# DYNAMICS AND MODULATION OF SYNAPTIC TRANSMISSION IN THE MAMMALIAN CNS

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# DYNAMICS AND MODULATION OF SYNAPTIC TRANSMISSION IN THE MAMMALIAN CNS

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EDITORIAL
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# Editorial: Dynamics and Modulation of Synaptic Transmission in the Mammalian CNS

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**Keywords: synaptic plasticity, synaptic transmission, neuronal-glial interactions, dendritic spine, neural circuitries formation**

## Editorial on the Research Topic

## Dynamics and Modulation of Synaptic Transmission in the Mammalian CNS

Structural and functional specializations of neurons and glial cells, and the dynamical nature of their connections, allow the precise modulation of homeostatic variables and the ongoing behavioral display. Synaptic transmission and plasticity are key cellular processes that enable the nervous system to process information, respond and adapt to a changing environment and internal milieu. In the last few years, the field of synaptic plasticity/modulation has undergone dramatic advances, in particular in the study of the mammalian central nervous system.

This Research Topic provides a state-of-the-art and comprehensive collection of studies focusing on the various mechanisms for modulation of synaptic formation and transmission. Articles depict relevant data about the molecular and structural basis of neuronal cell signaling, plasticity of dendritic spines, synaptic and extrasynaptic transmission, neuronal-glial interactions, modulation of chemical transmitters release, and their functional role, receptor types, and signal transduction up to the level of neural network properties under physiological conditions or related to brain disorders.

Petralia et al. reviewed the structural features to classify invaginating structures at chemical synapses into three categories in the central and peripheral nervous system. Ultrastructural data demonstrate the existence of these elements in presynaptic axonal terminals, postsynaptic spines or dendrites, and glial processes. Spinules and related structures compose the first type of protrusions, which do not present synaptic active zones, whereas the other two categories show active zones within the invagination. Postsynaptic spines that protrude directly into the presynaptic terminal exemplify the second type. Presynaptic terminals that protrude directly into the postsynaptic structure, such as at the neuromuscular junction, represent the third type. Evidence indicate that these specialized invaginating structures have to be carefully considered when further evaluating the mechanisms for neuronal cell signaling.

Mederos et al. reviewed the existing data supporting the crucial roles of astrocytes in synaptic function. These authors described the correlation between structurally- and functionally- different astrocyte populations, by highlighting the current data regarding the heterogeneity of anatomical, molecular, and functional properties of astrocyte–neuron communication. This article critically demonstrates the specialized role of these glial cells in the synaptic transmission and plasticity in different brain areas.



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Del-Bel and De-Miguel reviewed the mechanisms of extrasynaptic release of transmitters, by exocytosis or diffusion, from the soma, axon, and dendrites in the absence of postsynaptic counterparts. These authors compared the mechanisms of classic transmitters release, peptides, nitric oxide, and cannabinoids and explored how extrasynaptic transmission interacts to modulate visual sensitivity and blood flow. It is commented on the importance of considering extrasynaptic communication as an important component to understand the function of different neural circuits.

Repetto et al. provided original data into specific roles of individual domains of the multidomain protein Neurobeachin (Nbea) in spine formation, postsynaptic neurotransmitter receptor targeting, and actin distribution. By using live cell imaging and patch-clamp electrophysiology, these authors monitored the structure and function of spinous synapses in primary hippocampal neurons of wild type and Nbea KO mutant mice. They showed the function of specific domains of Nbea in restoring normal dendritic spine density and surface targeting of AMPAR subunits, and in regulating filopodia extension. Interestingly, as the heterozygous mutations in Nbea occur in autistic patients, these findings provides new understanding of the mechanism underlying neuropsychiatric disorders associated with impairments of spine function.

Aubrey and Supplisson provided original data on the heterogeneous signaling at GABA and glycine co-releasing terminals. Using cultured spinal neurons and a combination of loose-patch and whole-cell electrophysiology, these authors have demonstrated that miniature inhibitory postsynaptic currents (mIPSC) originated from terminals containing both GABA and glycine. Their modeling approach predicts that, when glycine gradually replaces GABA in synaptic vesicles, "the redistribution between the peak amplitude and charge transfer of mIPSCs acts to maintain the strength of inhibition while increasing the temporal precision of signaling." These new data add considerably to the interpretation of changes in synaptic signaling, the strength and plasticity of inhibitory transmission, and the functional role for the corelease of GABA and glycine at central synapses.

Cheng et al. used genetic, electrophysiological, and pharmacological approaches in cultured hippocampal neurons to determine the role of protein kinases and synapsins in two forms of activity dependent, short-term synaptic plasticity that enhance neurotransmitter release. In this original research article, authors provided novel evidence that PKA and synapsins, apparently the main substrates of PKA, are important for augmentation of spontaneous glutamate release at excitatory synapses. These results elucidate important signaling pathways involved in these two forms of short-term plasticity.

Fontes-Dutra et al. showed original results on altered localization of parvalbumin expressing neurons and reduced level of gephyrin in primary sensory cortex in the valproic acid animal model of autism spectrum disorder (ASD). Their results highlighted the importance of changes in GABAergic transmission during brain development. In addition, they demonstrated that resveratrol could have an important effect in rescuing the gephyrin expression in the studied rats.

Rajamani et al. reviewed the role of the neuropeptide oxytocin (OXT) as modulator of synaptic plasticity and neural activity in circuits that regulate social behavior in neurodevelopmental disorders, including ASD. This work highlights studies that report specific alterations in the OXT system in rodent models and explored the potential convergence between the OXT system and genes associated with brain disorders, focusing on the SHANK3 gene. These authors showed evidence supporting the hypothesis that failure of the OXT system during early development could affect social behavior by altering synaptic activity and plasticity.

Rigas et al. report original findings on the impact of early life seizures (ELS) on the network dynamics of the mouse neocortex. Single or multiple seizures were induced using pentylenetetrazole at two different brain developmental stages (postnatal days 9–15 or 19–23), and cortical electrophysiology was assessed by comparing spontaneous network activity (in the form of recurring Up states) in slices of the primary motor and the somatosensory areas of animals at adulthood. Interestingly, results showed that long lasting changes induced by seizures depend on their severity, are region-specific, and are related to the brain maturation period at which the seizures are induced. For example, "single intermittent ELS at P19–23 had no effect on Up state activity, but multiple seizures induced during the same period caused a significant change in the spectral content of spontaneous Up states."

Quillfeldt provides a Hypothesis and Theory article, discussing a series of findings in which systems consolidation temporal framework changes according to the nature of the behavioral task interposed between the training and the remote test. He proposes a hypothetical "reset" mechanism acting upon a fixed/limited pool of plastic synapses in the CA1 hippocampal area, according to their level of occupancy, to explain the observed temporal flexibility of systems consolidation. The occupancy/reset theory conceives putative CA1 synapse populations with different levels of ability to reset, providing not only a common basis for both synaptic and systems consolidation, but also explaining the different dynamics of episodic and semantic memories.

These current articles highlighted relevant mechanisms of synaptic activity with physiological and/or pathological implications. Indeed, the spatiotemporal modulation of synaptic function is crucial for emergent properties of neural cells, networks, and behaviors. This is a broad and vivid research area. We foresee new approaches and working hypotheses from the data of this Research Topic to the endeavor of understanding the functional organization of the central nervous system.

## AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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# Invaginating Structures in Mammalian Synapses

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Invaginating structures at chemical synapses in the mammalian nervous system exist in presynaptic axon terminals, postsynaptic spines or dendrites, and glial processes. These invaginating structures can be divided into three categories. The first category includes slender protrusions invaginating into axonal terminals, postsynaptic spines, or glial processes. Best known examples of this category are spinules extending from postsynaptic spines into presynaptic terminals in forebrain synapses. Another example of this category are protrusions from inhibitory presynaptic terminals invaginating into postsynaptic neuronal somas. Regardless of the direction and location, the invaginating structures of the first category do not have synaptic active zones within the invagination. The second category includes postsynaptic spines invaginating into presynaptic terminals, whereas the third category includes presynaptic terminals invaginating into postsynaptic spines or dendrites. Unlike the first category, the second and third categories have active zones within the invagination. An example of the second category are mossy terminal synapses of the hippocampal CA3 region, in which enlarged spine-like structures invaginate partly or entirely into mossy terminals. An example of the third category is the neuromuscular junction (NMJ) where substantial invaginations of the presynaptic terminals invaginate into the muscle fibers. In the retina, rod and cone synapses have invaginating processes from horizontal and bipolar cells. Because horizontal cells act both as post and presynaptic structures, their invaginating processes represent both the second and third category. These invaginating structures likely play broad yet specialized roles in modulating neuronal cell signaling.

**Keywords: CA3, horizontal cell, retina, neuromuscular, ephaptic, spinule, cannabinoid, indented**

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## INTRODUCTION

The classic image of a neuronal synapse with a bulbous presynaptic terminal separated from a postsynaptic dendrite shaft or spine (**Figure 1A**; Shepherd, 2004) is often, in reality, complicated by various invaginating structures. Even sponges, which seem to lack definitive neurons and chemical synapses, can have neuron-like cells with elongate processes making invaginating contacts with other cell processes; perhaps these invaginating contacts represent rudimentary chemical synapses. Some cubozoan jellyfish possess highly developed eyes with photoreceptor synapses that have complex invaginating postsynaptic spines. In fact, almost all major groups of animals, invertebrate and vertebrate, have a variety of invaginating structures at many of their synapses.

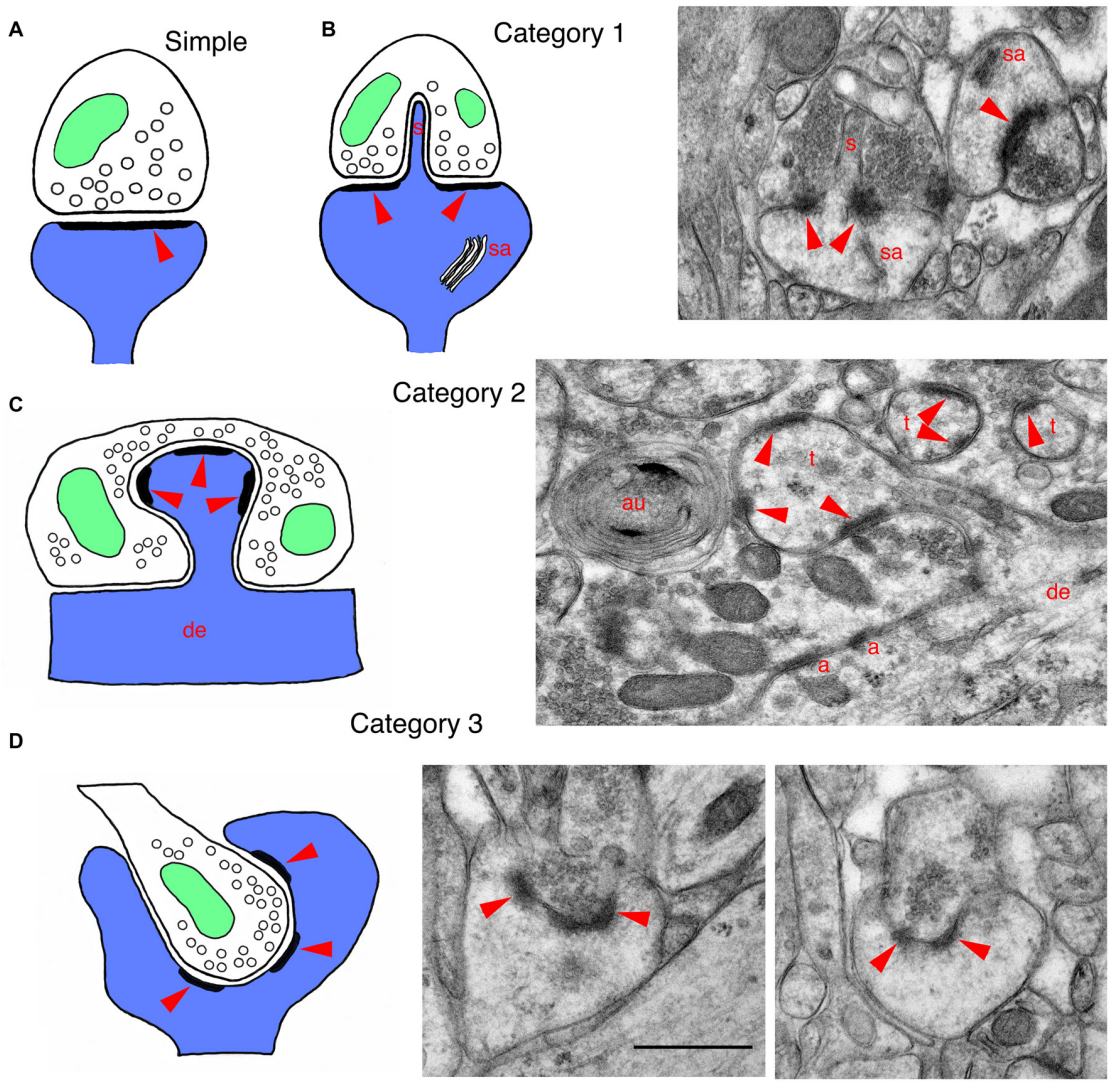


**FIGURE 1 |** Drawings and EM micrographs illustrating basic examples of the three categories of invaginating structures in mammalian synapses. In all drawings, postsynaptic structures are blue, presynaptic structures are white, mitochondria are green and red arrowheads indicate the postsynaptic densities of synapses. **(A)** A typical or regular postsynaptic spine forming a synapse with a presynaptic terminal. Arrowhead indicates the postsynaptic density (PSD) that is opposite the active zone, where synaptic vesicles fuse with the presynaptic membrane. **(B)** A drawing of a **category 1** invaginating structure shows a large mushroom spine with a spinule (s) that invaginates into the presynaptic terminal; mushroom spines often have a spine apparatus (sa). These large spines with spinules are associated with plasticity and spatial learning. The EM micrograph shows a spinule from a mushroom spine, invaginating into the presynaptic terminal (molecular layer of the dentate gyrus of adult rat). **(C)** A drawing of a **category 2** invaginating structure shows a postsynaptic spine protruding from a dendrite and invaginating into a presynaptic terminal. The EM micrograph was taken from the CA3 region of an adult hippocampus. It shows a Mossy fiber terminal (MFT) forming synapses on the spine-like thorny excrescences (t) extending from the apical dendrites of pyramidal cell neurons. The MFTs also form adherens junctions (a; a.k.a. attachment plaques) with the apical dendrite (de). Note also that a cluster of synaptic vesicles has been enwrapped by phagophores to form an autophagosome (au; Petralia et al., 2011; Vijayan and Verstreken, 2017). **(D)** A drawing of a **category 3** invaginating structure shows a presynaptic terminal invaginating into a postsynaptic process. The EM micrographs were taken from an adult rat dentate gyrus, and show cup spines with partially invaginating presynaptic terminals. The small terminal on the left is almost fully below the edge of the cup, while the terminal on the right is only partially invaginated; in some examples of cup spines described in the literature, the presynaptic terminal can be fully invaginating into the spine (see text for details). Note that tissue for EM in **Figures 1**, **2** was prepared using freeze substitution, and sections were stained with uranyl acetate and lead citrate (Petralia and Wenthold, 1999; Petralia et al., 2010). Scale bar in the two figures is 500 nm. Animal procedures were performed in accordance with guidelines approved by the institute Animal Care and Use Committee and NIH.

These invaginating structures can originate from the postsynaptic process, the presynaptic terminal, or glial processes. Many types of invaginating structures do not contain or contact active zones (for example, **Figure 1B**). These active zone-free invaginating structures have been given various names including spinules, varicosities, and protrusions. In contrast, active zone-associated invaginating structures can be derived from postsynaptic processes that include postsynaptic spines and spine-like structures, or from part or all of the presynaptic terminal. We have previously described three categories of

invaginating structures in all animals (Petralia et al., 2015, 2016, 2017). In this short review, we focus on the three categories in mammals, and update the literature. We also discuss how all these invaginations can be essential for precise signaling events among neurons, and contribute to synaptic signaling.

# EXAMPLES OF INVAGINATING STRUCTURES AT MAMMALIAN SYNAPSES

## Category 1. Invaginating Spinules and Related Structures

These invaginating protrusions can be derived from the postsynaptic, presynaptic or glial components of synapses. Although active zones often lie adjacent to the invaginating structures, they do not have any active zone within the invagination (see **Figure 1B**).

### Postsynaptic

In mammals, postsynaptic spinules have been described best in rat hippocampus, but spinules are found in other parts of the brain such as cerebral cortex and cerebellum (**Figures 1**, **2**; Blanque et al., 2015; Petralia et al., 2015; Familtsev et al., 2016; Rodriguez-Moreno et al., 2017). In the adult rat CA1 stratum radiatum, Westrum and Blackstad (1962) found that spinules are 25–100 nm wide and 75–150 nm long, but Spacek and Harris (2004) found greater variation in size (diameters from <8 nm to 150 nm), with some dendritic spine spinules longer than 0.75 μm; and Tao-Cheng et al. (2009) found postsynaptic spinules as long as 0.5 μm in hippocampal slice cultures. Postsynaptic spinules include those that invaginate into presynaptic terminals, as well as some that invaginate into adjacent axonal or glial processes (Spacek and Harris, 2004). Often the tip of the spinule is surrounded by a coated pit in the opposing cell membrane (hippocampus (Westrum and Blackstad, 1962; Spacek and Harris, 2004; Yao et al., 2005; Tao-Cheng et al., 2009); cerebellum (Eckenhoff and Pysh, 1979)). Spacek and Harris (2004) suggest that: "...spinules provide a general mechanism for signaling and remodeling throughout the brain". Postsynaptic spinules are involved in synaptic plasticity that occurs during all stages of life, from early postnatal development to old age; one of the best studied examples of synaptic plasticity that involves spinules occurs in large, mushroom-shaped spines of the hippocampus (reviewed in Geinisman et al., 1994; Petralia et al., 2014). Typically, during plasticity such as that initiated by long-term potentiation (LTP), the mushroom spine grows in size (and adds postsynaptic receptor molecules) and a perforation forms in the center of the postsynaptic density (PSD). At the perforation, the postsynaptic membrane may begin to invaginate into the presynaptic terminal as a spinule (**Figure 1B**); eventually, the PSD may separate into pieces (segmentation) as the spine continues to grow. These spines may go through cycles of enlargement and shrinkage associated with activity and aging. Also, the associated spinules undergo rapid turnover during sustained synaptic activity; this may be a mechanism of membrane retrieval by the presynaptic terminal to compensate for excessive growth of spine membrane induced by activity (Tao-Cheng et al., 2009).

### Presynaptic

In adult rat hippocampus stratum radiatum, as described above, most spinules originate from postsynaptic structures, but Spacek and Harris (2004) found that about 12% grow from axons and invaginate into other axons or glia. In several regions of the limbic system, such as the globus pallidus, axon terminals can interlock with each other along their lateral surfaces via large processes called pseudopodial indentations (Boyne and Tarrant, 1982). These might function as "variable diffusion traps" that control ions in the extracellular spaces between adjacent terminals, thus probably influencing their membrane potentials. In the dentate gyrus, entorhinal cortex and basolateral amygdala, some inhibitory GABAergic terminals extend short invaginating projections into the postsynaptic neuron; the presynaptic membrane contains cannabinoid receptors, and the invaginating projection opposes a part of the postsynaptic membrane that is rich in an enzyme, DGLα, that synthesizes an endogenous cannabinoid (**Figure 2A**; Yoshida et al., 2011; Omiya et al., 2015). This structure mediates a retrograde cannabinoid signal producing specific tonic inhibition of synaptic activity. Other interesting examples of presynaptic invaginating structures include thin spinules in early postnatal rodents extending from auditory hair cells and from cerebellar parallel fibers, into postsynaptic afferent processes or Purkinje cell dendrites, respectively (see Petralia et al., 2015). Also, Brusco et al. (2014) shows examples of both presynaptic and postsynaptic spinules in the amygdala.

### Glial

Glial-derived invaginating projections are common in invertebrates and some lower vertebrates, including at synapses and associated with other parts of neurons (Petralia et al., 2015), but relatively few have been described in mammals. In the cat, Schwann cell processes from the surrounding myelin sheath can extend small invaginating processes into spiral ganglion neurons (Adamo and Daigneault, 1973). Various kinds of glial processes commonly invaginate into axons of mammals (Spencer and Thomas, 1974). One kind involves invaginating "tongues" or "protrusions" originating from surrounding Schwann or oligodendrocyte cytoplasm; these processes appear to ensheath and remove groups of abnormal axonal organelles, and are more common in diseased axons (Spencer and Thomas, 1974). Small spinules also can invaginate into axons from surrounding glia; these typically end in coated pits in axons; Novotny (1984) suggests that glia utilize these structures to transfer substances essential for axonal function.

## Category 2. Invaginating Postsynaptic Spines

These postsynaptic spines protrude directly into the presynaptic terminal and contain active zones within the invagination (**Figure 1C**).

The best examples in mammals, in the hippocampal CA3 region and retina, are described separately. Other

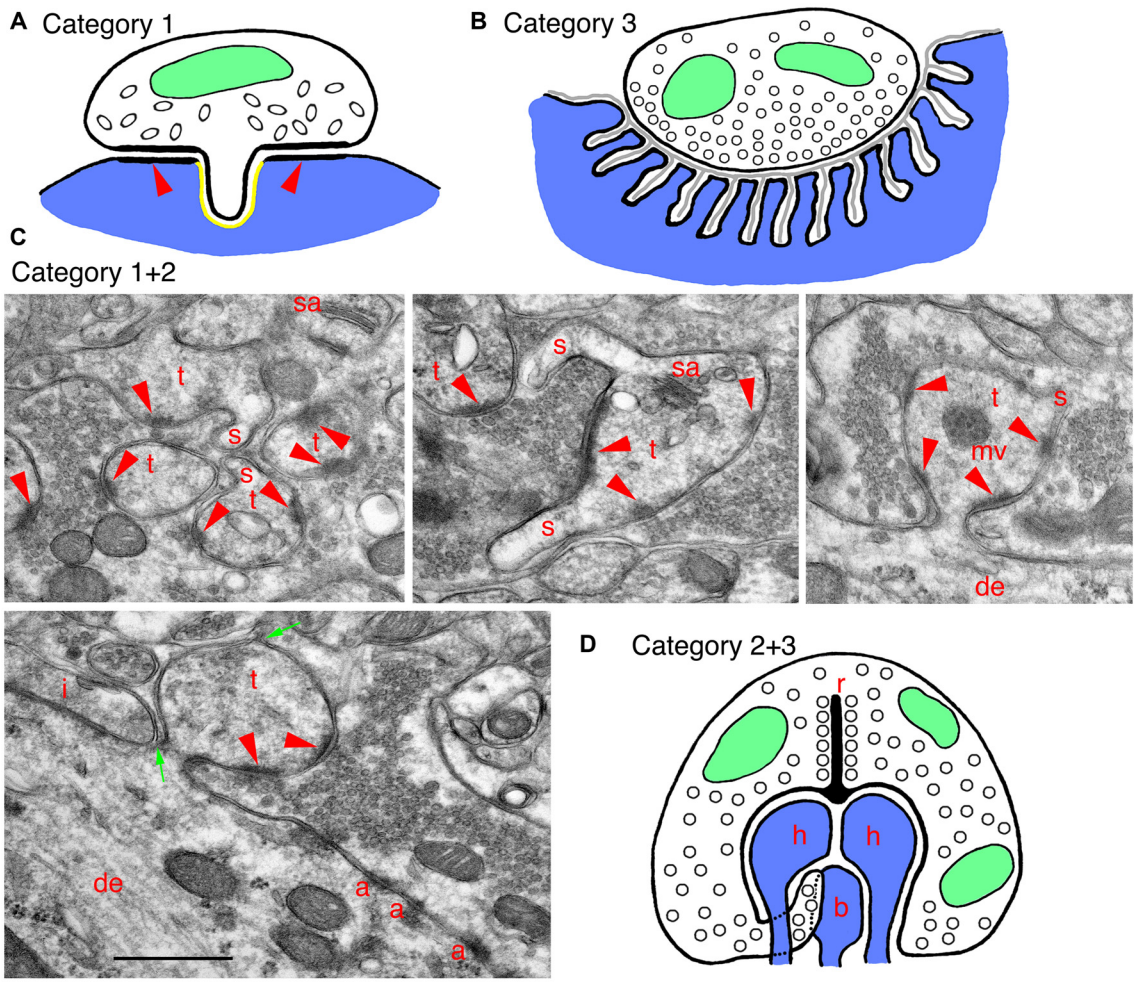


**FIGURE 2 |** Examples of specialized invaginating structures. **(A)** A drawing of an unusual example of a **category 1** invaginating structure: a presynaptic terminal invaginates into the postsynaptic soma. The synapse has characteristics of inhibitory terminals with a less prominent PSD (arrowheads) and oval-shaped synaptic vesicles. The GABA receptors are on the postsynaptic membrane, lining the PSD (arrowheads) and ringing the invagination, and there are endocannabinoid synthetic enzymes on the postsynaptic side of the invagination (shown in yellow). Endocannabinoid release activates cannabinoid receptors in the presynaptic membrane, and these then mediate retrograde suppression of neurotransmitter release from the terminal. **(B)** A drawing of a specialized example of a **category 3** invaginating structure: a generalized mammalian neuromuscular junction (NMJ). In this example, the presynaptic terminal is only partly invaginated (indented) into the muscle fiber. The indention is lined with deep subjunctional folds in the postsynaptic membrane. A thin basal lamina (gray) extends within the synaptic cleft and into the folds. **(C)** EM micrographs show examples of invaginating structures combining **categories 1 and 2**. These are from the CA3 MFT region, as described in **Figure 1C**. Thorny excrescences (*category 2*) also commonly invaginate spinules (s; *category 1*) into the MFTs, especially apparent in the upper three micrographs. Note in the left, lower micrograph how thin portions of the MFT shown between the two green arrows surround part of the invaginated thorny excrescence. A tiny spinule is barely visible near the top green arrow. Note also how this MFT isolates the thorny excrescence surface from possible spillover from an adjacent inhibitory terminal (i); the latter is identified by the elongate symmetrical density as well as by some obscure pleomorphic synaptic vesicles (compare to the more distinctive and rounder excitatory synaptic vesicles in the MFTs). Common organelles in the thorny excrescences include the spine apparatus (sa) and multivesicular body (mv). **(D)** A drawing of a specialized invaginating structure combining **categories 2 and 3**. A photoreceptor terminal-synaptic ribbon (r) contacts a deep invagination containing postsynaptic processes (*category 2*) from horizontal (h) and bipolar (b) neurons, as well as projections from the terminal. Rod terminals in mammals usually have a single invaginated ribbon/active zone with two horizontal and two bipolar cell processes, as well as "fingers" of rod cytoplasm, while cone terminals have multiple invaginated ribbon/active zones, each with two horizontal and 1–2 bipolar cell processes (Rao-Mirotznik et al., 1995; Sterling and Demb, 2004; Petralia et al., 2017). The horizontal cell processes also may act as invaginating presynaptic terminals (*category 3*) since they send a retrograde signal to the photoreceptor terminal, mediating a feedback mechanism.

interesting examples of invaginating spines include: spines invaginating into early-postnatal developing auditory hair cells of the mouse and into giant terminals called endbulbs of Held in the anteroventral cochlear nucleus of the early postnatal cat, some invaginating filopodia-like spines in the red nucleus, and those forming some crest synapses (for details, see Petralia et al., 2016). Note that various structures called filopodia are common in the nervous system; they look like spinules, only are larger—usually >100 nm wide and >1 μm long, and usually are not invaginating. Like spines, filopodia contain

actin filaments; in contrast, the content of spinules is generally diffuse and poorly defined, and it may be difficult to distinguish wide spinules from thin filopodia. Some filopodia may mediate synaptogenesis of spine synapses and be important components of synaptic plasticity and learning (Fiala et al., 1998; Ozcan, 2017). Crest synapses are particularly unusual, and consist of a flattened, disk-shaped spine with synaptic active zones on the two sides, either invaginating into a single terminal or having two terminals—one per side; they are found scattered throughout the central nervous system (Akert et al., 1967; Petralia et al., 2016). In addition, afferents to taste bud cells often form spine-like indented or invaginated synapses in many mammals (Royer and Kinnamon, 1988, 1991; Witt and Reutter, 1996); the more deeply invaginating ones are described as finger-like projections or processes (Royer and Kinnamon, 1988, 1991).

## Category 3. Invaginating Presynaptic Terminals

These presynaptic terminals protrude directly into the postsynaptic structure (spine or dendrite) and contain active zones within the invagination (**Figure 1D**).

A modest variety of invaginating presynaptic terminal structures occur, including in developing auditory nerve endbulbs on neuron somas in the cat, vestibular nerve terminals on neuron somas of the rat lateral vestibular nucleus, crested dendrites in the rat interpeduncular nucleus, and cup-shaped spines (see Petralia et al., 2017); also, terminals often partially invaginate (deep indention) into neuron somas in the monkey lateral geniculate nucleus (Saavedra et al., 1968). The crested dendrite is a unique dendritic structure containing several crest spines joined with invaginating presynaptic terminals, found in the interpeduncular nucleus of the rat (Murray et al., 1979). A number of studies have described cup-shaped spines in the cerebral cortex and hippocampus of mammals. Basically, the spine appears to wrap around the smaller presynaptic terminal; the best examples are seen in the rat hippocampal dentate gyrus (**Figure 1D**; Desmond and Levy, 1983; Frotscher and Léránth, 1986). Presence of cup spines may be affected by neuronal plasticity and they may be more frequent in slice and neuronal cell cultures (Mitchell et al., 2012; Petralia et al., 2017; and unpublished data).

### Neuromuscular and Secretomotor Endings

In neuromuscular junctions (NMJs) of most animals, invertebrate and vertebrate, presynaptic terminals are indenting or invaginating into muscle fibers; thus, some kinds of terminals are found in a shallow, elongate indention ("gutter") on the surface of the fiber, while others are invaginating completely into the fiber (**Figure 2B**; Petralia et al., 2017). Most skeletal muscles in mammals have twitch fibers, defined by their ability to propagate an action potential along the fiber from the NMJ. In skeletal muscles of mammals (and in vertebrates in general), the NMJ postsynaptic membrane (muscle fiber) is often highly folded into subjunctional folds. This is designed to separate the acetylcholine neurotransmitter receptors on the crests of the folds from the sodium channels at the bottom of the folds, as well as align the receptors with the presynaptic active zones (York and Zheng, 2017). The overall arrangement serves to amplify the response to a relatively small amount of neurotransmitter; this is especially efficient in humans compared to mice and rats, since humans have a relatively smaller NMJ area and larger area of folds compared to mice and rats (and even more so compared to frogs; Martin, 1994; Slater, 2008). In addition, an increased depth of the indention or invagination appears to be tied somewhat to greater depth and complexity of the subjunctional folds, and this also could be related to the response speed of the muscle fibers (e.g., for fast vs. slow twitch fibers; Ellisman et al., 1976; Petralia et al., 2017). Various other kinds of muscle fibers have NMJs that can be indented or invaginated, including the slow (tonic) muscle fibers of ear and extraocular muscles, and muscle spindles, cardiac muscle and smooth muscle in internal organs; they also are found at motor nerve endings in exocrine and endocrine gland cells (reviewed in detail in Petralia et al., 2017).

## Category 1+2. Hippocampal Excrescences

Mossy fiber terminal (MFT) synapses in the CA3 area (and also hilus) of the hippocampus form unusual synapses with invaginating postsynaptic, spine-like processes called thorny excrescences (*category 2*; Petralia et al., 2015, 2016; also Reberger et al., 2018). They seem to be a specialization largely unique to mammals, although some similar structures are present in lizards (reviewed in Petralia et al., 2016). The large excrescences can contain some structures that are usually absent in typical spines, such as mitochondria, multivesicular bodies (**Figures 1C**, **2C**), ribosomes and a few microtubules. The excrescences are plastic structures and can form new invaginating extensions with new active zones following LTP (Zhao et al., 2012). MFTs originate from granule cells of the dentate gyrus. These specialized synapses may have evolved in mammals to mediate higher abilities for pattern separation of episodic memory (Treves et al., 2008; Schmidt et al., 2012). The distinct advantage of the invagination is evident in the MFT-thorny excrescence structure. Basically, it forms a very large, continuous synaptic membrane compartment with multiple active zones and excludes any glial processes. This special enclosed synaptic environment facilitates presynaptic diffusion of calcium, spillover of neurotransmitter to reach postsynaptic receptors at multiple active zones, and the spread of zinc co-released from the synaptic vesicles with glutamate (Li et al., 2001; Rollenhagen et al., 2007). So, the invaginated environment keeps some components in and excludes others. The circuitry is complicated and will not be described here, but this unusual synapse is "designed to have a higher net probability of release than most other cortical synapses…" (Henze et al., 2000). Hints of a similar design can be found elsewhere. Thus, dendritic excrescences in the rat somatosensory thalamus (ventrobasal complex) are multiple-branched spines somewhat simpler than the hippocampal thorny excrescences (Spacek and Lieberman, 1974); a similar arrangement may occur in the hamster dorsal lateral geniculate nucleus (So et al., 1985). They invaginate deeply into the large presynaptic terminal, that

is stitched to the dendrite shaft via adherens-like junctions, reminiscent of the hippocampal MFTs.

In adult rats, spinules (*category 1*) are common on excrescences (**Figure 2C**; Petralia et al., 2011). Spatial (water maze) training increases the size of the excrescences and the number of spinules, and some spinules may even form bridges between individual thorns of the excrescences (Stewart et al., 2005); environmental enrichment also increases growth and complexity of the excrescences (Gogolla et al., 2009). Spinules may appear to form a contiguous sequence of structures with autophagosomes in the MFT (Petralia et al., 2011), suggesting that the spinules are involved in turnover of the excrescence membrane during activity, as suggested by Tao-Cheng et al. (2009) for hippocampus spinules in general. Interestingly, MFT spinules and autophagosomes label prominently with antibodies to the sonic hedgehog (Shh) signaling receptors, patched and smoothened, perhaps indicating a role for Shh in trans-synaptic signaling at the MFT synapse (Petralia et al., 2011); Shh also promotes autophagy in synaptic terminals (Petralia et al., 2013). Similarly, autophagy of the Wnt-signal mediator, disheveled, is implicated in regulation of Wnt signaling (Gao et al., 2010). In fact, the increased growth/complexity of thorny excrescences in mice exposed to an enriched environment is correlated with an increase in Wnt in the CA3; and it is likely that enhanced behavioral experience increases local signaling of Wnt at these synapses (Gogolla et al., 2009).

## Category 2+3. Photoreceptor Terminals

Photoreceptor terminals of many animals, both invertebrate and vertebrate, typically have invaginating postsynaptic processes (Petralia et al., 2016). In the retina of mammals, as for most vertebrates, the presynaptic terminal active zones of the rod and cone photoreceptor terminals usually have a deep invagination (**Figure 2D**) typically with 3–4 postsynaptic processes (spines or spine-like processes; *category 2*) including two from horizontal cell neurons and one or two from bipolar cell neurons (Sterling and Demb, 2004). The neurotransmitter glutamate is released from vesicles associated with ribbon-shaped, dense presynaptic structures (i.e., the synaptic ribbon) and diffuses to reach various populations of postsynaptic receptors placed at different distances from the active zone; in cones at least, this includes some receptors below the invagination (Haverkamp et al., 2000, 2001; Sterling and Demb, 2004). The complex structure of the invagination thus can serve to separate different receptor populations at various distances to control responses according to activity, glutamate release volume and subsequent extent of spillover.

Interestingly, at least the horizontal cell processes also appear to act as invaginating presynaptic terminals (*category 3*); these can be presynaptic to both the photoreceptor terminal and the bipolar cell processes. It is common to find numerous vesicles in the invaginated horizontal cell processes; good examples are found in rats, monkeys and humans; in addition, there is good evidence that the latter processes can be GABAergic (Petralia et al., 2017). However, definitive synapses between presynaptic horizontal cell processes and postsynaptic photoreceptor plus bipolar processes only have been described in the human (Linberg and Fisher, 1988). There is considerable evidence that horizontal processes provide a feedback mechanism to the photoreceptor cell synapse, but the details of the mechanism are debated; generally, this is believed to involve one or more of the following: GABA, protons (pH) and ephaptic transmission (close-range changes in electrical field; Gardner et al., 2015; Kramer and Davenport, 2015; Chapot et al., 2017). Ephaptic transmission might involve connexin hemichannels; these have been found in horizontal cell processes in fish, but it is not clear if they are present in mammalian horizontal cell processes (Klaassen et al., 2011; Gardner et al., 2015; Kramer and Davenport, 2015). Alternatively, both ephaptic and pH-mediated transmission in horizontal cells could be mediated via pannexin-based channels (Kranz et al., 2013; Cenedese et al., 2017; Chapot et al., 2017). In our own studies, we found preliminary evidence of GABA receptor immunogold labeling between horizontal cell processes and adjacent structures, including rod cytoplasmic fingers (Petralia et al., 2017). While GABA transmission, if it occurs, is assumed to involve postsynaptic GABA receptors on the photoreceptor terminal, some evidence indicates that these are autoreceptors on the horizontal cell processes, and they mediate a pH-based feedback (Hirano et al., 2016).

# CONCLUSION

Invaginating structures are common at synapses and are associated either with developmental plasticity or are integral to the mature synapse structure. In some cases, like the hippocampal MFTs or NMJs, mammals may show particularly well-developed invaginating synaptic structures, reflecting perhaps evolutionary enhancements in the mammalian brain and in brain-muscle coordination. The three categories differ in structure, but in all cases, the invaginated synapse forms a special, enclosed environment that allows wide movement of neurotransmitters and/or other chemicals while excluding other substances, leading to modifications in neurotransmission or selective chemical signaling between the neurons. The invagination also may be specialized for signaling via ephaptic conduction. This has been studied so far in only a few areas such as the retina, but it is likely a widespread mechanism for synaptic modulation, as noted by Gardner et al. (2015).

# AUTHOR CONTRIBUTIONS

RSP, MPM and PJY wrote the manuscript and RSP, Y-XW and PJY contributed to the figures.

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frontiers in Synaptic Neuroscience

REVIEW
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# Astrocyte–Neuron Networks: A Multilane Highway of Signaling for Homeostatic Brain Function

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Research on glial cells over the past 30 years has confirmed the critical role of astrocytes in pathophysiological brain states. However, most of our knowledge about astrocyte physiology and of the interactions between astrocytes and neurons is based on the premises that astrocytes constitute a homogeneous cell type, without considering the particular properties of the circuits or brain nuclei in which the astrocytes are located. Therefore, we argue that more-sophisticated experiments are required to elucidate the specific features of astrocytes in different brain regions, and even within different layers of a particular circuit. Thus, in addition to considering the diverse mechanisms used by astrocytes to communicate with neurons and synaptic partners, it is necessary to take into account the cellular heterogeneity that likely contributes to the outcomes of astrocyte–neuron signaling. In this review article, we briefly summarize the current data regarding the anatomical, molecular and functional properties of astrocyte–neuron communication, as well as the heterogeneity within this communication.

**Keywords: astrocytes, behavior, neuron–glia signaling, synaptic plasticity, heterogeneity**

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## INTRODUCTION

A fundamental property of the mammalian brain is its ability to modify its function based on experience, and thereby to alter subsequent behavior. By changing the strength of transmission at preexisting synapses, transient experiences can be incorporated into the neuronal circuits as persistent memory traces during both development and adulthood. As such, synaptic plasticity is a fundamental mechanism that supports brain function (Buzsáki and Chrobak, 2005). Among the different factors that regulate synaptic plasticity, glial cells have been found to be key players in maintenance of synapse homeostasis (Eroglu and Barres, 2010). The biggest challenge when studying the effects of glial cells on brain activity is isolating the different cell-type components, i.e., neurons vs. glia. Recent research advances using various strategies, such as pharmacological or genetic manipulation and gene expression from viral vectors (Nimmerjahn and Bergles, 2015; Oliveira et al., 2015; Ben Haim and Rowitch, 2017), have allowed researchers to elucidate the role of glial cells in several aspects of brain function, and such knowledge may lead to the development of new therapies and biomarkers for many types of neurological dysfunction (Almad and Maragakis, 2018).

Astrocytes, after oligodendrocytes, constitute the major glial cell population in the mammalian brain (Herculano-Houzel et al., 2015). Since the tripartite synapse concept emerged in 1999 (Araque et al., 1999), data from numerous studies have supported the notion that astrocytes are involved

in tight regulation of synaptic transmission (Eroglu and Barres, 2010; Perea et al., 2014; Bazargani and Attwell, 2016). Given that astrocytes have been revealed as strategic cells for controlling neuronal activity, it is crucial to understand the properties and functions of these cells. Astrocytes are now recognized as a markedly heterogeneous group comprising different morphologically specialized cells, such as protoplasmic astrocytes, fibrous astrocytes, perivascular glia, velate astrocytes, Müller cells or Bergman glia, which show particular molecular profiles and which have been extensively reviewed previously (Matyash and Kettenmann, 2010; Reichenbach et al., 2010; Farmer and Murai, 2017). Additionally, there are significant differences between human astrocytes and their rodent counterparts, i.e., the gene expression pattern (Zhang et al., 2016), size and complexity of cellular architecture (Oberheim et al., 2009), and faster calcium dynamics (Oberheim et al., 2009) indicate specialization of glial cells in the human brain that may contribute to the distinctive neurological capabilities that make humans different from other mammals (Han et al., 2013). It is not yet quite clear how those differences account for the higher functions of the human brain (Min et al., 2012; Vasile et al., 2017).

One of the key factors that regulates intracellular signaling in astrocytes is calcium ($Ca^{2+}$). However, because the controversies regarding astrocyte $Ca^{2+}$ signaling and synaptic plasticity, which have been revised in recent excellent reports (Araque et al., 2014; Rusakov, 2015; Fiacco and McCarthy, 2018; Savtchouk and Volterra, 2018), $Ca^{2+}$ signals will not be further discussed in this review article.

The goal of the present review is to highlight the existing data supporting the critical roles of astrocytes in synaptic function, and how those roles may be determined by structurally and functionally different astrocyte populations.

## ION HOMEOSTASIS AND NEUROTRANSMITTER UPTAKE

Astrocytes tightly enwrap neuronal cell bodies, axons, dendrites and synapses (Montagnese et al., 1988; Ventura and Harris, 1999; Khan et al., 2001; Witcher et al., 2010), and their endfoot processes associate with vascular endothelial cells and pericytes (Liebner et al., 2018), being ideally positioned to monitor and regulate both synaptic activity and blood brain barrier. The close association between astrocytes and neuronal synapses is a critical factor required for the maintenance of brain homeostasis (Perea et al., 2009). Astrocytes predominantly show potassium ($K^+$) conductance (Kuffler and Nicholls, 1966; Hertz et al., 2013), which is mainly due to the inwardly rectifying $K^+$ (Kir) channels that control the hyperpolarized resting potential of astrocytes (Seifert et al., 2009). Among other important channels/ion transporters (e.g., aquaporin-4, chloride channels, $Na^+$-$Ca^{2+}$ exchangers; Haj-Yasein et al., 2011b; Halnes et al., 2013), high densities of Kir4.1 channels have been found on thin processes that face the synapses, thus allowing rapid uptake of $K^+$ from the synaptic cleft and redistribution of $K^+$ in the extracellular space during neuronal activity (Kuffler and Nicholls, 1966; Seifert et al., 2018). Indeed, reduced levels of Kir4.1 protein expression in astrocytes lead to elevated extracellular levels of $K^+$ and neuronal membrane depolarization, which has been related to multiple sclerosis, amyotrophic lateral sclerosis, epilepsy and Huntington's disease (Haj-Yasein et al., 2011a; Jiang et al., 2016; Dossi et al., 2018). $K^+$ buffering has been well studied in the retina, where Müller cells show an enriched distribution of Kir channels in endfoot processes (Newman, 1984, 1993). The water channel aquaporin-4 is also highly expressed at the same subcellular domains (Nagelhus et al., 1999), indicating that $K^+$ uptake generates parallel water fluxes that are required to dissipate such osmotic changes. Additionally, it has been shown in optic nerve and hippocampus that $Na^+/K^+$-ATPase activity efficiently contributes to the clearance of $K^+$ following neuronal activity (Ransom et al., 2000; D'Ambrosio et al., 2002; Larsen et al., 2014), indicating that astrocytes may use a combination of different mechanisms to control extracellular $K^+$ levels.

Astrocytic membranes are enriched in glutamate and gamma-aminobutyric acid (GABA) transporters that are differentially expressed throughout the adult brain. These transporters serve as an efficient mechanism for clearing these neurotransmitters (NTs) from the extracellular space after neuronal activity (Borden, 1996; Bergles and Jahr, 1997; Danbolt, 2001). In fact, the expression of glutamate transporter 1 (GLT-1) and glutamate–aspartate transporter (GLAST) prevents glutamate-derived excitotoxicity during neuronal regular synaptic transmission (Danbolt, 2001); and under glutamatergic over-excitation, such as that observed in conditions like epilepsy or brain trauma (Tanaka et al., 1997; Goodrich et al., 2013). Although these transporters are distributed throughout the brain, the highest levels of GLT-1 are found in the hippocampus and the neocortex; while GLAST is enhanced in the cerebellum (Chaudhry et al., 1995; Lehre and Danbolt, 1998), and retina (Rauen et al., 1996; Lehre et al., 1997). Additionally, two populations of astrocytes have been described, based on the predominant expression of particular glutamate transporters in the hippocampus (Matthias et al., 2003). Interestingly, by modulating the expression levels and surface diffusion of glutamate transporters, astrocytes influence synaptic transmission by controlling the glutamate spillover beyond the synapse. Such glutamate spillover can activate extrasynaptic metabotropic glutamate receptors (Huang et al., 2004), which shape the kinetics of excitatory postsynaptic currents (EPSCs; Murphy-Royal et al., 2015). Hence, changes in EPSCs have important effects on the local and temporal integration of synaptic inputs by neuronal networks, and consequently on synaptic plasticity. Therefore, glutamate transporters not only support synaptic homeostasis, but also contribute, at least in part, to plasticity processes at the synaptic levels (reviewed by Rose et al., 2017).

Interestingly, the GABA transporters (GATs) GAT-1 and GAT-3 show particular cellular and sub-cellular distributions throughout the brain (Ribak et al., 1996; Boisvert et al., 2018). GAT-3 is the most abundant GAT in astrocytes and is localized in astrocytic processes that are adjacent to synapses and cell bodies, but are also close to basal and apical dendrites (Boddum et al., 2016), while GAT-1 can be found in distal astrocytic processes and is more abundant in neurons (Borden, 1996; Scimemi, 2014).

Activation of GAT-3 results in a rise in $Na^+$ concentrations in hippocampal astrocytes and a consequent increase in intracellular $Ca^{2+}$ through the action of $Na^+/Ca^{2+}$ exchangers (Doengi et al., 2009). Thus, GABA-uptake by astrocytic GAT-3 can stimulate the release of ATP/adenosine that contributes to downregulation of the excitatory synaptic transmission, and provides a mechanism for homeostatic regulation of synaptic activity in the hippocampus (Boddum et al., 2016). In the thalamus, GAT-1 and GAT-3 occupy different domains within the astrocytic membrane, with GAT-1 being located closer to synaptic contacts than GAT-3 (Beenhakker and Huguenard, 2010); this implies that these transporters might play different roles in GABAergic synaptic function. For instance, research suggests that GAT-1 reduces GABA spillover from the synaptic cleft, while GAT-3 controls the extrasynaptic GABA tone, thus regulating tonic inhibition (Beenhakker and Huguenard, 2010). There is a causal relationship between intracellular $Ca^{2+}$ levels and GAT-3 expression in striatal astrocytes (Yu et al., 2018). Downregulation of $Ca^{2+}$ signaling enhances membrane expression of GAT-3, resulting in the reduction of GABAergic tone and abnormal repetitive behavioral phenotypes in mice (Yu et al., 2018) that are related to human psychiatric disorders.

Together with glutamate and GABA uptake, a transient increase in intracellular $Na^+$ concentration occurs (Gadea and López-Colomé, 2001a,b). That $Na^+$ local boost can be buffered through gap junctions to neighboring astrocytes acting as an intercellular signaling molecule (Rose and Ransom, 1997; Kirischuk et al., 2007). Considering that $Na^+$ is also co-transported with other transmitters and molecules, changes in the intracellular $Na^+$ concentration are directly related with changes in synaptic transmission (Karus et al., 2015), and the activity of $Na^+/K^+$-ATPase, linking $Na^+$ homeostasis to metabolic functions in astrocytes (for review see Chatton et al., 2016).

Therefore, astrocytes are powerful regulators of synaptic activity by combining the extent of synapse coverage and the expression level of ion channels and neurotransmitter transporters at their cell membrane.

Nevertheless, it is important to note that astrocytes do not ensheath all synapses (Ventura and Harris, 1999; Witcher et al., 2010; Chung et al., 2015a). Moreover, the astrocytic coverage of synapses is a highly dynamic process that changes throughout development and adulthood (Chung et al., 2015a; Heller and Rusakov, 2015). Thus, in layer IV of the somatosensory cortex in adult mice, 90% of excitatory synapses are in contact with astrocytes (Bernardinelli et al., 2014a), as compared to 60%–90% of these synapses in the hippocampus (Ventura and Harris, 1999). In the cerebellum, only *ca*. 15% of mossy fiber synapses on granule cells are in contact with astrocyte processes; in contrast, the climbing fibers show *ca*. 85% of synapses covered by astroglial processes, and *ca*. 65% of parallel fiber synapses are also in relatively close contact with Bergmann glia (Xu-Friedman et al., 2001). Additionally, changes in astrocyte–synapse associations can be induced by different neuronal activity levels (Genoud et al., 2006; Bernardinelli et al., 2014b; Perez-Alvarez et al., 2014) and by a range of physiological conditions, including starvation and satiety (Panatier et al., 2006; Theodosis et al., 2008; Chung et al., 2015a). Hence, structural changes in the astrocytic processes can greatly impact the glial network signaling as well as its relationship with synapses, which will shift the function of neuronal circuits.

## ASTROCYTE NETWORKS

Astrocytes are enriched in gap junctions, which are formed by connexins (Cxs; Nagy et al., 1999). Cx43 and Cx30 are the main Cxs expressed by astrocytes (Nagy et al., 1999). Through gap junctions, which allow intercellular diffusion of ions, second messengers and small molecules of up to *ca*. 1.8 kDa (Kumar and Gilula, 1996), astrocytes form broad cellular networks that involve hundreds of astrocytes (Giaume et al., 2010; Pannasch et al., 2011). In fact, astrocytic intercellular diffusion has been reported for cyclic AMP, inositol-1,4,5-trisphosphate (InsP3), $Ca^{2+}$, glutamate, ATP and energy metabolites (glucose, glucose-6-phosphate and lactate; Tabernero et al., 2006; Harris, 2007). Prior research has demonstrated that the intact function of local astrocyte networks is critical for complex cerebral functions, including sleep–wake cycle regulation, sensory functions, cognition and behavior (for a review see Oliveira et al., 2015; Charvériat et al., 2017). Interestingly, such astrocytic networks show selective and preferential coupling, meaning that not all neighboring astrocytes are functionally connected by gap junctions (Houades et al., 2008; Roux et al., 2011). Based on data of intracellular loading of tracers/reporters in single cells, it has been shown that astrocytes occupy non-overlapping territories, that is, they have independent domains that are established during development (Bushong et al., 2002; Ogata and Kosaka, 2002). However, it remains unclear whether the preferential connectivity between subsets of astrocytes is determined by a common astrocyte progeny during embryonic development or by local factors. Studies focused on astrocyte lineage have revealed that multiple astrocyte clones derived from single precursor cells coexist in the adult cortex, where these clones establish spatially restricted domains that contain up to 40 astrocytes (García-Marqués and López-Mascaraque, 2013). Cx43 is expressed from early in development in radial glial cells; however, Cx30 is expressed postnatally in rodent astrocytes around the third postnatal week (Kunzelmann et al., 1999; Nagy and Rash, 2000). Such different expression of Cxs generates additional differences in the intercellular connectivity of astrocyte networks, with implications in metabolic states (glucose and lactate supply) and synaptic transmission (Rouach et al., 2008). Moreover, gap-junction connectivity is highly sensitive to changes in phosphorylation/dephosphorylation pathways, intracellular calcium levels, pH and redox-related variations (Sáez et al., 2014). Altogether, the data support the existence of plasticity within astrocyte networks. Because astrocytes form large circuits, further studies are required to understand how signals detected within particular astrocytic domains work either locally to affect a few synapses from the same neuron, or remotely to regulate synapses that possibly belong to different neurons or circuits. Future research should also clarify the molecular mechanisms underlying the complex actions of astrocyte–synapse communication in brain circuits.

## ASTROCYTES: MASTER REGULATORS OF SYNAPTIC ACTIVITY

Intracellular $Ca^{2+}$ signals, driven by endogenous signaling or neuronal activity, are also related to the release of active substances, called gliotransmitters (GTs), which target the synapse via vesicular-dependent (Araque et al., 2000, 2014; Bezzi et al., 2004; Bowser and Khakh, 2007; Parpura and Zorec, 2010) and -independent mechanisms (Duan et al., 2003; Hamilton and Attwell, 2010; Lee et al., 2010; Woo et al., 2012). Although there are controversies regarding the astrocytic expression of different components required for vesicular transmitter release (Schwarz et al., 2017; Bohmbach et al., 2018), several studies have elucidated the mechanisms underlying the dynamic regulation of synaptic transmission by astrocyte activity; this topic has been extensively reviewed (Araque et al., 2014; Bazargani and Attwell, 2016; Allen and Eroglu, 2017).

By releasing glutamate, D-serine, GABA, ATP, adenosine, or tumor necrosis factor-alpha, among others, astrocytes control the basal tone of synaptic activity and the threshold for synaptic plasticity (Beattie et al., 2002; Angulo et al., 2004; Fellin et al., 2004; Jourdain et al., 2007; Perea and Araque, 2007; Henneberger et al., 2010; Bonansco et al., 2011; Di Castro et al., 2011; Panatier et al., 2011; Chen et al., 2013; Shigetomi et al., 2013; Gómez-Gonzalo et al., 2015; De Pittà and Brunel, 2016; Petrelli et al., 2018). One hippocampal astrocyte ensheaths approximately 120,000 synapses (Bushong et al., 2002) belonging to different cell types (excitatory vs. inhibitory neurons) and circuits, and that astrocyte might be able to detect the NTs released from all of those synapses. Indeed, glutamatergic synaptic activation of astrocytes stimulates the release of glutamate, D-serine, ATP, or adenosine, which, through the activation of pre- and postsynaptic receptors sets the threshold for basal synaptic transmission (Bonansco et al., 2011; Panatier et al., 2011), and enhances short- and long-term glutamatergic synaptic plasticity (Jourdain et al., 2007; Perea and Araque, 2007; Henneberger et al., 2010). GABAergic activity stimulates astrocyte $Ca^{2+}$ signaling (Mariotti et al., 2016, 2018; Perea et al., 2016), which induces the release of ATP and adenosine, decreasing the excitatory synaptic tone (Serrano et al., 2006; Covelo and Araque, 2018). Interestingly, hippocampal astrocytes can contribute to neuronal information processing by decoding GABAergic synaptic activity based on frequency and duration of interneuron firing (Perea et al., 2016; Covelo and Araque, 2018). Such decoding dictates whether astrocytes release either glutamate, which enhances excitatory synaptic activity (Perea et al., 2016), or ATP/adenosine, which reduces excitatory synaptic strength (Covelo and Araque, 2018).

Pyramidal cell activity can also engage astrocytes through endocannabinoid (eCB) signaling. eCBs play a critical role in short- and long-term plasticity at both excitatory and inhibitory synapses, mainly via retrograde signaling (Kano, 2014). However, growing evidence indicates that astrocytes participate in eCB signaling, with the postsynaptic activity-dependent release of eCBs stimulating $Ca^{2+}$ signaling in surrounding astrocytes, ultimately influencing glutamatergic synaptic transmission (Navarrete and Araque, 2010; Min and Nevian, 2012; Gómez-Gonzalo et al., 2015; Martín et al., 2015; Andrade-Talavera et al., 2016; Martin-Fernandez et al., 2017; Robin et al., 2018). In fact, research has shown that eCB-astrocyte activation stimulates the release of glutamate, which enhances synaptic strength, with both short-term (Navarrete and Araque, 2010; Martín et al., 2015; Martin-Fernandez et al., 2017) and long-term effects (Gómez-Gonzalo et al., 2015). Moreover, D-serine is released in response to eCB-astrocyte activation, and by stimulating synaptic N-methyl-D-aspartate receptors (NMDARs), actively contributes to hippocampal long-term potentiation (LTP; Robin et al., 2018) and spike timing-dependent long-term depression (tLTD; Min and Nevian, 2012; Andrade-Talavera et al., 2016). Therefore, eCBs that mainly depress synaptic transmission can, by activating astrocytes, exert opposite or additive effects on excitatory synaptic transmission in different brain areas, such as the hippocampus. This has important homeostatic effects that contribute to achieving coordinated activity among neuronal ensembles. Another important factor released by astrocytes is S100β, a $Ca^{2+}$ binding protein, that is able to induce neuronal bursting and engages rhythmic activity both in the dorsal part of the trigeminal main sensory nucleus (NVsnpr; Morquette et al., 2015), and in the prefrontal cortex (Brockett et al., 2018). Additionally, astrocytic S100β enhances synchrony between theta and gamma cortical oscillations and improves cognitive flexibility (Brockett et al., 2018), indicating the behavioral impact of GTs.

