated (8, 9). Moreover, pregnenolone, a cholesterol metabolite used by neurons for the biosynthesis of P4 and E2, is also produced by the glia (10, 11). This implies that the actions of sex hormones in neuronal plasticity are the result of adrenal, gonadal, and brain local synthesis.

E2 and P4 effects depend on the signaling pathway they activate, which can be either through intracellular receptors (classical mechanism) or membrane receptors (nonclassical mechanism) (12). Female sex hormone receptors are expressed in different brain areas, such as the hippocampus, hypothalamus, cortex, cerebellum, medial amygdala, substantia nigra, and ventral tegmental area (13–18). In the classical mechanism of action, sex hormones enter the cell and interact with their intracellular receptors, progesterone receptors A and B (PR-A and PR-B), and estrogen receptors α and β (ERα and ERβ). In the cell, the receptors are associated with chaperones like the heat shock proteins 70 and 90 (Hsp70/90). The ligand–receptor interaction induces conformational changes in the latter that promotes the receptor phosphorylation, dissociation of the Hsp70/90 complex, and dimerization. The active receptor binds to specific DNA sequences named hormone response elements (HREs) located within the regulatory regions of target genes. The receptor also recruits coactivators and chromatin remodeling complexes that enhance the attachment of the basal transcription machinery to induce gene expression. Genes that lack HRE can be hormonally induced through the interaction of the receptor with transcription factors like Sp1 and Ap1 (19–22). Once the receptor dissociates from the DNA, it is marked for degradation through the 26S proteasome (23, 24).

Hormone receptor activation can also induce diverse signaling pathways like those mediated by MAPKs, PI3K/Akt, and PKC (25–27), regulate second messenger cascades (28) or modulate the actions of neurotransmitter receptors (29). These mechanisms are regulated through PR and ER located in the cytoplasm, nucleus, or plasma membrane (30–32) or through other membrane receptors that have different biochemical and pharmacological properties (33, 34). These signaling pathways may eventually induce gene transcription. The different mechanisms of action of sex hormones may account for the diverse signaling profiles observed in various brain regions.

The effects of E2 and P4 in the brain depend on hormonal levels and receptor expression. The levels of P4 and E2 fluctuate throughout the life span of the rat modifying different parts of the CNS and causing diverse alterations in brain anatomy, physiology, and behavior (35, 36). E2- and P4-induced plasticity occurs when neuronal cells dynamically respond to hormonal stimuli by modifying its connectivity network and biochemical composition. Brain plasticity can be long lasting, and even the same stimuli can induce different plastic responses at different ages (37). The most dramatic change induced by sex hormones in brain is the driving of its sexual differentiation. During the fetal–neonatal period, sex hormones permanently modify the brain architecture (13, 38). Neurogenesis, cell differentiation, synaptogenesis, axon guidance, myelination, cell migration, and cell death are some of the main mechanisms occurring during sexual differentiation of the brain. These mechanisms alter the brain area, volume, cell number, cytoarchitecture, cell activity, synaptic connectivity, and neurochemical content (1, 39).

After brain differentiation, sex hormone levels in the brain are transitory and fluctuating, and induce the continuous functional adaptation of the CNS throughout the life span of the animal, particularly in females (35, 40). The main periods where sex hormone levels fluctuate during the life span are the beginning of puberty, reproductive cycles, pregnancy, and menopause. During these phases, alterations in the number of neurons and synapses, glial complexity, morphological variations in dendrites and synapses, and changes in neurotransmitter levels have been reported (41–44). These changes promote neuronal and glial remodeling that is critical for cognition, learning, and memory. For example, spatial working memory varies during rat pregnancy, and the memory retention enhanced by E2 is maintained by P4 (45, 46). Further data show that E2 and P4 modify neuronal morphology of the hippocampus of rats and monkeys, an important region for memory consolidation (47, 48). It has been recently reported that P4 enhances object recognition memory consolidation through mTOR and Wnt signaling (49). There is also evidence that both E2 and P4 can modulate GABAergic, dopaminergic, glutamatergic, and serotoninergic neurotransmission, as well as the release of a variety of growth factors from the astroglia (50–52). Sex hormones also modify the outgrowth of astrocytic processes and the amount of neuronal membranes they can cover, facilitating neuronal synaptic connectivity and plasticity (3, 51, 53). Morphological changes induced by E2 and P4 in the brain as well as the cytoskeletal proteins participating in brain plasticity, which are modulated by sex hormones, are reviewed in detail in the next sections.

SEX HORMONES MODIFY THE NUMBER, SIZE, AND BIOCHEMICAL CHARACTERISTICS OF DENDRITIC SPINES

The effects of E2 and P4 on neuronal plasticity are related to adaptive changes in the structure and function of neurons that may contribute to learning, memory, and recovery after diverse insults (1). Reorganizational effects of sex hormones on neuronal circuitry involve different morphological events, including changes in dendritic length (54, 55) and neuronal membrane organization

related to synaptic and dendritic spine formation (56). Dendritic spines, first described by Ramón y Cajal in 1888 (57, 58), are small protrusions of the dendritic membrane of neurons that are specialized in synaptic transmission. They consist of an actin-rich head attached to the neuron by a thin neck and contain the necessary postsynaptic machinery to receive the input of an excitatory synapse. Dendritic spines and synapses can be stable or change dynamically, even in very short time lapses, in their morphology and biochemical composition upon different stimuli (59, 60). Sex hormones have been shown to alter the structure and function of these neuronal structures through both rapid and long-term mechanisms (32, 61).

Recent studies show that E2 can modify synaptic plasticity and dendritic spine formation in hippocampal neurons through rapid signaling cascades, such as MAPKs, PI3K/Akt, and PKC (43, 62), which can also involve the activation of ERα (63–65). Signaling pathways such as ERK1/2 and Akt have been reported to be essential for E2-mediated spinogenesis in primary cortical neurons, and the activation of ERβ can mimic the rapid E2-induced spinogenesis and synaptogenesis. These results suggest that in cortical neurons, E2 via ERβ promotes neuronal cell remodeling by increasing the number of excitatory synapses (66). The same study showed that 30 min of E2 treatment induces the recruitment of postsynaptic density protein 95 (PSD-95) to the newly formed dendritic spines, while in the nascent, synapses promotes the recruitment of the *N*-methyl-D-aspartate (NMDA) receptor subunit GluN1 (66). These proteins are essential for the formation of new synaptic contacts, suggesting that E2 promotes the recruitment of the required proteins to allow pre and postsynapses to form connections. Other studies show that E2 promotes the phosphorylation of NMDA receptors through the activation of the src tyrosine kinase/MAPK pathway, and thus enhances long-term potentiation (LTP) of synapse transmission (67). Also, cyclic changes in E2 levels during the estrous cycle of rats are associated with changes in the state of NR2 subunit tyrosine phosphorylation of NMDA receptors in the hippocampus and alter LTP (68). In addition to E2, rapid effects of P4 have been reported in primary cultures of cortical neurons, where P4 increases the density and number of dendritic spines through changes in cell cytoskeleton components (69). The rapid effects of P4 on dendritic spines have been proposed to occur through the activation of GABA receptors and through the recently described PR membrane component 1 (65, 70).

Non-rapid effects of sex hormones in the brain have also been observed, and reports show that they induce the formation of excitatory synapses both *in vitro* and *in vivo* (47, 48), thus modulating LTP (71, 72). For example, ovariectomized rats treated with E2 for 48 h showed an enhanced density of apical dendritic spines in the CA1 region of the hippocampus that was related to an increase in the number of functional synapses (73). Interestingly, the density of dendritic spines in the hippocampal pyramidal cells changes during the estrous cycle of the rat; more spines are observed during the afternoon of the proestrus and the morning of the diestrus when E2 and P4 levels are high (5, 74). Moreover, Kato and colleagues demonstrated that the concentration of E2 in the hippocampus correlates with the serum concentration observed during the estrous cycle (5). However, hormone levels in the brain vary between newborn female and male animals (7, 75), suggesting the importance of considering the developmental stage and sex of the animal for a better evaluation of the observed hormone effects. Other studies show that adult male rats have more spines than female animals in the medial nucleus of the amygdala, and that the density of these spines varies throughout the estrous cycle of virgin rats, showing fewer spines during the proestrus and estrus phases when compared to diestrus (76, 77). Remarkably, the inhibition of E2 synthesis in females but not in males results in LTP and synapse loss in hippocampal slices (78, 79), which points toward an important effect of local E2 synthesis on synaptic plasticity.

E2 also induces the formation of neural pathways during fetal and neonatal life that modulate the activity of synapses in adulthood (80). The role of P4 in synaptic plasticity is less studied, but it has been reported that in cerebellar slices of neonatal rats, P4 promotes dendritic outgrowth and synaptogenesis in Purkinje neurons contributing to the formation of new neuronal connections in this structure (81). Immature cerebellar Purkinje cells treated with P4 for 24 h increased the dendritic length and spine density but this effect was not observed in mature cells. The effect was blocked when cells were treated with PR antagonist RU486, which suggest a classical mechanism of action for this hormone in the cerebellum (70). Interestingly, chronic treatment with P4 (60 days) decreases hippocampal synaptic transmission and LTP in hippocampal slices from ovariectomized adult rats (65). These data suggest that in mature cells, P4 effects on dendritic spine formation and LTP are less clear than for E2. With respect to the importance of the glia, primary cultures of rat astrocytes treated with P4 for 24 h express higher levels of agrin, a protein shown to be important for synapse formation. The P4-induced increase in agrin in astrocytes enhances synapse formation in hippocampal neurons (82). These data show the strong relation between glia and neurons that can be modulated by sex hormones. Many of these changes observed in the adult brain eventually converge on the cell cytoskeleton. Neuronal and glial cytoskeletal reorganization depends on its own dynamic nature and on the expression, regulation, and activity of the proteins associated with it.

THE CYTOSKELETON IN NEURONAL PLASTICITY

The neuronal cytoskeleton is divided into three specific structural complexes with different properties: neurofilaments (NFs) or intermediate filaments, MTs, and microfilaments (MFs), each one with a specific composition and organization, and even a particular cell type or subcellular localization. NFs are heteropolymers composed of heavy, medium, and light NFs protein chains. NFs are very abundant in neuronal axons and have extremely elastic fibrous properties that help to maintain the asymmetrical shape of the neuronal cell and to regulate the axon diameter and growth (83). In addition to NFs, MTs are mainly located in the neuronal axon, where microtubule-associated proteins (MAPs) like Tau help to stabilize them. MTs are composed of heterodimers of α and β tubulin that give them an intrinsic polarity important for their dynamic nature (84). MTs and their MAPs (MAP1B, MAP2,

etc.) participate in the promotion of neurite extension, the induction of distinctive morphologies between axons and dendrites, axonal transport, neuronal plasticity, and neuronal degeneration (85). Lastly, MFs are constituted by actin filaments, and their polymerization dynamic is closely associated with the activity of actin-binding proteins (ABPs) like drebrin and ADF/cofilin. MFs are involved in a broad range of aspects that are crucial for the establishment and the correct function of synapses, axonal cones growth, shape, size, remodeling of dendritic spines, and protein trafficking (86).

The neuronal cell shape, the dendritic spines, and synapse morphology, as well as the speed of synapse growth, can be hormonally modulated (87–89). Morphological changes depend on the cell cytoskeleton, and its dynamic regulation helps to shape these diverse neuronal structures. Experimental evidence suggests that MFs and MTs play a prominent role in the establishment and stability balance in neuronal structures, such as synapses and dendritic spines, which are constantly constructed and modified throughout life (90, 91). Dendrites and their spines have important implications in neuronal activity. Cytoskeleton studies show that MFs are highly accumulated in the dendrite spines where a pool of dynamic MFs is located at the tip of the spine, while a pool of stable drebrin–actin filaments is located in the spine core. These stable drebrin–actin filaments interact with dynamic MTs whose presence is enhanced by synaptic activity. The interplay between MFs and MTs is therefore important for the temporal and local regulation of spine morphology (2, 92, 93). These cytoskeleton rearrangements are controlled by members of the Rho family of GTPases (e.g., RhoA, Rac1), which regulate the activity of different cytoskeleton-associated proteins such as MAPs and ABPs (94, 95).

Synaptic connections are very important for neuronal communication, so they are highly regulated. Astrocytes are active players in neuronal transmission and plasticity. They can extend their projections to surround neuronal somata, dendrites, and synapses. Actually, the majority of synapses are ensheathed by astrocytes providing the support for the organization and well functioning of the synaptic connections (**Figure 1**) (96). Astrocytic processes have in their structure bundles of intermediate filaments constituted by glial fibrillary acidic protein (GFAP). These projections gradually form a network that infiltrates the brain tissue in order to effectively associate with neuronal synapses (97, 98).

Different cytoskeletal proteins are modified when morphological plastic changes occur in the brain in response to diverse stimuli. Gonadal sex hormones are known to affect diverse morphological processes as mentioned in the text, so we will further review the effects of E2 and P4 on three of the main cytoskeletal proteins present in CNS cells.

E2 AND P4 PROMOTE THE REMODELING OF THE ACTIN CYTOSKELETON

Actin is a highly conserved protein involved in many important cellular processes, including contraction, cytokinesis, transport of vesicles and organelles, cell signaling processes, establishment and maintenance of cell junctions and cell shape, cell movement, and synaptic plasticity (99, 100). These actin features are mainly attributed to filamentous actin, which represents the major cytoskeletal component of dendritic spines (101). Hence, the morphological changes in spine shape, size, and number are determined by local actin dynamics (102). The overall process of cytoskeleton remodeling, including the formation of new MFs and their interaction with the plasma membrane, depends on the participation of diverse ABPs.

P4 and E2 are key modulators of cell morphology and movement in diverse cellular types, including neurons (103–106). Most of the events leading to cytoskeletal rearrangement are rapidly performed by changes in the phosphorylation state of ABPs. A key protein that controls actin remodeling is the WASP-family verprolin homologous protein 1 (WAVE1) whose activation by phosphorylation is essential to regulate actin polymerization through the actin-related protein Arp2/3 complex (107, 108). In this regard, E2 and P4 administration to rat cortical neurons leads to WAVE1 phosphorylation on 310, 397, and 441 serine residues. Phospho-WAVE1 is then redistributed toward the cell membrane at the sites of dendritic spine formation. An ERα rapid extranuclear signaling activates GTPase Rac1, which recruits the cyclin-dependent kinase 5 triggering WAVE1 phosphorylation. E2 also induces actin remodeling via the activation of ABP moesin through the RhoA and ROCK2 pathway (109). Moesin phosphorylation on Thr558 is essential to link the actin cytoskeleton to a variety of membrane-anchoring proteins, such as CD43 and CD44 (110, 111). Rat cortical neurons treated with E2 and P4 exhibit an increase in phosphorylation of moesin, which impacts the formation of neuronal spines (69, 109). Actin polymerization in dendritic spines of rat hippocampal slices has been linked to E2 activation of RhoA pathway that leads to the inhibition of the filament-severing protein cofilin (112). Interestingly, treatment of hippocampal slices with aromatase inhibitor letrozole promotes actin filaments depolymerization as a result of cofilin activation, thus leading to synapse loss (113). Also, it has been reported a transient spine density increase in cortical neurons treated with E2 dependent on a Rap/AF-6/ERK1/2 pathway (114). Another study reported that E2 induced an increase in the length of dendrites in the central nucleus of the amygdala and in the hypothalamic ventromedial nucleus that was due to the inactivation of cofilin and variations in the composition of GluA1 and GluA2 subunits of the AMPA receptors (87). The changes in the actin cytoskeleton suggest a possible relation between dendrite and dendritic spine remodeling and changes in animal behavior regulated by E2.

There is evidence that demonstrates that P4 increases the outspread of the neuronal growth cones of dorsal ganglia neurons, an effect related to morphological changes in the components of the actin cytoskeleton. The enhanced cytoskeletal dynamic within the growth cone after P4 treatment occurred through a classical mechanism of action because the effect was blocked by the administration of PR antagonist RU486 (115). These data show that E2 and P4 induce morphological changes in shape, size, and number of neuronal spines, and that these changes are determined by actin dynamics, suggesting a continuous plastic transformation (**Figure 1**).

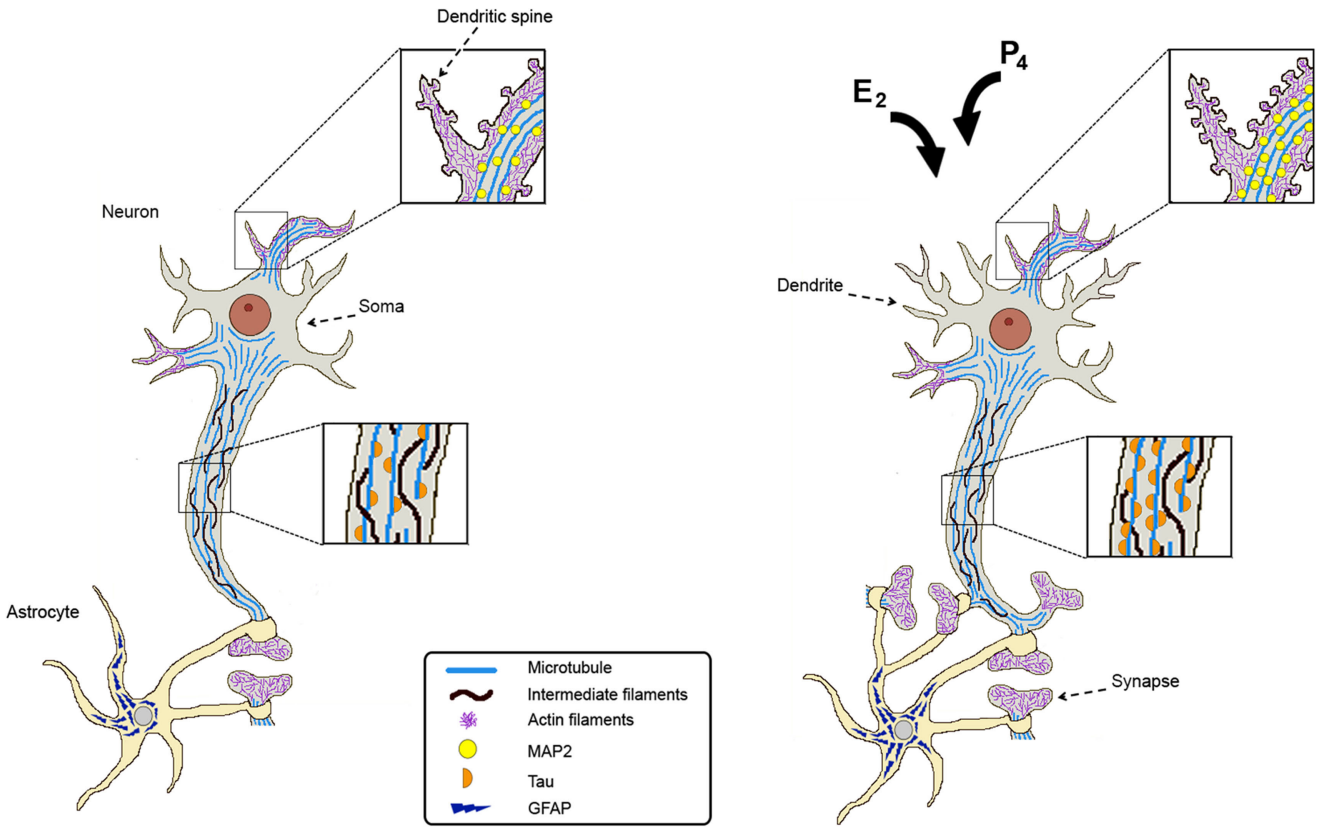

FIGURE 1 | Estradiol and progesterone regulate the expression of cytoskeletal proteins and promote neuronal plasticity. Cytoskeletal proteins are indicated as follows: microtubules at dendritic spines, soma, and axon; intermediate filaments along the axon; actin filaments at dendrites; Tau along the axon, MAP2 at dendrites and dendritic spines; and GFAP in astrocytes. After estradiol (E2) and/or progesterone (P4) treatments, cytoskeletal proteins increase their content in a region-specific manner and this correlates with an increase in the number of dendrites, dendritic spines, and synaptic contacts. Hormonal stimuli also increase astrocytes ensheathing the synapses providing the support for the organization and well functioning of synaptic connections.

E2 AND P4 EFFECTS ON MICROTUBULE-ASSOCIATED PROTEIN 2 AND TAU EXPRESSION

Microtubule-associated proteins regulate MTs dynamics by selectively binding to distinct conformations of polymerized and unpolymerized tubulin. Among them, the structural MAPs stabilize the MTs by binding along the length of the MT (116). In the brain, the main structural MAPs are MAP1, MAP2, and Tau, each one presenting several isoforms. Neuronal MAPs are differentially expressed during brain development: MAP1B is expressed in early stages of newly forming axons, MAP1A is expressed in mature axons, and both MAP2 and Tau isoforms are expressed during development and adulthood, predominantly in dendrites and axons, respectively (117, 118). In particular, Tau isoforms are of clinical relevance, given that they are the major component of paired helical filaments found in Alzheimer's disease (AD) and other brain diseases (85, 119).

It has been reported that MAP2 is preferentially located at the shaft of dendrites, where it may have the capacity to regulate morphological plasticity at a slow rate when compared to the rapid morphological changes regulated by actin filaments in dendritic spines (91). In the CA1 region of the hippocampus of MAP2-deficient mice, apical dendrites were shorter than those of wild-type animals (120, 121), suggesting an important role for MAP2 in dendrite elongation.

The expression pattern of MAPs and their correlation with ultrastructural changes induced by ovarian steroids have been observed in different brain areas and under specific hormonal and developmental conditions (89, 122–124). In medial basal hypothalamic neurons maintained for 4 days *in vitro* (DIV), E2 increased the levels of the 58-kDa Tau isoform but it did not change that of tubulin; by 7 DIV, E2 also increased the content of MAP1 and MAP2 (125). In cultured hypothalamic dissociated neurons, E2 exerted differential effects on neurite outgrowth depending on gender: the induction and differentiation of axons occur later in time, and cells develop fewer and shorter primary neurites in female fetuses compared with neurons taken from male fetuses. A comparable increase in Tau and MAP2 expression was observed in neuronal cultures obtained from both female and male rats (126). Another study showed that in dissociated cell cultures form embryonic rat medial amygdala, E2 induces

the differentiation of axons after 21 DIV and increases the total dendritic length of the cultured neurons. These changes were correlated with the respective increase in Tau and MAP2 expression but not with that of α-tubulin (127).

In the hippocampus of ovariectomized rats, an increase in MAP2 protein content has been reported after the treatment with E2, P4, or both hormones for 24 and 48 h, with no changes in the frontal cortex. Interestingly, these hormones did not modify MAP2 mRNA content in the hippocampus. These data suggest that MAP2 is involved in the structural changes induced by E2 and P4 in hippocampus and that its expression is regulated at a postranscriptional level (123). Interestingly, it has been demonstrated that the chronic administration of ovarian hormones immediately after ovariectomy modifies the content of MAP2 and Tau in the hippocampus and prefrontal cortex of the rat. Short- (2 weeks) and long-term (18 weeks) treatments with E2 or P4 had different and even opposing effects on MAP2 and Tau expression. None of the proteins changed its content in the prefrontal cortex in response to E2, but remarkably, P4 decreased MAP2 after short-term treatment and increased both MAP2 and Tau in this brain region after a long-term treatment. In the hippocampus, short- and long-term treatments with E2 increased MAP2 content, while P4 did it only after a short-term treatment (128). These data suggest that P4 regulates MAP2 expression depending on the brain region and the exposure time to the hormone, and it would be interesting to study P4 effects in E2-primed animals. Other authors have found similar tissue-specific effects with P4. For instance, in ovariectomized rats, an acute injection of P4 had no effect on Tau expression in the hypothalamus after 24 h, while it induced a decrease in the cerebellum (129). Another study reported that after P4 treatment for 3 days, the loss of MAP2 induced by acute spinal cord injury was attenuated, suggesting that P4 is partially responsible for preserving neuronal ultrastructure at the peripheral level (130). These studies highlight the importance of the type and length of treatment, the doses of E2 and P4 used and as well as the brain region studied; a summary of these results are shown in **Table 1**.

During pregnancy, circulating sex hormones are increased in the rat; E2 levels are two-fold and P4 three-fold higher compared with the hormone levels during proestrus day (131, 132). The brain displays diverse morphophysiological changes during pregnancy including cell plasticity (36, 45, 133). Furthermore, in the medial preoptic area (POA), late pregnant rats have bigger neuronal somata than ovariectomized rats (134), suggesting that E2 and P4 play an important role in neuronal morphology. Changes in the expression of MAP2 and Tau in the hippocampus and POA were evaluated during rat gestation and the beginning of lactation. In the hippocampus of pregnant rats, the content of MAP2 decreased during pregnancy, contrary to ovariectomized rats treated with P4 during 2 weeks (128, 132). These differences in P4 effects suggest a very fine regulation of MAP2 protein expression that depends on the characteristics of the hormonal stimulus. In addition, no significant changes in MAP2 content were detected in POA through rat pregnancy, suggesting that tissue-specific factors are involved in the regulation of MAP2 expression (132), which could be related to the different roles that have specific brain areas in the behavioral patterns observed throughout pregnancy.

Differences in Tau protein content and in its phosphorylation pattern in different brain regions may be related to Tau key role in the dynamic remodeling of neuronal cytoskeleton observed during gestation. Tau content and its phosphorylation are modified in a tissue-specific manner in the pregnant rat (132). In the hypothalamus, the hippocampus, and the cerebellum, Tau content diminished on gestation day 14 compared to gestation day 2, and only in the cerebellum and the hippocampus, this decrease was sustained until day 18 of pregnancy. Phosphorylated Tau at Ser396 (PhosphoTau) progressively augmented in the hippocampus, the hypothalamus, and the cerebellum throughout pregnancy, whereas in POA, the content of PhosphoTau decreased on day 21 of gestation (135). Tau phosphorylation at Ser396 results in tubulin depolymerization and MTs destabilization (136). Recent data show that Tau has an important role in synaptic plasticity in the hippocampus and that Ser396 phosphorylation is required for long-term depression (LTD), which is associated with the weakening of synaptic connections (137). LTD is important for certain cognitive processes like novelty discrimination and behavioral flexibility (138), which are fundamental for the pregnant rat.

Changes in MAP2 and Tau expression have been seen even after days of E2 or P4 treatment (1 day and 18 weeks), suggesting a classical mechanism of action where intracellular PR and ER are involved. However, not only MAP2 and Tau are under sex hormones influence, there are other proteins involved in synaptogenesis (neuroligins) or in spine density formation (PSD-95), whose expression also depends on P4 and E2 levels. Neuroligin-2 expression in the uterus is upregulated after 3 days of treatment with E2, P4, or E2 + P4 (139). Six-hour of E2 treatment stimulates the phosphorylation of Akt, as well as the phosphorylation of the translation initiation factor 4E binding protein 1. In turn, the activation of these signaling intermediates promotes the increase in the translation of PSD-95 in cultured neuronal cells (140). These data demonstrate that E2 and P4 induce the expression of different proteins involved in neuronal plasticity by different mechanisms of action.

SEX HORMONES AND THEIR IMPACT ON GLIAL FIBRILLARY ACIDIC PROTEIN EXPRESSION

Nowadays, it is evident that astrocytes respond to various stimuli by increasing their intracellular calcium levels, releasing gliotransmitters (141) or rapidly extending their projections (97). The large astrocytic processes have bundles of intermediate filaments that have GFAP as one of their principal constituent. GFAP has been implicated in cell motility (142), astrocyte proliferation (143), directional mobility of vesicles (144), the integrity of the blood–brain barrier, myelination (145), neuroprotection, and brain plasticity (146, 147).

Glial fibrillary acidic protein expression can be modified by factors such as neuronal damage, stress, age, or hormones (148). Sex hormones can regulate the astrocyte number during rat

TABLE 1 | Changes in MAP2A and Tau protein content in the hippocampus and frontal cortex of ovariectomized rats after acute and chronic E2 and P4 treatments.

Brain area	Time of treatment	E2		Time of treatment	P4		Reference
		MAP2A	Tau		MAP2A	Tau	
Hippocampus	48 h	Increase	Increase	24 h	Increase	Increase	Reyna-Neyra et al. (123)
Frontal cortex		NC	NC		NC	NC	
Hippocampus	2 weeks	Increase	NC	2 weeks	Increase	NC	Camacho-Arroyo et al. (128)
Frontal cortex		NC	NC		Decrease	NC	
Hippocampus	18 weeks	Increase	NC	18 weeks	NC	NC	Camacho-Arroyo et al. (128)
Frontal cortex		NC	NC		Increase	Increase	

The study of two brain regions and the modifications in protein content after acute (24 and 48 h) and chronic (2 and 18 weeks) hormone treatments.
NC, no change in protein content.

hippocampal development (149), enhance the extension of GFAP immunoreactive processes in astrocytes from hippocampal slices *in vitro* (150), and modulate astrocyte reaction after brain injury (151, 152). Interestingly, GFAP fluctuates during the estrous cycle of the rat and has a marked sex difference, at least in the hippocampus. The CA1, CA3, and dentate gyrus regions of the hippocampus had an increase in GFAP immunoreactivity during proestrus (high levels of P4 and E2) compared to male animals and diestrus females. During proestrus, astrocyte morphology changed to rounded cell bodies with numerous and short processes, whereas cells with stellate shape with few and long processes were present in the hippocampus of males and diestrus females (153).

During pregnancy and the beginning of lactation, a differential expression pattern of GFAP was found in the brain. Gómora-Arrati and coworkers analyzed GFAP expression on days 2, 14, 18, and 21 of gestation and the second day of lactation (L2) of the rat because of the marked changes in E2 and P4 levels observed in these days. It was found that in the hippocampus, GFAP content showed a constant increase of 25% throughout pregnancy and L2, while in the cerebellum, it first decreased more than 30% during pregnancy and later increased on L2 (41%). Interestingly, GFAP content increased in the frontal cortex and hypothalamus on gestational days 14 and 18, respectively. Then, a subsequent decrease was observed in the following days of pregnancy that persisted until L2 in the hypothalamus, in the cortex increased (42%) in L2. Contrary, a dramatic decrease in GFAP content was observed in POA on day 14 followed by an increase that was maintained throughout the rest of the studied days. These data suggest a differential expression of GFAP that should be associated with changes in brain function during these reproductive stages (154). Other reports showed that the chronic administration of P4 in ovariectomized rats resulted in a reduction of GFAP content in the hippocampus (128). This result contrasts with that observed under physiological conditions, highlighting the importance of hormonal concentration and exposure time on the content of GFAP in the brain.

E2 also modulates astrocytic form and function in the hypothalamus of rodents during development and adulthood. In the developing arcuate nucleus, E2 increased stellation of astrocytes through increases in neuronal GABA synthesis (155). Likewise, E2 positively regulates the length of GFAP-positive processes through ERα activation in astrocytes of ovariectomized animals (156). Still, there is no evidence whether E2-induced changes in astrocytes morphology are indirect effects of the E2 stimulation of neighboring neurons. Other reports show that in ovariectomized rats with entorhinal cortex lesions, E2 replacement inhibits the increase in GFAP (mRNA and protein level) and enhances neurite outgrowth. It is proposed that the decrease in GFAP alters the organization of laminin and this increases the fibrillary extracellular matrix supporting axonal growth (157). In adult castrated male rats, GFAP expression increased in the hippocampus, however, high levels of E2 prevented this castration-induced increase in GFAP (148). Interestingly, as evidence described herein shows that most of the effects of steroid hormones on GFAP expression are long term, and the data suggest that both P4 and E2 dynamically modify both the content and the distribution of GFAP.

SEX HORMONES IN NEUROGENESIS, NEUROPROTECTION AND DISEASE

E2 and P4 have been shown to exert both neuroprotective and neuroregenerative roles in several models of brain damage (158–161). Neurogenesis in the adult animal occurs in the cells lining the subventricular zone and the dentate gyrus of the hippocampus, where cells can remain quiescent or be activated to finally produce neuronal progenitor cells that later migrate into diverse brain regions (37, 162). It has been observed that neurogenesis in the dentate gyrus is higher in female animals than in males, probably because of the variations in gonadal hormones (163). Also, chronic treatment (21 days) of ovariectomized rats with E2 + P4 increased neurogenesis in the dentate gyrus (164). Regarding brain damage, E2 can induce neurogenesis post stroke in the adult animal (165) that could be through the activation of ERs (166, 167), and P4 has been reported to increase neurological functions after a traumatic brain injury (168). As a prerequisite for neuronal transmission, the new neurons need to have a well-defined axon and dendrites, which is known as neuronal polarization. The cytoskeleton is fundamental for the process of neuronal polarization (169) and as described in this review, sex hormones can modulate the expression and regulation of important proteins of the cytoskeleton.

The neuroprotective effects of sex hormones have been observed under different brain insults and diseases. In an

ischemic model, P4 reduces neurite growth inhibitory proteins like RhoA and Nogo-A, and E2 diminishes the loss of neurons and synapses from de CA1 hippocampal region (170, 171). In neurodegenerative diseases like AD, E2 administration reduces the expression of β-amyloid precursor protein, which is cleaved into amyloid beta (Aβ) and accumulated in plaques in the brain (172, 173). Aβ is involved in the generation of AD and it has been reported that estrogens can reduce its concentrations in the brain (174). Also, the formation of tangles of Tau protein caused by its abnormal phosphorylation, another AD characteristic, has been shown to be counteracted by E2 (175, 176). In fact, some studies have demonstrated that E2 therapy reduces the risk of presenting this neurodegenerative disease in women as well as diminishes the cognitive impairment associated with it (177, 178). Neuroprotective properties of E2 and P4 have also been observed *in vitro* in neuronal models of cell death induced by glutamate in hippocampal and cortical neurons (179, 180). Both E2 and P4 can induce recovery from neurodegeneration by increasing the synthesis of myelin components in both Schwann cells and oligodendrocytes (10, 181, 182). In fact, P4 promotes the expression of the myelin basic protein in cultured rat oligodendrocytes (183, 184). Taken together, sex hormones promote the recovery of brain tissue upon an insult and also protect against neurodegenerative diseases.

CONCLUSION

E2 and P4 play a key role in different neuronal and glial cell functions that involve changes in synaptic plasticity, and therefore in cell structure (**Figure 1**). These sex steroids induce changes in the brain cells cytoskeleton in addition to the content and activity of cytoskeletal proteins, such as MAP2, TAU, and GFAP. However, these changes significantly vary depending on sex, age, cerebral region, as well as the dose and length of exposure to these hormones.

PERSPECTIVES

There are several promising research areas that will give us a better understanding of the participation of sex steroid hormone action in cytoskeletal proteins regulation. The knowledge of the action mechanisms used by sex hormones to modulate cytoskeleton and therefore synaptic plasticity will be important to understand how learning and memory skills change during puberty, reproductive cycle, pregnancy, lactation, and menopause.

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Replacement of Asymmetric Synaptic Profiles in the Molecular Layer of Dentate Gyrus Following Cycloheximide in the Pilocarpine Model in Rats

*Simone Bittencourt[1], Luciene Covolan[1], Clement Hamani[2], Beatriz M. Longo[1], Flávio P. Faria[3], Edna Freymuller[3], Ole P. Ottersen[4] and Luiz E. Mello[1]**

[1] Department of Physiology, Universidade Federal de São Paulo, São Paulo, Brazil, [2] Division of Neurosurgery, Toronto Western Hospital, Toronto, ON, Canada, [3] Electron Microscopy Center, Universidade Federal de São Paulo, São Paulo, Brazil, [4] Department of Anatomy, Centre for Molecular Biology and Neuroscience, University of Oslo, Oslo, Norway

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***Correspondence:**
Luiz E. Mello
lemello@unifesp.br

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Bittencourt S, Covolan L, Hamani C, Longo BM, Faria FP, Freymuller E, Ottersen OP and Mello LE (2015) Replacement of Asymmetric Synaptic Profiles in the Molecular Layer of Dentate Gyrus Following Cycloheximide in the Pilocarpine Model in Rats. Front. Psychiatry 6:157. doi: 10.3389/fpsyt.2015.00157

Mossy fiber sprouting is among the best-studied forms of post-lesional synaptic plasticity and is regarded by many as contributory to seizures in both humans and animal models of epilepsy. It is not known whether mossy fiber sprouting increases the number of synapses in the molecular layer or merely replaces lost contacts. Using the pilocarpine (Pilo) model of *status epilepticus* to induce mossy fiber sprouting, and cycloheximide (CHX) to block this sprouting, we evaluated at the ultrastructural level the number and type of asymmetric synaptic contacts in the molecular layer of the dentate gyrus. As expected, whereas Pilo-treated rats had dense silver grain deposits in the inner molecular layer (IML) (reflecting mossy fiber sprouting), pilocarpine + cycloheximide (CHX + Pilo)-treated animals did not differ from controls. Both groups of treated rats (Pilo group and CHX + Pilo group) had reduced density of asymmetric synaptic profiles (putative excitatory synaptic contacts), which was greater for CHX-treated animals. For both treated groups, the loss of excitatory synaptic contacts was even greater in the outer molecular layer than in the best-studied IML (in which mossy fiber sprouting occurs). These results indicate that mossy fiber sprouting tends to replace lost synaptic contacts rather than increase the absolute number of contacts. We speculate that the overall result is more consistent with restored rather than with increased excitability.

Keywords: mossy fiber sprouting, asymmetric synaptic profiles, epilepsy, synaptic plasticity, cycloheximide

INTRODUCTION

The functional consequences of mossy fiber sprouting in the epileptic tissue have been interpreted as either contributing to (1–3) or counteracting epileptic seizures (4–6). The controversial nature of this synaptic reorganization has generated at least three hypotheses: the mossy fiber sprouting hypothesis (7) that holds sprouting as a major factor underlying hippocampal hyperexcitability; the dormant basket cell hypothesis (6, 8) that emphasizes the importance of changes in inhibitory activity; and the irritable mossy cell hypothesis (9) that focuses on the hyperexcitability of mossy

cells. The apparent inconsistency of the data derives partly from the limited perspective of most anatomical studies, which focus primarily on a single-synaptic input (e.g., sprouted mossy fibers as opposed to data from field neuronal recordings).

Dentate granule cells receive predominant input from the medial septum, entorhinal cortex, and hilus [for review, see Ref. (10)]. In this context, while most studies in this area have concentrated on mossy fiber sprouting, it is clear that synaptic changes from sources other than granule cells might also contribute to the development of epileptogenesis. These, however, remain largely unknown (11) due the lack of staining methods to specifically characterize such terminals (in contrast to the Timm's staining technique used to study mossy fiber terminals). One approach to address this issue is to study synaptic terminals that innervate the dentate molecular layer at the ultrastructural level.

We have previously demonstrated that induction of *status epilepticus (SE)* in the presence of cycloheximide (CHX) is associated with marked reduction of hilar mossy cell loss in mice and rats (13). Indeed, there is little or no sprouting of mossy fibers (granule cell axons) in animals subject to *SE* under the presence of CHX (14–16). Here, we investigated whether mossy fiber sprouting, and additional synaptic reorganization of the dentate molecular layer, would be associated with an increase in the number of asymmetric synaptic profiles, or simply with synaptic replacement.

MATERIALS AND METHODS

Animals and Protocol for Pilocarpine Induction of Chronic Seizures

All experimental protocols were approved by the Animal Care and Use Ethics Committee of UNIFESP and were performed in accordance with the Society for Neuroscience guidelines for animal research. Male Wistar rats ($n = 30$, 200–250 g) were kept on standard light/dark cycle (12/12 h) with lights on at 7:00 a.m. Animals had free access to rat chow pellets (Nuvilab) and tap water. Seizures were induced by injections of pilocarpine hydrochloride (Pilo, 320 mg/kg, i.p. Merck). Scopolamine methyl bromide (1 mg/kg, i.p., Sigma) was administered 30 min prior to Pilo to reduce its peripheral effects. In addition, one group of animals also received CHX (1 mg/kg, i.p., Sigma) 30 min prior to Pilo (CHX + Pilo). All animals developing *SE* received thionembutal (25 mg/kg, i.p., Cristalia, Brazil) 90 min later, as previously described (17). Four months after *SE*, animals were sacrificed and had their brains processed, as described below.

Tissue Processing

Two different protocols were performed 120 days after *SE* induction (*Experiment 1* and *Experiment 2*); for each protocol, we analyzed five animals per group. For *Experiment 1*, five animals from each group (Pilo, CHX + Pilo, and control) were transcardially perfused with 500 mL sulfide solution (4% glutaraldehyde, 0.1% Na_2S, 0.002% $CaCl_2$, in 0.12M Millonig's phosphate buffer, pH7.3) (18). One hour later, brains were processed according to the Timm's staining method for the ultrastructural evaluation of silver grains in synaptic terminals of the hippocampal dentate molecular layer. After removal from the skull, brains were placed in the same fixative solution for 24 h at 4°C. Coronal sections (100 μm thick) were cut on a vibratome (Vibratome Series 1000 Sectioning System) and transferred to a fresh developing solution (60 mL gum Arabic 50%, 10 mL of a 2M citrate buffer; 15 mL hydroquinone 5.67%, and 15 mL silver lactate 0.73%) for 1.5 h in the dark, under constant agitation, and subsequently processed for electron microscopy (EM).