These representative examples show the complex and refined effects of astrocyte-released transmitters on neuronal activity. Nevertheless, it is important to keep in mind that astrocytes can also respond to other neuromodulators, such as norepinephrine (Bekar et al., 2008; Paukert et al., 2014), acetylcholine (Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012; Papouin et al., 2017), dopamine (Jennings et al., 2017), and molecules derived from the neuroendocrine system (Fuente-Martin et al., 2013; Kim et al., 2014), possibly in different fashions depending on the nature of NTs and affecting particular neural circuits that rule behavioral outputs. For example, astrocytes in the hypothalamus respond to the hormones leptin, ghrelin, and insulin, and regulate neuronal activity by releasing ATP (Kim et al., 2014; García-Cáceres et al., 2016), controlling the food consumption. Astrocytes form the dorsal suprachiasmatic nucleus (SCN) and show an anti-phase oscillatory activity compared to neurons, being more active during the night and reducing neuronal firing by the release of glutamate (Brancaccio et al., 2017). Hence, SCN astrocytes show high $Ca^{2+}$ activity at night and release high levels of glutamate into the extracellular space, activating presynaptic NMDARs in SCN neurons, which in turn increases the GABAergic tone across the circuit. However, during daytime extracellular levels of glutamate are reduced by an increased glutamate uptake, and consequently GABAergic tone is reduced, facilitating neuronal firing (Brancaccio et al., 2017). Astrocytes also participate in sleep homeostasis, which is regulated by the accumulation of adenosine (Halassa et al., 2009; Brown et al., 2012). By releasing ATP/adenosine and glutamate, astrocytes regulate cortical states and induce the transition into slow neuronal oscillations associated with sleep

(Fellin et al., 2009; Poskanzer and Yuste, 2016; Clasadonte et al., 2017). In this spirit, the lymphatic-like pathway organized by astrocytes and blood vessels in the central nervous system, the "glymphatic" hypothesis (Xie et al., 2013; Plog and Nedergaard, 2018), suggests a significant impact of astrocyte activity during sleep in terms of the clearance of different solutes accumulated during wakefulness. Additionally, glymphatic system seems to be critical for the distribution of nutrients and metabolic homeostasis throughout the brain (Lundgaard et al., 2017), and an enhanced glymphatic clearance has been related with the reduced lactate levels in the brain that usually accompany the transition from wakefulness to sleep (Lundgaard et al., 2017). Therefore, the opposite and complementary neuron–astrocyte signals mutually support the mammalian circadian clock.

Interestingly, the disruption of the glymphatic system has been related with the accumulation of toxic species in the brain, such as amyloid β (Xie et al., 2013). Glymphatic system dysfunctions have been found in murine models that resemble human type 2 diabetes, which also show accumulation of misaggregated proteins (Jiang et al., 2017). Whether glymphatic system alterations and the accumulation of waste in the paravascular space drive the cognitive deficits associated with Alzheimer Disease (AD) or diabetes (Yaffe et al., 2004; Moheet et al., 2015) is under debate (Bacyinski et al., 2017).

## PLASTICITY AT ASTROCYTE–NEURON NETWORKS

Along with the changes noted in synapses, astrocytes are also sensitive to plasticity processes. Indeed, structural changes, based on the number of synapses covered by astrocyte processes, have been reported in the hippocampus, hypothalamus and cerebellum (Haber et al., 2006; Lippman et al., 2008; Theodosis et al., 2008). Structural imaging studies have shown that fine astrocyte processes have a high motility rate, changing their shape at a time-scale of minutes (Haber et al., 2006; Bernardinelli et al., 2014b; Perez-Alvarez et al., 2014), and can be influenced by learning paradigms, i.e., LTP protocols (Bernardinelli et al., 2014b; Perez-Alvarez et al., 2014). Moreover, after sustained afferent inputs, astrocytes display functional changes based on up/down regulation of membrane ion channels, and neurotransmitter receptors and transporters, showing similar plasticity phenomena to their neuronal counterparts. After using protocols that induce neuronal LTP, hippocampal astrocytes (Pita-Almenar et al., 2006, 2012) show enhanced ability to take up glutamate from adjacent synapses. *In vivo*, whisker stimulation that stimulates LTP in somatosensory cortical neurons also induces an increase of the expression of GLAST and GLT1 in cortical astrocytes (Genoud et al., 2006). In contrast, sustained depression of glutamate transporter currents and AMPA-mediated currents are expressed by Bergmann glia at low frequencies, which typically trigger LTP in Purkinje neurons (Bellamy and Ogden, 2006; Balakrishnan and Bellamy, 2009; Wang et al., 2014). Functional changes are seen not only in terms of neuron-to-astrocyte signaling, that is, the capability of astrocytes to sense and respond to neuronal activity, but also in terms of astrocyte-to-neuron communication. Thus, astrocytes from the ventrobasal (VB) thalamus are capable of adapting their actions on thalamic neurons when protocols for synaptic plasticity are applied to both the peripheral somatosensory and corticothalamic glutamatergic inputs (Pirttimaki et al., 2011). Repetitive stimulation of those pathways leads to a sustained increase in glutamate release from astrocytes, which persists for several minutes after the offset of the stimulus (*ca*. 60 min). Such enhanced gliotransmission affects the nearby thalamic neurons through NMDA receptor activation for long periods, boosting the time window for synaptic plasticity (Pirttimaki et al., 2011). These facts indicate that astrocytes are endowed with mechanisms that allow them to integrate synaptic information and store it for a period of time; therefore, astrocytes are able to memorize synaptic events that will have an impact on subsequent neuronal activity. Hence, astrocytic plasticity is an activity-dependent and input-specific process that is tightly controlled by synaptic activity. However, concomitantly neuronal signaling is dynamically modulated by the surrounding astrocytes, reinforcing the concept that brain function relies on interdependent neuron–astrocyte signaling.

## ASTROCYTE HETEROGENEITY

To improve our understanding of brain circuits, it is essential to identify the properties and functions of each of their components. Neurons consist of several subtypes that are defined by their morphology, genetic profile, electrophysiological properties and input/target regions (Bota and Swanson, 2007). Recent data indicate that astrocytes are also a highly heterogeneous cell group with precise neural circuit specializations, especially when considering the wide range of transporters, membrane receptors, protein expression and functions that they exhibit (Zhang and Barres, 2010; Freeman and Rowitch, 2013; Khakh and Sofroniew, 2015). For example, a recent study on astrocyte diversity, which employed state-of-the-art optical, anatomical, electrophysiological, transcriptomic and proteomic approaches, revealed that dorsal striatal and hippocampal astrocytes (stratum radiatum) show significant differences in the sizes of their barium-sensitive $K^+$ currents, as well as differences in the spontaneously and synaptically evoked G protein-coupled receptor-mediated $Ca^{2+}$ signals (Chai et al., 2017). Interestingly, hippocampal and striatal astrocytes show different territory sizes, with the territory size being larger for striatal astrocytes, although hippocampal astrocytes display significantly greater and closer physical interactions with excitatory synapses than do astrocytes in the striatum (Chai et al., 2017). Striatal astrocytes are enriched for expression of the aldehyde dehydrogenase 5 family member A1 (Aldh5a1), a protein involved in GABA degradation, which seems highly relevant to a circuit mainly composed of GABAergic medium spiny neurons (Chai et al., 2017). Likewise, astrocytes from the dorsal striatum show functional selectivity in terms of neuronal cell-type activity by responding with variations in $Ca^{2+}$ to the signaling of a particular type of medium spiny neuron (D1 or D2; Martín et al., 2015). By releasing glutamate, astrocytes activated by D1 or D2 neurons will specifically signal only the same type

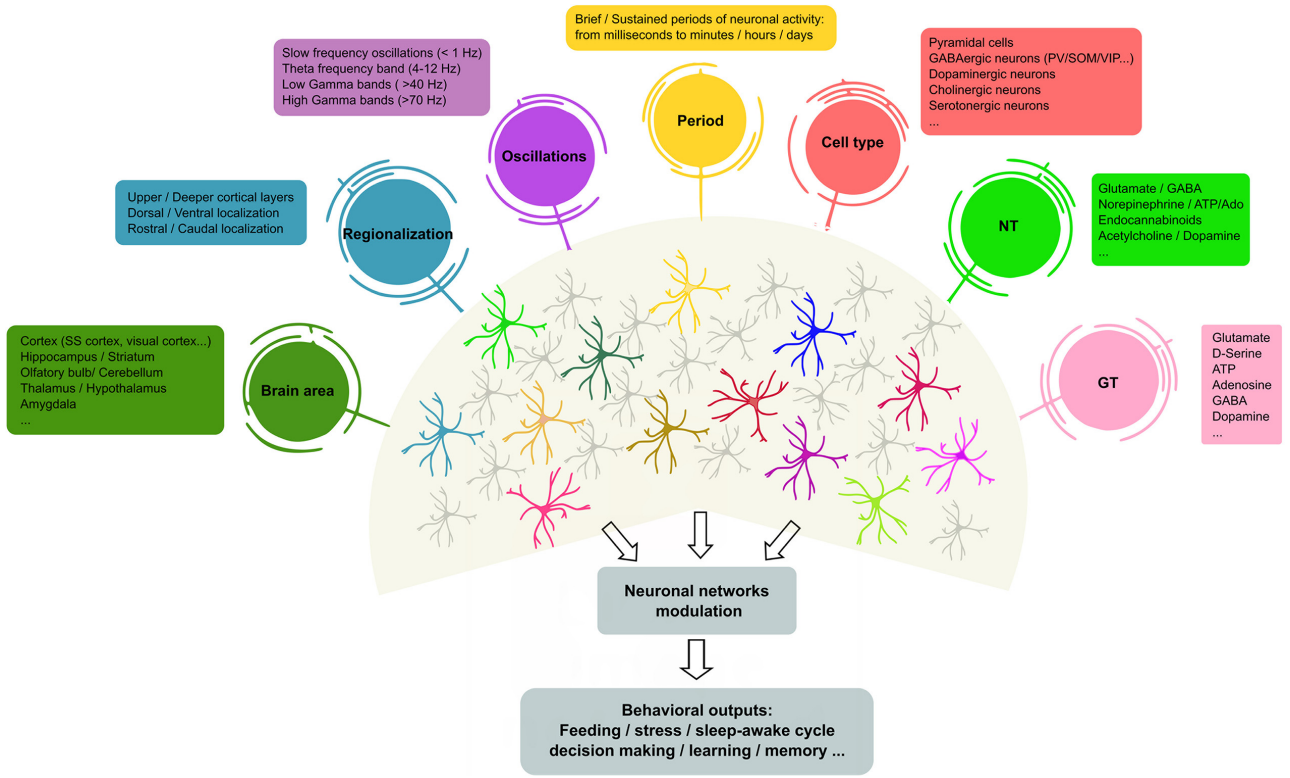


**FIGURE 1 |** Heterogeneity of astrocyte–neuron networks. Astrocytes are able to respond to different NTs, cell types in different brain areas and under diverse patterns of stimulation. In turn, astrocytes modulate the activity of ion channels and neurotransmitter transporters in their membranes, but also release different active substances (so-called GTs, e.g., glutamate, gamma-aminobutyric acid (GABA), adenosine and D-serine, among others) that contribute to shape neuronal activity. However, it remains unclear whether the same or different astrocyte subtypes mediate these differential effects. The scheme illustrates recent evidence of the molecular and functional diversity of astrocytes that may underlie the outcomes of brain activity. In this context, single astrocytes can be affected by more than one physiological feature (represented as colored circles that connect to astrocytes), or subsets of astrocytes may show preferential links with particular physiological features, suggesting the existence of different astrocyte populations that exhibit specific properties (astrocyte color code). NTs, neurotransmitters; GTs, gliotransmitters; SS, somatosensory; PV, parvalbumin positive cells; SOM, somatostatin positive cells; VIP, vasoactive-intestinal peptide.

of neuron, implying that astrocyte–synapse signaling is largely cell-type specific (Martín et al., 2015). Compared with the striatum, hippocampal astrocytes are enriched for expression of glial fibrillary acidic protein (GFAP), Cx 43 and glutamine synthetase (Chai et al., 2017), which are likely involved in both glutamate metabolism and astrocyte connectivity in a circuit with strong oscillatory activity. Astrocytes from another dopaminergic nucleus, the ventral tegmental area (VTA), also show specific features that differentiate them from cortical and hippocampal astrocytes. VTA astrocytes show morphological differences, smaller somata and less tissue coverage by their processes, as well as electrical membrane property differences, and reduced expression of Kir4.1 channels (Xin et al., 2018). Furthermore, although gap junction coupling between astrocytes and oligodendrocytes is also present in the hippocampus and cortex, it is significant higher in the VTA region (Xin et al., 2018), which could impact the metabolic states of the dopaminergic neurons and their axons that exhibit tonic firing activity.

Remarkably, one of the molecular markers usually used to identify astrocytes, GFAP, shows different isoforms (α, β, γ, δ and κ) that are variably expressed in astrocytes across different brain regions (Middeldorp and Hol, 2011). Indeed, the cortex shows limited detectable levels of GFAP-labeled astrocytes, mostly located in layer 1 and in deep layers; as well as in the thalamus and other subcortical regions. In contrast, the hippocampus displays a high number of astrocytes expressing detectable levels of GFAP, which is considered to indicate astrocytic molecular diversity. Additionally, developmental and regional differences can be found in terms of the expression of the GLTs GLT-1 and GLAST, which show a dominant expression in different astrocyte populations (Regan et al., 2007). Hence, much effort has been expended in quantitative analysis of the molecular profiles of astrocytes in different brain regions. An integrated transcriptional analysis has been performed, taking advantage of some of the most common proteins expressed by astrocytes, such as GFAP, aquaporin-4, S100β, glutamine synthetase, GLT-1 and Aldh1L1 (Bachoo et al., 2004; Zhang et al., 2016; John Lin et al., 2017; Morel et al., 2017). In this spirit, the astroglial mRNA expression patterns have been examined along the dorsoventral axis, including the cortex, hippocampus, thalamus, hypothalamus,

caudate-putamen and nucleus accumbens. These studies revealed opposite profiles between dorsal (cortex and hippocampus) and ventral (thalamus and hypothalamus) regions, i.e., the extracellular matrix protein, secreted protein acidic and rich in cysteine (SPARC) is selectively highly expressed in the hypothalamus/thalamus, while its levels are very low in the cortex/hippocampus (Morel et al., 2017). Additionally, astrocytes promote neurite growth and synaptic maturation of neurons from the same region, that is, subcortical neurons develop larger neurites when they are co-cultured with astrocytes from subcortical regions than with cortical astrocytes (Morel et al., 2017), which suggest that astrocyte modulation of synaptogenesis and synaptic activity is determined by neuronal cell type (Christopherson et al., 2005), but also specific brain areas (Morel et al., 2017).

It is important to establish whether astrocytes located at specific layers within a cortical circuit express different properties. Neurons display layer-specific subtypes that play particular roles in cortical circuitry. Therefore, it is possible that astrocytes show similar layer segregation to support and regulate such circuitry. A recent study on the somatosensory cortex found that, compared to astrocytes in deeper layers, astrocytes located in the upper layers differentially express several molecules related to morphogenesis, synaptic regulation and metabolism (Lanjakornsiripan et al., 2018). Astrocytes from layer 2/3 occupy a larger volume than do astrocytes at layers 4–6 and 1, likely due to greater astrocytic process arborization, thus supporting the notion that astrocytes in different layers possess distinct morphological features. Similarly, astrocytes located at layer 2/3 show more-extensive ensheathment of the synaptic clefts than do astrocytes in layer 6 (Lanjakornsiripan et al., 2018). Additionally, functional differences between astrocytes from different cortical layers have been described *in vivo* (Takata and Hirase, 2008). Astrocytes located in layer 1 show different spontaneous astrocytic $Ca^{2+}$ dynamics than those from layer 2/3; for instance, the average frequency of somatic $Ca^{2+}$ events is higher in layer 1 than in layer 2/3, and the magnitude of those $Ca^{2+}$ responses differ (Takata and Hirase, 2008); however, astrocytic membrane potential was similar for all layers (Mishima and Hirase, 2010). Hence, the diverse territorial volume of cortical astrocytes and particular $Ca^{2+}$ dynamics at different layers might differentially influence the surrounding synapses, yielding layer differences in astrocyte–synapse interactions, ultimately establishing functional heterogeneity through the modulation of glutamate/GABA clearance and the release of active substances that affect synaptic transmission and plasticity.

Surprisingly, such layer-specific distribution is dictated by neuronal migration during development. Indeed, the layer-specific orientation of neocortical astrocytes depends on reelin (Lanjakornsiripan et al., 2018), a protein secreted predominantly from Cajal-Retzius neurons located in layer 1 that regulates the migration of cortical neurons (D'Arcangelo et al., 1995; Katsuyama and Terashima, 2009). This indicates that the existence of neuronal layers is a requirement for establishing layer-specific features of mature cortical astrocytes (Lanjakornsiripan et al., 2018). Furthermore, signaling of the neuron-derived sonic hedgehog (Shh) protein also regulates the molecular and functional profile of astrocytes across different brain regions (Farmer et al., 2016). Hence, Shh signaling in cerebellar Bergman glia promotes glutamatergic signals, enhancing expression of GLTs (GLAST) and AMPA receptors; additionally, potassium homeostasis (Kir4.1) might be related to the dense glutamatergic inputs onto Purkinje cells in the molecular layer. In contrast, cortical and hippocampal astrocytes use Shh signaling for preferential regulation of Kir4.1 channels (Farmer et al., 2016), which are related to potassium buffering. Therefore, such astrocyte regionalization seems to be dictated not only by endogenous astrocytic molecular programs, but also by neuronal signals during development. Thus, neuron–astrocyte signaling dynamically cooperates to generate astrocyte heterogeneity, and ultimately guarantees that mature astrocytes are appropriately specialized to fit the requirements of particular neural circuits.

It is important to note that astrocyte diversity might get even more complex across species. Critical molecular and anatomical differences have been found between rodent and human astrocytes (Oberheim et al., 2009; Zhang et al., 2016; Vasile et al., 2017). As an example, while a single rodent astrocyte can cover up to 120,000 synapses, a human astrocyte might cover from *ca*. 270,000 to 2 million synapses within a single domain (Bushong et al., 2002; Oberheim et al., 2009). Consequently, it is tempting to speculate that astrocytic changes in channel or transporter expression, GTs or extension of astrocytic domains will deeply impact synapses. Astrocytes by enhancing or decreasing synaptic strength would regulate the operational capabilities of human neuronal networks, and might contribute to the higher functions of the human brain.

Collectively, these recent data indicate that astrocytes are not a homogeneous cell type, but rather are circuit-specialized cells that allow for focused astrocyte–synapse signaling, with critical consequences for information-coding in particular layers, circuits and regions in the adult brain (see **Figure 1**). Such astrocyte heterogeneity also provides new variables to the operational codes used by neural circuits that govern complex behavioral responses in health and disease. Therefore, our current knowledge of astrocyte physiology and its impact in synaptic function supports the idea that neuron–glia networks are complex systems that are regionally regulated, with particular structural and functional features.

## CONCLUSIONS

The aim of this review article was to provide an update on the central components that underlie the heterogeneity of astrocyte–neuron signaling, which supports the wide range of functional consequences of astrocytes on synaptic transmission and behavior. Current data show that astrocytes, via expression of ion channels, neurotransmitter receptors, subcellular $Ca^{2+}$ dynamics, GTs release and structural changes of the cell body and fine processes, critically contribute to shape neuronal transmission. However, the full scenario of what particular features trigger molecular, structural and functional changes in astrocytes is unknown. Yet, future studies applying new approaches and methodology are required to reveal the precise

mechanisms that rule astrocyte heterogeneity in different brain regions, which help to address some open questions in the field: (i) which features of astrocyte physiology are driven by neuronal activity and which others are inherent to astrocytes? (ii) what are the boundaries of brain homeostasis? That is, to what extent astrocytes can adapt themselves to neuronal changes to keep brain homeostasis; and vice versa, to what extent synapses can adapt themselves to astrocytic changes. These aims emphasize whether it is considered that altered balance of astrocyte–neuronal signaling might underlie numerous neuropathological states (AD, Huntington disease, epilepsy, major depression; for review see Lundgaard et al., 2014; Chung et al., 2015b; Koyama, 2015). Therefore, a deeper knowledge of astrocyte physiology and astrocyte–neuron networks is necessary to reveal the dynamic and complex organization of the brain circuits underlying animal behavior in health and disease.

## AUTHOR CONTRIBUTIONS

SM, CG-A and GP wrote the article. All authors discussed the manuscript and approved the submitted version.

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# Extrasynaptic Neurotransmission Mediated by Exocytosis and Diffusive Release of Transmitter Substances

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This review article deals with the mechanisms of extrasynaptic release of transmitter substances, namely the release from the soma, axon and dendrites in the absence of postsynaptic counterparts. Extrasynaptic release occurs by exocytosis or diffusion. Spillover from the synaptic cleft also contributes to extrasynaptic neurotransmission. Here, we first describe two well-known examples of exocytosis from the neuronal soma, which may release copious amounts of transmitter for up to hundreds of seconds after electrical stimulation. The mechanisms for somatic exocytosis of the low molecular weight transmitter serotonin, and the peptides oxytocin and vasopressin have been studied in detail. Serotonin release from leech neurons and oxytocin and vasopressin from rodent neurons have a common multi-step mechanism, which is completely different from that for exocytosis from presynaptic endings. Most transmitters and peptides released extrasynaptically seem to follow this same mechanism. Extrasynaptic exocytosis may occur onto glial cells, which act as intermediaries for long-term and long-distance transmission. The second part of this review article focuses on the release upon synthesis of the representative diffusible molecules nitric oxide (NO) and endocannabinoids. Diffusible molecules are synthesized "on demand" from postsynaptic terminals in response to electrical activity and intracellular calcium elevations. Their effects include the retrograde modulation of presynaptic electrical activity and transmitter release. Extrasynaptic neurotransmission is well exemplified in the retina. Light-evoked extrasynaptic communication sets the gain for visual responses and integrates the activity of neurons, glia and blood vessels. Understanding how extrasynaptic communication changes the function of hard-wired circuits has become fundamental to understand the function of the nervous system.

**Keywords: transmitter release, exocytosis, extrasynaptic transmission, volume transmission, diffusive transmitters**

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## INTRODUCTION

The demonstration by Santiago Ramon y Cajal of the existence of stereotyped circuits in the nervous system, followed by the discovery that acetylcholine, adrenaline and noradrenaline are released by nerve terminals by Elliot (1904), Loewi (1921) and Dale et al. (1936) set the basis for the discoveries by Bernard Katz and his colleagues on the fundamental

mechanism for synaptic communication (for review see Katz, 1996). The later discovery of electrical junctions (Furshpan and Potter, 1959) complemented the dominating concept that neuronal circuits function in a hard-wired manner. However, with time it also became clear that the input/output relationship of neuronal circuits varies depending on the previous patterns of electrical activity. Short- and long-term synaptic plasticity explains of some transitory changes in the strength of the circuit connectivity. However, extrasynaptic communication explains the integral modulation of whole neuronal circuits, glia and blood vessels in periods ranging from seconds to hours.

The classical observations of Dalstrom and Fuxe that the serotonin cell bodies in the Raphe nucleus are surrounded by free serotonin (reviewed by Fuxe et al., 2007; Borroto-Escuela et al., 2015), and the observation by Paton and Vizi (1969) that biogenic amines inhibit non-synaptically the cholinergic transmission onto muscle fibers suggested that transmitters may act extrasynaptically. The evidence was soon expanded to other transmitters and peptides, thus leading to the mechanistic concept of volume transmission, defined by Fuxe et al. (2007) as a form of communication mediated by extracellular diffusion of transmitter substances through the extracellular space (for review see Borroto-Escuela et al., 2015). Indirect evidence for the somatic release of transmitters came from experiments by Dun and Minota (1982) showing that electrical stimulation of the soma of peripheral neurons changed the membrane potential in a non-synaptic manner. Direct demonstrations of the extrasynaptic release of all sorts of low molecular transmitters and peptides came later, from experiments in central and peripheral neurons of vertebrates and invertebrates (for review see Trueta and De-Miguel, 2012). This bulk of evidence lead to the term of "extrasynaptic communication" to define volume transmission in response to transmitter liberation from extrasynaptic sites, in the soma, dendrites and axons (De-Miguel and Nicholls, 2015). Extrasynaptic release occurs in the absence of postsynaptic counterparts. In addition, synaptically-released transmitters, for example, dopamine, noradrenaline or glutamate (Zhang and Sulzer, 2003; Rice and Cragg, 2008; Courtney and Ford, 2014) spillover from the synaptic cleft and reach extrasynaptic receptors, thus contributing to extrasynaptic communication within small volumes of tissue. Glial cells are integral components of extrasynaptic communication by responding to transmitters and peptides and releasing the same or others. As will be seen below, the capillary blood flow is a target for extrasynaptic modulation.

Extrasynaptic release of transmitters occurs in central and peripheral neurons of vertebrates and invertebrates. Most low molecular weight transmitters and different peptides are released extrasynaptically (Trueta and De-Miguel, 2012). In addition, gases such as nitric oxide (NO), carbon monoxide (Queiroga et al., 2015) and hydrogen sulfide (Paul and Snyder, 2018), or the liposoluble endocannabinoid family (Iannotti et al., 2016; Lu and Mackie, 2016) and the hydrogen peroxide (Lee et al., 2015), are synthesized "*on demand,*" and reach their targets retrogradely (usually presynaptic), by diffusion.

In this mini-review article, we have assumed the immense task of comparing the mechanisms of release by exocytosis and by the synthesis of diffusible molecules. To achieve this goal, we first compare the best-known release mechanisms of classic transmitters, peptides, NO and cannabinoids. Then, we take advantage of the well-known retinal structure and function to give an account on how synaptic and extrasynaptic transmission interact to modulate visual sensitivity and blood flow.

## THE MECHANISM FOR EXTRASYNAPTIC EXOCYTOSIS

This section compares the extrasynaptic exocytosis of the low molecular weight transmitter serotonin and the peptides oxytocin and vasopressin. The release mechanism of both substances has been studied step by step in great detail. Somatic exocytosis of serotonin, resumed schematically in **Figure 1**, has been studied in the large soma of the classical Retzius neuron of the leech (De-Miguel et al., 2015); somato-dendritic oxytocin and vasopressin have been studied in thalamic mammalian neurons (Ludwig and Leng, 2006; Ludwig and Stern, 2015). The accessibility of both neuron types has permitted to apply diverse technical approaches in the search for direct experimental evidence on the exocytosis mechanism. The mechanism for both types of molecules are remarkable similar, and quite different from that for synaptic exocytosis. Since one example comes from release of a low molecular weight transmitter in an invertebrate and the other from peptides in mammals, the similarity predicts universal mechanistic steps governing somatic exocytosis. The cumulative evidence obtained from other central and peripheral neuron types from vertebrates and invertebrates, releasing low molecular transmitters or peptides strengthen this hypothesis (for review see Trueta and De-Miguel, 2012).

Serotonin, oxytocin and vasopressin are packed in large (∼100 nm) electrodense vesicles (a definition that stems from their appearance under the electron microscope) that rest at a distance from the plasma membrane (Coggeshall, 1972; Schimchowitsch et al., 1983). Single action potentials fail to evoke the large-scale exocytosis that characterizes these somata. However, rapid trains of impulses or large depolarizations, trigger a massive exocytosis that lasts for hundreds of seconds (Trueta et al., 2003; Ludwig and Stern, 2015). The frequencies of the trains of impulses that produce somatic exocytosis of serotonin are physiological, and can be evoked by mechanosensory stimulation to the skin (Velázquez-Ulloa et al., 2003).

The coupling between excitation and exocytosis incorporates a cascade of sequential steps. The large intracellular calcium transient produced mostly by its entry through L channels (Trueta et al., 2004; Tobin et al., 2011) activates an intracellular calcium-induced calcium release that generates a calcium "tsunami" that invades the whole soma (Sabatier et al., 1997; Ludwig et al., 2002; Tobin et al., 2011; Leon-Pinzon et al., 2014). As result, vesicles become transported actively to the plasma membrane across an actin cortex (Tobin and Ludwig, 2007; Tobin et al., 2011; De-Miguel et al., 2012). In serotonergic neurons, vesicle clusters are carried by molecular motors over 0.6–6.0 mm distances at 15–90 nm/s velocities (De-Miguel et al., 2012). This transport determines the characteristic long

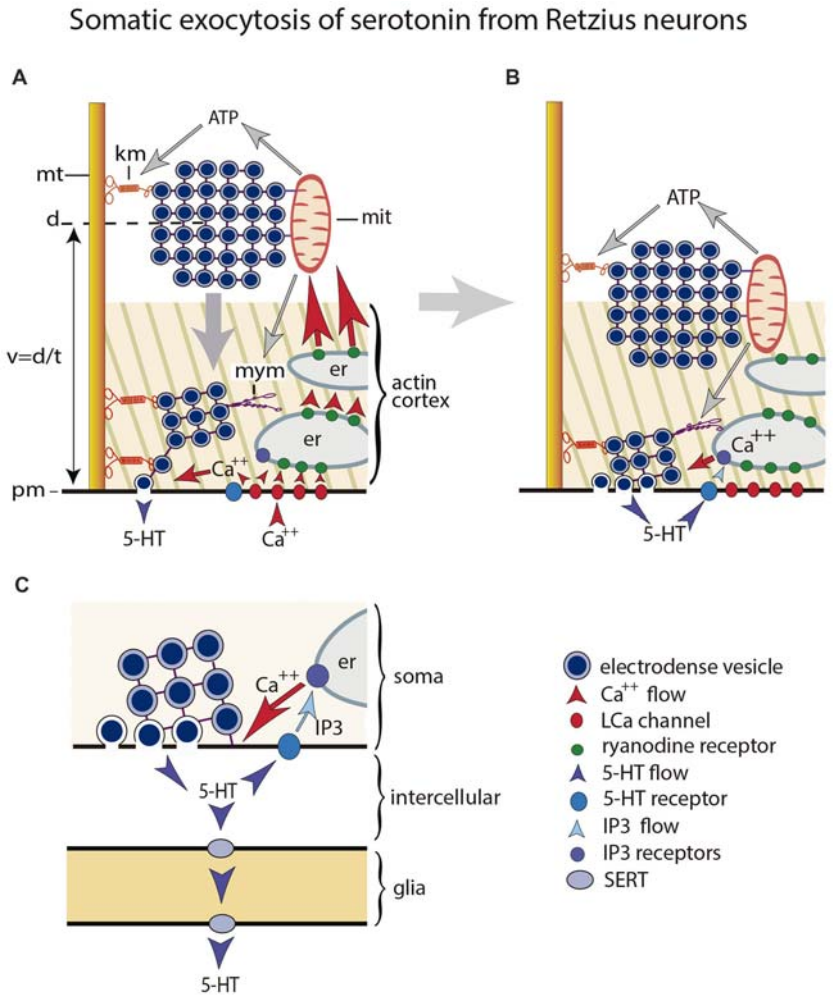


**FIGURE 1 |** Mechanism for somatic exocytosis of serotonin in leech Retzius neuron. **(A)** Vesicles forming clusters rest at different distances from the plasma membrane. Clusters are attached to microtubules via kinesin motors. At rest the actin cortex restricts vesicle mobilization. Clusters already inside the cortex spontaneously send vesicles to the plasma membrane where they release serotonin. Trains of electrical impulses promote calcium entry through L channels. Calcium induces calcium release from endoplasmic reticulum via ryanodine receptor activation. The amplified calcium wave arrives at the mitochondria, which responds producing ATP. The kinesin myosin motors become activated by ATP, thus transporting the vesicle clusters towards the plasma membrane. Electrical activity and calcium change the configuration of the actin cortex, which now becomes permeable for vesicle transport, with incorporation of myosin motors. The vesicle clusters are propelled towards the plasma. **(B)** Arrival of vesicle clusters at the plasma membrane occurs seconds after electrical activity and the intracellular calcium wave ended. The large-scale exocytosis is produced by a positive feedback loop established by serotonin released by the individual vesicles. The serotonin that has been released activates autoreceptors and phospholipase C. IP3 activates calcium release from the external layer of endoplasmic reticulum. This calcium maintains exocytosis until the last vesicles in the cluster fuse. **(C)** Amplified scheme of the positive feedback system, introducing the glia as serotonin transporter. Hypothetically this transport occurs via SER transporters that introduce serotonin when the internal concentration is low and release it at distal sites when the internal concentration is high.

latency of the large-scale somatic exocytosis, which starts seconds after the end of the calcium transient produced by electrical stimulation (Leon-Pinzon et al., 2014). Somatic exocytosis is maintained by a transmitter and calcium positive feedback loop (Wotjak et al., 1994; Leon-Pinzon et al., 2014). In serotonergic neurons, activation of 5-HT2 receptors by the serotonin that has been released increases the calcium concentration in the soma shell via the activation of phospholipase C and the production of IP3. This calcium elevation, promotes exocytosis as new vesicles arrive, but its localization prevents the transport of vesicle clusters resting more internally (Leon-Pinzon et al., 2014). The feedback loop ends when the last vesicles in the pool fuse with the plasma membrane. Strikingly enough, somatic exocytosis of serotonin in leech neurons occurs onto glial cells (Trueta et al., 2004). As will be seen below, in peripheral neurons and in retina, activation of glial cells by extrasynaptic exocytosis extends the duration and consequences of extrasynaptic communication.

Evidence from mammalian serotonergic Raphe neurons points to a similar mechanism for somatic exocytosis. The capacity of serotonin to emit fluorescence upon multiphoton excitation allowed the group of Sudipta Maiti (Kaushalya et al.,

2008; Sarkar et al., 2012) to study somatic exocytosis in Raphe neurons isolated from rodents. Depolarization with a high potassium extracellular solution triggers the mobilization of fluorescent serotonergic-containing spots to the plasma membrane followed by exocytosis. The size of these fluorescence spots resembles that of vesicle clusters in Retzius neurons.

Most neurotransmitters are released extrasynaptically and follow the mechanistic rules described above for serotonin and peptides (Trueta and De-Miguel, 2012). A well-studied example is dopamine release from amacrine cells in the retina of rodents and from invertebrate neurons (Chen et al., 1996; Puopolo et al., 2001). The somata and dendrites of dopaminergic neurons in the substantia nigra and the ventral tegmental area also release dopamine (Björklund and Lindvall, 1975; Geffen et al., 1976; Cheramy et al., 1981). However, there are also certain variations: somatic release of dopamine in basal ganglia seems to occur from clear vesicles (Jaffe et al., 1998). The functional effects of extrasynaptic release of dopamine in the retina are discussed below.

## EXTRASYNAPTIC TRANSMISSION MEDIATED BY THE SYNTHESIS OF DIFFUSIBLE MOLECULES: THE NITRERGIC AND ENDOCANNABINOID SYSTEMS

A separate set of diffusible transmitters is synthesized on demand upon increases of electrical activity and activation of certain G protein-coupled receptors, both of which produce increases of intracellular calcium concentration. Most diffusible neurotransmitters are liberated upon one or two rapid enzymatic steps after which they diffuse to the extracellular space. Therefore there is no defined mechanism for their release. Gases like NO, carbon monoxide and hydrogen sulfate diffuse freely across plasma membranes. They activate specific cellular and molecular targets (Bredt and Snyder, 1990; Wang, 2002). Another family of diffusible transmitters, the endocannabinoids, are the endogenous activators of the specific receptors that respond to chemicals produced by the plant cannabis. Endocannabinoids are also produced on demand by a receptor-stimulated cleavage of membrane phospholipid precursors. Once synthesized, endocannabinoids also diffuse rapidly out the neurons (Piomelli, 2003; Mechoulam and Parker, 2013).

## THE NITRIC OXIDE (NO) SYSTEM

Because of its physicochemical nature, NO is a volume transmitter (Agnati et al., 2010; Garthwaite, 2016). Increases in the intracellular calcium levels in the presence of the enzyme nitric-oxide-synthase (NOS), a calcium-calmodulin enzyme, produces NO from its precursor L-arginine (see Garthwaite et al., 1988; Bredt and Snyder, 1990; Garthwaite, 2008, 2016). The mechanism of NO inactivation remains unclear, although cytochrome P450 oxidoreductase and astrocytes seem to contribute (Hall et al., 2009; Rodriguez-Grande and Konsman, 2018).

The best-characterized stimulator of NO synthesis is the massive exocytosis of glutamate from hippocampal presynaptic endings (Garthwaite, 2008, 2016). During low presynaptic activity, glutamate activates mostly postsynaptic AMPA/kainate receptors. However, an increased glutamate release upon bursts of presynaptic action potentials promotes the opening of NMDA receptors. The pore of NMDA receptors is highly permeable to calcium that concentrates in the postsynaptic terminal. NMDA receptors and nNOS are associated by the postsynaptic density protein-95 (Brenman et al., 1996; Sattler et al., 1999). In this way, calcium influx activates the NOS/calmodulin complex that produces NO. The NO synthesis continues as long as the calcium levels are elevated. The NO synthesis is also activated by the cytoplasmic calcium increases in response to the activation of voltage-gated calcium channels or intracellular calcium release (Daniel et al., 1998).

Right after being synthesized, NO diffuses through aqueous and lipid environments, thus acting on pre- and postsynaptic targets. Unlike transmitters released by exocytosis, NO lacks enough chemical sophistication to activate specific receptor binding sites. However, it may activate the soluble enzyme guanylyl cyclase that converts guanosine-5′-triphosphate into cyclic guanosine-monophosphate (Arnold et al., 1977). NO also produces the nitrosylation of proteins and the generation of reactive oxygen species (Ahern et al., 2002).

The NO targets are presynaptic terminals, glia and blood vessels. For this reason NO is a retrograde transmitter. The physicochemical properties of NO allow its uniform diffusion bypassing most if not all anatomical constrains. NO may act in concert with other transmitters, producing subtle alterations in the function of ion channels and other proteins (for review see Steinert et al., 2010). Moreover, NO may link monoaminergic and glutamatergic transmission (West and Grace, 2000; Kiss and Vizi, 2001; Mitkovski et al., 2012). An excess of NO synthesis becomes neurotoxic by the formation of reactive oxygen species.

NO produces in rats and schizophrenic patients a rapid and long-lasting improvement of anxiety and depressive symptoms (Guimarães et al., 1994; Issy et al., 2011; Hallak et al., 2013). The inhibition of NO synthesis produces anxiolytic-, antidepressant-, anti-fear and anti-traumatic like effects (for review see Guimarães et al., 2005; Steinert et al., 2010; Paul and Snyder, 2018). In addition, reduces the L-DOPA-induced that follows the depletion of dopaminergic neurons in rodents and non-human primates (Del Bel et al., 2005; Padovan-Neto et al., 2009; Bortolanza et al., 2015; for review see Del-Bel et al., 2011). For these reason, all of these symptoms seem to have extrasynaptic communication components and open a field of study from the view of extrasynaptic communication.

## THE ENDOCANNABINOID SYSTEM

Endocannabinoids also contribute to extrasynaptic communication. The production of cannabinoids also occurs on demand in postsynaptic endings (Kano et al., 2009; Castillo et al., 2012; Iannotti et al., 2016; Lu and Mackie, 2016), upon increases of electrical activity (Piomelli, 2003; Mechoulam and Parker, 2013). Constitutive membrane phospholipids like di-and

tri-acylglycerols are metabolized intracellularly by a calcium-dependent diacylglycerol-lipase, to produce the best-known cannabinoids: anandamide and 2-arachidonoylglycerol (2-AG; for review, see Bisogno et al., 2005). Anandamide is synthesized upon activation of phospholipase β-coupled- glutamate, muscarinic or dopamine receptors. The synthesis of 2-AG requires an intracellular calcium elevation, as it happens for NO. The increases in the calcium concentration activate the enzyme N-arachidonoyl-phosphatidyl ethanolamine-specific phospholipase. Anandamide and 2-AG leak passively by diffusing throughout lipid membranes. However, a rapid and selective carrier accelerates this process in neurons and glial cells (Beltramo et al., 1997). Endocannabinoids are also secreted in extracellular membrane vesicles originated in microglial cells (Gabrielli et al., 2015).

Endocannabinoids are retrograde modulators of synaptic function by acting on specific presynaptic and glial cannabinoid receptors (reviewed in Hashimotodani et al., 2007; Kano et al., 2009). The type 1 (CB1) and type 2 (CB2) specific cannabinoid receptors belong to the G protein-coupled receptor family. A third cannabinoid receptor is the transient receptor potential vanilloid type 1 (TRPV1). Endocannabinoids may also modulate synapses by using glial cells as intermediaries. In addition, glial cells also produce endocannabinoids (Stella, 2010).

Termination of the endocannabinoid signaling occurs through a carrier-mediated transport into cells, followed by intracellular degradation (Piomelli, 2003; Iannotti et al., 2016; Lu and Mackie, 2016). 2AG is mostly degraded in presynapse by the enzyme monoacylglycerol lipase (Dinh et al., 2002; Marrs et al., 2010), while anandamide is degraded mostly in postsynapses by the fatty acid amide hydrolase to produce arachidonic acid and ethanolamine (Di Marzo et al., 1994; for review, see Iannotti et al., 2016; Lu and Mackie, 2016).

When we fell down as children, our grandmothers massaged our hurt knees to reduce our pain. A good explanation for grandmother's empirical knowledge came from experiments made in leech. Endocannabinoids released upon stimulation of touch- or pressure-mechanosensory neurons innervating the skin, act on TRPV receptors to decrease nociceptive synaptic transmission and increase the responses of the touch and pressure sensory connections (Summers et al., 2017).

Endocannabinoids modulate the excitatory and inhibitory synaptic strength of sensorymotor pathways (Pedrazzi et al., 2015). However, that endocannabinoid receptors appear more prominently in inhibitory terminals suggest their function to reduce over-excitability (Freund et al., 2003; Chevaleyre et al., 2006; for review, see Iannotti et al., 2016; Lu and Mackie, 2016). Endocanncabinoids inhibit transmitter release by closing calcium channels, opening $K^+$ channels, inhibiting adenylyl cyclase and stimulating protein kinases (Kano et al., 2009; Castillo et al., 2012). In addition, activation of CB1 receptors increase the spontaneous firing of noradrenergic, serotonergic and dopaminergic neurons, and increases the synthesis of these neurotransmitters (Mechoulam and Parker, 2013). Endocannabinoids also improve certain regeneration processes (Kwiatkoski et al., 2012) and increase neurogenesis (Campos et al., 2016). The endocannabinoid system may also be neuroprotector and a target to control neurodegenerative and neuropsychiatric diseases (for review see Campos et al., 2012, 2016).

## INTEGRATION OF SYNAPTIC AND EXTRASYNAPTIC TRANSMISSION IN THE RETINA

In this section, we will use the excellent possibilities offered by the histological organization and supercomputing power of the retina to exemplify how extrasynaptic communication integrates the function of neurons, glia and blood vessels. The link between extrasynaptic communication and its effects in the retina has been widely studied for dopamine, with some examples in the contribution of NO and cannabinoids. However, these substances suffice to exemplify the wide spectrum of concerted extrasynaptic communication actions that modulate function in a well-known neural tissue.

A bright light shone onto a retinal receptive field evokes electric signals in photoreceptors. On their way to the ganglion cells, interactions with bipolar, horizontal and amacrine cells, produce the characteristic "on" and "off" visual responses. In addition, activation of amacrine cells evoke the extrasynaptic exocytosis of dopamine (Puopolo et al., 2001) and GABA (Hirasawa et al., 2009). The mechanism for dopamine release (Puopolo et al., 2001) is as described for serotonin and oxytocin. Through volume transmission, dopamine increases the gain of the sensory field by three complementary effects: (a) potentiating the activity of glutamate receptors in bipolar and horizontal cells (Knapp and Dowling, 1987; Maguire and Werblin, 1994); (b) reducing the diameter of the visual field by uncoupling horizontal cells (Piccolino et al., 1984; DeVries and Schwartz, 1989); and (c) uncoupling the connections of AII rod amacrine cells and modifying the center-surround balance in ganglion cells (Daw et al., 1990).

Increases in the extracellular concentration of transmitters, activates the retinal glia—the Muller cells. The Muller cells respond by releasing ATP through a special type of channels, the pannexins (Dahl, 2015). ATP depresses the electrical activity of ganglion cells and evokes vasodilation of blood vessels (Newman, 2015).

Diffusible transmitters also contribute to retinal function. The blockade of NO synthesis increases blood pressure (Deussen et al., 1993). In addition, the activation of cannabinoid receptors reduces L calcium and K currents in cones while increases L currents and reduces K currents in rhodes (Straiker et al., 1999).

## DISCUSSION AND PERSPECTIVES

Extrasynaptic transmission is multivariate in every region of the nervous system. Several modes and sites of transmitter release exist different neurons. In addition, one neuron can be modulated by different transmitters. All modes of extrasynaptic release are triggered by increases of electrical activity, followed by large increases in the intracellular calcium concentration. Signaling is slow when compared to synaptic communication,

which occurs within half a millisecond. The threshold and amount of each extrasynaptic mode of release are coded by the frequency and duration of the stream of action potentials. In return, extrasynaptically released transmitters modulate, and in most cases reduce, the neuronal electrical activity.

Axons and dendrites contain clusters of clear and dense core vesicles anchored at different distances of the plasma membrane. These diverse configurations endow neurons with regional release possibilities. Passage of electrical activity along the neuron may then trigger different modes of exocytosis. Depending on the transmitter released and the region where release occurs, extrasynaptic exocytosis may have different timing and regional effects.

The ample catalog of transmitter molecules and extrasynaptic receptors contributing to extrasynaptic signaling adds a wide range of activity-dependent physiological responses to neuronal circuits. This contributes to explain the diversity of circuit responses, depending on the activity levels. Extrasynaptic communication incorporates glia, which adds feedback communication to neurons, releases chemical messages and regulates blood flow. Therefore, to understand the function of the nervous system, it is now essential to understand the roles of extrasynaptic neurotransmission.

Several pertinent questions that can be addressed now concern how many release modes a single neuron has? How release from different neuronal compartments modulates activity locally? How extrasynaptic release produces a self-modulation? How can we relate extrasynaptic neurotransmission to motivation, modulation, state-dependence or activity-dependence? Although not touched for the case of extrasynaptic exocytosis, an important question that can now be posted is the contribution of extrasynaptic communication in normal and diseased brain. Several examples were discussed for diffusible transmitters. For some diseases like depression or Parkinson's, the demonstrations of the role of extrasynaptic exocytosis of low molecular transmitters and peptides seems around the corner.

## AUTHOR CONTRIBUTIONS

ED-B and FD-M contributed equally to each step of the preparation of this manuscript.

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frontiers in Synaptic Neuroscience

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# Molecular Dissection of Neurobeachin Function at Excitatory Synapses

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Spines are small protrusions from dendrites where most excitatory synapses reside. Changes in number, shape, and size of dendritic spines often reflect changes of neural activity in entire circuits or at individual synapses, making spines key structures of synaptic plasticity. Neurobeachin is a multidomain protein with roles in spine formation, postsynaptic neurotransmitter receptor targeting and actin distribution. However, the contributions of individual domains of Neurobeachin to these functions is poorly understood. Here, we used mostly live cell imaging and patch-clamp electrophysiology to monitor morphology and function of spinous synapses in primary hippocampal neurons. We demonstrate that a recombinant full-length Neurobeachin from humans can restore mushroom spine density and excitatory postsynaptic currents in neurons of Neurobeachin-deficient mice. We then probed the role of individual domains of Neurobeachin by comparing them to the full-length molecule in rescue experiments of knockout neurons. We show that the combined PH-BEACH domain complex is highly localized in spine heads, and that it is sufficient to restore normal spine density and surface targeting of postsynaptic AMPA receptors. In addition, we report that the Armadillo domain facilitates the formation of filopodia, long dendritic protrusions which often precede the development of mature spines, whereas the PKA-binding site appears as a negative regulator of filopodial extension. Thus, our results indicate that individual domains of Neurobeachin sustain important and specific roles in the regulation of spinous synapses. Since heterozygous mutations in Neurobeachin occur in autistic patients, the results will also improve our understanding of pathomechanism in neuropsychiatric disorders associated with impairments of spine function.

**Keywords: synaptic transmission, dendritic spine, autism, AMPA receptor, neurobeachin, hippocampus**

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## INTRODUCTION

Dendritic spines (DSs) are small, actin-enriched protrusions that arise from neuronal dendrites and receive most of the excitatory input in brain circuitries (Hering and Sheng, 2001; Hotulainen and Hoogenraad, 2010; Segal, 2010). DS play a pivotal role during synaptic transmission because of their ability to compartmentalize biochemical signaling and integrate presynaptic input (Bourne and Harris, 2008; Cingolani and Goda, 2008; Yuste, 2011). To accomplish this, DS undergo

adaptive changes during development, in response to sensory stimuli or during cognitive processes such as learning and memory (Bhatt et al., 2009; Holtmaat and Svoboda, 2009; Lin and Koleske, 2010; Berry and Nedivi, 2017). Mature spines typically display a mushroom-shaped morphology with a well-distinguishable head and neck that contain the postsynaptic signaling machinery (Harris and Weinberg, 2012). Accordingly, numerous studies revealed that spine morphology and their function are mutually dependent (Kasai et al., 2010; Sala and Segal, 2014; Moyer and Zuo, 2018). In addition, important evidence has linked several neuropsychiatric disorders and behavioral deficits to the impairment of different spine types (Lin and Koleske, 2010; Penzes et al., 2011; Joensuu et al., 2018).

We and other groups have previously addressed the function of the neuronal BEACH (beige and Chediak-Higashi) protein Neurobeachin (Nbea), and observed that Nbea is involved in DS formation, synaptogenesis, and synaptic transmission in different species such as mouse (Su et al., 2004; Medrihan et al., 2009; Niesmann et al., 2011; Nair et al., 2013), zebrafish (Miller et al., 2015), and Drosophila (Volders et al., 2012; Wise et al., 2015). Mammalian Nbea is a large (327 kDa), cytosolic multidomain protein peripherally associated with neuronal membranes (Wang et al., 2000), and concentrated at the *trans*-Golgi network, post-Golgi vesicles, and at synaptic contacts (Wang et al., 2000; Niesmann et al., 2011; Volders et al., 2012; Nair et al., 2013). This localization is reflected by the morphological and functional defects found at synapses throughout the central and peripheral nervous system of Nbea knockout (KO) mice (Su et al., 2004; Medrihan et al., 2009; Niesmann et al., 2011; Nair et al., 2013). It is also consistent with the idea that Nbea regulates trafficking of glutamate and $GABA_A$ receptors to postsynaptic sites (Nair et al., 2013; Farzana et al., 2016).

Nbea contains at least seven distinct protein motifs or domains (Cullinane et al., 2013): First, an A-kinase anchoring protein (AKAP) motif that binds to PKA (Wang et al., 2000); second, a conserved BEACH domain that is in tight complex with, third, a Pleckstrin-Homology domain (PH) (Jogl et al., 2002; Gebauer et al., 2004); fourth, an Armadillo repeat domain originally proposed as "domain-of-unknown-function" (DUF4704); fifth, a Concanavalin A-like lectin domain (Burgess et al., 2009); sixth, another DUF domain (DUF1088) with a putative nuclear localization signal (Tuand et al., 2016); and finally, four C-terminal tryptophan-aspartic acid (WD 40) repeats. A binding study with recombinant mouse Nbea showed that the c-terminus including DUF1088-PH-BEACH-WD40 binds to SAP102. A single point mutation that disrupts the tight complex of PH and BEACH domain in full-length Nbea prevents SAP102 binding, although the isolated PH-BEACH domain does not bind to SAP102 (Lauks et al., 2012). Moreover, since human and mouse Nbea share 98% identical residues with some domains reaching 100% (Lectin, AKAP motif, and WD40 repeats), we hypothesized that it is possible to analyze human disease related single site mutations in neuronal cultures from mice. This high homology also allows to use detailed structural data of the PH-BEACH domain derived from human Nbea (Jogl et al., 2002).

Several reports have emphasized the medical importance of Nbea in humans. Nbea spans a common fragile site on human chromosome 13q13 that was found disrupted by translocation in idiopathic cases of non-familial autism (Castermans et al., 2003, 2010). In support, Nbea haploinsufficiency in mice recapitulates the human disorder including autism-like behaviors and social deficits (Nuytens et al., 2013). In addition, there are mutations in closely related proteins, for example in NBEAL2 that cause platelet abnormalities with impaired secretory α-granule biogenesis in patients (Albers et al., 2011; Gunay-Aygun et al., 2011; Kahr et al., 2011), and in LRBA which are linked to immunological deficit and autoimmunity (Lopez-Herrera et al., 2012). In an effort to address putative pathomechanisms, we have therefore investigated the contribution of recombinant full-length human Nbea and its individual domains to synaptic function in our present study. Specifically, we tested the ability of human Nbea domains to rescue the functional and morphological defects apparent at synapses of primary hippocampal neurons from Nbea KO mice. We show that expression of PH-BEACH domains of Nbea is sufficient to restore normal DS density and surface targeting of AMPAR subunits. In addition, we identify a cooperation of the Armadillo domain and PKA binding motif in regulating filopodia extension in opposite directions. Together, our data provide the first insights into specific roles of individual Nbea domains in synapse function.

# MATERIALS AND METHODS

## Animals

Wild-type and Nbea KO mutant mice of either sex were used for neuronal cultures derived from timed-pregnant dams at E17. Animal experiments were performed at the University of Münster in accordance with government regulations for animal welfare and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV, NRW, Germany), license numbers 84-02.05.20.11.209 and 84-02.04.2015.A423.

## Cell Culture

Dissociated primary neurons were prepared in Hank's Balanced Salt Solution (HBSS) from hippocampi as described (Neupert et al., 2015). Briefly, cell suspensions obtained after 0.25% trypsin and trituration were plated onto 18 mm glass coverslips (Menzel-Glaeser, Braunschweig, Germany) coated with poly-L-lysine (Sigma) at a density of 55,000 cells/coverslip. After 4 h at 37°C in plating medium (MEM, 10% horse serum, 0.6% glucose, 1 mM sodium pyruvate), coverslips were inverted onto a 70–80% confluent monolayer of astrocytes grown in 12-well plates (Falcon), and incubated in Neurobasal medium supplemented with B27, 0.5 mM glutamine and 12.5 μM glutamate. After 3 days, media were refreshed with Neurobasal medium supplemented with B27, 0.5 mM glutamine and 5 μM AraC. Cultures were maintained at 37°C in a humidified incubator with an atmosphere of 95% air and 5% $CO_2$. Neurons were transfected at days *in vitro* (DIV) 14–16 using lipofectamine (Thermo Fisher Scientific, Waltham, MA, United States), and experiments performed between DIV 17–21. Since endogenous Nbea is highly

expressed during the period of intense DS formation before synapse maturation is fully established (Nwabuisi-Heath et al., 2012; Nair et al., 2013), our transfection strategy was actually designed to fit to the time course of expression of the endogenous protein.

Autaptic cultures of hippocampal neurons were prepared on micro islands of astrocytes as described previously (Burgalossi et al., 2012). In brief, astrocytes were obtained from mouse cortices from P0 WT animals using digestion with 0.25% trypsin (Gibco) for 20 min at 37°C. The cells were plated in T75 culture flasks in DMEM medium (Gibco) containing 10% FBS (PAA) and penicillin/streptomycin (Gibco). The medium was exchanged the day after plating, and cells were allowed to grow for 7–10 days. Following this, cells were collected from the flask using trypsin digestion and plated at a density of 12,000 cells/well on 32 mm coverslips. The coverslips used for micro island cultures were first coated with agarose (Sigma-Aldrich), and then with a coating solution containing poly-D-lysine (Sigma-Aldrich), acetic acid, and collagen (BD), using a custom-made stamp to generate 400 μm × 400 μm substrate islands. Hippocampi from embryonic day 18 (E18) mouse embryos were dissected free of meninges and separately collected in ice-cold Hanks Buffered Salt Solution (HBSS; Gibco). They were incubated in papain solution containing 2 mg cysteine, 10 ml DMEM (Gibco), 1 mM $CaCl_2$, and 0.5 mM EDTA, along with 20–25 units of papain (Worthington Biomedical Corporation) for 45 min for at 37°C. After washing, cells were triturated and counted in a Fuchs-Rosenthal or Neubauer chamber. The cells were plated in pre-warmed Neurobasal medium (Gibco) supplemented with B-27 (Gibco), glutamax (Gibco) and penicillin/streptomycin (Gibco) at a density of 4,000 cells/well on a 32 mm coverslip for micro island cultures.

## Dendritic Spine Analysis

Dendritic spines were analyzed from cytosolic marker t-dimer-RFP transfected neurons. RFP-positive neurons were imaged with a confocal spinning disk Axio Observer-Z1 (Visitron) with an EMCCD camera (ImagEM 512 CCD, Hamamatsu), using 63x Plan-Neofluar oil immersion objectives. 0.5 μm Z-stacks were acquired, with a maximal distance of 1.5 μm from the focus plane. All images related to GFP and 647 nm channels were acquired with the same laser power, exposure time and camera gain settings. Using ImageJ software, maximum intensity projections of Z-stacks were generated for the analysis, and mushrooms spines and filopodia protrusions quantified on all first and second order dendrites for each imaged cell. The total length of these dendrites was measured and all mushroom and filopodia protrusions were counted to obtain the number of protrusion/20 μm dendritic length (Repetto et al., 2014). Data for number of protrusions/20 μm per cell are based on about 350–500 protrusions for each neuron. For the quantification of Nbea domains in the spine head, regions of interests (ROIs) were manually drawn with ImageJ software around at least 50 spine heads per neuron. In this experiment, only mushroom spines with clearly visible heads were selected randomly from the first and second order dendrites. After background subtraction, the ROIs series was copied over the fluorescent images correspondent to the GFP signal to obtain a fluorescence intensity value of GFP fused domain in DS head. In addition, for the quantification of the individual Nbea domains in dendritic shafts, 20 μm long dendritic windows excluding the spines were manually delineated on t-dimer-RFP transfected neurons. At least three first order and three second order dendrites were randomly selected for each neuron.

For FRAP experiments, WT and Nbea KO hippocampal neurons were transfected at DIV 14 with EGFP-Actin, cloned under the control of the human synapsin promoter. Transfected cells were imaged with the spinning disk confocal setup described above. The spine heads were bleached with a laser beam at 20% power and images were acquired every 2 s to measure EGFP-actin recovery. To improve the reliability of measurements, each spine was imaged twice with a recovery interval of at least 5 min. After background subtraction, fluorescence intensity in DS was measured for each time point. The series of fluorescence intensity values was fitted to a first order exponential equation using the FRAP profiler plug-in available in ImageJ software. After the exponential curve fitting, the halftime recovery of EGFP-Actin in spine head, indicating actin turnover rate was extracted and analyzed.

To monitor the dynamics of spines under basal conditions, WT and Nbea KO hippocampal neurons were transfected at DIV 14 with GFP or Life-Actin fused to RFP (Riedl et al., 2008). Live imaging confocal microscopy of DS was performed with the same confocal setup described above and acquiring up to six Z-stacks every 15 s for 30 min. Single spines in each time point were analyzed extracting the coordinates of the center of mass (COM) using ImageJ software and the particles analysis tool. The derivative in respect of time for the $x$ and $y$ coordinates values was calculated and the absolute value was derived. The sum of the absolute values for $x$ and $y$ coordinates, indicating the COM displacement was calculated and used for statistical analysis.

## Immunocytochemistry and GluA2 Surface Staining

For surface staining of GluA2 subunit of AMPA receptor the experiment was carried out as reported (Aoto et al., 2013). Briefly, live DIV 21 neurons were washed quickly in PBS supplemented with 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 4% sucrose (PBS-MC). Monoclonal mouse anti-GluA2 antibody (Millipore MAB397) against an extracellular epitope of GluA2 AMPA receptor subunit was incubated for 10 min at 37°C in incubator. After a brief wash in cold PBS-MC, cells were fixed with 4% paraformaldehyde/4% sucrose for 15 min and blocked in detergent free 10% normal goat serum PBS solution. To analyze the surface GluA2 fluorescence intensity, rectangular windows of at least 20 μm length were manually drawn around RFP transfected dendrites. These regions were copied to the corresponding fluorescent image of the anti-GluA2 labeling (Alexa 647 goat-anti-mouse IgG) and, after background subtraction, an intensity value was obtained.

For immunocytochemistry of hippocampal cultures, DIV 21 neurons were fixed with 4% paraformaldehyde/4% sucrose for 8 min, washed with PBS, blocked in 10% normal goat serum

(NGS), 0.1% Triton-X100/PBS for 1 h, and incubated with Alexa Fluor Phalloidin 633 (Thermo Fisher Scientific) staining F-Actin for 1 h at RT. After additional washings in PBS, coverslips were embedded in mounting medium (Dako).

## Expression Vectors

We used a mammalian expression vector containing the human synapsin 1 promoter and inserted EGFP to create hSYN1-EGFP-C. To generate GFP-Nbea (Nbea_Human, Q8NFP9), we introduced MluI and NotI sites to flank CMV-YFP in human YFP-Nbea (IMAGE clone 100069291) using quikchange primers (MM11-M, 5′-GCC ATG CAT TAG TTA TTA ACG CGT TAG TAA TCA ATT ACG GGG-3′, +MluI; MM11-N, 5′-GTA CAA ACT TGT TGA TGA TCG CGG CCG CGA CTT GTA CAG CTC GTC CAT GCC G-3′, +NotI). The MluI/NotI fragment was replaced by a correspondent PCR amplicon containing hSYN1-EGFP amplified from hSYN1-EGFP-C using primers (MM11-131, 5′-TTA TTA ACG CGT CTA GAC TGC AGA GGG CCC TGC GTA TGA GT-3′, +MluI; MM11-132, 5′-GAT GAT CGC GGC CGC GAC TTG TAC AGC TCG TCC ATG CCG AGA GTG-3′, +NotI) resulting in hSYN1-EGFP-Nbea. An EcoRI fragment of GFP-Nbea was subcloned into pbluescript to delete coding region of PKA site 1091-EVESLLDNVYSAAVEKLQN-1109 using quikchange primer (MM12-45, 5′-GGT GGA GAG AAT GGT GCA CTA GTG AAT GTA CAT GGA AGT GTT GG-3′, +SpeI) and re-ligated to obtain GFP-NbeaΔPKA. Single N-terminal EGFP-fused Nbea domains were obtained by PCR using following primers for lectin 231-TFN…STF-416 (MM12-68, 5′-GCC TGC AGG TCG ACA CTT TTT TCA ATT TCC CTG GTT GTA GCG C-3′, +SalI; MM12-69, 5′-GGA TCC TCT AGA GAA GGT ACT CTT ATA TCC AGG TCC TAA CTG ATG AAT TGC-3′, +XbaI), ARM-long 505-LFA…QEE-1156 (MM12-47, 5′-GCC TGC AGG TCG ACC TTT TTG CCC AAT TGG ATA ATA GGC AGC TCA ATG-3′, +SalI; MM12-48, 5′-ATC CTC TAG ATT CCT CCT GTT TGG GTA TTT TAT CAA AGA GAA ATG-3′, +XbaI), DUF1088 1966-EGR…EGD-2133 (MM12-84, 5′-CTG CAG GTC GAC GAA GGA AGA TTA CTG TGC CAT GCT ATG AAG-3′, +SalI; MM12-85, 5′-GAT CCT CTA GAG TCT CCT TCC AGC ATA AGT TCT GTC TC-3′, +XbaI), PH-BEACH 2150-GPV…PPR-2563 (MM12-70, 5′-GCC TGC AGG TCG ACG GCC CAG TGG TTC TCA GCA CCC CTG-3′, +SalI; MM12-71, 5′-GGA TCC TCT AGA CCG AGG CGG ATG TGG CTC AAT AAG CAA CTG-3′, +XbaI), WD40 2718-GHW…HYE-2941 (MM13-01, 5′-CTG CAG GTC GAC GGC CAT TGG GAT GTG GTC ACT TG-3′, +SalI; MM13-02, GAT CCT CTA GAC TCA TAA TGC CAC CGA TTA AAA TCT ATA TTA AAA GC-3′, +XbaI) and insertion into hSYN1-EGFP-C. Arm-coreΔPKA was generated by introducing two STOP signals after Arm-core domain 505-LFA…YHA-423 in Arm-long using quikchange primer (MM16-13, 5′-CAA GAG GAG GAA AAC ATA TGA TAA AAA AAG GGA AAG AAA GGG-3′, +NdeI). All mutations were performed by Quickchange method (Agilent) using shown forward and correspondent reverse complement primer including named silent restriction site. All constructs were validated by sequencing (GATC, Konstanz).

## Electrophysiological Recordings

For patch clamp recordings, coverslips with cultured neurons (DIV 17–20) were placed in a recording chamber mounted to an inverted microscope (Observer.A1, Zeiss, Oberkochen, Germany) and superfused at 1.0–1.5 ml/min with bath solution at room temperature (≈21°C), containing (in mM): NaCl 145, KCl 3, $MgCl_2$ 1.5, $CaCl_2$ 1.5, glucose 11, HEPES 10; pH 7.4 adjusted with NaOH; to isolate excitatory miniature postsynaptic currents (mEPSCs), 10 μM bicuculline and 0.5 μM tetrodotoxin were added. Patch pipettes (borosilicate glass, 1.5 mm outer diameter; Hilgenberg, Malsfeld, Germany) were pulled by a two-stage electrode puller (PIP 6, HEKA Elektronik, Lambrecht, Germany), showing resistances of 2–3 MΩ when filled with pipette solution containing (in mM): 140 K-gluconate, 1 $CaCl_2$, 2 $MgCl_2$, 4 Na-ATP, 0.5 Na-GTP, 10 EGTA, 10 Hepes, pH 7.3. Whole-cell currents were recorded with an EPC 10 USB Double patch-clamp amplifier and Patchmaster software (HEKA Elektronik). Signals were filtered at 3 kHz and digitized at 10 kHz, series resistance was compensated by about 60–80%. Cells were held at −70 mV in whole-cell configuration, series resistance and membrane capacitance compensated. For evaluation of EPSCs, more than 100 consecutive EPSCs per cell from continuous recordings (100 s) were chosen and analyzed with Minianalysis software (Synaptosoft, Decatur, GA, United States).

Autaptic neurons (11–14 DIV) were whole-cell voltage clamped at −70 mV with an Axoclamp amplifier under the control of the Clampex program 10.1. All analyses were performed using Axograph X. The experiments were performed using a patch-pipette solution containing (in mM) 136 KCl, 17.8 Hepes, 1 EGTA, 0.6 $MgCl_2$, 4 NaATP, 0.3 mM $Na_2GTP$, 15 creatine phosphate, and 5 U/mL phosphocreatine kinase (315–320 mOsmol/L, pH 7.4). The extracellular solution used for all recordings contained (in mM) 140 NaCl, 2.4 KCl, 10 Hepes, 10 glucose, 4 $CaCl_2$ and 4 $MgCl_2$ (320 mOsml/liter), pH 7.3. Evoked EPSCs were induced by depolarizing the cell from −70 to 0 mV at a frequency of 0.2 Hz. Short-term plasticity was evaluated by recording PSCs during a 10 Hz stimulation train. The series resistance was compensated by about 60–80%.

## Statistical Analysis

No statistical methods were engaged to predetermine sample size, instead we based our experimental design on numbers reported in previous studies (Niesmann et al., 2011; Nair et al., 2013). Statistical tests were performed either with GraphPad Prism (GraphPad Software, La Jolla, CA, United States) or Excel (Microsoft). If samples met criteria for normality, we used a Student's *t*-test to compare two groups, and a one-sided ANOVA for more than two groups. If ANOVAs were significant, we used a *post hoc* Tukey's multiple comparisons test to compare groups. Data are presented as means ± SEM. Significance levels were as indicated in figures: $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

## Structural Modeling

Neurobeachin is a multidomain protein with largely unknown molecular fold. The crystal structure of the PH and BEACH domain complex has been solved (PDB: 1MI1) and a model

for the lectin-like domain is available (Burgess et al., 2009). We have used the coordinates described in that study (PDB_ID: 1AF9) to model the lectin-like domain. A common 7-bladed beta propeller (PDB_ID: 2CE8 and 1K8K) served as template for Nbea WD40 repeats. We used threading method phyre2 (Kelley et al., 2015) to generate a model of an armadillo domain of high confidence (>97%) that was built mainly from importin-α-1 coordinates as template (PDB_ID:2JDQ). Using the Arm-long sequence which includes the AKAP motif, phyre2 gave the same Arm-core but with an extended helical domain and an AKAP helix attached to the Arm-core. The remainders, including DUF1088, are domains with low structural complexity. We used phyre2 to generate compact space-filling models to complete the full-length structure of Nbea.

# RESULTS

## Full-Length Nbea Restores Synaptic Transmission in Knockout Neurons

Homozygous Nbea KO mice die after birth by asphyxiation due to defects of synaptic transmission at neuromuscular junctions and synapses in the respiratory network (Su et al., 2004; Medrihan et al., 2009). Here, we analyzed the role of Nbea by performing rescue experiments in primary hippocampal neurons cultured from individual fetal KO mice. We focused our analysis on excitatory synapses because of emerging evidence that interaction partners and signaling pathways of Nbea may differ at inhibitory contacts (del Pino et al., 2011; Farzana et al., 2016).

To probe the ability of a newly constructed full-length human Nbea (Nbea FL) to rescue major functional impairments of excitatory KO synapses observed earlier (Medrihan et al., 2009; Niesmann et al., 2011; Nair et al., 2013), we first recorded miniature excitatory postsynaptic currents (mEPSC) from neuronal cultures of Nbea KO and control animals in presence of TTX and bicuculline (**Figures 1A–F**). Consistent with the previous reports, Nbea KO neurons showed a faster mEPSC rise time (WT: 0.85 ± 0.05 ms, $N$ = 15; KO: 0.67 ± 0.05, $N$ = 14; $P$ = 0.045) (**Figure 1C**) and a faster decay time (WT: 2.68 ± 0.16 ms, $N$ = 15; KO: 2.079 ± 0.14, $N$ = 14; $P$ = 0.01) (**Figure 1D**) that are in agreement with the shift from mature spinous to shaft synapses when Nbea is deleted because a faster kinetic could be caused by lack of dendritic filtering in thin spine necks (Araya et al., 2006; Niesmann et al., 2011). A significantly longer interevent interval time of Nbea KO mEPSCs compared to WT was also observed in our experiments (WT: 241.2 ± 44.3 ms, $N$ = 15; KO: 451.1 ± 78.5, $N$ = 14; $P$ = 0.012) (**Figure 1E**), whereas we did not see significant differences in the amplitude of mEPSCs (WT: −32.75 ± 3.9 pA, $N$ = 15; KO: −36.53 ± 4.2, $N$ = 14, $P$ = 0.995) (**Figure 1F**). Strikingly, transfection of a human EGFP-tagged full-length Nbea construct into KO neurons was able to rescue completely the defects in rise time (KO + NbeaFL: 0.95 ± 0.09 ms, $N$ = 13; $P_{WT}$ = 0.31; $P_{KO}$ = 0.004), decay time (KO + NbeaFL: 2.85 ± 0.22 ms, $N$ = 13; $P_{WT}$ = 0.48; $P_{KO}$ = 0.001) and interevent intervals (KO + NbeaFL: 221.8 ± 39.18 ms, $N$ = 13; $P_{WT}$ = 0.82; $P_{KO}$ = 0.008) of mEPSCs (**Figures 1C–E**). These data suggest that the phenotype was specific for the Nbea deletion and was not caused by compensatory mechanisms.

Previous analyses of Nbea-deficient neurons found that in contrast to spontaneous release events (**Figure 1F**), the amplitudes of evoked postsynaptic responses from excitatory synapses are affected more strongly by the lack of Nbea, reflecting the impaired surface targeting of AMPAR subunits that limits the number of available postsynaptic receptors (Nair et al., 2013). While a smaller amount of glutamate receptors in Nbea KO neurons may be enough to respond to the release by single vesicles with normal amplitudes, the response to action potential induced glutamate release is more than saturating the reduced number of postsynaptic receptors in Nbea KO neurons. Here, we confirmed the dramatic reduction of evoked EPSCs (eEPSC) in autaptic hippocampal neurons (**Figure 2A**), a model system in which single neurons grow on top of small glia cell islands to allow superior analysis of pre- and postsynaptic properties of synaptic transmission (Rosenmund et al., 1993; Burgalossi et al., 2012). The eEPSC amplitudes of Nbea KO neurons, mock-transfected with GFP as control, was strikingly smaller than those of wild-type neurons (WT: 4.63 ± 0.82 nA, $N$ = 7; KO: 0.33 ± 0.068, $P$ = 0.0003; $N$ = 12) (**Figure 2B**). We now validated that this is a specific effect by expressing the full-length Nbea in KO neurons, which was able to restore eEPSC amplitudes back to normal levels (KO + NbeaFL: 3.56 ± 0.71 nA, $N$ = 12; $P_{WT}$ = 0.65; $P_{KO}$ = 0.001). Together, these results demonstrate that expression of a full-length human Nbea in KO neurons restores the defects in both spontaneous and action potential-driven excitatory transmission. In addition, to screen for putative presynaptic defects that might depend on Nbea deletion, short-term plasticity was analyzed in the hippocampal autaptic cultures. eEPSC amplitudes in KO, KO transfected with Nbea FL and KO transfected with NbeaΔPKA depressed progressively during 10 Hz stimulation trains to a comparable steady-state depression level of about 50% as WT control neurons (**Figures 2C,D**). Moreover, comparing the first and second responses of these stimulation series, no significant differences in paired-pulse ratio were observed (WT: 0.80 ± 0.04, $N$ = 5; KO: 0.76 ± 0.05, $N$ = 7; KO + NbeaFL: 0.86 ± 0.11, $N$ = 11; KO + NbeaΔPKA: 0.83 ± 0.07, $N$ = 6). Thus, in extension of a previous study that emphasized more a presynaptic role of Nbea at synapses of the brainstem (Medrihan et al., 2009), our current data confirm the preponderance of postsynaptic defects in hippocampal neurons in absence of Nbea (Nair et al., 2013).

A successful rescue analysis as shown above not only attests to the specificity of the phenotype observed in null mutant neurons but opens the possibility to dissect the molecular basis of the process. Nbea's PKA binding site probably contributes to its function, and rescue experiments have been carried out (Farzana et al., 2016) by deleting the 19 residues long putative AKAP motif (Wang et al., 2000) in recombinant Nbea. Experiments with exogenously applied neurotransmitters suggested that the AKAP motif of Nbea is required for the regulation of GABA-induced postsynaptic responses but not for glutamate responses (Farzana et al., 2016). To probe the role of

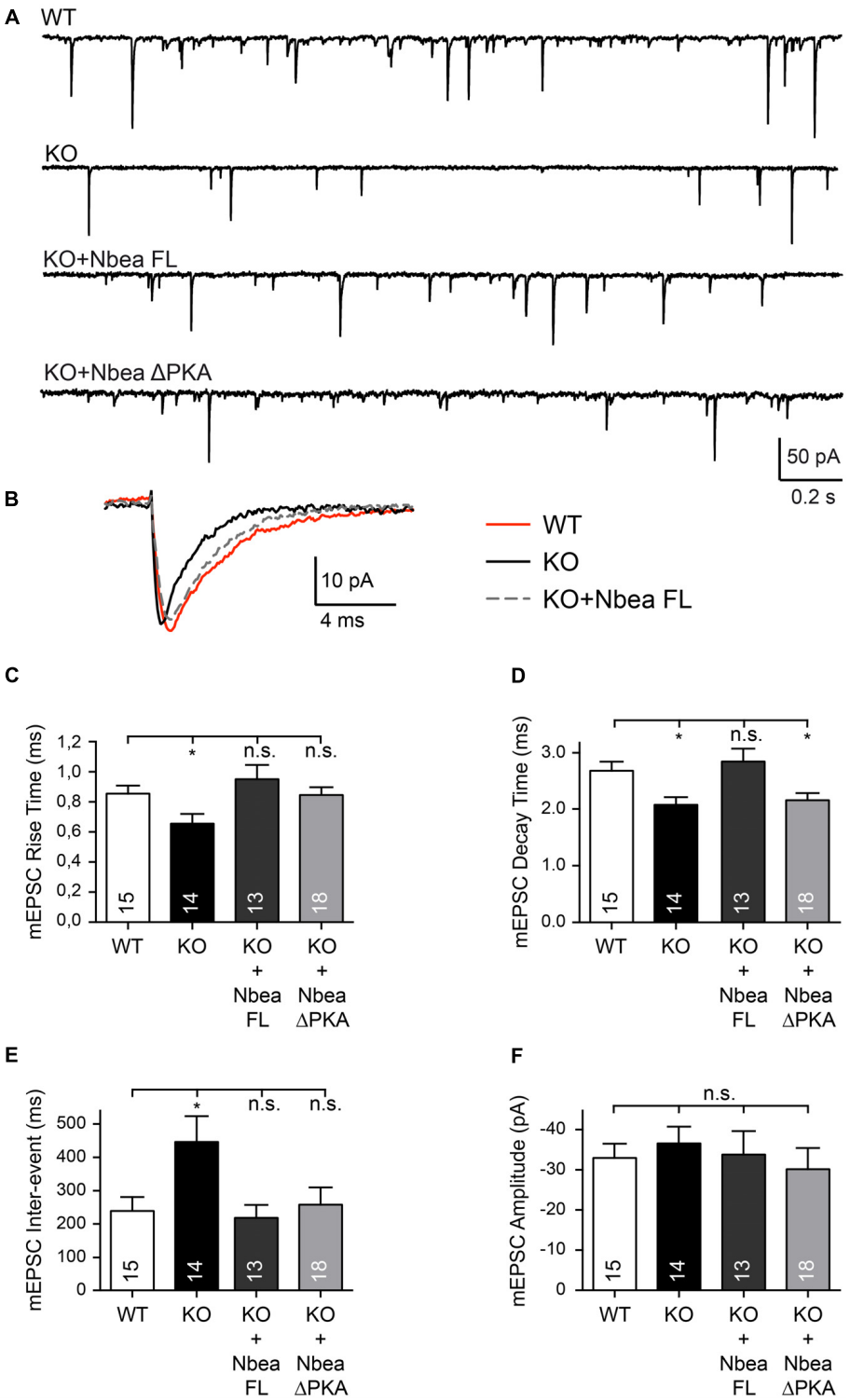


**FIGURE 1 |** Full-length human Nbea restores EPSC defects in null-mutant neurons. **(A)** Representative traces of continuous recordings of mEPSCs from cultured hippocampal neurons of WT, Nbea KO (KO), KO transfected with human full-length Nbea (KO + Nbea FL) and KO transfected with NbeaΔPKA (KO + NbeaΔPKA). **(B)** Averaged traces of mEPSCs recorded from WT (red line), KO (black), and KO + Nbea FL (dashed) neurons indicate faster kinetics in Nbea KO that can be rescued by Nbea FL. **(C–F)** Quantitative analysis of rise time **(C)**, decay time **(D)**, inter-event interval **(E)** and amplitude **(F)** of mEPSCs in WT, KO, KO + Nbea FL and KO + NbeaΔPKA. Digits in bars give the number of recorded neurons; from each neuron more than 100 consecutive mEPSCs were analyzed for evaluation. Data are means ± SEM; $^*P < 0.05$, n.s., not significant; by one-way ANOVA with Tukey's multiple comparisons test.

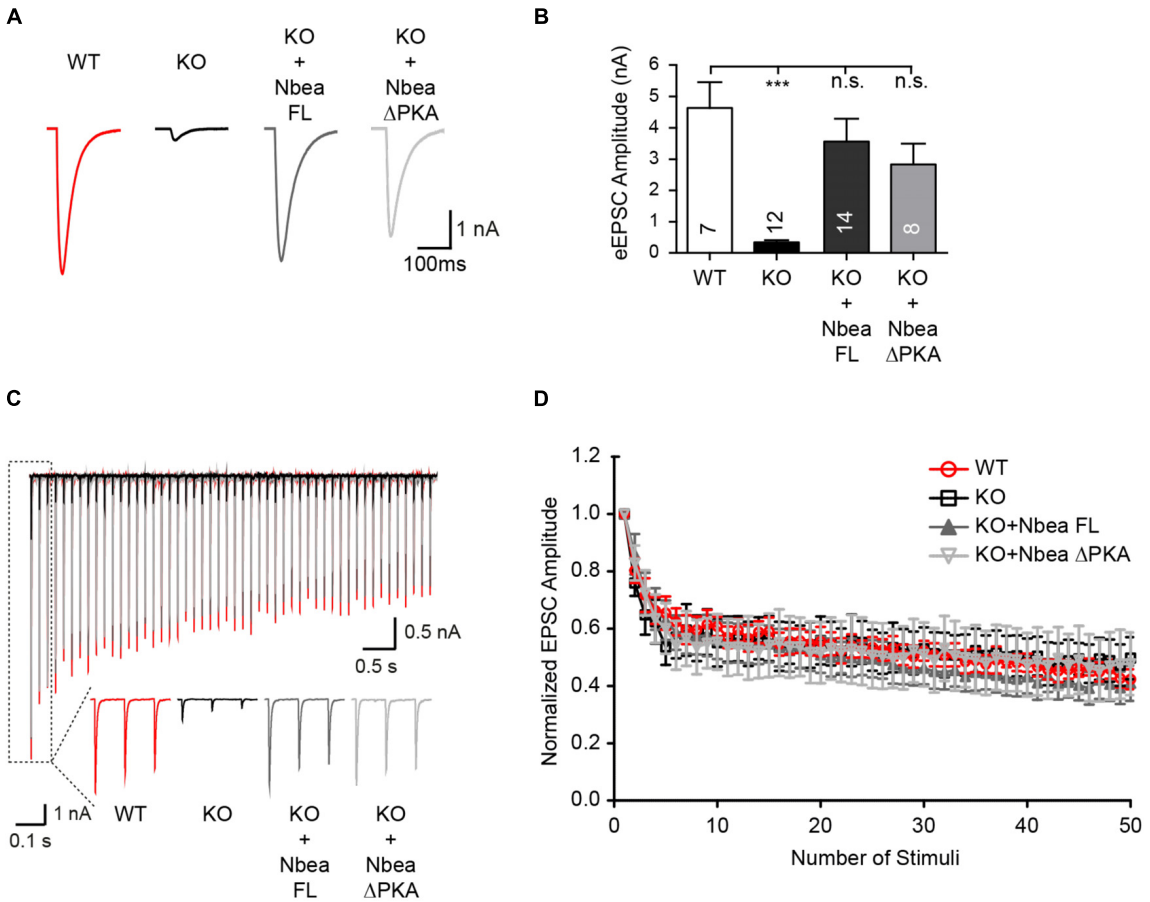


**FIGURE 2 |** Evoked EPSCs at autaptic synapses are restored by full-length Nbea and NbeaΔPKA. **(A)** Sample traces of evoked EPSCs recorded from individual hippocampal neurons grown on micro islands that form autapses. The eEPSCs shown are from untransfected WT, from Nbea KO neurons mock transfected with GFP alone, and transfected with Nbea FL or NbeaΔPKA constructs. **(B)** Average values of eEPSC amplitudes from WT, KO/GFP, Nbea FL, and NbeaΔPKA. Data are means ± SEM; ***$P < 0.001$, n.s., not significant; by one-way ANOVA with Tukey's multiple comparisons test. **(C)** Overlay of representative traces of eEPSCs during AP stimulation trains (10 Hz) from WT, KO/GFP, KO + Nbea FL and KO + NbeaΔPKA; insets depict magnifications of the response to the first three stimuli. **(D)** Change in EPSC amplitudes during a 10 Hz stimulation train in WT, KO, KO + Nbea FL and KO + NbeaΔPKA autaptic glutamatergic neurons. $N_{WT}$ = 5 neurons, $N_{KO}$ = 7 neurons, $N_{KO+NbeaFL}$ = 11 neurons, $N_{KO+Nbea\,\Delta PKA}$ = 6 neurons.

the PKA binding site in spontaneous and evoked transmission in our excitatory hippocampal neurons, we deleted the motif in the human Nbea construct (NbeaΔPKA; lacking residues 1091–1109: EVESLLDNVYSAAVEKLQN) and repeated the rescue experiments. The NbeaΔPKA mutant was expressed at levels comparable to the full-length Nbea in our hippocampal neurons as predicted from previous reports (Lauks et al., 2012; Farzana et al., 2016). Nbea lacking the PKA binding site was able to fully restore the increased interevent interval time of KO mEPSCs (NbeaΔPKA: 260 ± 53 ms, $N$ = 18, $P_{WT}$ = 0.81; $P_{KO}$ = 0.017; **Figure 1E**), and almost completely rescued the shorter mEPSC rise time (NbeaΔPKA: 0.84 ± 0.05 ms, $N$ = 18, $P_{WT}$ = 0.91; $P_{KO}$ = 0.047; **Figure 1E**) and the reduced amplitude of eEPSCs (NbeaΔPKA: 2.83 ± 0.66 nA, $N$ = 8, $P_{WT}$ = 0.32; $P_{KO}$ = 0.045; **Figures 2A,B**). Although it remains unclear why the NbeaΔPKA construct was less effective on the decay time of mEPSCs (NbeaΔPKA: 2.17 ± 0.13 ms, $N$ = 18, $P_{WT}$ = 0.022; $P_{KO}$ = 0.69), it emerges from these data that the PKA binding site is not critically required for the role of Nbea in excitatory synaptic transmission.

## PH-BEACH Domain Affects Synaptic Targeting of AMPA Receptors and Controls Spine Density

The amino acid sequence of Nbea includes a Concanavalin A-like lectin domain (Lectin, blue in **Figures 3A,B**) near the N-terminus, followed by an Armadillo repeat (Armadillo, turquoise in **Figures 3A,B**), the AKAP motif addressed above (PKA binding site, magenta in **Figures 3A,B**), a "domain-of-unknown-function" (DUF1088, orange in **Figures 3A,B**), the namesake complex of PH-BEACH domains (PH-BEACH, yellow and red in **Figures 3A,B**) and C-terminal WD40 repeats (WD, green in **Figures 3A,B**). For rescue experiments using these individual Nbea domains, we focused on the lower postsynaptic AMPA receptor availability (Nair et al., 2013) and the reduced

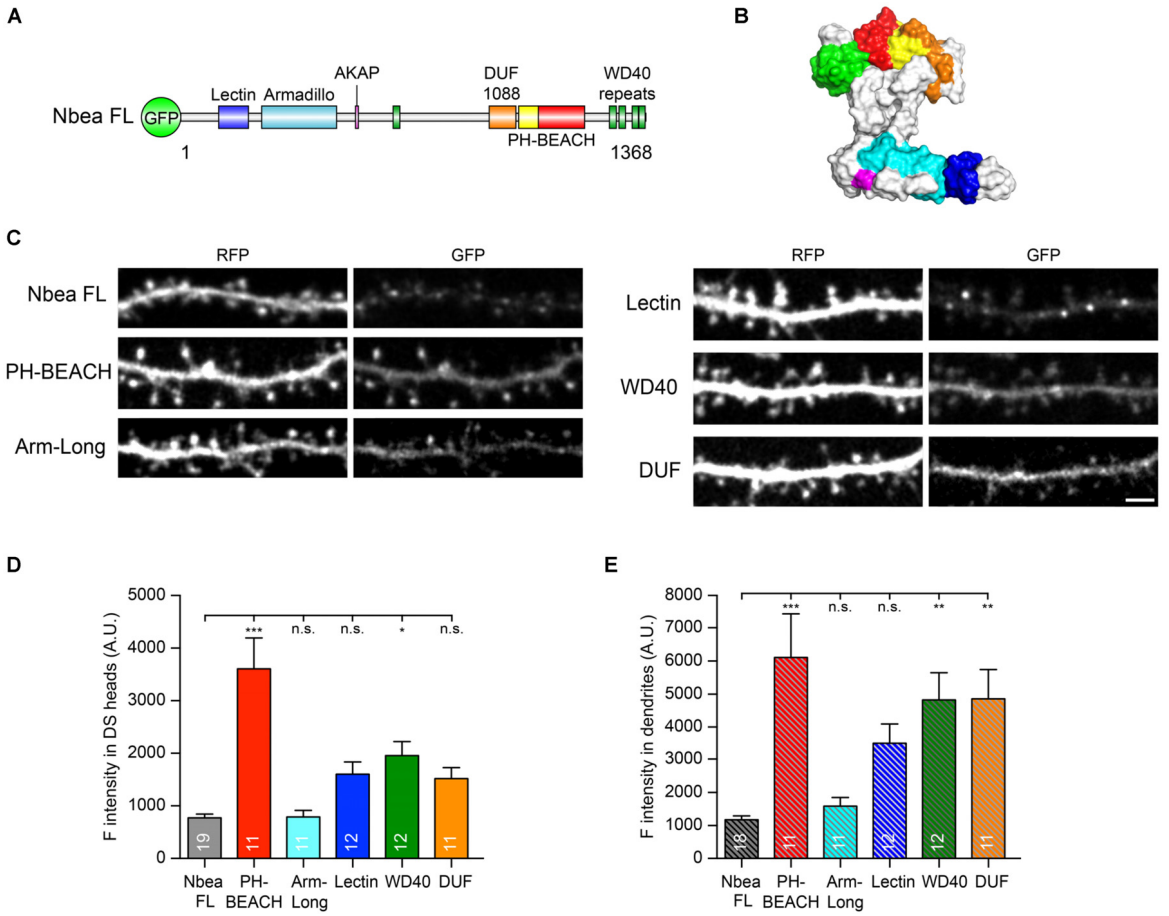


**FIGURE 3 |** Individual Nbea domains are able to localize to dendrites and spines. **(A)** Nbea motif architecture depicting its multidomain composition. GFP, N-terminally fused EGFP tag; Lectin, concanavalin A-like lectin domain; Armadillo, armadillo repeat; AKAP, A-kinase anchoring protein; DUF1088, domain-of-unknown-function; PH, pleckstrin homology-like domain; BEACH, *B*eige *a*nd *C*hediak-*H*igashi domain; WD40, tryptophan-aspartic acid repeats. **(B)** Predicted structural model of Nbea with domains color coded as in **(A)**. See "Materials and Methods" section for details on templates and modeling procedure. **(C)** Representative images of dendrites from primary hippocampal neurons transfected with cytosolic marker t-dimer-RFP and isolated GFP-tagged Nbea domains as indicated. Scale bar: 2.5 μm. **(D)** Quantification of fluorescence intensity of individual Nbea domains in dendritic spine (DS) heads. Data are means ± SEM (arbitrary units, A.U.). *N*, number of neurons (in bars); quantification was carried out on at least 50 spines per neuron. One-way ANOVA with Tukey's multiple comparisons test; n.s., not significant, $^{*}P < 0.05$, $^{***}P < 0.001$. **(E)** Quantification of fluorescence intensity of individual Nbea domains in dendritic shaft. Data are means ± SEM (arbitrary units, A.U.). *N*, number of neurons (in bars); quantification was carried out on at least four 20 μm-long dendritic windows per neuron. One-way ANOVA with Tukey's multiple comparisons test; n.s., not significant, $^{**}P < 0.01$, $^{***}P < 0.001$.

number of mature DSs (Niesmann et al., 2011) in Nbea KO neurons. As a prerequisite for such assays, we asked if and how efficiently individual domains were targeted to the postsynaptic compartment of DSs in comparison to full-length Nbea.

To investigate the subcellular targeting, we generated GFP-tagged expression constructs under the control of human synapsin 1 promoter, analogous to the full-length Nbea construct used in the electrophysiological experiments above (**Figures 1**, **2**). Nbea KO neurons were co-transfected with GFP-fused Nbea domain constructs and a soluble cytosolic marker to identify DS morphology, t-dimer-RFP. Interestingly, all individual domains reached dendritic branches and spines (**Figure 3C**) and showed at least the expression levels of full-length Nbea (**Figure 3D**). The Armadillo domain (Arm-long in **Figure 3C**) showed a fluorescence intensity in spine heads that was almost identical to full-length Nbea (Nbea FL: 784.3 ± 65.3 A.U., $N = 19$; Arm-long: 788.7 ± 126.7, $N = 11$, $P_{\mathrm{NbeaFL}} = 0.99$; **Figure 3D**). Lectin, WD40 and DUF domains showed a tendency of higher fluorescence intensity in spine heads compared to full-length Nbea but only WD40 reached a significant difference (Lectin: 1,604 ± 230, $N = 12$, $P_{\mathrm{NbeaFL}} = 0.19$; WD40: 1,955 ± 268, $N = 12$ $P_{\mathrm{NbeaFL}} = 0.012$; DUF: 1,519 ± 208, $N = 11$ $P_{\mathrm{NbeaFL}} = 0.34$; **Figure 3D**). Importantly, transfection of the PH-BEACH domain of Nbea resulted in the highest fluorescence intensity compared to full-length Nbea (PH-BEACH: 3,606 ± 586.2, $P_{\mathrm{NbeaFL}} < 0.0001$; **Figure 3D**). In addition, we compared the expression of these individual domains to full-length Nbea in the dendritic shaft which mimicked the expression in the spine head (**Figure 3E**). However, expression of Lectin, WD40 and DUF domains was more similar to PH-BEACH in the

dendritic shaft (PH-BEACH: 6,122 ± 1,323, $N$ = 11; Lectin: 3,505 ± 584, $N$ = 12, $P_{PH-BEACH}$ = 0.131; WD40: 4,823 ± 826, $N$ = 12, $P_{PH-BEACH}$ = 0.808; DUF: 4,859 ± 892, $N$ = 11, $P_{PH-BEACH}$ = 0.839; P values are from multiple comparison ANOVA). In contrast, Nbea FL and Armadillo domain showed low expression in dendritic shafts (Nbea FL: 1,181 ± 104, $N$ = 19; Arm-Long: 1,592 ± 250, $N$ = 12; **Figure 3E**). Together, these data indicate that all Nbea domains were reliably expressed in DSs, making their use in rescue assays meaningful. Moreover, the high levels of PH-BEACH in spine heads pointed to a particularly prominent role in regulating postsynaptic functions of excitatory synapses.

Since the phenotype of reduced amplitudes of EPSCs in Nbea KO neurons has been explained by an impaired surface targeting of AMPA receptor GluA2 subunits (Nair et al., 2013; Farzana et al., 2016), we asked if an individual domain of Nbea is able to re-establish normal dendritic GluA2 surface expression in KO neurons. To address this question, we performed live labeling of the surface population of endogenous GluA2 subunits in wild-type and Nbea null mutant neurons (**Figure 4A**). Nbea-deficient dendrites showed a diminished GluA2 surface staining compared to controls (WT: 309 ± 15 A.U., $N$ = 133/40; KO: 244.3 ± 8.9, N = 134/39; $P$ = 0.0036; **Figure 4B**), confirming an earlier report (Nair et al., 2013). The impairment could be fully rescued upon transfection of full-length Nbea in KO neurons (KO + Nbea FL: 335.6 ± 16.7 A.U., $N$ = 93/31; $P$ = 0.88; **Figures 4A,B**), attesting to the specificity of the phenotype. Most importantly, we found that expression of the PH-BEACH domain of Nbea alone in KO neurons was sufficient to restore GluA2 surface targeting back to control levels (KO + PH-BEACH: 336.5 ± 19.0 A.U., $N$ = 60/20; $P$ = 0.933; **Figures 3A,B**). These results identify the PH-BEACH complex of Nbea as the critical domain responsible for the impaired targeting of GluA2 receptors in KO neurons.

We then asked if the PH-BEACH domain is also able to restore the normal number of DSs which was reduced in Nbea KO neurons (Niesmann et al., 2011) and which could possibly be linked to the GluA2 receptor defect (Nair et al., 2013; Farzana et al., 2016). To visualize the morphology of control and mutant hippocampal neurons, we transfected the cytosolic marker t-dimer-RFP (**Figure 5A**). Consistent with the earlier

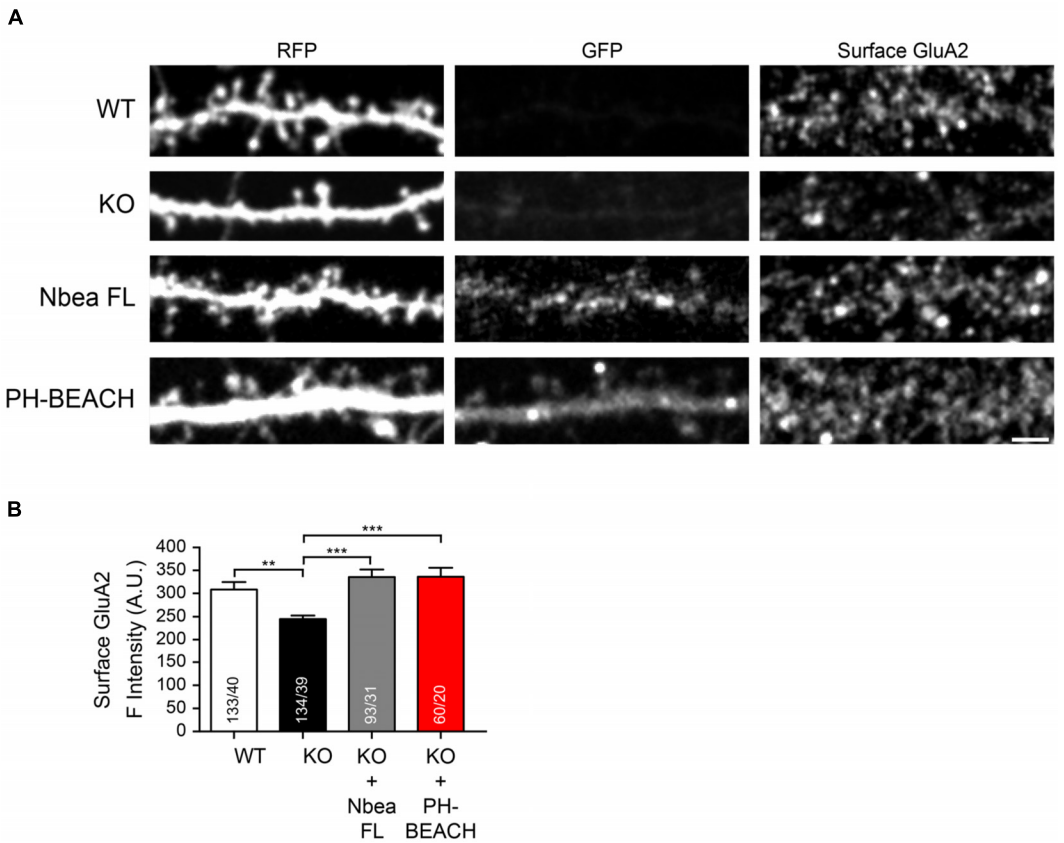


**FIGURE 4 |** PH-BEACH domain restores surface targeting of GluA2 receptors in Nbea-deficient neurons. **(A)** Representative dendrites of primary hippocampal neurons from wild-type (WT) and Nbea null-mutant (KO) mice transfected with cytosolic marker t-dimer-RFP alone and in combination with GFP-tagged full-length Nbea (KO + Nbea FL) or the PH-BEACH domain (KO + PH-BEACH). Right panels, surface populations of GluA2 receptor subunits visualized by live labeling of neurons with an antibody directed against an extracellular epitope of the GluA2 subunit. Scale bar: 2.5 μm. **(B)** Quantification of surface GluA2 fluorescence intensity measured on dendrites of WT, KO, KO + Nbea FL and KO + PH-BEACH neurons. Data are means ± SEM. $N$, number of dendritic window/neurons (in bars); $^{**}P < 0.01$, $^{***}P < 0.001$, by one-way ANOVA with Tukey's multiple comparisons test.

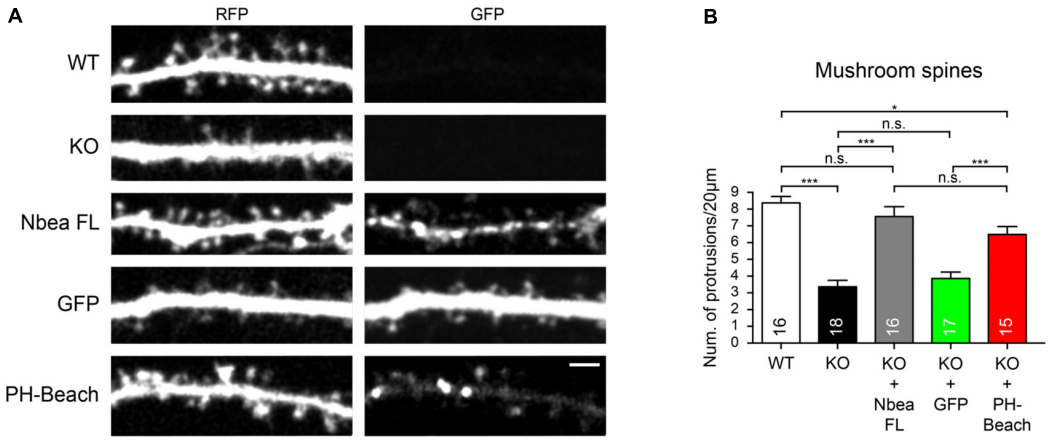


**FIGURE 5 |** PH-BEACH domain of Nbea is sufficient to rescue the defect on mushroom spines. **(A)** Representative dendrites of primary hippocampal neurons from wild-type (WT) and Nbea null-mutant (KO) mice transfected with cytosolic marker t-dimer-RFP alone and in combination with GFP-tagged full-length Nbea (KO + Nbea FL) or PH-BEACH domain (KO + PH-BEACH). As additional control, KO neurons were mock transfected with GFP alone (KO + GFP). Scale bar: 2.5 µm. **(B)** Quantification of the number of mature mushroom-like DSs in different genotypes and upon expression of different constructs as indicated. Data are means ± SEM; *N,* number of neurons (in bars). Quantification was carried out analyzing the whole dendritic tree for each neuron. $^{*}P < 0.05$ $^{***}P < 0.001$, n.s., not significant by one-way ANOVA with Tukey's multiple comparisons test.

report (Niesmann et al., 2011), we determined a more than 50% reduction in the number of mature, mushroom-shaped DS in absence of Nbea (WT: 8.38 ± 0.38 protrusions/20 µm, $N = 16$; KO: 3.36 ± 0.38, $N = 18$; $P < 0.001$; **Figure 4B**). This impairment could be fully restored by expression of full-length Nbea in KO neurons (KO + NbeaFL: 7.56 ± 0.59 protrusions/20 µm, $N = 16$; $P_{WT} = 0.693$; **Figures 5A,B**). Strikingly, expression of the PH-BEACH domain alone was also able to double the number of DS compared to KO neurons (KO + PH-BEACH: 6.46 ± 0.46 protrusions/20 µm, $N = 15$; $P_{KO} < 0.0001$; **Figures 5A,B**), reaching numbers that came close to full-length Nbea (**Figure 5B**, $P_{KO+NbeaFL} = 0.432$). To exclude any off-target effects from the presence of a GFP epitope tag in our constructs, we additionally analyzed the spine numbers in Nbea KO neurons expressing a GFP without any Nbea sequence attached as control. No difference was found compared to KO neurons (KO + GFP: 3.85 ± 0.38 protrusions/20 µm, $N = 17$; $P_{KO} = 0.926$; **Figures 5A,B**), suggesting that our approach was reliable and all changes observed were due to the Nbea moiety. In contrast to mushroom spines, no significant difference of the number of stubby spines could be observed in WT, Nbea KO neurons, KO transfected with Nbea FL and KO transfected with GFP ($P = 0.08$; ANOVA). Thus, our results indicate that the PH-BEACH complex of Nbea is sufficient to restore normal numbers of mature mushroom-shaped DS in KO neurons.

## PKA Binding Site and Armadillo Domains Regulate Filopodia Formation

The prominent effect of the PH-BEACH domain in restoring mushroom spine numbers shown above does not exclude, however, that other Nbea domains also contribute to the regulation of this or other classes of dendritic protrusions (**Figure 6A**). We first tested if the deletion of the PKA binding domain has an impact on DS by employing the NbeaΔPKA mutation that we used before to rescue the EPSCs (**Figure 1**). In line with our results obtained with the individual PH-BEACH domain (**Figures 4**, **5**), which is fully contained in the NbeaΔPKA construct and distant to the ΔPKA deletion site, the removal of AKAP did not affect the ability of Nbea to rescue mature DS numbers when transfected into KO neurons (NbeaΔPKA: 7.17 ± 0.56 protrusions/20 µm, $N = 14$; $P_{WT} = 0.458$ **Figures 6C,D**). However, we observed during the analysis of the dendritic tree of NbeaΔPKA transfected KO neurons (**Figure 6C**) that there were numerous thin and elongated protrusions called "filopodia" (**Figure 6A**). This is an interesting observation because larger numbers of filopodia occur during development and under conditions of reduced neural activity (Petrak et al., 2005). In our study, we classified every protrusion longer than 2 µm that lacked a well-defined head as "filopodium," accepting that our class of filopodia might include some long spines but avoiding the problems associated with the resolution and orientation necessary to detect very small heads at the end of filopodial-like protrusions (Tonnesen et al., 2014). In fact, quantification of these protrusions showed that expression of NbeaΔPKA mutant more than doubled the number of filopodia in KO neurons (KO: 1.50 ± 0.16 protrusions/20 µm, $N = 18$, KO + NbeaΔPKA: 3.25 ± 0.19, $N = 14$; $P < 0.0001$; **Figure 6E**). These data may indicate that the PKA binding of Nbea could serve an unexpected role in negatively regulating the formation of filopodial-like dendritic protrusions.

PKA binding motifs fold into an amphipathic helix that binds a PKA RII dimer (Gold et al., 2013) and is freely accessible and not part of a larger domain (Smith et al., 2013). The helix anchors PKA, but the remaining domains of a particular AKAP define targeting to compartments and subsequent functions of that complex (Wong and Scott, 2004). In Nbea, an Armadillo

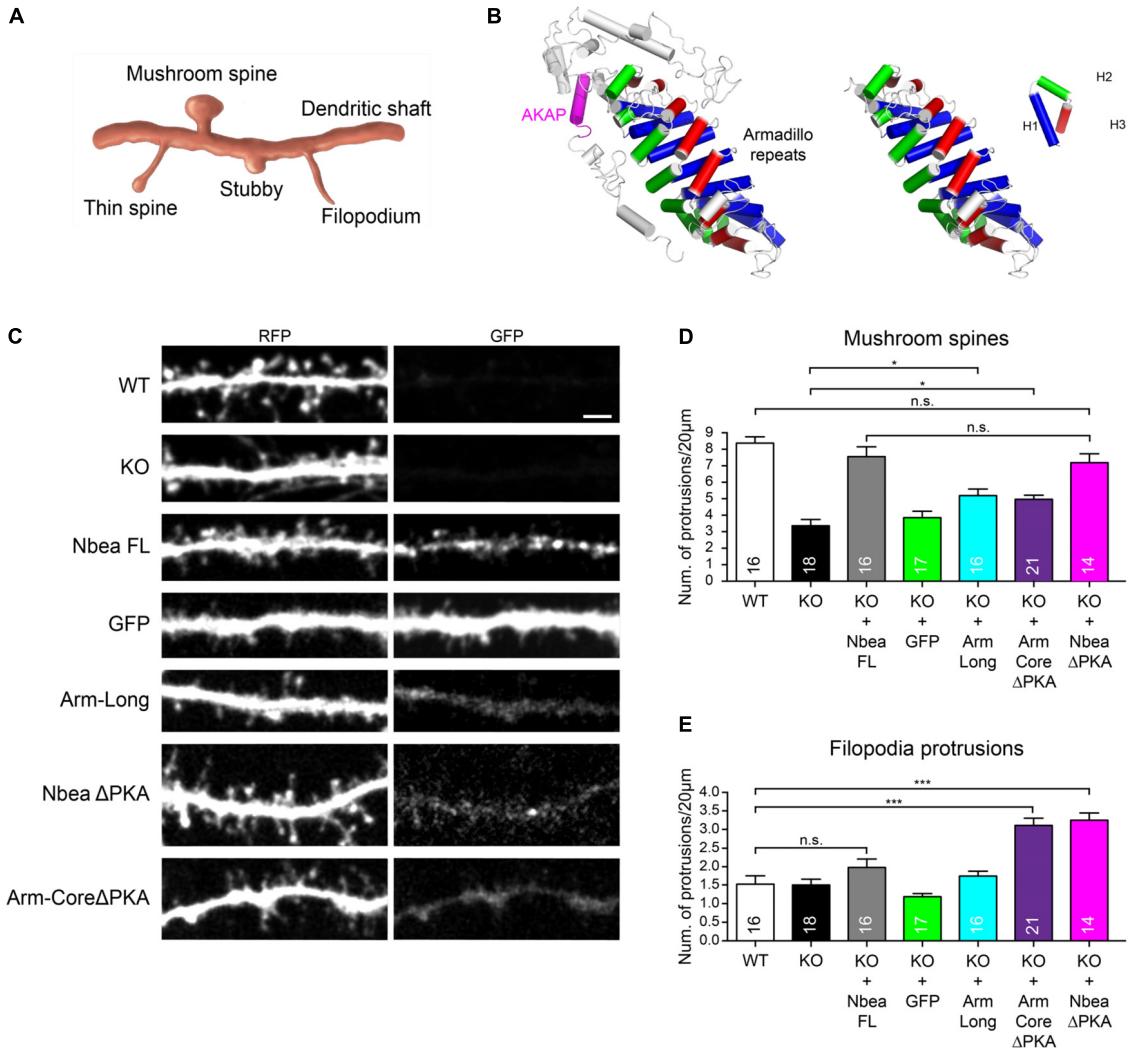


**FIGURE 6 |** Armadillo domain and AKAP motif of Nbea affect filopodia formation. **(A)** Scheme depicting major categories of DS morphology, including mature mushroom spines, long thin spines, stubby spines, and filopodia. **(B)** Structural model of Arm-Long sequences (left) including the AKAP helix (magenta) and Arm-Core (right). An Armadillo domain consists of eight repeats of three helices H1 (blue), H2 (green), and H3 (red). The AKAP domain of Nbea is present in the Arm-Long construct but deleted in the Arm-Core ΔPKA construct **(C–E)**. **(C)** Representative dendrites of primary hippocampal neurons from wild-type (WT) and Nbea null-mutant (KO) mice transfected with cytosolic marker t-dimer-RFP alone and in combination with GFP-tagged full-length Nbea (KO + Nbea FL), Armadillo domain with AKAP motif (KO + Arm-Long), Armadillo domain without AKAP (KO + Arm-CoreΔPKA) and full-length Nbea lacking the AKAP (KO + NbeaΔPKA). As additional control, KO neurons were mock transfected with GFP alone (KO + GFP). Scale bar: 2.5 µm. **(D)** Quantification of number of mushroom-shaped DS in WT and Nbea KO neurons and upon expression of Nbea domains as detailed in **(C)**. Values for WT, KO, KO + FL, and KO + GFP are the same as in **Figure 4B** and displayed here again to allow for easy comparison with individual domains. Data are means ± SEM, *N*, number of neurons (in bars). Quantification was carried out by analyzing the whole dendritic tree for each t-dimer-RFP transfected neuron. $^{*}P < 0.05$, n.s., not significant by one-way ANOVA with Tukey's multiple comparisons test. **(E)** Quantification of filopodial protrusions in WT and Nbea KO neurons and upon expression of Nbea domains as detailed in **(C,D)**. Data are means ± SEM, *N*, number of neurons (in bars). Quantification was carried out analyzing the whole dendritic tree for each t-dimer-RFP transfected neuron. $^{***}P < 0.001$, n.s., not significant by one-way ANOVA with Tukey's multiple comparisons test.

domain resides next to the PKA RII binding site. To explore the effect on dendritic protrusions in more detail, we decided to compare the influence of a long Armadillo construct containing this PKA binding sequence (**Figure 6B** left, Arm-long) to an Armadillo "core domain" without PKA binding sequence (**Figure 6B** right, Arm-coreΔPKA). When expressed in Nbea KO neurons (**Figure 6C**), the Arm-long and the Arm-coreΔPKA domains had only weak effects on the number of mushroom DS (Arm-Long: 5.16 ± 0.39 protrusions/20 µm, $N = 16$; Arm-coreΔPKA: 4.95 ± 0.24, $N = 21$; $P = 0.040$ and $P_{KO} = 0.062$; **Figure 6D**). This confirms the importance of the PH-BEACH domain, not contained in the Arm constructs, to promote strongly formation of mature DS (**Figure 5**). However, we discovered that the Armadillo domain was able to increase the number of filopodia but only if the PKA binding site was missing. The Arm-coreΔPKA domain increased significantly the

number of filopodia in Nbea-deficient neurons compared to all controls (Arm-coreΔPKA: 3.11 ± 0.19 protrusions/20 μm, $N$ = 21; $P_{WT}$ < 0.0001; **Figures 6C,E**), whereas the Arm-long domain failed to induce filopodia (Arm-long: 1.75 ± 0.13 protrusions/20 μm, $N$ = 16; $P_{WT}$ = 0.981; **Figure 6E**). Together, these results suggest that the Armadillo domain and PKA binding site of Nbea reciprocally regulate the number of filopodia on dendrites, with Armadillo showing a facilitating influence while the PKA site serves as a negative regulator.

## Actin Cytoskeletal Organization in Absence of Nbea

Actin is the major cytoskeletal component of DSs and changes in the morphology of spines critically depend on the remodeling of the actin cytoskeleton (Frost et al., 2010; Hotulainen and Hoogenraad, 2010). Since DSs are an important substrate of activity-dependent dynamic remodeling in the brain (Hofer et al., 2009; Kwon and Sabatini, 2011), we decided to analyze if lack of Nbea affects the basal motility of DS. Hippocampal wild-type and Nbea-deficient neurons were transfected with Lifeact to specifically stain polymerized filamentous F-actin (Riedl et al., 2008) (**Figures 7A,B**) and with cytosolic GFP to visualize the entire volume of spines (**Figures 7C,D**). We then imaged spines for 30 min every 15 s and calculated the center of mass (COM) displacement over time for individual spines (**Figures 7A,C**). Our quantitative analysis revealed that deletion of Nbea caused both reduced F-actin related (WT: 0.128 ± 0.004, $N$ = 62/13 A.U.; KO: 0.103 ± 0.003, $N$ = 62/12; $P$ < 0.0001; **Figure 7B**) and volume-based motility (WT: 0.133 ± 0.008, $N$ = 66/11 A.U.; KO: 0.0985 ± 0.008, $N$ = 66/11; $P$ = 0.008; **Figure 7D**) of mutant spine heads compared to controls. These data suggest that the basal motility of DSs in KO mice is impaired, raising the question if and how the actin cytoskeleton might be regulated by Nbea.