Another set of five animals for each group (Pilo, CHX + Pilo, and control) was used to evaluate synaptic profiles in the dentate gyrus molecular layer (*Experiment 2*). Under deep anesthesia with thionembutal (50 mg/kg, i.p.), rats were transcardially perfused with 500 mL of modified Karnovsky solution at 4°C (2.5% glutaraldehyde, 2% formaldehyde in 0.1M phosphate buffer, pH 7.4), for at least 1 h. One hour later, brains were removed from the skull, immersed in the same fixative solution for at (19) least 24 h at 4°C, and subsequently processed for EM.

Electron Microscopy Procedures

Tissue specimens were obtained from the right dorsal hippocampus (corresponding approximately to levels 28–31 of Swanson's rat brain atlas) (20). Samples remained overnight in a 0.1M pH 7.4 cacodylate buffer solution [$Na[CH_3]^2.AsO_2.3H_2O$], at 4°C. After rinsing, specimens were postfixed with 1% OsO_4 sodium cacodylate buffer, washed in sodium cacodylate buffer, and kept overnight in uranyl acetate. The specimens were then dehydrated in a series of ethanol baths, placed in propylene oxide, transferred to pure Epon resin, and placed in vacuum for 4 h. The block polymerization took place at 60°C for at least 2 days. Seventy-nonometer-thick (silver interference color) and 90-nm-thick (gold interference color) sections were cut (Ultracut S/FC S, Reichert) for *experiments* 2 and 1, respectively. These sections were then stained with 2% uranyl acetate and lead citrate.

Methodological Considerations

The criteria used to identify ultrastructural synaptic profiles have been previously described (21, 22). Briefly, synaptic profiles were identified by cleft material between parallel membranes of a presynaptic element using at least two spherical vesicles and a postsynaptic element with a postsynaptic density (PSD). Active zones were distinguished from puncta adhaerentia by the lack of a pronounced presynaptic thickening and the usual presence of apposed presynaptic vesicles. We restricted our evaluation to asymmetric synaptic profiles (excitatory synapses) of the dentate molecular layer. The asymmetric synaptic profiles were classified as perforated and non-perforated based on shape of the PSD (23–25). These were categorized as: PSD1 (non-perforated type 1 synapses), synaptic profiles with a single-synaptic bouton associated to a continuous disk shape; PSD2 or PSD3 (perforated types 2 or 3 synapses), synaptic profiles with two or more PSDs, respectively. We have also evaluated the number of synaptic profiles located on dendritic spines versus those located on dendritic shafts. Dendritic spines were discerned from dendritic shafts by morphological features of spines. As an example, the dendritic shaft cytoplasm contains microtubules, mitochondria, and a multivesicular body, while the cytoplasm of the spine consists

of stacks of smooth endoplasmic reticulum interdigitated by electron-dense plates (26, 27) (**Figure 1**).

The number of non-perforated and perforated synapses is not shown in absolute values because a quantification of this order would require the analysis of serial sections. The basic assumption for counting synaptic profiles at various single sections is the probability that different planes of section would be equally distributed across groups.

The area of silver grain electron-dense deposits from the Timm's reaction was evaluated by quantitative densitometric stereological analysis, through a cross test system, in approximately the same area of that used for counting the asymmetric synaptic profiles (approximately 1,713 μm^2 per layer per animal). Although one could consider desirable to obtain quantitative estimates of the relative number of silver grain-containing sprouted fibers, technical limitations in applying the Timm's method at an ultrastructural level make such estimates potentially unreliable. Moreover, the size and density of silver grains is not linearly related to the concentration of zinc within a terminal. It is important to emphasize that data obtained with Timm's staining for EM did not allow a clear definition of synaptic membranes. In some of the sections, however, we could observe silver grain clusters around dendritic shafts (putative mossy fiber sprouting terminals) with a non-perforated asymmetric synaptic profile (**Figure 1**). This is in agreement with a previous study using Timm's staining in kainate-treated rats (28).

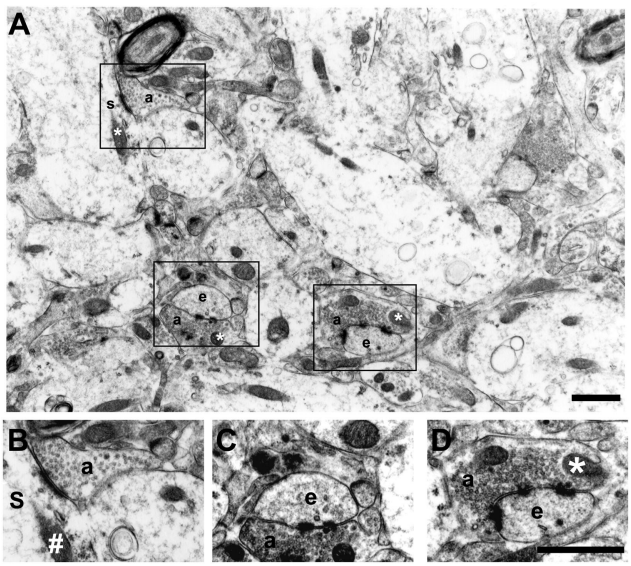

FIGURE 1 | Electron micrograph of the inner molecular layer showing asymmetric synaptic profiles and their localization in dendritic spines or shafts (A). **(B–D)** are higher magnification views of different synaptic contacts: PSD1 [**(B)** – non-perforated type 1, synaptic profiles with a single-synaptic bouton]; PSD2 [**(C)** – perforated type 2, synaptic profiles with two postsynaptic densities]; and PSD3 [**(D)** – perforated type 3, synaptic profiles with more than two postsynaptic densities]. Note the spherical presynaptic vesicles and mitochondrion (*) in the axon terminal (a) contacting a dendritic spine (e) and a dendritic shaft (s) with a mitochondria (#). Scale bars, 1.25 μm.

Analysis of Synaptic Profiles

In each experiment, six randomly selected photographs were taken from every examined tissue field [in the inner molecular layer (IML) and outer molecular layer (OML)]. Each 285 μm^2 field was photographed at 5000× and amplified to 15,000×. Quantification of synaptic profiles was performed only in non-overlapping areas (free of large blood vessels, tissue tears, or folds), by counting all asymmetric synaptic profiles in an area of 1,712.88 μm^2 per dentate molecular layer/animal. Synaptic profiles on the exclusion lines were not counted. The IML and OML boundaries were determined for each section. The rat dentate molecular layer has a 250 μm thickness on average (10). The innermost 50–70 μm are often considered the IML, whereas the outermost 150–200 μm comprise the OML. While this subdivision tends to ignore the intermediate molecular layer, it provides a well-defined distinction between the IML and OML with no chance of sampling overlapping fields. In addition, the characterization of the connectivity of the intermediate molecular layer has always been demonstrated as being similar to that of the OML. Bearing this in mind, we chose to describe our data as a fraction of the afferent connections, dividing the dentate gyrus molecular layer in inner and outer counterparts.

Comparative width analysis of the dentate molecular layer (from dentate granule cell layer to hippocampal fissure) using 100-μm-thick coronal sections revealed that this layer had a similar width in all experimental groups, indicating that there was no differential tissue shrinkage in controls, Pilo-, and CHX + Pilo-treated animals.

The estimation of the area of silver grains-impregnation and that of the density of asymmetric synaptic profiles were determined with a stereological test system method and an unbiased counting frame, respectively (29). The test system was applied as a mask over the final enlarged electron micrograph prints for estimating the area of silver grains. The space between the points of the test system was 11 mm, corresponding to a tissue area of 0.85 μm^2 (0.92 μm × 0.92 μm).

Statistical Analysis

Results are presented as mean ± SEM. Comparisons between parameters were carried out by one-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test, using the Statistica 7 software. Significance was set at $P < 0.05$.

RESULTS

Spontaneous recurrent epileptic seizures were first observed at approximately 15–21 days after *SE* in all animals. However, given that we did not perform a complete 24/7 seizure assessment, it is possible that spontaneous seizures may have emerged earlier.

The comparative width analysis of the dentate molecular layer (from dentate granule cell layer to hippocampal fissure) using 100-μm-thick coronal sections revealed that this layer had a similar width in all experimental groups, indicating that there was no differential tissue shrinkage in control, Pilo-, and CHX + Pilo-treated animals.

CHX + Pilo Treatment Reduced Deposits of Silver Grains in the IML

At the EM level, the Timm's sulfide silver method did not reveal any silver grains outside the hilus in the dentate gyrus of control animals (**Figures 2A** and **3A**). This finding is in agreement with previous light (13, 14, 30–32) and EM studies (28, 33, 34). By contrast, all Pilo-treated animals had silver grains in the IML but not in the OML (**Figures 2C,D**, respectively). A detailed examination of the density of silver grains indicated that the higher staining score mainly stemmed from those IML areas closer to the granule cell layer (up to 10 μm apart from the granule cells, **Figure 3B**), where it was about twice as high as the one recorded in more distant IML regions (50–70 μm from the granule cell layer, **Figure 3B**). In the IML of Pilo group, silver grains were mostly deposited on terminal axons where asymmetric synaptic profiles could be identified (**Figure 3B**). In CHX + Pilo-treated animals, the density of silver grains occupying the IML was only 7% of that observed in Pilo-treated animals and similar to that found in the control group (**Figures 2A** and **2E** respectively). In the hilus, the intensity of silver grains labeling did not differ among the three groups of animals. No animal, irrespective of group, showed silver grains in the OML (**Figures 2B,D,F**).

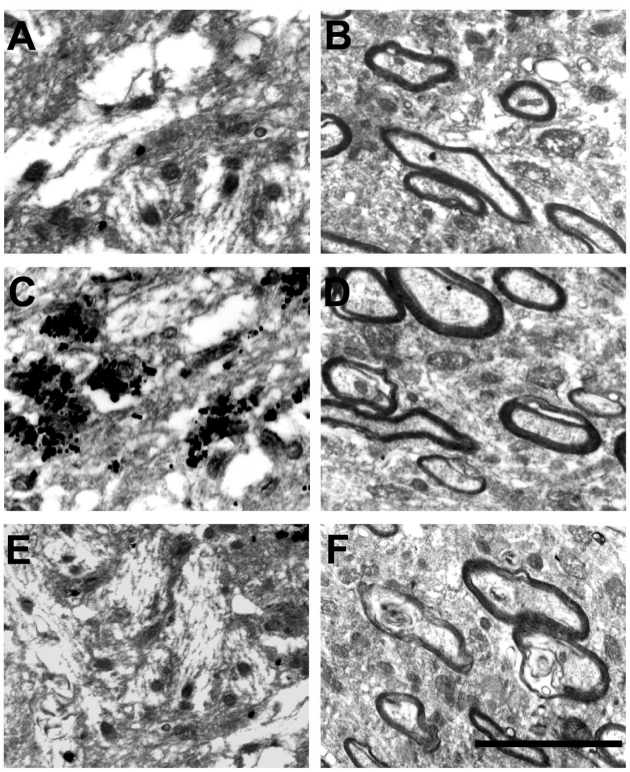

FIGURE 2 | Electron micrographs of the dentate gyrus molecular layer in controls (A,B), Pilo-treated (C,D) and CHX + Pilo-treated animals (E,F). **(A,C,E)** represent the inner molecular layer (IML). **(B,D,F)** represent the outer molecular layer (OML). Silver grain dots in the IML were only observed in Pilo-treated animals **(C)**. These profiles have not been found in the outer molecular layer of any of the groups **(B,D,F)**. Scale bar, 150 nm.

Reduced Density of Asymmetric Synaptic Profiles in Epileptic Rats

The analysis of asymmetric synaptic profiles in the dentate molecular layer (IML + OML) of the control group revealed a density of 23.64 ± 0.59/100 μm^2. This was significantly reduced by 8 and 20% in Pilo and CHX + Pilo groups, respectively. It is noteworthy that when considering only the IML, Pilo-treated animals had a synaptic density similar to that of controls (−4.5%). By contrast, animals in the CHX + Pilo group had significant loss of synaptic profiles when compared to control (−14%) and Pilo (–9.5%) groups. **Figure 4** summarizes results from **Table 1**. In the OML, a significant loss in the density of asymmetric synaptic profiles was found in both Pilo (–11%) and CHX + Pilo (–26%) treated rats, as compared with controls. These data suggest that the influence of CHX to inhibit the growth and/or formation of new asymmetric synaptic contacts after Pilo treatment occurs particularly in the OML, as the loss of asymmetric synaptic profiles in this region was twice as high as that observed in the IML.

Distribution of PSD1, PSD2, and PSD3 Asymmetric Synaptic Profiles in the Dentate Molecular Layer

In all groups (Pilo, Pilo + CHX, and controls), PSD1 was the most abundant contact type, followed by PSD2 and PSD3. The control group had a mean of 21.36 ± 0.58 PSD1; 1.92 ± 0.14 PSD2, and 0.37 ± 0.05 PSD3 synaptic profiles/100 μm^2, respectively. This corresponded to 90, 8, and 2% of the asymmetric synaptic profiles found in the dentate molecular layer (**Table 1**). A similar distribution of synaptic profiles was also seen in Pilo- and CHX + Pilo-treated animals, though with lower absolute values. Significant reductions in the number of PSD1 (21%, $P < 0.001$) were recorded in the CHX + Pilo group as compared to control group. In the same group, we found that the densities of PSD1 were diminished in the IML ($P < 0.01$, as compared to controls) and in the OML ($P < 0.001$ and $P < 0.01$, as compared to control and Pilo groups, respectively). Significant reductions in the number of PSD2 were found in the Pilo group (20%, $P < 0.05$ compared to controls), particularly in the OML ($P < 0.01$, as compared to both controls or CHX + Pilo-treated rats). By contrast, no differences were found in the PSD3 counts across groups (see **Table 1**). In summary, Pilo-treated animals had a lower density of PSD2 profiles in the OML, whereas the CHX + Pilo group had less PSD1 in both the IML and OML as compared to control group.

Synaptic Reorganization Preferentially Involved Spine Synapses Rather than Reorganizations on Shaft Synapses

Irrespective of the treatment (control, Pilo-, or CHX + Pilo-treated animals), synapses in the dentate molecular layer were largely located on dendritic spines rather than shafts (**Table 2**), as previously reported (35, 36). In control animals, 87, 7, and 1% of the synapses in the molecular layer were PSD1, PSD2, and PSD3, respectively. The remaining 5% of synapses were located on dendritic shafts. While a similar frequency was recorded

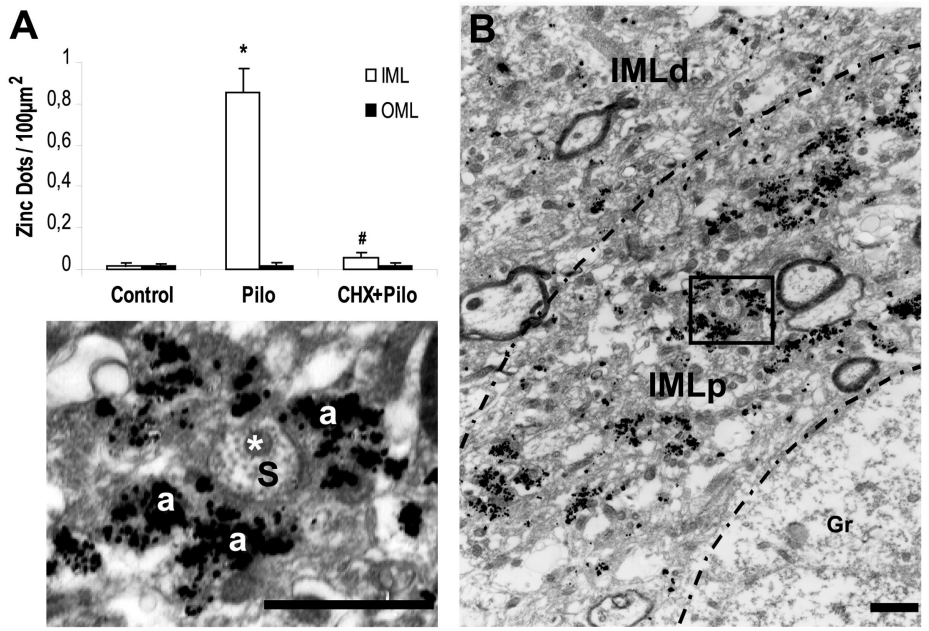

FIGURE 3 | (A) Density of silver grains staining per 100 μm^2 within dentate molecular layer of control, pilocarpine (Pilo)- and cycloheximide + pilocarpine (CHX + Pilo)-treated animals. IML, inner molecular layer; OML, outer molecular layer. *$P < 0.001$; compared to controls; #$P < 0.001$; compared to the Pilo group. **(B)** Sections at the level of the inner molecular layer staining for mossy fiber sprouting of Pilo-treated animal. Note the greater silver grains staining in the molecular layer more proximal (IMLp) to the granule cell layer (Gr) as compared to the more distal portion of the inner molecular layer (IMLd). In these higher magnification views, histochemically reactive silver grains could easily be localized on the asymmetric synapse contacts. Axon terminal (a) and dendritic shaft (s) with mitochondria (*). IMLp, Inner molecular layer proximal; IMLd, inner molecular layer distal. Scale bars, 1.25 µm.

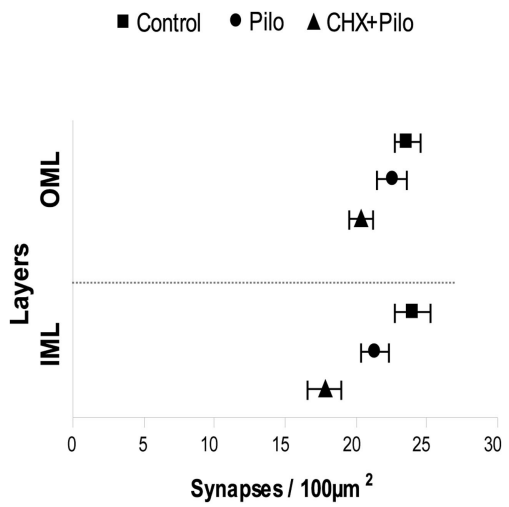

FIGURE 4 | Means and 95% confidence interval of the total number of synapses obtained using six photomicrographs per layer (IML and OML) in five animals per group. IML, inner molecular layer; OML, outer molecular layer.

TABLE 1 | Mean number of asymmetric synaptic profiles per 100 μm^2 in different layers of the dentate gyrus.

Type of synapses	Groups	Molecular layer	
		Inner	Outer
PSD 1	Control	21.43 ± 0.66	21.29 ± 0.98
	Pilo	20.38 ± 0.79	19.52 ± 0.66
	CHX + Pilo	18.54 ± 0.62**	15.16 ± 0.86***,##
PSD 2	Control	1.67 ± 0.19	2.17 ± 0.21
	Pilo	1.74 ± 0.17	1.32 ± 0.19**
	CHX + Pilo	1.40 ± 0.15	2.18 ± 0.26#
PSD 3	Control	0.36 ± 0.07	0.37 ± 0.07
	Pilo	0.29 ± 0.06	0.37 ± 0.09
	CHX + Pilo	0.25 ± 0.04	0.35 ± 0.08

Data expressed as mean ± SEM. ANOVA followed by Newman–Keuls.
***$P < 0.01$, and ***$P < 0.001$ as compared to controls.*
#$P < 0.05$ and ##$P < 0.01$ as compared to Pilo group.
Each group was comprised of five animals; for each animal, six slices were analyzed.

in the Pilo-treated group, this was not the case for rats given CHX + Pilo, which had a relatively lower frequency of PSD1 and a higher number of PSD2 profiles as compared to the other groups (**Table 2**).

In the IML of control animals, 96% of the asymmetric synaptic profiles contacted dendritic spines, while corresponding values for Pilo and CHX + Pilo animals were 92 and 93%, respectively. In the OML, 94, 93, and 94% of the asymmetric synaptic profiles occurred on dendritic spines of control, Pilo, and CHX + Pilo animals, respectively (**Table 2**). In the IML, the frequency of PSD1 and PSD3 asymmetric synaptic profiles on dendritic shafts of epileptic animals (Pilo and CHX–Pilo groups) was greater than

TABLE 2 | Frequency of dendritic spines and dendritic shafts (PSD1, 2, and 3) in the dentate molecular layer.

Groups	Type of synapses	IML		OML		Total of asymmetric synaptic profiles (to each group)
n = 5		Spines	Shafts	Spines	Shafts	
Control	PSD1	618 (44%)	26 (1.8	606 (43%)	34 (2.4%)	1284/1421 (90.4%)
Pilo		565 (43%)	48 (3.7%)***	545 (42%)	42 (3.2%)*	1200/1312 (91.5%)
CHX + Pilo		519 (46%)	38 (3.3%)***	428 (38%)**,##	27 (2.4%)#	1012/1138 (88.9%)
Control	PSD2	47 (3.3%)	3 (0.2%)	58 (4.1%)	7 (0.5%)	115/1421 (8.1%)
Pilo		48 (3.7%)	5 (0.4%)	36 (2.7%)**	4 (0.3%)	93/1312 (7.1%)
CHX + Pilo		39 (3.4%)	3 (0.3%)	62 (5.5%)**,###	4 (0.4%)	108/1138 (9.5%)
Control	PSD3	11 (0.8%)	0 (0%)	8 (0.6%)	3 (0.2%)	22/1421 (1.6%)
Pilo		7 (0.5%)	1 (0.1%)*	10 (0.8%)	1 (0.1%)	19/1312 (1.5%)
CHX + Pilo		6 (0.5%)	2 (0.2%)*	8 (0.7%)	2 (0.2%)	18/1138 (1.6%)

Data expressed as frequency of synaptic profiles.
Chi-square.
**P < 0.05, **P < 0.01, and ***P < 0.001 as compared to controls.*
#P < 0.05, ##P < 0.01, and ###P < 0.001 as compared to Pilo group.
Each group was comprised of five animals; for each animal, six slices were analyzed.

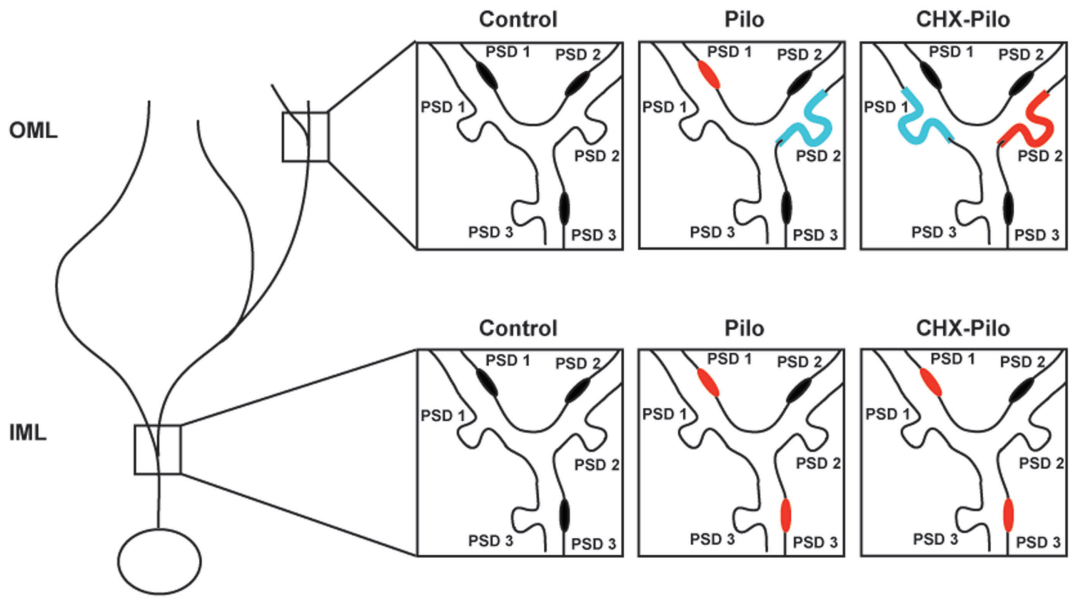

FIGURE 5 | Schematic view of the changes in the synaptic profiles of the different experimental groups as compared to controls. Synaptic profiles located on the shaft or spine of dendrites in inner (IML) or outer (OML) molecular layer were identified as PSD1, PSD2, or PSD3. Red denotes type and location of synaptic profile showing a significant increase in density as compared to control animals. Blue represents synaptic types and location that were significantly decreased as compared to control animals. Pilo – animals subjected to pilocarpine-induced *status epilepticus*. CHX + Pilo – animals subjected to pilocarpine-induced *status epilepticus* and co-injected with cycloheximide.

controls ($P < 0.001$ and $P < 0.05$, respectively). In the OML, the frequency of PSD2 asymmetric synaptic profiles in Pilo group was significantly lower than in controls ($P < 0.01$). In CHX + PILO group, while the frequency of PSD1 asymmetric synaptic profiles was significantly reduced ($P < 0.01$), PDS2 synaptic profiles were significantly increased ($P < 0.01$) as compared to controls.

Thus, while control animals lacked PSD3 profiles on dendritic shafts in the IML, these could be seen in both epileptic groups (Pilo and CHX + Pilo) (**Table 2**). Moreover, for Pilo-treated animals, PSD1 profiles apposing dendritic shafts in the OML were more numerous ($P < 0.05$) than in controls. By contrast, however, CHX–Pilo-treated animals had less PSD1 ($P < 0.05$) in the OML than Pilo-treated animals (**Table 1**).

Figure 5 summarizes the significant results of synaptic profiles and dendritic location. In general, the most conspicuous result of our study was that the density of all types of asymmetric synaptic

profiles in the epileptic groups was remarkably similar to that registered in controls. There were, however, a few noticeable differences. In Pilo animals, PSD1 contacts were distributed in both IML and OML dendritic shafts, whereas PSD3 contacts were only observed in the IML dendritic shafts. In CHX-treated animals, PSD1 and PSD3 contacts were distributed in IML dendritic shafts, whereas PSD2 contacts were only observed in OML dendritic spines. Finally, comparison of the Pilo and CHX–Pilo groups showed that the latter had a decrease in PSD1 (in both spines and shafts) and an increase in PSD2 (in spines only) in the OML.

DISCUSSION

The main findings of our study are the following: (1) the density of silver grains in the IML of animals receiving CHX + Pilo was much reduced when compared to that recorded in rats given Pilo alone. (2) CHX + Pilo treatment led to a significant reduction in the density of asymmetric synaptic profiles in the IML and OML (14 and 26%, respectively), whereas animals treated with Pilo did not differ from controls (4.5% for IML and 11% for OML). (3) Both Pilo and CHX + Pilo had an altered distribution of asymmetric synaptic profiles types (e.g., PSD1, PSD2, PSD3) in the dentate molecular layer as compared to controls. The current estimate of 96% of asymmetric synaptic profiles apposing dendritic spines in the IML of control animals is in agreement with previous findings from Buckmaster and colleagues (37). Overall, these findings support and expand our previous observations, suggesting that CHX blocks *SE*-induced supragranular mossy fiber sprouting (14, 15, 38). Indeed, here we not only showed a dramatic reduction of putative Timm-stained mossy fiber terminals in the IML of CHX + Pilo-treated animals but also a reduction in the number of asymmetric synaptic profiles in the IML and OML, and a shift in the type of synaptic terminals present in the same area. The assembly of synaptic profiles with different synaptic efficacies in Pilo- and CHX + Pilo-treated animals may significantly affect information processing in the dentate gyrus.

Silver Grain Deposits and Asymmetric Synaptic Profiles in the Dentate Molecular Layer

The *SE*-related hilar cell loss in the Pilo model is intense (17, 39) and has been considered to be critical for the development of subsequent mossy fiber sprouting given that hilar neurons represent 36% of all inputs to the dentate IML (40). Our current demonstration of a similar number of IML asymmetric synaptic profiles in both control and Pilo-treated animals further indicates that the synaptic reorganization observed in mossy fiber sprouting represents a tendency to replace lost synaptic contacts rather than the establishment of additional synaptic contacts. In the CHX + Pilo group, we did not find a correspondent loss of asymmetric synaptic profiles, despite the 93% reduction in Timm's IML labeling. Considering that CHX inhibited the mossy fiber sprouting from hilus, the similarity in the number of asymmetric synaptic profiles between control and CHX + Pilo animals could be an indication of synaptic plasticity from other sources, such as entorhinal cortex, given that this structure is a major source of afferent projections to the dentate gyrus (41). On the other hand, CHX may have protected the hilus from damage, given that the loss of hilar (as usually suggested) and entorhinal neurons are often a pre-requisite for mossy fibers to sprout in animal model of epilepsy, including Pilo (39, 42, 43). In fact, neuronal loss in the hilus (44) and entorhinal cortex (unpublished data) is less intense in CHX + Pilo than in Pilo animals. However, the present study is not sufficient to specifically elucidate whether the effects stem from CHX-related cell protection in the hilus, entorhinal cortex, or both. Our own previous findings in Pilo-treated animals provided evidence indicative of a protective role of CHX over hilar mossy cells in mice (12) and rats (13).

Synaptic Profile Morphology

Ganeshina and collaborators (2004) demonstrated that perforated synapses (PSD2 and PSD3) have an invariably higher concentration of AMPA receptors than non-perforated synapses (~660% more) and have 80% more immunoreactive NMDA receptors than non-perforated synapses (PSD1) in the hippocampal CA1 stratum radiatum. Moreover, ~35% of non-perforated synapses do not show any immunoreactivity for AMPA receptors (45), and may thus be considered "silent" synapses (46, 47). Therefore, perforated synapses may evoke synaptic responses with AMPA and NMDA receptors-mediated components of an exceptional magnitude and thereby contribute to an enhancement of synaptic transmission.

It is widely accepted that perforated (PSD2 and PSD3) synapses are much more efficient in impulse transmission than non-perforated (PSD1) synapses (45, 48–55). In the present work, the decreased number of PSD1 in the IML and OML of the CHX + Pilo group may suggest a reduced excitability. To the same extent, however, only Pilo-treated animals had a significant loss of the more effective PSD2 synaptic contacts (39% less compared to control group) in the OML (**Table 1**). Thus, the reduction in the number of perforated synapses (PSD2) in Pilo animals might have a greater impact in reducing the excitability of the OML than the reduction of non-perforated synapses in CHX + Pilo animals.

Interpreting changes in synaptic morphology is not an easy task. The total number of PSD1 in our study was one order of magnitude greater than that of PSD2 and two- to fivefold greater than that of PSD3. Therefore, a small change of 10% in the frequency of PSD1 synapses could indicate a decrease or increase of 150 synapses. By contrast, changes in only 15 PSD2 synapses may result in a similar 10% increase or decrease in the number of synapses. Of course, given that we did not perform any functional evaluation in the current study, these are merely assumptions based on anatomical data.

The reduction of silver grain profiles seen at the ultrastructural level in the IML of CHX + Pilo-treated animals, as compared to Pilo-treated animals, represents important additional evidence of CHX ability to block mossy fiber sprouting, confirming our previous findings (14, 16) despite contrasting data (56, 57). Our results suggest that the dentate molecular layer synaptic reorganization that follows *SE* is a fine tuned process, which might be more suitable to restore dentate function than increasing excitation.

AUTHOR CONTRIBUTIONS

SB manipulated animals and performed the EM procedures, discussed results, and wrote the first draft of the manuscript; FF and EF helped in the EM procedures, CH, BL, and OO discussed results and commented on discussion. LC and LM conceived the experimental design, participated in discussion of results and the final writing of the manuscript. All authors read and approved the final manuscript.

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Electrophysiological Evidence for a Direct Link between the Main and Accessory Olfactory Bulbs in the Adult Rat

*Victor Vargas-Barroso, Benito Ordaz-Sánchez, Fernando Peña-Ortega and Jorge A. Larriva-Sahd **

Neurobiología del Desarrollo y Neurofisiología, Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro, México

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Alberto A. Rasia-Filho, Federal University of Health Sciences/Brazil, Brazil

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****Correspondence:***
Jorge A. Larriva-Sahd
jlsneuro@unam.mx

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It is accepted that the main- and accessory- olfactory systems exhibit overlapping responses to pheromones and odorants. We performed whole-cell patch-clamp recordings in adult rat olfactory bulb slices to define a possible interaction between the first central relay of these systems: the accessory olfactory bulb (AOB) and the main olfactory bulb (MOB). This was tested by applying electrical field stimulation in the dorsal part of the MOB while recording large principal cells (LPCs) of the anterior AOB (aAOB). Additional recordings of LPCs were performed at either side of the plane of intersection between the aAOB and posterior-AOB (pAOB) halves, or *linea alba*, while applying field stimulation to the opposite half. A total of 92 recorded neurons were filled during whole-cell recordings with biocytin and studied at the light microscope. Neurons located in the aAOB ($n = 6$, 8%) send axon collaterals to the MOB since they were antidromically activated in the presence of glutamate receptor antagonists (APV and CNQX). Recorded LPCs evoked orthodromic excitatory post-synaptic responses ($n = 6$, aAOB; $n = 1$, pAOB) or antidromic action potentials ($n = 8$, aAOB; $n = 7$, pAOB) when applying field stimulation to the opposite half of the recording site (e.g., recording in aAOB; stimulating in pAOB, and vice-versa). Observation of the filled neurons revealed that indeed, LPCs send axon branches that cross the *linea alba* to resolve in the internal cellular layer. Additionally, LPCs of the aAOB send axon collaterals to dorsal-MOB territory. Notably, while performing AOB recordings we found a sub-population of neurons (24% of the total) that exhibited voltage-dependent bursts of action potentials. Our findings support the existence of: 1. a direct projection from aAOB LPCs to dorsal-MOB, 2. physiologically active synapses linking aAOB and pAOB, and 3. pacemaker-like neurons in both AOB halves. This work was presented in the form of an Abstract on SfN 2014 (719.14/EE17).

Keywords: accessory olfactory bulb, main olfactory bulb, bursting, patch-clamp

INTRODUCTION

Successful decoding of complex environmental stimuli by the nervous system relies upon central convergence of primary sensory systems. An important example of this is the interaction between the main- (MOS) and accessory olfactory (AOS) systems (Suárez et al., 2012; Baum and Larriva-Sahd, 2014). In fact, volatile and pheromonal stimuli that are sensed by the MOS and AOS, respectively, bring about functionally and behaviorally overlapping responses in these systems (Sam et al., 2001; Trinh and Storm, 2003; Lin et al., 2004; Xu et al., 2005; Spehr et al., 2006; Larriva-Sahd, 2008, 2012b). In the absence of a structural interaction between the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) or between the main- (MOB) and accessory olfactory (AOB) bulbs, synergistic responses of the MOS and AOB are largely attributed to the anatomical overlap beyond these primary and secondary sensory structures, respectively (Boehm et al., 2005; Kang et al., 2011).

At the cell receptor level, volatile stimuli bind distinct sets of olfactory receptors in the MOE (Buck and Axel, 1991; Mori et al., 1999) while pheromonal cues are actively pumped into the VNO (Mann, 1961; Meredith and O'Connell, 1979), where sensory cells are activated by the later (Leinders-Zufall et al., 2000; Boschat et al., 2002; Del Punta et al., 2002). Furthermore, sensory cells in the MOE and VNO project their central processes to the MOB (Ramón y Cajal, 1890) and AOB (Barber and Raisman, 1974; Larriva-Sahd, 2008), respectively. Mitral neurons in the MOB project to secondary olfactory areas (Ojima et al., 1986; Stettler and Axel, 2009; Kang et al., 2011), whereas in the AOB, large principal cells (LPCs; Larriva-Sahd, 2008) project to the so-called vomeronasal amygdala (Scalia and Winans, 1975; Boehm et al., 2005; Mohedano-Moriano et al., 2007; Kang et al., 2011).

It has been shown that the AOB is directly implicated in decoding pheromonal stimuli (Leinders-Zufall et al., 2000; Boschat et al., 2002; Del Punta et al., 2002; Luo et al., 2003) and that these chemical cues are detected by two sub-systems within the AOB (Imamura et al., 1985; Mori et al., 1987). For instance, VNO sensory cells distribute in two layers, apical and basal (Dulac and Axel, 1995) in which G protein expression (Berghard and Buck, 1996) and projection targets toward the AOB (Mori et al., 1987) vary as a function of location. More specifically, apical cells that express vomeronasal receptor 1 (V1R) family of receptors (Dulac and Axel, 1995) and most members of the formyl-peptide receptor (FPR) family present in the VNO (Liberles et al., 2009; Riviere et al., 2009), project their axons to the anterior half of the AOB (aAOB), whereas basal cells that express members of the vomeronasal 2 receptor (V2R) family (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997) project to the posterior half of the AOB (pAOB; Mori et al., 1987; Schwarting et al., 1994). Further, apical cells synapsing in the aAOB bind pheromones present in the urine of conspecifics (Leinders-Zufall et al., 2000; Boschat et al., 2002; Del Punta et al., 2002), some of which are sulfated steroids (Nodari et al., 2008; Hammen et al., 2014), whereas basal cells that synapse in the pAOB bind high molecular weight molecules such as major urinary proteins (MUPS; Chamero et al., 2007), as well as peptide ligands of major histocompatibility complex (MHC) proteins (Leinders-Zufall et al., 2004) and peptides derived from extraorbital lacrimal glands (Kimoto et al., 2005). Thus, these relatively independent streams of information processing within the AOS deal with specific semiochemicals and may have different implications for behavior and survival. It has even been shown that their projections into the brain are also partially segregated (Mohedano-Moriano et al., 2007), and as with a putative interaction between the MOB and the AOB, direct crosstalk between the two AOB halves remains to be demonstrated.

Fundamental for the present study is that anatomical overlap between the MOS and AOS has only been documented in secondary and tertiary projections arising from the MOB and AOB to the basal forebrain (Boehm et al., 2005; Kang et al., 2011; Mohedano-Moriano et al., 2012) The parceled, yet parallel, central path of vomeronasal, and main olfactory projections led to the dualistic notion that the MOS and AOS constitute distinct sensory systems (Raisman, 1972; Scalia and Winans, 1975). More recently, a growing number of observations which in most respects, confirmed earlier ones, have depicted varying degrees of structural overlap in both secondary relays of the MOS (nucleus of the lateral accessory tract, anterior cortical nucleus of the amygdala, and piriform-amygdaloid transitional zone of the amygdala; Shammah-Lagnado and Negrao, 1981; Dong et al., 2001) and AOS (ventral anterior, bed nucleus of the accessory olfactory tract, and medial amygdaloid nucleus; see Hintiryan et al., 2012). Furthermore, third order olfactory and vomeronasal recipient areas converge in the basal forebrain (see Dong et al., 2001; Dong and Swanson, 2004). In contrast, tract tracing studies aimed at defining a possible direct connection between the mammalian MOB and AOB have been unsuccessful (Price, 1973; Hintiryan et al., 2012, but see Martínez-García et al., 1991).

Thus, although these data may be the starting point for a new integrated hypothesis concerning chemosensory detection and processing, there has not been any information regarding the anatomical substrate of interactions at the level of the olfactory bulbs (but see Martínez-García et al., 1991). With the aim of obtaining physiological evidence for interactions between the MOB and the AOB and between the two AOB halves, we performed whole-cell patch-clamp recordings of neurons in the aAOB and pAOB while applying electrical stimuli to the MOB and/or to the AOB opposite to the recording site. Given the numerous connectional and cytological differences between the MOB and the AOB mitral cells, we have adopted the term LPC to refer to the "mitral cell" of the former (see Larriva-Sahd,

Abbreviations: aAP, antidromic action potential; AP, action potential; aAOB, anterior accessory olfactory bulb; AC, acommodating; aCSF, artificial cerebro-spinal fluid; ADP, after-depolarization; AHP, after-hyperpolarization; AOB, accessory olfactory bulb; AOS, accessory olfactory system; ECL, external cellular layer; EPSPs, excitatory post-synaptic potentials; eT, external tufted cells; FPR, formyl-peptide receptor; LA, línea alba; LOT, lateral olfactory tract; LPC, large principal cell; maCSF, modified artificial cerebro-spinal fluid; MUPs, major urinary proteins; MHC, major histocompatibility complex; MOE, main olfactory epithelium; MOS, main olfactory system; MOB, main olfactory bulb; NAC, non-acommodating; OR, olfactory receptor; pAOB, posterior accessory olfactory bulb; Vm_V, resting membrane potential; VNO, vomeronasal organ; VR1, vomeronasal receptor 1; VR2, vomeronasal receptor 2.

2008). Our results provide evidence that a subset of aAOB LPCs project to the dorsal-posterior MOB and that both AOB halves are synaptically connected. In addition, single cell recordings disclosed that, like in the MOB (Hayar et al., 2004a,b; Liu and Shipley, 2008), the AOB harbors a population of neurons with voltage-dependent intrinsic bursting activity.

MATERIALS AND METHODS

Animals

For this study, male Wistar rats of 8 weeks of age were used for whole-cell recordings. Animal manipulation and sacrifice were performed under the guidelines and with the approval of the Animal Research Committee of our Institute, which endeavors to minimize pain and suffering to the experimental subjects.

Olfactory Bulb Slice Preparation

Rats were anesthetized with pentobarbital (63 mg/Kg) and perfused intracardially with an ice-cold modified artificial cerebrospinal fluid (aCSF) that contained (in mM): 238 sucrose, 3 KCl, 2.5 $MgCl_2$, 25 $NaHCO_3$, and 30 D-glucose (pH 7.4; Alvarado-Martínez et al., 2013). The bulbs were removed and placed in normal aCSF equilibrated with carbogen (95% O_2 and 5% CO_2). The aCSF contained (in mM): 119 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, and 30 D-glucose (pH 7.4). The medial end of either the right or the left bulb was glued to a block of agar, mounted on a vibratome (Leica VT1000S), and serially cut into 350-μm thick slices obtained on a sagittal plane. Two or three slices per bulb were allowed to recover for 1 h in aCSF bubbled with carbogen at room temperature. Finally, a single slice was transferred to a recording chamber (3 ml) located on a Nomarski-DIC-equipped microscope (Eclipse E600FN; Nikon, Melville, NY). The chamber was continuously superfused with oxygenated aCSF ($32 \pm 0.5^{\circ}C$) at a flow rate of 17 ml/min. A total volume of 30 ml of recirculating medium was maintained both in the chamber and the tubing system.

Electrophysiological Recordings

Whole-cell patch-clamp recordings were obtained using the visual patch-clamp technique with an Axo-clamp 2B amplifier (Axon Instruments, Foster City, CA; Peña et al., 2010). Cells were aimed for and recorded based on their location within the external cellular layer of the AOB; those situated in the aAOB ($n = 72$) constitute the bulk of the recordings, and a small sample of pAOB ($n = 20$) neurons were also recorded. Patch electrodes (4–8 MΩ) were pulled from filamented borosilicate glass tubes (G150F-4; Warner Instruments, Handem, CT) and filled with a solution containing (in mM): 140 K-gluconic acid, 10 EGTA, 2 $MgCl_2$, 10 HEPES, 2 of Na_2ATP, 2 of LiGTP, and 1% biocytin, pH 7.4. Recordings performed with either lithium (i.e., LiGTP) or magnesium (i.e., MgGTP) salts dissolved in our internal solution, yielded to comparable traces as depicted in **Supplementary Figure 1**.

The discharge pattern and intrinsic properties of each recorded cell were disclosed by injecting 1-s-long hyperpolarizing and depolarizing current steps. During these current injections a given neuron always remained at the same membrane potential (variable between neurons), those neurons with high spontaneous activity were hyperpolarized until they became silent, all recordings were performed in the current-clamp mode. As we did not correct for liquid junction potentials, our membrane voltage values may change in a range of 10–15 mV. The electrophysiological variables measured for each neuron were: **resting membrane potential (Vm)**, the trans-membrane voltage measured immediately after obtaining stable whole-cell configuration; **action potential (AP) threshold**, measured as the most negative voltage value reached by the cell prior to the beginning of the inflection for the all-or-none AP, for each cell at least five depolarizing current injections were done to test this variable, always maintaining Vm; **sag potential**, measured as the difference between the peak voltage displacement and the steady-state voltage evoked by a 1-s hyperpolarizing current injection that drove the Vm beyond −80 mV and up to −100 mV; differences $\geq$ 2 mV were considered as sag potentials, all measurements were obtained from at least five current injections; **membrane time constant (τm)**, calculated by fitting an exponential curve to the decay phase of a depolarizing sub-threshold stimulus; **AP frequency**, measured as the number of APs during a 1-s suprathreshold depolarizing stimulus. First, we defined the threshold depolarizing stimulus as the minimal current able to reliably evoke at least one AP, and then we applied twice that current to evoke the suprathreshold spike train used for the quantifications; **rheobase,** which is the current required to elicit at least one AP; **spike frequency adaptation**, measured as *t2*/*t1*, where *t2* is the time between the peaks of the last two APs of the suprathreshold spike train, and *t1* is the period between the peaks of the first two APs of the suprathreshold spike train, values >1 indicate accommodation, values <1 indicate acceleration, whereas values = 1 indicate steady-state firing; **spike-width**, measured as the width (ms) of the first AP of the suprathreshold spike train, at 50% of its maximal amplitude; **input resistance**, measured in response to an hyperpolarizing stimulus.

For the neurons that exhibited voltage-dependent bursts of APs, we measured (60 measurements for each) **inter-burst interval**, defined here as the time between the peak of the last AP of a burst and the peak of the first AP of the next burst; **burst duration**, defined as the time between the first and the last AP in a single burst, and **bursting frequency**, defined as the number of AP in a burst per unit of time (seconds), before and after incubation with CNQX and D-APV (20 μM each for all experiments; Sigma, St. Louis MO). We searched for statistical significance within cells and also compared the mean values of each parameter measured in five neurons before and after drug application using paired *t*-tests.

To assess connectivity between the MOB and AOB and within the AOB, field stimulation was applied to the dorsal MOB, situated immediately anterior to the olfactory limbus (Larriva-Sahd, 2012b) and to the pAOB when recording in aAOB, whereas dorsal MOB or pAOB field stimulation was applied when recording in aAOB. The field electrical stimuli were delivered with a concentric bipolar electrode, which had an inter-polar distance of 50 μm at the tip (Peña et al., 2002, 2010; Zavala-Tecuapetla et al., 2014). Brief square current pulses (100 μs, 0.05 Hz) were applied, while the stimulus intensity was varied according to the response elicited. We utilized a sampling

frequency of 1 KHz and did corroborative experiments sampling at 10 KHz. Recordings were performed with a HS-2 headstage (Molecular devices) which has a gain of 0.01 MU. Signals were recorded on a computer using an analog-to-digital converter (BNC-2110, National Instruments) and stored on a personal computer using a custom-made program (Lemus-Aguilar et al., 2006) and an acquisition system from National Instruments (Austin, TX).

Histochemistry

To identify recorded neurons (1 per slice in most cases), 1% biocytin was included in the pipette solution (Zavala-Tecuapetla et al., 2014). Recordings lasted between 30 and 120 min; during that time biocytin diffused into distal processes (Zavala-Tecuapetla et al., 2014). Once the recording was finalized, the pipette was gently removed to avoid damage of the neuron's somata, and slices were fixed for at least 2 days in 0.1 M phosphate-buffered saline (pH 7.4, 4°C) with 4% paraformaldehyde and 1% picric acid (Zavala-Tecuapetla et al., 2014). Next, slices were thoroughly washed three times in 1x KPBS to remove excessive fixative, endogenous peroxidase activity was blocked by incubating slices for 60 min in 3% H_2O_2 diluted in PBS, slices were then washed again three times with PBS and stained overnight in TBS containing Triton X-100 and avidin-biotin-peroxidase complex (1:100; Vector Laboratories, Burlingham, CA) at room temperature. The next day, after washing three times with KPBS, slices were incubated in 3,3′-diaminobenzidine (DAB) tetrahydrochloride (0.05%) and H_2O_2 (0.003%) in TBS (Zavala-Tecuapetla et al., 2014). In some cases, slices were further cleared in order to fully visualize distal axons. Briefly, slices were dehydrated in graded methyl-alcohols followed by a 30-min incubation in absolute methanol and benzyl alcohol: benzyl-benzoate (BABB) solution (1:2; MP Biomedicals, Aurora, Ohio). Finally, the slices were mounted on slides to visualize the reaction product of the bound horseradish peroxidase using the light microscope.

Data Analysis

Neuronal somata, dendrites, axons, and collaterals were reproduced with a camera lucida adapted to a Zeiss Axioplan 2 microscope, utilizing 10x, 40x, and 100x objectives (NA = 0.3, 1.0, and 1.4, respectively) or digitized and measured with a personal computer aided by Kontron 400 software. Measurements included somata largest and transverse axes, somata area (expressed in μm and μm^2, respectively) and number of glomerular dendrites. These measurements were made in 44 successfully recovered and visualized neurons; data are expressed as means ± SEM unless stated otherwise.

RESULTS

Electrophysiological Properties of AOB Large Principal Cells

In the present study we report whole-cell patch-clamp recordings of 92 neurons of the anterior and posterior regions of the AOB made with the primary goal of determining connectivity principles within the AOB and between the latter and the MOB. When recordings were performed on the aAOB ($n = 72$), field electrical stimulation was applied in the dorsal-posterior region of the MOB and in the pAOB, whereas recordings from the pAOB ($n = 20$) were accompanied by field electric stimulation of the aAOB and the MOB. Twenty-eight neurons (30%) elicited an antidromic and/or an orthodromic response following stimulation of the regions mentioned (**Figures 5–7**; see below).

For the entire population of recorded neurons we measured specific electrophysiological variables (see Methods Section and **Figure 1**). For each neuron recorded, we first applied a series of depolarizing and hyperpolarizing current injections (**Figure 1A**) in order to obtain the following population parameters (mean ± SEM): **Vm** = 63.5 ± 0.51 mV; **τm** = 85.4 ± 5.2 ms; **Rheobase** = 129.7 ± 15 pA; **AP threshold** = 45.45 ± 0.86 mV; **spike frequency adaptation** = 1.23 ± 0.07; **half-width** = 2.72 ± 0.12 ms; **Input resistance** = 88.43 ± 5 MΩ; **AP frequency** = 10.21 ± 0.51 Hz. **Figure 1B** shows frequency histograms of the physiological variables measured. We found that from the entire population of recorded neurons only 40% ($n = 37$) of them displayed a sag potential (I_h current; **Figure 1C**). Twenty-eight were from the aAOB and nine from the pAOB. This feature has been shown to be an important network affiliation signature in the MOB (Angelo et al., 2012). We have confirmed some intrinsic electrophysiological characteristics of AOB LPCs, which respond to threshold-surpassing stimuli with few APs and have a significant delay in their AP onset (see **Figure 1B**), these properties differ from those seen in mitral cells of the MOB (Zibman et al., 2011). We also find neurons that exhibit accommodating, non-accommodating, and steady-state firing responses as reported previously (see **Figures 1A,B**); (Zibman et al., 2011).

Additionally, we found that 42% of the recorded neurons ($n = 39$) showed a characteristic after-hyperpolarization (AHP; 3.2 ± 0.31 mV) following the train of APs evoked by the suprathreshold depolarizing current steps (**Figures 2A,C**). Conversely, 17% of the neurons recorded ($n = 16$) displayed after-depolarizations (ADP), for which we measured the amplitude of the voltage displacements (3.94 ± 0.52 Mv; **Figure 2C**). In nine of these cells such ADPs reached a plateau that elicited spiking activity for 1–10 s (**Figure 2B**). Only six cells displayed both AHP and ADP, with the AHP emerging just at the end of the ADP (**Figure 2B**).

Large Principal Cells of the AOB Discharge Voltage-Dependent Rhythmic Bursts of Action Potentials

We describe and confirm here the presence of neurons with intrinsic voltage-dependent rhythmic discharge patterns (Gorin, 2014; Gorin and Spehr, 2014; **Figure 3**) in the rat's AOB. Of the total of 92 recorded neurons, 22 (24%) of them displayed rhythmic bursts of voltage-dependent APs. We recorded 19 neurons with such characteristics in the aAOB and three in the pAOB (26 and 15%, respectively; **Figure 3A**). For some of these neurons ($n = 5$) we measured the inter-burst interval (3.21 ± 0.27 s), the duration of the burst (1.41 ± 0.24 s in control) and

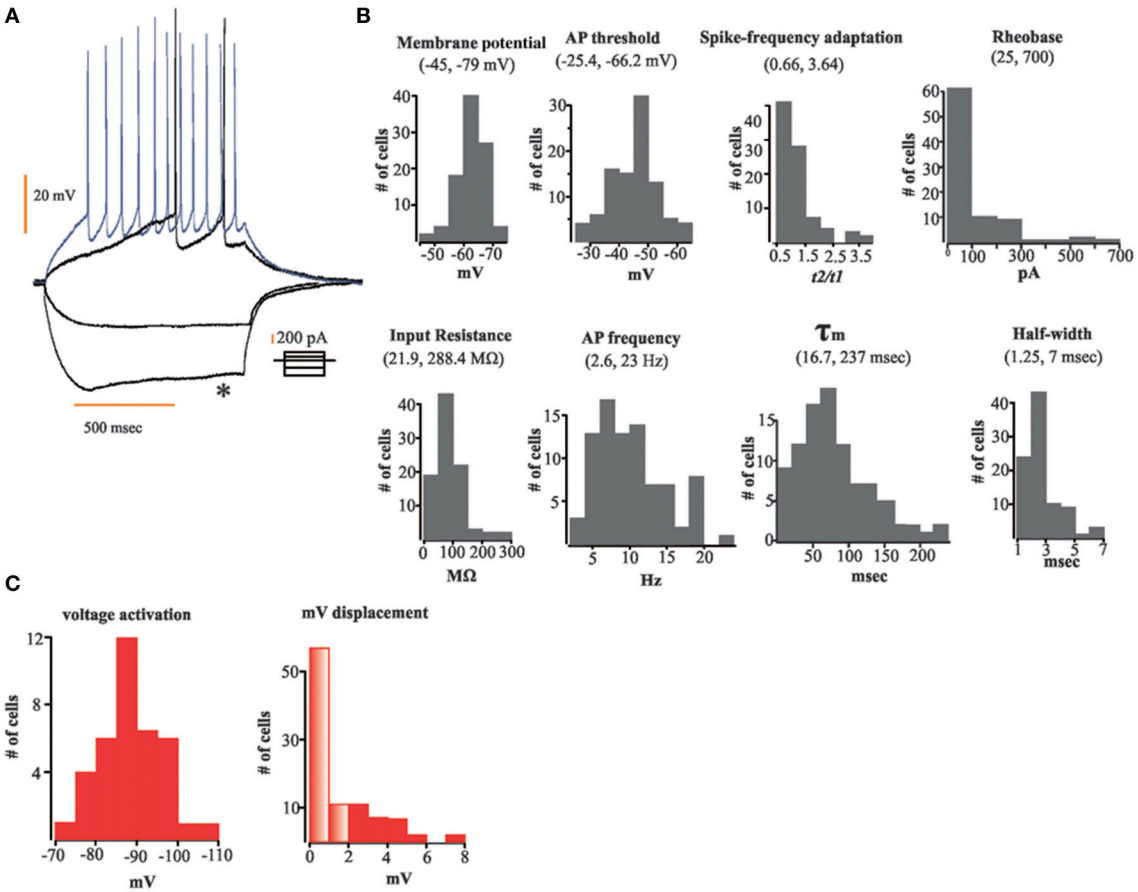

FIGURE 1 | Data distribution (histograms) of the electrophysiological variables measured. (A) Representative traces of our current injection protocol; depolarizing and hyperpolarizing current steps were applied to each neuron to disclose their electric properties; note sag potential (asterisk). **(B)** Histograms of electrophysiological variables measured; from top to bottom and left to right: membrane potential, action potential (AP), threshold, spike-frequency adaptation, Rheobase, input resistance, AP frequency, τm, and half-width. **(C)** Frequency histograms of voltage activation (left) and voltage displacement (right) of neurons showing sag potential ($n = 35$); transparent bars designate number of cells without a sag potential (differences ≤ 2 mV between peak and steady-state membrane voltage upon hyperpolarizing current injection).

the bursting frequency (15.02 ± 1.91 Hz; **Figure 3C**), before and after bath-applying the glutamatergic inhibitors CNQX and APV (20 μM; Zavala-Tecuapetla et al., 2008, 2014). After blocking fast glutamatergic transmission, the rhythmic burst firing persisted, which led us to conclude that these voltage dependent bursts were generated endogenously (Peña et al., 2004; Zavala-Tecuapetla et al., 2008, 2014).

All "rhythmic" neurons that were successfully recovered for morphological analysis ($n = 8$) exhibited morphological features corresponding to LPCs (**Figures 4B–D**), which consist of the presence of one or more glomerular dendrites and an axon entering the lateral olfactory tract (LOT). Moreover, "rhythmic" LPCs often had elaborate dendritic arborizations innervating more than one glomerulus (**Figure 4D**) and, one of them seemed to be tributary of a glomerular complex far beyond aAOB confines (**Figure 4I**). It is also noteworthy that half (3/6) of the AOB neurons that elicited antidromic APs (aAPs) upon MOB electric stimulation exhibited oscillatory discharge patterns spontaneously (see below, **Figures 3**, **5**).

Cytology

The majority of visualized neurons ($n = 41$; **Figure 4**) corresponded to LPCs of either anterior or posterior AOB halves and they were classified according to their anatomical characteristics, namely, their size (Takami and Graziadei, 1991), morphology (i.e., glomerular dendrites), and axonal arborization, which frequently incorporates and proceeds in the LOT (Mohedano-Moriano et al., 2007; Larriva-Sahd, 2008). We measured somata length (20.41 ± 0.87 μm), width (12.47 ± 0.64 μm), area (204.44 ± 18.44 μm^2), and number of glomerular dendrites (3.1 ± 0.23) for the neurons that were successfully recovered for morphological characterization ($n = 44$).

As described elsewhere (Larriva-Sahd, 2008), LPCs visualized here were scattered along the external cellular layer (ECL) of the AOB providing glomerular dendrites to more than one glomerulus (**Figure 4**), and, occasionally, to up to six glomeruli. These are important differences between mitral and LPCs (Hayar et al., 2004a; Larriva-Sahd, 2008; Zibman et al., 2011).

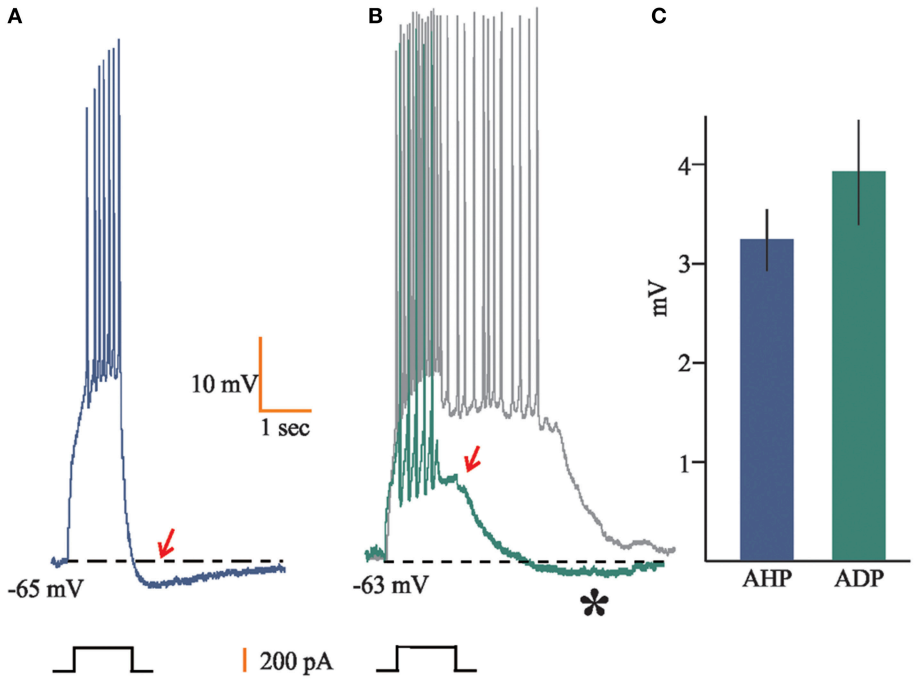

FIGURE 2 | Afterpotentials in accessory olfactory bulb (AOB) neurons. (A) 42% of the neurons recorded ($n = 39$) displayed after hyperpolarization (AHP) (arrow) upon depolarizing current steps, **(B)** whereas seven and nine cells (17% in total) showed after depolarization (ADP) (arrow) or persistent firing (gray trace), respectively. Some cells ($n = 6$) also displayed slowly activating AHP following the ADP (asterisk). **(C)** Mean ± SEM of voltage displacements for AHP and ADP population values.

aAOB Large Principal Cells Send Axon Collaterals to the Dorsal MOB

For the neurons recorded in the aAOB ($n = 72$), we found that a proportion of them ($n = 6$, 8%) responded to MOB electrical stimulation with aAPs (**Figure 5B**, left). aAPs were evoked in an all-or-none fashion and had a very short latency (1.97 ± 0.02 ms). These responses persisted in spite of the presence of the glutamate inhibitors CNQX and APV (20 μM) (**Figure 5B**, center), disclosing the non-synaptic nature of the evoked APs; furthermore, collision tests prevented aAP generation after inducing a somatic AP (**Figure 5B**, right), which led us to the conclusion that LPCs in the aAOB must send axons to the dorsal region of the MOB.

Indeed, the *post-hoc* visualization of the neurons with antidromic responses revealed that they emit axon collaterals directed toward the MOB (e.g., the stimulation site; **Figure 5C**). The somato-dendritic features of the neurons that responded with aAPs resembled those of LPCs of the AOB (Takami and Graziadei, 1991; Larriva-Sahd, 2008); moreover, the presence of glomerular dendrites and their distinctive axon entering the LOT served as an unequivocal determinant of cell identity; hence, we conclude that there is a sub-population of LPCs of the aAOB that sends axon collaterals to the the dorsal-posterior region of the MOB. Interestingly, three of the six neurons that were antidromically activated by applying electrical stimuli to the MOB, displayed a voltage-dependent burst discharge pattern that was maintained in the presence of glutamatergic inhibitors (**Figure 5D**).

Anterior and Posterior Halves of the AOB are Reciprocally Connected

When we applied electrical stimuli in the pAOB while recording in the aAOB (**Figure 6A**) we found that neurons responded with either orthodromic excitatory post-synaptic potentials (EPSPs; $n = 6$, 7.5%; **Figure 6B**) or with aAPs ($n = 8$, 11%; **Figure 7A**, left). The half-width of the EPSPs was of 120.89 ± 18.65 (mean ± SEM) which may be due to the combined activation of NMDA and non-NMDA receptors (Forsythe and Westbrook, 1988; Trombley and Westbrook, 1990; Maccaferri and Dingledine, 2002). Five out of six neurons had latencies of 3.07 ± 0.12 (mean ± sd), failure rates of 8 ± 4%, and a shock-to-shock variability (synaptic jitter) ranging from 45 to 130 μs, which may be suggestive of monosynaptic connection (Doyle and Andresen, 2001). We further evaluated if these responses were mediated by glutamatergic transmission by bath-applying CNQX and APV (20 μM) into the recording chamber and found that indeed, EPSPs were completely abolished after the pharmacological blockade of excitatory synaptic transmission (**Figure 6B**). This strongly suggests that there might be a monosynaptic connection arising from pAOB neurons. Two of the six neurons that elicited EPSPs upon activation of their opposite half displayed morphological features consistent with those of interneurons, namely, a dense peri-somatic axonal arborization that avoids the LOT and the lack of glomerular dendrites (**Figure 6C**); whereas the remaining neurons exhibited LPC morphology.

For the neurons recorded in the aAOB that evoked aAPs due to the electrical activation of the pAOB, we found that these

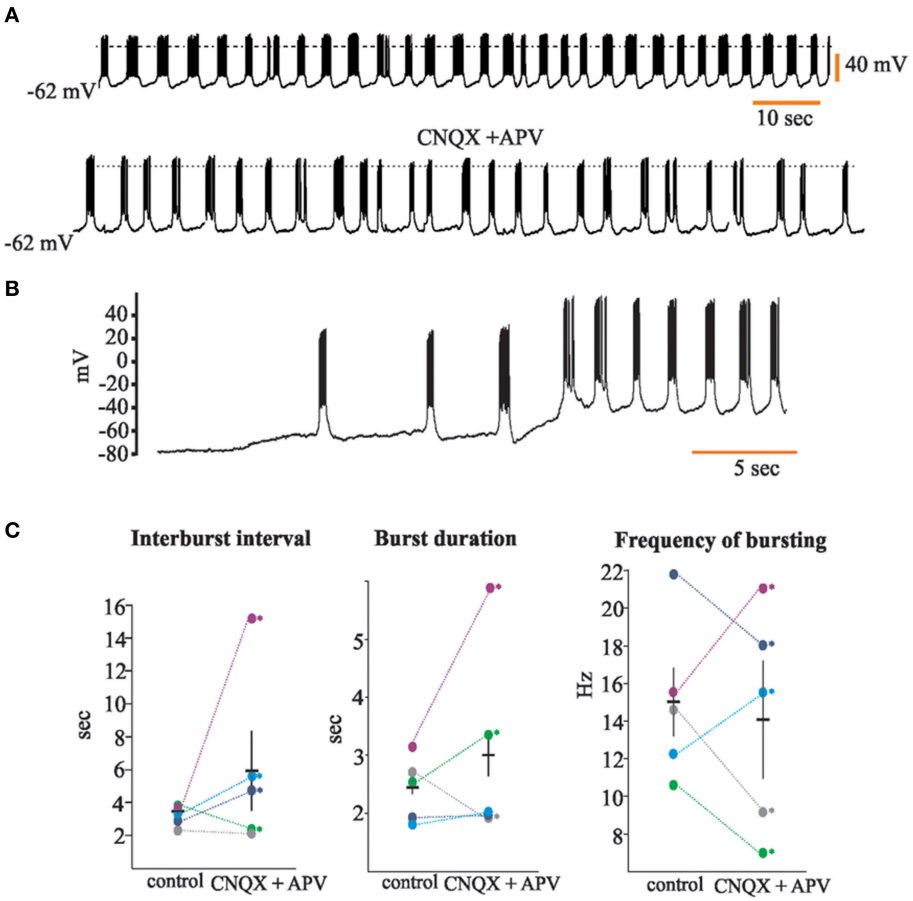

FIGURE 3 | Accessory olfactory bulb (AOB) neurons display voltage-dependent bursts of action potentials. (A) Trace of spontaneous activity from a representative "rhythmic" neuron of the AOB (top); when cells are incubated with glutamatergic antagonists CNQX and APV (20 μM), rhythmic bursting persists (bottom); dotted line is 0 mV **(B)** Rhythmic firing of AOB neurons is voltage-dependent. **(C)** Dot plots showing analysis for inter-burst interval (left), burst duration (center), and bursting frequency (right). Each color represents a single neuron ($n = 5$). Statistical differences in control vs. CNQX + APV were only found within single neurons (asterisks).

responses persisted in the presence of glutamatergic inhibitors and also, collision tests resulted in annihilation of the evoked aAPs (**Figure 7A**). Furthermore, histological inspection of these cells showed that they correspond to LPCs that display axon collaterals that cross the *línea alba* (LA; see Larriva-Sahd, 2008) and may influence pAOB neurons before incorporating into the LOT (**Figure 7B**).

We last performed a subset of recordings in the pAOB in order to determine if the synaptic activation seen in aAOB when stimulating the pAOB is a reciprocal event. Thus, we recorded from cells in the pAOB ($n = 20$) while applying electrical stimuli to the aAOB. Indeed, neurons in the pAOB responded to the electrical stimulus with either aAP or with orthodromic EPSPs ($n = 7$ and 1, respectively). As in the previous experiments, the aAPs were prevented in collision tests, and they persisted in the presence of glutamatergic inhibitors, whereas the EPSPs were abolished by CNQX and APV bath application. Altogether these results lead us to conclude that both AOB halves are linked by glutamatergic synapses mediated by the LPCs of both halves, whose common target may be other LPCs or putative short-axon neurons.

DISCUSSION

Here, we investigated the inner circuitry of the AOB and its interaction with the MOB. Previous structural and physiological studies suggested that, in sharp contrast with the MOB, the AOB is parceled along the antero-posterior axis into two distinct halves. Both AOB halves are interconnected by at least two sets of distinct processes, namely, LPC axons and its dendrites (Larriva-Sahd, 2008).

LPC axons zigzag in the rostro-caudal direction providing defined sets of collaterals to both halves; accessory dendrites, on the other hand, pierce the LA to resolve in the neuropil of the adjacent AOB half. Since axon collaterals issued by the LPC axon appear to terminate on interneurons in the opposite AOB half, as suggested by the Golgi technique, a basic circuitry between the two AOB halves was furnished (Larriva-Sahd, 2008). With this

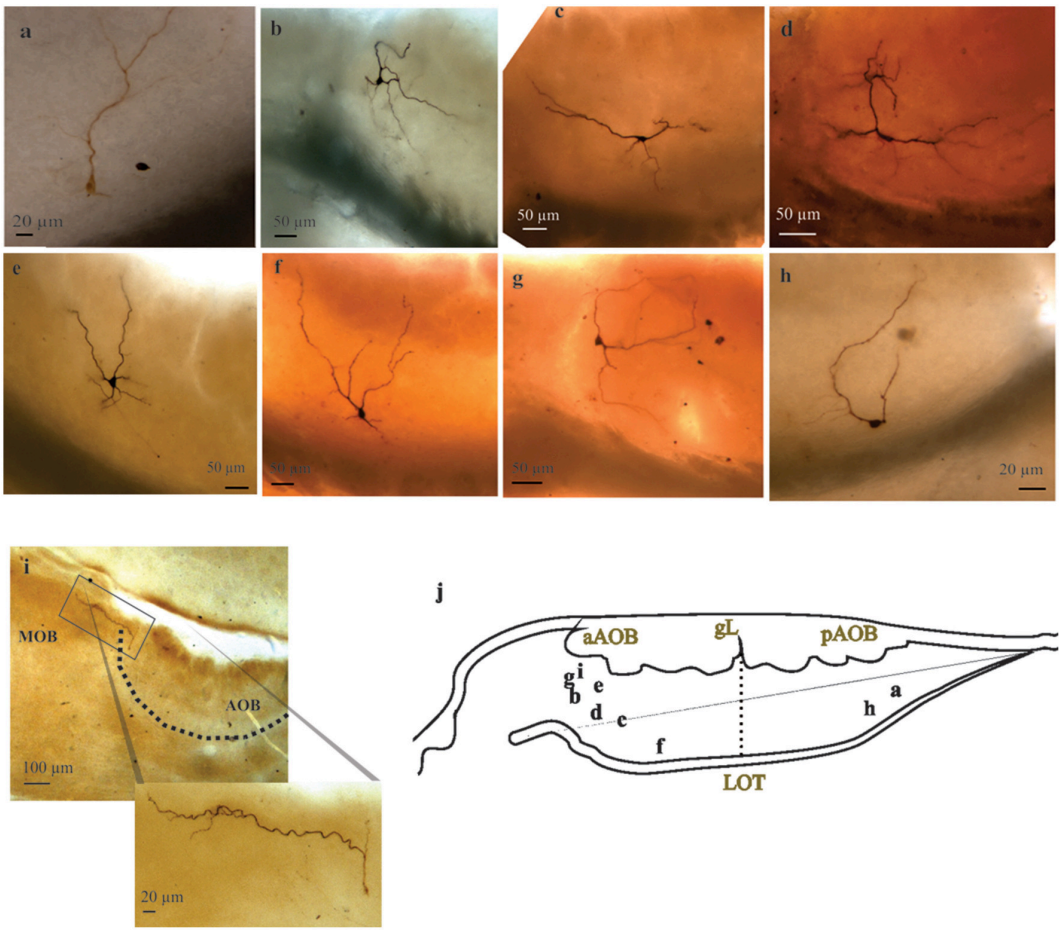

FIGURE 4 | Large principal cells of anterior and posterior halves of the accessory olfactory bulb (AOB). (A–I) Representative pictures of recorded AOB-large principal cells (LPCs). Note the variable morphology of this population of principal cells and their distinct degrees of glomerular innervation. Neuron in ***i*** is a "rhythmic" neuron, note its far reaching dendrite leaving the AOB. **(J)** Camera lucida drawing showing the approximate position of the neuron's somata.

background, *in vitro* whole-cell patch-clamp recordings of adult rat olfactory bulb were utilized in the present study to depict possible synaptic interactions between the aAOB and pAOB. Recordings confirmed that principal cells of both halves project collaterals piercing the LA to resolve in the opposite AOB half.

The presence and unique intrinsic properties (Gorin, 2014; Gorin and Spehr, 2014) of AOB "rhythmic" LPCs (**Figure 3**) suggests that they may play a significant role in information processing within the AOB. Furthermore, a subset of the oscillatory aAOB LPCs sends axon collaterals to the dorsal part of the MOB, which suggested us the possibility that there might be a direct, functional synaptic link between the aAOB and dorsal MOB. Santiago Ramón y Cajal defined that granule cells in the homonymous layer represent the converging site of extrinsic modulatory influences on the mitral cell (Larriva-Sahd, 2012a). This, coupled with our neurophysiological evidence suggests that APs generated by LPC pheromonal recruitment may have, in turn, a granule cell-mediated influence on the MOB-mitral cell (see Martínez-García et al., 1991 and Pressler and Strowbridge, 2006).

Both AOB Halves Possess a Sub-Population of "Rhythmic" Large Principal Cells

The confirmation (Gorin, 2014; Gorin and Spehr, 2014) that a set of LPCs corresponds to typical "pacemaker" neurons is potentially important in the context of both AOB circuitry and its projection to the MOB; 24% of our sample corresponds to "rhythmic neurons." This electrophysiological profile is generated endogenously in LPCs of the rat's AOB (**Figure 3A**), and it is voltage-dependent (**Figure 3B**). Pacemaker-like cells have been previously found in the MOB, where external tufted neurons (eT) fire intrinsically generating rhythmic bursts of action potentials (Hayar et al., 2004a,b; Liu and Shipley, 2008), and recently, Golgi cells of the MOB granule layer have also been reported to fire state-dependent rhythmic discharges (Pressler et al., 2013).

LPCs of the AOB oscillate at much higher frequencies (15.6 ± 1.91 Hz) than their counterparts in the MOB (see **Figure 3**), as it is reported that Golgi and eT cells fire near the θ frequency (Hayar

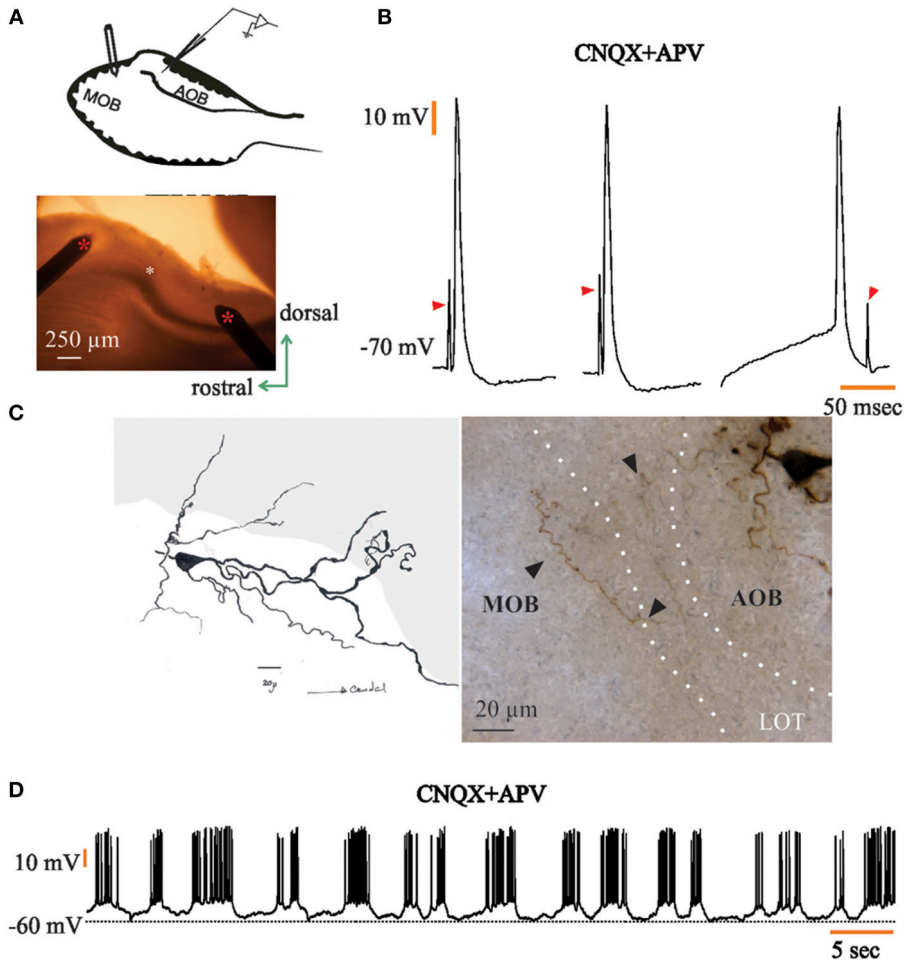

FIGURE 5 | Large principal cells of the anterior half of the accessory olfactory bulb (aAOB) send axon collaterals to the dorsal main olfactory bulb (MOB). (A) Schematic of the recording setup (up) and picture taken the day the neuron in ***c*** was recorded (red asterisks, stimulating electrodes; white asterisk, tip of the recording electrode). **(B)** Antidromic action potentials elicited by dorsal-MOB stimulation (left); the evoked antidromic responses persist in the presence of 20 µM of CNQX and APV (center); collision tests annihilated the evoked response (right); arrowheads indicate stimulating artifact. **(C)** Drawing (left) and photomontage (right) of the LPC recorded; note that the axon (arrowheads) that has been omitted in the drawing leaves aAOB territory and heads toward the MOB. Orientation is the same as in ***a***. **(D)** The neuron recorded fires rhythmic bursts of action potentials in the presence of CNQX and APV (20 µM).

et al., 2004a; Pressler et al., 2013), which suggests that these rhythmic discharges parallel the sniffing cycles. In contrast, access of stimuli to the VNO is aided by mechanical (Meredith and O'Connell, 1979) and behavioral (Mann, 1961) processes. Thus, the dissimilar dynamics of central processing reflected by the bursting frequency may also underlie the functional differences observed between the two systems.

Another important difference between AOB and MOB "rhythmic" cells is their dendritic arborization. In fact, almost all eT cells have only one glomerular dendrite that extensively ramifies within a single tributary glomerulus (Hayar et al., 2004a) which contrasts to the numerous glomerular dendrites we have seen in our LPCs (3.1 ± 0.23). Moreover, glomerular dendrites often were committed to at least two glomeruli and ramified less profusely within the glomerular domain (see **Figure 4**; see Larriva-Sahd, 2008).

The functional significance of the rhythmic activity observed in the AOB remains to be determined. However, due to the fact that each glomerulus may receive sensory input from more than one receptor type (Belluscio et al., 1999; Wagner et al., 2006) and that each LPC innervates more than one glomerulus (Larriva-Sahd, 2008), it is possible that these cells may serve as network synchronizers (Hayar et al., 2004b; Peña et al., 2004; Ramírez et al., 2004).

Whatever the post-synaptic effect(s) of the episodic bursting of LPCs on their eventual targets might be, they are committed to pheromone detection (Leinders-Zufall et al., 2000; Boschat et al., 2002; Del Punta et al., 2002; Luo et al., 2003) and some LPCs exhibiting "rhythmic" discharges project to the MOB. Hence, it is plausible that certain pheromones may have a modulatory influence on the latter via the AOB-MOB interaction documented here. Although in our sample (n = 5) of "rhythmic" cells treated with glutamatergic inhibitors we did not find statistical differences in bursting properties before and after bath-applying CNQX and APV (**Figure 3C**), it is clear that glutamatergic modulation may affect certain parameters of

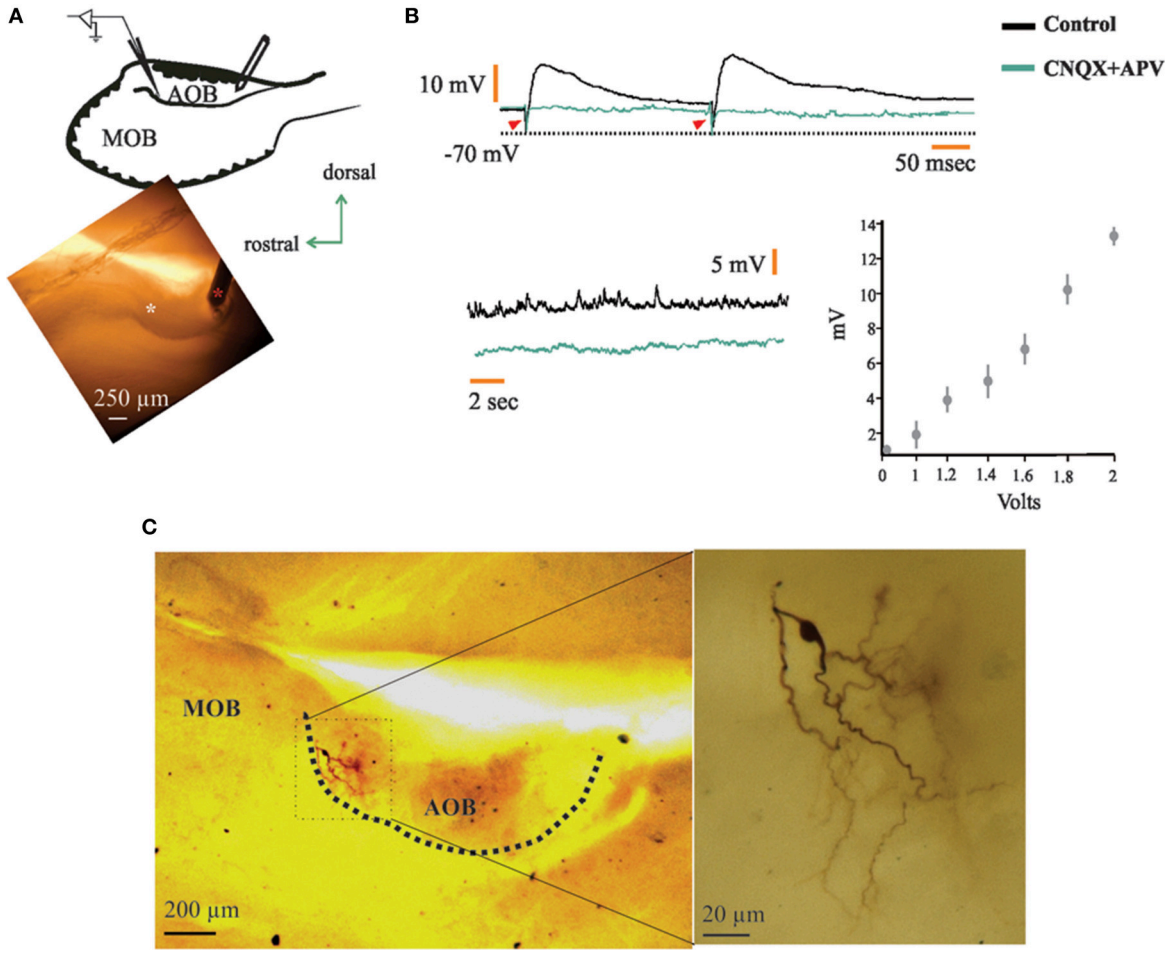

FIGURE 6 | Neurons in the anterior half of the accessory olfactory bulb (aAOB) receive synaptic input from posterior half (pAOB) neurons. (A) Schematic of the recording setup (left) and picture taken the day the neuron in ***c*** was recorded (red asterisk, stimulating electrode; white asterisk, tip of recording electrode). **(B)** Top: upon pAOB paired-pulse stimulation, neurons ($n = 6$) responded with excitatory postsynaptic potentials (EPSPs) that were completely abolished in the presence of CNQX and APV (20 µM; arrowheads indicate stimulating artifact). Bottom: spontaneous EPSPs before and after CNQX and APV incubation (left) and plot showing EPSP response amplitude vs. stimulus intensity (right; $n = 6$). **(C)** Picture of the neuron at low (left) and high (right) magnifications, note the very anterior position of the cell recorded (top left), which has a morphology resembling that of a short-axon neuron.

their rhythmic bursting differentially (see **Figure 3C**). Moreover, we recorded two neurons in which both synaptic excitation and inhibition were blocked and the rhythmic activity was still observed (data not shown).