In support of the importance of the actin cytoskeleton, we previously obtained indirect evidence that the actin organization may be involved in the spine phenotype of Nbea KO neurons (Niesmann et al., 2011). We now built on this study and demonstrate that KO neurons contain increased areas of phalloidin-labeled clusters compared to WT (WT: 16.44 ± 4.61 μm$^2$, $N$ = 18; KO: 57.68 ± μm$^2$, $N$ = 19; $P$ < 0.01; **Figures 8A,B**). Moreover, Nbea KO cultures contained an increased percentage of neurons with such phalloidin-stained actin clusters that were mostly found ectopically in the soma (WT: 8.66 ± 1.84%, $N$ = 20; KO: 22.68 ± 2.02, $N$ = 20; $P$ < 0.001; **Figure 8C**). Our findings on DS numbers, filopodia formation and basal spine motility raised the question if Nbea acts directly on the actin cytoskeleton, for example, by modulating actin polymerization rates in DS. To analyze if changes in actin polymerization rates were responsible for the spine phenotype, we performed FRAP experiments on single

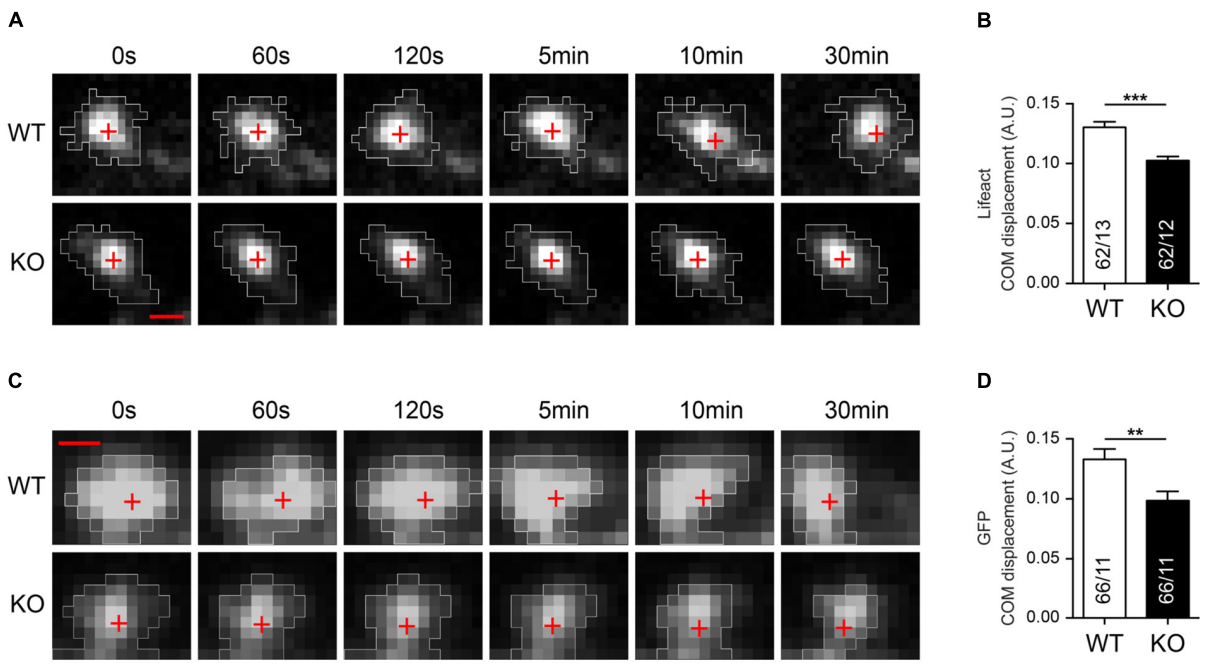


**FIGURE 7 |** Impaired basal motility of Nbea-deficient DSs. **(A)** Representative image frames of WT and Nbea KO spines at different time points and transfected with Lifeact fused to RFP. The fluorescent signal of Lifeact-RFP highlights specifically polymerized filamentous F-Actin. White areas represent regions taken in consideration for center of mass (COM) displacement analysis in each time point. Red crosses depict the position of COM at each time point. **(B)** Quantification of COM displacement in WT and Nbea KO single spine. Data are means ± SEM, $N$, number of spines/neurons (in bars); ***$P$ < 0.001 by unpaired $t$-test. **(C,D)** Similar analysis to **(A,B)** using an alternative label of the spine volume, cytosolic GFP expressed in WT and Nbea KO neurons. White areas represent regions taken in consideration for COM displacement analysis in each time point. Red crosses depict the position of COM at each time point. Data are means ± SEM, $N$, number of spines/neurons (in bars); **$P$ < 0.01 by unpaired $t$-test. Scale bar: 1 μm; pixel size = 0.211 μm.

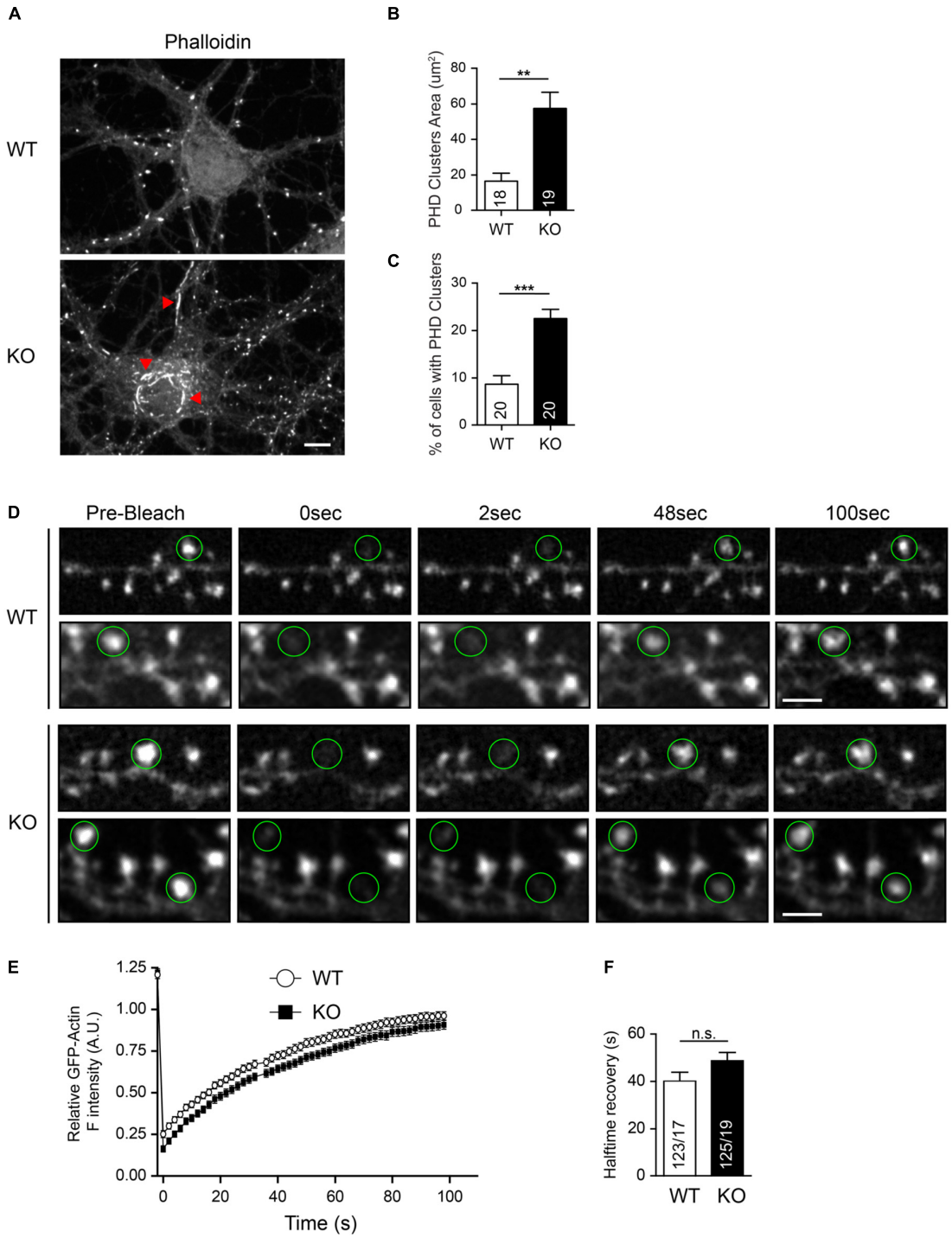


**FIGURE 8 |** Normal actin polymerization and turnover in Nbea-deficient DSs. **(A)** Representative images of hippocampal WT and Nbea KO neurons stained with fluorescent phalloidin. Red arrowheads in images point to ectopic actin clusters mostly in the soma and soma-near large dendrites. Scale bar: 5 μm. **(B)** Quantification of actin cluster areas detected by phalloidin staining. Data are means ± SEM, *N*, number of neurons (in bars); $^{***}P < 0.001$ by unpaired *t*-test. **(C)** Quantification of the percentage of cells with visible phalloidin-stained actin clusters in WT and KO cultures. Data are means ± SEM, *N,* number of visual fields counted (in bars); $^{***}P < 0.001$ by unpaired *t*-test. **(D)** Representative images of FRAP experiments of wild-type (WT) and Nbea-deficient (KO) spines at different time points. Samples show GFP-actin transfected dendrites at the indicated time points after bleaching, pre-Bleach indicates spines before quenching. Green circles, region selected for bleaching around spine heads. Scale bar: 2.5 μm. **(E,F)** Quantification of FRAP data with first order exponential equation fitting of relative fluorescence intensity of GFP-Actin values over time **(E)**. Halftime recovery of GFP-Actin was derived from exponential curves **(F)**. Data are means ± SEM, *N,* number of spines/neurons (in bars); n.s., not significant by unpaired *t*-test.

spines by expressing GFP-actin in wild-type and Nbea-deficient neurons (**Figure 8D**). However, KO spines showed no differences in fluorescence intensity recovery curves compared to controls (**Figure 8E**). Moreover, an analysis of the halftime of GFP-actin fluorescence recovery of WT and Nbea KO DS revealed only a tendency, albeit not significant, toward longer recovery halftime (WT: 20.16 ± 1.79 s, $N$ = 123/17; KO: 24.38 ± 1.72, $N$ = 125/19; $P$ = 0.097; **Figure 8F**). These results indicate that Nbea does not, or at least not prominently, regulate the actin polymerization dynamics in DS. Future research will have to test other mechanisms, for example related to branching of the dendritic actin cytoskeleton, that may mediate the important effects of Nbea on spinous synapses.

## DISCUSSION

Here, we report that expression of full-length human Nbea is able to restore normal excitatory postsynaptic currents in neurons lacking endogenous Nbea. We demonstrate that an isolated motif of the multidomain protein Nbea, PH-BEACH, is sufficient to correct one deficiency of Nbea KO neurons, the impaired surface targeting of GluA2 AMPA receptors. Moreover, both full-length Nbea and PH-BEACH alone are able to restore a normal density of mature mushroom spine numbers at excitatory synapses, whereas the PKA RII binding site and the Armadillo domain of Nbea reciprocally regulate filopodial extension, a transient class of dendritic protrusions.

### PH-BEACH Domain Mediates Key Functions of Nbea

Our study aimed at the identification of functions that are specific for individual domains of Nbea, a membrane-associated protein classified by its BEACH domain. Crystallization of BEACH domains remained elusive until the sequence was extended by 130 residues to the N-terminus (Jogl et al., 2002). This additional sequence was an untypical PH domain, revealing a unique insertion of two helices into the canonical fold motif. Furthermore, it lacks a lipid binding loop consisting of basic residues that coordinate phosphate ions of phosphatidylinositol phosphates (PIP) in other PH domains as in GRP1 (PDB_ID: 1FHW, Ferguson et al., 2000). Rather, the loop in PH of Nbea is made of hydrophobic residues that form a hydrophobic cluster with the BEACH domain (Jogl et al., 2002; Gebauer et al., 2004). A surface plasmon resonance binding assay of isolated recombinant domains confirmed their tight complex and determined the PH-BEACH domain tandem as a structural unit (Gebauer et al., 2004). Consistent with the missing phosphate binding loop, PIP binding was not immediately detectable for PH-BEACH domains of Nbea and LRBA (Jogl et al., 2002; Gebauer et al., 2004) but was present for FAN, another member of the family that consists solely of PH-BEACH and WD domains. The basic residues identified for PIP binding of FAN are located untypically at the surface of the PH domain (Haubert et al., 2007) and are not conserved in Nbea or LRBA. This feature is reminiscent of a similar tight PH-phosphotyrosine phosphatase (PTP) tandem, where a phosphoinositol phosphate binds to the PTP domain, but not to the PH (Begley et al., 2006). These authors concluded based on the structure that the PH domain has contact to PIPs, albeit with low affinity. By analogy, the PH-BEACH domains of Nbea and LRBA, despite forming tight structural unit, might still attach to PIPs as they contain basic surface regions. This putative low affinity PIP interaction has to await experimental confirmation but could be relevant to the effect of the PH-BEACH domain on GluA2 targeting to the postsynaptic membrane. This is an important question since no other binding partners have been identified yet for the PH-BEACH domain within Nbea or in any other PH-BEACH containing molecule (Cullinane et al., 2013).

More structural insight came from a study showing the C-terminal half of Nbea binds to SAP102, a MAGUK molecule involved in trafficking of ionotropic receptors (Lauks et al., 2012). Although the PH-BEACH domain is contained in the C-terminal half along with the DUF and WD domains (compare **Figure 3A**), the isolated PH-BEACH domain failed to bind to SAP102 (Lauks et al., 2012). Moreover, a point mutation introduced into the PH-BEACH domain (E2218R) of their C-terminal Nbea construct, which possibly disrupted the tight structural unit between PH and BEACH (Jogl et al., 2002), also prevented SAP102 binding. These data suggest that the PH-BEACH domain itself does not contain a SAP102 binding site but that the integrity of PH-BEACH determines the conformation of the entire C-terminus. To facilitate future experimental work, we therefore propose a structural model of full-length Nbea (**Figure 3B**) that is based on a compact C-terminus in which the PH-BEACH domain interacts with the flanking DUF and WD domains. In support, there is another WD repeat upstream of the DUF that can be identified by sequence homology (residues 1326–1368, UniProt). This repeat might form a 5-bladed WD propeller fold together with the four WD repeats (WD40) at the C-terminus (green in **Figure 3B**) that should clamp the C-terminus even tighter into a compact domain arrangement.

In spite of the predicted compact structure of the C-terminus of Nbea, we observed that the PH-BEACH can be functional as an individual unit: it localized prominently to the spine head (**Figure 3**), rescued the surface expression of GluR2 AMPA receptors (**Figure 4**) and restored normal numbers of mature mushroom spines (**Figure 5**). Consistent with a prominent role at excitatory spine synapses, the E2218R mutation in PH-BEACH of full-length Nbea impaired excitatory but not inhibitory neurotransmission (Farzana et al., 2016). While this defect can be interpreted by the loss of SAP102 binding (Farzana et al., 2016), our results emphasize an alternative role of PH-BEACH at the postsynaptic density that is independent of SAP102. The latter is corroborated by the fact that glutamate receptor signaling was unchanged in SAP102 null mutant neurons (Farzana et al., 2016).

### Role of PKA Binding Site in Nbea

Our rescue experiments with the isolated PH-BEACH domain implicated that its role is independent of the PKA RII binding site of Nbea, a hallmark of the protein (Wang et al., 2000). Consistently, we found that deletion of the binding site (NbeaΔPKA) does not abolish the ability of full-length Nbea to rescue most properties of EPSCs (**Figures 1**, **2**) or mushroom

spine numbers (**Figure 5**). At the same time, we discovered a new role for the PKA RII binding site: we observed increased filopodia formation in its absence (shown by NbeaΔPKA and ARM-coreΔPKA constructs), whereas full-length Nbea and the armadillo domain with PKA RII binding site behaved normally (**Figure 5**). It can be concluded that binding of PKA to Nbea prevents or limits filopodia formation which by default might be promoted by the armadillo domain. In fact, PKA dependent processes have been shown to facilitate filopodia formation in other studies (Chen et al., 2003; Lin et al., 2007). Similarly, other armadillo domains, for example from δ-catenin, are also involved in filopodia formation (Abu-Elneel et al., 2008). Since many AKAPs are integrated in protein networks involving cdc42 (Poelmans et al., 2013), Nbea might regulate the protrusive activity for filopodia extension through the cdc42-cofilin-actin pathway.

Our electrophysiological analysis of the NbeaΔPKA mutant revealed another relevant aspect of the impaired regulation of filopodia because we observed that NbeaΔPKA restores mEPSCs rise time and inter-event interval but not decay time. Recent work correlated the imbalanced number of filopodia protrusions with EPSCs impairments: for example, knock-down of class II Myosin motor heavy chain *MyH7B* in rat hippocampal neurons causes irregularly shaped DS heads with filopodia protrusions and impaired mEPSCs (Rubio et al., 2011). Conversely, neurons depleted of the 4E-binding-protein-2 (4E-BP2), a repressor of mRNA translation, displayed lower number of filopodia protrusion and increased evoked and miniature EPSCs (Ran et al., 2013). The higher number of filopodia seen upon expression of NbeaΔPKA in Nbea KO neurons could therefore explain its inability to rescue decay time of mEPSCs due to the absence of a functional postsynaptic density in filopodia protrusions.

The question of additional targets of the PKA signaling of Nbea remains open. The PKA signaling pathway in DSs is known, for example, to lead to reduced phosphorylation of GluA1 as was shown for the melanocortin GPCR receptor. MC4R stimulates surface GluA1 trafficking through phosphorylation at Ser845 in a Gα-cAMP/PKA-dependent manner (Shen et al., 2013). Possibly, a similar mechanism may affect the serine phosphorylation of GluA2 subunits which were reduced in surface trafficking in Nbea KO neurons. While experimental proof for such a scenario is lacking, at least PKC is able to phosphorylate GluA2 at serine 880 and PKA phosphorylation of GluA1 induces exocytosis to extrasynaptic sites and primes AMPA receptors for CamKII dependent synaptic delivery (Henley et al., 2011). Although deletion of Nbea revealed a particularly strong reduction of GluA2 subunit at the surface (**Figure 4** and Nair et al., 2013), diminished surface levels of GluA1 and GluK2/3 kainate receptors have also been described (Nair et al., 2013). Thus, the PKA binding site of Nbea might be critical for the fine-tuning of the phosphorylation status of several AMPA receptor subunits, collectively leading to the impaired mEPSCs decay time and imbalance of different classes of DSs.

Finally, the PKA signaling of Nbea could also involve synaptopodin, a key molecule of the spine apparatus (Deller et al., 2003), which is ectopically retained in the *trans*-Golgi network together with actin clusters in Nbea KO neurons (Niesmann et al., 2011). Synaptopodin has recently been described as PKA substrate essential for the regulation of NMDA receptor-dependent synaptic plasticity and spine expansion upon chemical LTP induction (Faul et al., 2008; Zhang et al., 2013). Thus, Nbea could recruit a molecular complex regulating synaptopodin phosphorylation through its interaction with PKA RIIα subunit, and thereby inducing actin cytoskeleton remodeling. In presence of our NbeaΔPKA mutant that is unable to interact with PKA, synaptopodin regulation could be lost or impaired, leading to defective actin cytoskeleton remodeling and filopodia formation. On the other hand, if Nbea influences via its interaction with PKA the amount of active NMDA channels in the postsynapse, then the lack of rescue of the mEPSC decay time by NbeaΔPKA may reflect the inability of this truncated form to support the slower NMDA-component of the postsynaptic current. Although our results showed that the actin polymerization and turnover rate are normal in Nbea KO neurons, the impairment of the actin cytoskeleton in DSs could involve other aspects such as actin branching or capping, which future research will have to address.

## AUTHOR CONTRIBUTIONS

DR, JB, CL, LN, WG, HR, and CR generated the data. DR, JB, JR, CR, and MM analyzed the data. MK provided mutant mice. DR, JR, and MM designed the study. DR and MM wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Synaptic Neuroscience

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# Heterogeneous Signaling at GABA and Glycine Co-releasing Terminals

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The corelease of several neurotransmitters from a single synaptic vesicle has been observed at many central synapses. Nevertheless, the signaling synergy offered by cotransmission and the mechanisms that maintain the optimal release and detection of neurotransmitters at mixed synapses remain poorly understood, thus limiting our ability to interpret changes in synaptic signaling and identify molecules important for plasticity. In the brainstem and spinal cord, GABA and glycine cotransmission is facilitated by a shared vesicular transporter VIAAT (also named VGAT), and occurs at many immature inhibitory synapses. As sensory and motor networks mature, GABA/glycine cotransmission is generally replaced by either pure glycinergic or GABAergic transmission, and the functional role for the continued corelease of GABA and glycine is unclear. Whether or not, and how, the GABA/glycine content is balanced in VIAAT-expressing vesicles from the same terminal, and how loading variability effects the strength of inhibitory transmission is not known. Here, we use a combination of loose-patch (LP) and whole-cell (WC) electrophysiology in cultured spinal neurons of GlyT2:eGFP mice to sample miniature inhibitory post synaptic currents (mIPSCs) that originate from individual GABA/glycine co-releasing synapses and develop a modeling approach to illustrate the gradual change in mIPSC phenotypes as glycine replaces GABA in vesicles. As a consistent GABA/glycine balance is predicted if VIAAT has access to both amino-acids, we test whether vesicle exocytosis from a single terminal evokes a homogeneous population of mixed mIPSCs. We recorded mIPSCs from 18 individual synapses and detected glycine-only mIPSCs in 4/18 synapses sampled. The rest (14/18) were co-releasing synapses that had a significant proportion of mixed GABA/glycine mIPSCs with a characteristic biphasic decay. The majority (9/14) of co-releasing synapses did not have a homogenous phenotype, but instead signaled with a combination of mixed and pure mIPSCs, suggesting that there is variability in the loading and/or storage of GABA and glycine at the level of individual vesicles. Our modeling predicts that when glycine replaces GABA in synaptic vesicles, the redistribution between the peak amplitude and charge transfer of mIPSCs acts to maintain the strength of inhibition while increasing the temporal precision of signaling.

**Keywords: GABA, glycine, inhibitory neurotransmission, VIAAT, VGAT, GlyT2-eGFP mouse, cotransmission, quantal release**

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## INTRODUCTION

A well-established and long accepted postulate known as "Dale's principle" proposes that neurons have chemical unity for signaling (Strata and Harvey, 1999; Tritsch et al., 2016) —meaning that they communicate by releasing the same neurotransmitter at all their synapses. When the quantal nature of synaptic transmission was later discovered, application of Dale's principle implied that each presynaptic terminal shares the particular set of enzymes and transporters that are required for the synthesis, recapture and accumulation of a specific neurotransmitter(s) into the synaptic vesicles, which are recycled and refilled locally before being reused (Hnasko and Edwards, 2012). Now it is clear that many central synapses corelease multiple neurotransmitters, expanding the repertoire of chemical signaling (Vaaga et al., 2014; Granger et al., 2017) and the extent of presynaptic coordination require to uphold Dale's principal.

In the hindbrain, GABA and glycine are coreleased from single vesicles (Jonas et al., 1998; Lu et al., 2008). Although GABA and glycine have separate presynaptic supply mechanisms, postsynaptic receptors, and modulators, they share the same vesicular transporter, VIAAT (Wojcik et al., 2006; Aubrey et al., 2007) and when GABA/glycine are coreleased from the same single vesicle, mixed miniature inhibitory postsynaptic currents (mIPSCs) have a characteristic biphasic time course (Jonas et al., 1998; Keller et al., 2001; Russier et al., 2002). The regulation of GABA/glycine corelease is known to play a significant role during the maturation of spinal inhibitory circuits (Chéry and de Koninck, 1999; Keller et al., 2001; Russier et al., 2002; Coull et al., 2003; Rahman et al., 2013; Medelin et al., 2016), as well as in other hind-brain regions including the auditory nucleus (Kotak et al., 1998; Lu et al., 2008; Fischl and Burger, 2014; Nerlich et al., 2014) and cerebellum (Dugué et al., 2005; Rousseau et al., 2012). Early biochemical examinations of GABA and glycine uptake into synaptosomes (an isolated synaptic terminal preparation) indicated that both amino acids compete for the same vesicular transporter (Fykse and Fonnum, 1988; Burger et al., 1991; Christensen and Fonnum, 1991). This idea was endorsed when VIAAT was found in both GABAergic and glycinergic terminals (McIntire et al., 1997; Sagné et al., 1997) and taken together, these data suggested that the vesicular content is determined by the relative occupancy of GABA/glycine at VIAAT (Gasnier, 2000) and therefore, will be primarily determined by the relative presynaptic cytosolic concentrations of the two inhibitory neurotransmitters.

In active terminals, GABA is synthesized from glutamate by two isoforms of the glutamate decarboxylase enzyme (GAD65/67; Martin and Rimvall, 1993; Kakizaki et al., 2015), whereas the concentrative power of GlyT2, a 3 $Na^+$-coupled glycine transporter (Roux and Supplisson, 2000), is required to increase basal glycine concentration to the appropriate levels for VIAAT uptake (Gomeza et al., 2003; Rousseau et al., 2008; Apostolides and Trussell, 2013). Experimental evidence confirms that the cytosolic concentration of glycine and GABA does indeed influence synaptic vesicle content (Rousseau et al., 2008; Apostolides and Trussell, 2013; Ishibashi et al., 2013). Furthermore, two *in vitro* studies have succeeded in sampling the inhibitory transmitter content of VIAAT-expressing vesicles at single terminals and found evidence that the mIPSCs phenotypes were not homogenous. Instead, GABA-, glycine- and mixed mIPSCs were all detected at some individual synapses, suggesting that the signaling phenotype of a single terminal may considerably vary from vesicle to vesicle (Katsurabayashi et al., 2004; Aubrey et al., 2007; **Supplementary Figure S1**).

Here, we record mIPSCs in networks of culture spinal cord neurons and extract the subpopulation of mIPSCs that originate from an individual terminal by simultaneously recording mIPSCs with a loose-cell patch-clamp electrode placed over a single pre-synaptic varicosity. Then, we examine whether vesicles originating from the same co-releasing terminal evoke homogenous postsynaptic currents, as expected if all of a presynaptic terminal's vesicles contain a similar concentration of GABA and glycine. In addition, we develop a simulation model of cotransmission to examine how mIPSC peak amplitude and charge transfer change as function of glycine vs. GABA release. Using activation kinetic schemes of GlyR and $GABA_AR$, our simulations show that alterations in vesicular content would not compromised the strength, but rather would shape the time-course of postsynaptic inhibition.

## MATERIALS AND METHODS

### Embryonic Mouse Spinal Cord Neurons

Primary cultures of spinal cord neurons were prepared as described in Hanus et al. (2004) from embryonic day 13 or 14 (E13–14) C57BL/6J wild-type or heterozygous GlyT2-EGFP mouse pups (Zeilhofer et al., 2005). Embryos were obtained by cesarean section from pregnant mice anesthetized by intraperitoneal injection of ketamine-xylazine (100 and 10 mg/kg) and killed by cervical dislocation. Spinal cords were dissected under sterile conditions into PBS with 33 mM glucose at pH = 7.4 and then incubated in trypsin/EDTA solution (0.05% v/v, Sigma, St. Louis, MO, USA) for 10 min at 37°C. Cells were dissociated mechanically in a modified L15 Leibowitz's medium (Life Technologies, Cergy Pontoise, France) and plated at a density of $1.0 \times 10^5$ cells/$cm^2$ on sterilized glass coverslips coated with 60 μg/ml poly D-L ornithine and with medium containing 5% inactivated fetal calf serum (Sigma, St. Louis, MO, USA). To insure easy visualization of a few eGFP-GlyT2 positive axons and their boutons, each coverslip was composed of 5%–10% neurons from GlyT2-eGFP mice; the remaining 90%–95% neurons were from unlabeled WT littermates. Cells were maintained at 37°C in 5% $CO_2$ in serum-free Neurobasal™ medium containing supplement B27 (Invitrogen, Carlsbad, CA, USA; Brewer et al., 1993) for up to 3 weeks. Medium was changed every 4–5 days.

### Electrophysiology

Whole-cell (WC) patch clamp recordings of spinal cord neurons (14–22 DIV) were performed at 30°C. Voltage-clamp was imposed by a Multiclamp 700B amplifier controlled by pCLAMP 9 or 10 acquisition software (Molecular Devices). Currents were filtered at 4 kHz and sampled at 20 kHz using a Digidata

1440A (Molecular Devices). Neurons were continuously bathed with an external solution containing (mM): NaCl 140, KCl 2.4, $CaCl_2$ 2, $MgCl_2$ 2, Glucose 10, HEPES 10, pH = 7.4. WC patch clamped mIPSCs were recorded in the presence of 0.2 μM of the sodium channel blocker tetrodotoxin (TTX) and 2 μM of the benzodiazepine flunitrazepam. AMPA and NMDA receptors were blocked with 2 μM NBQX (6-nitro-7-sulfamoylbenzo[f]quinoxaline-2, 3-dione) and 5 μM MK-801, respectively. When indicated $GABA_A$ receptors ($GABA_A$Rs) were blocked with 5 μM gabazine (SR 95531), and glycine receptors (GlyRs) were blocked with 1 μM strychnine. The majority of whole cell mIPSCs have fast rise times, and a few cells were found to have a correlation between their glycinergic mIPCS rise and decay time constants, suggesting that dendritic filtering of mIPSCs from more distant synapses influences the kinetic of some, but not all, events included in the WC mIPSC data (**Supplementary Figure S2C**; Gardner et al., 1999). There was no evidence of dendritic filtering at any of the single synapse mIPSCs, as expected for events that originated from synaptic terminals that are on, or very close to, the neuronal soma (**Supplementary Figure S2C**).

Patch-pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Maisfeld, Germany) and pipettes for WC recording had typical resistances of 4–6 MΩ. The mIPSCs were recorded at a holding potential ($V_H$) of −70 mV (taking into account the junction potential) using pipettes filled with a standard internal solution containing (in mM): CsCl 140, $CaCl_2$ 1, EGTA 10, BAPTA 1, $MgCl_2$ 1, Mg-ATP 4, QX314-Cl 5, Hepes 10, adjusted to pH 7.4 with CsOH. Loose-patch (LP) recording pipettes had resistances of 1–2 MΩ and LP mIPSCs were recorded at 0 mV. One out of every 10 attempts at obtaining a LP recording were successful. LP pipettes were filled with the high calcium external solution (4 mM $Ca^{2+}$; 0 mM $Mg^{2+}$), 0.2 μM TTX, 2 μM flunitrazepam, 2 μM NBQX, 5 μM MK-801. Baclofen, a $GABA_B$R agonist, was added to the extracellular solution to reduce the frequency of mIPSC when required (O'Brien et al., 2004). Unless indicated, drugs were purchased from Sigma (St. Louis, MO, USA) or Tocris Bioscience (Bristol, UK).

## Data Analysis

Miniature synaptic currents were detected semi-automatically and analyzed using SpAcAn, a custom-made integral detection Igor package developed by Guillaume Dugué and Charly Rousseau and available at www.spacan.net. or in AxoGraph. LP and WC mIPSCs whose peak currents occurred simultaneously (<0.6 ms) and were verified to have a smooth rise and decay phase, as well as a strongly correlated peak amplitudes, were included in the analysis. Electrophysiological results are reported as mean ± SEM. All statistical tests were nonparametric and performed using Prism software unless indicated. The Mann–Whitney and Wilcoxon Matched-Pairs test was used to assess differences between two independent and two related samples respectively. For all tests, the number of asterisks (*) in the figures corresponds to level of significance: $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$.

Populations of glycine and GABA mIPSCs recorded in the presence of SR 95331 or strychinine, respectively, were classified as glycinergic, GABAergic or mixed on the basis of their peak amplitude to charge transfer relationship and then fitted with a linear regression and 95% prediction intervals. We also attempted to classify mIPSC events by fitting their currents with biexponential and monoexponential fits (Jonas et al., 1993; Rahman et al., 2013) but found that the peak amplitude to charge relationship robust and fast (**Supplementary Figure S2**). We set a conservative limit of ≥30% to considering groups of single synapse mIPSCs as a separate population. This value was based on our pharmacological characterization of glycine and GABA mIPSCs.

## Numerical Simulation

The set of linear differential equations that defined the GlyR and $GABA_A$R kinetic models was numerically solved in Mathematica 11 using the Q-matrix approach (Colqhoun and Hawkes, 1995). The current amplitude corresponds to the summation of 120 GlyRs and 60 $GABA_A$Rs, with elementary currents of 1.05 and 3 pA for the $GABA_A$R mono- and di-liganded receptors, respectively, and 5 pA for GlyR. The rate constants are indicated in **Figure 3A**, that correspond to the values determined by Burzomato et al. (2004) for GlyR and Labrakakis et al. (2014) for $GABA_A$R except for the dissociation rate constants that were slightly increased to match the experimental time course of the mIPSC and peak-charge amplitude relationship.

We used the simplified assumption of a monoexponential time course for the transient of GABA and glycine (AAN: amino-acid neurotransmitter) with a piecewise function:

$$[\mathrm{AAN}]_t = \begin{cases} 0 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{if } t<t_0 \\ [\mathrm{AAN}]_{peak} e^{-(t-t_0)/\tau} \ldots \text{if } t \geq t_0 \end{cases}$$

The time constant of the transient in **Figure 3** was τ = 0.7 ms and the $[AAN]_{peak} = [Gly]_{peak} + [GABA]_{peak}$ = 3 mM (Beato, 2008). The time offset ($t_0$) was 2 ms and the time of integration was 200 ms.

The simulated mixed mIPSC corresponds to the algebraic sum of the glycine and GABA mIPSCs. We used the FindMinimum and NIntegrate functions of Mathematica 11 (Wolfram Research) to determine the peak amplitude and charge transfer of simulated mIPSCs.

The parameter α set the relative glycine concentration in the transient:

$$\alpha = \frac{[Gly]_{tr}}{[Gly]_{tr} + [GABA]_{tr}}$$

In **Figure 4** and **Supplementary Figure S3**, we examined the distribution of mIPSC phenotypes that were simulated with a broad variation in $[Gly]_{peak}$ and $[GABA]_{peak}$ and/or $GABA_A$ and GlyR. Normal distributions of $[Gly]_{peak}$, $[GABA]_{peak}$ and/or $GABA_A$ and GlyR were generated using the Random Variate and NormalDistribution functions of Mathematica with $[Gly]_{peak} = \mathcal{N}[3\alpha, (3\alpha\ CV)^2]$ and $[GABA]_{peak} = \mathcal{N}[3(1-\alpha), (3(1-\alpha)CV)^2]$. Accordingly, the average $[AAN]_{peak}$ was constant (3 mM) in all simulations.

## Ethics Statements

This study was carried out in accordance with the recommendations of guidelines of the Centre National de la Recherche Scientifique. The protocol was approved by the CNRS under number 02235.02 of the general agreement C750520.

# RESULTS

## Identification of mIPSCs From Individual Terminals Using a Combination of Loose-Patch and Whole-Cell Electrophysiology in Cultured Spinal Neurons From GlyT2-eGFP Mice

We combined the spinal cords of wild-type and GlyT2-eGFP (5–10%) embryos (Zeilhofer et al., 2005) and made low-density cultures of eGFP-expressing neurons that were used for electrophysiological experiments at DIV14–21. When viewed under fluorescent light, we observed thin, parallel neurites with beaded varicosities (1.05 ± 0.2 μm, $n$ = 30), suggestive of axons (**Figure 1A**). The GlyT2-eGFP$^+$ beaded varicosities rapidly accumulated the styryl dye FM 4–64, a marker of cycling presynaptic vesicles (**Figure 1B**), confirming that they correspond to presynaptic terminals.

Next, we voltage-clamped ($V_H$ = −70 mV) the cell body of an eGFP$^-$ neuron that appeared to have direct contact with one or a few eGFP$^+$ varicosities at or near to its soma, and recorded WC mIPSCs in the presence of TTX (**Figure 1C**). WC mIPSCs are heterogeneous because they correspond to the postsynaptic neurons response to the spontaneous release of single synaptic vesicle at one of its many inhibitory terminals (Hubbard et al., 1967). To identify the specific subset of mIPSCs that originated from a single GlyT2-eGFP$^+$ varicosity, we simultaneously recorded extracellular mIPSCs from a second LP recording electrode ($V_H$ = 0 mV) placed directly over a single synapse, identified by its eGFP$^+$ varicosity, located at or near to the soma of the recorded neurons (**Figure 1C**; Forti et al., 1997). Single-synapse mIPSCs were identified from whole cell mIPSCs (inward) that were time- and amplitude-locked to extracellular LP mIPSCs (outward, **Figures 1C,D**). LP-mIPSCs were never

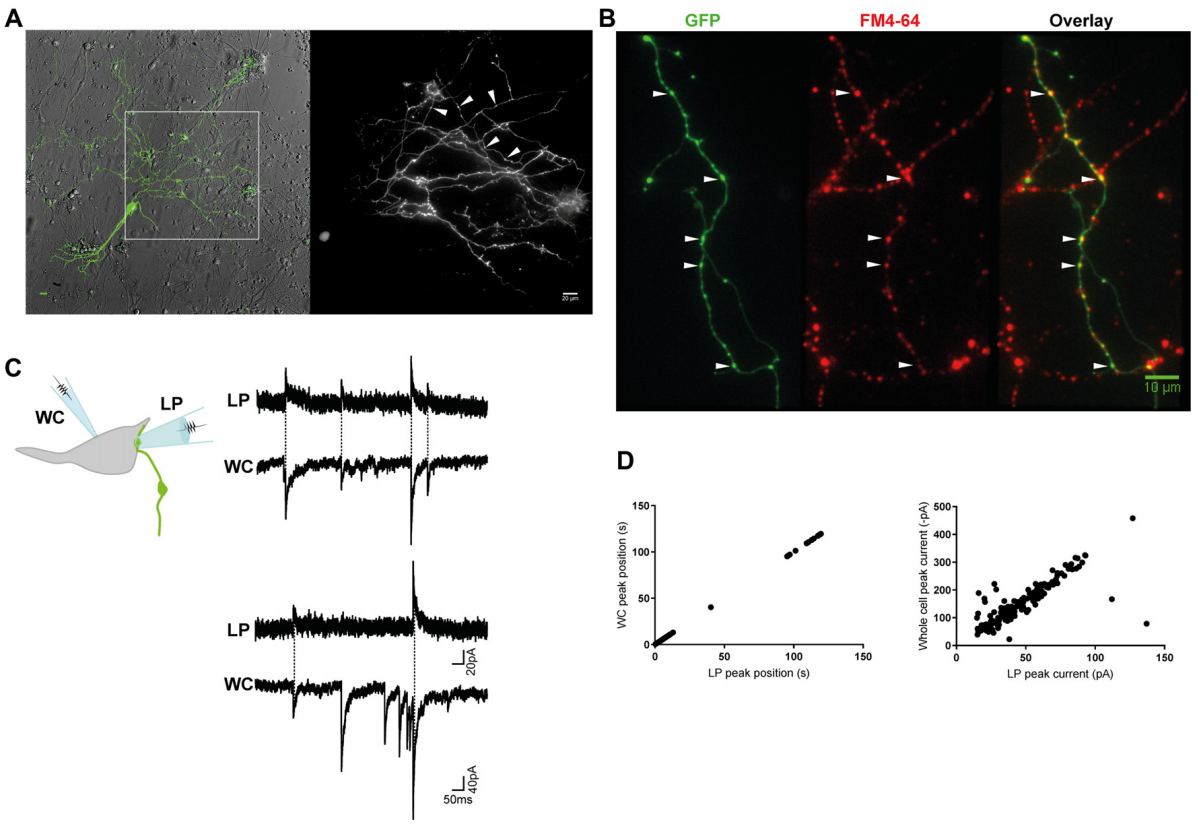


**FIGURE 1 |** Miniature inhibitory postsynaptic currents (mIPSCs) were recorded from individual inhibitory terminals. **(A)** GlyT2-eGFP$^+$ neurons and neurites were visible in our spinal cord cultures. eGFP$^+$ neurites with a parallel structure and beaded varicosities (*) were labeled with FM 4–64 dye **(B)**, confirming they are glycine-containing presynaptic terminals. Scale bars are 10 μm. **(C)** Cartoon of the paired recording configuration that allowed identification of mIPSCs that originated from an individual inhibitory terminal (top). Postsynaptic mIPSCs were simultaneously recorded with the whole-cell patch clamp pipette (WC) and the loose-patch clamped pipette (LP). The traces (middle and bottom) are a continuous 2 s recording from one WC-LP pair. **(D)** The LP and whole cell mIPSCs from the same recording as displayed in **(C)**, were strongly correlated in time and amplitude, as expected from an extracellular and intracellular measurement of the same signal.

detected if the LP-pipette was not centered precisely on the GlyT2-eGFP$^{+}$ varicosity, consistent with the prediction that the LP-pipette will detect less than 1% of currents that originate outside the LP pipette (Forti et al., 1997) and suggestive that detection of current from two independent synases is unlikely.

## GABA- and Glycine-Only mIPSCs Events Were Defined Based on Their Peak Amplitude to Charge Relationship

Although GABA and glycine activate ligand-gated chloride channels with distinct biophysical and pharmacological properties, GABAergic and glycinergic mIPSCs are difficult to separate because of their overlapping receptor kinetics (Burzomato et al., 2004; Labrakakis et al., 2014). We used flunitrazepam, a positive allosteric modulator of $GABA_A$ receptors, to selectively slow the decay of GABAergic mIPSCs and allow a crude separation of GABA vs. glycine mIPSCs based on their peak amplitude and charge transfer (**Figures 2A,B**). Nevertheless, the two mIPSC populations were highly variable both within and between neurons (**Figure 2C**; $n$ = 15 cells, 600 mIPSC events each). The variability is attributable to the presence of multiple independent inhibitory connections with potential differences in: receptor numbers, receptor subunit composition, synaptic architecture, as well as synaptic vesicle content (Ropert et al., 1990; Frerking et al., 1995; Auger and Marty, 1997; Nusser et al., 1997) and dendritic filtering (Gardner et al., 1999). This variability was not reduced by analyzing data from neurons prepared from the same animals, or on the same number of days *in vitro* (data not shown). Linear regressions of the peak amplitude vs. charge data and 95% prediction intervals frame the GABA and glycine mIPSC populations and were used to classify pure and mixed phenotypes (**Figure 2C**). When we recorded whole cell mIPSCs in the absence of strychnine and SR 95531, and selected mIPSC events with clear biphasic decay currents characteristic of mixed GABA/glycine cotransmission (Jonas et al., 1998; Lu et al., 2008), these events mapped in-between the GABA and glycine mIPSC prediction intervals on graphs of peak amplitude vs. charge (**Figure 2D**) or within the GABA mIPSC prediction intervals (**Figure 2E**). Thus, the linear regression and 95% prediction intervals of the glycine peak to charge

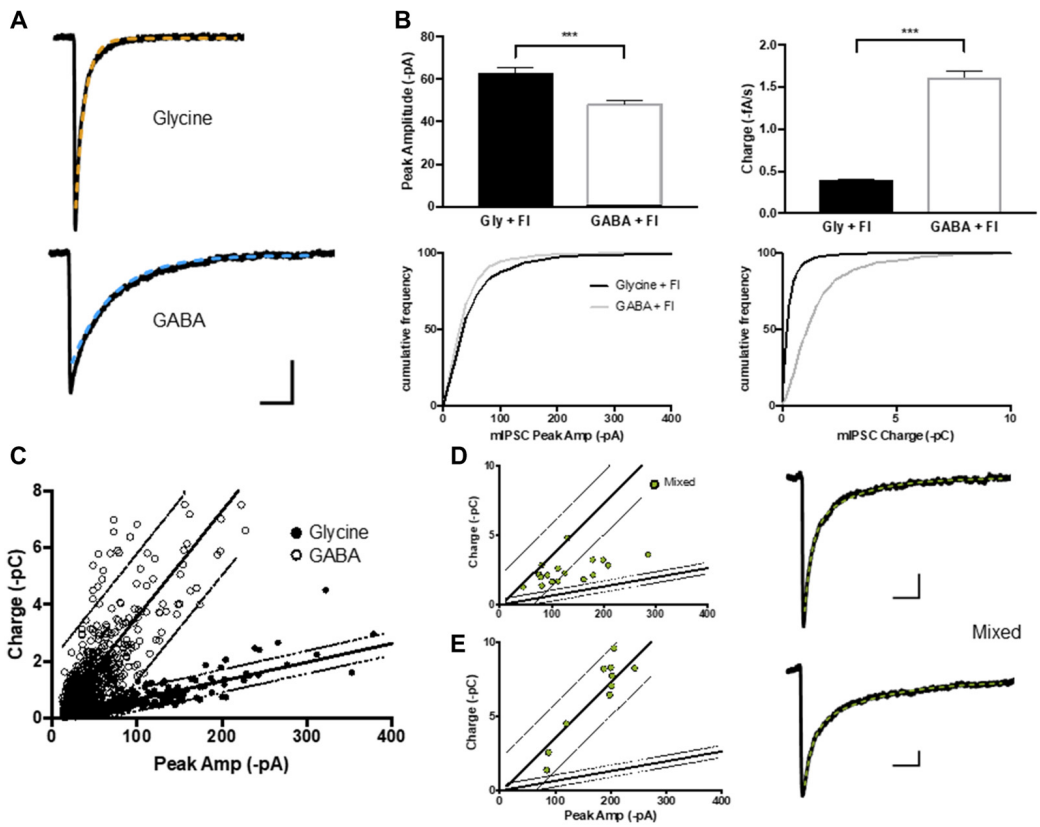


**FIGURE 2 |** GABA, glycine and mIPSCs were defined on the basis of their peak current and charge transfer. **(A)** Average current traces of glycinergic and GABAergic mIPCs were recorded in whole-cell configuration in the presence strychnine or SR95331 and decays fitted with a single exponential (orange and blue) respectively. **(B,C)** The peak current (I) to charge ratio of GABA and glycine mIPSC ($n$ = 600 events from 15 neurons each) were used to define 95% prediction intervals (gray lines) for these two mIPSC phenotypes. **(D,E)** Two example populations of mixed mIPSCs recorded in the absence of strychnine or SR 95331 and selected on the basis of their biphasic decay current (double exponential fit (green)). Mixed mIPSCs mapped between the two 95% prediction intervals or were misidentified as GABAergic, depending on the size of their GABA component.

data provides functional limits to define glycinergic and mixed mIPSCs. In contrast, the GABAergic mIPSC data was not able to differentiating between mixed and GABAergic mIPSCs, especially when mixed mIPSC currents had a large GABA component (**Figure 2E**).

To explore all possible phenotype outcomes in response to variable GABA and glycine vesicular release, we simulate glycinergic, GABAergic and mixed mIPSCs with activation kinetic models for $GABA_AR$ and GlyR that have been previously established (Burzomato et al., 2004; Labrakakis et al., 2014; **Figures 3A,B**). We limited our simulations to the simplest, linear substitution of GABA by glycine, keeping a constant [neurotransmitter release] ($AAN_{peak}$ = 3 mM, $\tau_r$= 0.7 ms (Beato, 2008), **Figure 3C**). Therefore, increasing α potentiates the glycine component and decreases the GABA component of mixed mIPSCs (**Figures 3D–F**) in such a way that the increase in peak amplitude may compensate for the reduction in charge transfer, thus preserving the strength of synaptic inhibition (**Figure 3G**).

To map a more realistic distribution of inhibitory phenotypes, we plot the peak-charge amplitude distribution of simulated mIPSCs for a randomly generated number of postsynaptic receptors and peak concentration transients (see "Materials and Methods" section). First, we plot the smooth density histograms for 200 simulated mIPSCs when there is a unique, pre- or post-synaptic source of variability with a coefficient of variation of 0.2 ($CV_R$ = 0.2 or $CV_{AAN}$ = 0.2, **Supplementary Figure S3**). **Supplementary Figure S3** illustrates that a lower variability of the GABA mIPSCs is expected when α values are low, because $GABA_AR$ receptors are almost saturated by 3 mM GABA transient ($EC_{50}$ = 0.81 mM for $GABA_AR$ and 2.68 mM for GlyR, with comparable apparent hill-coefficients of 2.17 and 2.06, respectively). Then we map the distribution of 500 randomly simulated mIPSCs with average α values ranging from 0 (pure GABA) to 1 (pure Glycine) by 0.05 step increments (**Figure 4A**), with high pre- ($CV_R$ = 0.4, **Figure 4B**) and post- ($CV_{AAN}$ = 0.4, **Figure 4C**) synaptic variability. By definition, the simulated mIPSCs are mixed and have

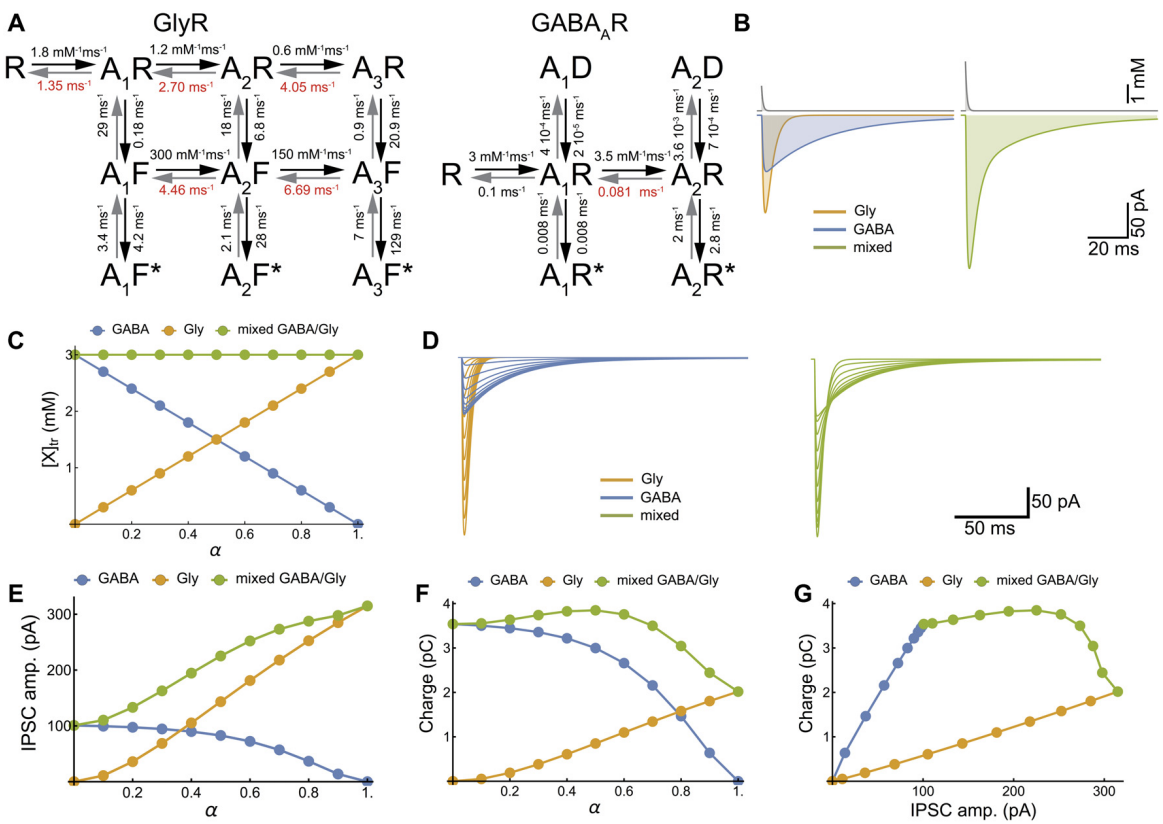


**FIGURE 3 |** Model of cotransmission shows that swapping glycine for GABA in the neurotransmitter transient maintains the strength of synaptic inhibition. **(A)** Kinetic schemes for the activation of GlyR (Burzomato et al., 2004) and $GABA_AR$ (Jones and Westbrook, 1995; Labrakakis et al., 2014) that were used for the numerical simulations. We adopted the set of rate constants determined by Burzomato et al. (2004) and Labrakakis et al. (2014), with the exception of the dissociation rate constants, indicated in red, that were adjusted for matching the time course of our recorded mIPSCs ($\tau$ = 6.4 ms and 34.3 ms for glycine and GABA IPSC respectively). **(B)** Numerical simulations of the mIPSCs evoked by concentration transient (1.5 mM) of glycine or GABA applied alone (left) or together (right). The concentration scale is the total concentration of neurotransmitter. **(C)** Inverse relationships of GABA and glycine concentrations for increasing values of α, which is the fraction of glycine released during each transient, assuming constant vesicular release (3 mM). **(D–G)** Numerical simulations of glycinergic, GABAergic and mixed mIPSCs when α increased from 0 to 1 by increments of 0.1, showing the current traces **(D)**, the IPSC peak amplitudes **(E)**, the IPSC charge transfer **(F)** and the IPSC charge-amplitude relationships **(G)**.

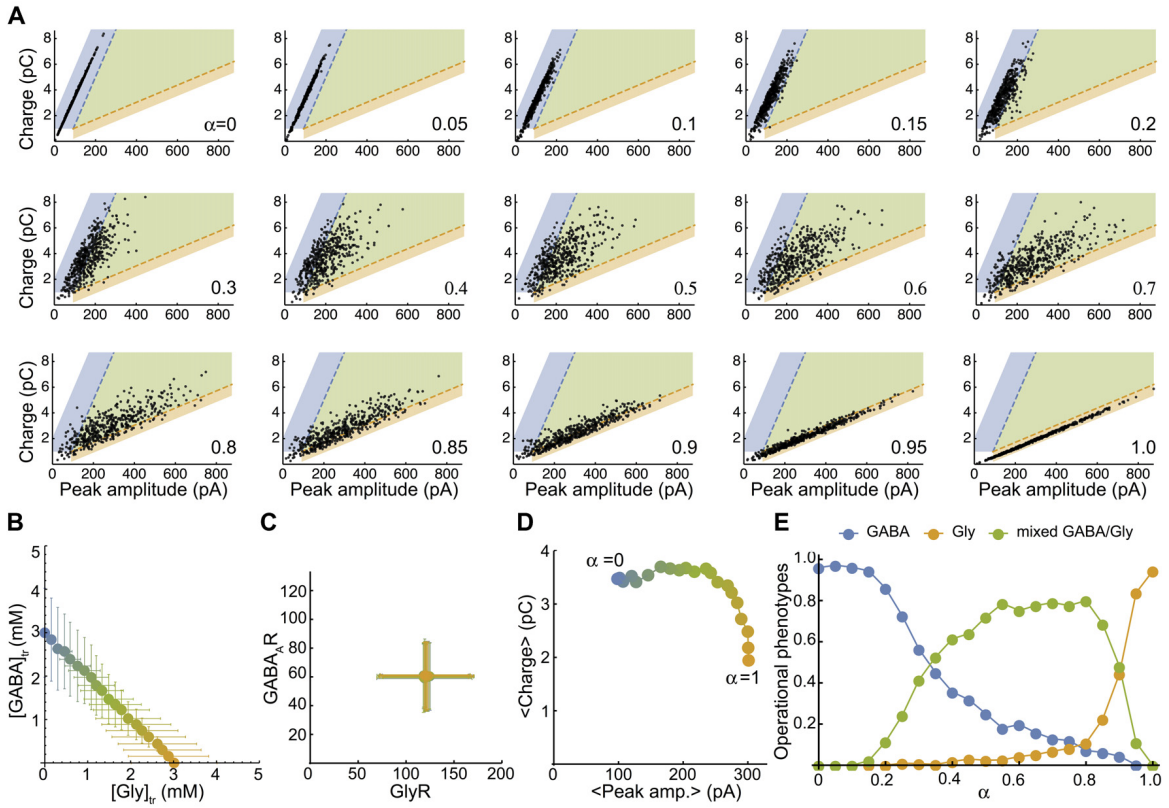


**FIGURE 4 |** The simulated mixed mIPSC phenotypes shifts as function of the proportion of glycine and GABA coreleased. **(A)** Simulated-mIPSC charge-amplitude distribution for increasing α values. Each panel shows the charge-amplitude distribution of 500 randomly simulated mIPSCs for the α value indicated. We defined three areas as GABAergic (blue), glycinergic (orange) and mixed (green) to operationally sort individual mIPSC phenotypes with similar threshold criteria that were used experimentally. **(B)** Parametric plot of the average GABA-glycine peak concentration in the transient as function of α. Each value was randomly generated from normal distributions as function of α with $CV_{AAN}$ = 0.4 ([gly]: 3 α ± 1.2 α mM and [GABA] = 3 (1−α) ± 1.2 (1-α) mM). **(C)** Plot of the average number of $GABA_AR$-GlyR randomly generated from normal distributions with $CV_R$ = 0.4 (120 ± 48 GlyRs and 60 ± 24 $GABA_ARs$). **(D)** Plot of the average charge-peak amplitude. **(E)** Proportions of GABAergic, glycinergic and mixed phenotypes of simulated mIPSCs as function of α. Each point in **(B–D)** represents the average value of the distribution for an α value and the color blend is a linear function of α between pure GABA (3 mM, blue, a = 0) and pure glycine (3 mM, orange, α = 1). **Figure 4A** has been reproduced from Aubrey et al. (2007).

two components when 0 < α < 1 and would therefore be arbitrarily qualified as mixed, even if one component is small and the mIPSC resembles pure GABAergic or pure glycinergic events. Therefore, we delimit three areas in the charge-peak amplitude plot (**Figure 4A**) for the attribution of GABAergic (blue area), glycinergic (orange area) and mixed phenotypes (green area), based on the 95% prediction from the linear regression of the experimental values for charge-peak amplitude. The average peak-charge amplitudes for each α values follow the model predictions (**Figure 4D**) and the repartition of mIPSC phenotypes as function of α (**Figure 4E**) shows that 10% GABA release (α = 0.9) reduces the glycinergic component of the mixed mIPSCs by 50%, whereas in contrast a similar reduction in GABAergic phenotypes is not achieved until α = 0.3. This finding is consistent with our experimental data (**Figures 2D,E**) and indicates that mixed mIPSCs with small glycine components are likely to be characterized as a GABAergic phenotype. Overall, the model confirms that neurotransmitter release by vesicles containing higher proportions of glycine than GABA will evoke mixed mIPSCs that have intermediate peak amplitude-charge relationships, falling in-between the GABA and glycine prediction intervals defined by our experimental data. When α = 0.45–0.85 the model predicts that mixed phenotypes will be the dominant phenotype, with <20% of mixed events being mischaracterized.