Electrophysiological Properties of Large Principal Cells in the AOB

As already mentioned, the majority of neurons recorded and successfully visualized corresponded to LPCs and, although not our major goal in this research, we defined some of their electrophysiological characteristics that might be relevant. For instance, we found that principal cells in the AOB in either half may have persistent firing activity upon cessation of stimuli. Neurons exhibiting such properties, have been shown both *in vitro* (Shpak et al., 2012) and *in vivo* (Luo et al., 2003; **Figure 2B**). It has been suggested that these intrinsic properties may be associated with social context decoding (Shpak et al., 2012). Furthermore, some of these cells fire persistently and some others display ADPs that do not develop into persistent firing. Both characteristics result from distinct biophysical mechanisms (Shpak et al., 2015) and may also be modulated by basal forebrain cholinergic inputs (Smith and Araneda, 2010; Shpak et al., 2015). Because of both the basic physiological properties of these neurons and the extrinsic modulatory influence they receive, it is conceivable that they are involved in decoding complex sensory cues.

Axonal Link between the Accessory- and Main-Olfactory Bulbs

The existence of the axonal projection described here from the aAOB to the dorsal-posterior MOB (**Figure 5**) may represent one anatomical substrate accounting for the functional cross-talk observed between the main- and accessory-olfactory systems (Xu et al., 2005; see Baum and Larriva-Sahd, 2014). Consistent with this interpretation is that the dorsal MOB, a region receiving axonal collaterals from the AOB (**Figure 5**), has been implicated in the expression of social behaviors in mice (Matsuo et al., 2015).

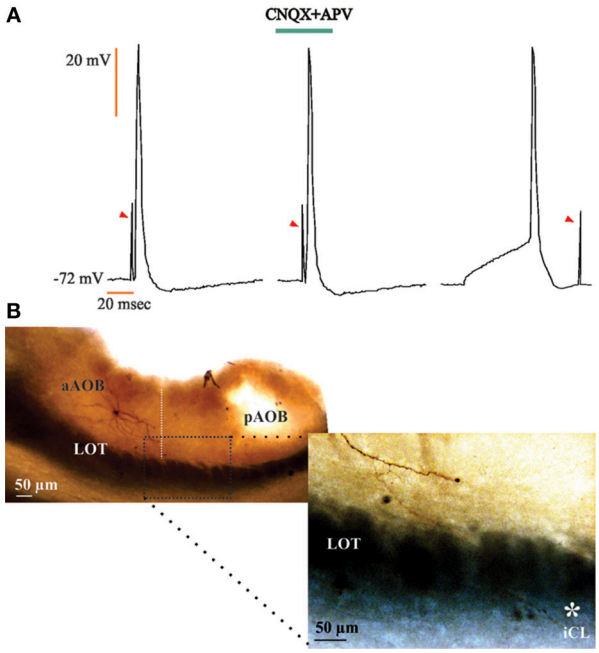

FIGURE 7 | Large principal cells in the anterior half of the accessory olfactory bulb (aAOB) send axons to the homonymous cells in the posterior half (pAOB). (A) Action potentials evoked by opposite half AOB field stimulation (left); these responses persist in the presence of CNQX and APV (20 µM; center) and were always collided (right). Arrowheads: stimulation artifact. **(B)** Low power photograph of anterior and posterior AOB halves (upper left); an aAOB neuron sends a collateral fiber* that resolves in the opposite internal cellular layer (ICL; lower right).

Potentially relevant is the observation that at least a set of LPCs displaying "rhythmic" activity projects from the aAOB to the dorsal MOB. Hence, these cells may imprint a pre-synaptic, synchronizing activity upon the MOB. Moreover, the fact that at least one of the recorded rhythmic neurons issued a long dendrite encompassing what seems to be a modified MOB glomerular complex (Shinoda et al., 1989; see **Figure 4I**) suggests convergence of olfactory and vomeronasal afferents into a single LPC. If there is a reciprocal (i.e., between the MOB to AOB) projection remains to be determined; however, electrolytic damage of the dorsal MOB resulted in orthograde degeneration in the AOB neuropil (Larriva-Sahd, 2008), suggesting a mutual connectivity between them. Further, reciprocal connections between the AOB and the MOB have been reported in the reptile *P. hispanica* (Martínez-García et al., 1991). Thus far, we assume that the projections of the aAOB to the MOB are a numerically small contingent of fibers, although a systematic search for MOB projections other than from its dorsal-posterior region is required.

The Accessory Olfactory Bulb Halves are Reciprocally Connected by Large Principal Cells

The initial observation in Golgi-impregnated specimens (Larriva-Sahd, 2008) regarding axon distribution and collateralization at either side of the LA suggested that this distinct cell type represents the substrate for a functional interaction between the two AOB halves. This notion became a central hypothesis to be tested here. Whole-cell recordings performed at either side of the LA proved that LPCs are mutually connected. Visualization of LPCs following recordings revealed that their axon collaterals distribute in the adjacent AOB half (**Figure 7**). Furthermore, a set of distinct dendrites traversing the LA may also represent a structural link between the AOB halves (Larriva-Sahd, 2008). While a first choice strategy to define neuron to neuron interactions is the recording of cell pairs, this turned out to be technically inaccessible, at least in our hands. In fact, LPCs laying at either side of LA are far apart (>300 µm; Larriva-Sahd, 2008), which significantly lowers the probability of successfully recording synaptically linked cells (McGarry et al., 2010).

Histological inspection of neurons that elicited EPSPs following stimulation of the opposite half suggests that some of them correspond to interneurons. Although a systematic study of neurons synaptically linked with the opposite AOB half is required, some neurons studied here exhibited dense peri-somatic axonal arborizations and absence of glomerular dendrites (**Figure 6C**). Thereby suggesting that short axon neurons mediate between LPCs in either AOB side (**Figure 8**). Hence, the structural evidence suggested earlier and confirmed here, coupled with the present physiological observations offers a normative foundation for the eventual understanding of the cellular interactions implicated in central pheromonal decoding.

Recently, an important imaging study of presynaptic calcium activity upon glomeruli, showed that AOB neurons are selectively tuned to the sex, strain, and species of urine samples (Hammen et al., 2014). Moreover, it has even been assumed that the AOB has a "modular organization" based on the defined sensory innervation of glomeruli by vomeronasal sensory neurons with similar receptive properties.

Like in the cerebral cortex, axon collaterals of principal cells recruit neighboring interneurons to define distinct functional clusters or domains (Lorente de Nó, 1949; Larriva-Sahd, 2008). The same seems to apply to LPCs that, by a set of tiny axon collaterals may recruit both, neighboring interneurons (i.e., columns or functional modules) or homonymous cells in their opposite half. Indeed, an *in vivo* study has highlighted the importance of lateral inhibition phenomena for AOB (Luo et al., 2003).

CONCLUSIONS

There is a novel direct axonal (i.e., collateral fibers) projection from aAOB neurons into dorsal MOB territory. Second, LPCs at either side of the LA send collateral axons and terminals to their opposite side, which may establish mono-synaptic contacts with LPCs and/or putative interneurons (see **Figure 8**). Lastly, there is a sub-population of "rhythmic" neurons that fire voltage-dependent bursts of action potentials. These neurons reside in both halves of the AOB.

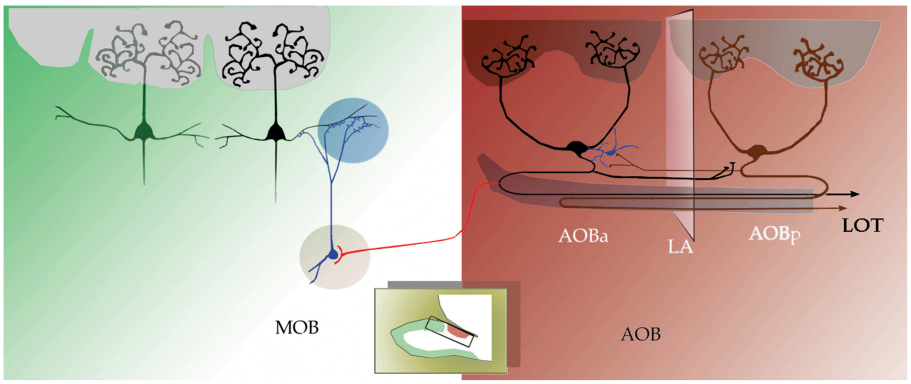

FIGURE 8 | Schematic representation of the results reported here; drawing of a sagittal view of the rat's OB. Anterior and posterior halves of the accessory olfactory bulb (aAOB and pAOB, respectively) are interconnected by large principal cells (black, dark red) that send axons (in black and dark red) and collaterals across the *línea alba*. These axons may influence putative interneurons (blue) and this communication is reciprocal. Conversely, aAOB LPCs send collateral fibers (red) into dorsal-MOB territory.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00518

Supplementary Figure 1 | Comparable recordings of APs with MgGTP instead of LiGTP in the internal solution. (A) Single APs obtained with LiGTP in our internal solution (black traces) and replacing the former with MgGTP (light blue); note the different time scales. **(B)** "Rhythmic" LPC of the aAOB recorded with an internal solution containing MgGTP.

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Interneurons: Role in Maintaining and Restoring Synaptic Plasticity

*Maria Elisa Calcagnotto**

Neurophysiology and Neurochemistry of Neuronal Excitability and Synaptic Plasticity Laboratory, Biochemistry Department, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Keywords: GABA, interneurons, synaptic plasticity, brain oscillations, cell replacement

Inhibitory circuits play an important role in synaptic plasticity during development and adulthood. Changes in interneuronal activity induce structural and synaptic rearrangements of inhibitory interneurons, network oscillations, and homeostatic plasticity. In addition to epileptic seizures, deficits in the inhibitory system lead to aberrant information processing and cognitive impairment in various neurological disorders. Studies exploring the structural and functional plasticity of interneurons are essential, not only to understand the mechanisms underlying normal development and behavior but also, to identify the etiology of different psychiatric and neurological disorders to pursue new therapies. Here, it will be discussed the role of inhibitory circuit in the synaptic plasticity, and how cellular replacement strategies can remodel changes in circuit function and homeostasis in the context of brain repair.

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***Correspondence:**
Maria Elisa Calcagnotto
elisa.calcagnotto@ufrgs.br

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INTERNEURON DIVERSITY AND ORIGIN

Most of the gamma-aminobutyric acid-containing (GABAergic) interneurons in the cerebral cortex and hippocampus originate from three progenitor regions in the embryonic subpallium: caudal ganglionic eminence (CGE), medial ganglionic eminence (MGE), and preoptic area (POA) (1–4). Each progenitor region produces a particular group of interneurons, although some interneuron classes may emerge from different progenitor domains. In the cortex and in the hippocampus, the MGE produces most of the interneurons including fast spiking-parvalbumin (FS-PV)-expressing basket and chandelier cells and somatostatin (SOM)-expressing interneurons with or without coexpression of calretinin (CR), neuropeptide-Y (NPY), or reelin. CGE generates cholecystokinin (CCK), CR, vasointestinal peptide (VIP), reelin, and neurogliaform cells, but not SOM-expressing interneurons. Some interneurons coexpress CR and VIP, whereas others coexpress NPY and reelin. POA originates a small population of reelin and/or NPY-expressing neurons. Recent studies indicate that this region may also give rise to a small fraction of PV- and SOM-expressing cortical interneurons whose development does not depend on Lhx6 function (3). These inhibitory interneurons play key roles in regulating local circuit activity and synaptic plasticity (5).

INTERNEURONS ORCHESTRATING SYNAPTIC PLASTICITY AND OSCILLATIONS

In the cortex and hippocampus, interneurons subtypes differ in their functional connectivity and generate differentially timed inhibition at distinct sites of postsynaptic cells (6–8). Interneurons are perfectly positioned to synchronize network activity. Some target dendritic domains (e.g., SOM-, NPY-, or CB-expressing interneurons) to control the efficacy and plasticity of excitatory inputs onto principal neurons (7). In CA1 hippocampal region, the oriens-lacunosum moleculare (O-LM) and bistratified interneurons, both expressing PV and SOM, are dendritic targeting cells (9). Others target perisomatic compartments (i.e., soma, axon initial segment, and thick proximal dendrites) (e.g., FS-PV-expressing basket cells or CCK-expressing interneurons) (6) to control the output

and consequently synchronize the firing rate of principal cells action potentials (10–12). The hippocampal CA1 pyramidal cells soma receive abundant GABAergic inputs from basket cells (13) that are able to control the ability of inputs to generate action potential and to synchronize the pyramidal neurons firing rate (10). Chandelier cells target the axon initial segments of several pyramidal neurons and also contribute to the output synchronization of CA1 principal cells (14). In the mature cortex, a single basket interneuron is able to form characteristic perisomatic synapses (15) with hundreds of pyramidal neurons (16). Each pyramidal neuron, in its turn, can receive inputs from multiple basket cells (16, 17). The postnatal maturation of perisomatic innervation is essential to synchronize pyramidal neurons activity within cortical circuits. In addition to perisomatic inhibition, other FS-PV-expressing interneurons also innervate either axons (chandelier cells) (18) or dendrites (O-LM and bistratified cells) of target cells (7, 9, 19, 20). All these GABAergic inputs in specific subcellular domains play an important role in brain oscillations and plasticity. In particular, the FS-PV-expressing interneurons are known to be crucial in gamma (30–80 Hz) oscillations in the cortex and hippocampus (5, 21, 22), involved in cognition and information processing (23). Accordingly, the blockage of synaptic output of hippocampal PV-expressing interneurons impairs spatial working memory (24). Moreover, the O-LM and bistratified cells that fire in phase with theta oscillations generate global dendritic inhibition, mediate network-state-dependent inhibition on specific parts of pyramidal neuron dendrites, are targeted by afferents from the medial septal region, and are crucial for hippocampal rhythm generation in behaving animal (9, 19, 20). During gamma oscillations, bistratified cells seem to participate in the transmission of the CA3-dependent gamma component to CA1 (19, 20).

Besides the direct control of inhibition, synchrony and plasticity on principal cells, different interneuron subtypes exhibit a wide range of responses to different neuromodulatory inputs, leading to changes in net inhibition, synchronization, and synaptic plasticity (25). The innervation pattern of O-LM cells, which includes the excitation by fast cholinergic transmission, the inhibition of distal dendrites, and the disinhibition of proximal dendrites of pyramidal neurons in CA1, enables them to modulate synaptic efficiency and plasticity of entorhinal cortex and CA3 inputs (19).

Interneuron subtypes differ in their functional connectivity to the principal cells in cortex and hippocampus (6, 8), providing different functional outcome for action potential generation from principal cells (7). This can be exemplified by an elegant work from Ledri and colleagues, where the rupture of hypersynchronization was achieved, by controlling the activity of large populations of interneurons rather than a single population of PV- or SOM-expressing interneurons in the hippocampus, using optogenetics (12). The inhibition of PV-expressing interneurons target by optogenetics also seems to suppress gamma power, while its stimulation elicited gamma oscillations in downstream pyramidal neurons (22), therefore controlling network oscillation.

As mentioned above, GABAergic interneurons targeting dendritic domains control the efficacy and plasticity of excitatory inputs onto principal neurons. This dendritic remodeling of inhibitory neurons, either in normal or pathological conditions, affects activity-dependent modulation of neuronal connectivity within local circuits (26). Reduction in dendritic ramification and decreased axonal length has been described in interneurons in schizophrenia (27) and animal models of epilepsy (28). However, decrease in density and substantial increase in size, axonal reorganization, and aberrant synaptic connections of remaining and newborn SOM-expressing interneurons were observed in hippocampal CA1 and dentate gyrus of animal models of epilepsy (29, 30). This reorganized circuitry synchronizes granule cells activity and decreases seizure threshold, despite the increment in number of GABAergic terminals, contributing to the inhibitory dysfunction in epilepsy. It seems to be an abundant but dysfunctional attempt to compensate the decreased inhibitory input to granule cells after epileptogenic injuries (29, 30). Thus, changes in the synaptic reorganization of SOM-expressing interneurons can disrupt network organization and increase excitation levels.

Together with the interneuronal network connectivity at different cell domains, modulatory inputs and dendritic remodeling, the intrinsic properties of interneurons are particularly crucial to generate and control neuronal oscillations and plasticity (31). For example, the intrinsic properties of FS-PV-expressing interneurons and fast $GABA_A$ receptor kinetics are likely to be needed to achieve precise timing gamma oscillations in cortex and hippocampus (21, 22). Additionally, different GABAergic interneuron subtypes fire independently and innervate distinct postsynaptic domains at different time points. These coordinated synaptic interactions actively orchestrate the precise input/output information to generate and control neuronal activity during network oscillations in different developmental and behavioral states (8).

INTERNEURON REPLACEMENT RESTORES SYNAPTIC PLASTICITY AND NETWORK OSCILLATION

As we discussed earlier, GABA transmission has an important role in synaptic plasticity. Moreover, it is essential to regulate plasticity during critical periods of brain development. Changes in inhibition create an environment with aberrant neuronal network that is associated with neurodevelopmental (29, 30, 32–35). Interestingly, some of the most consistent findings in autism spectrum disorders, schizophrenia, epilepsy, and cognitive disorders consist in SOM- and PV-expressing interneurons dysfunction in the brain, including O-LM cells (29, 30, 36–39). Thus, by controlling GABAergic activity, it could be possible to maintain or rescue normal network oscillations and synaptic plasticity.

To address this issue, cell replacement using MGE precursors (source of PV- and SOM-expressing interneurons) has been performed in animal models. The studies had shown that MGE-derived cells are able to survive, differentiate in mature interneurons, migrate, and functionally integrate through the host brain parenchyma with low risk of promoting brain tumors. The integrated mature interneurons modify the neuronal network, rescuing the normal functional inhibition and the synaptic plasticity (40–44). Some examples are studies using MGE

precursors grafted into brain of neonate distal less homeobox 1-deficient mice (*Dlx1*$^{-/-}$) (34) and cyclin D2 knockout mice (*Ccnd2*$^{-/-}$) (43). *Dlx1*$^{-/-}$ mice exhibit late-onset interneuron loss and reduced inhibition (34), with consequent deficit in interneuronal network, altered gamma frequency oscillations (GFOs), and dysfunction in homeostatic plasticity and seizures (44). The authors demonstrated that MGE-derived cells reduced seizure severity, restored inhibition, normalize gamma oscillations, and reversed the homeostatic changes in excitatory synaptic activity hippocampal long-term potentiation (44). In the *Ccnd2*$^{-/-}$ mice that display deficit in PV-expressing interneurons, hippocampal disinhibition, increased ventral tegmental area dopamine neuronal activity, and cognitive impairment, the MGE-grafted cells were able to differentiate in long-range survival GABAergic interneurons distributed through the hippocampus and reverse the psychosis and cognitive phenotypes (43).

The maturation of GABAergic interneurons, specifically PV- and SOM-expressing subtypes, has been strongly implicated in critical period of plasticity, such as the development of visual cortex (45, 46). It has been shown that PV- and SOM-expressing interneurons, derived from MGE-precursors grafted into neonate brain, were able to induce ocular dominance plasticity shortly after the normal critical period. Inhibitory-grafted neurons reorganize the cortical circuitry by introducing a new set of weak inhibitory synapses (47), rather than simply enhancing the endogenous mature inhibitory synaptic strength. This pattern of numerous and weak connections is consistent with the form of developing inhibition during the critical period (48). However, suppression of PV-expressing neurons in visual cortex in adult mice also induced plasticity and beyond critical period (49). Therefore, new plasticity can be induced when inhibitory precursors were grafted into visual cortex during development and in adult mice (50–52). The plasticity process seems to be a direct result of modification in neural circuit induced by PV- and SOM-expressing cells integrated into the primary visual cortex. Some suggested mechanism are increased connectivity; increased expression of $GABA_A$ receptors containing the α1 subunit, abundant at synapses mediated by PV-expressing interneurons (46); and changes in maturation of interneurons (47, 51).

Changes in GABAergic function mediated by PV- and SOM-expressing interneurons impair synaptic plasticity and disrupt network organization and normal brain oscillations. However, by temporal and selective coordination of interneuronal activity, it is possible to modulate interneuronal network and increase GABA release on different subcellular domains of target cells to maintain or rescue normal network oscillations and synaptic plasticity. The investigation of PV- and SOM-expressing interneurons, using strategies that modify interneuronal network and regulate inhibitory input/output of such interneurons at different time points, as selective target them or using precursor cells grafts, are helping us to better understand the role of these specific interneuron subtypes in controlling and restoring synaptic plasticity and brain oscillations during development and adulthood.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Examining the role of vasopressin in the modulation of parental and sexual behaviors

*Josi Maria Zimmermann-Peruzatto[1], Virgínia Meneghini Lazzari[2], Ana Carolina de Moura[2], Silvana Almeida[2] and Márcia Giovenardi[1,2]**

[1] *Programa de Pós-Graduação em Fisiologia, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil,* [2] *Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil*

Vasopressin (VP) and VP-like neuropeptides are evolutionarily stable peptides found in all vertebrate species. In non-mammalian vertebrates, vasotocin (VT) plays a role similar to mammalian VP, whereas mesotocin and isotocin are functionally similar to mammalian oxytocin (OT). Here, we review the involvement of VP in brain circuits, synaptic plasticity, evolution, and function, highlighting the role of VP in social behavior. In all studied species, VP is encoded on chromosome 20p13, and in mammals, VP is produced in specific hypothalamic nuclei and released by the posterior pituitary. The role of VP is mediated by the stimulation of the V_{1a}, V_{1b}, and V_2 receptors as well as the oxytocinergic and purinergic receptors. VT and VP functions are usually related to osmotic and cardiovascular homeostasis when acting peripherally. However, these neuropeptides are also critically involved in the central modulation of social behavior displays, such as pairing recognition, pair-bonding, social memory, sexual behavior, parental care, and maternal and aggressive behavior. Evidence suggests that these effects are primarily mediated by V_{1a} receptor in specific brain circuits that provide important information for the onset and control of social behaviors in normal and pathological conditions.

Keywords: evolutionary lineage VP, vasopressin-like, V_{1a} receptor, V_{1b} receptor, social behaviors

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Elliott Albers, Georgia State University, USA
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****Correspondence:***
Márcia Giovenardi, Sarmento Leite Street 245/308, Division of Physiology, Federal University of Health Science, Porto Alegre, Rio Grande do Sul 90050-170, Brazil
giovenardi.marcia@gmail.com

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General Aspects

Vasopressin-like (VP-like) and oxytocin-like (OT-like) peptides have been isolated from invertebrates and vertebrates in more than 100 species (1). Approximately 700 million years ago, the ancestral gene encoding the precursor protein diverged between the invertebrate and vertebrate families (2). Generally, all vertebrate species express a VP-like and OT-like peptide.

The lineage of VP-like peptides is evolutionarily stable. In fishes, amphibians, reptiles, and birds, vasotocin (VT) shares similar roles with mammalian arginine vasopressin (VP), whereas mesotocin and teleost isotocin are functionally similar to mammalian oxytocin (OT) (3).

The gene expression and regulation of these peptides is conserved among vertebrates (3). The chemical structure of VP and OT differs by only two of nine amino acid residues (4, 5). The VP/OT superfamily can be traced back to different types of invertebrates, such as annelids and mollusks (6). There is a homology of 80% between VP and OT, but these neuropeptides have distinct physiological activities. The receptors for VP-like and OT-like peptides have been described in invertebrates (6) and vertebrates (7).

Both VP and OT are produced in the hypothalamus, released in the neurohypophysis, and distributed throughout the brain (8, 9). In 1895, the vasopressor effect of the neurohypophyseal extract was attributed to the neurohypophysis gland (10). However, two decades later, the antidiuretic effect of the pituitary extract was demonstrated (11). The isolation of VP in the fifties confirmed that the same neuropeptide is synthesized in the neurohypophysis gland and possesses antidiuretic and vasopressor effects (12).

This review focuses on the involvement of VP in brain circuits, synaptic plasticity, evolution, and function, highlighting the role of VP in parental and sexual behaviors.

Vasopressin and Vasopressin-Like Peptides

Gene Structure

Nucleotide sequences encoding the VP and OT hormones are highly homologous. In all species, VP and OT are located on the same chromosome, 20p13, but encoded by different genes separated by a segment of DNA only 12 kb long (13). The similarity in the intron–exon structures of the two genes and opposite orientation suggest recent gene duplication (14).

Synthesis and Release

Vasopressin is a nonapeptide with a disulfide bridge between two cysteine amino acids and is synthesized in a smaller amount by parvocellular neurons, primarily by magnocellular neurons of the hypothalamus in paraventricular nucleus (PVN) and supraoptic nucleus (SON) (15). These nuclei send axons to the neuro pituitary along the supraoptic–hypophyseal tract. In a prohormone state, VP migrates along the supraoptic–hypophyseal tract to the neurohypophysis gland, where it is released into circulation (16).

The PVN and SON receive afferent nerve impulses from receptors in the left atrium, aortic arch, and carotid sinuses via the vagus nerve. PVN and SON receive osmotic input from the lamina terminalis, which is excluded from the blood–brain barrier and is thus affected by systemic osmolality. Furthermore, Holmes et al. (16) suggested that in the rat brain, extrahypothalamic structures, such as the bed nucleus of the stria terminalis (BNST), the medial amygdala, nucleus of the locus coeruleus, hippocampus, and choroid plexus, in addition to the hypothalamus, are able to synthesize VP.

The anterior pituitary gland also releases VP but in smaller quantities. VP can activate the hypothalamic–pituitary–adrenal axis by stimulating the V_{1b} receptor ($V_{1b}R$) and controlling the liberation of adrenocorticotropic hormone (ACTH) (17), whereas the V_{1a} receptor ($V_{1a}R$) controls the synthesis and release of cortisol in the adrenal cortex (18).

Vasopressin Receptors

The functions of VP are modulated by stimulation of G-protein-coupled receptors (GPRCs) from each independent tissue. They are classified as $V_{1a}R$, $V_{1b}R$ (also known as V_3R), V_2R, oxytocinergic (OTR), and P_2 purinergic (P_2R) receptors (19, 20). In mammals, the VPRs are widely distributed in the brain (21, 22).

Vasopressin can bind to all of these receptors but not with the same affinity. The receptors, which use G-proteins as transducer signals across the cell membranes, have seven hydrophobic transmembrane domains, four extracellular domains, and four intracellular domains (23). Neurotransmitters, hormones, and chemokines indicate the courses of VP action, whereas local mediators signal to the four main G-protein families to regulate metabolic enzymes, ion channels, and transcriptional regulators. The different types of VPR extracellular signals refer to specific G-proteins. Several important hormones interact with the G_i pathway, which is characterized by inhibition of adenylyl cyclase (24).

Agonist of VPR is a substance that initiates a physiological response through specific interactions with G-protein-coupled receptor kinases and protein kinase C present in the carboxyl termini of the receptors (25). The VP signal is transmitted through guanine nucleotide-binding proteins (G-proteins) (26), such as G_s and $G_{q/11}$ subtypes (24).

$V_{1a}R$ Receptor

$V_{1a}R$ is primarily found on vascular smooth muscle and causes vasoconstriction by an increase in intracellular calcium via the phosphatidyl-inositol-bisphosphonate cascade. Studies in rats have shown that $V_{1a}R$ is also located in the brain, myocardium, gonads, cervical ganglion, liver, blood vessels, kidney, spleen, renal medulla, and platelets (20–22, 27–29); however, the physiologic roles of VP remain unknown in many tissues.

$V_{1b}R$ Receptor

$V_{1b}R$ is localized in pituitary gland, olfactory bulb, septum, hippocampus, pancreatic beta cells, and adrenal medulla and induces the release of hormones (20, 30–32). The phylogenetic analysis showed that $V_{1b}R$ diverged early from the $V_{1a}R$ sequences and presented the closest relationship with the OTR (31). $V_{1b}R$ is highly expressed in the anterior pituitary where it is thought to play a role in costimulating the neuroendocrine response to stress (33). The VP causes secretion of ACTH, which is important for the induction and phenotype maintenance of ACTH-secreting tumors mediated through G_s, G_i, and $G_{q/11}$ (34). Studies have shown that $V_{1b}R$ gene expression may thus be a marker of the corticotroph phenotype and can be used to help shed light on the pathophysiological mechanism of ectopic ACTH syndrome (30, 35).

V_2 Receptor

V_2R is located on vascular smooth muscle cells, vascular endothelium, and the collecting ducts of the renal medulla (20). The hydro-osmotic or antidiuretic effect of VP occurs via activation of V_2R (36, 37). VP adjusts water homeostasis regulation of the fast shuttling of aquaporin 2 to the cell surface and stimulates the synthesis of mRNA encoding this protein (38, 39).

Phillips et al. (28) evaluated V_1Rs and V_2R binding sites *in vitro* using selective radioligands and demonstrated that there was no vasoconstrictor activity of V_2R in the endothelium, liver, brain, spinal cord, sympathetic ganglia, heart, or vascular smooth muscle. In this study, specific binding was only identified in the kidney, which is consistent with the known distribution of antidiuretic V_2Rs on renal collecting tubules (28).

Other Receptors

Both VP and OT can bind with OTR but not with the same affinity. OTRs are coupled to $G_{q/11}$ class binding proteins, which stimulate phospholipase C activity (34).

P_2 purinergic receptors also belong to the seven-transmembrane domain GPCR superfamily. Its role was confirmed in cardiac endothelium because VP exhibited effects through activation of P_2Rs (40).

Vasopressin and Synaptic Plasticity

Vasotocin/vasopressin neuropeptides affect several sex-typical and species-specific behaviors and produce an integrational neural substrate for the dynamic regulation of these behaviors via endocrine and sensory stimuli. Different types of social behaviors are influenced by VT and VP, influencing the objectives of many neuroanatomical studies of VT/VP distribution and central nervous system (CNS) targets (4, 41, 42). There is a sexually dimorphic vasopressinergic extrahypothalamic network that plays a key role in the modulation of behavior by VP (43), and sex differences in VP distribution were first demonstrated in rats (44).

In vertebrates, behaviors such as reproductive, smell recognition, social communication, pair-bonding, parental care, and aggressive behavior are also modulated by VT/VP (41, 45, 46). In different species of vertebrates, both central and peripheral VT/VP administration stimulate spawning behavior, phonotaxis, sexual receptivity, lordosis, courtship, and mating (4, 41, 47).

In the rat brain, V_{1a}R is believed to play the predominant role in regulating behavior. V_{1a}R is found in neural networks involved in responses related to social behavior and exhibits considerable plasticity (45, 46, 48). V_{1a}R is expressed in olfactory bulb, hippocampal dentate gyrus, cerebellum, septal nuclei, accumbens nucleus, arcuate nuclei, suprachiasmatic nuclei, and periventricular nuclei and the lateral hypothalamic area, parvocellular paraventricular and anteroventral nucleus of the thalamus, circumventricular organs including the pineal, and the subfornical organ (49, 50). Further, V_{1b}R also participates in the neural regulation of social behaviors (51), but has received much less attention due to a lack of specific drugs (52, 53). V_{1b}R transcripts and immunoreactive cell bodies are localized to the cerebellum, cerebral cortex, hippocampus, olfactory bulb (including in the area periglomerular), PVN, piriform cortex layer II, red nucleus, septum, and suprachiasmatic nucleus (54–61).

Vasopressinergic as well as oxytocinergic systems can be modulated by circulating gonadal steroids and together are involved in behavioral regulation (4, 41, 62, 63). Sex hormones affect the quantity and localization of the VT/VP receptors in the brain (64). Previous studies (64, 65) have shown that behavioral responses, such as courtship behavior in newts, aggression in hamsters, and parental behaviors in voles (66), depend on VT/VP and the presence of gonadal steroids.

Role of Vasopressin in Parental and Sexual Behavior

The ability of an animal to recognize intra- and interspecific individuals is essential for all complex relationships. Several studies (5, 46, 63, 67–71) have shown that VP participates in the modulation of non-social and social behaviors.

In this sense, vasopressinergic neurons play an important role in coding information from social contact (47). In rodents, social information is primarily mediated by the exchange of olfactory information, and there is evidence that VP signaling is important in brain areas where olfactory information is processed. Wacker et al. (51) described populations of vasopressinergic neurons in the main and accessory olfactory bulbs and anterior olfactory nucleus that are involved in processing social odor cues. Pharmacological studies have shown that VP administration improves social recognition in both sexes (58, 63). If applied bilaterally in the olfactory bulbs, extending the memory retention interval for the recognition of male rat odor is extended (72).

In rodents, intracerebroventricular microinjections of VP agonists facilitate social memory and can significantly extend social recognition and memory consolidation for as much as 120 min (73, 74). V_{1a}R antagonists produce marked effects on learning, memory, and social behaviors (59, 63). Intracerebroventricular and intraseptal microinjection of V_{1a}R antagonists block social recognition, and VP microinjection can rescue deficits in social recognition in Brattleboro rats that lack VP (74–77).

Bielsky et al. (45) demonstrated that V_{1a}R knockout mice (V_{1a}RKO) display an enormous deficit in social recognition, providing strong evidence that V_{1a}R is essential for this action. Similarly, V_{1b}R knockout mice (V_{1b}RKO) also presented deficits in the social memory test (52, 53). However, the increased V_{1a}R expression in the lateral septum (LS) facilitated social behavior (78).

Among other social behaviors, the parental behavior exerts an essential role in the behavioral development of offspring (79). Maternal care is best studied; however, pup-directed positive behaviors, such as retrieval and kyphosis (huddling), are exhibited by no-parturient animals and are characteristics of socially monogamous or cooperative breeding species. Male parental care has been primarily studied in relation to the vasopressinergic system, which is sexually dimorphic and androgen-dependent because testosterone promotes VP synthesis (79).

Paternal care is highly demonstrated by prairie vole males (80, 81). Lim and Young (82) demonstrated that the infusion of VP (0.1 ng) directly into the LS of these animals can enhance licking/grooming (LG) behavior toward pups and that this behavior is blocked by the use of a V_{1a}R antagonist (80). The father LG behaviors and retrieving the pups exert a pivotal role on the development of the VP systems and aggressive behavior of their adult offspring (79). In adult male rats, LG behaviors increase the levels of V_{1a}R binding within the amygdale nucleus (46).

Besides the parental behavior, the VP system is also an important mediator of maternal behavior. In the peripartum and lactation periods, there is an increase in the expression of mRNA, receptors, and density/binding of VP in the maternal brain. This increase contributes to the adaptations that develop in the female maternal care. Previous studies (83–85) have already suggested a role for VP facilitating maternal care, but more recent studies showed substantial evidence of this neuropeptide facilitating maternal behavior [for review, see Bosch and Newman (86)].

Furthermore, in females, $V_{1a}R$ density was significantly correlated with postpartum LG of the offspring (87).

Most studies are based on infusion of VP and $V_{1a}R$ antagonists in specific areas of the CNS participating in the neural circuitry of maternal behavior and thus demonstrating the modulation of VP in this behavior [for review, see Ref. (86, 88–91)].

Moreover, previous studies (92, 93) with HAB (high anxiety-related behavior) and LAB (low anxiety-related behavior) dams also reinforce that central VP modulates the maternal behavior because the blocking of $V_{1a}R$ by repeated acute intracerebroventricular administration of a selective antagonist promoved decrease arched back nursing and the time the dam spent with the pups in HAB dams.

In conjunction with these findings, the involvement of $V_{1b}R$ in the modulation of maternal behavior was demonstrated in V_{1b}RKO mice (94), which showed that lactating females who received the $V_{1b}R$ antagonist in the lateral ventricle decreased the nursing and interaction with their pups.

In addition to maternal care, lactating rats exhibit aggressive behavior that is observed during the first two postpartum weeks and which aims to protect the offspring against a potentially dangerous intruder (95, 96). The role of VP in aggression has received attention, and previous studies (86, 88, 89, 91, 97–100) provide substantial evidence for VP promoting maternal aggressive behavior. Neuroanatomical studies with multiparous rats revealed significant increases in $V_{1a}R$ mRNA expression in the amygdala, SON, and LS in females on the fifth postpartum day when compared with primiparous rats (101, 102).

Bosch and Neumann (90) demonstrated that microinjection of a selective $V_{1a}R$ antagonist bilaterally into the BNST reduced maternal aggression (91) and the VP within the central nucleus of the amygdala (CeA) was positively correlated with the increased offensive behavior (90). Furthermore, other study by Bosch and Neumann (92) reported that in HAB rats VP promotes maternal aggression, and Lonstein and Gammie (103) showed the increased expression of the VP gene in the PVN. On the other hand, studies with Sprague-Dawley lactating rats showed different results, as the intracerebroventricular infusions of VP reduced maternal aggression, while treatments with an $V_{1a}R$ antagonist increased maternal aggression during early lactation (88).

Vasopressin-deficient Brattleboro rats exhibit reduced aggressive maternal behavior and reduced attacks in males without sexual experience against intruders (99). In other study on female pregnancy (51, 104), increasing $V_{1a}R$ levels in the PVN, CeA, and LS were positively correlated with aggressive behavior. The fluctuations observed in the OTR and $V_{1a}R$ in important areas of the CNS appear to regulate maternal aggression during the peripartum period. In a pharmacological study, microinjection of a selective $V_{1a}R$ antagonist bilaterally into the BNST reduced maternal aggression behavior but did not alter maternal care (105).

Furthermore, VP may be a new target for studies on treatments involving $V_{1a}R$ antagonists or synthetic VP to promote maternal care or suppress aggression in lactating females exposed to chronic stress-associated disorders (98). Using a model of VP-deficient mothers, Fodor et al. (105) demonstrated that these rats have decreased LG behavior and act less depressive. Thus, they suggest that VP antagonists could be an option for future studies on postpartum depression; however, the possible side effects of maternal neglect require further investigation (90, 105).

Some findings suggest that $V_{1b}R$ might also be involved in the modulation of aggressive behavior in both females and males. V_{1b}RKO lactating mice showed an increased latency and decreased number of attacks against intruders when compared with wild-type mothers (97). The defensive behavior was studied in V_{1b}RKO male mice and increased social behavioral responses were observed (106). In support, V_{1b}RKO male mice also had impaired attack behavior toward a conspecific (97).

The role of this nonapeptide in sexual behavior has also been described in the past decades, and previous studies showed that VT/VP modulates specific types of vocalization in rats and squirrel monkeys (107). VT/VP central administration enhances pair-bonding and smell-recognition behaviors as a key feature for the onset of sexual behavior (66, 107).

Since the 1980s, studies have described the importance of vasopressinergic projections in both sexes in relation to sexual behavior. In this context, Meyerson et al. (104) administered an antagonist of VPRs into the lateral ventricles of female Sprague-Dawley rats in the neonatal period; this treatment induced a persistent increase in VP content and facilitated female sexual behavior. This study provided functional and immunocytochemical evidence of the importance of VP in female sexual behavior. In the same decade, Södersten et al. (108) reported that intracerebroventricular injections of VP inhibit sexual behavior in receptive female Wistar rats. These findings were reinforced by Pedersen and Boccia (109), which suggested that VP influences ovarian steroid activation of female sexual behavior via interactions with OT.