## A Combination of Mixed and Glycinergic mIPSCs Were Detected at Some Individual Terminals

Individual-terminal mIPSCs were recorded in the absence of strychnine and SR 95331, and analyzed when the peak amplitude and charge values were measurable in ≥25 LP-linked mIPSCs. We classified the mIPSC phenotype from 18 individual terminals. In 4 of the 18 synapses only glycine mIPSC were detected (**Figure 5A**), with a reduced co-efficients of variation (CV) compared to glycine mIPSCs from whole cell recording (0.42 ± 0.5, range 0.30–0.51; and 0.65 ± 0.05, range

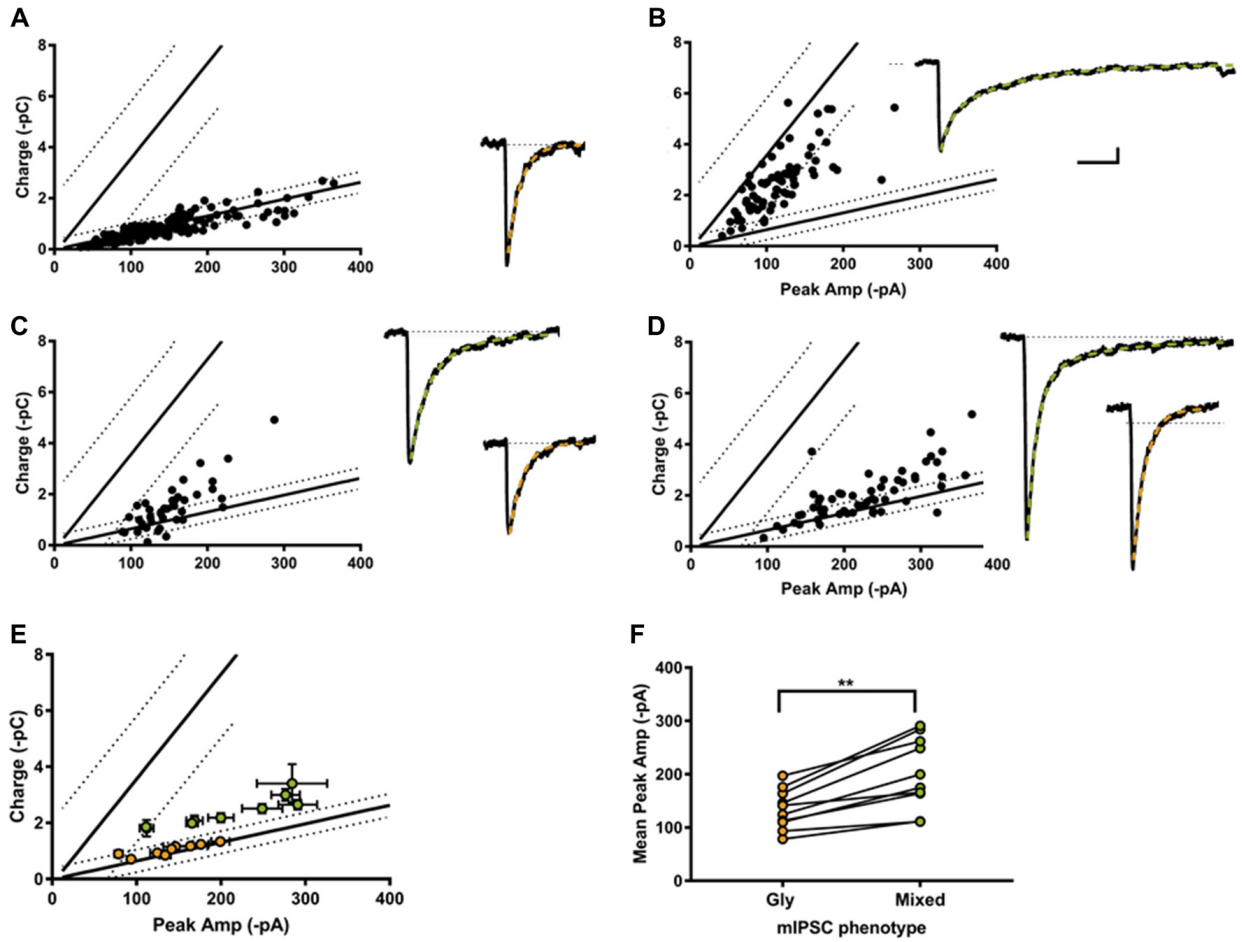


**FIGURE 5 |** Most individual inhibitory synapses had a combination phenotype comprised of both glycine and mixed mIPSCs. Examples of single synapse peak amplitude-charge relationships (left) and average mIPSCs (10 events each, right) with their decay current fitted with a single (orange) or double exponential (green). 4/18 of the recorded synapses were purely glycinergic **(A)**, and 5/18 were purely mixed **(B)**. The other 9/18 synapses displayed a combination of mixed and glycine mIPSCs **(C,D)**. **(E)** Average peak current-charge relationship of the glycine (orange) and mixed (green) mIPSCs identified in the nine combination synapses. **(F)** Glycine mIPSC peak amplitudes were always smaller than the mixed mIPSC peak amplitude, as expected from a compound GABA+glycine mIPSC event.

0.25–0.86, respectively). The rest (14/18) were cotransmission terminals, with a substantial proportion of mixed mIPSCs. According to our classification system, we found that 36% (5/14) of the cotransmission terminals we sampled had a homogenous mixed mIPSC phenotype as hypothesized (**Figure 5B**). The majority (64%, 9/14) had a heterogeneous phenotype made up of a combination of mixed and glycine-only mIPSCs (**Figures 5C,D**). In these combination synapses, the mixed eIPSCs were always larger than glycine-only mIPSCs, as predicted for an additive current, and the mixed current was carried primarily by glycine (**Figures 5E,F**). Examination of the raw mIPSCs currents grouped into their designated phenotypes (averaged from 10 consecutive events), demonstrates that phenotypically glycinergic mIPSCs have characteristic fast monophasic decay kinetic (**Figures 5A,C,D**). In contrast, mIPSCs events that fell into the mixed or GABA phenotype regions all had a discernable biphasic kinetics (**Figures 5B–D**) indicating this population corresponded to mixed events. Together, these data supports the identification of distinct mixed and pure mIPSC phenotypes in combination synapses.

## DISCUSSION

### Heterogeneity of GABA/Glycine Vesicular Content as a Presynaptic Source of mIPSC Variability

Because identifying the glycine and GABA components in individual mixed IPSCs is not straightforward, we previously used a giant-synapse, made from a secretory BON cell and a sniffer HEK cell, to sample all vesicular phenotypes when a VIAAT-expressing BON cell had access to both neurotransmitters (Aubrey et al., 2007). In this cellular model, sniffer HEK cells expressed GABA and glycine receptors that had cationic and anionic permeability respectively, thus

GABA- and glycine-evoked currents had opposite polarities and could be identified unambiguously (Aubrey et al., 2007, **Supplementary Figures S1A–C**). Surprisingly, the distribution of pure and mixed events from individual co-releasing BON cells suggested heterologous vesicular GABA/glycine content (Aubrey et al., 2007, **Supplementary Figure S1C**), as reported in acutely dissociated spinal cord neurons (Katsurabayashi et al., 2004). We can excluded a significant contribution of the sniffer cell in the BON/HEK model because scanning across the entire sniffer HEK cell surface with brief iontophoretic applications of a fixed [GABA] and [glycine] evoked stable biphasic currents (**Supplementary Figures S1D,E**), again supporting heterogeneous vesicular content that favors glycine- or GABA-like mIPSCs.

In the present work, we combined LP extracellular recordings with WC recordings to sample mIPSCs from single inhibitory varicosity in spinal cord cultures from GlyT2:EGFP mice. We have shown previously that evoked IPSCs from GlyT2-expressing neurons are predominately mixed, with a larger glycinergic component (Rousseau et al., 2008). As expected, we detected mixed mIPSCs in the majority (78%) of GlyT2-GFP$^+$ synapses sampled and found that most mixed GABA/glycine synapses signaled with a combination of pure and mixed mIPSCs. This phenotypic heterogeneity may have a presynaptic origin that results from variability in the accumulation and packaging of GABA and glycine in VIAAT-containing synaptic vesicles, similar to the BON/HEK model system (Aubrey et al., 2007; **Supplementary Figure S1**), however we cannot rule out a postsynaptic origin because inhibitory synapses are dynamic structures (Choquet and Triller, 2013), with glycine and $GABA_A$ receptors being constantly trapped or exchanged between gephyrin containing nano-domains (Calamai et al., 2009; Maric et al., 2014; Tyagarajan and Fritschy, 2014; Alvarez, 2017; Pennacchietti et al., 2017). In addition, varicosities are larger structures than the small en passant boutons of typical central synapses that may contain multiple active zones facing postsynaptic clusters with different receptor compositions (Nusser et al., 1997; Biró et al., 2006; Lévi et al., 2008). When we generated simulated mIPSCs including a pre- and a post-synaptic source of variability (**Supplementary Figure S3**), we found that both were able to recapitulate the mostly glycinergic mIPSC distributions observed experimentally. We did not attempt to differentiate the source of variability any further in the present work.

## mIPSC Detection Bias

The distribution of mIPSC phenotypes, which is based on their peak to charge relationship, indicates that single varicosities predominantly signal with glycine in these cultures ($\alpha \geq 0.7$). This dominant glycinergic phenotype was anticipated because: 1. all the synapses sampled had GlyT2-eGFP$^+$ presynaptic varicosities. 2. Glycine mIPSCs have faster kinetics and larger peak amplitude than GABA mIPSCs. As a result, 3. time-integration of mIPSCs was more consistently resolved for glycine events than for GABA events because often GABA mIPSCs that did not return to baseline before a subsequent mIPSC occurred and thus could not be reliably integrated. 4. Mixed mIPSCs with a high or low $\alpha$-values are likely to be mis-classified, although mixed mIPSCs with small GABA components ($\alpha > 0.9$) were more easily detected than those with small glycine components ($\alpha < 0.3$). 5. Finally, mIPSCs with larger peak amplitudes, typically carried by glycine currents in these cultures, were more likely to be detected by the low resistance, LP recordings.

## A Model to Study Cotransmission

We modeled mixed mIPSCs as the summation of the $GABA_A$Rs and GlyRs mediated currents activated by a fast synaptic transient of GABA and glycine. Each randomly generated transient aims to represent the possible content of one VIAAT expressing vesicle. As the time course of glycine and GABA in the synaptic cleft have been thoroughly modeled (Overstreet and Westbrook, 2003; Beato, 2008), we used a fixed average peak concentration of 3 mM and a synaptic transient time-constant of 0.7 ms for the simulation. We fixed the peak-concentration transient because the amount of neurotransmitter stored in a vesicle may be limited by VIAAT thermodynamics, VIAAT kinetics or set by other vesicular factors (Edwards, 2007).

We used kinetic models and rate constants well-established for both receptors (Burzomato et al., 2004; Labrakakis et al., 2014), and assumed for simplicity that glycine and GABA act independently, although it has been shown that GABA/glycine corelease accelerates the kinetics of glycinergic IPSCs in pure glycinergic synapses (Lu et al., 2008). The distribution of randomly generated simulated mIPSCs on the charge-peak amplitude plot provided a graphical overview of all transitions between pure GABAergic and pure glycinergic phenotypes when the proportion of glycine in the neurotransmitter transient is sequentially increased. We operationally distinguished pure and mixed phenotypes for individual mIPSCs based on their location on three experimentally determined areas in the charge-peak amplitude plot. The distribution of "pure" GABAergic, mixed and "pure" glycinergic phenotypes shows that "pure" glycinergic phenotype is only compatible with pure glycine release since $\alpha = 0.95$ (about 150 $\mu$M GABA and 2.85 mM Glycine) is enough to evoked mixed phenotypes. In contrast "pure" GABAergic phenotypes are reported up to $\alpha = 0.2$ (about 2.4 mM GABA and 0.6 mM glycine), indicating that minor glycine corelease may more easily go unnoticed than minor GABA corelease. This asymmetry reflects the apparent saturation of $GABA_A$Rs with a 3 mM GABA transient.

## Functional Roles for GABA/Glycine Corelease

GABA/glycine co-signaling is habitually thought to be a property of immature inhibitory synapses in the spinal cord, brainstem and granular layer of the cerebellum that diminishes during postnatal development, with few exceptions (Inquimbert et al., 2007; Bhumbra et al., 2012). Indeed mixed GABA/glycine cotransmission was initially detected in juvenile rat spinal and brainstem motoneurons (Jonas et al., 1998; O'Brien and Berger,

1999; Russier et al., 2002) and lamina I and II of the spinal cord dorsal horn (Keller et al., 2001). Furthermore, a critical shift toward pure glycinergic transmission takes place during the postnatal maturation in spinal cord locomotor centers (Gao et al., 2001) and brainstem auditory nuclei where the large amplitude and rapid decay of glycinergic IPSCs provide the microsecond precision needed for the processing and the coding of acoustic information (Brand et al., 2002; Nabekura et al., 2004).

However, corelease with or without cotransmission persists in adults at some synapses, suggesting a continuing specific need for each neurotransmitter. In the superficial lamina of the spinal cord, GABA and glycine cotransmission is replaced by pure glycinergic transmission after P23 (Keller et al., 2001). Vesicular corelease persists in into adulthood however, and coreleased GABA influences the release probability by acting at presynaptic $GABA_BR$ (Chéry and de Koninck, 2000). Furthermore, extrasynaptic $GABA_A$ receptor signals can be detected at higher stimulation intensities (Chéry and de Koninck, 1999). At physiological temperatures evidence suggests that GABA and glycine corelease may not contribute to spillover and tonic inhibition in the dorsal horn of the spinal cord, as the diffusion of GABA and glycine out of the synaptic cleft is readily controlled by transporters in this region (Mitchell et al., 2007). Nevertheless, spillover of synaptically released glycine has been shown to potentiate the NMDA responses in the superficial dorsal horn of the spinal cord, by increasing the occupancy of the NMDA receptor coagonist site (Ahmadi et al., 2003). The same developmental pattern of inhibitory transmitter use has been reported in the rat MNTB, where GABA/glycine cotransmission is detected until P9–P12 after which time pure glycinergic transmission dominates (Awatramani et al., 2005). Again vesicular corelease persists in older animals, and coreleased GABA has been shown to shorten glycinergic mIPSC decay times here (Lu et al., 2008). In the central nucleus of the inferior colliculus of the auditory midbrain, GABA and glycine cotransmission persists in brain slices from P19 to P35 mice. Coreleased GABA and glycine evoke IPSCs with nearly identical amplitude and time course, suggesting that GABA and glycine are operationally fully interchangeable in this nucleus, thus securing inhibitory signaling by redundancy (Moore and Trussell, 2017). Finally, in the cerebellum of juvenile rats, inhibitory corelease occurs between Golgi cells and vestibulocerebellar Unipolar Brush Cells (Dugué et al., 2005; Rousseau et al., 2012), and once again evidence suggests that the transmission phenotype is under postsynaptic control. Interestingly, the phenotype of transmission in this region is not fixed developmentally, but seems to be coordinated with the phenotype of the glutamatergic input onto the Unipolar Brush Cell (Rousseau et al., 2012).

Our data suggests that a dynamic vesicular GABA/glycine balance at individual synapses may contribute to the tuning of phasic synaptic inhibition in spinal neurons. If the origin of the variability is indeed the neurotransmitter content in presynaptic vesicles, then glycine IPSC kinetics (Lu et al., 2008) and the probability of transmitter corelease (where $GABA_B$ receptors are expressed (Chéry and de Koninck, 1999, 2000) would also contribute to this fine tuning.

## Possible Mechanisms for Vesicular GABA/Glycine Variations at Single Terminals

The mechanisms that specify or regulate the vesicular GABA/glycine content at mixed synapses are not well understood. As previously discussed, changes to GABA (Mathews and Diamond, 2003; Wang et al., 2013) and glycine supply/resupply (Rousseau et al., 2008; Apostolides and Trussell, 2013; Ishibashi et al., 2013) can strongly shift the GABA/glycine balance and alterations in IPSC characteristics can be observed within minutes. Indeed, metabolic alteration such as these have been implicated in disease states including chronic pain (Coull et al., 2003; Imlach et al., 2016; Takazawa et al., 2017) and amyotrophic lateral sclerosis (Medelin et al., 2016).

With an apparent lower affinity for glycine than for GABA (about 25 and 6 mM, respectively), VIAAT uptake is likely to be rate limiting and a major source of variability for vesicular loading (Burger et al., 1991; Gasnier, 2000; Edwards, 2007; Farsi et al., 2016; Takamori, 2016). In addition, VIAAT has presumably the lowest driving force for uphill transport among the vesicular transporters (Edwards, 2007), being coupled to the exchange of a single $H^+$ (Hell et al., 1991; McIntire et al., 1997; Farsi et al., 2016) and with smaller protonmotive force (Egashira et al., 2016). If VIAAT kinetic is limiting for filling vesicles, as suggested by a slow recovery from synaptic depression (Katsurabayashi et al., 2004; Rousseau et al., 2008; Wang et al., 2013; Yamashita et al., 2018), then the initial vesicular content may continue to change as VIAAT exchanges a fast filling neurotransmitter (presumably GABA) with cytosolic glycine, providing opportunity for alterations in the transient neurotransmitter composition.

Other potential mechanisms include protein-protein interactions between the transmitter supply machinery and different populations of vesicles (Jin et al., 2003), differential modulation of the pool of cycling vesicle in response to presynaptic GABA or glycine supply (Mathews and Diamond, 2003; Wang et al., 2013; Truckenbrodt et al., 2018) or differential modes of GABA and glycine uptake by VIAAT (Aubrey et al., 2007). Given the diverse and highly regulated roles of the presynaptic terminal, this list is in not exhaustive.

In summary, this work adds to a growing body of evidence that suggest that the presynaptic terminal and neurotransmitter corelease can significantly regulate synaptic transmission. We present evidence that the GABA/glycine balance at individual co-releasing terminals is variable, and predict these variations would serve to fine tune the timing of inhibition and the integration of sensory inputs. Given the complexity and high level of control the presynapse has over synaptic vesicle release (Rizzoli and Betz, 2004; Edwards, 2007; Südhof, 2012), small, dynamic alternations in the balance of GABA/glycine signaling are possible and likely to contribute to synaptic plasticity.

Cotransmission is known to shape synaptic plasticity of other brain regions (Vaaga et al., 2014; Tritsch et al., 2016). For example, in the lateral habenula the GABA component of GABA/glutamate co-releasing inputs from the basal ganglia are selectively reduced in an animal model of depression.

This deficiency is restored following sustained treatment with the antidepressant citalopram (Shabel et al., 2014). A similar reduction in the GABA component of mixed GABA/glutamate release has been observed in the same region in animal models of cocaine withdrawal, where it is attributed to a selective decrease in VIAAT expression in vesicles (Meye et al., 2016).

## AUTHOR CONTRIBUTIONS

SS and KA conceived and planned the experiments. KA carried out the experiments and their analysis. SS carried out the modeling and simulations. SS and KA contributed to the interpretation of the results and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnsyn.2018.00040/full#supplementary-material

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# Molecular Mechanisms of Short-Term Plasticity: Role of Synapsin Phosphorylation in Augmentation and Potentiation of Spontaneous Glutamate Release

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We used genetic and pharmacological approaches to identify the signaling pathways involved in augmentation and potentiation, two forms of activity dependent, short-term synaptic plasticity that enhance neurotransmitter release. Trains of presynaptic action potentials produced a robust increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs). Following the end of the stimulus, mEPSC frequency followed a bi-exponential decay back to basal levels. The time constants of decay identified these two exponential components as the decay of augmentation and potentiation, respectively. Augmentation increased mEPSC frequency by 9.3-fold, while potentiation increased mEPSC frequency by 2.4-fold. In synapsin triple-knockout (TKO) neurons, augmentation was reduced by 83% and potentiation was reduced by 74%, suggesting that synapsins are key signaling elements in both forms of plasticity. To examine the synapsin isoforms involved, we expressed individual synapsin isoforms in TKO neurons. While synapsin IIIa rescued both augmentation and potentiation, none of the other synapsin isoforms produced statistically significant amounts of rescue. To determine the involvement of protein kinases in these two forms of short-term plasticity, we examined the effects of inhibitors of protein kinases A (PKA) and C (PKC). While inhibition of PKC had little effect, PKA inhibition reduced augmentation by 76% and potentiation by 60%. Further, elevation of intracellular cAMP concentration, by either forskolin or IBMX, greatly increased mEPSC frequency and occluded the amount of augmentation and potentiation evoked by electrical stimulation. Finally, mutating a PKA phosphorylation site to non-phosphorylatable alanine largely abolished the ability of synapsin IIIa to rescue both augmentation and potentiation. Together, these results indicate that PKA activation is required for both augmentation and potentiation of spontaneous neurotransmitter release and that PKA-mediated phosphorylation of synapsin IIIa underlies both forms of presynaptic short-term plasticity.

**Keywords: post-tetanic potentiation, synapsins, neurotransmitter release, PKA, synaptic plasticity**

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## INTRODUCTION

Numerous forms of activity-dependent synaptic plasticity enable dynamic changes in the properties of neural circuits (Zucker and Regehr, 2002; Abbott and Regehr, 2004; Jackman and Regehr, 2017; Nicoll, 2017). Bouts of high-frequency synaptic activity generate augmentation and potentiation (often called post-tetanic potentiation, or PTP), two forms of short-term plasticity that enhance neurotransmitter release for tens of seconds to minutes (Magleby and Zengel, 1976a,b; Fioravante and Regehr, 2011; Regehr, 2012). Augmentation and potentiation apparently enhance neurotransmitter release via a variety of presynaptic mechanisms, including increasing quantal release probability (Kalkstein and Magleby, 2004; Zhao and Klein, 2004; Korogod et al., 2007; Lee et al., 2008; Valente et al., 2012), enhancing the readily releasable pool of synaptic vesicles (Zhao and Klein, 2004; Habets and Borst, 2007; Lee et al., 2008; Valente et al., 2012) and/or other mechanisms (Habets and Borst, 2006; Humeau et al., 2007; Korogod et al., 2007; Neher and Sakaba, 2008; He et al., 2009).

It is well established that both augmentation and potentiation are triggered by a transient rise in calcium concentration within the presynaptic terminal (Erulkar and Rahamimoff, 1978; Kretz et al., 1982; Swandulla et al., 1991; Delaney and Tank, 1994; Kamiya and Zucker, 1994; Regehr et al., 1994; Brager et al., 2003; Kalkstein and Magleby, 2004; Habets and Borst, 2005; Korogod et al., 2005). However, the downstream effectors within these activity-dependent calcium signaling pathways remain unclear. For the case of augmentation, studies have indicated an important role for munc13, a calcium-sensitive regulator of the SNARE proteins that mediate neurotransmitter release (Rosenmund et al., 2002; Gioia et al., 2016). In contrast, a variety of calcium-regulated protein kinase pathways have been implicated in potentiation (Alle et al., 2001; Brager et al., 2002; Sweatt, 2004; Korogod et al., 2007; Fioravante et al., 2011; Lee et al., 2010). Among these, protein kinases C (PKC) and A (PKA) have received the most attention. Substantial evidence indicates that PKC activity is required for potentiation at the calyx of Held synapse (Korogod et al., 2007; Fioravante et al., 2011). However, potentiation at other synapses is independent of PKC (Wang et al., 2016). At still other synapses, inhibiting PKA activity prevents potentiation, also suggesting a role for this protein kinase in potentiation (Alle et al., 2001; Valente et al., 2012).

The downstream targets of these protein kinases are also unclear. Members of the synapsin gene family are leading candidates. Synapsins are a family of vesicle-associated proteins, encoded by three genes, that regulate synaptic vesicle dynamics and neurotransmitter release (Greengard et al., 1993; Rosahl et al., 1995; Cesca et al., 2010; Song and Augustine, 2015). Synapsins are substrates of several protein kinases, including PKA, calcium-calmodulin regulated protein kinases and the mitogen-activated protein kinase (Jovanovic et al., 1996; Jovanovic et al., 2001; Chi et al., 2003; Kohansal-Nodehi et al., 2016). Therefore, synapsins could serve as downstream effectors to regulate neurotransmitter release during potentiation. Indeed, knock-out of the synapsin I gene partially reduces potentiation in cultured hippocampal neurons (Valente et al., 2012), while deletion of both synapsin I and II genes reduces potentiation in the hippocampus (Rosahl et al., 1995) and injection of anti-synapsin antibodies reduces potentiation at *Aplysia* synapses (Humeau et al., 2001). These studies suggest that synapsins and their phosphorylation play an important role in potentiation.

Here we have done experiments in cultured hippocampal neurons to clarify the roles of protein kinases and synapsins in synaptic augmentation and potentiation. Pharmacological experiments indicate that PKA is important for both augmentation and potentiation of spontaneous glutamate release at excitatory synapses. Synapsins also are important because augmentation and potentiation are greatly reduced by knock-out of all three synapsin genes. Further, synapsins apparently are the main substrates of PKA because mutation of a PKA phosphorylation site in synapsin IIIa largely abolished the ability of this isoform to rescue augmentation and potentiation in synapsin knock-out neurons. Our results lead to a new model for the signaling pathways involved in these two forms of short-term plasticity.

## MATERIALS AND METHODS

### Hippocampal Neuronal Cultures

Homozygous synapsin triple-knockout (TKO) mice and matching triple wild-type (TWT) mice were produced as described previously (Gitler et al., 2004a,b). The procedures used to maintain and use these mice were approved by our institutional Animal Care and Use Committees. Newborn pups (postnatal day 0–1) were used to prepare dissociated hippocampal neurons. Microisland cultures were prepared from these neurons as described in Bekkers and Stevens (1991), with the addition of glia feeder cells to promote neuronal survival. Neurons were allowed to mature for 10–14 days before being used for electrophysiological recordings.

### Electrophysiological Data Acquisition and Analysis

To record spontaneous miniature excitatory postsynaptic currents (mEPSCs), whole-cell patch-clamp recordings were made from single neurons on microislands (Gitler et al., 2004a). Patch pipettes (4–6 MOhm) were filled with intracellular solution containing (in mM): 50 K-glutamate, 71 K-gluconate (Fluka, Buchs, Switzerland), 15 NaCl, 6 $MgCl_2$, 0.5 EGTA, 5 $Na_2ATP$, 0.3 $Na_2GTP$, and 20 HEPES-KOH, pH 7.3 (285 mOsm). The extracellular solution contained (in mM): 150 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose, and 10 HEPES-NaOH, pH 7.3 (310 mOsm). All materials were from Sigma, unless specified otherwise. An EPC-9D amplifier (HEKA, Lambrecht/Pfalz, Germany) was used to voltage clamp neurons at a holding potential of −70 mV. Under these conditions, spontaneous EPCSs are solely due to mEPSCs that were blocked by the AMPA receptor antagonist, CNQX (20 μM). Spontaneous synaptic events were first detected automatically, with an amplitude threshold of 8 pA, using the MiniAnalysis program (Synaptosoft, Decatur, GA, United States), and then subsequently manually

screened to remove any residual artifacts. mEPSC frequency was measured within 5 s bins.

"Presynaptic" action potentials were evoked by using the recording pipette to depolarize the neuron to +40 mV for 0.5 ms. To measure the amplitudes of augmentation and potentiation evoked by a train of such stimuli (50 Hz, 2 s), we first normalized the response by dividing mEPSC frequency at each time point following the stimulus train by the basal frequency of mEPSCs prior to the stimulus (as in **Figure 1B**). We then fitted the normalized mEPSC frequency for each timepoint, *t*, with a 2-exponential decay function:

$$f(t) = A_1 e^{\frac{-t}{\tau_1}} + A_2 e^{\frac{-t}{\tau_2}} + f_0$$

where $A_1$ represents the amplitude of augmentation and $A_2$ represents the amplitude of potentiation, $\tau_1$ is the time constant of augmentation and $\tau_2$ is the time constant of potentiation, and $f_0$ is the mean baseline frequency of mEPSCs. Using this equation, the two components were mathematically separated and the contributions of each component were independently defined. Thus, the $A_1$ and $A_2$ amplitude values reflect the increases in mEPSC frequency independently contributed by each process.

Photoactivation of opto-Gs (Airan et al., 2009) was done with blue light (470 ± 20 nm) from a mercury lamp, with light flash duration controlled by an electronic shutter (Uniblitz).

Differences between experimental parameters measured in two groups were tested for statistical significance using the Student's *t*-test. For comparisons across more than two experimental groups, we first performed a normality test (Kolmogorov–Smirnov test) to determine whether the data were normally distributed. All datasets were found to be normally distributed, permitting the use of parametric statistical tests. Specifically, data were analyzed by using a one-way ANOVA to determine whether there were any significant differences between groups, followed by the *post hoc* Holm–Bonferroni method to control for the familywise error rate associated with multiple comparisons. Throughout the "Results" section, the outcome of these statistical analyses are reported as both t and *p*-values.

## Viral Expression of Synapsin Isoforms

EGFP-tagged synapsin Ia, Ib, IIa, IIb, and IIIa were subcloned into a pFUGW shuttle vector, where the inserted synapsin genes were driven by the human polyubiquitin-C promoter. Site-directed mutagenesis was done using Quikchange kit (Stratagene). Lentivirus was then prepared as described in Lois et al. (2002). Opto-β2-AR plasmid (Airan et al., 2009) was a generous gift from Dr. K Deisseroth. Neurons were infected after 3–4 days in culture, with a 1:3 multiplicity of infection, and studied 7–10 days post-infection. Song and Augustine (2016) have reported that cultured TKO neurons virally infected with various synapsin constructs express exogenous synapsin isoforms 1.3 to 2.5-fold greater than the expression of endogenous synapsins in TWT neurons, indicating mild overexpression in virally infected TKO neurons. Electrical recordings were made only from neurons that were infected, based on visible expression of GFP-tagged synapsins. Thus, 100% of the "presynaptic" cells that we stimulated were transfected.

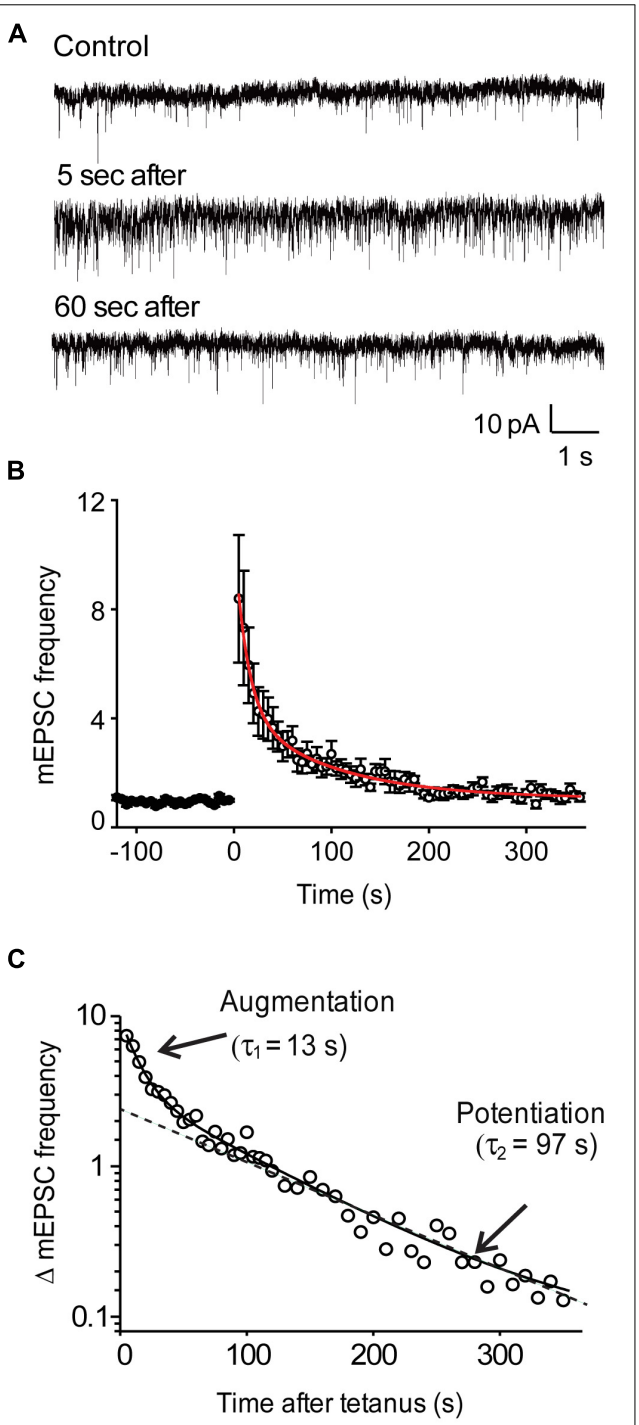


**FIGURE 1 |** Activity-dependent enhancement of miniature EPSC frequency in wild-type hippocampal neurons. **(A)** Representative recordings of mEPSCs before, as well as 5 and 60 s after the end of a stimulus train (50 Hz, 2 s). **(B)** Time course of mEPSC frequency changes produced by stimulation (at time = 0). Points indicate mean values of mEPSC frequency ($n$ = 9), normalized to baseline values measured prior to the stimulus train, and error bars indicate ± SEM. Red curve indicates fit of bi-exponential decay function. **(C)** Semi-logarithmic plot of the post-tetanic changes in mEPSC frequency shown in panel **(B)**. Solid curve indicates bi-exponential decay function, while dashed line indicates time course of slower potentiation component.

## RESULTS

Our experiments measured augmentation and potentiation of spontaneous transmitter release at excitatory synapses of microisland-cultured hippocampal neurons. The advantage of measuring synaptic plasticity via spontaneous release, rather than by measuring release evoked by presynaptic action potentials, is that this approach circumvents several confounds – such as activity-dependent changes in quantal size (He et al., 2009; Fioravante et al., 2011), presynaptic action potential waveform (Habets and Borst, 2005), or presynaptic calcium currents (Habets and Borst, 2006) – that make it difficult to interpret measurements of action-potential evoked synaptic responses. Previous work has established that the kinetics of synaptic augmentation and potentiation of spontaneous transmitter release are very similar to the kinetics of action-potential evoked transmitter release (Erulkar and Rahamimoff, 1978; Zengel and Magleby, 1981; Eliot et al., 1994).

### Augmentation and Potentiation of Spontaneous Transmitter Release

When recording synaptic responses, the rapid kinetics of the autaptic excitatory postsynaptic currents (EPSCs) evoked by brief depolarizations could be used to identify glutamatergic synapses (Gitler et al., 2004a). To measure the rate of spontaneous glutamate release at these excitatory synapses, we monitored the frequency of mEPSCs before and after trains of depolarizing stimuli (50 Hz, 2 s). Application of such tetanic stimuli produced a robust increase in the frequency of mEPSCs in wild-type neurons (**Figure 1A**). Following the end of the stimulus, mEPSC frequency declined back to baseline levels over a few hundred seconds (**Figure 1B**). This post-tetanic decay of mEPSC frequency could be fit with the sum of two exponential functions (red line in **Figure 1B**), one with a time constant of 13.2 ± 1.7 s ($n$ = 9) and a second with a slower time constant of 97.1 ± 15.6 s ($n$ = 9). These time constants identify these two components as the decay of augmentation and potentiation (Zengel and Magleby, 1982; Regehr, 2012). The bi-exponential decay of mEPSC frequency after a tetanus was readily visualized when plotted on semi-logarithmic coordinates, making both the decay of augmentation and potentiation appear linear (**Figure 1C**). The augmentation component increased mEPSC frequency by 931% ± 221% ($n$ = 7), while the potentiation component increased mEPSC frequency by 245% ± 140% ($n$ = 7).

### Synapsins Are Required for Augmentation and Potentiation

Synapsins are the most abundant phosphoprotein in the brain (Hilfiker et al., 1999, 2005) and are known to be phosphorylated after tetanic stimuli and other forms of prolonged depolarization (Greengard et al., 1993; Kohansal-Nodehi et al., 2016). Given previous evidence indicating a role for both synapsins and protein phosphorylation in potentiation (see references in "Introduction" section), we hypothesized that synapsins could play a role in short-term plasticity by serving as protein kinase substrates.

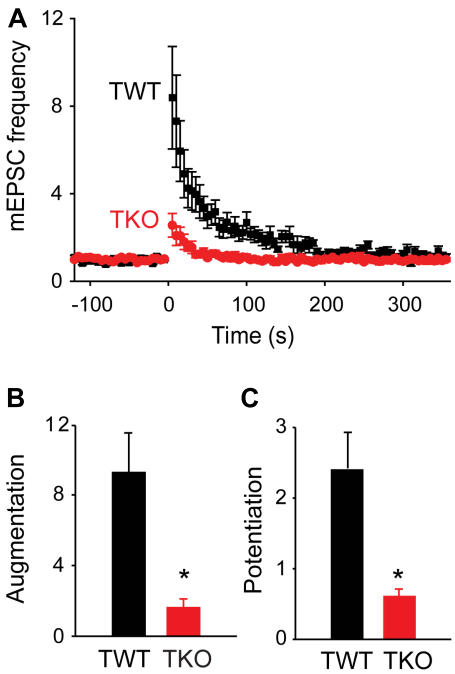


**FIGURE 2 |** Role of synapsins in augmentation and potentiation. **(A)** Time course of changes in normalized mEPSC frequency produced by electrical activity (50 Hz, 2 s stimulation) in TWT (black) and TKO (red) neurons. Points indicate mean values of mEPSC frequency ($n$ = 7), normalized to baseline values measured prior to the stimulus train, and error bars indicate ± SEM. **(B)** Peak amount of augmentation of mEPSC frequency, calculated from exponential fits to the data in panel **(A)**, in TWT (black) and TKO (red) neurons. **(C)** Amount of potentiation of mEPSC frequency in TWT (black) and TKO (red) neurons. Values in panles **(B,C)** indicate means and error bars indicate ± SEM, while asterisks indicate significant differences ($p$ < 0.05) between TWO and TKO.

To evaluate the role of synapsins, we compared augmentation and potentiation in neurons from synapsin TKO and TWT mice. In TKO neurons, both augmentation and potentiation were significantly reduced (**Figure 2A**, $n$ = 7). The amplitude of the augmentation component was reduced by 82.6% (to a 162% ± 50% increase in mEPSC frequency; $p$ = 0.006, Student's $t$-test; **Figure 2B**), while the amplitude of the potentiation component was reduced by 74.6% (to a 62.2% ± 12% increase in mEPSC frequency; $p$ = 0.01, Student's $t$-test; **Figure 2C**) The kinetics of the remaining augmentation (time constant = 18.0 ± 4.6 s, $n$ = 7) and potentiation (time constant = 149 ± 55 s, $n$ = 7) were similar to control values ($p$ = 0.83, Student's $t$-test). The substantial attenuation of augmentation and potentiation observed in TKO neurons indicates that synapsins play important roles in both of these types of short-term synaptic plasticity.

In TKO mice, all synapsin isoforms are eliminated. To identify the specific synapsin isoforms involved in augmentation and potentiation, we determined which isoforms could rescue the reductions in these forms of short-term plasticity observed in TKO neurons. For this purpose, we infected TKO neurons with lentivirus encoding GFP-tagged versions of five synapsin (syn)

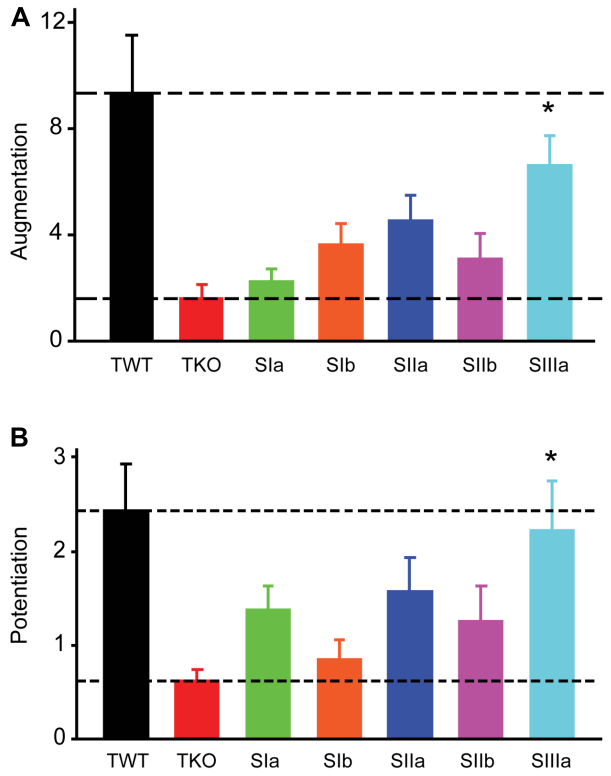


**FIGURE 3 |** Rescue of augmentation and potentiation defects in TKO neurons by synapsin isoforms. **(A)** Amplitude of augmentation in TWT (black) and TKO (red) neurons, as well as in TKO neurons expressing indicated synapsin isoforms. **(B)** Amplitude of potentiation in the same conditions shown in panel **(A)**. Values in panels **(A,B)** indicate means, determined as in **Figure 2**, error bars indicate ± SEM, and asterisks indicate significant differences compared to values measured in TKO neurons (lower dashed lines).

isoforms: synIa, Ib, IIa, IIb and IIIa (Gitler et al., 2008). GFP-tagged synapsins have been shown to function normally in terms of synaptic targeting (Gitler et al., 2004a,b), phosphorylation by protein kinases (Chi et al., 2003), and their ability to rescue both glutamatergic (Gitler et al., 2008) and GABAergic (Song and Augustine, 2016) synaptic transmission in synapsin TKO neurons. Synapsin isoforms differed in their ability to rescue synaptic augmentation (**Figure 3A**) and potentiation (**Figure 3B**). In TKO neurons expressing synIIIa, the amplitude of augmentation was increased ($p < 0.001$, $t = 3.86$; $n = 8$), as was the amplitude of potentiation ($p = 0.001$, $t = 3.45$; $n = 8$). However, synIIIa did not fully restore augmentation or potentiation in TKO neurons to the levels measured in TWT neurons (TWT vs. TKO-synIIIa: $p = 0.24$ for augmentation and $p = 0.14$ for potentiation). In contrast, the amplitude of augmentation was not significantly rescued in TKO neurons expressing synIa ($p = 0.62$, $t = 0.50$; $n = 10$), synIb ($p = 0.13$, $t = 1.50$; $n = 7$), synIIa ($p = 0.03$, $t = 2.18$; $n = 7$), or synIIb ($p = 0.22$, $t = 1.21$; $n = 11$). Similarly, the amplitude of potentiation was not significantly rescued by synIa ($p = 0.14$, $t = 1.52$; $n = 10$), synIb ($p = 0.68$, $t = 0.41$; $n = 7$), synIIa ($p = 0.08$, $t = 1.75$; $n = 7$), or synIIb ($p = 0.20$, $t = 1.29$; $n = 11$). These results indicate that synapsins play an important role in augmentation and potentiation, with the synapsin IIIa isoform having the greatest ability to rescue both the augmentation and potentiation phenotypes of TKO neurons.

## Differential Roles of PKA and PKC

To define the potential role of protein kinase(s) in augmentation and potentiation, we examined the effects of protein kinase inhibitors on augmentation and potentiation in TWT neurons. Because PKC has been implicated in augmentation and/or potentiation at a number of synapses (Beierlein et al., 2007; Korogod et al., 2007; Fioravante et al., 2011; Genc et al., 2014), we first tested bisindolylmaleimide (BIM; 0.5 μM). This drug inhibits PKC by blocking its ATP-binding site (Gassel et al., 2004) and has been shown to block PTP of evoked EPSCs at hippocampal CA3-CA1 synapses (Brager et al., 2003). However, we did not observe a significant reduction in either augmentation (**Figure 4A**; $p = 0.34$, $t = -0.97$; $n = 4$) or potentiation (**Figure 4B**; $p = 0.68$, $t = -0.41$; $n = 4$) of spontaneous glutamate release following treatment with BIM. This suggests that PKC is not involved in either augmentation or potentiation of spontaneous glutamate release in cultured hippocampal neurons. This conclusion is consistent with findings at hippocampal mossy fiber synapses (Wang et al., 2016) and also is consistent with the fact that PKC does not phosphorylate synapsins (Hilfiker et al., 2005).

All synapsin isoforms, including synIIIa, are known to be phosphorylated by PKA (Hosaka et al., 1999). Given the role of PKA in augmentation and/or potentiation at numerous synapses (Kuromi and Kidokoro, 2000; Alle et al., 2001; Fiumara et al., 2004), we next determined the involvement of PKA in synapsin-dependent augmentation and potentiation. We first examined the effects of Rp-cAMPS, a membrane-permeant cyclic AMP analog that binds to the regulatory subunit of PKA and prevents activation of the catalytic subunit that phosphorylates PKA substrates (de Wit et al., 1982; Van Haastert et al., 1984; Rothermel and Parker Botelho, 1988; Dostmann et al., 1990).

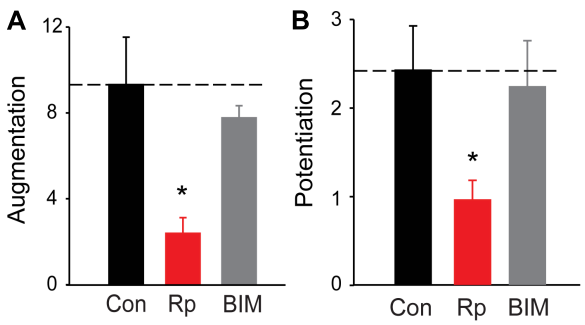


**FIGURE 4 |** Roles of PKA and PKC in augmentation and potentiation. **(A)** Amplitude of augmentation measured in TWT neurons in control conditions (black), as well as in the presence of the PKA inhibitor Rp-cAMP (red) and the PKC inhibitor BIM (gray). **(B)** Amplitude of potentiation the same conditions indicated in panel **(A)**. Values indicate means, determined as in **Figure 2**, error bars indicate ± SEM, and asterisks indicate significant differences compared to values measured in control conditions (dashed lines).

Bath application of Rp-cAMPS (25 μM) reduced the amplitude of both augmentation (by 74.4%; $p = 0.0012$, $t = -3.65$; $n = 8$) and potentiation (by 60.4%; $p = 0.02$, $t = -2.47$; $n = 8$), as shown in **Figure 4**. The degree of reduction of both augmentation and potentiation by Rp-cAMPS is roughly similar to the degree of reduction produced by loss of synapsins (**Figure 2**). These results suggest that activation of PKA is important for synapsin-dependent synaptic plasticity.

We next examined the effects of activating PKA by elevating intracellular cAMP concentration. We began by treating the cultured neurons with forskolin, which elevates cAMP concentration by activating adenylyl cyclase (Seamon et al., 1983). Application of forskolin (10 μM) caused a time-dependent increase in mEPSC frequency (**Figure 5A**) which at its peak increased mEPSC frequency by approximately 3-fold over the basal level (**Figure 5C**). Thus, increasing PKA activity potentiates spontaneous transmitter release, as reported previously at many synapses (Yoshihara et al., 2000; Sakaba and Neher, 2001; Kaneko and Takahashi, 2004; Miura et al., 2012). Delivery of a train of stimuli (50 Hz, 2 s) in the presence of forskolin reduced augmentation and potentiation (**Figure 5A**, solid triangles and **Figure 6A**, red points). On average, the amplitude of augmentation was reduced by 73.5% (**Figure 6B**; $p = 0.004$, $t = -4.62$; $n = 7$) and potentiation was reduced by 69.2% (**Figure 6C**; $p = 0.02$, $t = -2.51$; $n = 7$). Thus, elevation of cAMP levels enhanced spontaneous transmitter release and occluded both the augmentation and potentiation of spontaneous release produced by electrical activity.

Elevation of cAMP levels by treatment with the inhibitor IBMX, which blocks the phosphodiesterase responsible for degradation of cAMP (Deth and Lynch, 1981; Leroy et al., 2008), produced similar effects. Application of IBMX (0.5 mM) caused a time-dependent increase in mEPSC frequency (**Figure 5B**); at its peak, IBMX increased mEPSC frequency by approximately 4-fold over basal levels (**Figure 5C**) and reduced the increase in mEPSC frequency evoked by a train of electrical stimuli (**Figure 5B**, solid triangles and **Figure 6A**, green points). The mean reductions in the amplitude of augmentation (87.8%; $p = 0.03$, $t = -3.73$; $n = 7$) and potentiation (67.5%; $p = 0.009$, $t = -2.78$; $n = 7$) were comparable to the effects of forskolin treatment (**Figures 6B,C**). Taken together, these results suggest that activation of PKA by cAMP is involved in both forms of short-term synaptic plasticity.

## Synapsins as PKA Substrates During Synaptic Plasticity

To define the temporal relationship between PKA activation and synaptic plasticity, we next made a time-resolved jump in cAMP concentration within the presynaptic terminal. For this purpose, we expressed in the cultured neurons opto-β2-AR, a light-sensitive, chimeric G-protein coupled receptor that produces a rapid elevation in cAMP concentration in response to blue light (Airan et al., 2009). In TWT neurons expressing opto-β2-AR, a brief light flash (470 ± 20 nm, 30 s duration) produced a transient increase in mEPSC frequency (**Figure 7A**). mEPSC frequency gradually increased during the light flash, reached a peak almost immediately after the end of the flash,

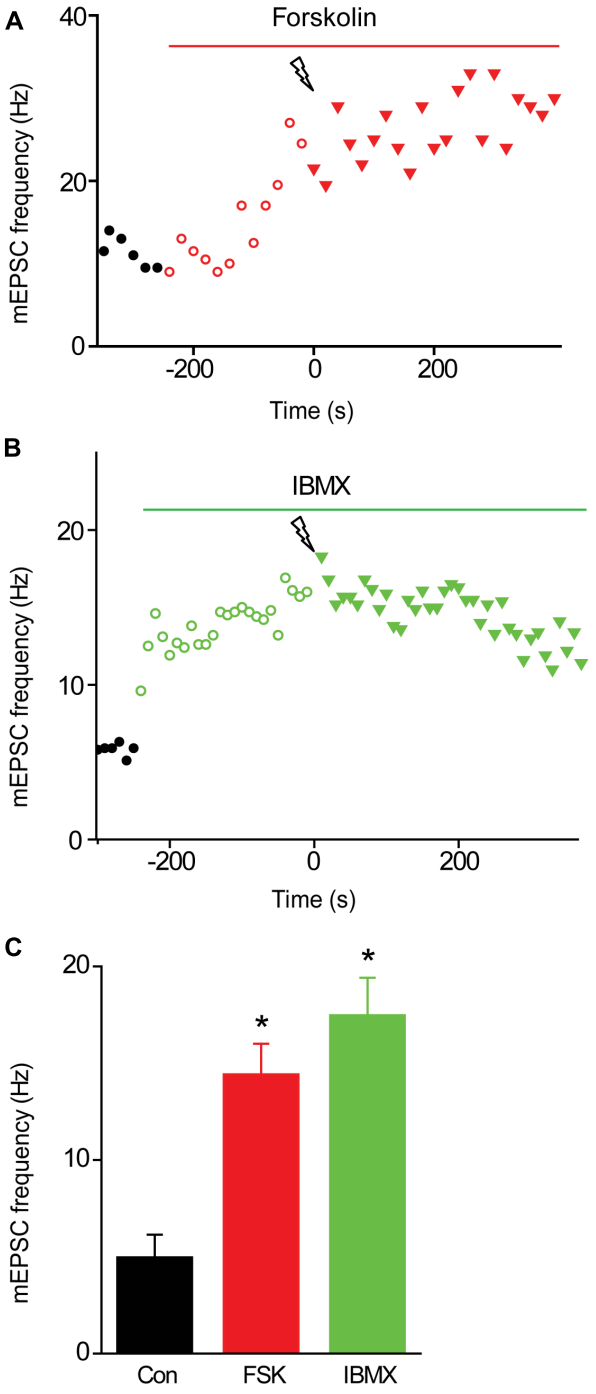


**FIGURE 5 |** Effects of elevating presynaptic cAMP levels on mEPSC frequency in TWT neurons. **(A)** Time course of changes in normalized mEPSC frequency (red symbols) produced by application of forskolin (10 μM) during time indicated by red bar. Electrical stimulation (50 Hz, 2 s) was applied at time = 0. **(B)** Time course of changes in mEPSC frequency (green symbols) produced by application of IBMX (0.5 mM) during time indicated by green bar. Electrical stimulation (50 Hz, 2 s) was applied at time = 0. **(C)** Mean values of mEPSC frequency measured in control conditions (black), as well as in the presence of forskolin (FSK; red) and IBMX (green). Values indicate means, error bars indicate ±SEM, and asterisks indicate significant differences compared to values measured in control conditions.

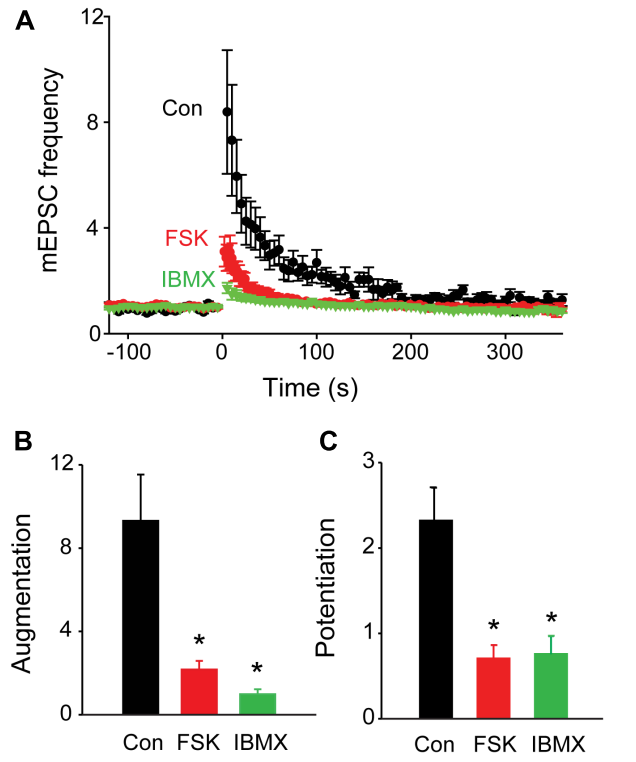


**FIGURE 6 |** Occlusion of synaptic plasticity following elevation of presynaptic cAMP levels. **(A)** Time course of changes in normalized mEPSC frequency produced by electrical activity (50 Hz, 2 s stimulation) in TWT neurons in control conditions (black), as well as in the presence of forskolin (FSK; 10 μM; red) and IBMX (green). Points indicate mean values of mEPSC frequency ($n$ = 5), normalized to baseline values measured prior to the stimulus train, and error bars indicate ±SEM. **(B)** Peak amount of augmentation of mEPSC frequency, calculated from exponential fits to the data in panel **(A)**, in control (black), forskolin (red), and IBMX (green). **(C)** Amount of potentiation of mEPSC frequency in same conditions described in panel **(B)**. Values in panels **(B,C)** indicate means and error bars indicate ±SEM, while asterisks indicate significant differences between control and forskolin or control and IBMX.