In males, Bohus (110) observed that VP agonists reversed the decrease of sexual behavior after castration, but injections of VP into LS of males did not dramatically alter sexual behavior (111, 112). In the 1990s, the role of VP in the modulation of male sexual behavior was hypothesized to be due to the refractory period in rats and consequently be an inhibitor of sexual behavior in males (112). In 1993, Winslow et al. (66) reported that VP is necessary to partner preference formation in monogamous prairie vole. However, these studies commonly focused on differences in neurotransmitter systems in brain structure between sexes instead of the role of the neurotransmitter on sexual behavior. Knowledge about differences in cell density, neurotransmitter content, receptors distribution, and vasopressinergic projections in rats and voles between sexes does not necessarily cause sex differences in sexual behavior (113).

Knockout animals were also used to investigate the role of VP and its receptors in sexual behavior. V_{1b}RKO mice exhibited deficits in social behaviors that require olfactory function, including aggression and social recognition, but these animals had normal sexual behavior (52). The BNST is a sexually dimorphic structure that can be involved in the control of male sexual behavior because males have more VP neurons and denser projections from this area and in the medial amygdaloid nucleus than females (114). However, studies about VP innervation have focused on female sexual behavior. VP innervation from

the LS inhibits sexual behavior in females; thus, hypothetically, the higher levels of VP in males are correlated with less lordosis behavior (115).

In humans, VP has been reported as a selective enhancer of recognition of sexual cues in a behavioral task administered to males (116). Additionally, Argiolas and Melis (117) conducted an elegant review about the control of sexual behavior by neuropeptides in the species studied thus far, including rats, mice, monkeys, and humans (117), and describe VP as an ineffective neuropeptide on copulatory behavior in males but as an inhibitor of lordosis in female sexual behavior.

The role of VP in sexual behavior remains unclear. Controversial results can be explained by the different methods applied and the interactions of this peptide with others, which should always be considered.

In conclusion, the lineage for VP or VP-like neuropeptides is evolutionarily stable and present in all vertebrate species. The neural distribution of VP neurons and its projections and also the distribution of VPRs are widely investigated for more than 30 years. These extensive data collected shows that male and female have important differences in the VP distribution across the CNS. The role of VP in osmotic and cardiovascular homeostasis is well established in the literature; however, the neuropeptides VT/VP are also critically involved in the modulation of social behaviors in many species. In mammals, the parental and sexual behaviors are mediated by VT/VP system and mainly by $V_{1a}R$ in specific brain circuits that provide important information for the onset and control of these behaviors in normal and pathological conditions. Future investigations must be conducted to further elucidate the involvement of $V_{1b}R$ modulating social behaviors.

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Endocrine and Cognitive Adaptations to Cope with Stress in Immature Common Marmosets (*Callithrix jacchus*): Sex and Age Matter

Maria Bernardete Cordeiro de Sousa[1], Ana Cecília de Menezes Galvão[2], Carla Jéssica Rodrigues Sales[2], Dijenaide Chaves de Castro[2] and Nicole Leite Galvão-Coelho[3]*

[1] Brain Institute, Universidade Federal do Rio Grande do Norte, Natal, Brazil, [2] Graduate Program in Psychobiology, Universidade Federal do Rio Grande do Norte, Natal, Brazil, [3] Department of Physiology, Universidade Federal do Rio Grande do Norte, Natal, Brazil

Phenotypic sex differences in primates are associated with body differentiation during the early stages of life, expressed in both physiological and behavioral features. Hormones seem to play a pivotal role in creating a range of responses to meet environmental and social demands, resulting in better reactions to cope with challenges to survival and reproduction. Steroid hormones actively participate in neuroplasticity and steroids from both gonads and neurons seem to be involved in behavioral modulation in primates. Indirect evidence suggests the participation of sexual steroids in dimorphism of the stress response in common marmosets. This species is an important experimental model in psychiatry, and we found a dual profile for cortisol in the transition from juvenile to subadult, with females showing higher levels. Immature males and females at 6 and 9 months of age moved alone from the family group to a new cage, over a 21-day period, expressed distinct patterns of cortisol variation with respect to range and duration of response. Additional evidence showed that at 12 months of age, males and females buffered the hypothalamic–pituitary–adrenal axis during chronic stress. Moreover, chronic stressed juvenile marmoset males showed better cognitive performance in working memory tests and motivation when compared to those submitted to short-term stress living in family groups. Thus, as cortisol profile seems to be sexually dimorphic before adulthood, age and sex are critical variables to consider in approaches that require immature marmosets in their experimental protocols. Moreover, available cognitive tests should be scrutinized to allow better investigation of cognitive traits in this species.

Keywords: stress coping, cortisol, cognition, development, non-human primate model

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****Correspondence:***
Maria Bernardete Cordeiro de Sousa
mbcsousa@neuro.ufrn.br

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INTRODUCTION

During evolution, animals developed mechanisms to cope with challenging situations that are influenced by developmental phases and sex. In this context, steroid hormones seem to play a pivotal role in creating a range of responses to meet environmental and social demands, resulting in better reactions to cope with challenges to survival and reproduction. Their effects act on brain plasticity, changing neural circuitry constructed on a genetic, physical, and social environment

basis, and involve synaptic sprout, spine growth, and trophic actions (1). Before the effects of steroids on the brain were known, the classification criteria of sexual dimorphism in males and females were based on sex chromosomes, gamete type, and morphology of the reproductive system. However, Phoenix et al. (2) demonstrated that female guinea pigs will display masculinized sexual behavior in adulthood when exposed to androgens during intrauterine development, indicating that the brain is susceptible to permanent hormonal changes in early life, a phenomenon they called the organizational effect. They also suggested that hormonal outcome may influence vertebrate morphology and behavior during other life stages, such as puberty and reproductive age (activational effects). These concepts were revised, and the organizational effects of hormones are currently considered to occur throughout life, and sex differences are expressed in conjunction with genetic (3, 4) and/or epigenetic mechanisms (5, 6), in addition to hormonal activity in the organs (7). These differences have repercussions on specific sex features that contribute to the sexual attributes of both sexes (8).

In humans, the biological basis of sex differences includes morphological, physiological, and behavioral changes, the last mechanism involving cognitive functions such as social and gender identity, preferred recreational activity, choice of social partners, as well as prevalence of genetic syndromes, cognitive performance, and susceptibility to drugs (8). This challenging multidimensional emotional and cognitive processing creates expectations in terms of new models to study psychiatric disorders (9–12).

The use of common marmosets as a model in studies involving emotional response to distress began around 20 years ago (13, 14), but has increased considerably this century, since their significant potential was revealed in terms of etiological, predictive, reactive, and functional validations to respond to human psychogenic and physical stressors (9, 15, 16). This species is also known for its sociobiology (17), with a social system based on dominance hierarchy, where reproduction is restricted to dominant pairs living in extended groups of 3–15 animals (18–21). Thus, behavioral and physiological studies using these animals are particularly enriching since biological data can be analyzed in the context of evolutionary pressures (ultimate and proximate). Approaches that investigate social isolation may challenge these social animals and trigger pathologies after long-term exposure that induces structural, behavioral, and cognitive changes (22). In this respect, sex and age are modulators of stress, which is expressed differently depending on the nature of the stressors (23).

On the other hand, differences in stress response across the lifespan are associated with different brain maturity levels in the specific areas implicated in neural organization of stress response and also involve experience in interpreting the harmfulness of the stressor (24). Clinical evidence for the dissimilarity in physical and mental illness in males and females after chronic exposure to stress should be considered in research involving human beings (25). Additionally, the evidence that stress has a different impact depending on critical periods of increased plasticity in the nervous system over the lifespan is a compelling factor for using animal models to study this issue (26–28).

Therefore, since adults exhibit dimorphic response to stress, where males respond to social isolation with a higher level of cortisol than females, we hypothesize that this difference may occur before adulthood. Thus, in this study, we investigated whether the cortisol profile of immature male and female common marmosets exhibits a dimorphic pattern, in addition to analyzing hormonal profiles in both undisturbed and challenging situations. We also investigated the influence of acute and chronic stress on the cognition of juvenile males.

MATERIALS AND METHODS

Animals

Three experimental procedures using immature common marmoset (*Callithrix jacchus*) males and females were performed to investigate: (a) cortisol profile during natural development of male and female common marmosets living in their family groups (FGs) from birth until the onset of adulthood; (b) cortisol response to the paradigm of social isolation for 3 weeks in immature males and females at three age stages, according to the classification proposed by Leão et al. (29): juvenile I (6 months), juvenile II (9 months), and subadult (12 months); and (c) cortisol and cognitive performance after chronic social isolation for 4 months across the juvenile stage of males.

All animals used in the three experimental procedures were housed in the Laboratory for Advanced Primate Studies (LEAP, formerly Núcleo de Primatologia) at the Federal University of Rio Grande do Norte (UFRN), in cages under natural lighting, humidity, and temperature conditions. Animals received twice-a-day feedings that included seasonal fruits such as banana, papaya, melon, and mango, as well as potato and a protein potage containing milk, oats, egg, and bread. Water was available *ad libitum*. Animals were habituated to the presence of the researchers prior to the study, and a veterinarian provided health care throughout the experiment. The animals were treated in accordance with the criteria established by Brazilian Institute for the Environment and Renewable Resources (IBAMA) in Normative Instruction No. 169/2008 and Law No. 11.794, October 8, 2008, of the National Commission for Animal Care (CONCEA/Brazil). The LEAP follows international *ex situ* maintenance standards defined by the Animal Behavior Society and International Primatological Society. The studies were approved by the UFRN Ethics Committee for the use of animals (CEUA). The age, sex, and number of animals used in the three experimental protocols are shown in **Table 1**.

Experimental Procedures

Experiment I – Cortisol Levels Across Common Marmosets' Developmental Ages

Ten common marmosets (*C. jacchus*), six females and four males, were followed from birth to 16 months (early adulthood), encompassing infancy, juvenile, and subadult stages. They lived in their FGs in individual outdoor cages and were submitted to fecal sampling once a week.

TABLE 1 | Number of animals used in the three experimental procedures by sex and/or age.

Experiment	Males	Females
I – Animals living in their family groups monitored for 16 months from birth to early adulthood	4	6
II – Animals were moved from their families and monitored over a 3-month period at the following ages		
Juvenile I at 6 months	4	4
Juvenile II at 9 months	5	5
Subadult at 12 months	4	6
III – Animals were monitored for 5 months starting at juvenile I (from 7 to 11 months) under two conditions		
Living with their families for 5 months	5	–
Living with their families for 1 month and isolated for 4 months	5	–

Experiment II – Cortisol Response to Social Isolation of Immature Males and Females

Thirteen immature common marmoset males and females were used in this experiment. The animals were divided according to the age classification proposed by Leão et al. (29) into juvenile I (>4–7; 6 months: four males and four females), juvenile II (>7–10 months; 9 months: five males and five females), and subadult (>10–15 months; 12 months: four males and six females) (**Table 1**). The study consisted of three phases: (i) Baseline phase (Bp) – animals were living in their FG, in cages measuring 3 m × 2 m × 2 m, with feces collected on two consecutive days to measure fecal cortisol before isolation; (ii) Isolation phase (Ip) – animals were socially isolated in a new cage measuring 2 m × 1 m × 1 m, for 21 days and fecal collections were performed on the first 2 days (Ip1: days 1 and 2) and last 2 days (Ip2: days 20 and 21) of the isolation period. During this phase, the animals were in auditory and olfactory, but not visual, contact with their conspecifics; (iii) Reunion phase (Rp) – animals were returned to their family cages and fecal samples were collected during the first 2 days after reunion. Food and water were freely available during all phases of the experiment.

Experiment III – Effects of Chronic Social Isolation on Cortisol and Cognition in Juvenile Males

Cortisol

Ten juvenile I males, aged 7 months, were followed until the age of 11 months (**Table 1**) to investigate cortisol response and cognitive impairment after chronic social isolation compared to the control group. Animals were divided into two groups: (i) FG: composed of five animals living for 5 months within their FG in cages measuring 3 m × 2 m × 2 m; (ii) Isolated group (IG): consisting of five animals that were followed during the first month living in their FGs and 4 months after social isolation in an individual cage, measuring 2 m × 1 m × 1 m. The duration of social isolation was in accordance with other studies on chronic stress in non-human primates (22, 30). During the experimental period, the animals in both situations had auditory and olfactory, but not visual, contact with other conspecifics. Food and water were available without restriction. For both groups, fecal samples were collected on alternate days in the first month, considered the Bp and feces were collected daily during the first week (seven samples per animal) of the four successive months (W2, W3, W4, W5a); only in the fifth month were feces collected in the last week (W5b).

Memory Tests

In Experiment III, two reverse learning memory tests – (1) object test and (2) box test – were used to assess working memory in all animals of the IG and FG groups. None of the animals had been submitted to cognitive tests. The object memory test involves memory discrimination and was adapted from Roberts and Wallis (31). Easy-to-handle three-dimensional plastic objects of different shapes and colors (**Figure 1A**) were used. During the tests, two different objects were placed on top of two plates presented to the animals through feed drawers located below the unidirectional visor, without animal interaction with the experimenter (**Figure 1B**). In the direct phase, the same object was paired with food reward and was repeatedly presented with different objects on the empty plate throughout this phase of the trials. After the animal learned the task, the next phase (reverse) began. In the reverse phase, the animals had to learn that the food was associated with the object that had been changed during the trials.

The box memory test is a spatial memory test adapted from Murai et al. (32). A cuboid plexiglass box measuring 30 cm × 30 cm × 30 cm, with a 17-cm circular hole on one side, was used (**Figure 2**). This experimental apparatus was placed inside the cage and the experimenter observed the performance of the animals through a unidirectional visor in the front wall of the cage. During direct phase tests, the animal had to find the side of the box containing the hole, which was always facing upward, and obtain the reward. After the animal learned the task, the next phase (reverse) was initiated. In the reverse phase, the animal had to find the new side of the box where the opening was located and obtain the reward.

These tests were conducted inside the cage where the animals were housed. To test FG animals, they were rapidly separated, for 45 min at most, from their families into an adjoining cage divided by a solid wood plate. Before the beginning of the tests, in W5b, all animals were habituated to the experimental apparatus and FG animal acute family separation (10–15 min) was necessary during this protocol. All animals were randomly handled six times, three times in the morning and three times in the afternoon, over 3 days within 1 week.

After the 5-month period, memory tests were applied to animals from both IG and FG groups over the next 3 days. Object tests were conducted in the morning (between 7:30 and 9:00 a.m.) and box tests in the afternoon (between 2:00 and 4:00 p.m.) on the same day, always 2 h after the animals were fed. Trial duration was at most 3 min and the delay between trials was approximately 5 s. The animals were submitted to 15 trials for each of the direct and reverse phases of both object and box memory tests. In both phases of the two tests, it was expected that the animal would learn where the food reward was. When the animal achieved six consecutive correct responses, it was considered to have learned the task, which was then interrupted. In order to properly execute the reverse-phase task, the animal had to inhibit the learning

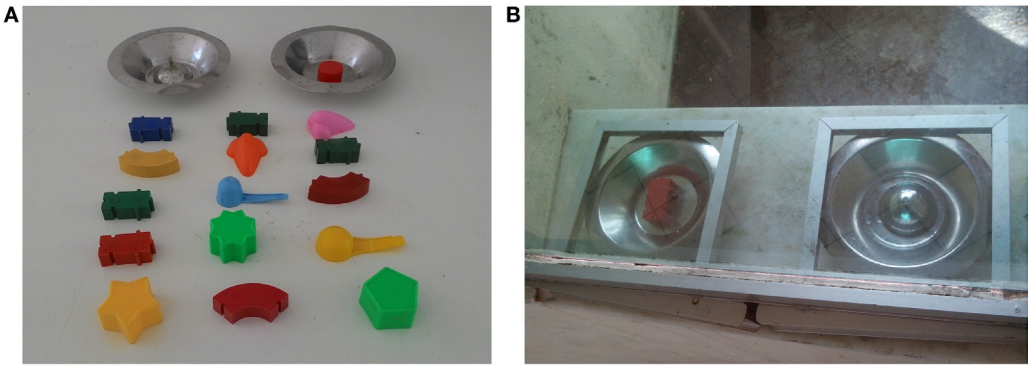

FIGURE 1 | (A) Plates and **(B)** objects that were used for memory tests.

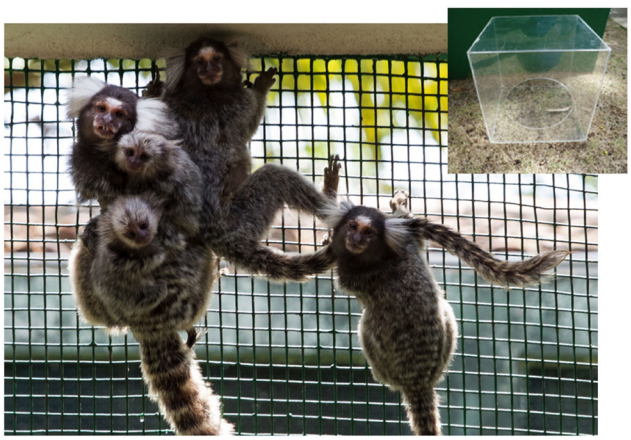

FIGURE 2 | The plexiglass container used for box memory tests at top right; common marmoset family living in the LEAP colony.

of the direct phase. The test results were calculated as learning indices (H = hits; E = errors) and motivation index (Li = lack of interest).

Fecal Cortisol Measurement

In Experiment I, fecal samples were taken in the morning and afternoon, since diurnal variation of cortisol during development must be monitored. In the other two experiments, cortisol was quantified from fecal samples collected at similar times, between 6:00 and 9:00 a.m., to avoid circadian variation in hormone profile (33). In all experiments, the observer was aware of defecation through a unidirectional visor. When the animal defecated samples were collected, identified, and stored in small glass tubes at −4°C until hydrolysis and solvolysis procedures for cortisol extraction and measurements.

For Experiment I, fecal collection was possible only when infants were 1 month of age or older. All processing of cortisol extraction and measurements using immune-enzymatic (ELISA) competitive assays were according to Sousa and Ziegler (34). Intra and interassay coefficients of variation were 1.97 and 3.39%; 23.40 and 29.40%; and 1.23 and 29.35% for Experiment I, II, and III, respectively.

Statistical Analysis

Experiment I

For the study of cortisol during development for the same individual across the lifespan, repetitive measures are observed. Moreover, the time of day and observations of a same animal are correlated. In this type of analysis, it is important that the models include possible dependencies among observations of the same individual. The changes in cortisol at different age phases are an example of these variations, since the focus is on evaluating hormonal changes across the phases. Therefore, it is necessary to simultaneously fit the model to the structure of the general mean as well as both intra and interindividual variability.

Logarithmic transformation of the hormonal data was performed to obtain good model fit and achieve normalization and variability constancy of the residues, demonstrating suitable model fit. After the significance of the age factor was detected, Tukey's test for multiple comparisons among means of the age stages was used.

Experiment II

After normalization of hormonal data by logarithmic transformation, the ANOVA parametric test for repeated measures and Fisher's *post hoc* test were applied to investigate cortisol variations using sex and age as independent variables and phases of the study as dependent variable. An outlier value of four standard deviations above the mean was excluded from one male aged 6 months.

Experiment III

After normalization of hormonal data by logarithmic transformation, the ANOVA parametric test for repeated measures and Fisher's *post hoc* were used to investigate cortisol variations between the groups (IG and FG) and independent variables through the phases (Bp, W2, W3, W4, W5a, and W5b). For memory tests, hit rates, errors, and lack of interest were transformed into continuous data for use in the non-parametric Mann–Whitney test to

investigate the difference between FG and IG in each phase (forward and reverse) of the test separately. The Spearman correlation test was performed using mean cortisol levels in W5b versus the sum of the indices for learning or motivation. An outlier value of four standard deviations above the mean was excluded from one animal in W3.

The significance level for all variables in the three experiments was set at $p \leq 0.05$. A near-significant trend when p was between $0.05 < p \leq 0.07$ was considered.

RESULTS

Experiment I – Cortisol Levels Across Common Marmoset Developmental Ages

The developing fluctuation patterns for fecal cortisol in males ($n = 4$) and females ($n = 6$) are shown in **Figure 3**. As previously stated, fecal collection was possible only after animals reached the infantile II stage and includes samples from the morning and afternoon. Thus, it is expected that cortisol levels reach higher levels than those observed for protocols where fecal collections include only samples from the morning, since cortisol excretion is significantly higher in the afternoon (34).

Mean total cortisol values are higher during the infantile II stage (>1–3 months) when compared to successive age-related development stages (ANOVA, $p < 0.05$) and were different for both sexes at juvenile II and subadult, with a significant decrease during the infantile III (>3–4 months) stage, which was statistically significant only for females when compared to the previous phase. From the juvenile stage to the end of the study, when animals were classified as subadults (>15–16 months), age-related cortisol excretion in females was higher than that of males at juvenile II (Tukey's test $p = 0.036$) and subadult ($p = 0.004$) ages.

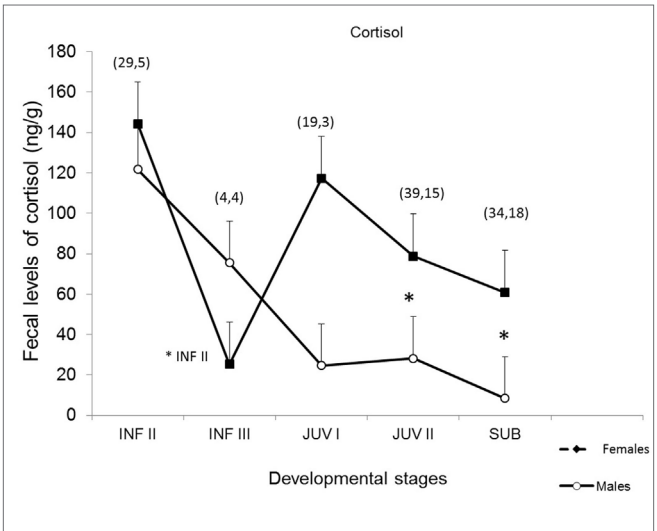

FIGURE 3 | Fecal cortisol in male ($n = 4$) and female ($n = 6$) common marmosets across developmental phases (INF I, II, III: infantile I, infantile II, infantile III; JUV I, II: juvenile I, juvenile II; SUB: subadult) [based on Ref. (29)]. Tukey's test was performed, $p < 0.05$. The symbol "* INF II" indicates significant statistical difference between ages INF II and INF III in females; The symbol "*" indicates significant statistical differences between males and females at juvenile II and subadult ages.

Experiment II – Cortisol Response to Social Isolation of Immature Males and Females

In Experiment II, we used immature animals at juvenile I ($n = 8/4$ males), juvenile II ($n = 10/5$ males), and subadult ($n = 10/4$ males) development stages living in their FGs, isolated in cages and reunited with their family. A significant interaction among sex, age, and phases was found (ANOVA; Sex × Ages × Phases: $F = 2.20$, $p = 0.04$) as shown in **Figure 4**.

Six-month-old males showed no variations in fecal cortisol levels across the phases (*post hoc* Fisher; Bp*Ip1, $p = 0.8$; Bp*Ip2, $p = 0.7$; Bp*Rp, $p = 0.3$; Ip1*Ip2, $p = 0.9$; Ip1*Rp, $p = 0.2$; Ip2*Rp, $p = 0.19$) (**Figure 4**). Nine-month-old males exhibited a statistically significant increase in cortisol levels during the initial isolation phase (Ip1) with respect to baseline (Bp) (*post hoc* Fisher; Bp*Ip1, $p = 0.04$) and a statistical tendency to reduction in the Rp with respect to Ip1 (*post hoc* Fisher; Bp*Ip2, $p = 0.13$; Bp*Rp, $p = 0.8$; Ip1*Ip2, $p = 0.60$; Ip1*Rp, $p = 0.07$; Ip2*Rp, $p = 0.18$) (**Figure 4**). Subadults (12 months) showed a statistical tendency to reduction in cortisol levels in the final isolation phase (Ip2) compared with baseline (Bp) (*post hoc* Fisher; Bp*Ip1, $p = 0.13$; Bp*Ip2, $p = 0.06$; Bp*Rp, $p = 0.34$; Ip1*Ip2, $p = 0.75$; Ip1*Rp, $p = 0.5$; Ip2*Rp, $p = 0.3$) (**Figure 4**).

For females, six-month-old animals showed a significant reduction in fecal cortisol levels in Ip2 with respect to Ip1 and Rp (*Post hoc* Fisher; Bp*Ip1, $p = 0.59$; Bp*Ip2, $p = 0.1$; Bp*Rp, $p = 0.3$; Ip1*Ip2, $p = 0.03$; Ip1*Rp, $p = 0.73$; Ip2*Rp, $p = 0.01$). Nine-month-old females exhibited a statistical tendency to increasing cortisol levels during the final isolation phase (Ip2) with respect to the initial isolation phase (Ip1) (*Post hoc* Fisher; Bp*Ip1, $p = 0.6$; Bp*Ip2, $p = 0.15$; Bp*Rp, $p = 0.9$; Ip1*Ip2, $p = 0.06$; Ip1*Rp, $p = 0.6$; Ip2*Rp, $p = 0.1$). Subadult (12 months) females showed a significant reduction in cortisol levels in the Rp compared with baseline (Bp) and Ip1 (*Post hoc* Fisher; Bp*Ip1, $p = 0.65$; Bp*Ip2, $p = 0.13$; Bp* Rp, $p = 0.01$; Ip1*Ip2, $p = 0.2$; Ip1*Rp, $p = 0.04$; Ip2*Rp, $p = 0.3$) (**Figure 4**).

Considering the entire set of results, it is noteworthy that young animals exhibit distinct cortisol response patterns in relation to sex and age, although no typical pattern could be characterized. In general, more immature males and females (juvenile I and II) exhibited different cortisol levels during the isolation phase with different intensities and temporalities, whereas subadult males and females showed a similar reduction in cortisol levels across experimental phases, as demonstrated by significant results and trends observed after statistical analysis.

Experiment III – Effects of Chronic Social Isolation on Cortisol and Cognition in Juvenile Males

Cortisol

Cortisol levels in the animals living with their families (FG) showed no statistically significant variations during the study (ANOVA, Fisher *post hoc* – W2*Bp: $p = 0.96$; W2*W3: $p = 0.94$;

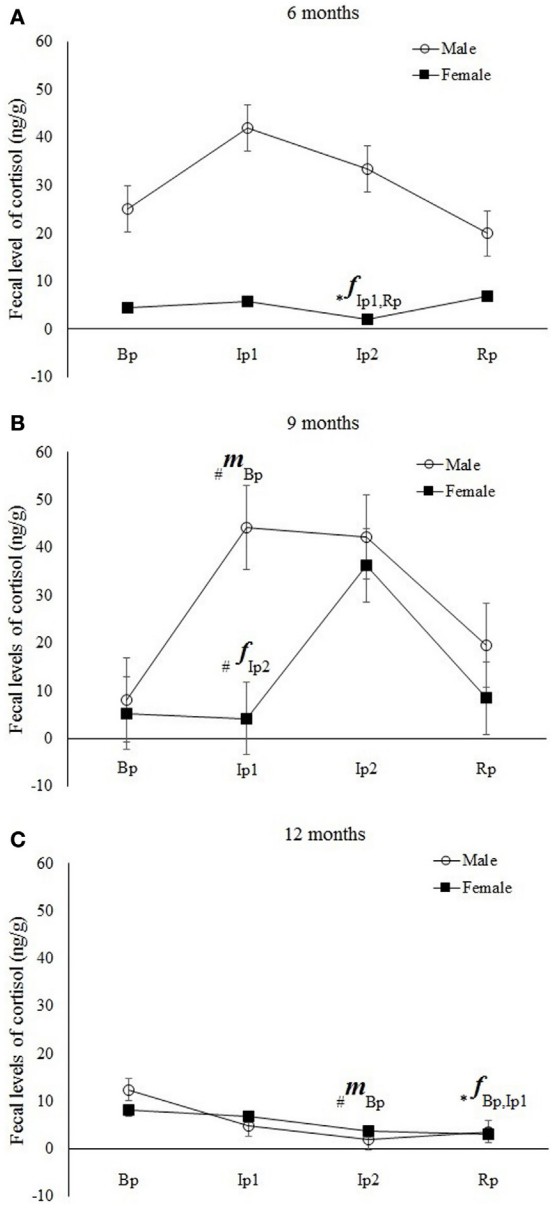

FIGURE 4 | Mean (±SE) of fecal cortisol levels (ng/g) in *Callithrix jacchus* in the Experiment II (Bp = baseline phase; Ip1 = initial isolation phase; Ip2 = late isolation phase; and Rp = reunion phase) at (A) 6 months, (B) 9 months, and (C) 12 months of age. A multivariate ANOVA test for repeated measures was performed with the Fisher posttest, $p < 0.05$. The symbol "*" indicates significant statistical differences and "#" a statistical tendency to differences in cortisol levels between the respective study phase and phases represented beside the symbol. The letters "*f*" and "*m*" indicate females and males, respectively.

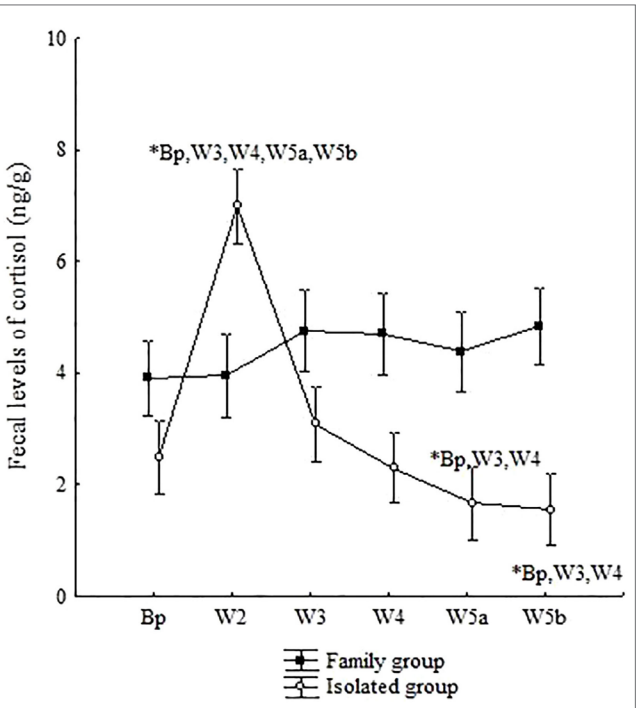

FIGURE 5 | Mean (±SE) of fecal cortisol levels (ng/g) in common marmoset males in Experiment III, living in family groups (FG) or under chronic social isolation for 4 months (IG). Bp = baseline phase; W2 = first week of second month; W3 = first week of third month; W4 = first week of fourth month; W5a = first week of fifth month; and W5b = last week of fifth month of isolation. A multivariate ANOVA test for repeated measures was performed using the Fisher posttest, $p < 0.05$. The symbol "*" indicates a significant difference in cortisol levels between the respective study phase and those represented beside the symbol.

W2*W4: $p = 0.8$; W2*W5a: $p = 0.82$; W2*W5b: $p = 0.48$; W3*Bp: $p = 0.91$; W3*W4: $p = 0.86$; W3*W5a: $p = 0.8$; W3*W5b: $p = 0.53$; W4*Bp: $p = 0.77$; W4*W5a: $p = 0.98$; W4*W5b: $p = 0.65$; W5a*Bp: $p = 0.78$; W5a*W5b: $p = 0.64$; W5b*Bp: $p = 0.44$) (**Figure 5**).

For the animals that were separated from their FG, a sharp rise in cortisol was observed in W2, with higher hormone levels in this phase when compared with all other phases of the study (ANOVA, Fisher *post hoc* – W2*Bp: $p = 0.001$; W2*W3: $p = 0.001$; W2*W4: $p = 0.001$; W2*W5a: $p = 0.001$; W2*W5b: $p = 0.001$). The decreasing cortisol profile during the isolation period was characterized by a gradual reduction across the phases, with the lowest concentrations in W5a and W5b in relation to baseline levels (Fisher *post hoc* – W3*Bp: $p = 0.51$; W3*W4: $p = 0.39$; W3*W5a: $p = 0.001$; W3*W5b: $p = 0.004$; W4*Bp: $p = 0.83$; W4*W5a: $p = 0.005$; W4*W5b: $p = 0.04$; W5a*Bp: $p = 0.003$; W5a*W5b: $p = 0.40$; W5b*Bp: $p = 0.02$) (**Figure 5**).

In short, it is interesting to observe that marmosets under chronic social isolation (IG) showed a different cortisol pattern in relation to that of the FG, ranging from an increase at the beginning of the isolation period to decreased levels in the final isolation phases.

Memory Tests

Object Memory Tests

The performance of FG and IG animals differed in the object memory tests, with higher hit (H) (Mann–Whitney $U = 45$, $p = 0.004$) and error (E) (Mann–Whitney $U = 36$, $p = 0.001$) rates for IG, whereas FG exhibited higher lack of interest (Li) levels than IG (Mann–Whitney $U = 30$, $p = 0.0001$) (**Figure 6A**). Since

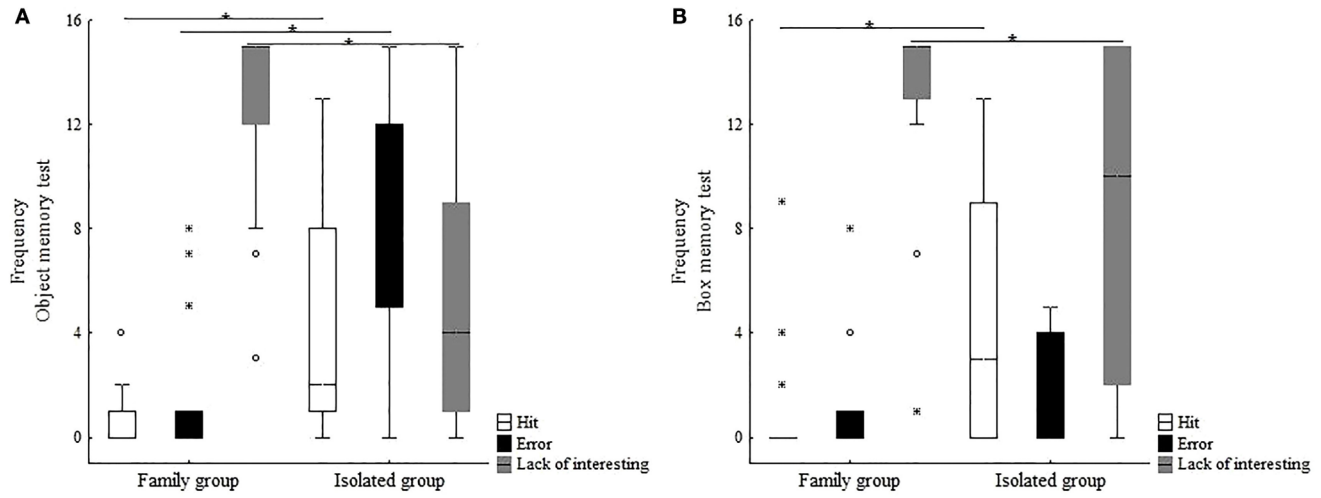

FIGURE 6 | Indexes of hits, errors, and lack of interest investigated in immature common marmosets at subadult age living with their families (FG) or in chronic social isolation (IG): (A) object memory tests and (B) box memory tests.

none of the animals in either group was able to learn the direct task, the reverse phase was not presented.

For both FG and IG groups, negative correlations between cortisol levels in the last week of study (W5b) and AI (Spearman correlation, FG: $r_s = -0.9$; IG: $r_s = -0.9$) were found.

Box Memory Test

When the animals were resubmitted to the direct phase of the box memory test (**Figure 6B**), significantly higher H rates were observed for IG (Mann–Whitney $U = 57$, $p = 0.021$), whereas FG displayed higher Li (Mann–Whitney $U = 65$, $p = 0.05$). No significant intergroup difference was found in the number of E (Mann–Whitney $U = 103$, $p = 0.713$). Only 3 of the 10 IG animals learned the direct task and started the reverse phase. However, none learned the reverse task in the time period used.

Again, for both groups (FG and IG), negative correlations between cortisol levels in the last week of study (W5b) and AI (Spearman correlation, FG = $r_s = -0.97$; IG = $r_s = -0.89$) were observed. Additionally, for IG, a negative correlation between cortisol and hits (Spearman correlation: $r_s = -0.9$) and a positive correlation with errors (Spearman correlation: $r_s = 0.9$) were recorded.

DISCUSSION

In this study, we investigated the age and sex of common marmosets (*C. jacchus*), a non-human primate widely used as an experimental model to obtain new information to better understand basal cortisol profile and postnatal stress exposure during developmental stages. We found a different profile for cortisol during marmoset development, with females showing low levels at infantile III, and higher at both juvenile II and subadult ages when compared to that of males. Immature males and females at 6 and 9 months of age moved alone from the FG to a new cage, over a 21-day period, expressed distinct patterns of cortisol variation with respect to range and duration of response but during juvenile to subadult transition (at 12 months) both males and females buffered similarly the hypothalamic–pituitary–adrenal (HPA) axis during chronic stress. Moreover, during juvenile to subadult transition, the patterns of cortisol reduction were maintained in males under social isolation for 4 months and were associated to better cognitive performance. The results for memory testing showed that acute and chronic stress impaired working memory and marmosets were more motivated to perform these tests when under chronic isolation.

Common marmosets, like other New World primates, exhibit extremely high cortisol levels and adaptations of the adrenal reticular zone, in terms of morphology and functionality that are different in males and females (35, 36). These authors demonstrated that the adrenals of neonatal marmosets exhibit functional secretion of the C19 steroid, as observed in humans, but adult males do not develop a functional reticular zone (35). They also found that anovulatory subordinates and ovariectomized common marmoset females displayed elevated cytochrome b5 (cytb5) expression in the adrenal reticular zone, generating significant ACTH-responsive DHEA production, suggesting both gonadal and sex differences in adrenocortical regulation.

Experiment I sought to characterize the cortisol production pattern in common marmoset males and females, from birth to early adulthood at the intermediate ages between infancy and adulthood, living in FGs in an undisturbed condition. The main findings included similar cortisol levels in infantile II, a significant decrease in cortisol production during infantile III, and a further increase that occurs only in females, with males maintaining low cortisol levels in juvenile II to subadulthood. In a study where common marmosets were also followed from birth to adulthood to investigate pituitary–adrenal basal function (27), ACTH and cortisol plasma levels were elevated in newborns and

infants (4 weeks) compared to 2-month-old infants, juveniles (6 months), subadults (12 months), and adults, but no intersex differences were observed. These general age differences are in accordance with the current study, but male and female cortisol differences across development were found in the present study and might have resulted from the fact that these authors did not collect samples from common marmosets at 4 months of age, thereby missing these transient changes at infantile III age. This result suggests that differences in the functioning between adrenals of male and female common marmosets might start early in life, during the transition between infantile and juvenile stages, and could be a consequence of different female needs for developing physiological systems, including differentiating the behavioral expression of their sexual strategies and/or different demands for immediate reproductive maturation. Gonadal activation in common marmoset males starts at around 4 months, i.e., around the infantile III stage, and this decrease observed only for females may be due to puberty steroid production and puberty changes at 6–7 months (37), sexual maturity occurring at approximately 16 months. For females, estradiol peaks start at around 6 months, and progesterone increases at about 13 months, with sexual maturity occurring at approximately 16 months (38, 39). However, studies in which females were paired with adult males showed that they were able to ovulate at the age of 10 months (40). Sex steroid production by these same animals was studied by Castro (41), who found that sex hormone secretion starts at around 5 months and puberty (sex steroid production in amounts close to those of adults) at 5–7 months, for both sexes, suggesting a common range for gonadal maturation in males and females. Thus, these results might be indicating a surge of an early dimorphic pattern of cortisol production in marmosets that is also expressed in adults, when the HPA axis of males is more responsible to separation than that of females, probably associated with different reproductive strategies (42).

In Experiment II, immature males and females in juvenile I and II and subadult stages were separated from FGs, and after reunion showed differences in cortisol profile. Common marmoset males in juvenile I, aged 6 months, exhibited a changeable pattern of cortisol over the course of the study (21 days). Females also showed a changeable profile of cortisol with a slight reduction in the last phase of isolation when compared to previous isolation and reunion phases. Juvenile II males and females, aged 9 months, also showed changeable cortisol profile but characterized by increased and distinct patterns of temporality. These findings seem to be compatible with development of the mechanisms that occur at this age related to maturation and integration of both HPA and HPG axes. Some studies suggest that maturation of the HPG axis is a major factor for the emergence of sexual dimorphism in the HPA axis (43). Thus, the sexual immaturity of animals observed in this study might have produced the variable cortisol profile. Alternatively, the small sample size may also have contributed to this variance. Assessing the temporal dynamics of stress response is important in determining the costs and benefits of this reaction (44). The return to baseline is an important component of the stress response because the deactivation systems reduce the risk of diseases associated with chronic stress (45, 46). Adjustments to feedback control systems during ontogenesis contribute to calibrating the stress response, making it more adaptive to that particular situation (47). In a related species of marmoset (*Callithrix geoffroy*), in which an acute stress protocol (8 h of isolation from the FG) was used for males and females at 6, 12, and 18 months (48), no significant sex differences were found in cortisol at any age. However, they observed age-related differences in cortisol variation over the 8 h, with the highest, intermediate, and lowest reactivity at 6, 12, and 18 months, respectively. Nonetheless, the evolution of cortisol profile for all animals was similar, with an increasing pattern from the first 2–4 h after isolation that remained high but stable during the last 4 h. Baseline cortisol values, which recovered 1 h after animals were returned to family cages, were similar at the three different ages.