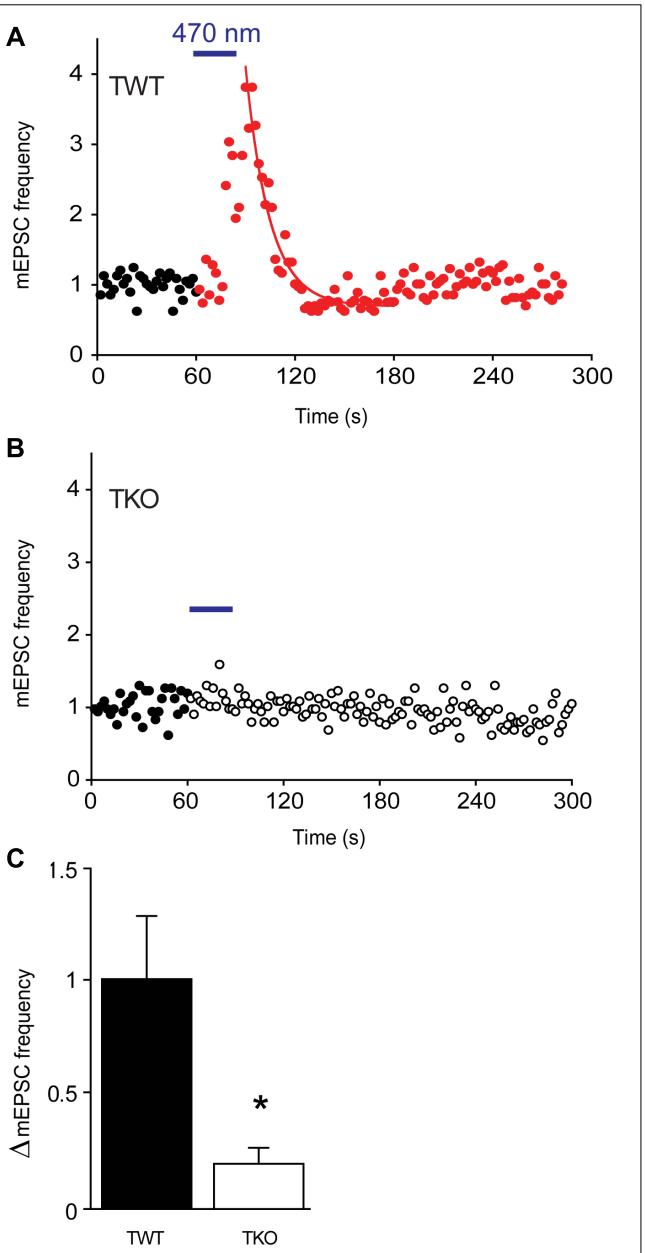


**FIGURE 7 |** Changes in mEPSC frequency produced by optogenetic elevation of presynaptic cAMP levels. **(A)** Time course of normalized mEPSC frequency in response to illumination (470 nm light, 30 s at blue bar) of a TWT neuron expressing opto-β2-AR. Red points indicate mEPSC frequency, normalized to baseline values, measured following the start of the light flash. Smooth curve indicates an exponential fit to the decay of mEPSC frequency following the end of the light flash. **(B)** Same for a TKO neuron expressing opto-β2-AR. Open symbols indicate normalized mEPSC frequency measured following the start of the light flash. **(C)** Mean increase in mEPSC frequency produced by activation of opto-β2-AR in WT (black) and TKO (white) neurons. Error bars indicate ±SEM, while asterisks indicate significant differences.

and exponentially decayed back to baseline levels afterward. The mean increase in mEPSC frequency was 97.3 ± 28.8% (**Figure 7C**; $n$ = 6) and the time constant for decay of mEPSC frequency after the flash was 35.8 ± 10.6 s ($n$ = 6), a decay time course that is intermediate between those of augmentation and potentiation. In TKO neurons expressing opto-β2-AR, the same light flash produced a significantly smaller ($p$ = 0.03, Student's $t$-test, $n$ = 3) increase in mEPSC frequency (**Figure 7B**), with mean increase of 19.7 ± 6.8% (**Figure 7C**; $n$ = 3). Thus, a rapid jump in presynaptic cAMP concentration was capable of generating an increase in spontaneous glutamate release that resembles augmentation and potentiation. Further, similar to augmentation and potentiation, this increase depends upon synapsins.

Finally, we asked which sites on synapsins are phosphorylated by PKA to produce augmentation and potentiation. Synapsin IIIa, the isoform most effective in rescuing augmentation and potentiation in TKO neurons (**Figure 3**), contains a known PKA phosphorylation site (serine 9). We mutated this serine into non-phosphorylatable alanine to prevent PKA from phosphorylating this residue. Expression of synapsin IIIa in TKO neurons rescued

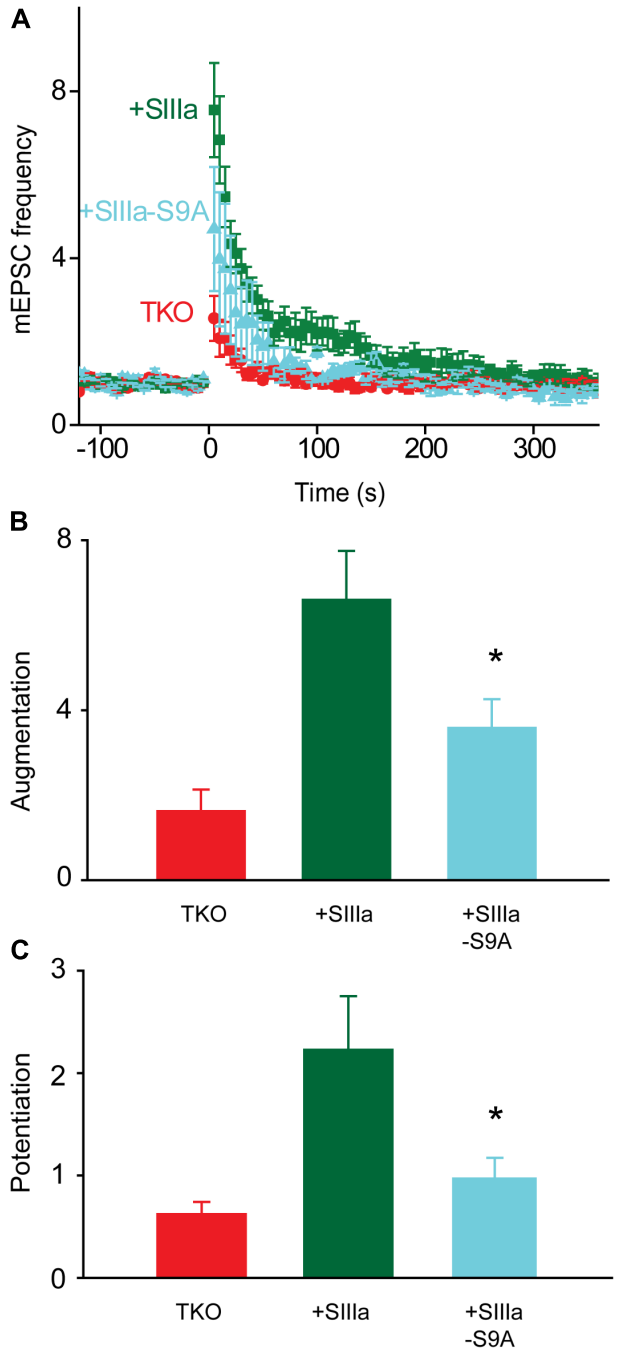


**FIGURE 8 |** PKA regulation of synapsin IIIa rescue of TKO phenotype. **(A)** Time course of changes in mEPSC frequency produced by electrical activity (50 Hz, 2 s stimulation) in TKO neurons (red) and in TKO neurons expressing wild-type synapsin IIIa (+SIIIa; green) or phosphorylation-deficient synapsin IIIa S9A (+SIIIa-S9A; cyan). Points indicate mean values of mEPSC frequency ($n$ = 5), normalized to baseline values measured prior to the stimulus train, and error bars indicate ±SEM. **(B)** Peak amount of augmentation of mEPSC frequency, calculated from exponential fits to the data in panel **(A)**, in TKO neurons (red) and in TKO neurons expressing wild-type synapsin IIIa (green) or phosphorylation-deficient synapsin IIIa S9A (cyan). Values in panels **(B,C)** indicate means and error bars indicate ± SEM, while asterisks indicate significant differences compared to TKO + SynIIIa neurons.

both augmentation and potentiation (**Figure 8A**), as indicated in **Figure 3**. However, the rescue of augmentation and potentiation in TKO neurons by synapsin IIIa was greatly reduced by the synIIIa-S9A mutant (**Figure 8A**). This was true for both the augmentation (**Figure 8B**; 60.6% reduction; $p = 0.03$, $t = -2.37$; $n = 6$) and potentiation (**Figure 8C**; 77.5% reduction; $p = 0.01$, $t = -2.73$; $n = 4$). This indicates that the PKA phosphorylation site of synapsin IIIa is critical for rescue of augmentation and potentiation in TKO neurons. The same mutation in the PKA phosphorylation site of synapsin IIa also eliminated the partial rescue produced by synIIa (data not shown). Taken together, we conclude that synapsin IIIa is important for augmentation and potentiation, specifically by serving as a substrate for PKA to transiently enhance the rate of spontaneous glutamate release in response to repetitive presynaptic activity.

## DISCUSSION

Augmentation and potentiation are two forms of short-term plasticity that enhance neurotransmitter release for seconds to minutes following a bout of presynaptic activity. Here we have examined the molecular signaling underlying these two forms of plasticity in cultured hippocampal neurons. We found that PKA, but not PKC, is involved in regulation of both augmentation and potentiation of spontaneous glutamate release. Further, this kinase seems to act by phosphorylating synapsins, specifically the synapsin IIIa isoform.

### Roles of Protein Kinases in Augmentation and Potentiation

While it is well-established that both augmentation and potentiation are triggered by transient rises in presynaptic calcium concentration, the involvement of downstream protein kinase signaling in these forms of plasticity is much less clear. While PKC clearly plays a role in potentiation at the glutamatergic calyx of Held synapse (Korogod et al., 2007; Fioravante et al., 2011), the role of this kinase in potentiation of hippocampal synapses is uncertain. Both our data (**Figure 4B**) and those of Wang et al. (2016) indicate that potentiation of glutamate release onto hippocampal pyramidal cells does not require PKC. However, PKC may be involved in potentiation at glutamatergic synapses onto hippocampal interneurons (Alle et al., 2001). Thus, there seem to be clear differences in the importance of PKC for potentiation at different synapses, even within the same brain area.

We have established several lines of evidence implicating PKA in potentiation of excitatory synapses onto hippocampal pyramidal cells. First, a PKA inhibitor reduced potentiation (**Figure 4B**). Second, two different pharmacological treatments that elevate cAMP levels enhanced spontaneous glutamate release (**Figure 5**) and occluded potentiation (**Figures 6A,C**). Third, transient optogenetic elevation of cAMP levels evoked a transient, potentiation-like enhancement of spontaneous release (**Figures 7A,C**). Finally, deletion of a PKA phosphorylation site in synapsin IIIa reduced the ability of this isoform to rescue the loss of potentiation observed in synapsin TKO neurons (**Figure 8**).

Thus, we conclude that PKA is important for potentiation of spontaneous release at these synapses. This is consistent with observations that inhibitors of PKA reduce potentiation of glutamatergic synapses onto hippocampal interneurons (Alle et al., 2001) and at excitatory synapses of *Helix* (Fiumara et al., 2007) and *Aplysia* (Khoutorsky and Spira, 2009). Although PKA is not directly activated by calcium, adenylyl cyclase is activated by $Ca^{2+}$/calmodulin (Hanoune and Defer, 2001; Wang and Zhang, 2012) and this could allow PKA to be activated during a tetanus.

Remarkably, we found that PKA also is important for augmentation of spontaneous glutamate release at excitatory synapses onto hippocampal pyramidal cells. While there have been few studies of the molecular mechanisms of augmentation, to date most analyses suggest that augmentation results from calcium directly binding to calcium-regulated proteins such as munc13 (Rosenmund et al., 2002; Gioia et al., 2016) rather than from kinase-mediated signaling. Thus, our findings open a new window into the signaling processes underlying augmentation. For example, our observation that elevation of presynaptic cAMP levels alone causes an enhancement of transmitter release that lasts longer than augmentation (**Figure 7A**) suggests that the decay of augmentation could be accelerated by an activity-dependent decay in the levels of synapsin phosphorylation. A plausible hypothesis for such a mechanism would be calcium-dependent activation of the protein phosphatase, calcineurin, which is able to dephosphorylate synapsins (King et al., 1984).

## Synapsin Isoforms and Synaptic Plasticity

Synapsins are known to control synaptic vesicle mobilization during periods of intense synaptic activity, such as the type of activity that elicits augmentation and potentiation. Further, phosphorylation regulates the binding affinity of synapsins for synaptic vesicles and cytoskeletal elements (Greengard et al., 1993; Hilfiker et al., 1999; Hosaka et al., 1999; Cesca et al., 2010). Given the roles of protein phosphorylation in both augmentation and potentiation, it is therefore possible that synapsins could serve as downstream targets of protein kinase signaling during short-term synaptic plasticity. Consistent with this possibility, previous work has shown that potentiation is reduced both by genetic deletion of synapsins at mouse excitatory synapses (Rosahl et al., 1995; Valente et al., 2012) and by antibody neutralization of synapsin at *Aplysia* inhibitory synapses (Humeau et al., 2001). We have extended these findings by showing that both augmentation and potentiation are almost completely eliminated at excitatory hippocampal synapses of synapsin TKO neurons (**Figure 2**). Further, we have systematically evaluated the ability of each synapsin isoform to support these forms of short-term synaptic plasticity (**Figure 3**) and the role of PKA phosphorylation in rescue of augmentation and potentiation by synapsin IIIa (**Figures 7**, **8**).

We found that not all synapsin isoforms are involved in augmentation and potentiation of excitatory transmission: only synapsin IIIa was capable of significantly rescuing these forms of synaptic plasticity in synapsin TKO neurons. This extends previous work indicating that synapsin isoforms differ in their physiological functions (Song and Augustine, 2015). Our finding that synapsin IIa partially rescued augmentation and potentiation, an effect that did not reach statistical significance, is consistent with a previous study showing a partial loss of potentiation in synapsin II knock-out mice (Rosahl et al., 1995). Our results extend the earlier finding by indicating that the loss of synapsin IIa, rather than synapsin IIb, is likely responsible for the defect in potentiation. We found that synapsin IIIa had the greatest ability to rescue short-term plasticity, almost completely rescuing potentiation and largely rescuing augmentation (**Figure 3**). While synapsin IIIa is predominantly expressed during early neuronal development (Ferreira et al., 2000), it is also known to regulate neurotransmitter release in more mature neurons (Feng et al., 2002; Kile et al., 2010; Song and Augustine, 2016).

While mutation of the PKA phosphorylation site, serine 9, reduced the ability of synapsin IIIa to rescue augmentation and potentiation in TKO neurons, this mutation did not completely lower these forms of synaptic plasticity down to the levels observed in control TKO neurons. This could indicate a role for other phosphorylation sites in the regulation of synapsin IIIa function during augmentation and potentiation. In addition to this PKA phosphorylation site, synapsin IIIa also possesses a unique MAPK phosphorylation site within its J domain. Given the role of MAPK phosphorylation in regulating vesicle trafficking (Chi et al., 2003) and potentiation (Schenk et al., 2005; Khoutorsky and Spira, 2009; Giachello et al., 2010) during synaptic activity, it is possible that this kinase could also phosphorylate synapsin IIIa during augmentation and potentiation. Other evidence implicates still other protein kinases, such as calcium/calmodulin-dependent protein kinases (Jin and Hawkins, 2003; Fiumara et al., 2007, but see Malinow et al., 1988; Stevens et al., 1994). Thus, it is possible that multiple protein kinases regulate augmentation and potentiation by phosphorylating synapsins, with the complement of relevant kinases likely to vary according to the type of synapse as well as the amount of synaptic activity (Chi et al., 2003; Yamagata and Nairn, 2015).

Phosphorylation of synapsin I by PKA is a key regulator of synaptic vesicle exocytosis and recycling (Chi et al., 2003; Menegon et al., 2006). However, we found that synapsin Ia only modestly rescued potentiation, an effect that did not reach statistical significance, and did not rescue augmentation at all. Further, synapsin Ib could not rescue either form of synaptic plasticity. These results are consistent with a report that potentiation is normal in the hippocampal CA1 region of synapsin I KO mice (Rosahl et al., 1995). Our results also fit with the observation of Valente et al. (2012) that loss of synapsin I causes a partial loss of potentiation in cultured hippocampal neurons, an effect that was rescued by synapsin Ia. Our results are also consistent with the observation that overexpression of a *Helix* synapsin resembling synapsin I has no effect on augmentation (Fiumara et al., 2007).

## Mechanisms of Synapsin Action in Synaptic Plasticity

As described in the "Introduction" section, multiple mechanisms are involved in the enhancement of neurotransmitter release during augmentation and potentiation. It is not yet clear which, if any, of these mechanisms involve PKA and synapsin IIIa. PKA-mediated phosphorylation could allow synapsin IIIa to dissociate from synaptic vesicles within the reserve pool, thereby mobilizing these vesicles to enhance their availability to participate in glutamate release (Kuromi and Kidokoro, 2000). This model is attractive because PKA-mediated phosphorylation of synapsin IIIa controls both augmentation and potentiation, both of which ultimately depend upon mobilization of synaptic vesicles from the reserve pool. However, synapsin IIa is the only isoform that can maintain vesicles within the reserve pool; synapsin IIIa has no significant ability rescue the defect in vesicle reserve pool size observed in TKO neurons (Gitler et al., 2008). The fact that synapsin IIIa can rescue augmentation and potentiation in TKO neurons, without rescuing the reserve pool, argues that the regulatory role of synapsins in short-term synaptic plasticity does not arise from an effect on the reserve pool. Alternatively, it is known that synapsins can produce activity-stimulated increases in the number of active release sites (Humeau et al., 2007) as well as having other effects on the readily releasable pool of synaptic vesicles (Hilfiker et al., 1998; Humeau et al., 2001; Medrihan et al., 2013; Song and Augustine, 2016). Further work will be required to determine whether these or other mechanisms permit PKA-mediated phosphorylation of synapsins to regulate neurotransmitter release during augmentation and potentiation.

## ETHICS STATEMENT

The procedures used to maintain and use mice were approved by Duke University Institutional Animal Care and Use Committee, Biopolis Institutional Animal Care and Use Committee, and Nanyang Technological University Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

QC and GA designed the experiments. QC and SHS performed the experiments and analyzed the data. All authors wrote the paper.

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# Resveratrol Prevents Cellular and Behavioral Sensory Alterations in the Animal Model of Autism Induced by Valproic Acid

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Autism spectrum disorder (ASD) is characterized by impairments in both social communication and interaction and repetitive or stereotyped behaviors. Although its etiology remains unknown, genetic and environmental risk factors have been associated with this disorder, including the exposure to valproic acid (VPA) during pregnancy. Resveratrol (RSV) is an anti-inflammatory and antioxidant molecule known to prevent social impairments in the VPA animal model of autism. This study aimed to analyze the effects of prenatal exposure to VPA, as well as possible preventive effects of RSV, on sensory behavior, the localization of GABAergic parvalbumin ($PV^+$) neurons in sensory brain regions and the expression of proteins of excitatory and inhibitory synapses. Pregnant rats were treated daily with RSV (3.6 mg/kg) from E6.5 to E18.5 and injected with VPA (600 mg/kg) in the E12.5. Male pups were analyzed in Nest Seeking (NS) behavior and in whisker nuisance task (WNT). At P30, the tissues were removed and analyzed by immunofluorescence and western blotting. Our data showed for the first time an altered localization of $PV^+$-neurons in primary sensory cortex and amygdala. We also showed a reduced level of gephyrin in the primary somatosensory area (PSSA) of VPA animals. The treatment with RSV prevented all the aforementioned alterations triggered by VPA. Our data shed light on the relevance of sensory component in ASD and highlights the interplay between RSV and VPA animal model as an important tool to investigate the pathophysiology of ASD.

**Keywords: animal model, GABA, inhibition, parvalbumin, resveratrol, sensory, synaptic proteins, VPA**

## INTRODUCTION

Autism spectrum disorder (ASD) is a highly prevalent neurodevelopmental condition affecting 1 in 68 children aged 8 years in the USA (American Psychiatry Association (APA), 2013) and is characterized, according to the DSM-5, by a behavioral dyad composed by impairments in communication and social interaction and repetitive or stereotyped behaviors (American Psychiatry Association (APA), 2013). Although many interesting theories have been recently proposed (Patterson, 2009; Lucchina and Depino, 2013; Sandin et al., 2014; Gottfried et al., 2015), the ASD etiology remains unknown. This in turn hinders the discovery of new biomarkers and treatments, making ASD a significant individual and societal challenge (Bambini-Junior et al., 2014a; Anderson, 2015; Hu et al., 2017; Masi et al., 2017).

Autism has a high genetic heritability, which can be demonstrated by the high agreement of ASD development in monozygotic twins (reaching values of up to 90%; Dietert et al., 2011), compared to a concordance rate of about 10% in dizygotic twins (Miles, 2011; Yoo, 2015). In addition, some environmental factors are also associated to ASD, including prenatal exposure to valproic acid (VPA; Rodier et al., 1997; Christensen et al., 2013; Smith and Brown, 2014). Thus, based on these clinical observations, an animal model of autism by prenatal exposure to VPA was developed. Since then, it has been extensively validated, demonstrating a myriad of behavioral (Schneider and Przewłocki, 2005; Haddad et al., 2009; Dendrinos et al., 2011; Favre et al., 2013; Gottfried et al., 2013; Roullet et al., 2013; Mabunga et al., 2015), molecular (Roullet et al., 2010; Gottfried et al., 2013), morphological (Rodier et al., 1997; Dendrinos et al., 2011; Favre et al., 2013; Gottfried et al., 2013) and electrophysiological autistic-like features (Dawson et al., 2005; Markram et al., 2008; Rinaldi et al., 2008).

Sensory impairments are one of the most prevalent comorbidities associated with ASD and are identified in more than 90% of patients (Geschwind, 2009). Indeed, hyper- or hyporeactivity to sensory input have been used as one of the four behavioral patterns observed to evaluate restricted, repetitive patterns of behavior, interests, or activities in ASD (American Psychiatry Association (APA), 2013). Common deficits include hyper-responsiveness to non-harmful stimuli (e. g., visual, tactile and auditory) and hypo-responsiveness to harmful (nociceptive) stimuli (American Psychiatry Association (APA), 2013). Studies investigating how sensory stimuli are processed and integrated in patients with ASD and animal models of autism are scarce. Yet, the impairments in the perception of the environment possibly affect both social and repetitive behaviors (Nienborg and Cumming, 2010; Dendrinos et al., 2011; Wöhr et al., 2015) and the sensory deficits can also be valuable for diagnostic purposes (Marco et al., 2011). The prompted Ayres proposed a Sensory integration (SI) theory to explain ASD and other neurological disorders (Cummins, 1991). Actually, previous studies showed correlations between sensory misprocessing in cortical and subcortical regions with altered excitatory/inhibitory balance and disorganization of cortical columnar and laminar pattern in autistic individuals (Spence and Schneider, 2009; Stoner et al., 2014; Khan et al., 2015).

Given the abnormal sensory behaviors present in the animal model of autism induced by VPA, we asked if the neuronal organization in the primary somatosensory area (PSSA) was affected by exposure to VPA. Interneurons have a major role in brain circuits and organization, acting as either switches or pattern generators and providing refinement to the countless connections present in the brain (Xu et al., 2010; Chu and Anderson, 2015). GABAergic neurons expressing parvalbumin ($PV^+$-neurons) are the most common interneurons in the cortex, comprising 40% of the total interneuron population (Staiger et al., 2009; Xu et al., 2010; Rudy et al., 2011) and playing important roles in social memory, attention, integration of different sensory areas (Gogolla et al., 2009; Unichenko et al., 2017) and notably providing a relevancy filter in sensory processing (Yang et al., 2017). Therefore, we addressed the question of whether the $PV^+$-neuron distribution was altered in somatosensory cortex and amygdala in the VPA model and if there was a concomitant change in inhibitory and excitatory synaptic markers in this region.

We recently showed that prenatal treatment with resveratrol (RSV), a polyphenol compound presenting antioxidant and anti-inflammatory properties, prevents altered social behavior in the VPA animal model of autism (Bambini-Junior et al., 2014b). Considering that the social impairments of the VPA animals could be explained, at least partially, by excitatory/inhibitory imbalance in the sensory cortices and amygdala, we asked if RSV could prevent these alterations.

## MATERIALS AND METHODS

### Animals

Wistar rats were obtained from Center of Reproduction and Experimentation of Laboratory Animals (CREAL) and maintained under a standard 12/12-h light/dark cycle (light cycle starting at 7 am and ending at 7 pm) at a constant temperature of 22 ± 1°C. The animals had *ad libitum* access to food and water, and were handled in accordance with the guidelines established by the National Council for the Control of Animal Experimentation (CONCEA) of Brazil. This project was approved by the ethics committee of the Federal University of Rio Grande do Sul (CEUA-UFRGS #31872) and by the Clinical Hospital of Porto Alegre (HCPA-FIPE #160477).

Animals were mated overnight and pregnancy was verified by next morning through presence of spermatozoa in the vaginal smear. This was considered the embryonic day 0.5 (E0.5). Pregnant rats were divided into four groups according to the treatment they received: Control, RSV, VPA, or RSV+VPA. From E6.5 to E18.5, the pregnant females received a daily subcutaneously injection of RSV (Fluxome, Stenløse, Denmark) at 3.6 mg/kg or dimethyl sulfoxide (DMSO, equivalent volume of RSV injection) as previously described (Bambini-Junior et al., 2014b). On E12.5, rats received a single intraperitoneal injection with either

VPA at 600 mg/kg (Acros Organics, NJ, USA) or saline solution 0.9%.

## Behavioral Tasks

### Nest Seeking Behavior

We assessed the nest-seeking (NS) response mediated by olfactory discrimination as described previously (Schneider and Przewłocki, 2005) at the postnatal day 10 (P10). All litter (males and females) was evaluated, since sex is very difficult to determine during behavioral tests before P10 and one would have to manipulate the litter, which could introduce a stress component. The apparatus used was a plastic container (30 × 20 × 13 cm) that was divided in thirds and had the lateral sections covered with wood shavings, but leaving a clear uncovered center. One side is filled with the home-cage bedding (nest shavings) and in the other side with sterilized shavings. The pup was placed in the center of the apparatus and the latency to reach the nest shavings and the time to make any choice was registered. The total time of the test was limited to 60 s. In order to maintain the smell of the litter/mother, we did not change the shavings in the home-cage in the 2 days preceding the test.

### Whisker Nuisance Task (WNT)

During this test, the animal behavioral response to direct vibrissae stimulation was observed in P30 animals. All tests and analyses were performed blindly. Since this is a sensory test, prior to testing, animals were familiarized with handling of the experimenter and habituated with the empty housing (57.1 × 39.4 × 15.2 cm) coated with an absorbent pad. To perform the test, the vibrissae are stimulated with a wooden toothpick for three consecutive periods of 5 min (15 min in total) with a 30 s interval between stimulation (**Figures 1E,F**). Animals were scored according to a scale developed by McNamara et al. (2010), in which freezing, stance and body position, breathing, whisker position, whisking response, evading stimulation, response to stick presentation and grooming are classified from 0 to 2 according to the response (0 = absent/typical, 1 = present/light response and 2 = profound/accentuated response; Supplementary Table S1). The sum of all scores is then calculated. Low scores (0–4) indicate normal responses, in which the animal is calm or indifferent to stimulation. High scores (8–16) indicate abnormal responses to stimulation, in which the animal freezes, shakes, or is aggressive (McNamara et al., 2010).

## Tissue Preparation and Analysis

### Transcardiac Perfusion

P30 male rats were anesthetized (75 mg/kg ketamine +10 mg/kg xylazine) and subjected to transcardiac perfusion with 0.9%-NaCl solution followed by 1.5%-paraformaldehyde and 4%-paraformaldehyde solutions before the removal of the brain. The tissues were post-fixed for 4 h in a 4%-paraformaldehyde solution and subsequently cryoprotected by sequential immersion in 15% and 30%-sucrose solutions in PBS (the tissue was kept in each solution until complete submersion).

### Immunofluorescence

Brains were embedded in Tissue-Tek® and kept in −80°C ultra-freezer until further processing. Coronal brain slices (25 μm) were obtained in cryostat (Leica Microsystems GmbH) and a rat brain atlas (Paxinos and Watson, 1997) was utilized to identify sections containing the PSSA and the amygdala. We obtained the slices both from PSSA and amygdalar region according to the Paxinos rat atlas, using the following markers: Bregma (anteroposterior): −3.48 mm, interaural: 5.52 mm (Figure 62 from Paxinos rat atlas). After the immunofluorescence protocol from these slices described above, we delimited the regions as it follows: for PSSA we first localized the CA2 hippocampal region. Then moved laterally from this region until finding cortical border, where we can find the barrel fields, a specific region for whisker sensory processing. We localized the layer II/III and IV/V examining the cytoarchitecture of neuronal composition positive to NeuN labeling, as described in literature (Narayanan et al., 2017). For amygdalar region, we first localized the external capsule, a white matter region easily identified by DAPI staining. Then, we moved dorsoventrally to the end of this white matter region and, medially to this point, we identified lateral amygdalar region. We consider the amygdalar region since the histological contour of lateral amygdalar complex is difficult to determine.

The immunostaining procedure was performed in the following steps: (1) exposure to vapors of 4%-paraformaldehyde (10 min); (2) three washes with PBS 0.1 M buffer (5 min each); (3) permeabilization with PBS-Triton 0.1% (10 min); (4) three washes with PBS 0.1 M buffer (5 min each); (5) antigen retrieval suing citrate buffer at 60°C (1 h); (6) two washes with PBS-Triton 0.1% (5 min each); (7) blocking with BSA 5% in PBS-Triton 0.1% (1 h); (8) incubation with primary antibodies—diluted to 1:500 in blocking solution—for 48 h at 4°C; (9) five washes with PBS 0.1 M buffer (3 min each); (10) incubation with both secondary antibodies anti-mouse and anti-rabbit—diluted to 1:2000 in blocking solution) for 2 h at room temperature; (11) five washes with PBS 0.1 M buffer (3 min each); (12) incubation with DAPI solution (10 min); and (13) five washes with PBS 0.1 M buffer (3 min each) followed by addition of mounting medium with fluorshield and coverslip. The list of antibodies used in this work is available in the Supplementary Table S2.

Images were obtained with at least eight times per image (dimension: 635.9 × 635.9 microns) in a confocal microscope (Olympus FluoView FV1000 confocal laser scanning) of the Electron Microscopy Core. Processing and quantification of all tomes obtained from two to four tissue sections per glass slide were performed using the ImageJ software with the Cell Counter plug-in. The neuronal quantification results are shown in absolute number of $NeuN^+$ labeled cells (total neuronal cells), $PV^+$ and $NeuN^+$ labeled cells (PV-neuronal cells) and in density of $PV^+$-neuronal cells (which is the ratio between absolute number of $PV^+$-neuronal cells by the total number of

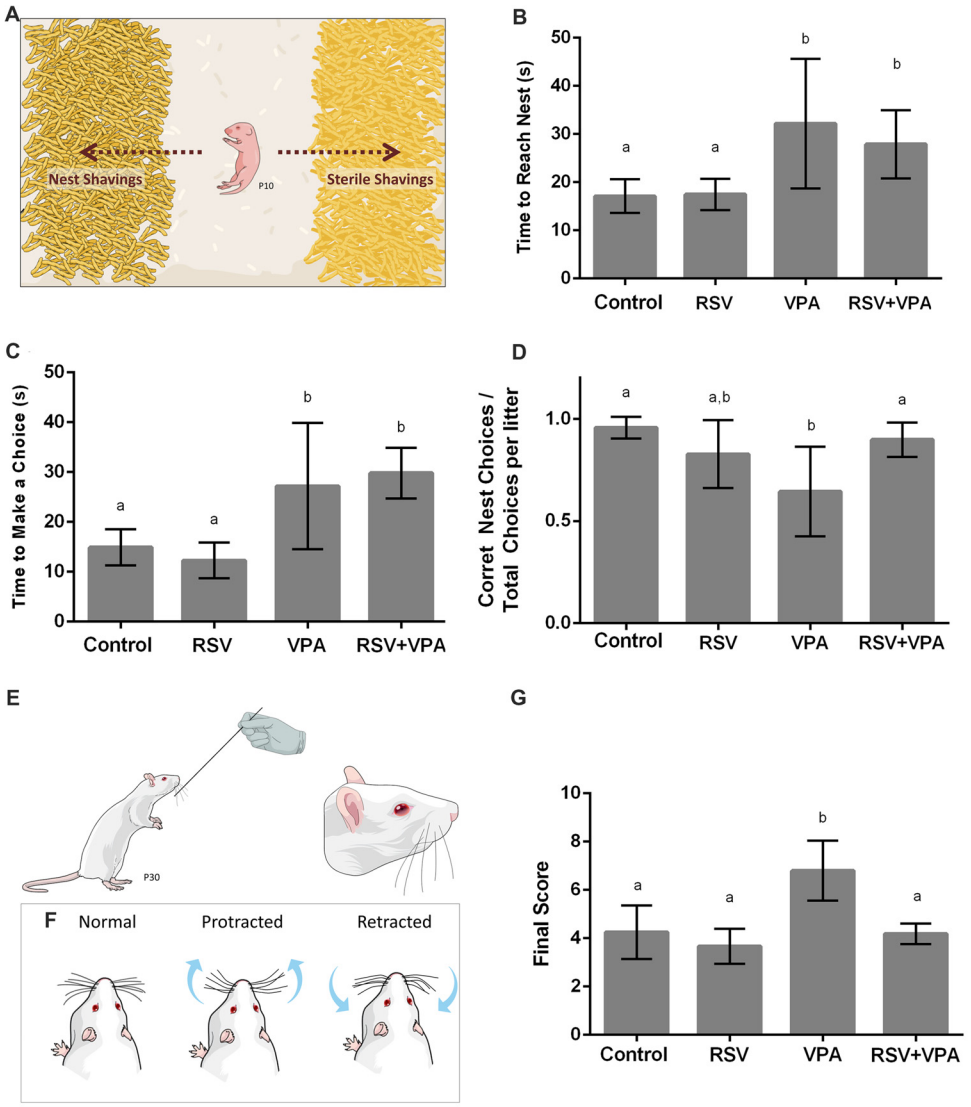


**FIGURE 1 |** Animals from the valproic acid (VPA) model of autism demonstrate impaired sensorial behavior, which is counteracted by resveratrol (RSV). **(A)** Representative illustration from the Nest Seeking (NS) test. RSV administration could not prevent all behavioral alterations in VPA groups, which presented an increasing time to reach the nest **(B)** and increased latency to make any choice **(C)**. Whilst VPA induced significant decrease in the accuracy of the choice, prenatal RSV treatment prevented this feature **(D)**. **(E,F)** Representative illustration of the stimulation in the Whisker Nuisance Task (WNT; **E**) and possible position of whiskers: protracted (curiosity), retracted (avoidance) or not showing any particular response **(F)**. **(G)** Behavioral analysis of WNT, demonstrating the hyper-responsiveness of VPA animals, a behavior prevent by RSV treatment. Different letters indicate statistically significant differences between groups. Values plotted are Mean ± standard deviation (SD). Statistical analysis: ordinary one-way analysis of variance (ANOVA) followed by Bonferroni.

neuronal cells) for layer II/III, layer IV/V and all layers (II/III and IV/V). The number of $PV^+$-neurons, total neurons and total cells were counted by an observer blind to the animal group.

## Eletrophoresis and Western Blotting

Amygdala region (AmR) and PSSA were surgically isolated and fresh tissues homogenates were prepared in lysis buffer. Protein concentration was measured by Lowry method (Lowry et al., 1951), equal amounts of protein (40 μg) were loaded in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After overnight incubation with the primary antibody at 4°C (Anti-PSD95, Anti-gephyrin, Anti-synaptophysin or Anti- β-actin; Supplementary Table S3), membranes were incubated with the proper secondary antibody conjugated to HRP (Donkey anti-mouse-IgG HRP or Goat anti-rabbit-IgG HRP) at room temperature for 1:30 h. No stripping of the membranes was performed (Supplementary Figures S1, S2). The SuperSignal West Pico reagent (Thermo Fisher Scientific) was used and its chemiluminescence was detected using the

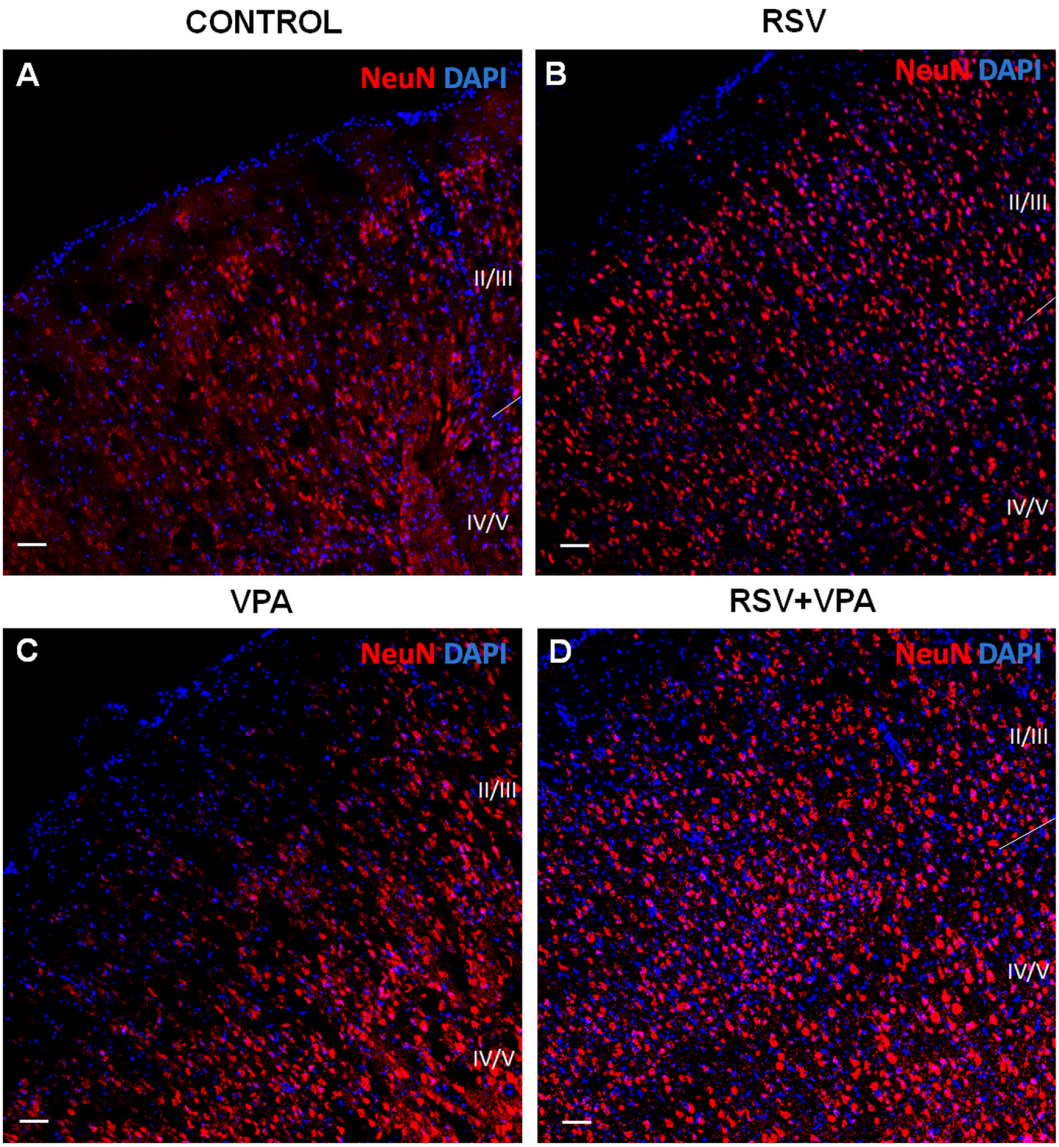


**FIGURE 2 |** RSV prevents the impairments in cortical organization of the primary somatosensory area (PSSA) induced by VPA. **(A–D)** Cell nuclei—blue (DAPI) and total neurons—red (NeuN$^+$). Layers II-III and IV-V can be distinctly visualized by its specific cellularity, enabling a qualitative analysis of the cortical laminar organization between the groups. Scale bar = 50 μm.

ImageQuant LAS 4000 immunodetector (GE HealthCare Life Sciences).

## Statistical Analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* multi comparison test was performed using the IBM SPSS software (version 20.0). Data are reported as mean ± standard deviation (SD), considering significant when $p < 0.05$. In graphic representation, different letters indicate statistically significant differences between the experimental groups. The total number of animals analyzed in each experiment was four animals from at least four different litters per group.

# RESULTS

## In the Nest Seeking Behavior, RSV Prevents the Reduction of Accuracy But Not the Increase in Latency for Choice Induced by VPA

In order to evaluate the effects of VPA and RSV on olfactory discrimination, P10 pups were tested for NS behavior (**Figure 1A**). The following parameters were evaluated: latency to reach the nest shavings, latency to make any choice (nest or sterile shavings) and percentage of correct choices (reach the nest shavings) per litter. The latency to reach the nest

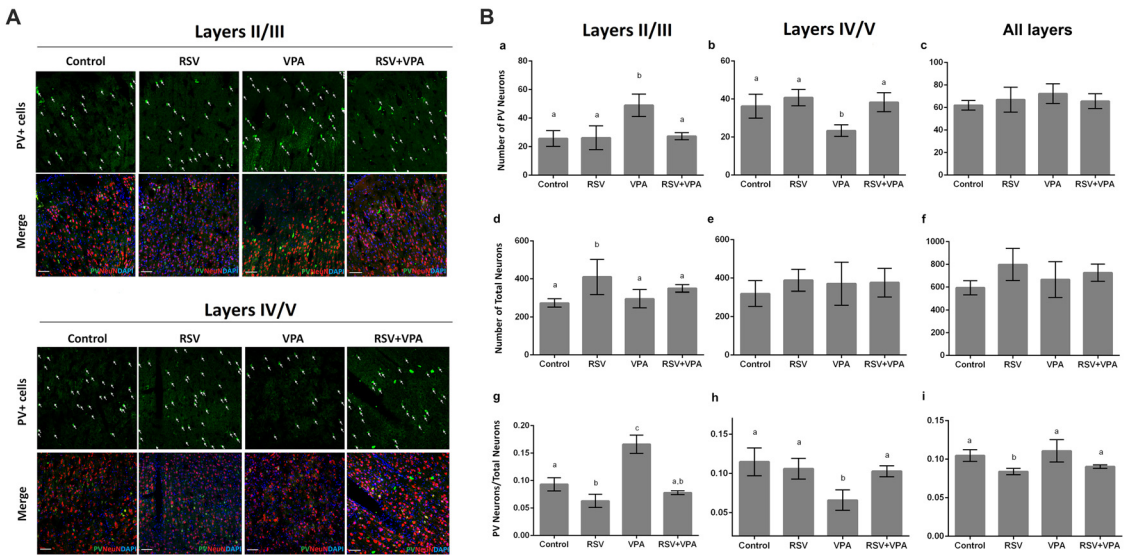


**FIGURE 3 |** RSV averts the abnormal distribution of PV+ GABAergic neurons in the PSSA of animals of the VPA model of autism. Cell nuclei—blue (DAPI), total neurons—red (NeuN$^+$) and GABAergic parvalbumin (PV$^+$) neurons—green. **(A)** Representative immunofluorescence images of PSSA. Layer II-III and IV-V are separated in order to improve visualization of cell distribution. In each case, the first micrograph presents the green channel showing PV$^+$ cells distribution in the indicated groups, followed by the merge image of three channels below (green, red and blue). **(Ba–i)** Quantitative analysis of PV$^+$ cells in layer II-III and IV-V. Different letters indicate statistically significant differences between groups. Values plotted are Mean ± SD. Statistical analysis: ordinary one-way ANOVA followed by Bonferroni. Scale bar = 50 μm.

shavings (**Figure 1B**) was increased in animals of the groups VPA ($p$ = 0.0063) and RSV+VPA ($p$ = 0.0486) when compared to the control group (Control: 17.1 ± 3.4, RSV: 17.4 ± 3.2, VPA: 32.1 ± 13.4, RSV+VPA: 27.8 ± 7.0, $F$ = 6.8). In a similar way, the latency to make any choice (**Figure 1C**) was also increased in VPA ($p$ = 0.0116) and RSV+VPA ($p$ = 0.0011) groups compared to the control (Control: 14.8 ± 3.6, RSV: 12.2 ± 3.5, VPA: 27.2 ± 12.6, RSV+VPA: 29.8 ± 5.1, $F$ = 13.05). Thus, VPA delayed the general response time of the animals, which was not prevented by RSV. There were no significant differences between groups in latency to reach sterile shavings and in the total time spent by the pups in either of the shavings (data not shown). However, prenatal administration of RSV successfully prevented the reduction of percentage of correct choices (**Figure 1D**) seen in the VPA group (Control: 0.95 ± 0.05, RSV: 0.8 ± 0.16, VPA: 0.6 ± 0.22, RSV+VPA: 0.89 ± 0.08, $F$ = 5.9; $p$ = 0.0020, VPA vs. Control and $p$ = 0.0252, VPA vs. RSV+VPA).

## RSV Prevents the Abnormal Response to Direct Whisker Stimulation Observed in Rats of the VPA Model of Autism

Since ASD is usually associated with several impairments in SI, we sought to investigate the behavioral response of rats of the VPA model of autism in the Whisker Nuisance Task (WNT; **Figure 1E**). Animals from the VPA group increased WNT scores (**Figure 1G**) as compared to the control group ($p$ = 0.0027, VPA compared to the control group). Strikingly, prenatal treatment with RSV averted the over-responsiveness induced by VPA (Control: 4.2 ± 1.1, RSV: 3.6 ± 0.72, VPA: 6.8 ± 1.2, RSV+VPA: 4.2 ± 0.42, $F$ = 12.4; $p$ = 0.0006, VPA vs. RSV+VPA group).

## RSV Counteracts the VPA Effects on Neuronal Organization in the Primary Somatosensory Cortex, Promoting Typical Laminar Distribution and Localization of PV$^+$-Neurons

As shown in **Figures 2A,B**, the control and RSV groups presented a typical cortical organization, with a high number of medium-sized pyramidal neurons in layer II-III and granular and largest pyramidal neurons in layer IV-V, and a low cellularity between these layers. However, the VPA exposure induced visible alterations in cellular organization, increasing the space between layer I and layer II-III while drastically reducing the spacing between layer II-III and layer IV-V (**Figure 2C**). Interestingly, prenatal RSV treatment was able to prevent the VPA effects on cortical organization (**Figure 2D**).

We then asked if there was any change in the number of PV$^+$- neurons in layer II-III and layer IV-V of the PSSA. Representative micrographs of the layer II-III (**a–d**) and layer IV-V (**e–h**) are shown in **Figure 3A**. Our quantitative analysis revealed a significant increase in number of PV$^+$ neurons in layer II-III of the VPA group, when compared to control animals ($p$ = 0.0029). In addition, as showed in **Figure 3Ba**, RSV treatment successfully prevented this alteration (Control: 25.7 ± 5.5, RSV: 26.2 ± 8.3, VPA: 49 ± 7.8, RSV+VPA: 27.3 ± 2.5, $F$ = 11.1; $p$ = 0.0087, VPA compared to RSV+VPA

group). On the other hand, animals of the VPA group showed reduced numbers ($p$ = 0.0180) of PV$^+$-neurons in layer IV-V, as compared to the control group (**Figure 3Bb**). Interestingly, RSV was also able to prevent this change (Control: 36.2 ± 6.3, RSV: 40.7 ± 4.2, VPA: 23.3 ± 3.3, RSV+VPA: 38.3 ± 5.03, $F$ = 10.2; $p$ = 0.0110, VPA compared to the RSV+VPA group). No significant differences were found in the sum of PV$^+$-neurons of all cortical layers, suggesting an impairment of neuron localization rather than overall quantity (**Figure 3Bc**).

We also evaluated the total number of neurons in layer II-III and layer IV-V of PSSA using immunostaining with the NeuN marker. Our results pointed out for an effect of RSV exposure ($p$ = 0.0328), increasing the total number of neurons in layer II-III (**Figures 3Bd–f**), in comparison to the control group (Control: 274.3 ± 21.8, RSV: 410.5 ± 91.9, VPA: 296.3 ± 47.5, RSV+VPA: 350 ± 19.9, $F$ = 4.7).

When normalizing the number of PV$^+$-neurons to the total number of neurons, the VPA group presented a significantly increased ratio in layer II/III ($p < 0.0001$), which was prevented by RSV ($p < 0.0001$, VPA compared to the RSV+VPA group). Surprisingly, the RSV treatment *per se* reduced the ratio PV$^+$-neurons/total neurons (**Figure 3Bg**; $p$ = 0.0356) in this same region when compared to control (Control: 0.09319 ± 0.01211, RSV: 0.063 ± 0.01, VPA: 0.16 ± 0.016, RSV+VPA: 0.07 ± 0.003, $F$ = 52.4). We also observed a significant decrease in the PV$^+$-neurons/total neurons ratio in the VPA group compared to controls (**Figure 3Bh**; $p$ = 0.0023). Importantly, this alteration was also prevented by RSV (Control: 0.11 ± 0.02, RSV: 0.11 ± 0.01, VPA: 0.06 ± 0.01, RSV+VPA: 0.1 ± 0.007, $F$ = 9.8; $p$ = 0.0290 to VPA vs. RSV+VPA). When combining all layers, we only observed an effect of RSV (**Figure 3Bi**), reducing the PV$^+$-neurons/total neurons ratio (Control: 0.10 ± 0.007, RSV: 0.08 ± 0.004, VPA: 0.11 ± 0.014, RSV+VPA: 0.09 ± 0.002, $F$ = 7.5; $p$ = 0.0438). Thus, our immunofluorescence data suggests a protective effect of RSV for the laminar organization and correct distribution of PV$^+$-neurons in animals of the VPA model of autism.

## RSV Reestablishes a Typical Proportion of PV$^+$-Neurons in the Amygdala

Given the importance of the AmR to attribute affective content to sensory information, we also evaluated the quantity of PV$^+$-neurons in this region. **Figures 4Aa–d** shows illustrative micrographs from PV$^+$-neuron distribution in the amygdala of our four experimental groups. As showed in **Figure 4Ba**, no significant differences were observed in the number of PV$^+$-cells (Control: 33.8 ± 6.6, RSV: 25.6 ± 7.5, VPA: 24.2 ± 8.5, RSV+VPA: 32.1 ± 3.5, $p$ = 26, $F$ = 1.55) or (**Figure 4Bb**) in the total number of neuronal cells (Control: 221.8 ± 71.6, RSV: 413 ± 17.5, VPA: 402.6 ± 97.9, RSV+VPA: 277.3 ± 46.3, $p$ = 0.2119, $F$ = 1.79). Nevertheless, we observed a significant reduction in the PV$^+$-neurons/total neurons ratio (**Figure 4Bc**) in the RSV ($p$ = 0.0040) and VPA groups ($p$ = 0.0015), when compared to the control

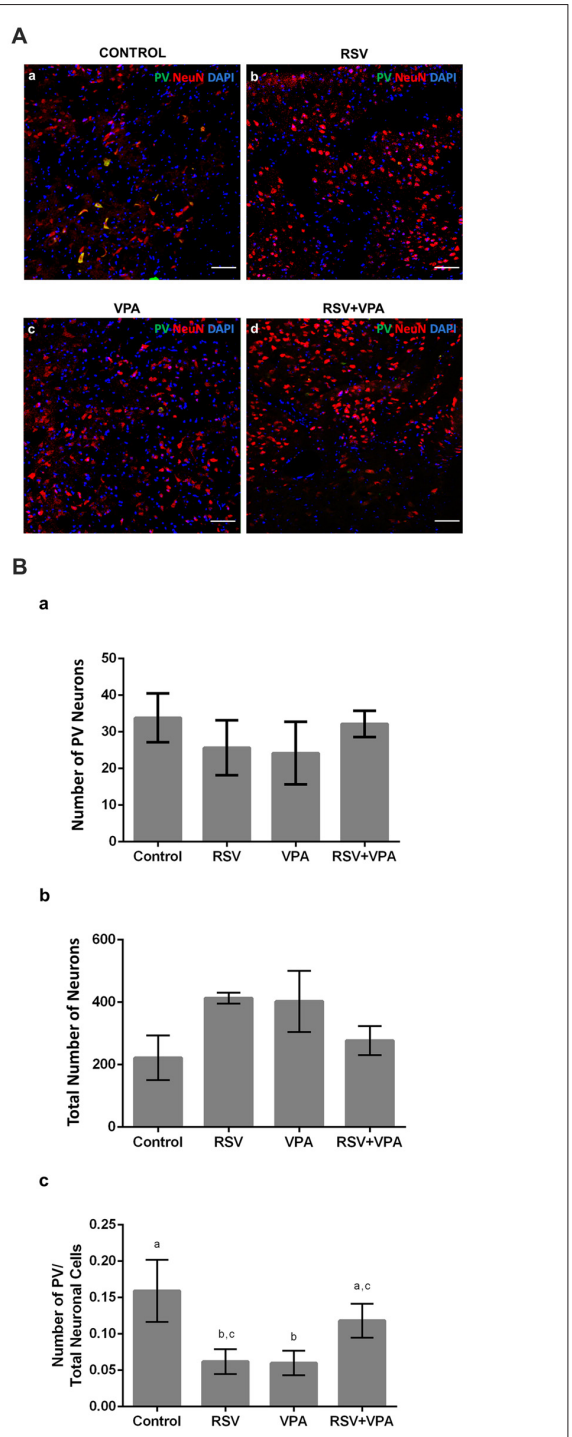


**FIGURE 4 |** RSV restores the correct proportion of PV+ neurons in the amygdala of rats of the animal model of autism induced by VPA. Cell nuclei—blue (DAPI), total neurons—red (NeuN$^+$) and GABAergic PV$^+$ neurons—green (PV$^+$). **(Aa–d)** Representative immunofluorescence images of the amygdala Region (AmR). Cell distribution and organization can be visualized along the basolateral portion of AmR. **(Ba–c)** Quantitative analysis of the density of PV$^+$ neurons in the AmR. Different letters indicate statistically significant differences between groups. Mean ± SD were represented. Statistical analysis: ordinary one-way ANOVA followed by Bonferroni. Scale bar = 50 μm.

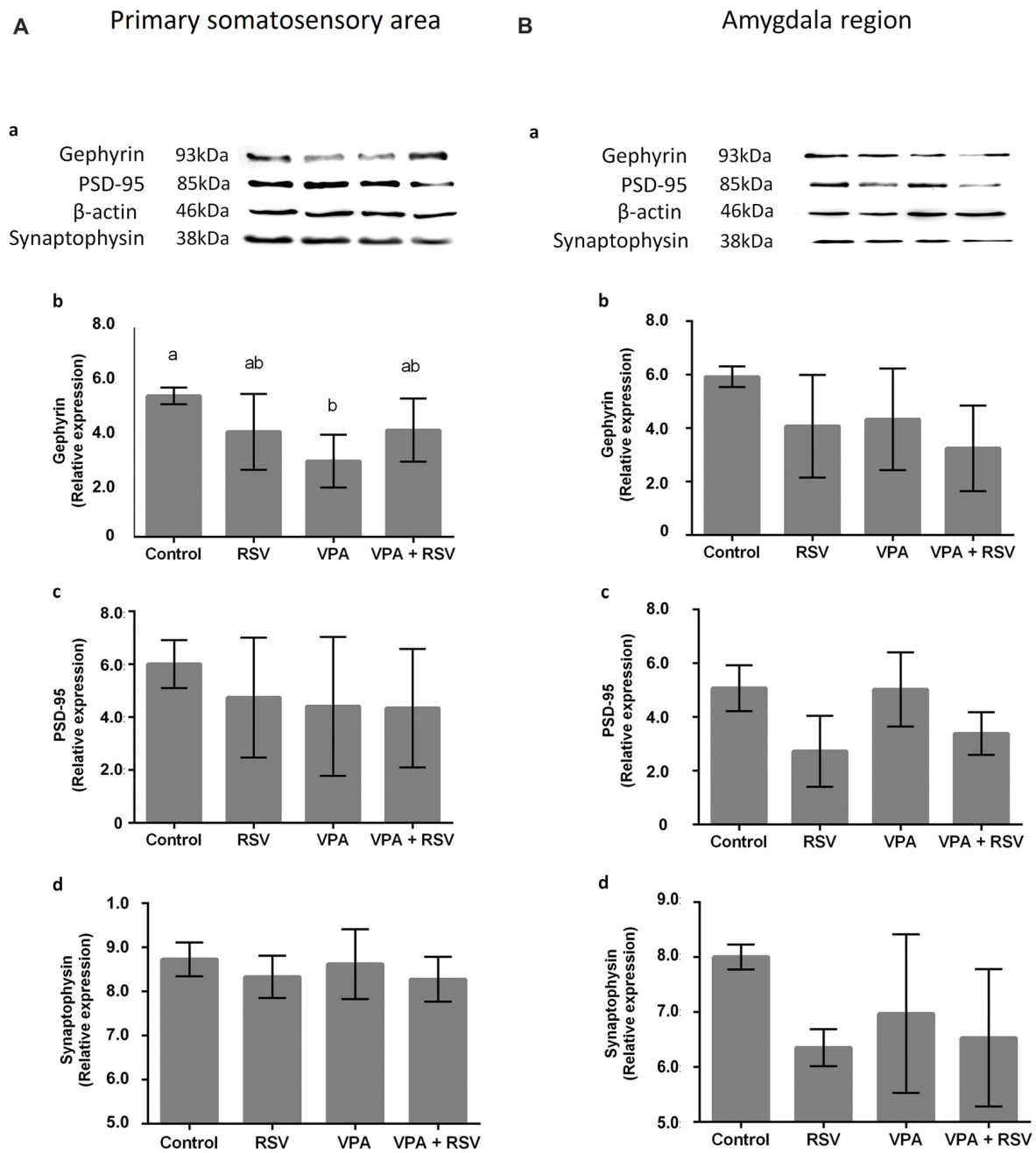


**FIGURE 5 |** Effects of prenatal exposure to VPA and RSV in synaptic proteins in PSSA and AmR. Representative images of Western blotting for synaptic proteins are shown in **(Aa)** from PSSA and in **(Ba)** from AmR. Protein quantification of gephyrin, PSD-95 and synaptophysin are shown in **(Ab–d)** for PSSA and in **(Bb–d)** for AmR, respectively. Different letters indicate statistically significant differences between groups. Mean ± SD were plotted. Statistical analysis: ordinary One-Way ANOVA followed by Bonferroni.

group. Interestingly, RSV was able to totally prevent the VPA effect (Control: 0.16 ± 0.04, RSV: 0.06 ± 0.017, VPA: 0.059 ± 0.016, RSV+VPA: 0.12 ± 0.02, $F = 12.09$). Here, our results demonstrate interesting and complex effects of RSV and VPA exposures in the AmR in which the combined actions of VPA and RSV normalized the $PV^+$/total neurons ratio.

## VPA and RSV Modulate Synaptic Proteins in Primary Somatosensory Area (PSSA) and Amygdala Region (AmR)

Next, we asked if the neuronal reorganization promoted by VPA and prevented by RSV in PSSA and AmR influenced the overall expression of synaptic proteins. We evaluated proteins

from excitatory (PSD-95) and inhibitory (gephyrin) synapses, as well as synaptophysin, an ubiquitous pre-synaptic component. Illustrative western blot images are shown in **Figure 5Aa** (PSSA) and **Figure 5Ba** for (AmR). No significant differences were observed between groups in PSD-95 levels in PSSA (**Figure 5Ac**) or AmR (**Figure 5Bc**). On the other hand, gephyrin levels were reduced in the PSSA of VPA animals compared to the control group (Control: $5.306 \times 10^7 \pm 3.123 \times 10^6$, RSV: $3.959 \times 10^7 \pm 1.426 \times 10^7$, VPA: $2.859 \times 10^7 \pm 9.937 \times 10^7$, RSV+VPA: $4.020 \times 10^7 \pm 1.188 \times 10^7$, $F = 3.54$; $p = 0.0305$). In the PSSA of RSV+VPA animals, gephyrin is expressed at intermediate levels between the control and VPA groups, not being statistically different from either one (**Figure 5Ab**). No significant differences were observed in the expression of gephyrin in the AmR of our experimental groups (**Figure 5Bb**). The synaptophysin and PSD-95 expression levels are not significantly different between groups in PSSA (**Figure 5Ad**) or AmR (**Figure 5Bd**).

## DISCUSSION

The response to sensory stimuli is altered in more than 90% of ASD patients resulting in great impairment in synaptic transmission and processing, affecting the health of these individuals (Coskun et al., 2009; Geschwind, 2009). These alterations include hyper-responsiveness to auditory, visual and tactile stimuli and hypo responsiveness to nociceptive stimuli. An interesting hypothesis suggests a perturbation in the processing and integration of the sensory information between different areas, resulting in local and global alterations, from neurotransmitter release, to the neural network, leading to disruption of sensory perception (Coskun et al., 2013; Supekar et al., 2013). Here we report, for the first time, that RSV prevented the alterations caused by VPA in the WNT, a behavioral test that evaluates quality of response to direct whisker stimulation. In addition to the striking result in the present work, RSV was able to preserve the cortical laminar patterning and the distribution of $PV^+$-neurons affected by VPA in the PSSA, the brain area related to tactile processing of the whiskers (Chen-Bee et al., 2012). These results corroborate similar findings in the literature in other brain regions involved in sensory processing, such as the superior colliculus, presenting dysfunctional sensory processing characterized by an inability to filter sensory information and impairments in GABAergic synaptic transmission, particularly simultaneously arriving multimodal inputs (Dendrinos et al., 2011).

The behavior evaluated in the NS test is of extreme importance for the development of rats, which need major care from their mothers to survive. This attachment behavior is present in many species (Broad et al., 2006) and combines a sensory component (olfactory, in rodents) with an affective memory, creating a mother-offspring connection that allows young animals (who can barely see or hear at this age) to find the source of food and protection. The AmR is an important area for primary attachment behaviors and other emotional responses, such as aversive behavior (Landers and Sullivan, 2012; Rigon et al., 2016). In the context of ASD, it is known that many alterations were already described in the amygdala, including hyper reactivity (Markram et al., 2008; Lin et al., 2013), enlargement and hyper cellularity (Markram et al., 2008; Ecker et al., 2015), elevated NMDA receptor levels and enhanced postsynaptic long-term potentiation (LTP; Rinaldi et al., 2007). Therefore, our hypothesis highlights the possibility that an alteration in this region is causing the impairment identified in the NS task and can be related to the outcomes in social development. Interestingly, studies have demonstrated that the AmR seems to develop faster in animals of the VPA model of autism. It results in premature maturation of fear responses, caused by the hyper reactivity, hyper plasticity and deficits in inhibitory system found in the lateral amygdala, which could lead to an early termination of the attachment learning period (Markram et al., 2008; Landers and Sullivan, 2012; Barrett et al., 2017).

We report in our present work that RSV reestablishes the typical proportion of GABAergic $PV^+$-neurons in the amygdala of VPA animals, which might be crucial for proper inhibition of responses in the amygdala (McDonald and Betette, 2001; Woodruff and Sah, 2007; Bocchio et al., 2015). The majority of $PV^+$-neurons originate in the medial ganglionic eminence and migrate to their target regions in the brain (Guo and Anton, 2014). The ganglionic eminence is a temporary brain structure first observed at E11.5 in rodents (Lavdas et al., 1999; Anderson et al., 2001; Marín et al., 2001) with the first interneurons starting their tangential migration towards the cortex at E12.5 in mice (Kelsom and Lu, 2013). This coincides with our hypothesis of the time point of VPA administration and opens the intriguing possibility that VPA interferes either with GABAergic interneuron specification and proliferation or their migration to the cortical layers and their survival in the cortex, promoting the increase of $PV^+$-neuronal density in layer II/III and the decrease of this subpopulation in layer IV/V. In fact, studies with 7 days old mice of the VPA model of autism present a reduced number of $BrdU^+$ cells (generated at E12.5) in the deep layers of the somatosensory and prefrontal cortices, indicating several impairments in cortical migration related to cortical areas (Kataoka et al., 2013).

A decreased number of $PV^+$-neurons and of PV mRNA was observed in several animal models related to ASD (Gogolla et al., 2009; Wöhr et al., 2015; Lauber et al., 2016) and a recent study demonstrated reduction in the number of $PV^+$-neuros in the medial prefrontal cortex of individuals with autism (Hashemi et al., 2017). Even though the significance of the reduction of $PV^+$-neurons in ASD remains unclear, PV knockout mice display behavioral phenotypes related to all the core symptoms present in ASD patients, such as abnormal reciprocal social interactions, altered ultrasonic vocalization and presence of repetitive/stereotyped patterns of behavior (Wöhr et al., 2015). Moreover, there is an increase of apoptosis in the developing neocortex 12 h and 24 h after VPA exposure, accompanied by a reduction in proliferation in the ganglionic eminences (Kataoka et al., 2013). This can likely have widespread and long-lasting consequences to brain organization, since, during development, GABAergic neurons are excitatory and promote the maturation of neural networks (Le Magueresse and Monyer, 2013).

Finally, in western blotting analysis, we observed in this work a significant alteration in the expression level of the protein gephyrin (a key scaffolding protein of inhibitory synapses) in PSSA: VPA decreased its levels and RSV treatment was able to prevent this impairment. These findings corroborate previous studies showing an excitatory/inhibitory imbalance in cortical regions of animal models of ASD (Rinaldi et al., 2008; Gao and Penzes, 2015; Nelson and Valakh, 2015). Since, VPA affects the $PV^+$-neuron localization and distribution, this might lead to altered inhibitory synaptic distribution and organization, as seen in autistic individuals (Zikopoulos and Barbas, 2013; Gao and Penzes, 2015; Nelson and Valakh, 2015). Thus, the RSV prevention could be related to the excitation/inhibition balance restoration in the PSSA, leading to correct sensory perception/processing.

It is also possible that both RSV and VPA primarily exert independent actions in the developing nervous system, modulating neuronal proliferation, migration and establishment of synaptic connections. It is worth to mention that RSV is itself, a teratogen. Therefore, we are not proposing this approach as a "vaccine" to ASD but as a potential research tool, that could help to clarify specific mechanisms related to ASD etiology and pathophysiology.

Our results from behavioral, histological and protein analyses showed a relevant impact of RSV treatment in sensory aspects of the VPA animal model of autism. Thus, RSV can be used as a tool to study pathways related to ASD pathophysiology, and further investigation of VPA effects counteracted by RSV can help to shed light in molecular mechanisms involved in the etiology of ASD.

## CONCLUDING REMARKS

Taken together, our data showed important deficits in the processing and integration of sensory information in the VPA animal model of autism, corroborating the face validity of this model. Furthermore, the prenatal treatment of RSV successfully prevented sensory deficits in behavioral analyses, possibly by correcting altered $PV^+$-neuron localization and cortical organization impaired by VPA.

We suggest that not only the correct number, but also localization of $PV^+$-neurons throughout the PSSA cortical layers, might play important roles in proper sensory processing, refining the excitatory inputs. Additionally, in AmR, the correct balance of this neuronal subpopulation might be necessary to attribute the correct emotional load to sensory information, providing a refined and complex cognitive experience. Thus, the perturbation of PV by VPA may be an important player in the sensory behavioral deficits evaluated.

Since RSV appears as a promising molecule for investigation of ASD etiology and pathophysiology, it is important to explore whether these effects result from its anti-inflammatory or anti-oxidant properties or from previously unrecognized activities of this compound. Furthermore, it will be of upmost importance to investigate the opposite actions of VPA and RSV during embryonic development and in the pregnant female to characterize the molecular alterations involved in the triggering of autistic-like alterations in the VPA animal model of autism. Again, we think that our data support the possible therapeutic use of RSV, but future studies have to be done to show if RSV has any beneficial effect on the postnatal development of animals presenting autistic-like features.

## AUTHOR CONTRIBUTIONS

MF-D, CG, VB-J, RR and CH-P: experimental design and intellectual contribution. MF-D, JS-T, ID, GBS, GD-FN, MMH and GB-N: *in vivo* and *in vitro* analyses. MF-D, JS-T, ID, GBS, GD-FN, MMH, GB-N, CH-P, VB-J, RR and CG: data discussion and manuscript preparation.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnsyn.2018.00009/full#supplementary-material

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# Oxytocin as a Modulator of Synaptic Plasticity: Implications for Neurodevelopmental Disorders

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The neuropeptide oxytocin (OXT) is a crucial mediator of parturition and milk ejection and a major modulator of various social behaviors, including social recognition, aggression and parenting. In the past decade, there has been significant excitement around the possible use of OXT to treat behavioral deficits in neurodevelopmental disorders, including autism spectrum disorder (ASD). Yet, despite the fast move to clinical trials with OXT, little attention has been paid to the possibility that the OXT system in the brain is perturbed in these disorders and to what extent such perturbations may contribute to social behavior deficits. Large-scale whole-exome sequencing studies in subjects with ASD, along with biochemical and electrophysiological studies in animal models of the disorder, indicate several risk genes that play an essential role in brain synapses, suggesting that deficits in synaptic activity and plasticity underlie the pathophysiology in a considerable portion of these cases. OXT has been repeatedly shown, both *in vitro* and *in vivo*, to modify synaptic properties and plasticity and to modulate neural activity in circuits that regulate social behavior. Together, these findings led us to hypothesize that failure of the OXT system during early development, as a direct or indirect consequence of genetic mutations, may impact social behavior by altering synaptic activity and plasticity. In this article, we review the evidence that support our hypothesis.

**Keywords: oxytocin, synaptic plasticity, neurodevelopmental disorder, autism spectrum disorder (ASD), animal models for ASD**

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## INTRODUCTION

Behaviors are driven by diverse sets of functionally and anatomically connected brain regions that form brain circuits (Insel and Fernald, 2004; Goodson and Kabelik, 2009; Averbeck and Costa, 2017; Kohl et al., 2017; Roseberry and Kreitzer, 2017; Yang and Wang, 2017). Communications within brain circuits are not hard-wired but rather constantly adapting to the environment via neuromodulatory mechanisms. These mechanisms involve various neuromodulators, including neuropeptides, which exert their effect on neural ensembles to construct and modulate the circuit function and to shape a specific behavior (Marder, 2012; Nusbaum and Blitz, 2012). The last two decades have been enriched with studies exploring the behavioral effects of the pro-social

oxytocin (OXT) neuropeptide (Heinrichs et al., 2003, 2009; Kirsch et al., 2005; Guastella et al., 2008a,b; Marsh et al., 2010; Guastella and MacLeod, 2012) and its mechanisms of action (Blume et al., 2008; Jurek et al., 2012; van den Burg et al., 2015). In parallel, several clinical and preclinical studies have focused on the therapeutic potential of OXT, mainly to treat social behavior deficits (Guastella and Hickie, 2016; Wagner and Harony-Nicolas, 2017). However, little attention has been paid to the possible implication of the OXT system in neurodevelopmental disorders and to whether perturbation in OXT may contribute to the social behavior phenotype. The objective of this review is to provide a framework for the role OXT plays in modulating synaptic plasticity and its implication in neurodevelopmental disorders. We begin by summarizing studies that examined the role of OXT in regulating synaptic plasticity underlying behavior. We then highlight studies that report specific alterations in the OXT system in rodent models of neurodevelopmental disorders. Finally, we explore the potential convergence between the OXT system and genes associated with neurodevelopmental disorders, focusing on the *SHANK3* gene.

## THE OXYTOCIN SYSTEM

OXT is a neuropeptide that is exclusively synthesized in neurons residing in the paraventricular (PVN), supraoptic (SON) and accessory nuclei (AN) of the hypothalamus. These nuclei harbor two major types of OXT-producing cells: magno- and parvocellular neurons (Althammer and Grinevich, 2017). Magnocellular OXT neurons project to the posterior pituitary for OXT release into systemic circulation and concomitantly send axonal collaterals to a large proportion of forebrain regions. Parvocellular (or preautonomic) OXT neurons differ in their projections to the midbrain, brainstem and spinal cord, thus controlling autonomic and metabolic processes as well as processing of nociceptive and non-nociceptive information (see reviews: (Althammer and Grinevich, 2017; Boll et al., 2017; Poisbeau et al., 2017)).

In rodents, OXT action is conveyed through the OXT receptor (OXTR), whose expression shows a significant overlap with axon terminals of OXT neurons within the brain (Grinevich et al., 2016; Marlin and Froemke, 2017). OXTR is a G-protein-coupled receptor that is capable of enhancing (Knobloch et al., 2012; Stoop, 2012) or reducing (Eliava et al., 2016) the excitability of neural cells via distinct mechanisms, including its dual coupling to Gq or Go/Gi proteins (Gravati et al., 2010; Busnelli et al., 2012). Among the intracellular signaling pathways activated by the OXTRs is the mitogen-activated protein kinase (MAPK) cascade (van den Burg and Neumann, 2011), whose role in regulating specific behaviors is now beginning to emerge. For example, it has been demonstrated that the OXT anxiolytic effect requires OXTR/MEK/ERK signaling (Blume et al., 2008; Jurek et al., 2012; van den Burg et al., 2015), and that this anxiolytic pathway strictly requires the influx of extra-cellular calcium through transient receptor potential vanilloid (TRPV) channels (van den Burg et al., 2015).

## OXYTOCIN, SYNAPTIC PLASTICITY AND BEHAVIOR

Long-term potentiation (LTP) and long-term depression (LTD) are the most common forms of long-term synaptic plasticity. Both are long-lasting changes in synaptic strength induced by certain patterns of synaptic activity (Cooke and Bliss, 2006). LTP and LTD are considered as putative synaptic mechanisms that mediate learning and memory (Redondo and Morris, 2011). The effect of OXT on LTP was first demonstrated by Dubrovsky et al. (2002) in the rat hippocampus. The authors examined the effect of intracerebroventricular (ICV) administration of OXT (1 μg) on LTP induction *in vivo* using high-frequency tetanic stimulation in the dentate gyrus (DG) of anesthetized rats. They found that, in the presence of OXT, tetanic stimulation induced LTD rather than the expected LTP. We have reported a similar effect of OXT in the medial nucleus of the amygdala (MeA) in anesthetized rat. Specifically, we examined the effect of ICV OXT administration (1 μg) on synaptic plasticity induction by tetanic stimulation of the accessory olfactory bulb (AOB) (Gur et al., 2014) and demonstrated that OXT strongly augments LTD induction in the AOB-MeA pathway. The MeA is an essential component of the brain network that subserves social recognition memory (SRM) (Ferguson et al., 2002), a subtype of social memory that enables subjects to remember and distinguish individual conspecifics (Gheusi et al., 1994). In agreement with previous findings (Ferguson et al., 2001; Lukas et al., 2013), our study validated that OXT in the MeA is crucial for SRM and further suggested that the OXT mediated LTD in the AOB-MeA pathway is involved in consolidating long-term SRM (Gur et al., 2014). We have recently demonstrated that ICV administration of CRF-related peptide urocortin3 or 17β-estradiol 45 min before OXT administration induced LTP rather than LTD in the MeA in response to AOB stimulation, a result that suggests a bidirectional long-term plasticity in the AOB-MeA synaptic pathway (Frankiensztajn et al., 2018).

In 2003, Tomizawa et al. (2003) reported that OXT perfusion (1 μM) of mouse hippocampal slices enhanced the ability of subthreshold synaptic stimulation to induce long-lasting LTP (L-LTP) at Schaffer collateral-CA1 synapses. The authors also demonstrated that this induction was mediated by the activation of the MAPK cascade and phosphorylation of cyclic AMP-responsive element binding protein (CREB), suggesting that OXT induced neuronal plasticity in the hippocampus is transcription-dependent. In an attempt to correlate these findings with behavior, the authors demonstrated that ICV administration of OXT in virgin mice improved long-term spatial learning, a result that aligned with a previous discovery showing that spatial memory is enhanced during pregnancy, delivery and lactation, situations when OXT levels are substantially high (Kinsley et al., 1999). Similarly, Lin et al. (2012) demonstrated that

| Syndrome | Fragile X | Rett | Tuberous Sclerosis | Prader- Willi | Cortical Dysplasia and Focal Epilepsy | Phelan McDermid |
|---|---|---|---|---|---|---|
| Gene | *FMR 1* | *MECP2* | *TSC1 & TSC2* | *MAGEL2, MAGED1, & NECDIN* | *CNTNAP2* | *SHANK3* |
| Rodent model | Mouse & rat | Mouse | Mouse & rat | Mouse | Mouse | Mouse & rat |
| Synaptic plasticity | Altered LTP and/or LTD in CA1, cingulate cortex, amygdala, and visual cortex (Reviewed in Bhakar, Dolen, & Bear, 2012), and rat model results in Tian et al., 2017; Till et al., 2015). | Altered LTP and/or LTD in CA1 (Reviewed in Chung, Bey, & Jiang, 2012). | Impaired LTP and LTD in CA1 (Reviewed in Kirschstein, 2012). | N/A | N/A | Altered LTP and or LTD in CA1 in and altered Hippocampal-PFC LTP (Reviewed in (Harony-Nicolas, De Rubeis, Kolevzon, & Buxbaum, 2015), and rat model results in (Harony-Nicolas et al., 2017) |
| Oxytocin system | N/A | N/A | N/A | *Maged1* and *Magel2* deficient pups show a reduction in the production of mature oxytocin in the PVN. *Magel2* also show higher levels of mature oxytocin in the PVN, and increased innervation of target brain areas by these neurons. *Necdin*-deficient mice also show a significant reduction in the number of OT-neurons in the hypothalamus (reviewed in (Wagner & Harony-Nicolas, 2017) | Decreased oxytocin expression in the PVN and decreased oxytocin levels in brain extracts (Penagarikano et al., 2015). | N/A |
| Effect of oxytocin on synaptic plasticity deficits | N/A | N/A | N/A | N/A | N/A | Reversal of hippocampal LTP and hippocampal-mPFC LTP deficits (Harony-Nicolas et al., 2017) |
| Effect of oxytocin on behavioral deficits | N/A | N/A | N/A | Reversed of social behavior deficits in *Magel2*-deficient mice (Meziane et al., 2015) | Reversal of social behavioral deficits (Penagarikano et al., 2015). | Reversal of social recognition memory and attentional deficits (Harony-Nicolas et al., 2017). |

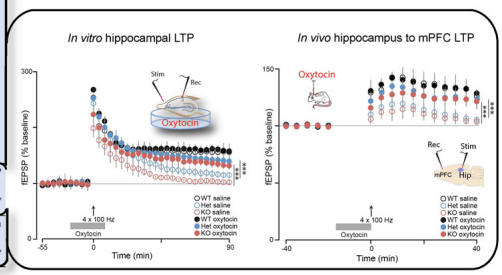


**FIGURE 1 |** The table summarizes the available knowledge on synaptic plasticity deficits, alteration in the oxytocin (OXT) system, and the effect of OXT administration on behavioral and/or synaptic plasticity deficits in six syndromes associated with autism spectrum disorder (ASD). Inset shows the effect of OXT on synaptic plasticity deficits in the *Shank3*-deficient rat model. Reproduced from Figure 6, Harony-Nicolas et al., 2017, eLife, published under the Creative Commons Attribution 4.0 International Public License CC BY 4.0; (https://creativecommons.org/licenses/by/4.0/). *FMR1*, Fragile X mental retardation; *MECP2*, Methyl-CpG-binding protein 2; *TSC1/2*, Tuberous Sclerosis 1/2; *MAGEL2*, MAGE Family Member L2; *MAGED1*, MAGE Family Member D1; *CNTNAP2*, contactin associated protein like 2; *SHANK3*, SH3 And Multiple Ankyrin Repeat Domains 3; LTP, Long-term potentiation; LTD, Long-term depression; mPFC, medial prefrontal cortex; PVN, paraventricular nucleus.

endogenous OXT contributes to the maintenance of late but not early phase LTP, which was induced by subthreshold stimulation. Furthermore, they showed that the OXT-induced enhancement of LTP is OXTR dependent and involves an EGFR-mediated rapid and persistent increase in the local translation of an atypical protein kinase C (PKC) isoform, thus describing a mechanism for OXT-dependent LTP. Notably, stress is known to have a prolonged negative effect on memory and synaptic plasticity (Kim et al., 2015). In rats, an uncontrollable stress experience following unpredictable and unescapable shocks causes impairment in hippocampal-dependent memory tasks and leads to deficits in both LTP and LTD (Foy et al., 1987; Shors et al., 1989; Xu et al., 1997; Kim et al., 2001). The effect of OXT on stress-induced impairments in synaptic plasticity and cognition has been recently addressed in two separate studies by the same group (Lee et al., 2015; Park et al., 2017). These studies demonstrated that administration of intranasal OXT before or after the stress event could reverse the LTP and LTD deficits observed in hippocampal slices as well as improve spatial memory impairments by activating OXTRs and regulating ERK activity.

The nucleus accumbens (NAc) is a key component of the mesocorticolimbic dopamine reward circuit and is known to be a target for synaptic plasticity-associated changes induced by drugs of abuse (Luscher and Malenka, 2011). Dolen et al. (2013) have recently shown that within the NAc, a region that is also implicated in social reward, OXT acts as a social reinforcement signal, and blocking OXTRs in the NAc inhibits the establishment of a preference for social cues. Furthermore, they showed that bath application of OXT (1 μM) induced a presynaptic LTD in NAc medium spiny neurons caused by decreased presynaptic neurotransmitter release probability. Using viral genetic tools, they demonstrated that presynaptic OXTRs on serotonergic axon terminals, arriving from the dorsal raphe nucleus to the NAc, are required for social reward and OXT-induced LTD, which they found to be dependent on the coordinated activity of OXT and serotonin (Dolen et al., 2013).

The effect of OXT on synaptic plasticity has also been studied in mouse brain slices from other brain regions. In mouse slices of infralimbic medial prefrontal cortex, OXT (100 nM) produced a significant suppression of basal glutamatergic neurotransmission through reduction of presynaptic glutamate release and lead to conversion of the activity dependent LTD to LTP. This OXT-dependent conversion is NMDA receptor-dependent and requires synaptic insertion of calcium-permeable AMPA receptors (Ninan, 2011). In slices of the AOB, OXT (0.2–2 μM) facilitated the induction of a NMDA receptor-dependent LTP in reciprocal synapses of excitatory mitral cells on inhibitory interneurons, thus enhancing inhibition on the mitral cells (Fang et al., 2008). In slices of the left auditory cortex, OXT induced LTP and led to increased spike firing (Mitre et al., 2016). Finally, although out of the scope of the current review that focuses on OXT and synaptic plasticity in the context of behavior, it is important to note that the effect

of OXT on synaptic plasticity has been also studied in the context of pain, where stimulation of OXT-PVN neurons or intrathecal OXT administration was shown to reduce or prevent LTP in spinal dorsal horn neurons and transiently interrupt the long-lasting LTP-mediated mechanical hyperalgesia (DeLaTorre et al., 2009).

## IMPLICATIONS FOR NEURODEVELOPMENTAL DISORDERS

Recent advances in genetic studies of autism spectrum disorder (ASD) and other neurodevelopmental disorder have implicated several risk genes that play an essential role in brain synapses (Xu et al., 2012; De Rubeis et al., 2014; Sanders et al., 2015), a finding that suggests that deficits in synaptic activity and plasticity may underlie the pathophysiology of these disorders in a considerable portion of the cases. Electrophysiological studies in animal models for ASD have supported this theory and have repeatedly shown that LTP and/or LTD are impaired in the vast majority of these models (Bhakar et al., 2012; Chung et al., 2012; Kirschstein, 2012; Harony-Nicolas et al., 2015, 2017; Till et al., 2015; Tian et al., 2017) (examples for are presented in **Figure 1**). The role that OXT plays in modulating synaptic plasticity led us to hypothesize that failure of the OXT system during early development may impact social behavior by altering synaptic plasticity in brain regions implicated in social behavior. Here we summarize several studies that propose that dysfunction in the OXT system early in life could account for the development of some of the social behavior symptoms.

### Prader-Willi Syndrome

Prader-Willi Syndrome (PWS) is a rare multisystem neurodevelopmental disorder that presents with abnormal clinical features during development, starting with severe hypotonia and feeding difficulties in infants followed by unrelenting feelings of hunger and consequently excessive eating and obesity problems later in life (Angulo et al., 2015). Individuals with PWS also present with intellectual disability and some features of ASD (Bennett et al., 2015). PWS results from the lack of expression of the paternal allele of several contiguous genes including *MKRN3*, *MAGEL2*, *MAGED1*, *NECDIN* and *SNURF-SNRPRN*. Notably, subjects with PWS have a significantly decreased number of PVN-OXT neurons and decreased levels of circulating OXT (Swaab et al., 1995; Hæybye, 2004). These alterations are suggested to underlie the obesity phenotype in PWS patients (reviewed in Sabatier et al., 2013). Mouse models for PWS present with several phenotypes, some of which mimic those observed in subjects with PWS. *Maged1*-deficient mice develop progressive obesity, show impaired social interaction and social memory and display alerted sexual behavior, increased anxiety and self-grooming. Notably, in these mice, the synthesis of mature OXT in the brain is also significantly decreased, and restoring OXT levels via acute peripheral administration of OXT reverses the social memory deficits (Dombret et al., 2012). Similarly, the *Magel2*-deficient pups also show a significant reduction in the levels of mature OXT peptide in the brain. These abnormalities are accompanied by deficits in social and learning behaviors that are reversed following subcutaneous administration of OXT at an early postnatal stage (Schaller et al., 2010; Meziane et al., 2015). Notably, *Magel2*-deficient mice also exhibit feeding difficulties, a phenotype that mirrors those observed in patients with PWS. Finally, in the *Necdin*-deficient mouse model, the number of hypothalamic OXT-neurons is also significantly decreased (Muscatelli et al., 2000). Together, these studies suggest that the alterations in OXT system may underlie the social behavior deficits observed in subjects with PWS and that OXT treatment may be beneficial to treat these deficits. Similarly, a recent clinical study showed that intranasal application of OXT in subjects with PWS under age of 6 months improves feeding and social skills (Tauber et al., 2017).

### *CNTNAP2*

Missense heterozygous mutations in the contactin-associated protein-like 2 (*CNTNAP2*) are implicated in cortical dysplasia-focal epilepsy (CFDE) syndrome and are associated with epilepsy, seizures, attention-deficit hyperactivity disorder (ADHD) and ASD (Strauss et al., 2006; Elia et al., 2010; Mefford et al., 2010; Rodenas-Cuadrado et al., 2014). The *CNTNAP2* gene encodes for the CASPR2 protein, which is a member of the neurexin superfamily. Presynaptic neurexins interact with members of the neuroligin family at the post synapse, and members of both protein families have been associated with ASD (Betancur et al., 2009). Similar to human subjects, mice with a *Cntnap2* mutation also have epileptic seizures and show deficits in ASD-associated behaviors (Brunner et al., 2015; Peñagarikano et al., 2015). Notably, *Cntnap2*-KO mice exhibit a significant and specific reduction in the number of OXT expressing cells in the PVN as well as in OXT concentrations in brain extracts. Single intraperitoneal or intranasal application of OT in these mice is sufficient to transiently rescue their social behavior deficits. Interestingly, early postnatal sub-chronic intranasal application of OXT alleviates their social behavior deficits and restores PVN-OXT neurons and brain OXT concentrations to wild-type levels (Peñagarikano et al., 2015). Together, these findings suggest that the OXT system may be affected in individuals with *CNTNAP2* mutations and that those individuals may particularly benefit from an early-life treatment with OXT.

### *Shank3*

We have recently produced and validated the *Shank3*-deficient rat model, a novel transgenic rat model for ASD and intellectual disability that harbors a mutation in the *Shank3* gene (Harony-Nicolas et al., 2017). Shank3 is a scaffolding protein at the postsynaptic density (PSD), which functions as a platform upon which other additional PSD proteins accrete (Grabrucker et al., 2011). In humans, deletions or mutations in the *SHANK3* gene lead to Phelan-McDermid Syndrome (PMS) with approximately 80% meeting criteria for ASD (Soorya

et al., 2013). Mouse models with *Shank3* gene mutations display ASD-related behavioral phenotypes, altered synaptic transmission and changes in neural plasticity and synaptic morphology (Harony-Nicolas et al., 2015). In rats, we found that *Shank3* mutations lead to deficits in attention, long- but not short-term SRM (Harony-Nicolas et al., 2017) and developmental social communication (Berg et al., 2018). We also found that these deficits are accompanied by impaired synaptic plasticity. Specifically, we showed that maintenance of LTP in the hippocampus (*in vitro*) and the hippocampal-PFC pathway (*in vivo*) is impaired in the *Shank3*-deficient rat. Finally, we demonstrated that acute ICV administration of OXT in these rats reversed both the behavioral and the *in vitro* and *in vivo* synaptic plasticity deficits (Harony-Nicolas et al., 2017). These findings were the first to report that OXT can reverse not only behavioral but also synaptic plasticity deficits in a genetic model for a neurodevelopmental disorder, suggesting that the reversal effect of OXT on synaptic plasticity, specifically LTP, may underlie its ameliorative effect on behavior. Further studies are needed to determine the effect of *SHANK3* mutations on the OXT system in order to understand if perturbation in this system could explain some of the observed behavioral phenotypes and plasticity-related changes.

## CONCLUDING REMARKS

Here, we provided an overview of the modulatory effects of OXT on synaptic activity that underlie diverse behaviors. We also reviewed findings from genetic rodent models of neurodevelopmental disorders that demonstrate alterations in the OXT system. Despite the major interest in the therapeutic potential of OXT to treat social behavior deficits, there is still a considerable gap in the knowledge about the plausible implication of the OXT system in the pathogenesis of neurodevelopmental disorders. To fill this gap, there is a need for future studies to investigate not only the effect of mutations associated with these disorders on the maturation of the OXT system during early development stages but also the integrity and functionality of this system during later postnatal windows and under different behavioral contexts. Many of the mutations associated with neurodevelopmental disorders reside in genes encoding for neural or synaptic proteins (Xu et al., 2012; De Rubeis et al., 2014; Sanders et al., 2015), suggesting that they may impact: (1) the development and maturation of OXT neurons; (2) the OXT projections; (3) the intrinsic properties of OXT neurons; and (4) the trafficking and release of OXT. An alteration in any of these elements could potentially lead to imbalanced OXT levels in the brain and/or a lack or diminished response by the OXT system to stimuli (e.g., stress or social stimuli). This consequence could affect synaptic activity and plasticity in target brain regions modulated by OXT and ultimately impair the behavioral response. The $CD38^{-/-}$ mouse model provides an example for how behavioral phenotypes can be attributed to deficits in OXT release. This model harbors a mutation in the CD38 gene that encodes for a transmembrane glycoprotein involved in OXT release. $CD38^{-/-}$ mice have reduced OXT plasma levels and increased number of large dense core vesicles (LDCVs), which package the OXT neuropeptide. These alterations are accompanied with impaired maternal nurturing and SRM, which can be rescued with OXT administration (Jin et al., 2007).

Our findings in the *Shank3*-deficient rat, where both the behavioral and the synaptic plasticity deficits are rescued with OXT administration (Harony-Nicolas et al., 2017), suggest that OXT delivery and/or release at target brain regions involved in social behavior may be impaired. Notably, OXT release is known to be reliant on rapid and transient depolymerization of actin filaments (Tobin et al., 2012), and *Shank3*-deficient mice show dysregulation of actin filaments via upregulation of cofilin, a known actin depolymerizing agent (Duffney et al., 2015). Based on these findings it is possible that mutations in the *SHANK3* gene would impact the actin cytoskeleton in OXT neurons, the release of OXT, and consequently, synaptic plasticity and behaviors that are modulated by OXT. To address this theory, there is a need for future studies to assess the central and peripheral OXT levels in this model and OXT levels following behavioral (e.g., social or stress stimuli), drug-induced (e.g., CCK8) or chemogenetic (designer receptors exclusively activated by designer drug; DREADDS) activation of OXT-neurons. Moreover, given that OXT neurons project to several brain targets, it is important to elucidate whether the effect of OXT on modulating synaptic activity persists across all projection targets to influence a circuit and whether some of these target regions are more vulnerable to genetic insults than others.

Importantly, given the etiological and phenotypic heterogeneity in ASD and neurodevelopmental disorders, we do not expect impairment in the OXT system to explain the behavioral phenotypes of all individuals with the disorder. This highlights the need for future studies in additional genetic models of neurodevelopmental disorders to (1) identify mutations that pose a deleterious effect on the OXT system and therefore converge on a shared pathophysiology and (2) to define the mechanistic interplay between these mutations and the OXT system. Findings from these studies will inform targeted treatments in human individuals carrying these pathogenic mutations.

## AUTHOR CONTRIBUTIONS

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# Long-Term Effects of Early Life Seizures on Endogenous Local Network Activity of the Mouse Neocortex

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Understanding the long term impact of early life seizures (ELS) is of vital importance both for researchers and clinicians. Most experimental studies of how seizures affect the developing brain have drawn their conclusions based on changes detected at the cellular or behavioral level, rather than on intermediate levels of analysis, such as the physiology of neuronal networks. Neurons work as part of networks and network dynamics integrate the function of molecules, cells and synapses in the emergent properties of brain circuits that reflect the balance of excitation and inhibition in the brain. Therefore, studying network dynamics could help bridge the cell-to-behavior gap in our understanding of the neurobiological effects of seizures. To this end we investigated the long-term effects of ELS on local network dynamics in mouse neocortex. By using the pentylenetetrazole (PTZ)-induced animal model of generalized seizures, single or multiple seizures were induced at two different developmental stages (P9–15 or P19–23) in order to examine how seizure severity and brain maturational status interact to affect the brain's vulnerability to ELS. Cortical physiology was assessed by comparing spontaneous network activity (in the form of recurring Up states) in brain slices of adult (>5 mo) mice. In these experiments we examined two distinct cortical regions, the primary motor (M1) and somatosensory (S1) cortex in order to investigate regional differences in vulnerability to ELS. We find that the effects of ELSs vary depending on (i) the severity of the seizures (e.g., single intermittent ELS at P19–23 had no effect on Up state activity, but multiple seizures induced during the same period caused a significant change in the spectral content of spontaneous Up states), (ii) the cortical area examined, and (iii) the developmental stage at which the seizures are administered. These results reveal that even moderate experiences of ELS can have long lasting age- and region-specific effects in local cortical network dynamics.

**Keywords: early-life, seizures, long-term effects, spontaneous, Up states, neocortex, mouse**

## INTRODUCTION

The balance between excitatory and inhibitory synapses in the cortex is critical for normal brain function and adaptive behavior. In the developing brain this balance is shifted in favor of excitation due to the delayed maturation of inhibitory circuits (Gaiarsa et al., 1995; Ben-Ari, 2006); a fact that makes neonates and juveniles more susceptible to seizures (Hauser and Kurland, 1975;

Olafsson et al., 2005), either spontaneous or in response to a number of different insults (Volpe, 1973). This becomes a clinical issue because early-life seizures are often associated with severe neurological and behavioral impairments in adult life, such as cognitive deficits and a higher propensity for epilepsy (Sillanpaa et al., 1998; Brunquell et al., 2002). However, the outcome of early-life seizures varies on an individual basis. Statistically, a third to half of affected children will fare well in adulthood, while the rest will either lead a sick life suffering from cognitive and neurological dysfunctions such as mental retardation, attention deficit disorders, behavioral disorders and epilepsy (17–40%) or will suffer premature death (16–30%) (Sillanpaa et al., 1998; Brunquell et al., 2002; Lombroso, 2007). This highly variable outcome emphasizes the need to understand the mechanisms that mediate the effects of seizures *per se*, i.e., dissociated from precursor and/or concurrent underlying pathologies, and from the effects of exposure to anti-epileptic drugs.

The study of the long-term effects of early-life seizures in humans is difficult and problematic due to the number of variables that influence the outcome (age of onset, etiology, seizure type, frequency and duration of seizures, genetics, environment, and pharmaceutical treatment), all of which are difficult to control in clinical studies (Haut et al., 2004; Holmes, 2005; Lombroso, 2007). Therefore, experimental models of early-life seizures are essential and rodents have been systematically used given the similarities to human seizures, in terms of electrical and behavioral parameters (Kubova and Moshe, 1994). For example, in both species, status epilepticus, the condition of persistent seizures, is manifested electrically with interictal and ictal discharges, and behaviorally with myoclonic seizures (Kubova and Moshe, 1994; Castro-Alamancos, 2000). In addition, humans and rodents have parallel behavioral profiles regarding the long-term effects of early seizures, as both develop cognitive deficits and a higher propensity for epilepsy (Holmes et al., 1998; Sillanpaa et al., 1998). Finally, just like humans, young rats and mice are much more prone to seizures than adults (Holmes, 2005).

Rodent studies have revealed a number of structural and/or functional effects of early-life seizures on the adult cortex, including: changes in neurogenesis (Holmes et al., 2002; Porter, 2008) or cell loss (Sankar et al., 2000) and synaptic re-organization (sprouting) of axons and terminals (Holmes and Ben-Ari, 1998; Holmes et al., 1998); modifications of glutamate and GABA receptors (Sanchez et al., 2001; Sogawa et al., 2001; Bo et al., 2004; Ni et al., 2004; Cornejo et al., 2007), changes in intrinsic properties (Villeneuve et al., 2000), or synaptic dynamics (Isaeva et al., 2006, 2009) of cortical cells, decreases in excitatory amino acid carrier (Zhang et al., 2004), and decreases in threshold for electrographic seizures (Santos et al., 2000; Isaeva et al., 2010). Moreover, respective behavioral studies in rodents have shown changes in behavior and cognition as reflected in deficits in learning and memory (Holmes et al., 1998; Huang et al., 1999; Chang et al., 2003; Karnam et al., 2009a,b) and sensory processing (Neill et al., 1996) indicating deficiencies in cortical function. However, the majority of these studies (a) have focused more on changes in either structure or behavior, rather than on alterations at the intermediate level of analysis, the physiology of neuronal circuits. This link is important in order to understand the underlying biological mechanisms that mediate early seizure effects. And (b) have focused much more on the effects of ELS on the hippocampus, rather than on the neocortex (Lombroso, 2007). Although this is understandable given the significance of the hippocampus in certain types of epilepsy, there is also evidence that brain regions differ, both in their sensitivity to seizures (Castro-Alamancos and Rigas, 2002; Rigas and Castro-Alamancos, 2004) and the resulting changes (Sankar et al., 1998; Kubova et al., 2001), implying that the effects of early-life seizures in the hippocampus cannot necessarily be generalized to the neocortex. This highlights an unmet need for studying the neocortex since this is the structure involved in most cognitive functions; in humans neonatal seizures typically involve the neocortex, and post-neonatal epilepsy is often of neocortical origin (Mizrahi and Clancy, 2000). Indeed, neonatal seizures more likely lead to epilepsy originating in neocortex than in hippocampus (Ronen et al., 2007). Therefore, progress in our understanding of the long-term effects of early seizures necessitates developing appropriate methods to evaluate the functional status of the neocortex (Lombroso, 2007).

Here we have examined the effect of chemically induced ELS on the endogenous cortical activity in brain slices from mouse cortex. Previous studies, including our own have shown that local recurrent networks formed by excitatory and inhibitory connectivity in the neocortex generate stable and self-sustaining periods of persistent activity alternating with periods of no activity, called *Up* and *Down* states, respectively – a prominent feature of the cortical activity during slow wave sleep *in vivo* (Steriade et al., 1993, 2001; Sanchez-Vives and McCormick, 2000; Cossart et al., 2003; MacLean et al., 2005; Haider et al., 2006; Rigas and Castro-Alamancos, 2007, 2009; Poulet and Petersen, 2008; Rigas et al., 2015; Sigalas et al., 2015, 2017). Such activity is maintained in cortical slice preparations, in the absence of sensory inputs or active neuromodulation, indicating that it is chiefly the outcome of intrinsic properties of local networks and hence reflects the 'default' activity of the cortex (Yuste et al., 2005; Sanchez-Vives et al., 2017). Since Up states are synaptically mediated network events that reflect the balance of excitation and inhibition in the neocortex (Sanchez-Vives and McCormick, 2000; Shu et al., 2003; Hasenstaub et al., 2005) we investigated whether and how they may reflect the long-term effects of ELS in an attempt to extend findings of an earlier report on the acute effects of seizures on this type of activity (Gerkin et al., 2010). Furthermore, in order to evaluate the differential spatial and temporal vulnerability to early life seizures, we examined two distinct cortical areas and induced the seizures at two different developmental stages.

## MATERIALS AND METHODS

### Animals

C57Bl/6J mice were bred in the animal facility of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. The facility is registered as a

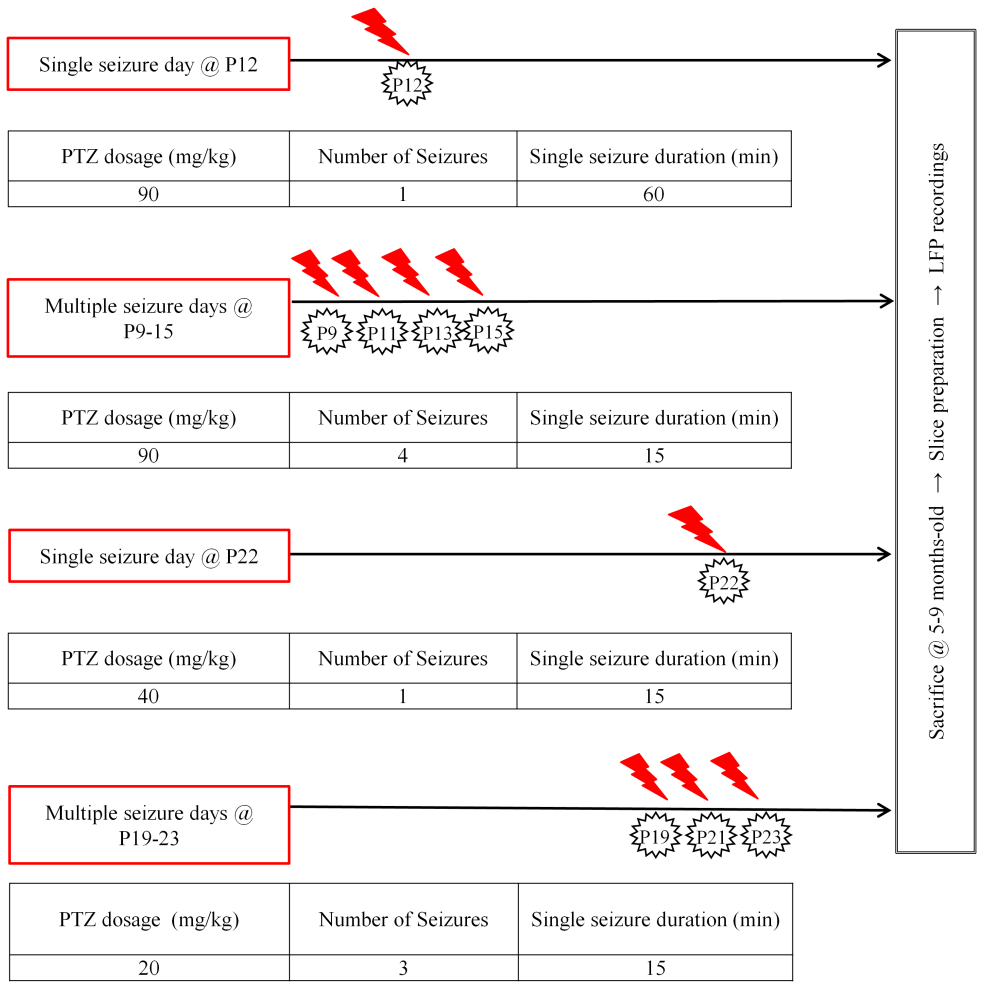


**FIGURE 1 |** Seizure induction. Diagrammatic representation of our seizure induction scheme as applied at two distinct developmental ages (postnatal ages: 9–15 days old, P9–15 and 19–23 days old, P19–23), either once (single) or repetitively (multiple); at longer (60 min) or shorter (15 min) durations. Protocols were adjusted depending on age and times of injections in order to achieve highest survival rates for aimed seizure intensity and duration. Modified figure from Rigas et al. (2015) in **Supplementary Figure 1**.

breeding and experimental facility according to the Presidential Decree of the Greek Democracy 160/91, which harmonizes the Greek national legislation with the European Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. The present study was approved by the Regional Veterinary Service, in accordance to the National legal framework for the protection of animals used for scientific purposes (reference number 2834/08-05-2013). Mice were weaned at 27 days postnatally (P27, considering P0 as the day of birth), housed in groups of 5–10, in 267 mm × 483 mm × 203 mm cages supplied with bedding material and kept at a 12–12 dark-light schedule. Food was provided *ad libitum*.

## Seizure Induction

We induced generalized seizures in young mice by injecting intraperitoneally (i.p.) the proconvulsant pentylenetetrazole (PTZ), a $GABA_AR$ antagonist. This method has been widely used in chemically induced acute animal models of generalized seizures (Kubova and Moshe, 1994) and does not necessarily result in chronic epilepsy (Kandratavicius et al., 2014). PTZ can produce either non-convulsive absence seizures or myoclonic seizures and can even lead to status epilepticus (SE) if given at sufficient amounts (Loscher, 1997; Pitkaenen et al., 2006). Moreover, the protocol of PTZ delivery is simple and the compound can be easily administered intraperitoneally, subcutaneously or intravenously. A major advantage of using PTZ is the lack of neuron loss in developing rodents, even after the induction of recurrent seizures (Holmes et al., 1999). On the other hand, other chemical seizure models such as kainate (kainic acid) and pilocarpine produce wide-spread brain damage (Loscher, 1997). Kainic acid has a direct excitotoxic effect on neuronal cells that makes it difficult to separate it from the seizure-induced neuronal damage (Rao et al., 2006; Reddy and Kuruba, 2013), whereas pilocarpine has a pattern of neuronal damage similar to the kainic acid model with greater

damage detected in the neocortex (Buckmaster et al., 2002; Reddy and Kuruba, 2013). Finally, PTZ has the advantage of being eliminated within 24 h from the animal without any known toxic or long-term direct effects (Loscher and Schmidt, 1988).

Single or recurrent seizures were induced in mice during two defined developmental periods: P9–15 and P19–23, which are the mouse equivalent of neonatal period and early childhood in humans, respectively (Lombroso, 2007; Dutta and Sengupta, 2016). Control mice received an equal volume of saline (0.9%) delivered through the same number of injections as PTZ-treated mice (single or multiple, depending on the protocol). Although we did not perform any systematic electrophysiological assessment, PTZ treated mice appeared behaviorally indistinguishable from their saline-treated littermates, with no obvious signs of spontaneous seizures later in life, in line with previous reports (Kandratavicius et al., 2014). Mice responded to i.p. PTZ injections with a continuum of behaviors categorized into stages ranging from 1 to 6, which we briefly describe as follows in accordance with Luttjohann et al. (2009): Stage 1 was characterized by sudden behavioral arrest and/or motionless staring; stage 2 by facial jerking with muzzle or muzzle and eye; stage 3 by neck jerks; stage 4 by clonic seizure in a sitting position; stage 5 by convulsions including clonic and/or tonic–clonic seizures while lying on the belly and/or pure tonic seizures and stage 6 by convulsions including clonic and/or tonic–clonic seizures while lying on the side and/or wild jumping. Our injection scheme at both developmental stages involved either *single* or *multiple* seizures as described in **Figure 1**. We considered "seizure" the full-blown *generalized* tonic-clonic seizure for 15 min. In cases where this was not achieved with a single PTZ injection, additional injections were given in order for each mouse to experience the required duration of generalized (stage 5–6) seizures. In order to define the doses of PTZ which would induce seizures of stage 6 with the lowest mortality rate we performed pilot studies with mice at P12, P22 (single seizure tests) and P9–P15, P19–P23 (multiple seizure tests) which were injected intraperitoneally with various dosages of PTZ (*Standardization of Seizure Induction Protocol*). Repetitive seizures were induced every second day in order to increase survival rates. In line with previous work, we also found that P9–15 and P19–23 animals could not be given equivalent per kilogram doses (P9–15 vs. P19–23: 90 vs. 40 mg/kg, respectively) as the CD50 (Convulsion Dose 50%: dose producing clonic convulsions in 50% of tested animals) of PTZ increases to a peak at the animal age of 12 days and then declines to the CD50 of 8-days-old (Vernadakis and Woodbury, 1969; McCaughran and Manetto, 1982). Moreover, we found that, as opposed to younger ages, mice at P19–23 had to be injected with different doses of PTZ depending on whether we aimed at single or multiple seizures (single: 40 mg/Kg, multiple: 20 mg/Kg).

## Standardization of Seizure Induction Protocol

Pilot experiments were performed in order to optimize the seizure induction protocol in terms of efficiency and viability of seizure induction, a task which was often complicated by our equally important goals of increasing animal survival and reducing their suffering. For example, in our intial attempt to compare the effects of a single prolonged seizure (status epilepticus, SE) at P12 with those of multiple (five) SE occurring on five consecutive days from P10 to P15, we realized that when mice of the multiple protocol were left to recover on their own, survival rates were very low (3 out of 9 mice: 33.3%) already by the third day of sequential seizures. In addition, we observed that even animals that survived the seizures sometimes died after being returned to their cage, if they had not recovered completely. For this reason we decided to end seizures in all animals of this group by injecting them with the anticonvulsant diazepam (DZP, 2 mg/Kg) and aimed at five consecutive days of seizures. Despite this measure we ended up again with extremely low survival rates by the fifth day (2 out of 11 mice: 18.2%). In addition, we realized that PTZ was less effective in inducing stage 5–6 seizures when administered daily. In particular, as opposed to the first day of injections, stages 5–6 seizures were harder to achieve on subsequent days; in addition, seizures had delayed onsets and were of shorter durations. Therefore, we decided to administer PTZ every second day, each time aiming at seizures of a total duration of 15 min. With this method, PTZ-injections were very effective in inducing generalized seizures with hardly any need for supplementary injections. Moreover, in order to maintain the two ELS periods clearly separated, we decided to begin earlier (on P9 instead of P10). With these measures survival rates increased to 86.7% for up to the first four days of seizures. However, surprisingly, none of the mice could survive through a fifth day of seizures (on P17), a fact that forced us to limit our protocol between ages P9 and P15. A similar strategy was followed for seizure injections in mice of the older age group.

Although PTZ injections in our final protocols induced seizures in all mice, we observed a clear distinction between younger and older mice as well as between single and multiple days of PTZ-injections regarding (i) how easily seizures were induced and (ii) how long they lasted. In particular, P9–15 mice usually responded with a full-blown seizure to even a single PTZ injection and would remain in stage 5–6 for a protracted period of time (>1 h) during the single seizure protocol or the first day of a series of injections (multiple seizure protocol). In addition, although seizures in these mice during subsequent days of the multiple seizures protocol would last less than an hour, they still tended to remain in stage 5–6 significantly longer than 15 min. Therefore, we had to always inject these P9–15 PTZ-treated mice with DZP (2 mg/kg) in order to terminate seizures either at 60 min for status epilepticus (SE) or at 15 min for the shorter duration seizures protocols. In contrast, mice in the older group (P19–23) for both single and multiple seizures would almost always need supplementary doses in order to reach stage 5–6 seizures and/or sustain them for the aimed total seizure duration of 15 min. Although in older mice seizures lasted less and they showed earlier spontaneous recovery compared to younger mice, we still injected all of them with the anticonvulsant DZP (2 mg/kg) in order to terminate their seizures once they reached (i.e., either lasted or summed up to) 15 min. Finally, since saline-treated mice were not injected with DZP, in order

to ensure that the electrophysiological phenotype of the PTZ-injected mice was not affected by DZP *per se*, we repeated the experiments in a separate group of animals that had received only DZP injections (2 mg/kg) and compared them to saline-treated mice. Importantly we found no differences in Up state activity between DZP- and saline-treated mice, suggesting that early-life administrations of DZP had no long-term effects on spontaneous cortical Up states (**Supplementary Text, Section 1, Tables 1–8**).

Younger and older mice also differed in lethality rates: P9–15 mice exhibited higher survival rates (90.9% for 1 × 60 min seizure at P12 and 86.7% for 4 × 15 min seizures at P9–15) compared to P19–23 mice (56.7% for 1 × 15 min seizure at P22 and 33.3% for 3 × 15 min seizures). In summary, protocols for seizure induction for the two age groups were adjusted accordingly in terms of both duration and number of seizures:

(a) The 60 min seizure was only applied in the younger mice. Instead, in P19–23 animals the single seizure duration was reduced to 15 min.

(b) Multiple seizures in both groups were induced on alternate days in order to increase survival rates. The number of seizures in the older group was restricted to three. As evident from the data (see Results section), this difference does not preclude valid comparisons between the two age groups.

(c) Given that four 15 min seizures in the P9–15 group had no effect on any of the parameters of Up state activity, we felt there was no justification to also include a group with one 15 min seizure in this age group.

## Brain Slice Preparation

Coronal brain slices (400 μm) from primary somatosensory cortex of the whiskers [i.e., barrel cortex, S1BF; Anterior-Posterior from Bregma (A/P): 0.58–1.58 mm, Medial-Lateral (M/L): 2.5–4 mm] or primary motor cortex (M1; A/P: 1.54–0.74 mm, M/L: 1–2.75 mm) were prepared from the right hemisphere of adult male mice (5–9 months old) (**Figure 2A**). After the mouse was sacrificed with cervical dislocation, we removed the brain and placed it in an oxygenated (95% $O_2$–5% $CO_2$) ice-cold dissection buffer containing, in mM: KCl 2.14; $NaH_2PO_4.H_2O$ 1.47; $NaHCO_3$ 27; $MgSO_4$ 2.2; D-Glucose10; Sucrose 200; and $CaCl_2.2H_2O$ 2; osmolarity (mean ± SD): 298 ± 5 mOsm, pH: 7.4. Brain slices were cut using a vibratome (VT 1000S, Leica) and placed in a holding chamber with artificial cerebrospinal fluid (ACSF) where they were left to recover at room temperature (RT: 24–26°C) for at least 1 h before transferred to the recording chamber. The ACSF contained (in mM): NaCl 126; KCl 3.53; $NaH_2PO_4.H2O$ 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; D-Glucose 10 and $CaCl_2.2H_2O$ 2 [osmolarity (mean ± SD): 317 ± 4 mOsm, pH: 7.4].