At 12 months of age, male and female marmosets showed a similar response, and a decrease in cortisol and below-baseline levels was observed in the two last phases, with statistical significance for females and statistical tendency for males. Buffering of the cortisol response to stressors around pubertal period was also observed in other studies using this animal model (49). Sachser et al. (50) argue that the adaptive value of this buffering supports migratory behavior, especially in females, which has been recorded from early adulthood in a number of species, such as occurs for common marmosets where females are the main sex to emigrate (42).

In Experiment III, immature marmoset males living in FGs throughout the study showed no statistically significant variation in cortisol levels across the phases. The non-significant, but important, increase in cortisol levels observed in FG in W5b with respect to W5a was possibly due to acute (10–15 min) family separation during the period of habituation to the experimental apparatus in memory tests, which occurred six times over 3 days during w5b. Smaller oscillations in cortisol levels in the IG were likely due to social support, which attenuates routine daily challenges (51). Galvão-Coelho et al. (11) reported on the benefits of social support among adult common marmoset males during periods of crises, reducing cortisol changes caused by social stressors.

On the other hand, cortisol was significantly altered in juvenile common marmoset males isolated from FGs for 8–11 months compared with those living in natal groups. In the first week of isolation, marmosets showed an increase in fecal cortisol, followed by a progressive drop over the ensuing months, exhibiting lower than baseline levels at the end of the experiment, when these animals were in their FGs. Different theories attempt to explain HPA axis hyporeactivity to stress. Some studies report that hypocortisolism is an adaptive and defensive mechanism during episodes of chronic HPA axis activation (52–54). Badanes et al. (55) found that chronic buffering of HPA activity may promote vulnerability, especially in critical periods of ontogenetic development such as the juvenile stage, in addition to facilitating subsequent emergence of psychiatric disorders such as depression. Moreover, recent studies have also associated hypocortisolism with a number of mental disorders, such as atypical and bipolar depression (56).

In addition to cortisol measurement, after 4 months of social separation, the animals were submitted to working memory tests. Males from the IG showed a negative correlation between cortisol

and hits and a positive correlation between cortisol and errors. These findings are supported by evidence of a biphasic modulation of stress and cortisol profile suggesting that an increase in cortisol might be contributing to the impairment on this type of memory in the prefrontal cortex (PFC), where working memory occurs (57). Additionally, negative correlations between cortisol and AI for both groups (FG and IG) indicate that increased cortisol reduced motivation to perform working memory tests. Marmosets living in FGs were briefly separated, for 45 min at most, to undergo memory (object and box) tests. The number of hits and interest in performing the tests were lower than in marmosets in chronic social isolation. The three main reasons for the FG performing worse and being less motivated than their IG counterparts in the present study are as follows: (i) acute stress provoked by separation during the tests themselves and/or by a residual effect resulting from acute separation in the preceding period of habituation, which appears to have been too short to reduce the stress response; (ii) the fact that stress situations may produce motivational impairment and that isolated animals are deprived of sensorial stimuli; and (iii) animals are more aware of changes in their environment.

This study was based on the methodological approach developed by Roberts and Wallis (31) (object) and Murai et al. (32) (box) for common marmosets, in which few experimental sessions were used. The overall poor performance of the two groups in both direct and mainly reverse tasks calls into question the protocol used in this study, since other experiments report different levels of training required to learn visual discrimination tasks. Yamazaki and Watanabe (58) demonstrated that adult common marmosets require 20–300 trials before learning tasks, whereas our study used a maximum of 15 attempts per day, over 3 days. Spinelli et al. (59) report that adult common marmosets usually commit around 50 errors before learning a direct task and about 100 before learning a reverse task. Moreover, with training, marmosets were able to perform visual discrimination tasks and learn reversal tasks in both automated and non-automated working memory tests (32, 60, 61). Thus, the limitations shown by the animals in the present study are likely due to the lower number of trials used in the protocol, as well as the stress induced by acute (isolation for habituation before tests) and/or chronic isolation, which poses an additional challenge, thereby reducing learning. Furthermore, studies suggest that species with cooperative reproductive systems, such as common marmosets, perform better in cognitive tasks involving social learning, such as communication, imitation, and cooperation, than in tasks using working memory, inhibitory control, and tools (62).

In summary, the findings of the present study showed that during common marmosets' development, cortisol profile is variable, becoming dimorphic in males and females as early as 4 months of age. In both sexes, a cortisol decrease occurs at 3 months, with females showing a more acute decrease and recovery, to below birth levels, but higher than that of males through juvenile II and subadult ages. Cortisol in males and females was more changeable over 21 days of social isolation at 6 and 9 months, showing differences between males and females, although no typical sex pattern could be characterized. Cortisol decreased in both female and male marmosets at 12 months of age after 21 days of social isolation. As shown in juvenile males under chronic social isolation for 4 months, cortisol decline was sustained during this period and marmosets in this situation displayed better cognitive performance and motivation when compared to those submitted to short-term stress living in FGs. Thus, since cortisol profile seems to be sexually dimorphic early in life, age and sex are critical variables to consider in approaches that require immature marmosets in their experimental protocols. Moreover, the cognitive tests available should be scrutinized to allow better investigation of cognitive traits in this species.

AUTHOR CONTRIBUTIONS

MS and NG-C designed the experiments; MS and DC carried out statistical analysis of Experiment I; NG-C and AG carried out statistical analysis of Experiment II; NG-C and AG carried out statistical analysis of Experiment III; MS and AG prepared figures; DC collected experimental data from Experiment I; CS collected experimental data from Experiment II; AG collected experimental data from Experiment III; and MS, NG-C, and AG prepared the manuscript.

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The impact of neuroimmune alterations in autism spectrum disorder

Carmem Gottfried[1,2], Victorio Bambini-Junior[1,2,3], Fiona Francis[4,5,6], Rudimar Riesgo[1,7] and Wilson Savino[3]**

[1] Translational Research Group in Autism Spectrum Disorder (GETTEA), Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [2] Research Group in Neuroglial Plasticity, Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [3] Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, [4] Sorbonne Université, Université Pierre et Marie Curie, Paris, France, [5] INSERM UMR-S 839, Paris, France, [6] Institut du Fer à Moulin, Paris, France, [7] Child Neurology Unit, Clinical Hospital of Porto Alegre, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

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***Correspondence:**
Carmem Gottfried,
Ramiro Barcelos, 2600-Anexo,
Porto Alegre, Rio Grande do Sul 90035003, Brazil
cgottfried@ufrgs.br;
Wilson Savino,
Laboratório de Pesquisa sobre o Timo,
Instituto Oswaldo Cruz,
Fundação Oswaldo Cruz,
Ave. Brasil 4365, Manguinhos,
Rio de Janeiro 21045-900, Brazil
savino.w@gmail.com

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Autism spectrum disorder (ASD) involves a complex interplay of both genetic and environmental risk factors, with immune alterations and synaptic connection deficiency in early life. In the past decade, studies of ASD have substantially increased, in both humans and animal models. Immunological imbalance (including autoimmunity) has been proposed as a major etiological component in ASD, taking into account increased levels of pro-inflammatory cytokines observed in postmortem brain from patients, as well as autoantibody production. Also, epidemiological studies have established a correlation of ASD with family history of autoimmune diseases; associations with major histocompatibility complex haplotypes and abnormal levels of immunological markers in the blood. Moreover, the use of animal models to study ASD is providing increasing information on the relationship between the immune system and the pathophysiology of ASD. Herein, we will discuss the accumulating literature for ASD, giving special attention to the relevant aspects of factors that may be related to the neuroimmune interface in the development of ASD, including changes in neuroplasticity.

Keywords: autism, neuroimmune interactions, environmental risk factors, rodent models, valproic acid

History of ASD Studies

The first use of the term "autistic" was in 1911, by the Swiss psychiatrist Eugen Bleuler (1857–1939), referring to the limitation of human relationships and the loss of contact with reality presented by patients with schizophrenia (1). The term was then adopted by the Austrian pediatrician Hans Asperger (1906–1980) working at the University Children's Hospital-Vienna, referring to "autistic psychopaths." Asperger was investigating a form of autism spectrum disorder (ASD) now known as Asperger syndrome and not widely recognized as a separate diagnosis until 1981. In 1943, the Austrian-American psychiatrist Leo Kanner (1894–1981) used the term "autistic disturbances of affective contact" to describe 11 children with behavior marked by difficulties in establishing affective and interpersonal contact (2). He reported a form distinct from other diseases, such as schizophrenia, and that seemed to affect patients from the beginning of their lives. In the following year, Hans Asperger described cases exhibiting some characteristics similar to autism, which included difficulty

in social communication, but without cognitive loss. For further reading, see Ref. (3), in which Asperger's 1944 manuscript was translated.

In 1980, the term "autism" was first inserted in the third edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-III). In 1994, the fourth edition of the DSM included new criteria due to the need to identify subgroups of individuals with autism, for both practical purposes and research, considering the subdivisions: typical autism, pervasive developmental disorder not otherwise specified (PDD-NOS), and Asperger syndrome (4).

In the fifth edition, DSM considered instead of three domains of autism symptoms (social impairment, language/communication impairment, and repetitive/restricted behaviors), only two categories: (1) social communication impairment and (2) restricted interest/repetitive behaviors. Also, the new classification eliminated the previously separate subcategories into the broad term ASD (5–7). To simplify reading, the term "autism" or "ASD" will be used throughout the text representing the entire spectrum.

As a developmental disorder, ASD includes different degrees of severity and males are more affected than females by a ratio 5:1 approximately (8).

The number of cases in children increased by 23% between 2006 and 2008, reaching 1:88 children under 8 years diagnosed with any of the spectrum subtypes, and increased by 78% when the 2008 data were compared with the data for 2002. The overall prevalence of ASD for 2010 in the United States of America was 1:68 children aged 8 years and there is a clear growth in the number of identified cases (8). This can be due to advances in the knowledge of the symptoms associated with improvement in diagnostic criteria, as well as increase of environmental risk factors (drugs, pollutants, stress, etc.), especially during pregnancy, which may be related to changes in lifestyle of the society (8). In any case, this high prevalence indicates that the subject requires emergency measures due to the high economic, social, and family cost. The *Autism Speaks* organization estimates in the USA that the current costs of ASD reach US$137 billion per year, a number that has increased more than threefold since 2006.

Clinical Approach and Molecular Phenotypes

There are two complementary issues in the clinical approach for autism. The first is the general management, including diagnosis and evaluation of the intensity level of eventual core behavioral symptoms (9). The second considers treatment options, such as psychopharmacotherapy and different types of non-medical treatments. It is important to consider that ASD symptoms usually change during the patient's lifetime, and therefore, it is crucial for clinicians to be aware of age-related differences. Future perspectives in the treatment of ASD will probably include immunomodulation, stem cell therapy, and other approaches, after careful randomized controlled trials attesting the corresponding efficiency of these various strategies.

Although a number of definitions and improvements have been made in ASD, the etiological aspects remain unclear. The growing number of publications, especially in the last decade, leaves no doubt of the multifactorial aspect of the spectrum and indicates a complex interplay between genetic/environmental factors and the immune system, including stimulation of immune cells, generation of autoantibodies, cytokine/chemokine imbalance, and increased permeability of the blood–brain barrier (BBB) favoring leukocyte migration into the brain tissue (10).

In addition to clinical knowledge related to ASD, intense efforts have been directed toward identifying genes that specifically cause or increase the risk of developing autism, through both large genome-wide association studies and investigation of new candidate genes (11–16). It is estimated that 400–1000 genes may be related to ASD and large-scale studies in ASD and respective families have allowed the identification of candidate genes that may be related to the development of this disorder. Single-gene polymorphisms have been associated with ASD (17, 18), including those affecting contactin-associated protein like 2 (*CNTNAP2*); SH3 and multiple ankyrin repeat domains 3 (SHANK3); neuroligin 3 (NLGN3/4); copy-number variations and chromosomal abnormalities, such as the 15q11–q13 duplication and 16p11.2 deletions or duplications.

Other ASD candidate genes include forkhead box P2 (FOXP2); suppression of tumorigenicity 7 (ST7); IMP2 inner mitochondrial membrane peptidase-like (IMMP2L); reelin (RELN) at 7q22–q33, gamma-amino butyric acid (GABA)A receptor subunit; and ubiquitin-protein ligase E3A (UBE3A) on chromosome 15q11–q13 (19).

Table 1 illustrates polymorphisms with correlation to gut–brain axis abnormalities. The communication between these two systems needs to be further studied in order to identify possible key elements involved in ASD symptomatology. We mention a few examples as follows. The protein MET is a pleiotropic tyrosine-kinase receptor that functions in both brain development and gastrointestinal repair. An important functional variant (rs1858830 allele "C") of the gene encoding this protein has been demonstrated to be strongly associated with autism, as seen in a group of patients that also presented gastrointestinal disturbances (20) and altered immune response (21). Also, it was demonstrated that MET protein levels were significantly decreased in the cerebral cortex from ASD cases, compared to healthy controls (22), suggesting a dysregulation of signaling that may contribute

TABLE 1 | Selected genes altered in ASD, correlated with immune function.

Gene	Protein	Function
MET	Receptor tyrosine kinase (MET)	
PTEN	Phosphatase and tensin homolog (PTEN)	
TSC1	Tuberous sclerosis complex-1 (TSC1)	Promote IL-12 increase and M2-macrophage conversion
TSC2	Tuberous sclerosis complex-2 (TSC2)	
HLA-DRB4	Major histocompatibility complex type II (MHC-II)	
MIF	Macrophage migration inhibitory factor (MIF)	Guide and control of immune response
C4B	Complement component 4B (C4B)	

to altered neural circuit formation and function. As the MET receptor plays important function in regulating immune responsiveness (21), it is conceivable that it has a simultaneous influence upon both brain development and gastrointestinal function.

Overall, these data indicate molecular phenotypes, genetic risk factors, and gastrointestinal abnormalities, with the gut–brain axis. This hypothesis emerges from the observation that MET expression is decreased in temporal cortex from postmortem ASD brains and that the endogenous MET ligand, hepatocyte growth factor (HGF) is decreased in the gastrointestinal tract from ASD patients (17).

In a second vein, specific haplotypes related to the polymorphism of the *SLC6A4* serotonin transporter (SERT) gene correlate with hyperfunctioning of serotonin transporter SERT in brain, in circulating platelets, and in enterocytes (17), further indicating interconnections between genetic risk factors for autism and gastrointestinal abnormalities. The *SLC64A* gene is found on chromosome 17q11–12 and encodes one of the SERT genes. The 5-hydroxytryptamine-transporter length polymorphism (5HTTLPR) of the *SLC64A* gene has been considered to be associated with abnormalities seen in serotonin transporter binding in ASD (17, 23, 24). Serotonin receptors have also been found in the gut mucous layer (25), indicating possible implications in ASD since drugs that alter serotonin levels are taken orally.

In future studies, it will be important to improve the understanding of the relationships between genetic variation and phenotype. In fact, the wide diversity of core features in ASD and a varied occurrence of comorbidities make diagnostic procedure and clinical management of the patient more difficult, presenting a complex range of brain alterations with important changes in the frontal cortex.

It should be pointed out that, in addition to genetic alterations, environmental risk factors (such as infections, and drug use) during key periods of embryonic/fetal development may be associated with triggering ASD (26). It was demonstrated that modeling a situation of maternal infection (by maternal immune activation, MIA) in mice leads to permanent immune dysregulation in the progeny animals, together with autistic-like symptoms.

Cortical Connectivity Dysfunction in ASD

Although a consensus concerning structural and functional abnormalities in ASD remains difficult, a number of studies on these topics bring together important data, as shown in **Table 2**. Several abnormalities have been identified, which may have a relationship with neuroimmune changes during development. These include subtle defects in cortical architecture, aggravated perhaps by perturbed critical period activity-dependent remodeling of the network. Such changes lead to white matter defects and connectivity problems, which can, in some cases, be linked to behavioral abnormalities, as discussed below.

As previously mentioned, structural abnormalities are likely to be developmental in origin but may have diverse causes. Yet, before entering into this issue, it seemed worthwhile to describe normal cortical development (**Box 1**), also described in Ref. (56).

Environmental risk factors acting during cortical development (*in utero* effects related to maternal infections, stress, other agents, such as pharmaceutics, alcohol and drugs of abuse, and postnatal experience-dependent activities), can, hence, have heterogeneous influences on the formation of cortical areas. For example, maternal autoimmunity, infection during pregnancy, maternal age and obesity, gestational diabetes, and the presence of maternal *MET* variant rs1858830 "C" allele have been associated with the triggering of ASD through maternal immune activation, which could manifests as changes in the maternal peripheral cytokine milieu, generation of immunoglobulin G (IgG) and autoantibodies reactive to fetal brain proteins in different areas, such as frontal cortex (59).

Increased brain size ("macrocephaly") in the first years of life is now firmly associated with ASD (60, 61). This may have its origins in increased numbers of neurons in some brain areas (as the prefrontal) compared to normal individuals (28), or more prominently in increased cell size (soma, axonal tracts, and dendrites), or in combination of both, in localized regions. Accordingly, differences are observed in ASD patients in gray or white matter volumes, both identified in MRI studies (62, 63). These increased volumes in ASD are associated with aberrant connectivity, which may combine over and under-connectivity. As mentioned below, structural and functional data revealed a connectivity disturbance, affecting frontal, fronto-temporal, fronto-limbic, fronto-parietal, and inter-hemispheric connections (31, 64).

Concerning potential gray matter abnormalities during childhood, in postmortem studies, 79 and 29% more neurons were identified in dorsolateral and medial prefrontal regions, respectively, in ASD (28). Furthermore, subtle defects in the radial organization and local densities of neurons ("minicolumns") in different cortical areas, including the frontal cortex have been identified in brains from both ASD children and adults (65, 66), reviewed by Zikopoulos and Barbas (31). Occasionally, nodular subependymal heterotopia has been identified in ASD (33), suggesting local progenitor or neuronal migration abnormalities, although this may be rare. In the adult, increased numbers of neurons are not obvious (31), although minicolumn changes, with subtle abnormal lamination have been identified occasionally in certain areas (35, 67).

Increased dendritic spine densities have also been observed notably in layer 2 of lateral prefrontal association areas (36). Parvalbumin positive interneurons have been shown in postmortem studies to be less numerous in dorsolateral prefrontal regions (31). Blurred white and gray matter boundaries are also regularly observed in ASD (37, 65), and this has been suggested in other situations (e.g., schizophrenia) to be due to an excess of interstitial neurons in the white matter, which may have their origin in the subplate (68). Overall, several neuronal density and distribution alterations, localized to certain areas, are associated with ASD.

A number of changes in brain seem to be related to late prenatal or early postnatal periods. Transiently enlarged white matter volumes (related to abnormal axonal tracts) have been documented in ASD infants exhibiting enlarged head circumferences. White matter volumes in these individuals then increase less slowly during childhood compared to control individuals [reviewed by Cassina et al. (69)]. Axonal tracts have been studied

TABLE 2 | Anatomical studies of brains from individual with ASD.

Phenotype	Brain area	Method	Studied	Age (autism)	Sex	Reference
Macrocephaly	Head	Head circumference	*n* = 208 probands, *n* = 147 parents, *n* = 149 siblings, and other controls	9.7 ± 5.4 years (3–47 years)	5.9M:1F	(27)
Neuron number	DL-PFC and M-PFC	Postmortem	*n* = 7 autistic, *n* = 6 control	2–16 years	Male	(28)
WM volume	Head	MRI	41 boys (13 autistic, 14 DLD, 14 normal control); 22 girls (7 DLD, 15 normal controls)	9.0 0.9 years (autistic), 8.2 1.6 years (DLD), and 9.1 1.2 years (controls)	Males and females	(29)
GM volume, Gyral thickness	Head-temporal and parietal lobes affected	MRI	*n* = 17 autism, *n* = 14 controls	8–12 years	Male	(30)
PV interneurons	DLPFC	Postmortem	*n* = 2 ASD *n* = 2 matched controls for age, sex, and hemisphere	30–44 years	Male	(31)
Minicolumns	Prefrontal (area 9) and Temporal (areas 21, 22) lobe	Postmortem	*n* = 9 autism brains and *n* = 4 and 5 controls	5–28 years	–	(32)
Neuron migration disorders	Brain	Postmortem	*n* = 13 autism, *n* = 14 controls	4–62 years	9 males 4 females	(33)
Cell density cortical layers	ACC	Postmortem	*n* = 9 autism brains and controls			(34)
Cortical layers	PCC FFG	Postmortem	*n* = 8–9 autism, *n* = 7–8 control	19–54 years (PCC), 14–32 years (FFG)	Male	(35)
Dendritic spines	Frontal, temporal and parietal (layer II), layer V (temporal)	Postmortem Golgi	*n* = 10 autism; *n* = 10 and 5 controls	10–45 years	Males	(36)
Gray-white matter boundary	STG, DL-frontal, and DL-parietal		*n* = 8 ASD, *n* = 8 control	10–45 years	Males	(37)
Axons	ACC, OFC, LPFC	Postmortem	*n* = 5 autism, *n* = 4 control	30–44 years	4 male, 1 female	(38)
Corpus callosum	CC	MRI	*n* = 253 autism, *n* = 250	Meta-analysis (10 studies)	Male >74%	(39)
Corpus callosum	CC	MRI	*n* = 17 autism, *n* = 17 control	16–54 years	Males and females (3)	(40)
Brain development	White matter	DTI (prospective, longitudinal)	*n* = 28 ASD, *n* = 64 control	6–24 months	Males and females	(41)
Brain	White matter (CC)	DTI (prospective, longitudinal)	*n* = 100 ASD, *n* = 56 controls	3–41 years	Males	(42)
Brain	White matter (CC)	DTI	*n* = 43 autism (or PDD, or ASD); *n* = 34 controls	7–33 years	Males	(43)
Brain	White matter and activation (ACC)	DTI and fMRI	*n* = 12 ASD (autism), PDD or Asperger; *n* = 14 control (6F)	20–40 years	Males and females (2)	(44)
Brain	White matter (arcuate)	DTI	*n* = 13 autism; *n* = 13 siblings, *n* = 11 controls	6–13 years	Males and females (2)	(45)
Brain	White matter (several areas)	DTI	*n* = 7 autism; *n* = 7 controls	11–18 years	Males and females (1)	(46)
Brain	Theory of mind areas	fMRI	*n* = 12 high functioning autism; *n* = 12 control (6F)	15–35 years	Males and females (2)	(47)
Brain	Several areas	MRI/DTI	*n* = 18 autism; *n* = 18 control	6–12 years	Males and females (2)	(48)
Brain	Language and spatial	fMRI	*n* = 12 autism; *n* = 13 control	Mean 22.5 years	Males and females (1)	(47)

(Continued)

TABLE 2 | Continued

Phenotype	Brain area	Method	Studied	Age (autism)	Sex	Reference
Brain	Working memory face recognition	fMRI	*n* = 11 high functioning autism; *n* = 11 controls	24.5 ± 10.2 years	Males	(49)
Brain	Reading comprehension	fMRI	*n* = 17 high functioning autism; *n* = 17 controls	–	–	(50)
Brain	Resting state	fMRI	*n* = 13 (6 autism; 6 Asperger; 1 PDD); *n* = 12 control	15–52 years	Males	(51)
Brain	Resting state	fMRI	*n* = 16 high functioning (6 autism, 2 Asperger; 8 PDD); *n* = 15 controls	13–17 years	Males and females (2)	(52)
Brain	Executive function (Tower of London task), CC size	fMRI	*n* = 18 high functioning autism; *n* = 18 controls	27.1 ± 11.9 years	Males and females (1)	(50)
Brain	Resting state	fMRI	*n* = 12 high functioning; *n* = 12 control	26 ± 5.93 years	Males and females (1)	(53)
Brain	Source recognition task	fMRI	*n* = 10 ASD 6 autism, 4 Asperger; *n* = 10 control	14–43 years	Males	(54)
Brain	Face processing	fMRI	*n* = 19 high functioning (8 autism, 9 Asperger, 2 PDD) and *n* = 21 control	23.5 ± 7.8 years	–	(55)

BOX 1 | General features of normal cortical development.

During early steps of cortical development, stem cells and progenitor cells divide in zones close to the cerebral ventricles before giving rise to neurons, which migrate long distances to reach the developing cortical plate. Future principal and inhibitory neurons are derived mainly from dorsal and ventral telencephalic regions, respectively. Critically timed neuronal activity is essential for circuit development, both intrinsic activity and sensory derived, affecting synaptogenesis and remodeling. Synaptic pruning removes unused and unwanted connections to refine the synaptic patterns. Timing is critical and activity-dependent processes contribute to spine turnover and maturation (57). Excitatory synapses are generally formed first, followed by inhibitory synapses. The temporal regulation of synaptogenesis is likely to be highly regulated for a correct excitatory: inhibitory balance. Myelination of mature neurons is another critical process ensuring correct functional connectivity in a timely fashion between neurons. In the primate, it has been shown that cortical areas take different amounts of time to form (58).

by confocal and electron microscopy in postmortem tissue (38), showing fewer large axons in the deep white matter of the anterior cingulate cortex (likely representing long-range cortico-cortical connections), a higher proportion of branched axons of medium caliber, and a significantly increased density of thinner branched, axons in the superficial white matter (likely connecting nearby areas). Other neuroimaging studies have shown reductions in the strength of long-distance connections, e.g., sensory input to prefrontal cortex and inter-hemispheric connections (40, 43, 70–72). Such defects would be expected to have quite severe network effects.

Travers et al. (73) (and references therein) summarize and compare 48 peer-reviewed diffusion tensor imaging (DTI) studies. Preliminary findings suggest that developmental trajectories of fractional anisotropy in ASD infants are also different from controls, and may mimic the accelerated brain volume phenotype (41, 73). Despite small sample sizes, the corpus callosum was found in several DTI studies to be reduced in volume [Ref. (43), see Figure 3 of Ref. (73)], and in one study the authors further found this result to be specific to patients who did not have macrocephaly (61). Interestingly and *vice versa*, callosal agenesis is also associated with autism-like symptoms. Concerning microstructure, fractional anisotropy was found reduced in anterior regions or across the length of the corpus callosum in multiple studies (42, 43, 73). This may be due to reduced myelination or larger axon diameter or reduced density. In some studies, this finding was associated with reduced performance IQ and reduced callosal volume (43). Differences were observed in ASD patients, concerning the cingulum bundles, which are primary inter-hemispheric-association pathways associated with executive function, connecting the medial cingulate cortex with temporal lobe structures, such as the hippocampus, consistent macrostructural, and reduced fractional anisotropy (44). Relatively concordant results of decreased fractional anisotropy were obtained at the beginning of the arcuate fasciculus (although heterogeneous results were obtained for the whole tract) in the region of the temporo-parietal junction and superior temporal gray matter (45, 46, 73). This latter region is associated with social perception, and gray matter structure and functional connectivity differences have also previously been identified (47). For the moment, relationships between DTI measures and ASD symptoms remain

only preliminary and future work with defined patient groups will deepen these correlations [see also Ref. (44)].

Studies using DTI also show differences in the cerebellar fibers that connect to various brain regions, demonstrating altered cerebellar feedback projections in ASD (74). In addition, neuropathological studies have also reported a decrease in Purkinje cell density in the cerebellum of ASD patients indicating that this abnormality may contribute to selected clinical features of the autism phenotype (75).

Functional magnetic resonance imaging (fMRI) studies are being used to assess synchronous activated and deactivated brain regions during cognitive tasks and in resting states in ASD patients [reviewed by Rathinam et al. (76)]. It appears that the most consistent functional results refers to a decreased connectivity between frontal and more posterior brain regions (in high-functioning patients), performing a variety of tasks. These include task integrating language comprehension (frontal) and spatial processing (parietal) (77); in working memory tasks related to face recognition [involving frontal executive and occipito-temporal fusiform gyrus regions (49)], and in reading comprehension requiring language comprehension and working memory (50). Similarly, frontal-posterior under-connectivity has also been found in studies of patients at rest, revealing hence spontaneous brain activity connections (51, 52, 78). These resting state studies suggest that abnormal connectivity may already exist in patient brains, not specifically related to different tasks, and perhaps indicating a structural basis for some differences, as suggested above. There is, however, some heterogeneity in other fMRI results, since some tasks in some patients have also shown frontal-posterior over-connectivity (79), fronto-frontal, or posterio-posterior over-connectivity in the resting state (53). Analyzing connections with other brain regions, e.g., subcortico-cortico, has also in several cases revealed over-connectivity [e.g., in task-independent tasks, Ref. (72) and references therein], or under-connectivity (55). A variety of other brain regions have been analyzed contributing to the variability of the results obtained [Ref. (80) and references therein]. In addition, transcranial ultrasonography may be a useful screening technique for children at potential risk of ASD, providing rapid, non-invasive evaluation of extra-axial fluid and cortical lesions (81). Further work, potentially involving new methods, may help to clarify under- or over-connectivity in different brain regions.

Whether or not these changes are related to neuroimmune interactions is a completely open field of investigation. In particular, it should be helpful to perform correlation studies between the above described changes with specific immune activation states, such as infections.

Crosstalk Between the CNS and the Immune System in ASD

The crosstalk involving the immune and nervous systems encompasses a complex and intricate pathway of signals with extensive communication between them in health and disease (82, 83). Cytokines and chemokines modulate brain function, as well as systemic and CNS responses to infection, injury, and inflammation (84). In fact, cytokines, such as TNF-α, IL-1β, IL-6, and TGF-β family, are able to modulate neuronal activity (85) and IL-6 promotes oligodendrocyte survival (86).

Pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-12, interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α), are involved in CNS pleiotropic effects during neurodevelopment (87) and have been extensively studied in patients with ASD. In a pioneer work indicating immune dysfunction in ASD (88), cell-mediated immune response was assessed *in vitro* by phytohemagglutinin (PHA) stimulation in lymphocyte cultures from 12 children with ASD and 13 control subjects: the ASD against neural antigens, produced by the mother during pregnancy (89–92), and that may induce changes in neural development and plasticity in the developing embryo/fetus.

Anti-double-stranded DNA antibodies and anti-nuclear antibodies were measured in the sera of 100 autistic children, aged between 4 and 11 years, in comparison to 100 healthy-matched children (93). In this study, the authors found increased levels of anti-double-strand DNA (34%) or anti-nuclear antibodies (25%) in ASD children. Furthermore, meta-analysis of data reported in patients with ASD clearly revealed alterations in different cytokines, both in plasma and in brain, as seen in **Table 3**.

Also, although ASD patients present reduced amounts of total IgM and IgG immunoglobulins contents, they exhibit increased levels of antibodies against various proteins expressed in the nervous tissue, e.g., serotonin receptors, myelin basic protein, heat shock protein, and glial fibrillary acidic protein (GFAP) (107, 108). Recently, the presence of autoantibodies against human neuronal progenitor cells (NPCs) was assayed in sera from children with ASD (109). Immunoreactivity against multiple NPC proteins of molecular sizes ranging from 55 to 210 kDa was found in the ASD group, significantly differing from control individuals. This is in keeping with the fact that in the mouse model of autism following maternal immune activation triggered by poly(I:C)-injection, offspring exhibited a reduction of 50% in the numbers of regulatory T lymphocytes ($CD4^+Foxp3^+CD25^+$) in the spleen (110), indicating a dysfunction in the regulation of the immune response in autism.

As mentioned above, studies in animal models indicate that maternal immune activation leads to autistic-like behavioral patterns in the offspring (111, 112). In addition to B and T cell abnormalities, changes in the innate immune response have been reported. Using *in vitro* experiments, it was demonstrated that ASD individuals have a reduced capacity of natural killer (NK) cells to kill K562 target cells (an immortalized myelogenous leukemia cell line) (113). Thus, it is likely that an aberrant group showed impaired lymphocyte PHA-induced proliferation when compared to control subjects.

In the following years, the hypothesis of autoimmunity involving the CNS was postulated as a key issue in the pathogenesis of autism and various clinical studies indicated a link between dysfunctional immune activity and ASD, including maternal immune abnormalities during early pregnancy (10, 114) and increased incidence of familial autoimmunity (115). Additionally, autoimmunity triggered by viral or bacterial infections has been considered as risk factor to ASD development (87, 116, 117). It has also been demonstrated in humans that family history of autoimmune disorders is more common in families of children with ASD (118). In addition, immune-mediated disorders during

TABLE 3 | Altered cytokines in autism spectrum disorder (ASD).

Cytokines	Level compared to control group	Source	Evaluated subjects	Reference
		INTERLEUKINS		
IL-1β	↑	Plasma	Children with ASD	(94)
	↑	Plasma	Children with ASD	(95)
	↑	Plasma	Adults with severe ASD	(96)
	↑	Blood cells	Children with ASD	(97)
	↑ (TLR2 or TLR4 stimulation)	Blood cells	Children with ASD	(98)
	↓ (TLR-9 stimulation)	Blood cells	Children with ASD	(98)
IL-6	↑	Plasma	Children with ASD	(94)
	↑	Plasma	Adults with severe autism	(96)
	↑	Blood cells	Children with ASD	(97)
	↑ (TLR2 or TLR4 stimulation)	Blood cells	Children with ASD	(98)
	↓ (TLR-9 stimulation)	Blood cells	Children with ASD	(98)
	↑	Lymphoblasts	Children with ASD	(99)
	↑	Cerebellum (postmortem)	Children with ASD	(100)
	↑	Brain (postmortem)	ASD subjects (children and adults)	(101)
	↑	Brain (postmortem)	ASD subjects (children and adults)	(102)
IL-12 P40	↑	Plasma	Children with ASD	(94)
		CHEMOKINES		
CCL2	↑	Brain (postmortem)	ASD subjects (children and adults)	(101)
		Plasma	Children with ASD	(94)
		TUMOR NECROSIS FACTOR		
TNF-α	↑	CSF	Children with ASD	(94)
		Brain (postmortem)	Children with ASD	(103)
		INTERFERON		
IFN-γ		Serum (mid-gestational)	Mothers giving birth to child with ASD	(6)
	↑	Whole blood and serum	Children with ASD	(104)
		Brain (postmortem)	ASD subjects (children and adults)	(103)
		GROWTH FACTORS		
TGF-β1	↓	Plasma	Children with ASD (Lower levels correlated with more severe behavioral scores)	(105)
	↓	Serum	Adults with ASD	(106)
BDNF	↑	Brain (postmortem)	ASD subjects (children and adults)	(101)
	↑	Plasma	Children with ASD	(94)

IK, interleukin; IFN, interferon; TGF, transforming growth factor; TLR, toll-like receptors.

pregnancy, such as allergy and psoriasis, are more frequent in mothers of children with ASD compared with mothers of children with typical development (119).

Yet, the biological mechanism(s) of maternal immune dysfunction that could be involved in triggering ASD remain(s) unclear. One possibility involves the generation of antibodies activity of these components of innate immunity may also contribute to atypical immune activity seen in patients with ASD.

Moreover, increased numbers of circulating monocytes, important precursors for macrophages, dendritic, and microglial cells, have been observed in the blood and in the postmortem brain tissue from ASD individuals, associated with the presence of perivascular macrophages (101, 120). Furthermore, analysis of cytokine serum levels in children with ASD revealed a representative profile of myeloid cell activation, with increased production of IL-14, IL-12p40, TNF-α, IL-1β, and IL-6 (94–97, 121). Also, increased level of TNF-α was found in cerebrospinal fluid of children with ASD (122).

In respect to caspases, a group of cysteinyl aspartate-specific proteases involved in apoptosis and several other cell functions, it has been shown that the activation of some members of the caspase family contributes to the differentiation of monocytes into macrophages, in the absence of cell death (123). Interestingly, the mRNA levels for caspases 1–5, 7, and 12 were significantly increased in ASD patients as compared to healthy subjects, suggesting a role of the caspase pathway in ASD clinical outcome and as potential diagnostic and/or as therapeutic tools (124). These studies will hopefully provide new insights in the mechanisms of caspase activation and abnormal differentiation of monocytes into macrophages in ASD.

Considering that monocytes are key elements for the immune response, these alterations may result in long-term immune alterations in ASD children, with adverse neuroimmune interactions, ultimately contributing to the ASD pathophysiology. Also, it was found increased expression levels of pro-inflammatory cytokines TNF-alpha and IL-6, and decreased Bcl2 expression in lymphoblasts (99) and decreased levels of TGF-β in plasma (105) and in serum (106) of autistic subjects.

Moreover, considering that increased levels of anti- and pro-inflammatory cytokines have been observed in ASD individuals (6), it is conceivable that cytokines are also involved in the pathophysiology of ASD.

Taking into account, the environmental *in utero* influence in triggering ASD changes in oxidative stress responses may also correlate with activation of the hypothalamic–pituitary–adrenal (HPA) axis. Upon activation, the hypothalamus secretes corticotropin-releasing hormone (CRH), stimulating the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn stimulates the cortex of the adrenal glands to release glucocorticoid, which plays an important role in adaptive responses (125), including immunosuppression. This response can signal to the organism under stressful events, such as environmental adverse factors *in utero*. In fact, patients with ASD present elevated blood levels of nitric oxide (NO), nitrites, and nitrates (126). These molecules might increase the permeability of BBB and intestinal permeability, as commonly found in autism (127). Furthermore, ASD patients have diminished antioxidant systems in plasma, including decreased amounts of glutathione (GSH), vitamins (A, C, and E), and antioxidant enzymes (superoxide dismutase and glutathione peroxidase) (128–130). The increase in oxidative stress can potentially induce dysfunction in the immune system, plasticity and function of the thymus and stimulate neuroinflammatory infiltrates. Potentially, this set of dysfunctions may be associated with the behavioral abnormalities, gastrointestinal disorders, and sleep disturbances present in autism. In an animal model of ASD induced by prenatal exposure to valproic acid (VPA), a reduced thymus size was observed in the VPA group, compared to the control animals (131), indicating that T-cell development can also be affected in autism, and may be at the origin of both T and B cell dysfunctions seen in ASD, including neuroinflammation.

One important point is that, although the well accepted fact that the CNS undergoes constant immune surveillance that takes place within the meningeal compartment (132), the real mechanism(s) that guide(s) the entrance and exit of immune cells from the CNS remains to be demonstrated. Recently, an interesting investigation revealed the presence of structures with functional lymphatic vessels lining the dural sinuses, in a place difficult to visualize and actually so far ignored. These structures present characteristics of lymphatic endothelial cells and are able to carry both fluid and immune cells from and into the cerebrospinal fluid. Importantly, these structures are connected to the deep cervical lymph nodes (132). From this view, it is clear that a new and important window of investigation starts, in the search for possible link(s) connecting triggering of ASD to immune system impairment and *vice versa*.

Evidence for Neuroimmune Interactions in ASD

The intercommunication between the brain and blood systems is followed by integrative exchanges, and the BBB permeability is variable, depending on the vessel type (artery, capillary, or vein) (8). During development, neurons, astrocytes, oligodendrocytes, and microglia intercommunicate in a paracrine/autocrine manner (133), withstand endocrine and immune systems influences, particularly during pregnancy, which can impair functions of the nervous system. Microglial cells in turn act as surveillance systems, with the capacity to respond phenotypically with varying degrees of activation to fluctuations in microenvironment stimuli or to transient or chronic damage, reaching the phagocytic state in the event of cell death (134). These cells also present dynamic movements or projections able to detect irregularities in neural microenvironments, in both intra and extracellular milieu and can increase in number by proliferation or through the entrance of macrophages into the brain (135).

In this vein, it was demonstrated by analysis of postmortem brain tissue that individuals with autism have an increased number of activated microglial cells (136).

Figure 1 illustrates alterations found in both blood and postmortem brains of patients with ASD, including blood/brain cell activation, autoantibody production, and alterations in levels of different molecules that can modify cell signaling, brain response, and BBB permeability. The associated neuroinflammatory process does support the hypothesis of neuroimmune interactions in the pathogenesis of ASD.

The analysis of postmortem brains from ASD individuals indicates changes in synaptic organization, dendritic arborization, neurotransmission (i.e., GABAergic, serotonergic, and glutamatergic pathways), and glial cells. Accordingly, recent studies suggested an important role for astrocytes and microglial cells in ASD, with alterations in GFAP expression (137), and increases of pro-inflammatory cytokines (6).

Molecules secreted by the brain's immune system may influence neurodevelopment. As already mentioned above, individuals with autism have a marked neuroinflammation, with microglial activation and increased NO, as well as production of chemokines and pro-inflammatory cytokines (6, 101). There is evidence that an increase of TNF-α is associated with stereotypic behaviors similar to those found in individuals with autism (138). Moreover, soluble cytokine receptors that are normally present in blood can regulate peripheral cytokine and lymphoid activity (139–141). Further elucidation and characterization of the molecular pathways that mediate soluble cytokine receptor signaling in ASD will promote new strategies for therapeutic interventions.

In addition, as demonstrated in **Table 1**, the genes *MET*, *PTEN*, *TSC1*, and *TSC2*, for example, encode proteins related to the phosphoinositide3-kinase (PI3K) pathway, which plays an important role in suppressing the production of the pro-inflammatory cytokine IL-12 (142). MET is important to the developing brain, particularly to the neocortex and cerebellum in two regions frequently compromised in autism (22). Also, alteration in this gene can be correlated with increased immune response, involving cytokine expression (21) and regulation by small RNAs (miRNA) (143), which are presently known to be associated with the immunological response, such as lymphocytic phenotypes or key points during hematopoiesis (144). In ASD, various miRNA are altered in the blood, providing new clues in the search for new molecular targets in the study of autism (145–147). One is miR-132, altered in both autism and schizophrenia, and that can participate in brain plasticity, connectivity, and regulation of immune responses (145).

Another area implicated in autism is the cerebellum, and immunological studies indicate increased levels of IL-6 in the cerebellum of ASD subjects, stimulating the formation of granule cell excitatory synapses, without affecting inhibitory synapses (100).

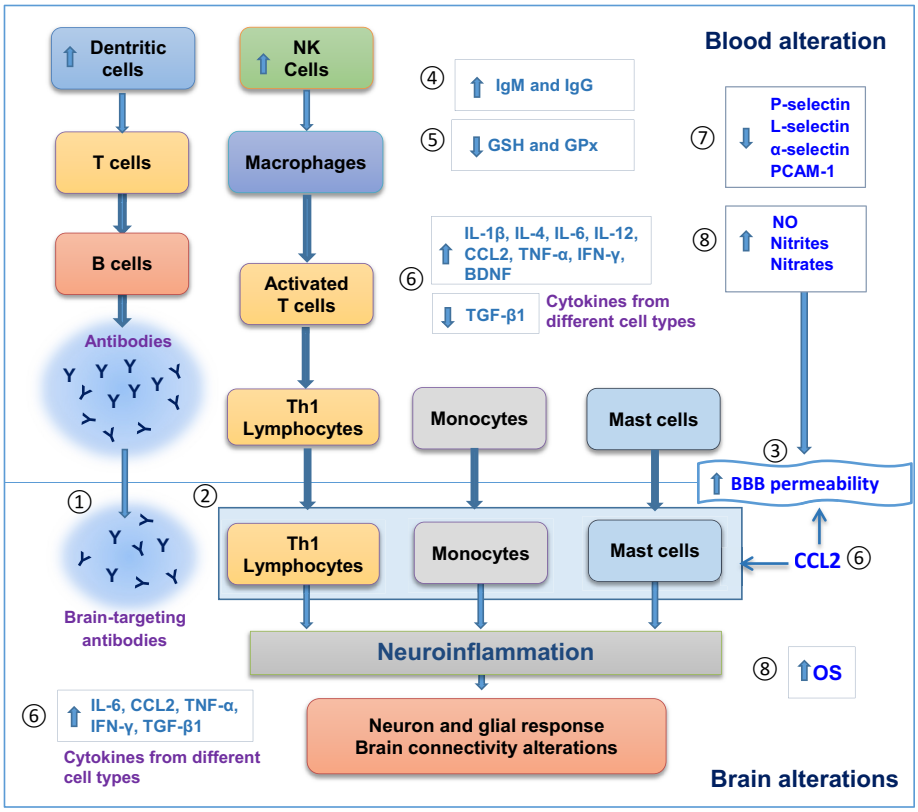

FIGURE 1 | Evidence for neuroimmune interactions in autism spectrum disorder (ASD). Blood and postmortem brain alterations in individuals with ASD. (1) Antibody production in blood against brain antigens. (2) Brain cell infiltration of Th1 lymphocytes, monocytes and mast cells. (3) Increase in blood brain barrier (BBB) permeability. (4) Increase in IgG and IgM levels. (5) Less antioxidant defenses. (6) Changes in cytokine levels. (7) Decrease in cell adhesion molecules, such as Selectins and PCAM-1. 8. Increase in oxidative stress. All these alterations can promote neuroinflammation, followed by neuron–glial response and brain connectivity dysfunction that ultimately can influence behavioral features in ASD. GSH, glutathione; GPx, glutathione peroxidase; NO, nitric oxide; Th, T-helper; OS, oxidative stress; CCL2, C–C motif chemokine 2.

A relevant point to be considered in the neuroimmune interactions occurring in autism is the fact that the intestinal mucosa of children with autism has a higher frequency of TNF-α^+ T cells and lower frequency of IL-10^+ T cells (148, 149). These studies indicate that such lymphocytes assume a pro-inflammatory profile, which corroborates with the increased levels of pro-inflammatory cytokines found in plasma and brain of patients with ASD.

Another important issue is the strong association between autism and allergic response involving mast cells, which correlates with various cellular processes, including allergic reactions enteric nervous system (ENS) (87, 150).

Increased plasma levels of IgG4 in children with ASD were also observed (151). These changes may be linked to changes in BBB permeability and also may influence neural plasticity and function, resulting in impairment in social interaction, communication, and behavior (87).

It is also important to consider cell adhesion molecules (CAM), which are present in endothelial cells, promoting a direct and selective interaction between blood cells and the cerebral endothelium (152). It is well known that CAMs play an important role in mediating the passage of T cells through endothelial barriers (153). These data indicate that the modulation of immune cell entry into the brain from patients with autism might also be a potential therapeutic target.

Working with the animal model of ASD induced by prenatal exposure of VPA, we recently demonstrated that the treatment of pregnant females with the antioxidant and anti-inflammatory resveratrol (RSV), before and after VPA exposure, prevented all behavioral impairments observed in the offspring (154). This is a naturally occurring phytochemical that was detected in 1963 in the dried roots of *Polygonum cuspidatum* (Itadori tea) and has been proposed as a pharmacological tool for neuroprotection against neuronal injury, including age-associated chronic diseases (155), ischemic brain damage (156), and cerebral models of stroke (157). For a systematic review and recommendations on the use of RSV, read (158).

Since similar alterations are also observed in the animal model induced by VPA (131, 159–161) and RSV exerts anti-inflammatory effects (158), future studies will be relevant to evaluate the influence of RSV in the immune system, particular in the ASD context. There is evidence for RSV use to establish immunological tolerance during treatment of autoimmune diseases that ablate or suppress the immune system. Specifically, RSV effect on tolerance

TABLE 4 | Selected findings in animal models related to ASD and immune system.

Model	Animal	Outcome, breakthrough or major finding	Reference
–	Mouse	Suggested that animal models of autoimmunity-associated behavioral syndrome (AABS) may be a useful model for the study of CNS involvement in human autoimmune diseases, e.g., autism	(164)
Neonatal rat infection with Borna disease virus	Rat	Abnormalities of early development; Increase locomotor activity; Increased stereotypies; Increased brain expression of mRNA for IL-1a, IL1-b, IL-6, TNF-α, and TNF-β	(165)
MIA	Mouse	Offspring display deficits in prepulse inhibition; deficiency in exploratory behavior and deficiency in social interaction	(166)
MIA	Mouse	Prepulse inhibition (PPI) and latent inhibition (LI) deficits were observed in the adult offspring. Coadministration of an anti-IL-6 antibody in the model of MIA prevented the behavioral changes. MIA in IL-6 knockout mice does not result in several of the behavioral changes seen in the offspring of wild-type mice after MIA	(167)
Prenatal exposure to VPA	Rat	Increased basal level of corticosterone, decreased weight of the thymus, decreased splenocytes proliferative response to concanavaline A, lower IFN-gamma/IL-10 ratio, and increased production of NO by peritoneal macrophages	(159)
Prenatal exposure to antibodies from mothers of children with autism	Mouse	Adult mice exposed *in utero* to IgG from mothers of children with autistic disorder displayed anxiety-like behavior and mice had alterations of sociability; evidence of cytokine and glial activation in embryonic brains	(168)
MIA	Rhesus monkey	Behavioral alterations in infants monkeys were observed, e.g., disruption of prepulse inhibition. Magnetic resonance imaging (MRI) revealed a significant 8.8% increase in global white matter volume distributed across many cortical regions compared to controls	(169)
MIA	Mouse	Pups born to maternal immune activation (MIA) mothers produce a lower rate of Ultrasonic vocalizations, decreased sociability and increased repetitive/stereotyped behavior	(170)
MIA	Mouse	Systemic deficit in CD4(+) TCRβ(+) Foxp3(+) CD25(+) T regulatory cells, increased IL 6 and IL-17 production by CD4(+) T cells, and elevated levels of peripheral Gr-1(+) cells; hematopoietic stem cells exhibit altered myeloid lineage potential and differentiation; behaviorally abnormal MA offspring that have been irradiated and transplanted with immunologically normal bone marrow from either MIA or control offspring no longer exhibit deficits in stereotyped/repetitive and anxiety-like behaviors	(110)
MIA	Rhesus monkey	Offspring exhibited abnormal responses to separation from their mothers, increased repetitive behaviors and inappropriately approaching and remaining in immediate proximity of an unfamiliar animal	(171)
Prenatal exposure to antibodies from mothers of children with autism	Mouse	Offspring displayed autistic-like stereotypical behavior in both marble burying and spontaneous grooming behaviors. Additionally, small alterations in social approach behavior were observed	(172)
MIA	Mouse	Following stimulation macrophages from offspring of poly(I:C) treated dams produced higher levels of IL-12, suggesting an increased M1 polarization. Also, macrophages from offspring of poly(I:C) treated dams exhibited a higher production of CCL3	(173)
MIA	Mouse	In the marble burying test of repetitive behavior, male offspring but not female offspring from both LPS and PolyIC-treated mothers showed increased marble burying	(174)
Prenatal exposure to VPA	Mouse	VPA mice present signs of chronic glial activation in the hippocampus and the cerebellum; When they are challenged LPS, they show an exacerbated inflammatory response, increased expression of pro-inflammatory cytokines in the spleen and higher corticosterone secretion to the blood	(112)
BTBR strain	Mouse	Levels of IgG isotypes deposited in fetal brain of BTBR mice were significantly higher than in FVB mice except for IgG1	(175)
BTBR strain	Mouse	Altered IgG levels were found, e.g., higher IgG1:IgG2a ratios; presence of brain-reactive IgG in the sera; levels of IgG1 deposited in the cerebellum, cortex, hippocampus or striatum of both BTBR male and female mice were significantly higher than in FVB counterpart	(176)
MIA	Mouse	Adult LPS-treated mice offspring had an elevated percentage of interferon (IFN)-γ(+) CD4(+) T cells and interleukin (IL)-17A(+) CD4(+) T cells in the spleen, IL-17A(+) CD4(+) T cells in the liver, and CD4(+) Foxp3(+) T cells in the spleen. LPS offspring CD4(+) T cells showed increased proliferation and an enhanced survival rate	(177)

is likely to be in the induction of Foxp3+ T cells and IL-10 expression, which are critical to development of T cells that are protective against autoimmune diseases, such as multiple sclerosis (162). In addition, the administration of RSV to mice developing experimental autoimmune encephalomyelitis – an animal model of human multiple sclerosis – increases expression of IL-10 and Foxp3 in T cells, the animal model of multiple sclerosis (163). In order to advance the knowledge related ASD development, it is important to also evaluate intracellular targets of VPA and RSV to clarify molecules and pathways affected by both. In this respect, we anticipate that further understanding of these molecular targets will be relevant to both therapeutic and etiological aspects of ASD. Similarly, such studies will hopefully help us to understand ASD-related epigenetic modulation and developmental alterations implicated in the neural and behavioral impairments.

In **Table 4**, we have summarized outcomes, breakthroughs, or major findings in animal models, relating to ASD and immune system activation. In the case of animal models of maternal immune activation, there is a cascade of inflammatory responses that are dependent on the pathogenic agent and can potentiate immune responses in offspring in a strain-dependent manner (111). It is hypothesized that pro-inflammatory cytokines, brain-reactive antibodies, and endocrine mediators, such as corticotropin-releasing factor and glucocorticoids participate in the etiology of autoimmunity-associated behavioral syndrome (164). Also, neonatal rat infection with Borna disease virus results in abnormalities of early development and increase in locomotor activity; stereotypies and brain expression of mRNA for IL-1α, IL1-β, IL-6, TNF-α, and TNF-β (165).

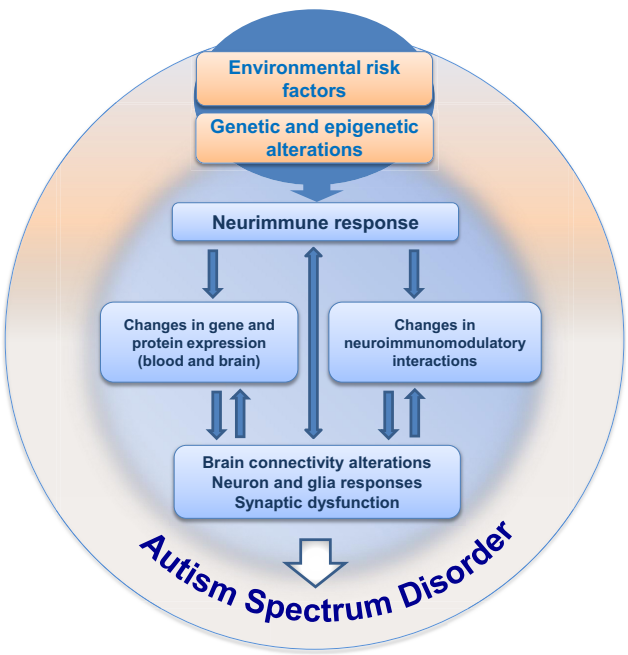

FIGURE 2 | Hypothesis for neuroimmune interactions in triggering the development of ASD. This hypothesis considers the presence of environmental risk factors during pregnancy, followed by immunoneuroendocrine response from the mother to the developing embryo/fetus. The risk factors (such as VPA) would influence central and peripheral neural responses in the context of a crosstalk with the immune system, followed by gradual changes in neural plasticity and function, resulting in behavioral impairment during development, ultimately leading to ASD.

Animal models of maternal infection have also been used to study behavioral impairments and brain alterations, such as maternal influenza infection (166), maternal immune activation (110, 167, 169–171, 173, 174, 177, 178), and prenatal exposure to antibodies (168, 172). In addition, the inbred BTBR T + tf/J (BTBR) mouse strain has been used as an animal model of core behavioral deficits in autism. BTBR mice exhibit repetitive behaviors and deficits in sociability and communication, presenting higher IgG1:IgG2a ratios and increased levels of IgG1 in brain (175, 176).

Summary and Outlook

Since the first descriptions of autism, 70 years of investigation have passed, with great efforts mainly in the last decade, bringing important information and knowledge on the mechanisms underlying ASD. Nevertheless, even with these advances, the etiology of ASD remains largely unknown and we are still searching for specific clinical marker(s) able to improve early diagnosis. We work on the hypothesis that integrating maternal–embryo systems will contribute to the understanding of ASD. One possibility, which was summarized here, concerns the hypothesis of neuroimmune interactions being involved in triggering ASD development, as schematically depicted in **Figure 2**. The presence of environmental risk factors during critical periods of embryonic/fetal development may influence the immune system in the mother, promoting localized or systemic inflammatory responses with the release of cytokines and hormonal molecules, which in turn, via neuroimmunomodulatory responses and crosstalk between circulatory and neural systems, may impair circuitry development, neuronal plasticity, and neuroglial function in the embryo/fetus. As immunological factors interfere with neural development since the embryonic period, and considering that inflammation or immune response may arise due to abnormal environmental interactions *in utero*, a better understanding of the neuroimmune changes that may underlie the pathogenesis or pathophysiology of ASD will hopefully have a large impact on the development of new clinical and therapeutic strategies to better deal with ASD.

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GENERAL COMMENTARY
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Commentary: The impact of neuroimmune alterations in autism spectrum disorder

Dario Siniscalco[1,2,3]*

[1] Department of Experimental Medicine, Second University of Naples, Naples, Italy, [2] Centre for Autism – La Forza del Silenzio, Caserta, Italy, [3] Cancellautismo – No Profit Association for Autism Care, Florence, Italy

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A commentary on

The impact of neuroimmune alterations in autism spectrum disorder
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The dramatic increasing prevalence of autism spectrum disorders (ASDs) (1), together with the influence on the quality of life and the lifetime societal cost of caring, has called for newest research on both the development of these diseases and the therapeutic options. Nowadays, it is well recognized that multifactorial and polygenic features (complex combination of genetic, epigenetic, and environmental interactions) characterize ASDs (2). Prenatal immune alterations and early inflammatory processes could be the autism etiological events. The authors Gottfried et al. (3) in this hypothesis-and-theory article discuss the recent findings in autism discovery. Starting from a brief historical way on autism development, the main topic of the article is to focus on the state-of-the-art of the novel findings in autism studies. The authors rightly highlight the newest challenging frontier of autism research: the neuroimmune axis alterations. These alterations are first evident in the cells early responsible for immune responses, as they are the precursors for macrophages, dendritic, and microglial cells: monocytes or peripheral blood mononuclear cells (PBMCs). These cells show strong dysfunctions in ASD children and are committed to a pro-inflammatory state, which in turn result in long-term immune alterations (4). In ASDs, altered PBMCs are responsible for elevated pro-inflammatory cytokine production. The up-regulation of inflammatory cytokines is also reflected in brain centers of autistic patients (5): the consequences are the induction of blood–brain barrier (the immunological interface between peripheral immune system and central nervous system) disruption. Changes in BBB permeability directly influence neural plasticity, connectivity and function, triggering impairments in social interaction, communication, and behavior (3). Immunological abnormalities also influence the gastrointestinal system and the microglial innate immune cells of the central nervous system (6). The authors also discuss the role of autoimmunity in the pathogenesis of autism. Familial or virus/bacteria-infected autoimmunity could be a risk factor for autism. Even if the exact cellular and molecular pathways responsible for the induction of neuroimmune alterations are still to be further clarify, a complex interaction among epigenetic and environmental risk factors (7) could trigger the neuroimmune abnormalities, such as abnormal neuron and glia responses.

Taken together, these autism-associated neuroimmune changes could help in identifying novel therapeutic target for a better future management of ASDs.

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Edited by:
Kathleen Dave, St. Luke's University Health Network, USA

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***Correspondence:**
Dario Siniscalco
dariosin@uab.edu

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REVIEW
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Adolescent Alcohol Exposure: Burden of Epigenetic Reprogramming, Synaptic Remodeling, and Adult Psychopathology

Evan J. Kyzar[1,2], *Christina Floreani*[1,2], *Tara L. Teppen*[1,2] *and Subhash C. Pandey*[1,2,3]*

[1] Department of Psychiatry, Center for Alcohol Research in Epigenetics, University of Illinois at Chicago, Chicago, IL, USA, [2] Jesse Brown Veterans Affairs Medical Center, Chicago, IL, USA, [3] Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, IL, USA

Adolescence represents a crucial phase of synaptic maturation characterized by molecular changes in the developing brain that shape normal behavioral patterns. Epigenetic mechanisms play an important role in these neuromaturation processes. Perturbations of normal epigenetic programming during adolescence by ethanol can disrupt these molecular events, leading to synaptic remodeling and abnormal adult behaviors. Repeated exposure to binge levels of alcohol increases the risk for alcohol use disorder (AUD) and comorbid psychopathology including anxiety in adulthood. Recent studies in the field clearly suggest that adolescent alcohol exposure causes widespread and persistent changes in epigenetic, neurotrophic, and neuroimmune pathways in the brain. These changes are manifested by altered synaptic remodeling and neurogenesis in key brain regions leading to adult psychopathology such as anxiety and alcoholism. This review details the molecular mechanisms underlying adolescent alcohol exposure-induced changes in synaptic plasticity and the development of alcohol addiction-related phenotypes in adulthood.

Keywords: adolescence, binge drinking, anxiety, epigenetics, neuroinflammation, neurogenesis, dendritic spines

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Alberto A. Rasia-Filho, Federal University of Health Sciences of Porto Alegre (UFCSPA), Brazil

Reviewed by:
Dorit Ron, University of California, San Francisco (UCSF), School of Medicine, USA
Mickael Naassila, University of Picardie Jules Verne, France

***Correspondence:**
Subhash C. Pandey
scpandey@uic.edu

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INTRODUCTION: ADOLESCENT ALCOHOL USE AND ALCOHOL USE DISORDERS

Alcohol exposure, whether acute or chronic in nature, produces profound morphological, structural, and molecular changes in the brain (Spiga et al., 2014a; Kyzar and Pandey, 2015). In the clinic, a pathological cycle occurs with repeated alcohol exposure that is manifested as persistent alcohol use in a chronically relapsing pattern despite related negative consequences (Koob, 2003; Hyman, 2005). The clinical manifestations of alcohol use disorders (AUD) appear to be fueled by neuroadaptations on the molecular level that affect synaptic plasticity on the cellular level and alter connectivity in specific neurocircuitry (Kyzar and Pandey, 2015). A growing understanding of AUD points to the importance of targeting the neurobiological mechanisms underlying synaptic plasticity in the study of AUD pathogenesis, as well as in the development of novel treatment options (Nixon and McClain, 2010; Koob et al., 2014; Kyzar and Pandey, 2015).

Recent estimates using DSM-V criteria reveal that the prevalence of AUD was 13.9% in the past year and 29.1% lifetime across all age groups (Grant et al., 2016). However, the same study found that 26.7% of respondents between the ages of 18 and 29 met criteria for AUD in the past year. Approximately 40% of adolescent drug-related visits to the emergency room involve alcohol, and nearly 10% of adolescents report drinking and driving (Grigsby et al., 2016). As drinking in adolescence is widespread, this phenomenon warrants detailed study because early use can lead to dependence and addiction later in life (Donovan, 2004; Nixon and McClain, 2010). Specifically, people who begin drinking before the age of 14 show an increased risk for alcohol abuse and dependence in adulthood (DeWit et al., 2000). Additionally, approximately 10–40% of adolescents across populations engage in frequent binge drinking, where 4 (for women) or 5 (for men) drinks are consumed over 2 h or fewer, and blood ethanol concentrations exceed 80 mg/dL (Miller et al., 2007; López-Caneda et al., 2014). Several preclinical studies also indicate ethanol exposure during early adolescence leads to heightened anxiety and higher alcohol intake in adulthood (Pandey et al., 2015; Van Skike et al., 2015). Adolescent alcohol use can lead to various stages of addiction that can be further exacerbated by other factors such as stress and comorbidity with depression and anxiety (**Figure 1**; Clark et al., 1997). Scientific investigation of binge alcohol use during the adolescent stage is critical, as it leads to an increased risk for psychiatric disorders including anxiety and alcoholism later in adulthood (**Figure 1**; Pandey et al., 2015). This review will critically address the notion that the persistent risks and effects of adolescent binge alcohol exposure are due, in part, to the altered structure and organization of synaptic connections possibly due to epigenetic reprogramming and related molecular mechanisms.

BEHAVIORAL EFFECTS OF ADOLESCENT ETHANOL EXPOSURE PERSIST TO ADULTHOOD

Behavioral Effects of Alcohol in Adolescents

Adolescents respond to alcohol in quantitatively different ways than adults, given the state of adolescent brain development and the molecular and synaptic correlates of this trajectory. In particular, both clinical and preclinical models support the notion that adolescents are more sensitive to the positive rewarding effects of acute alcohol exposure and less sensitive to negative aspects of alcohol intoxication (Donovan, 2004; Spear and Swartzwelder, 2014). However, adolescents and young adults appear to show greater neural reorganization and degeneration after binge alcohol use than their adult counterparts (Vetreno et al., 2014), suggesting that cellular and molecular mechanisms are involved in the differential responsiveness to ethanol exposure.

Adolescents are less sensitive to the adverse effects of alcohol consumption that often limit heavier drinking patterns in adults (Spear and Varlinskaya, 2005). Adolescents appear to be less sensitive to severe aspects of alcohol withdrawal such as seizures (Chung et al., 2008), which is dependent on the increased metabolic capacity of adolescents (Morris et al., 2010a). In rodents, adolescents show decreased anxiety during the withdrawal period from alcohol compared to adults (Slawecki et al., 2006). Adolescents are less sensitive to the anxiolytic effects of alcohol compared to adult rats (Sakharkar et al., 2012, 2014). In addition, the sedative and motor effects of alcohol exposure are less severe in adolescent animals when compared to adults (Little et al., 1996). Adolescents show an increased responsiveness to the rewarding and positive effects of alcohol consumption (Spear and Varlinskaya, 2005; Spear and Swartzwelder, 2014). Adolescents will self-administer alcohol to the point of tachycardia, unlike adults (Ristuccia and Spear, 2008). Increased alcohol-induced heart rate in young adults is associated with reduced subjective intoxication but increased alcohol-induced mood changes (Conrod et al., 2001), suggesting that the increased self-administration of alcohol by adolescents is connected to its rewarding effects. Alcohol also induces social facilitation more robustly in adolescent animals compared to adults (Varlinskaya and Spear, 2008).

Persistent Effects of Adolescent Alcohol Use on Adult Behavior

Adolescent binge-like alcohol exposure alters a number of different behaviors in animal models. For example, adolescent alcohol causes deficits in reversal learning, a measure of cognitive flexibility, at adulthood in a rat model. The same study found that adolescent ethanol conferred resistance to the extinction of ethanol self-administration in adulthood (Gass et al., 2014). It is interesting to note that adolescent intermittent ethanol exposure in rats produces anxiety-like behaviors during immediate withdrawal, a phenomenon that persists into adulthood (Sakharkar et al., 2014, 2016; Pandey et al., 2015). However, in adult rats chronic ethanol exposure also produces anxiety-like behaviors, but these behaviors disappear within a few days of last ethanol exposure (Pandey et al., 1999a; Zhang et al., 2007; Aujla et al., 2013).

Adolescent alcohol leads to long-term deficits in novel object recognition in adult animals (Vetreno and Crews, 2015). Multiple studies have shown increased ethanol consumption in adult rodents exposed to adolescent alcohol (Gilpin et al., 2012; Alaux-Cantin et al., 2013; Broadwater and Spear, 2013; Pandey et al., 2015), as well as decreases in alcohol-induced conditioned taste aversion in adulthood (Diaz-Granados and Graham, 2007). Adult mice exposed to binge-like alcohol during adolescence also show resistance to alcohol-induced sedation (Matthews et al., 2008), social impairment (Varlinskaya et al., 2014), and motor impairment (White et al., 2002), but increased levels of alcohol-induced social facilitation (Varlinskaya et al., 2014) and working memory deficits (White et al., 2000). These observations lend themselves to an emerging hypothesis in the field that prolonged and/or repeated binge exposure to alcohol during the critical developmental period of adolescence may cause the persistence of adolescent-like phenotypes, namely the increased

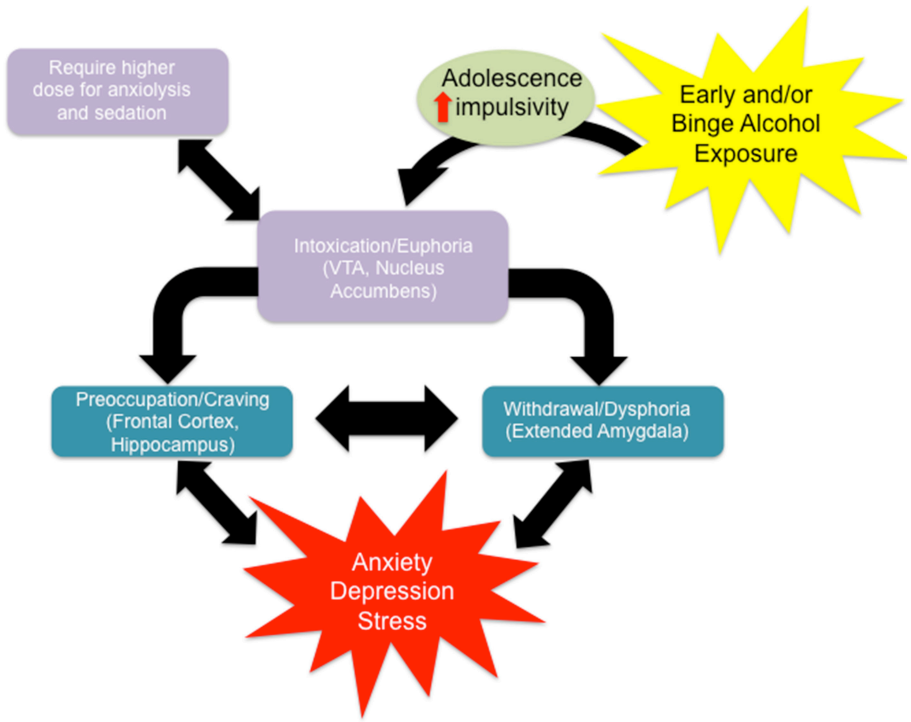

FIGURE 1 | Alcohol use disorder is characterized by the classical pattern of addiction, namely a cycle from a state of euphoria during alcohol intoxication to that of dysphoria during alcohol withdrawal to one of craving in the absence of acute intoxication. Craving, with or without withdrawal symptoms, is characterized by preoccupation with obtaining alcohol and anticipation of alcohol use, leading to relapse and a return to the intoxicated state. Certain individual clinical characteristics or psychosocial factors can exacerbate this cycle, driving alcohol use and addiction. For example, impulsivity renders an individual more sensitive to the immediate, rewarding effects of alcohol intake, with minimization of any longer term negative consequences, driving alcohol intake. Adolescence alone is characterized by an increased sensitivity to the rewarding effects of alcohol with a protection from the negative effects relative to aged counterparts, driving alcohol intake for this group. Adolescence is often characterized by impulsivity and together these characteristics facilitate alcohol intake. Also they require higher doses of ethanol to produce anxiolysis and sedation. Withdrawal from alcohol can both induce anxiety or depression symptoms and be worsened by comorbid mood disorders. Anxiety or depression can separately be exacerbated by other stressors, acute or chronic, and has been shown to be increased over the long term by environmental insults during development (early adversity). Stress and/or mood decompensation can also, in the absence of withdrawal dysphoria, directly stimulate craving and relapse, driving the cycle of addiction at either stage.

responsiveness to positive aspects and decreased responsiveness to negative aspects of intoxication, into adulthood (Spear and Swartzwelder, 2014).

ADOLESCENT NEUROMATURATION AND SYNAPTIC ORGANIZATION: RELEVANCE TO ALCOHOL EXPOSURE

The period of adolescence is particularly important for brain development and involves changes in synaptic structure, gene expression, and neurotransmission in crucial brain circuits responsible for emotion and cognition (Tau and Peterson, 2010). For example, reward seeking behaviors peak in adolescence well before the complete maturation of executive control and regulation networks (Keshavan et al., 2014). This "maturational lag" is underpinned by a complex concert of cellular and molecular changes in the brain involving various neurotransmitters and neural circuits (Keshavan et al., 2014).

Adolescents generally exhibit high levels of impulsive behavior (Keshavan et al., 2014), and are additionally highly sensitive to the rewarding effects and less sensitive to the sedative and anxiolytic effects of alcohol (Beck et al., 1993; Sakharkar et al., 2012, 2014; Spear and Swartzwelder, 2014). These characteristics may permit excessive alcohol intake during adolescence, and as mentioned above, adolescent alcohol exposure is a major risk factor for lifetime AUD prevalence (DeWit et al., 2000). Additionally, the earlier the onset of drinking during adolescence, the earlier and more severe the onset of adult AUD (Donovan, 2004; Dawson et al., 2008; Nixon and McClain, 2010). Adolescent alcohol may alter the normal processes of neuromaturation through biological mechanisms including epigenetics, arresting the behavioral development of impulse control and executive function, promoting the maintenance of adolescent-like impulsive use of alcohol into adulthood, intensifying the cycle of addiction, and potentially predisposing an individual to adult psychopathology (**Figures 1**, **2**; Crews et al., 2007; Pascual et al., 2009; Jacobus and Tapert, 2013; Conrod and Nikolaou, 2016).

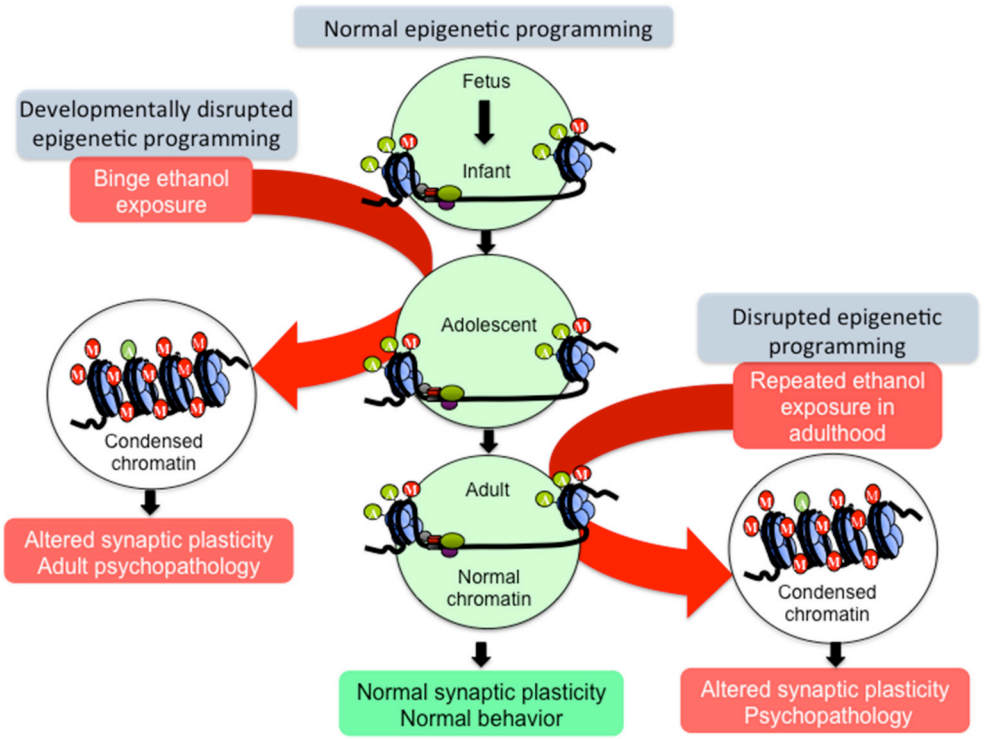

FIGURE 2 | Hypothetical model of epigenetic reprogramming by alcohol during adolescence. Adolescence is characterized by widespread changes in the developing brain. These changes are underpinned by molecular processes such as epigenetic programming, which turn specific genetic programs on to mediate cell fate determination, axonal outgrowth, dendrite formation and neuronal maturation. These processes, in turn, affect synaptic plasticity and neurocircuit function to shape normal behaviors. However, developmental perturbation by early life and/or binge alcohol exposure during this critical period can produce persistent effects on chromatin remodeling, synaptic plasticity, and brain function in adulthood, leading to alcohol use disorders (AUDs) and comorbid psychopathology including anxiety and depression. Adult exposure of alcohol can also disturb chromatin remodeling and plasticity, but some of these effects may not be long-lasting.

Neurocircuitry of Alcohol Exposure and Addiction

The neurocircuitry involved in alcohol abuse and the addiction cycle has recently been comprehensively reviewed (Koob and Volkow, 2010), but we will briefly outline this topic for our purposes. Addiction to alcohol and other drugs is posited to involve three main phases involving distinct brain circuits: (1) binge intoxication, (2) withdrawal/negative affective states, and (3) craving/preoccupation with drug intake (Koob and Volkow, 2010).

Each of these phases involves corresponding neuroadaptive changes in brain circuitry. For example, the ventral tegmental area (VTA), ventral pallidum, and nucleus accumbens (NAc) are involved in the initial binge/intoxication stage of drug and alcohol use. Ethanol directly increases the firing rate of dopaminergic VTA neurons (Brodie et al., 1990), and dopamine and $GABA_A$ receptors in the ventral pallidum are likely involved in the reinforcing effects of acute alcohol use (Melendez et al., 2004). Dendritic spine density in the VTA and NAc is critically involved in addiction to alcohol and other drugs of abuse (Spiga et al., 2014a), and chronic alcohol exposure affects synaptic organization in the NAc (Zhou et al., 2007; Spiga et al., 2014b).

The extended amygdala, composed of the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), and a transitional zone of the NAc shell (Alheid, 2003), is proposed to integrate brain stress and reward systems to produce negative affective states during the withdrawal stages of drug and alcohol use and addiction (Koob and Volkow, 2010). Extended amygdala regions receive inputs from the cortex, hippocampus and basolateral amygdala (BLA) and send projection fibers to the ventral pallidum, hypothalamus, and cortical structures (McDonald et al., 1999; Alheid, 2003). Extended amygdala structures display plasticity after exposure to alcohol. For example, repeated ethanol exposure leads to a decrease in dopamine and serotonin release in the NAc 8 h after last ethanol exposure (Weiss et al., 1996) that is again restored with ethanol exposure. Brain stress systems also contribute to neuroadaptations that develop with ethanol exposure to promote a maladapted baseline state of heightened arousal and anxiety-like behaviors in the absence of ethanol. This is, in part, due to ethanol-induced increases in the activity of the corticotrophin releasing factor (CRF) system in the CeA (Funk et al., 2006; Roberto et al., 2010). For example, infusion of a CRF antagonist into the CeA leads to a reduction of ethanol self-administration during withdrawal in ethanol-dependent rats (Funk et al., 2006).

CRF activity mediates the behavioral stress response and anxiety-like behaviors during both ethanol withdrawal and protracted abstinence (Funk et al., 2006; Heilig and Koob, 2007).

The third stage of alcohol addiction involves preoccupation with drug taking/intoxication and "craving." It should be noted that the concept of craving has not been successfully measured clinically and may not correlate with actual relapse (Sinha, 2013). This stage of the cycle is thought to involve neuroadaptation of cortical regions such as the prefrontal (PFC) and orbitofrontal cortex (OFC) as well as limbic structures with cortical connections (Koob and Volkow, 2010). For instance, cue-induced drug reinstatement likely involves reciprocal connections between the BLA and PFC (Schulteis et al., 2000; Everitt and Wolf, 2002), and the preoccupation stage of addiction also involves stress systems such as CRF in the extended amygdala circuitry (Shaham et al., 2003), as centrally-administered CRF antagonists can reduce ethanol self-administration following prolonged abstinence from chronic, high-dose ethanol vapor exposure (Valdez et al., 2002). However, addiction-related changes are not limited to these structures and occur in additional brain regions including the hippocampus (Nestler, 2002; Koob and Volkow, 2010). The changes in neural substrates in response to alcohol and other drugs of abuse demonstrate the remarkable structural and molecular plasticity of neurocircuitry, and many of these changes are susceptible to alterations during adolescence.

Adolescent Neurodevelopment in Addiction-Related Circuitry

Adolescence is marked by widespread changes in the developing brain that include the regions involved in alcohol addiction stated above. Brain white matter increases throughout adolescence as a function of ongoing myelination and gray matter reduction (Gogtay et al., 2004; Shaw et al., 2008). Total cortical volume initially increases during early adolescence before synaptic pruning occurs causing the cortex to decrease in size and reach normal adult proportions (Shaw et al., 2008). Dendritic spines are overproduced during initial neurogenesis and early development and are pruned away in an activity-dependent manner (Changeux and Danchin, 1976). It was once widely accepted that this synaptic pruning was mostly complete by the early adolescent stage of life (Bourne and Harris, 2008), but recent studies have shown that this process continues in cortical and limbic regions well into early adulthood in both humans (Petanjek et al., 2011; Goyal and Raichle, 2013) and animal models (Willing and Juraska, 2015; Johnson et al., 2016).

In addition to early life synaptic remodeling, the refinement of specific brain circuits corresponds to the behavioral changes seen in adolescence. For example, evolutionarily older structures such as the amygdala and hippocampus are among the first brain regions to reach adult-like levels of total volume and synaptic organization, while phylogenetically younger structures such as the PFC and associated cortical areas do not obtain adult-like stability until late adolescence or early adulthood (Gogtay et al., 2004; Shaw et al., 2008). This correlates roughly with an increase in reward-seeking behaviors during adolescence, including drug and alcohol use, as brain areas involved in executive function and complex decision-making have yet to mature while brain areas involved in anxiety and emotionality have reached histological and possibly functional maturity (Casey et al., 2011; Lebel and Beaulieu, 2011).

ALCOHOL-INDUCED EPIGENETIC REPROGRAMMING AND SYNAPTIC REMODELING

As alcohol exerts potent effects on the brain at the cellular and molecular level, early life alcohol exposure, and especially adolescent binge drinking, may prime the brain for alcohol-related psychopathology later in life via molecular mechanisms such as epigenetic reprogramming (**Figure 2**). Adolescence involves a concert of epigenetic cascades that prime the brain for functional changes occurring during this period, and some of these epigenetic processes are likely involved in the aforementioned behavioral and synaptic alterations seen during adolescence (Keshavan et al., 2014).

Epigenetics and the Developmental Response to Ethanol

"Epigenetics" is defined as a pattern of stable changes to the organization and function of a chromosome that result in a specific phenotype but do not change the underlying DNA sequence (Kouzarides, 2007; Berger et al., 2009). These phenotypes include the methylation and acetylation, among other added structural groups, of histone terminal tails around which DNA is wrapped, and also the addition of methyl groups to DNA itself (Kouzarides, 2007). These epigenetic chemical modifications exert effects on transcription, as DNA methylation is most often an inhibitory marker of closed, inaccessible chromatin while histone acetylation is considered a marker of open, active chromatin (Boyes and Bird, 1991; Gräff and Tsai, 2013). In contrast, histone methylation can either activate or inhibit transcription of the underlying DNA sequence depending on the specific histone protein residue that is modified. For instance, histone 3 lysine 4 (H3K4) methylation usually activates transcription while H3K9 methylation is repressive toward transcription (Kouzarides, 2007). Because epigenetic markers and the enzymes that add or remove these markers fluctuate in expression throughout development, it is hypothesized that perturbations during crucial developmental periods may cause widespread genetic dysregulation possibly due to epigenetic reprogramming, affecting normal developmental trajectories and causing the persistence and/or emergence of pathology in adulthood (**Figure 2**).

Numerous studies have shown that alcohol effects epigenetic pathways, leading to changes in gene expression, synaptic plasticity, dendritic spine morphology, and behavior (Kyzar and Pandey, 2015). Notably, acute alcohol increases dendritic spine density in the CeA and MeA, while withdrawal from alcohol causes a decrease in spinogenesis in the same regions (Pandey et al., 2008a; Moonat et al., 2013; You et al., 2014). These structural changes are correlated with anxiolysis in

response to acute alcohol and anxiety-like behavior during withdrawal, suggesting that the morphological changes in the amygdala are reflective of circuits underlying the expression of anxiety-like behavior. Interestingly, acute alcohol potently inhibits histone deacetylases (HDACs), epigenetic enzymes that remove acetyl groups from histone proteins, leading to increased histone acetylation that drives increased gene expression of crucial synaptic plasticity genes such as brain-derived neurotrophic factor (*Bdnf*) and activity-regulated cytoskeletal-associated protein (*Arc*) and subsequently increased dendritic spine density (Pandey et al., 2008a; Kyzar and Pandey, 2015). Again, these increases are associated with acute ethanol-induced anxiolysis (Moonat et al., 2013; Kyzar and Pandey, 2015). The opposite response is seen during alcohol withdrawal, with increased HDAC activity leading to decreased histone acetylation, decreased *Bdnf* and *Arc* expression and decreased dendritic spine density along with increased anxiety-like behaviors (Pandey et al., 2008a; You et al., 2014). Notably, alcohol-preferring (P) rats show an innately increased level of the histone deacetylase isoform 2 (HDAC2) that leads to decreased histone acetylation, decreased *Bdnf* and *Arc* expression and decreased spine density in the CeA and MeA. Infusion of an HDAC2 siRNA into the CeA reverses these molecular effects while normalizing the dendritic spine density, anxiety-like behaviors, and alcohol intake seen in these animals (Moonat et al., 2013). The notion that temporary inhibition of a single epigenetic enzyme can cause proximal effects on neuronal morphology underlines the impact of epigenetic processes on ongoing synaptic plasticity. Given these effects of alcohol exposure in adult animals, multiple studies have investigated both the acute and long-lasting effects of alcohol on brain epigenetic pathways in adolescence that affect dendritic morphology and behavioral phenotypes in adulthood (Sakharkar et al., 2014; Pandey et al., 2015).

Effects of Alcohol on Neuroepigenetics in Adolescents

Adolescent ethanol exposure causes widespread epigenetic changes, many of which are dose-dependent. Notably, adolescent animals appear to require a higher dose of alcohol than adults to inhibit HDAC activity in the amygdala and achieve anxiolysis acutely, as they are less sensitive to anxiolytic effects of ethanol (Spear and Varlinskaya, 2005; Walker and Ehlers, 2009; Sakharkar et al., 2012, 2014). Adolescent rats exposed to either one or two doses (24 h apart) of 2 g/kg ethanol show inhibition of HDACs and DNA methyltransferases (DNMTs; add methyl groups directly to DNA) in the amygdala and BNST (Sakharkar et al., 2014). Interestingly, two doses of 2 g/kg ethanol causes a decrease in *Dnmt3l* isoform expression in the amygdala but an increase in *Dnmt1* and *Dnmt3a* in the BNST of these same animals (Sakharkar et al., 2014). Binge-like exposure to alcohol during adolescence causes marked anxiety-like behaviors in adolescent rats 24 h after last alcohol exposure during the withdrawal period (Pandey et al., 2015). This anxiety was associated with increased global HDAC activity and increased HDAC2 and HDAC4 isoform expression in the CeA and MeA, leading to decreased levels of activating histone 3 lysine 9 (H3K9) acetylation (Pandey et al., 2015). Some effects of histone modifications, such as increased expression of HDAC2 and deficits in histone H3K9 acetylation in the amygdala, persist in adulthood. As HDAC2 has been shown to regulate spinogenesis and LTP (Guan et al., 2009), it is possible that the AIE-induced increase in HDAC2 expression in the CeA and MeA may be involved in reduced synaptic plasticity and psychopathology in adulthood (Pandey et al., 2015). Adolescent binge-like exposure also increases the activity of histone acetyltransferases (HATs; add acetyl groups to histone proteins) in the PFC and increases both histone acetylation and permissive H3K4 dimethylation at the promoters of the immediate-early and synaptic plasticity-related genes *Cfos*, *Cdk5*, and *Fosb* (Pascual et al., 2012). These genes have been implicated in the regulation of behavioral and neuronal plasticity (Nestler et al., 1999; Lai and Ip, 2009). Notably, pre-treatment with the HDAC inhibitor sodium butyrate increased the induction of HATs and promoter-specific histone acetylation by binge-like ethanol in the same study (Pascual et al., 2012). Binge-like alcohol exposure also increases total levels of acetylated H3K9, H4K5, and H4K12 while interestingly decreasing levels of H3K4 trimethylation 24 h after last exposure in the medial PFC of adolescent animals (Montesinos et al., 2016).

Epigenetic mechanisms also contribute to the effects of alcohol in clinical adolescent populations. As genetic risk does not fully explain the heritability of AUD and other addiction-related disorders, the influence of epigenetic environmental factors are posited to play a crucial role in AUD pathogenesis and disease progression (Kofink et al., 2013). A recent clinical study identified DNA hypermethylation at the 3′-protein-phosphatase-1G (*PPM1G*) gene as a risk factor for AUD in discordant monozygotic twins (Ruggeri et al., 2015). In an unrelated sample of adolescents, *PPM1G* hypermethylation was associated with impulsive behaviors and escalation of alcohol intake (Ruggeri et al., 2015). PPM1G interestingly dephosphorylates protein members of the microRNA processing pathway, another epigenetic mechanism responsible for gene regulation and implicated in synaptic organization (Petri et al., 2007; Smalheiser, 2008).

Persistent Effects of Adolescent Alcohol Use on Epigenetic Pathways in Adulthood

Many of the long-lasting effects of adolescent alcohol in the brain are likely to be mediated by altered epigenetic programming during crucial stages of development (**Figure 2**). In adulthood, this manifests as altered epigenetic architecture, specifically around genes that are important for synaptic plasticity such as *Bdnf* and *Arc*. Adolescent intermittent ethanol (AIE) exposure leads to long-lasting increases in global HDAC activity, as well as specific increases in HDAC2 protein and mRNA, in the amygdala at adulthood (Pandey et al., 2015). This is associated with decreased H3K9 acetylation globally and at the promoter regions of *Bdnf* and *Arc* and markedly reduced dendritic spines in the CeA and MeA, but not the BLA (Pandey et al., 2015). Notably, the increased alcohol preference and anxiety-like behaviors seen

in AIE-treated animals in adulthood are effectively reversed by treatment with the pan-HDAC inhibitor trichostatin A (TSA) (Pandey et al., 2015), further connecting epigenetic processes to brain morphology and behavior. The same alcohol exposure paradigm caused increased HDAC activity and decreased CREB binding protein (CBP) and histone H3K9 acetylation in the CA1, CA2, and CA3 regions of the hippocampus in adulthood. This was associated with a decrease in BDNF protein and H3 acetylation at the *Bdnf* exon IV promoter that were again reversed by TSA treatment (Sakharkar et al., 2016). Notably, HDAC2 overexpression in the hippocampus is directly linked to increased dendritic spine density, while decreased HDAC2 expression leads to decreased spines and synaptic plasticity as measured by LTP (Guan et al., 2009). Recent studies have linked AIE with increased hippocampal spine density, specifically increased immature dendritic spines, in adulthood (Risher et al., 2015), again highlighting the interrelatedness of epigenetics and synaptic function. AIE exposure additionally increased levels of acetylated H4K5 in the adult medial PFC, with no alterations seen in acetylated H3K9, acetylated H4K12, or trimethylated H3K4 residues, an effect that was abolished in toll-like receptor 4 (*Tlr4*) knockout animals (Montesinos et al., 2016). Therefore, epigenetic alterations induced by adolescent alcohol exposure may be brain region- and residue-specific. These results also suggest that the neuroimmune and epigenetic pathways are interrelated with regards to the lasting effects of adolescent binge-like ethanol, as TLR4 is altered by AIE and other methods of alcohol exposure (**Figure 3**).

The long-lasting alterations induced in the brain include decreases in BDNF expression in the amygdala and hippocampus of adults that were exposed to adolescent alcohol, and this decrease is regulated by epigenetic mechanisms (Pandey et al., 2015; Sakharkar et al., 2016). The decrease in BDNF and Arc is correlated with decreased dendritic spines in the CeA and MeA (Pandey et al., 2015). In addition to the regulation of *Bdnf* expression by histone acetylation, BDNF is also regulated by a number of different small non-coding RNAs, including microRNAs, that fine-tune cellular levels of this crucial neurotrophin (Numakawa et al., 2011). MicroRNAs are known to be altered in the brain of human alcoholics, as well as in several brain regions of preclinical models, and are likely involved in synaptic remodeling after alcohol exposure (Nunez and Mayfield, 2012; Pandey, 2016). The cyclic AMP response element binding protein (CREB) appears to regulate gene expression via a regulatory loop, with CREB/pCREB/CBP/p300 affecting epigenetic remodeling via histone acetylation, which in turn regulates the expression of crucial pathway genes such as *Cbp*. We recently observed that acute ethanol-induced anxiolysis appears to be regulated by decreased expression of microRNA-494, and increased expression of its target genes *Cbp* and *p300* in the amygdala of adult rats (Teppen et al., 2015). As CREB functions to regulate synaptic plasticity on its own and through the expression of CREB-target genes including *Bdnf* and *Arc*, we hypothesize that the CREB pathway may integrate signals from neuroimmune, neurotrophic, and epigenetic mechanisms to exert persistent effects on dendritic organization and remodeling in response to adolescent binge alcohol exposure (**Figure 3**; see below).

In addition to lasting effects on histone acetylation, histone methylation mechanisms have recently been examined in the adult amygdala after AIE exposure. mRNA of the neuron-specific splice variant of lysine demethylase 1 (Lsd1), termed *Lsd1 + 8a* for the inclusion of the mini-exon 8a, is decreased in the CeA and MeA of adult animals following binge-like alcohol exposure in adolescence (Kyzar et al., 2016). This leads to increased repressive H3K9 dimethylation in the CeA and

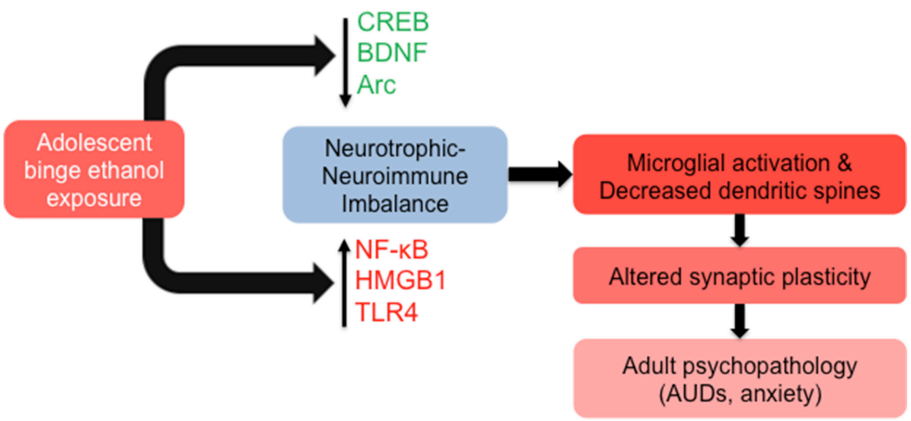

FIGURE 3 | Molecular and cellular signaling mechanisms in the brain leading to altered synaptic plasticity in adulthood after adolescent alcohol exposure. Adolescent binge alcohol exposure exerts effects in the brain long after alcohol has left the system. For example, adolescent alcohol causes a long-lasting decrease in key synaptic plasticity-related genes including cyclic AMP response element binding protein (CREB) pathway (CREB binding protein), brain-derived neurotrophic factor (BDNF), and activity-regulated cytoskeleton-associated protein (Arc). At the same time, adolescent binge alcohol exposure increases neuroimmune mediators including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), high mobility group box 1 (HMGB1) and toll-like receptor 4 (TLR4). The increase in neuroimmune factors and decrease in neurotrophic factors may be responsible for molecular imbalance in the adult brain that is mediated by increased microglial activation and decreased synaptic plasticity, leading to an increased risk for alcohol use disorders (AUDs) and related psychopathology such as anxiety.

MeA, but unchanged activating H3K4 dimethylation in the amygdala, which correlates with increased anxiety-like behaviors and possibly exerts effects on synaptic plasticity and morphology (Kyzar et al., 2016). Notably, the neuron-specific *Lsd1 + 8a* regulates neurite outgrowth during neuronal development, and recent *in vitro* studies suggest that this splice variant primarily works to demethylate H3K9 residues and not H3K4 (Zibetti et al., 2010; Laurent et al., 2015). The involvement of histone methylation mechanisms in the long-lasting effects of adolescent alcohol exposure highlights the need for investigation of additional epigenetic modifications after alcohol exposure, as some may be more sensitive to the perturbation of normal epigenetic and developmental trajectories and thus better targets for therapeutic intervention. For example, recent work shows that escalating alcohol consumption following ethanol vapor exposure causes progressive increases in DNA methylation at crucial synaptic plasticity genes such as synaptotagmin 2 (*Syt2*) in the medial PFC that is reversed by local infusion of a DNMT inhibitor (Barbier et al., 2015).

Alcohol-Induced Changes in the Adolescent Brain: Neurogenesis

Adult human alcoholic patients show characteristic structural brain abnormalities including presumed neurodegeneration in response to heavy and prolonged alcohol use (Crews and Nixon, 2009), and some of these effects may be regulated by epigenetic mechanisms. Patients with adolescent-onset AUD demonstrate marked reductions in total hippocampal and PFC volume (De Bellis et al., 2000; Medina et al., 2008), with hippocampal alterations uncharacteristic of adult-onset AUD. Studies in animal models have shown that a 4-day alcohol binge significantly reduces markers of neurogenesis in the hippocampus, including doublecortin (DCX) staining (a marker of immature neurons) and bromodeoxyuridine (BrdU) staining of DNA replication events (Morris et al., 2010b). It is possible that decreased adolescent neurogenesis may lead to the reductions in hippocampal volume in early-onset AUD patients (Donovan, 2004). Adolescent binge drinking damages white matter tracts in humans, and this effect correlates with estimated blood alcohol concentrations (Jacobus et al., 2009). The disruption in white matter integrity is also present in adolescents who show subclinical levels of binge drinking (i.e., not qualifying for AUD) (Jacobus et al., 2009). Alterations in hippocampal and PFC-related circuitry are manifested behaviorally by cognitive impairments in binge-drinking adolescents. For example, binge drinkers demonstrate decreased performance on simple verbal memory tasks while showing increased utilization of higher order cortical areas, possibly to compensate for decreased hippocampal and medial temporal lobe utilization (Tapert et al., 2004; Schweinsburg et al., 2010). Adolescent ethanol also exerts negative effects on learning and memory in animal models (Schulteis et al., 2008), suggesting that the behavioral manifestations of altered brain structure may be useful biomarkers for early intervention.

Persistent Effects of Adolescent Alcohol Use on Adult Neurogenesis

The persistent behavioral effects of adolescent alcohol are accompanied by marked effects on synaptic plasticity and neurotransmission. Adolescents show enhanced long-term potentiation (LTP), or the ability of synaptic activity to increase downstream signaling effects and ion flux during subsequent activation, at baseline compared to adult animals (Johnston, 1995), and consequently adolescents show a greater decrease in LTP after acute ethanol exposure than adult animals (Swartzwelder et al., 1995). Interestingly, animals exposed to binge-like ethanol (5 g/kg ethanol via intragastric gavage [i.g.] on a 2 days on, 1 day off, 2 days on, 2 days off schedule) during adolescence display enhanced LTP in the CA1 region of the hippocampus at adulthood (Risher et al., 2015). This is associated with increased dendritic spine density, but a predominance of immature spines and decreased synaptic connections and synaptic scaffolding proteins postsynaptic density 95 (PSD-95) and synapse-associated protein 102 (SAP-102) (Risher et al., 2015). Additional studies have shown a decrease in dendritic spine density in the CeA and MeA, which are primarily GABAergic in nature, in adult animals exposed to AIE via i.p. injections (Pandey et al., 2015). Notably, the BLA, which is primarily glutamatergic, did not show gross alterations in spines at adulthood, suggesting that a cell-type specific mechanism may target specific cells during high-dose adolescent alcohol consumption.

Neurogenesis is another phenomenon of brain plasticity that continues to occur in the adult brain in discrete locations including the subventricular zone and the granule cell layer of the hippocampal dentate gyrus (Eriksson et al., 1998). Numerous studies have reported a decrease in markers of neurogenesis, including DCX staining and Ki-67 staining (a marker of neural progenitor cell proliferation), in the hippocampus of adult animals exposed to intermittent binge-like alcohol by both i.p. and i.g. routes of administration in adolescence (Broadwater et al., 2014; Vetreno and Crews, 2015; Sakharkar et al., 2016), an effect that does not persist in adult rats exposed to similar amounts of ethanol (Broadwater et al., 2014). The decrease in neurogenesis in these animals may contribute to some of the long-lasting behavioral abnormalities, specifically those related to memory, cognition and anxiety, observed in AIE adult animals. Of particular interest is the increased nuclear HDAC activity and decreased markers of hippocampal neurogenesis (DCX and Ki-67) seen in adult rats exposed to AIE (Sakharkar et al., 2016). Similar to the epigenetic changes seen in these animals in the amygdala (Pandey et al., 2015), TSA administration reverses deficits in histone acetylation of the *Bdnf* gene and rescues the decrease in neurogenesis markers in the hippocampus (Sakharkar et al., 2016). These studies again emphasize the emerging role of epigenetics in regulating synaptic plasticity, and epigenetic drugs such as HDAC inhibitors may hold promise as therapeutic interventions for AUDs and other psychiatric disorders in adulthood (Pandey et al., 2008a, 2015; Kofink et al., 2013; You et al., 2014; Kyzar and Pandey, 2015).

ACUTE AND LASTING EFFECTS OF ADOLESCENT ALCOHOL ON NEUROTRANSMISSION AND NEUROIMMUNITY

Effects of Alcohol on Neurotransmission in Adolescents

The behavioral effects that differentiate the adolescent and adult response to ethanol are underpinned by cellular and molecular changes in the brain. For example, the decreased sensitivity to the negative aspects of alcohol intoxication may be a function of decreased facilitation of GABAergic inhibitory postsynaptic currents (iPSCs) in the hippocampus of adolescents vs. adult animals (Li et al., 2003, 2006). As GABA receptors play a crucial role in synaptic reorganization during neurogenesis and after exposure to stressful stimuli (Inoue et al., 2013), reduced alcohol-induced potentiation of iPSCs may contribute to alterations in dendritic dynamics following alcohol exposure.

The increased sensitivity to the positive effects of alcohol may be mediated by increased alcohol-induced dopamine release in the NAc in adolescents (Pascual et al., 2009). Following alcohol exposure, adolescent animals display increased baseline dopamine release in the NAc and decreased dopamine receptor D2 expression. Conversely, alcohol induces a decreased accumbal dopamine response in these animals (Pascual et al., 2009; Philpot et al., 2009). The increased basal dopamine, decreased alcohol-induced dopamine, and decreased D2 expression is hypothesized to contribute to a state of reward deficit, correlating with high levels of risk-taking behavior typically seen during adolescence (Koob et al., 2014). Normally, dopamine release in the NAc peaks during adolescence before falling to mature levels (Keshavan et al., 2014). Adolescent alcohol exposure may alter the normal developmental pattern of the VTA, NAc, and related systems via synaptic remodeling, which plays a crucial role in the switch from casual drug-taking to dependence for alcohol and other drugs of abuse (Spiga et al., 2014b).

Notably, dopamine interacts with glutamate in the NAc in an age-dependent manner. Dopamine appears to decrease the magnitude of NMDA-receptor mediated excitatory postsynaptic currents (ePSCs) in adolescents, but facilitate ePSCs in adult NAc slices in a mechanism dependent on presynaptic D1 receptors (Huppé-Gourgues and O'Donnell, 2012; Zhang et al., 2014). The glutamatergic system is heavily implicated in the synaptic pruning that occurs during development, contributing to the fluctuations in cortical volume seen during adolescence (Johnston, 1995; Crews et al., 2007). Adolescents show increased inhibition of NMDA-mediated synaptic activation compared to adult animals in the hippocampus (Swartzwelder et al., 1995). The NMDA receptor consists of multiple subunits that dimerize to produce mature receptors, with NR2A, NR2B, or another subunit coupling with the requisite subunit NR1. Interestingly, NR2B predominates in the developing brain and has a greater affinity for downstream calcium-related signaling mechanisms than NR2A, which increases in expression across development to become the most prominent subunit in the adult brain (Williams et al., 1993; Tovar and Westbrook, 1999). This developmental switch also plays a role in synaptic maturation and elimination (Barria and Malinow, 2005; Gambrill and Barria, 2011). Adolescent alcohol exposure changes the proportion of NR2A and NR2B receptors in multiple brain regions including the hippocampus, NAc, and cortical areas across multiple routes of administration including i.p. injection, vapor inhalation, and *ad libitum* consumption (Hargreaves et al., 2009; Pascual et al., 2009; Pian et al., 2010).

Neurotransmission Effects Caused by Adolescent Alcohol in Adulthood

Tonic inhibition by GABA, mostly mediated by extrasynaptic receptors, is also reduced in normal adolescent hippocampal slices when compared to adults, but is more sensitive to potentiation by ethanol (Fleming et al., 2007). Adult hippocampal slices taken from rats exposed to adolescent alcohol (5 g/kg ethanol via intragastric gavage [i.g.] on a 2 days on and 2 days off schedule) show the same pattern of baseline reduction and increased ethanol-induced potentiation of GABA-mediated tonic inhibition when compared to control animals (Fleming et al., 2012, 2013). However, the same exposure paradigm decreased adult hippocampal protein levels of the $GABA_A$ α4 receptor and the extrasynaptic $GABA_A$ δ receptor (Centanni et al., 2014). The $GABA_A$ δ receptor-mediated tonic inhibitory current is furthermore reduced in the adult prelimbic cortex of animals exposed to intermittent alcohol in adolescence despite the observation that expression of this receptor does not change (Centanni et al., 2016).

Adolescent alcohol exposure additionally exerts lasting effects on dopaminergic signaling. Adult animals exposed to moderate doses of ethanol in adolescence show increased stimulus-evoked mesolimbic dopamine release as measured by fast-scan cyclic voltammetry that may impact reward-associated decision making (Spoelder et al., 2015). AIE-exposed (via intermittent i.g. exposure) adult animals show decreased ethanol-evoked dopamine release in the NAc at adulthood (Shnitko et al., 2016). Additionally, adult rats show a stark decrease in markers of cholinergic (choline acetyltransferase; ChAT) neurons in the basal forebrain and dopaminergic (tyrosine hydroxylase; TH) neurons in the prelimbic cortex in adulthood after AIE via i.g. route of adminstration (Boutros et al., 2014; Vetreno et al., 2014). Notably, the decrease in ChAT staining in the basal forebrain is only persistent after adolescent binge exposure and not after exposure to the same dose and timing of alcohol exposure in early adulthood (Vetreno et al., 2014).

Effects of Alcohol on Neuroimmune Activation in Adolescents

Mediators of the immune response additionally play a role in the cellular response to alcohol, especially during adolescence (Crews and Vetreno, 2016). Younger age of alcohol drinking onset correlates with increased expression of immune-related genes including receptor for the advanced glycation end product (*RAGE*), high-mobility group box 1 (*HMGB1*), and the HMGB1 receptor *TLR4* in the postmortem brains of human alcoholics (Vetreno et al., 2013). Animal models show that binge ethanol

exposure (5 g/kg i.g. ethanol on a 2 days on-2 days off schedule) increases *TLR4* and *HMGB1* in the PFC of adolescent rats (Vetreno and Crews, 2012). An additional study demonstrated that binge-like alcohol increases *TLR2* and *TLR4* in the PFC along with the inflammatory cytokines tumor necrosis factor alpha (*TNFα*) and interleukin 1 beta (*IL-1β*) (Pascual et al., 2014). Both the HMGB1-TLR4 and IL-1β signaling cascades mediate increased flux through synaptic NMDA receptors that increases the likelihood for excitotoxicity (Viviani et al., 2003; Balosso et al., 2014). Chemokine (C-C motif) ligand 2 (*Ccl2*) gene expression is increased in the cortex of adolescent rats during withdrawal to a greater extent than adults (Harper et al., 2015), but an earlier study in mice found that *Ccl2* mRNA was increased in the adult but not adolescent cortex, hippocampus, and cerebellum (Kane et al., 2014). Interestingly, many of the epigenetic alterations seen in the brain of mice exposed to binge-like levels of alcohol were abolished in *Tlr4* knockout mice, suggesting that the epigenetic and neuroimmune systems interact during adolescent alcohol exposure (Montesinos et al., 2016).

Persistent Effects of Adolescent Alcohol Use on Neuroinflammation in Adulthood

Alcohol potently activates neuroimmune pathways in both the developing and adult brain, but some of these effects continue to persist after adolescent alcohol exposure into adulthood. The upregulation of HMGB1 and TLR4 by adolescent intermittent alcohol exposure (i.g) persists into adulthood in the cortex (Vetreno and Crews, 2012). Notably, mice lacking the *Tlr4* gene appear to be additionally resistant to the long-term lasting effects of AIE exposure on epigenetic remodeling and cognitive functioning (Montesinos et al., 2016). A 4-day alcohol binge during adolescence is sufficient to cause activation of microglial cells that persists for at least 30 days (McClain et al., 2011), which is important due to the well-studied effects of microglia on synaptic pruning and may play a role in ethanol-induced structural remodeling (Yang et al., 2014). Recent work suggests that lifetime ethanol exposure positively correlates with brain neuroimmune markers such as HMGB1 and TLR4 (Vetreno et al., 2013), and adolescent binge alcohol use may predispose an individual to an addictive cycle of alcohol abuse resulting in steadily increasing immune activation, likely mediated at least in part through epigenetic and chromatin regulation, and subsequent synaptic remodeling (**Figure 3**).

CONVERGENCE OF MOLECULAR AND SYNAPTIC TARGETS AFTER ADOLESCENT ALCOHOL EXPOSURE

Adolescent alcohol exposure causes specific long-term changes to neurocircuitry across the domains of immune function, neurogenesis, and epigenetic programming (Vetreno et al., 2013; Pandey et al., 2015; Vetreno and Crews, 2015; Sakharkar et al., 2016). Although the studies described above have generally focused on biological parameters relevant to one domain, in actuality these systems likely act in concert to exert lasting effects on synaptic function and behavior. The p65 isoform of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is increased in the adult medial PFC of mice exposed to binge-like ethanol (8 total i.p. injections of 3 g/kg ethanol on a 2 days on-2 days off schedule) in adolescence, an effect that is not present in *Tlr4* receptor knockout mice (Montesinos et al., 2016). These changes coincide with epigenetic alterations in the medial PFC that are not present in *Tlr4* knockout mice (Montesinos et al., 2016). Notably, NF-κB and TLR4 are known to interact with two genes that are crucially involved in the actions of alcohol: *CREB* and *BDNF* (Marini et al., 2004; Kaltschmidt et al., 2006).

CREB As a Master Regulator of Alcohol Action and Synaptic Plasicity

CREB is a gene transcription factor that binds to cAMP response elements (CRE) on DNA after phosphorylation and usually increases the transcription of downstream genes such as *Bdnf* and *Arc*. The molecular actions of CREB in the development of addiction have been extensively studied, providing evidence of a common molecular mechanism for addictive behaviors (Pandey, 2004; Nestler, 2005). As NF-κB is known to interact with CREB and influence synaptic plasticity (Marini et al., 2004; Kaltschmidt et al., 2006), these two transcription factors may act in convergence to remodel neural circuits and synapses after high-dose adolescent alcohol exposure. As described above, CREB also interacts with CBP and p300, both of which act as HATs, to remodel chromatin in response to alcohol exposure (Pandey et al., 2008a; Teppen et al., 2015), possibly integrating neuroimmune, epigenetic, and neurotrophic pathways to influence synaptic plasticity and behavior (**Figure 3**).

CREB functioning within the amygdala plays an integral role in the development and maintenance of AUD. It has been shown that alcohol-preferring (P) rats display innately heightened anxiety levels and excessive alcohol drinking behaviors, as well as lower levels of CREB and the functionally active phosphorylated form of CREB (pCREB) within the CeA and MeA when compared to non-preferring (NP) rats (Pandey et al., 1999b, 2005). Additionally, a single acute alcohol exposure (1 g/kg; i.p.) produces anxiolysis and activation of amygdalar CREB leading to increased expression of BDNF and dendritic spines in both P rats and an unselected stock of rats, but not in NP rats (Pandey et al., 2005, 2008b; Moonat et al., 2011). Furthermore, innately lower levels of CREB and pCREB have been found in the NAc shell in a genetic strain of alcohol preferring mice (C57BL/6J) as compared to non-preferring DBA/2J mice (Belknap et al., 1993; Misra and Pandey, 2003). Recently, we observed that decreased DNA demethylation mechanisms are associated with decreased *Bdnf* expression in the NAc shell while also promoting alcohol drinking behavior (Gavin et al., 2016). Several studies demonstrated that CREB phosphorylation increases during acute alcohol exposure, normalizes in response to chronic alcohol treatment, and decreases in amygdaloid structures during alcohol withdrawal, suggesting that CREB serves as a molecular switch in the CeA to regulate anxiety and alcohol drinking behaviors (Pandey et al., 2003, 2005, 2008b; Moonat et al., 2011).

CREB Target Genes and Alcohol

CREB-regulated pathways in the amygdala regulate alcohol-related behaviors (Pandey, 2004; Teppen et al., 2015). An important target of CREB, the neurotrophic factor BDNF, is activated by CREB plays important roles in neuronal development, neurogenesis, synaptic plasticity, and regulation of dendritic morphology (Poo, 2001). Interestingly, BDNF is regulated by its own signaling pathway via activation of tropomyosin receptor kinase B (TrkB) receptors and mitogen activated protein (MAP) kinases that induce CREB phosphorylation (Finkbeiner et al., 1997; Poo, 2001; Minichiello et al., 2002).

Ethanol increases *Bdnf* expression, and BDNF signaling pathways in specific brain regions play a profound role in regulating alcohol drinking and anxiety-like behaviors (McGough et al., 2004; Pandey et al., 2006; Prakash et al., 2008; You et al., 2014; Warnault et al., 2016). For example, decreased *Bdnf* expression in hippocampal and amygdaloid brain regions results in increased ethanol consumption, while increased *Bdnf* expression attenuates ethanol intake (McGough et al., 2004; Pandey et al., 2006). It has been shown recently that decreased function of BDNF in the medial prefrontal cortex due to a BDNF valine 68 to methionine (Val68Met, analogous to the human Val66Met) polymorphism increases alcohol-drinking behaviors in mice (Pandey, 2016; Warnault et al., 2016). BDNF expression and phosphorylation of members of its signaling cascade, namely MAP kinases and CREB, are increased in CeA and MeA by acute ethanol, which is normalized after chronic treatment and decreased significantly during ethanol withdrawal in rats (Pandey et al., 2008b). Moreover, intra-CeA infusion of BDNF attenuated anxiety-like behaviors that developed during ethanol withdrawal in rats (Pandey et al., 2008b). Further studies suggest that BDNF acts through Arc, which ultimately regulates spinogenesis as *Arc* expression is decreased in the CeA during ethanol withdrawal and normalized by BDNF infusion (Pandey et al., 2008b). It has been shown that *Arc* antisense oligodeoxynucleotide (ODN) infusion into CeA significantly decreases *Arc* expression, dendritic spines, and promotes anxiety-like and alcohol drinking behaviors in rats (Pandey et al., 2008b). The direct role of BDNF in various amygdaloid nuclei in anxiety-like and alcohol drinking behaviors has been well established (Pandey et al., 2006). Infusing BDNF antisense ODN directly into the CeA or MeA, but not in the BLA, is associated with increased ethanol consumption and provokes anxiety-like behaviors in rats. However, these behavioral changes were prevented following co-infusion of exogenous BDNF with the BDNF antisense ODN (Pandey et al., 2006). It is important to point out that BDNF antisense ODN infusion in each of the amygdaloid nuclei significantly decreased BDNF expression and phosphorylation of CREB and MAP kinases that were reversed by co-infusion with exogenous BDNF (Pandey et al., 2006). Baseline BDNF mRNA and protein levels are lower in the CeA, MeA, and bed nucleus of stria terminalis, but not BLA and NAc shell or core, of P vs. NP rats (Prakash et al., 2008). Moreover, acute ethanol exposure increased BDNF and Arc expression and dendritic spines in the CeA and MeA, but not BLA of P rats, leading to anxiolytic-like effects (Moonat et al., 2011). Several other studies have found that deficits in BDNF in PFC, hippocampal, and striatal brain regions are also involved in regulating alcohol consumption in animals (McGough et al., 2004; Logrip et al., 2015; Pandey, 2016; Warnault et al., 2016). Exogenous BDNF exposure appears to play a protective role in ethanol-induced cytotoxic damage in cultured neuronal cells (Sakai et al., 2005). Human studies show that decreased serum BDNF levels in alcoholics are associated with alcohol withdrawal symptoms (Joe et al., 2007; Huang et al., 2011), and *BDNF* gene polymorphisms have been linked to increased susceptibility to alcohol abuse (Uhl et al., 2001; Matsushita et al., 2004; Warnault et al., 2016). As discussed above AIE also produces long-lasting reductions in the expression of BDNF in the amygdaloid and hippocampal brain regions of rats during adulthood and these changes are correlated with phenotypes of anxiety and alcohol intake (Pandey et al., 2015; Sakharkar et al., 2016). Taken together, these results provide evidence of homeostatic mechanisms utilized by the BDNF signaling pathway in various key brain circuits in the regulation of alcohol addiction, and these mechanisms are critical in the development of alcohol dependence and promoting drinking (Pandey, 2016).

FUTURE DIRECTIONS AND CONCLUSION

Scientific inquiry into the adolescent alcohol exposure and its lasting effects has increased greatly in recent years. As human populations are often confounded with variable onset of drinking, polydrug use, and other factors, animal models have been utilized in the vast majority of mechanistic studies presented here. Additionally, these animal models have often used only male rats. As the influence of sex differences in alcohol response has recently been critically reviewed (Becker and Koob, 2016), it is crucial to use both male and female cohorts to examine the acute and lasting effects of adolescent alcohol abuse. Future studies should attempt to translate the most robust and reproducible preclinical epigenetic findings into human populations, possibly establishing novel treatments for AUDs.

As many of the studies cited herein have used different modes and schedules of alcohol exposure, it is important to note that some effects of adolescent exposure may be specific to routes of administration and exposure paradigms. However, a subset of data advocates that certain long-lasting effects of adolescent alcohol exposure (biochemical and behavioral) occur across treatment paradigms including i.p. injection, i.g. gavage, vapor exposure, and voluntary consumption, and are therefore likely the result of ethanol exposure itself (Hargreaves et al., 2009; Pascual et al., 2009; Pian et al., 2010; Gilpin et al., 2012; Boutros et al., 2014; Broadwater et al., 2014; Pandey et al., 2015; Vetreno and Crews, 2015; Sakharkar et al., 2016).

Taken together, early onset of alcohol use and repeated binge-like exposure during the critical developmental period of adolescence greatly increase the risk for later AUD diagnosis. Animal models have greatly increased our understanding of the biological mechanisms underlying synaptic plasticity associated with this risk. Adolescent alcohol increases neuroimmune signaling in the brain and persistently inhibits markers of neurogenesis in the hippocampus. Additionally, binge-like exposure to alcohol alters crucial epigenetic and neurotrophic

factors signaling in multiple brain regions involved in addiction. These molecular factors exert robust effects on neuronal morphology and synaptic plasticity. The altered dendritic spine density and synaptic signaling observed alter adolescent ethanol exposure contribute to alterations in neurocircuitry and behavior, and targeting these cellular mechanisms may lead to new treatments for AUDs and other related psychopathology (Kyzar and Pandey, 2015; Pandey et al., 2015; Kyzar et al., 2016).

AUTHOR CONTRIBUTIONS

The concept and idea of the review article was conceived by SP and then discussed with all authors. All authors (EK, CF, TT, and SP) contributed in the review of literature and writing of the article. The content of figures was conceived by SP and then prepared and edited by all authors.

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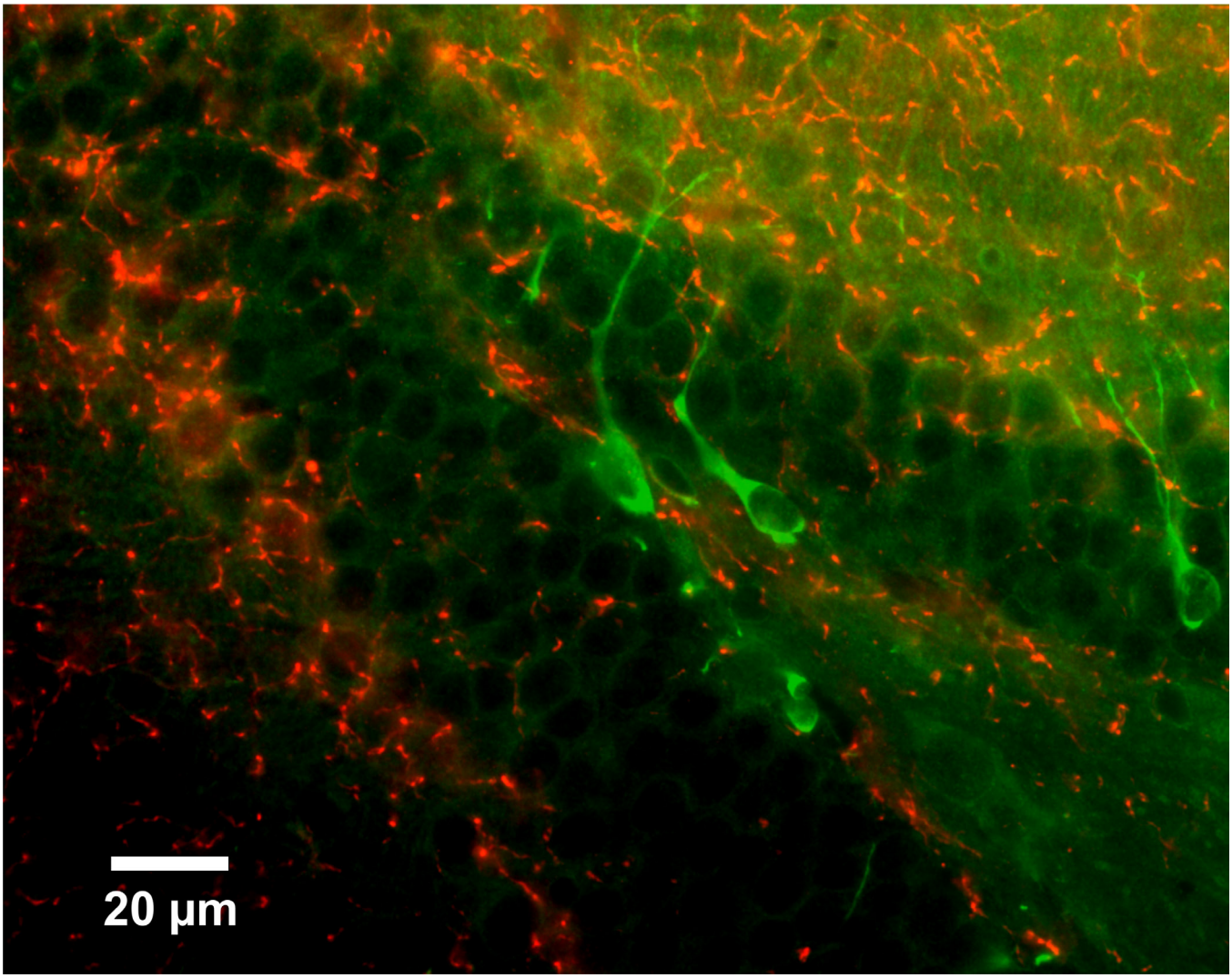
Conflict of Interest Statement: SP reports that a US patent application entitled "Histone acetyltransferase activators and histone deacetylase inhibitors in the treatment of alcoholism" (serial number 60/848237 filed on September 29th, 2006) is currently pending.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



Newly formed postmitotic neurons in the dentate gyrus.

Doublecortin (in green) was used for visualizing newly formed neurons. The cholinergic innervation (in red) of the dentate gyrus is visualized by using antibodies directed against choline acetyltransferase.

Image by Oliver von Bohlen und Halbach

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