## *In vitro* Electrophysiology

Following recovery, slices were transferred to a submerged type of chamber (Luigs and Neumann), where they were gravity-perfused at high flow rates (10–15 ml/min) to ensure optimal oxygenation of the cortical tissue (Hajos et al., 2009; Bregestovski and Bernard, 2012). Recordings were performed in "*in vivo* like" ACSF (whose composition was identical to above except for 1 instead of 2 mM $CaCl_2$), since this ionic buffer is thought to better mimic cerebrospinal fluid *in vivo* (Fishman, 1992; Somjen, 2004) and we and others have previously shown that under these conditions cortical slices are spontaneously active in the form of a slow wave-like oscillation composed of alternating Up and Down states (**Figure 2B**; Sanchez-Vives and McCormick, 2000; MacLean et al., 2005; Rigas and Castro-Alamancos, 2007; Mann et al., 2009; Fanselow and Connors, 2010). Recordings were performed at RT after an hour (1 h) of incubation in 1 mM [$CaCl_2$] ACSF buffer. To stabilize slices we modified our submerged chamber in order to included a surface of transparent silicone onto which up to four slices could be pinned. The advantage of this modification was that we could perform simultaneous

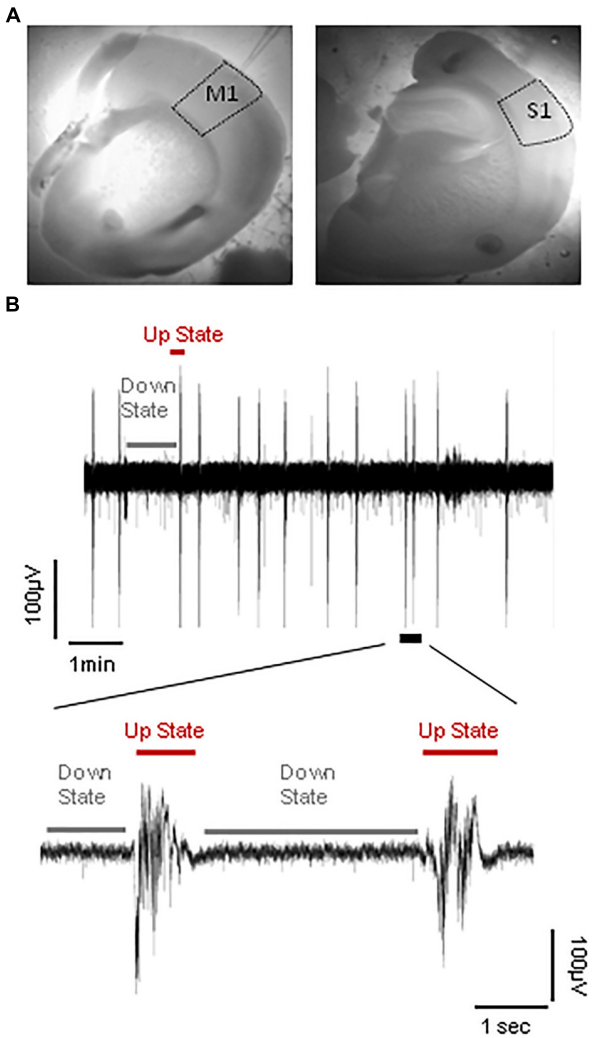


**FIGURE 2 |** Spontaneous cortical Up states *in vitro*. **(A)** Outlined areas of primary motor (M1) and primary somatosensory area of the whiskers (S1) from which recordings were obtained. **(B)** *Upper trace*: typical example of continuous field potential recordings of alternating Up and Down states performed *in vitro* from the S1 cortical slice of an adult (5–9 months old) mouse. *Lower trace*: A close-up of two individual Up state events indicated in the trace above.

recordings from different ages and/or brain regions and therefore maximize the yield of our experiments and achieve to directly compare different experimental groups under identical conditions.

Spontaneous network activity was assessed by means of local field potential (LFP) recordings (sampled at 10 kHz, band-passed filtered at 1 Hz–3 kHz) which were obtained from cortical layers II/III using low impedance (∼0.5 MΩ) glass pipettes filled with ACSF. Signals were acquired and amplified (MultiClamp 700B, Axon Instruments), digitized (InstruTech, ITC-18) and viewed on-line with appropriate software (AxoGraph). All reagents and drugs were purchased from Sigma except for KCl and K-gluconate, which were purchased from CARLO ERBA Reagents and Fluka, respectively.

## Data Analysis

For visualization and analysis of spontaneous LFP Up states, traces were exported to MATLAB format and analyzed with custom-made MATLAB scripts (LFPAnalyzer) that automatically detected the LFP events and marked their onsets and offsets as previously described (Rigas et al., 2015, 2017; Tsakanikas et al., 2017). In particular, preprocessing of the recordings included low-pass filtering at 200 Hz with a third order Butterworth filter and DC offset subtraction. Subsequently, detection of individual Up states was performed through the following automated steps: (a) the signal was transformed using the Hilbert Transform (Oppenheim and Schafer, 1998) and the Short-Time Energy Transform in parallel (Jalil et al., 2013), (b) a dynamic and data-driven threshold was then automatically estimated via Gaussian Mixture Modeling (McLachlan and Peel, 2000), and finally (c) the detected signal segments from each transformed signal were combined via an OR logical operation, resulting to the final LFP event (Tsakanikas et al., 2017).

In order to describe LFP Up states we employed a number of different parameters which were either measured or calculated and subsequently used for statistical analysis, as previously described (Rigas et al., 2015). In particular, home-made software was developed to automatically measure (i) *duration*, (ii) maximal negative peak (*amplitude*), (iii) *rectified area*, and (iv) *spectral power* of each detected Up state (Tsakanikas et al., 2017). Some of the measured parameters are depicted in **Supplementary Figure 1**. Furthermore, for each of our recordings we calculated (a) the *occurrence* of spontaneous events (i.e., number of events divided by the duration of the recording session) and (b) an overall Up state activity index calculated as the product of occurrence * mean rectified area of Up states within each LFP recording (*Up state index*). Occurrence is a measure of how frequently spontaneous Up states occur while the rectified area is an overall measure of LFP Up state size, which includes both their duration and amplitude. Finally, the power spectrum of each event, estimated on the basis of Fourier Transform coefficients, is presented in the conventionally described frequency bands: *delta* (1–4 Hz), *theta* (4–8 Hz), *alpha* (8–12 Hz), *beta* (12–30 Hz), and *gamma* (30–100 Hz) range and normalized to the total power of each event in the 1–200 Hz range. The normalization procedure allows a direct comparison of the % differences of power, since LFP events within or between recordings can differ significantly in both amplitude and duration and thus in absolute power value.

## Statistical Analysis

Statistical analyses were performed using SPSS (version 17) software. Sample size was defined based on the number of slices and data were tested for normality using the Shapiro-Wilk test. Measurements of normally distributed data ($p > 0.05$) are presented as their mean ± standard deviation (SD), whereas data that deviated from normality are presented as their median and interquartile range. Factorial analysis of variance (ANOVA) for multiple group comparisons was applied to both normally and not normally distributed data after transforming data according to the rules of the Aligned Rank Transformation (Wobbrock et al., 2011) using the ARTool software[1].

# RESULTS

We investigated the long-term effects of early life seizures (ELS) on spontaneous cortical Up states during adulthood. ELS were introduced at two different developmental stages: either P9–15 or P19–23, and activity was sampled from two distinct cortical areas, namely the primary somatosensory cortex of the whiskers (barrel cortex, S1) and the primary motor cortex (M1) which differ in both their function and cytoarchitecture (Welker, 1971, 1976; Donoghue and Wise, 1982; Castro-Alamancos et al., 1995, 2007; Castro-Alamancos and Rigas, 2002; Katzel et al., 2011; Herculano-Houzel et al., 2013).

## The Long-Term Effects of Early-Life Status Epilepticus (SE) on Local Cortical Network Activity Are Region-Specific

We first examined the effect of a 60 min status epilepticus (SE). SE has been defined as a state of continuous or recurrent seizures for at least 30 min with incomplete or no recovery between seizures (Lowenstein et al., 1999; Mitchell, 2002; Gaitanis and Drislane, 2003; Varelas and Mirski, 2009). This experiment was only possible during the earlier postnatal period (P9–15) because at later stages (P19-23) animal lethality was prohibitive. A two-way analysis of variance (two-way ANOVA) was conducted on the influence of two independent variables (seizures and cortex) on network dynamics of local spontaneous Up states as described by ten parameters, namely: occurrence, duration, amplitude, rectified area, Up state index, normalized delta, normalized theta, normalized alpha, normalized beta and normalized gamma (as described in *Materials and Methods*). *Seizures* included two levels [no seizures ("injections with saline") and seizures ("injections with PTZ")] and *cortex* also consisted of two levels (S1, M1). There was a highly significant interaction between the effects of seizure and cortex for occurrence [$F(1,42) = 9.071$, $p = 0.004$] and Up states index of spontaneous activity [$F(1,42) = 12.7$, $p = 0.001$]; and a marginally significant effect of the two on Up state amplitude [$F(1,42) = 4.172$, $p = 0.047$] and rectified area [$F(1,42) = 4.151$, $p = 0.048$] (**Supplementary**

[1]http://depts.washington.edu/aimgroup/proj/art/

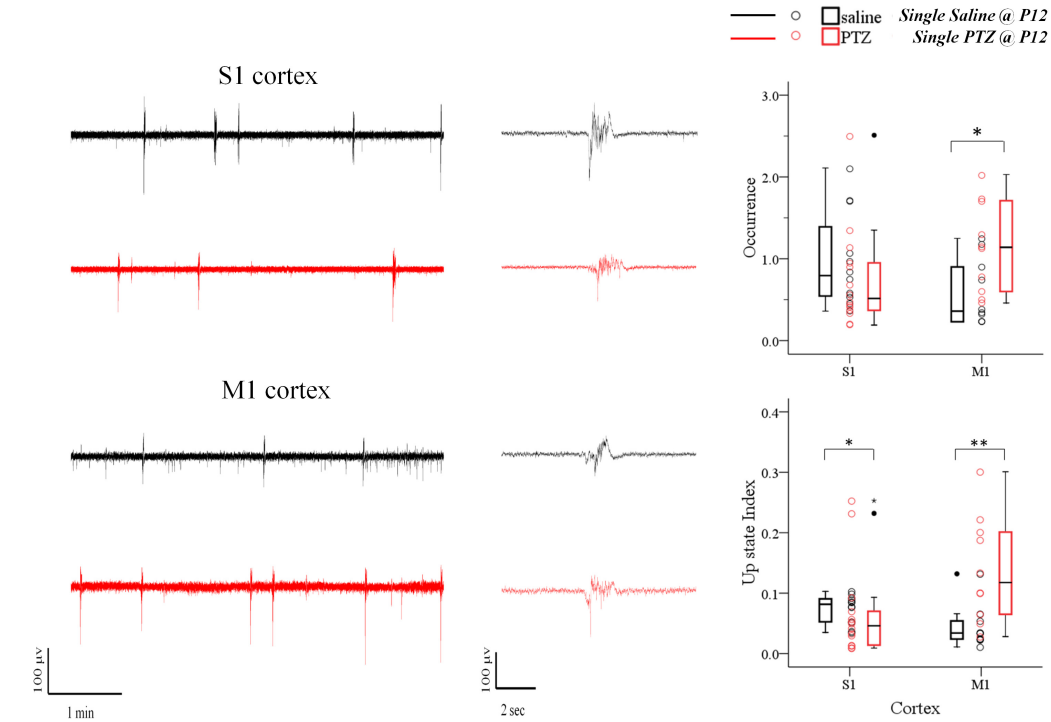


**FIGURE 3 |** The long-term effects of status epilepticus on spontaneous Up states are region-specific. Prolonged (60 min) seizures (status epilepticus, SE) induced by a single administration of PTZ at P12 mice cause a region-specific effect in the adult cortex since they enhance spontaneous Up states in M1, but decrease network activity in S1 cortex compared to their control (saline-injected) groups. **(A)** Examples of continuous 5 min recordings of spontaneous Up states from S1 and M1 cortex of adult mice injected at P12 either with saline (black traces) or PTZ (red traces). **(B)** Close-up of individual Up states. The first Up state of respective long recording on the left was selected and demonstrated at higher time resolution. **(C)** Population data comparing occurrence (*upper panel*) and Up state index (an overall metric for spontaneous cortical activity that integrates the occurrence with the rectified area of Up state events, *lower panel*) of spontaneous Up states in adult mice which had experienced SE at P12 with age-matched control mice. *Open circles* represent superimposed vertical scatterplots of individual data points of the control (saline, *black*) and treated (PTZ, *red*) groups of animals. *Box and Whisker plots* placed on each side of the scatterplots describe the spread of data for the saline- (black) and PTZ-treated (red) group showing interquartile ranges (length of *boxes*), upper and lower limits of data (*whiskers*), median (*line inside box*), extreme values (*stars*), and outliers (*filled circles*). *Asterisks* indicate levels of statistically significant differences between compared groups (*$p < 0.05$, **$p < 0.01$).

**Text, Section 2.1.2**, **Table 9**). Further simple effect analyses showed that SE during early life significantly increased the occurrence of spontaneous Up states in the adult M1 but not S1 cortex [Occurrence: *S1, saline ($N_{Animals}$ = 8, $N_{slices}$ = 12) vs. PTZ ($N_{Animals}$ = 9, $N_{slices}$ = 14): 0.80 (1.01) vs. 0.512 (0.64) Up states/min [median (interquartile range)], F(1,42) = 2.502, p = 0.121, Bonferroni; M1, saline ($N_{Animals}$ = 9, $N_{slices}$ = 10) vs. PTZ ($N_{Animals}$ = 7, $N_{slices}$ = 10): 0.36 (0.74) vs. 1.14 (1.14) Up states/min [median (interquartile range)], F(1,42) = 4.425, p = 0.041, Bonferroni,* **Figure 3** and **Supplementary Text, Section 2.2.1.1**]. The same analysis applied to Up state index (which reflects an overall metric for spontaneous cortical activity that integrates the occurrence with the rectified area of Up state events) confirmed the enhancement Up states dynamics in M1 but also revealed a significant effect in the opposite direction for S1 [Up state index: *S1, saline vs. PTZ: 0.08 (0.04) vs. 0.05 (0.06) [median (interquartile range)], F(1,42) = 5.576, p = 0.023, Bonferroni; M1, saline: 0.03 (0.03) vs. 0.12 (0.15) [median (interquartile range)], F(1,42) = 7.601, p = 0.009, Bonferroni,* **Figure 3** and **Supplementary Text, Section 2.2.1.2**]. On the contrary there was no significant interaction between the effects of seizures and cortex type for duration or spectral content of spontaneous Up states (**Supplementary Text, Section 2.1.2., Table 9**) and early life SE had no main effect on either Up state duration or spectral power (**Supplementary Text, Section 2.3., Table 10**). Taken together, these results indicate a region-specific long-term effect of prolonged status epilepticus in early life on the adult cortex.

## The Long-Term Effects of ELS on Local Cortical Network Activity Depend on the Duration of Individual Seizures, Rather Than on Their Frequency

Having documented the significant impact of prolonged ELS (60 min status epilepticus, SE) on the adult cortex we then wondered if this effect was dependent on the pattern and/or total duration of SE, i.e., whether multiple shorter duration seizures would also affect cortical network dynamics as single long-lasting SE did. This was prompted by the fact that, in

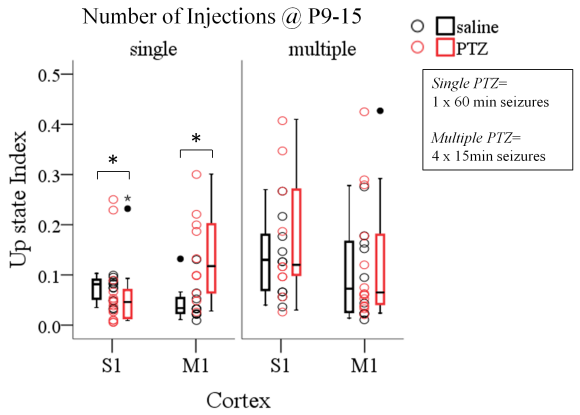


**FIGURE 4 |** The long-term effects of early life seizures on spontaneous Up states depend on their pattern. Single 60 min seizures, but not multiple seizures of equal total duration (4 min × 15 min) during early life (P9–15) significantly affect spontaneous Up states in the adult cortex. *Open circles* represent superimposed vertical scatterplots of individual data points of the control (saline, *black*) and treated (PTZ, *red*) groups of animals. *Box and Whisker plots* placed on each side of the scatterplots describe the spread of data for the saline- (black) and PTZ-treated (red) group showing interquartile ranges (length of *boxes*), upper and lower limits of data (*whiskers*), median (*line inside box*), extreme values (*stars*), and outliers (*filled circles*). *Asterisks* indicate levels of statistically significant differences between compared groups (*$p < 0.05$).

humans, the outcome of febrile seizures, commonly triggered by fever in infants and children, critically depends on their duration – with short seizures being benign compared to prolonged seizures (Annegers et al., 1987; French et al., 1993; Verity et al., 1998). Therefore, in a second group of pups we induced four 15 min seizures on every second day from P9–P15 and we tested their effect on spontaneous Up states during adulthood. To this end we conducted a three-way ANOVA on the influence of three independent variables (*injections*, *number of injections* and *cortex*) on the dynamics of spontaneous Up states. As previously described *injections* included two levels ["injections with saline" (i.e., no seizures) and "injections with PTZ" (i.e., seizures)], *cortex* consisted of two levels (S1, M1) and *number of injections* also had two levels (single and multiple). We found a significant interaction among the three factors for the overall index of spontaneous Up states (*Up state index*) [$F(1,82) = 4.458$, $p = 0.038$], but not for the other parameters tested (**Supplementary Text, Section 3.1**, **Tables 11–13**). Simple effect analysis revealed a significant effect of a single prolonged seizure on Up state network index in both cortices, as opposed to multiple seizures of equal total duration which had no effect on either cortex [Single Seizures: *S1, saline* ($N_{Animals} = 8$, $N_{slices} = 12$) *vs. PTZ* ($N_{Animals} = 9$, $N_{slices} = 14$)*: 0.08 (0.04) vs. 0.05 (0.06) [median (interquartile range)], F(1,82) = 5.154, Bonferroni, p = 0.026; M1, saline* ($N_{Animals} = 9$, $N_{slices} = 10$) *vs. PTZ* ($N_{Animals} = 7$, $N_{slices} = 10$)*: 0.03 (0.03) vs. 0.12 (0.15) [median (interquartile range)], F(1,82) = 7.025, Bonferroni, p = 0.010,* **Figure 4**; Multiple Seizures: *S1, saline* ($N_{Animals} = 7$, $N_{slices} = 10$) *vs. PTZ* ($N_{Animals} = 7$, $N_{slices} = 10$)*: 0.13 (0.12) vs. 0.12 (0.20) [median (interquartile range)], F(1,82) = 0.122, Bonferroni, p = 0.728; M1, saline* ($N_{Animals} = 6$, $N_{slices} = 10$) *vs. PTZ* ($N_{Animals} = 9$, $N_{slices} = 14$)*: 0.07 (0.14) vs. 0.07 (0.16) [median (interquartile range)], F(1,82) = 0.600, Bonferroni, p = 0.441,* **Figure 4** and **Supplementary Text, Section 3.2.1.1**]. Finally, a two-way ANOVA of the effects of *number of injections* and *injections* revealed no significant interaction for any Up states variable that we tested (**Supplementary Text, Section 3.3**, **Tables 14–16**). Therefore, although both protocols of induced seizures that we applied (single and multiple) were of equal total duration (4 min × 15 min vs. 60 min) our results indicate that long-lasting single seizures rather than multiple shorter seizures cause long-term effects in network dynamics emphasizing the importance of ELS duration over ELS frequency in determining their long-term impact.

## Younger Ages Are More Resilient to the Long-Term Effects of ELS on Local Cortical Network Activity

In order to investigate whether the effects of ELS on cortical network dynamics are age dependent, and given that the 60 min SE was not possible in older animals due to increased mortality rates (as described in *Materials and Methods*), we compared the effects of multiple seizures of shorter duration, implemented at either P9–15 or P19–23, in both cortices. For this we conducted a three-way ANOVA as before, of the effect of three independent variables: seizures (*injections*: saline and PTZ), age of seizures (*age of injections*: P9–15 and P19–23) and type of cortex (*cortex*: S1 and M1) on Up states dynamics as quantified by respective parameters. We found a significant interaction of the effects of these three factors on Up state spectral content at the theta [$F(1,71) = 4.620$, $p = 0.035$] and gamma [$F(1,71) = 4.184$, $p = 0.045$] range (**Supplementary Text, Section 4.1**, **Tables 17–19**). Subsequent simple effect analysis of *seizures* on the theta power revealed a significant age-specific increase of power after seizures induced at P19–23 but not at P9–15. In addition, this effect was region-specific since it occurred in S1 but not M1 cortex [P19-23: *S1, saline* ($N_{Animals} = 7$, $N_{slices} = 13$) *vs. PTZ* ($N_{Animals} = 6$, $N_{slices} = 11$)*:* 0.16 (0.02) *vs.* 0.21 (0.06) *[median (interquartile range)], F(1,71) = 14.206, Bonferroni, p < 0.001; M1, saline* ($N_{Animals} = 5$, $N_{slices} = 6$) *vs. PTZ* ($N_{Animals} = 4$, $N_{slices} = 5$)*:* 0.17 (0.10) *vs.* 0.15 (0.03) [*median (interquartile range)], F(1,71) = 0.458, Bonferroni, p = 0.501,* **Figure 5**; P9–15: *S1, saline* ($N_{Animals} = 7$, $N_{slices} = 10$) *vs. PTZ* ($N_{Animals} = 7$, $N_{slices} = 10$)*:* 0.13 (0.08) *vs.* 0.15 (0.04) *[median (interquartile range)], F(1,71) = 1.244, Bonferroni, p = 0.269; M1, saline* ($N_{Animals} = 6$, $N_{slices} = 10$) *vs. PTZ* ($N_{Animals} = 9$, $N_{slices} = 14$)*:* 0.10 (0.06) *vs.* 0.12 (0.04) *[median (interquartile range)], F(1,71) = 2.682, Bonferroni, p = 0.106,* **Figure 5** and **Supplementary Text, Section 4.2.1.1**]. A similar age- and region-specific effect of early multiple seizures on the adult cortex was also supported by respective simple effect analysis of seizures on the gamma power of Up states. In particular we found that multiple ELS significantly reduced the power of gamma frequencies in the adult S1,

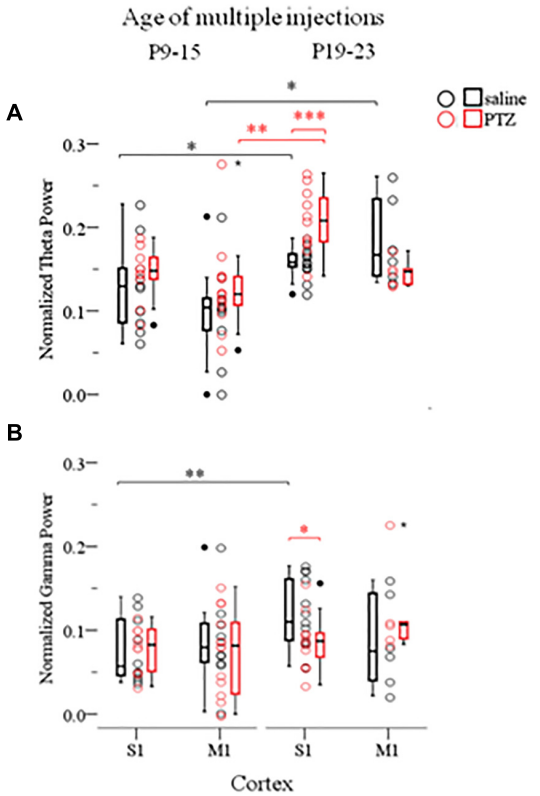


**FIGURE 5 |** The long-term effects of multiple ELSs on spontaneous Up states are age-specific. Multiple 15 min PTZ-induced seizures administered at P9–15 mice had no effect on the spectral content of spontaneous Up states in the theta **(A)** (4–8 Hz) and gamma **(B)** (30–100 Hz) range, in either primary sensory (S1) or motor (M1) cortex. On the contrary, multiple seizures at P19–25 significantly increased theta power, but reduced gamma power in S1, but not M1, cortex. Spectral power of Up states within each range was normalized to the total Up state power in the 1–200 Hz range. *Open circles* represent superimposed vertical scatterplots of individual data points of the control (saline, *black*) and treated (PTZ, *red*) groups of animals. *Box and Whisker plots* placed on each side of the scatterplots describe the spread of data for the saline- (black) and PTZ-treated (red) group showing interquartile ranges (length of *boxes*), upper and lower limits of data (*whiskers*), median (*line inside box*), extreme values (*stars*), and outliers (*filled circles*). *Asterisks* indicate levels of statistically significant differences between compared groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

but not M1 cortex, when they occurred at later (P19–23) rather than earlier (P9–15) developmental stages [P19–23: *S1, saline* ($N_{Animals}$ = 7, $N_{slices}$ = 13) *vs. PTZ* ($N_{Animals}$ = 6, $N_{slices}$ = 11): 0.11 (0.08) *vs.* 0.09 (0.04) *[median (interquartile range)], F(1,71) = 4.750, Bonferroni, p = 0.033; M1, saline* ($N_{Animals}$ = 5, $N_{slices}$ = 6) *vs. PTZ* ($N_{Animals}$ = 4, $N_{slices}$ = 5): 0.08 (0.11) *vs.* 0.11 (0.08) [*median (interquartile range)], F(1,71) = 0.391, Bonferroni, p = 0.534,* **Figure 5**; P9–15: *S1, saline* ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. PTZ* ($N_{Animals}$ = 7, $N_{slices}$ = 10): 0.06 (0.07) *vs.* 0.08 (0.05) *[median (interquartile range)], F(1,71) = 0.081, Bonferroni, p = 0.777; M1, saline* ($N_{Animals}$ = 6, $N_{slices}$ = 10) *vs. PTZ* ($N_{Animals}$ = 9, $N_{slices}$ = 14): 0.08 (0.05) *vs.* 0.08 (0.09) [*median (interquartile range)], F(1,71) = 0.391, Bonferroni, p = 0.534,* **Figure 5 Supplementary Text, Section 4.2.1.2**].

A simple effect analysis of the *age of injections* revealed a significant effect of age within the saline- and PTZ-treated groups for both theta and gamma power. In particular we found theta power of spontaneous Up states to be significantly higher in both S1 and M1 cortex of the adult mice that were injected with saline at P19-23 compared to saline-treated mice at P9–15 [Theta: *S1, saline, P9–15* ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. P19–23* ($N_{Animals}$ = 7, $N_{slices}$ = 13): *0.13 (0.08) vs. 0.16 (0.02) [median (interquartile range)], F(1,71) = 6.175, Bonferroni, p = 0.015; M1, saline, P9–15* ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. P19–23* ($N_{Animals}$ = 7, $N_{slices}$ = 13): 0.10 (0.06) vs. 0.17 (0.10) [median (interquartile range)], F(1,71) = 6.006, Bonferroni, p = 0.017, **Figure 5** and **Supplementary Text, Section 4.2.2.1**]. Respectively, we found that gamma power of spontaneous Up states in the adult S1 cortex was significantly lower in mice injected with saline at P19-23 compared to mice injected with saline at P9–15 [Gamma: *S1, saline, P9–15* ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. P19–23* ($N_{Animals}$ = 7, $N_{slices}$ = 13): 0.06 (0.07) *vs.* 0.11 (0.08) *[median (interquartile range)], F(1,71) = 7.589, Bonferroni, p = 0.007,* **Figure 5** and **Supplementary Text, Section 4.2.2.2**]. This age-dependent saline effect was an unexpected finding for which we have no satisfactory explanation given that recording conditions in all animal groups were identical. Moreover, this result compromises the aforementioned age-specific effect of early seizures on the gamma power of Up states in the adult S1 cortex and renders it inconclusive since simple effect analysis of age showed that gamma levels in adult mice treated with PTZ at P19-23 did not differ significantly from those that had received PTZ at P9–15 [Gamma: *S1, PTZ, P9–15* ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. P19–23* ($N_{Animals}$ = 7, $N_{slices}$ = 13): 0.08 (0.05) *vs.* 0.09 (0.04) *[median (interquartile range)], F(1,71) = 0.373, Bonferroni, p = 0.543,* **Figure 5** and **Supplementary Text, Section 4.2.2.2].** On the contrary, we would argue that the age- (and cortex-) specific effect of ELS on the theta content of spontaneous Up states in the adult S1 cortex, as concluded from our three-way ANOVA analysis, is a reliable result despite the age-dependent saline-effect revealed by simple effect analysis, since PTZ injections at P19–23 increased theta power in the adult S1 (but not M1) cortex compared not only to saline treatment at the same age, but also to PTZ injections at P9–15 [Theta: S1, PTZ, P9–15 ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. P19–23* ($N_{Animals}$ = 7, $N_{slices}$ = 13): 0.15 (0.04) *vs.* 0.21 (0.06) *[median (interquartile range)], F(1,71) = 12.938, Bonferroni, p = 0.001; M1, PTZ,* P9–15 ($N_{Animals}$ = 7, $N_{slices}$ = 10) *vs. P19–23* ($N_{Animals}$ = 7, $N_{slices}$ = 13): 0.12 (0.04) *vs.* 0.15 (0.03) *[median (interquartile range)], F(1,71) = 3.872, Bonferroni, p = 0.053,* **Figure 5** and **Supplementary Text, Section 4.2.2.1].** Finally, a two-way ANOVA of the effects of *age of injections* and *injections* on the remaining Up states variables (i.e., except for theta) revealed no significant interaction (**Supplementary Text, Section 4.3, Tables 20–22**). In conclusion, these results indicate older ages to be more vulnerable to the long-term effects of ELS, while they provide additional evidence that the effects of ELS are region specific.

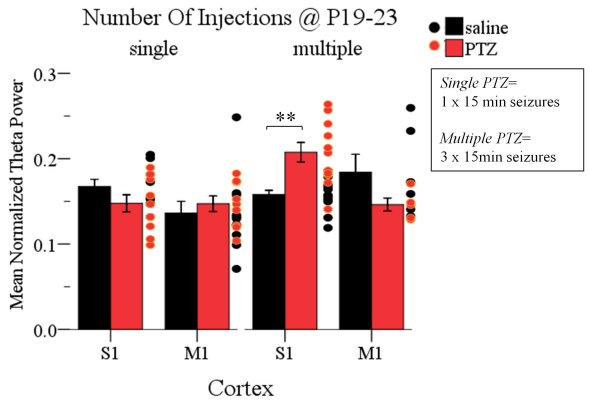


**FIGURE 6 |** The long-term effects of early life seizures on spontaneous Up states depend on their frequency. Multiple, but not single, 15 min seizures administered in P19-23 mice have a region-specific effect on the adult cortex since they significantly increased the theta (4–8 Hz) content of spontaneous Up states in the adult S1, but not M1, cortex compared to saline-treated groups. Spectral power of Up states in the theta range was normalized to the total Up state power in the 1–200 Hz range. *Dots* represent superimposed vertical scatterplots of individual data points of the control (saline, *black*) and treated (PTZ, *red*) groups of animals compared on the left. *Asterisks* indicate levels of statistically significant differences between compared groups (**$p < 0.01$).

## The Long-Term Effects of ELS on Cortical Dynamics Depend on Their Frequency

Finally, having documented that multiple intermittent seizures at P19-23 significantly enhance Up state theta power in the adult cortex, we asked whether a single seizure of the same duration (15 min) was enough to produce a similar effect. To this end we conducted a three-way ANOVA of the effect of the three independent variables: seizures (*injections*: saline and PTZ), number of seizures (*number of injections*: single and multiple) and type of cortex (*cortex*: S1 and M1) on Up states dynamics. We found a significant interaction of the effects of these three factors, which was specific for the spectral content of spontaneous Up states at the theta range [$F(1,66) = 13.195$, $p = 0.001$] (**Supplementary Text Section 5.1, Tables 23–25**). Subsequent simple effect analysis of *seizures* (*injections*) revealed a frequency-specific effect of seizures since multiple but not single seizures significantly increased the theta power in the adult cortex. In addition, this effect was cortex-specific since it was significant for S1 but marginally non-significant for M1 [Single: *S1, saline* ($N_{Animals} = 7$, $N_{slices} = 9$) *vs. PTZ* ($N_{Animals} = 8$, $N_{slices} = 10$): $0.17 \pm 0.03$ *vs.* $0.15 \pm 0.03$ *(mean ± SD), F(1,66) = 1.637, Bonferroni, p = 0.205; M1, saline* ($N_{Animals} = 8$, $N_{slices} = 11$) *vs. PTZ* ($N_{Animals} = 7$, $N_{slices} = 9$): $0.14 \pm 0.05$ *vs.* $0.15 \pm 0.03$ *(mean ± SD), F(1,66) = 0.523, Bonferroni, p = 0.472;* Multiple: *S1, saline* ($N_{Animals} = 7$, $N_{slices} = 13$) *vs. PTZ* ($N_{Animals} = 6$, $N_{slices} = 11$): $0.16 \pm 0.02$ *vs.* $0.21 \pm 0.04$ *(mean ± SD), F(1,66) = 13.157, Bonferroni, p = 0.001; M1, saline* ($N_{Animals} = 5$, $N_{slices} = 6$) *vs. PTZ* ($N_{Animals} = 4$, $N_{slices} = 5$): $0.18 \pm 0.05$ *vs.* $0.15 \pm 0.02$ *(mean ± SD), F(1,66) = 3.525, Bonferroni, p = 0.065;* **Figure 6** and **Supplementary Text, Section 5.2.1.1**]. Finally, in order to test for potential cortical-independent effects of severity of seizures (number of injections) on Up states, we conducted a two-way ANOVA of the effects of *number of injections* and *injections* on the remaining Up states variables (i.e., except for theta), which, however, revealed no significant interaction (**Supplementary Text, Section 5.3, Tables 26–28**). Overall our results show that the impact of short duration ELS increases with their frequency.

Taken together, our results provide evidence that early-life seizures can affect spontaneous local network activity recorded even months later, in the adult mouse neocortex. Importantly, we found this effect to vary depending on several factors such as: (a) the severity of seizures, (b) the cortical area tested, (c) the age at which seizures occurred, and finally (d) the physiological parameter measured (**Table 1**). We conclude that long-term effects of ELS on cortical network dynamics are *region-specific* and *age-specific*. In particular, prolonged single status epilepticus -but not multiple intermittent seizures- during the second postnatal week (P12) enhanced spontaneous Up states in motor but reduced those in sensory cortex. However, multiple intermittent seizures during the third (P19–23) but not the second (P9–15) postnatal week affected S1, but not M1, cortical networks by increasing their theta activity.

# DISCUSSION

## Endogenous Cortical Network Activity in the Form of Spontaneous Up States Is a Sensitive Neurophysiological Measure to Reflect Long-Term Effects of Early Life Seizures on the Cerebral Cortex

In this study we have shown that ELS can cause permanent changes in cortical network dynamics persisting well into adulthood. Experimentally induced seizures are routinely used to explore the impact of ELS on the developing brain. However, the results of such studies are often contradictory and have failed to provide a broad consensus on the effects of ELS on brain function and structure, or behavior (Wasterlain, 1997; Lado et al., 2000). This is at least partly due to the wide variability in experimental protocols including (a) the specific ages at which seizures are induced; (b) the seizure-induction methods (e.g., chemically, electrically, febrile-, and hypoxia-induced seizures); (c) the number of seizures (single vs. multiple); (d) the duration of individual seizures [prolonged (i.e., status epilepticus) vs. intermittent]; (e) the post-seizure intervals at which effects are investigated (immediate, medium- or long-term); and (f) the brain area under investigation. Furthermore, most studies that examine the effects of ELS focus on specific neuronal and/or synaptic elements, or overall behavioral phenotypes, rather than on the intermediate level of organization such as the neuronal network. Here, using the PTZ model we investigated how experimental seizures of distinct duration, number and age at which they occurred affected two distinct cortical areas during adulthood. To our knowledge this is the first attempt to examine

**TABLE 1 |** Summary of the effect of ELS at two developmental stages (P9–15 and P19–23) on spontaneous Up states in M1 and S1 cortex of the adult mouse.

| | | Age of seizures induction | | | |
|---|---|---|---|---|---|
| | | P9–15 | | P19–23 | |
| **Cortex** | | **S1** | **M1** | **S1** | **M1** |
| Type of seizure | Single 60 min | ↓Up state index | ↑Occurrence<br>↑Up state index | | |
| | Single 15 min | | | *No effect* | *No effect* |
| | Multiple 15 min | *No effect* | *No effect* | ↑Theta | *No effect* |

*Symbols indicate whether there was an increase (↑), decrease (↓) on respective parameter.*

the long-term outcome of ELS as a result of the independent or interactive effects of distinct factors, such as seizure severity, brain region and age of seizure onset, all tested within the same study.

It is also the first study to quantify the *long-term* effects of ELS on the endogenous cortical network activity in the form of recurring Up/Down states. This type of activity is characteristic of the slow oscillation (SO) and is observed both *in vivo*, during quiescence, but also *in vitro*, in the brain slice preparation (Steriade et al., 1993, 2001; Sanchez-Vives and McCormick, 2000; Cossart et al., 2003; MacLean et al., 2005; Haider et al., 2006; Rigas and Castro-Alamancos, 2007, 2009; Poulet and Petersen, 2008). The slow oscillation is considered the 'default' activity of the cortex rendering it a *central pattern generator* (Yuste et al., 2005; Sanchez-Vives et al., 2017). Hence, the SO and in particular its active components (i.e., the spontaneous Up states) provide the framework to assess the impact of early life events on local network dynamics of the adult cerebral cortex. Since Up states consist an emergent property of cortical microcircuits that involve both excitatory and inhibitory neurons (Steriade et al., 1993) they reflect the balanced contribution of excitation and inhibition of cortical networks (Sanchez-Vives and McCormick, 2000; Shu et al., 2003; Hasenstaub et al., 2005; Haider et al., 2006). To our knowledge this is the first report that ELS can have a long-term impact on spontaneous Up states, extending findings of an earlier report on the acute effects of seizures on this type of activity (Gerkin et al., 2010).

## The Effects of ELS Are Regionally Specific

Our results suggest that ELS affect the adult brain in a region-specific manner. In particular, we found a significant effect of cortex in both our main results: (a) prolonged seizures (status epilepticus, SE) at P12 significantly affected both primary motor (M1) and sensory (S1) cortices, but in opposite directions since they enhanced the occurrence and overall index of spontaneous Up states in M1, but decreased Up states index in S1. And (b) multiple intermittent seizures at P19-23 affected S1 but not M1 cortex in terms of the theta content of their Up states.

The idea that ELS may affect the brain in a region-specific manner is supported by a recent study showing that seizure activity in the early postnatal mouse neocortex for 2 h diminishes the rates of apoptosis in M1 but not S1 (Blanquie et al., 2017). Apoptosis plays a critical role in establishing neural circuits in the developing mammalian brain, by selectively eliminating a substantial portion of the initially overproduced neurons through cell death (Haydar et al., 1999). Moreover, there is a general consensus that spontaneous electrical activity, a widespread property of the developing cerebral cortex, plays a pro-survival role, while reduced activity increases the number of neurons undergoing apoptosis (Ikonomidou et al., 1999; Golbs et al., 2011; Murase et al., 2011; Lebedeva et al., 2017). Therefore, in regard with our own results, prolonged seizures (status epilepticus, SE) at P12 could for example compromise apoptotic processes in M1 specifically, leading to larger and/or denser neuronal assemblies in the cortex of PTZ-treated compared to non-treated mice, capable of producing spontaneous network events (i.e., Up states) at higher incidences. Moreover, evidence suggests that, as opposed to primary pyramidal neurons, the cell fate of developing cortical inhibitory interneurons is rather intrinsically programmed than activity-dependent (Sahara et al., 2012; Southwell et al., 2012). This property of inhibitory cells could, therefore, render them resistant to the excessive neural activity of seizures, thus, preserving them to normal adult levels. Such a scenario would explain to an extent why we found early SE to affect the occurrence of Up states but not their duration, since inhibition has been associated with modulation of Up state duration (Mann et al., 2009; Sanchez-Vives et al., 2010). Similar to our results, which differentiate the effect of early seizures depending on the variable of network dynamics tested, 20–40 min long seizures in 2 week old mice increased the occurrence of spontaneous Up states in the somatosensory cortex 24 h later without affecting their duration (Gerkin et al., 2010). The differential effect of ELS on Up state occurrence and duration could reflect the fact that previous studies, including our own, have shown that the mechanism of Up state generation (which is reflected in the Up state occurrence) is distinct from the mechanism of Up state termination (which is reflected in the Up state duration) (Mann et al., 2009; Sanchez-Vives et al., 2010; Sigalas et al., 2015). Finally, enhanced Up state activity in M1 cortex suggests an increased excitability of this cortical area which at its extreme could serve as the substrate for the development of spontaneous seizures (Ziburkus et al., 2013). Although none of our PTZ-treated mice appeared to develop epileptic behavior, ELS have been associated with epileptogenesis during adulthood (Sillanpaa et al., 1998; Brunquell et al., 2002). On the other hand, the reduced index of spontaneous Up states (*Up state index*) in S1 recorded in the brain of adult mice that had experienced SE indicates compromised network dynamics

which, in turn, could contribute to sensory processing and cognitive deficits associated with ELS (Neill et al., 1996; Holmes, 2016).

Our findings on the region-specific effects of ELS on the cerebral cortex suggest that the general concept of the "immature brain" may not be sufficient to predict neuronal vulnerability to ELS. Instead, regional levels of maturation might need to be taken into account (Lopez-Meraz et al., 2010). Accumulating evidence suggests that development is not necessarily synchronized among distinct cortical areas, but that structural and functional maturation progress in a caudal-to-rostral direction (Huttenlocher, 1990; Huttenlocher and Dabholkar, 1997; Kurth et al., 2010; Bianchi et al., 2013). In agreement, we have recently shown that endogenous Up state activity matures faster in S1 compared to M1 in the developing mouse (Rigas et al., 2015). In addition, physiological and anatomical findings also show that maturational levels are not uniform throughout the rat hippocampus, with CA1 maturing earlier than the dentate gyrus (Harris and Teyler, 1984; Bekenstein and Lothman, 1991a,b; Lowenstein and Alldredge, 1998). Therefore, one could expect distinct cortical regions to respond differently to a common seizure experience depending on their maturational stage.

## The Effects of ELS Depend on Seizure Frequency and Pattern

While the impact of recurrent seizures has been addressed experimentally (Stafstrom et al., 1992; Huang et al., 2002; Lai et al., 2002; Riviello et al., 2002; Karnam et al., 2009a,b), to our knowledge the direct comparison of their effect to that of either an individual seizure (i.e., addressing the issue of *seizure frequency*) or of a single prolonged seizure whose duration would equal the total duration of multiple seizures (i.e., addressing the issue of *seizure pattern*), has received scarce or no attention, respectively (Ni et al., 2005). Both issues are inherently associated with the on-going dilemma that neurologists face in the clinic on whether or when seizures should be treated or not. For example, it is debated whether early single or non-persistent seizures should be treated (Hughes, 2010). The dilemma rises from the fact that it is unclear whether the cognitive impairments often associated with early-seizures are due to the seizures *per se*, or are rather a consequence of either the underlying etiology of seizures, or of the antiepileptic drug therapy itself (Reeta et al., 2009, 2010). Hence, the relative merit of treating early-life non-persistent seizures with anti-epileptic drugs over leaving them untreated is currently not well understood.

As opposed to clinical observations or other experimental models in which seizure durations are difficult to control, in our study we were able to induce seizures in a well-regulated manner and thus test the effect of both seizure frequency and of seizure pattern. We found (a) a pattern effect of ELS: a single 60 min event of status epilepticus, but not multiple seizures of *equal total duration,* suffices to cause a persistent change in M1 and S1 network excitability in the mature brain (**Figure 4**). And (b) a frequency effect of ELS: multiple, but not single seizures during early life lead to a significant increase in the theta power of S1 network activity during adulthood (**Figure 6**). Hence, our results suggest that short duration seizures recurring over a brief period in early life may be more benign compared to even a single event of status epilepticus, in line with clinical observations for febrile seizures (Annegers et al., 1987; French et al., 1993; Verity et al., 1998). In addition, our findings are in agreement to data from other experimental models showing the developing brain to be resistant to single seizures (Albala et al., 1984; Nitecka et al., 1984; Sperber et al., 1991; Thurber et al., 1992; Liu et al., 1994; Sarkisian et al., 1997; Riviello et al., 2002), but differ from them since we show a significant effect of recurring ELS (**Figure 6**).

## The Long-Term Effects of ELS Depend on the Age at Which They Occur

Our data indicate that recurrent early seizures caused changes in the spectral content of spontaneous Up states in the S1 cortex of the adult brain (increase in theta power). This effect was both age- and region-specific since it occurred in the older but not the younger age group (P19–23 vs. P9–15) and in S1 but not M1 cortex (**Figure 5**). In a recent study of ours we traced changes in the spectral content of spontaneous Up states of the mouse S1 cortex during development and maturation. Interestingly, we found that the most prominent changes take place during the transition from the second to the third postnatal week: the power of lower frequencies (delta+theta) decrease and the power of higher frequencies (beta+gamma) increase (Rigas et al., 2015). We could therefore speculate that the age-dependent long-term effects of ELS on theta may rise from the fact that seizures act on uneven levels of theta activity (i.e., higher at P9–15 vs. lower at P19–23). In addition, this age-specific effect was cortex-dependent since only S1 cortex was affected. Our previous work has shown that the developmental trajectory of endogenous network dynamics is faster in S1 compared to M1 cortex, with the peak of Up state activity occurring at P19 vs. P30, in S1 vs. M1 cortex, respectively (Rigas et al., 2015). Therefore multiple ELS during P19-23 would act on distinct levels of network excitability in the two areas (higher in S1 and lower in M1), which in turn could contribute to the increased effect on the spectral content in the adult S1 but not M1 cortex.

The age-specific effect of ELS that we found is in agreement with several lines of evidence, both clinical and experimental, supporting a higher vulnerability of the older than the younger brain to seizures. In particular, clinical evidence suggests that status epilepticus (SE) in young children leads to lower mortality and better cognitive outcomes when compared to SE in adults and the elderly (Maytal et al., 1989; Lowenstein and Alldredge, 1998; Leppik et al., 2006; Towne, 2007). For example, many studies show that prolonged seizures are less likely to result to neuronal loss or synaptic rearrangement in the brain of infants

and children compared to the mature brain (Sperber et al., 1992; Holmes and Ben-Ari, 2001; Bender et al., 2003; Porter et al., 2004; Baram et al., 2011). Similarly, animal studies of experimental seizures have shown the immature hippocampus to be more resilient to seizure-induced neuronal cell death and synaptic reorganization (Sperber et al., 1991, 1999; Lado et al., 2000; Sperber and Moshe, 2001; Riviello et al., 2002). In the current study, however, we also wanted to explore whether the long term outcome of ELS in the immature brain differed depending on the specific *developmental stage* these occurred. To this end we tested developing mice at two ages: P9–15 and P19–23. Given that rodents are born prematurely compared to humans and that mice sexually mature around P30–35 (Safranski et al., 1993), the ages that we studied are equivalent to human infants and prepubertal children (Nehlig, 1997; Velisek and Moshe, 2002; Sengupta, 2013; Dutta and Sengupta, 2016). While the differential effects of seizures on the immature and adult brain are well established, less is known on whether and how the effects of ELS may differ depending on when they occur during development. For example, resistance of the human brain to seizures differs among developmental stages, gradually decreasing from infancy to childhood and adolescence as recently reviewed (Nickels, 2015). Respectively, animal studies have shown that the effects of ELS in rats differ depending on whether they occur before or after P20, with younger rats being more resilient (Stafstrom et al., 1992; Sayin et al., 2015). In addition, recent research has indicated the days P20–30 as a critical period for the long-term outcome of ELS, at least for the rat hippocampus (Sayin et al., 2015). Our results extend these studies and indicate that the broad divisions "mature vs. immature," or "adult vs. developing" are not adequate enough to explore differential brain vulnerability to seizures. Instead, this issue should be examined at a higher temporal resolution by examining specific and more restricted periods during development and maturation.

## Significance and Perspectives

The reported electrophysiological results consist the first, to our knowledge, evidence that seizures during early development may cause permanent changes in the local network dynamics of the adult neocortex. We therefore propose that spontaneous cortical network activity, in the form of recurring Up states, may serve as a neurophysiological measure to describe and study the long-term effects of ELS on cortical function and excitability, in both the lab and the clinic. The current results are part of a larger ongoing study that includes behavioral assessment as well as evaluation of brain cytoarchitecture in order to corroborate electrophysiological findings and explore the underlying mechanisms responsible for the differential effects.

Cortical neurons form recurrent networks which synchronize individual cells and are intrinsically active in the form of oscillating activity, visible at increasingly macroscopic neurophysiological levels: from single cells to LFPs; to the clinically relevant electroencephalography (EEG). Synchronized oscillating neuronal networks are viewed as the "middle ground" between single-neuron activity and behavior (Buzsaki and Draguhn, 2004). Although, research of experimental seizures has provided invaluable insights to the cellular and synaptic changes that ELS can cause to the adult cortex (as reviewed in the *Introduction*), whether, to what extent and how these changes actually contribute to higher levels of organization such as the *neuronal network*, as a *final functional pathway* that defines brain physiology and ultimately behavior, are issues that have received less attention. Our results support the idea that spontaneous Up states may provide the necessary framework to link molecular, cellular and synaptic changes to ELS-induced local network dynamics. Importantly, since this activity is present not only in the intact brain but also at the reduced level of the cortical slice, it also provides researchers with a useful experimental tool with which to explore the underlying cellular and synaptic mechanisms.

Spontaneous Up and Down states are the intracellular correlates of the *slow oscillation*, the electroencephalographic hallmark of quiescent states of the brain, such as non-REM sleep, anesthesia and quiet wakefulness (Steriade et al., 1993; Crochet and Petersen, 2006). It is noteworthy that *in vivo* recordings of spontaneous network cortical activity during rest are currently employed in the clinic for the discovery of biomarkers of psychiatric disorders (Wada et al., 1998; Kissler et al., 2000; Sorg et al., 2007; Gandal et al., 2010), while EEG recordings during sleep have been used to describe cortical development in humans (Buchmann et al., 2011). If replicated *in vivo*, the results of our study raise the interesting possibility that the parameters of the slow oscillation in EEG recordings may provide clinicians and researchers with *endophenotypes* of seizure-induced cortical malfunctions. By definition, an endophenotype is the biological manifestation of a disease at a *reduced* level of biological organization as opposed to the macro-level of behavior (Gottesman and Shields, 1973; Almasy and Blangero, 2001; Gottesman and Gould, 2003; Hasler et al., 2004; Gould and Gottesman, 2006). Thus, in order for biological research of mental disorders to proceed, it is essential to 'decompose' the disorder into simpler parameters that can serve as endophenotypes. In this perspective, studying the activity of local cortical microcircuits may provide useful insights toward understanding the brain pathology induced by ELS.

## AUTHOR CONTRIBUTIONS

PR contributed to the conception and design of the work; the acquisition, analysis, and interpretation of data; and drafting the manuscript and critically revising it. CS contributed to the acquisition of the data and critically revising the manuscript. MN, CP, AspK, and AnnKatri contributed to experiments of seizure induction. AniK contributed to data analysis and drafting the manuscript. LL contributed to data analysis, drafting the manuscript and critically revising it. KA and CZ contributed

to data analysis. IS contributed to the conception and design of the work, interpretation of data, and critically revising the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnsyn.2018.00043/full#supplementary-material

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# Temporal Flexibility of Systems Consolidation and the Synaptic Occupancy/Reset Theory (SORT): Cues About the Nature of the Engram

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The ability to adapt to new situations involves behavioral changes expressed either from an innate repertoire, or by acquiring experience through memory consolidation mechanisms, by far a much richer and flexible source of adaptation. Memory formation consists of two interrelated processes that take place at different spatial and temporal scales, *Synaptic Consolidation,* local plastic changes in the recruited neurons, and *Systems Consolidation, a* process of gradual reorganization of the explicit/declarative memory trace between hippocampus and the neocortex. In this review, we summarize some converging experimental results from our lab that support a normal temporal framework of memory systems consolidation as measured both from the anatomical and the psychological points of view, and propose a hypothetical model that explains these findings while predicting other phenomena. Then, the same experimental design was repeated interposing additional tasks between the training and the remote test to verify for any interference: we found that (a) when the animals were subject to a succession of new learnings, systems consolidation was accelerated, with the disengagement of the hippocampus taking place before the natural time point of this functional switch, but (b) when a few reactivation sessions reexposed the animal to the training context without the shock, systems consolidation was delayed, with the hippocampus prolonging its involvement in retrieval. We hypothesize that new learning recruits from a fixed number of plastic synapses in the CA1 area to store the engram index, while reconsolidation lead to a different outcome, in which additional synapses are made available. The first situation implies the need of a reset mechanism in order to free synapses needed for further learning, and explains the acceleration observed under intense learning activity, while the delay might be explained by a different process, able to generate extra free

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synapses: depending on the cognitive demands, it deals either with a fixed or a variable pool of available synapses. The Synaptic Occupancy/Reset Theory (SORT) emerged as an explanation for the temporal flexibility of systems consolidation, to encompass the two different dynamics of explicit memories, as well as to bridge both synaptic and systems consolidation in one single mechanism.

**Keywords: systems consolidation temporal framework, recent vs. remote memory, precision vs. generalization, hippocampus, neocortex, Synaptic Occupancy/Reset Theory (SORT)**

## MEMORY AND TIME

The ability to adapt to challenging new situations involves both physiological and behavioral changes, and behavior may either be expressed from an innate repertoire of stereotyped responses – which Fuster (1995) calls "phyletic memory" – or by the acquisition of experience through memory mechanisms, or even a combination of both (James, 1890). These two classes of cognitive functions, however, differ in several respects, and the second one – "individual memory" – is by far a much richer and flexible source of both adaptation and resilience (two complementary concepts according to Wong-Parodi et al., 2015), and, ultimately, might be the reason for the evolutionary success of vertebrates, specially the mammals.

Memory is an experience-based behavior modification. This is a purely operational definition that covers the basic types of memory that humans and non-human animals fully share, leaving imaginary and/or abstract constructions – whose relation to behavior is somewhat distant – aside for a while. In order to be preserved, it is generally accepted that this change demands the storage (and retrievability) of a physical trace that somehow embodies the experience (Craik, 2002). However, we still don't know how much (and exactly which) information is effectively stored, with possibilities varying from a simple set of reconstruction instructions (Bartlett, 1932; Neisser, 1967; Roediger and De Soto, 2015) up to a larger collection of detailed information.

Memory formation consists of two interrelated processes, equally referred to as *consolidation*, that take place at different spatial and temporal scales. *Synaptic* (or *Cellular*) *Consolidation* comes first and consists of local plastic changes in the recruited neurons *in each and every brain area involved* in order to re-structure synaptic connections, lasting from minutes to hours (Dudai, 1996). Over a much larger time scale, *Systems Consolidation* is the process of gradual reorganization of the explicit (non-episodic-like) memory trace in the NCTX, along with progressive independence from the HPC and its adjacent cortices – which in rats takes a few weeks, but in humans can take from months to years (Kim and Fanselow, 1992; McClelland et al., 1995; Dudai, 1996; Quillfeldt et al., 1996; Izquierdo et al., 1997; Frankland and Bontempi, 2005; Winocur et al., 2010; Wiltgen et al., 2010).

Evidence for memory systems consolidation began to emerge in studies with lesioned patients already in the 19th century (e.g., Ribot, 1881), but it was only after the paradigmatic case of patient H. M. (Henry Molaisson), described by Scoville and Milner (1957), that the HPC was singled out as a crucial structure for memory (McDonald and White, 1993; Squire et al., 1984; Squire, 2004). Lesions restricted to the MTL, that includes the hippocampal formation, resulted in temporally graded RA – the loss of the memories acquired more recently with some degree of preservation of the older ones, as well as a severe anterograde amnesia – the inability to code for new long-term memories (Squire and Bayley, 2007; Nadel et al., 2007).

## EPISODIC MEMORY IN TIME: CLASH BETWEEN FACTS AND THEORIES

Systems consolidation, with a functional "transition" between HPC and NCTX, has been mostly verified for the so-called explicit or declarative memory, which in humans involve two categories, episodic and semantic memories (Tulving, 1972; Cohen and Squire, 1980; Cohen, 1981; Graf and Schacter, 1985): however, human episodic memory have resisted to conform to this dynamics since it typically remains indefinitely dependent from the HPC – non-graded or "flat" temporal gradient RA (Nadel et al., 2007; Nadel and Hardt, 2011). Episodic memory is still at the fulcrum of a decades-old debate between two competing theories about temporal modifications undergone by explicit memories. The first, conventionally known as the SMSC (Squire and Alvarez, 1995), proposes that all long-term memories already consolidated at the synaptic level (i.e., after at least 6hs), in the beginning need the HPC to be retrieved, but this dependence will subside progressively, with memory processes becoming reliant upon neocortical circuits. SMSC holds that *all declarative* memories, be them of episodic or semantic nature, must have the same fate, becoming independent from the HPC. After an extensive review of the literature on human memory, however, it became clear that the remote episodic memories cannot usually be retrieved without the assistance of the HPC (Nadel and Moscovitch, 1997), which paved the way for the more

**Abbreviations:** ACC, anterior cingulate cortex; CA1, *Cornu Ammonis 1* area of the hippocampus; CA3, *Cornu Ammonis 3* area of the hippocampus; CFC, contextual fear conditioning; CTT, *Competitive Trace Theory*; DG, dentate gyrus area of the hippocampus; DRT, *Distributed Reinstatement Theory*; ERC, entorhinal cortex; GluN2B, subunit N2B of NMDA receptor; HIT, Hippocampal Memory Indexing Theory; HPC, hippocampus; HSAM, highly superior autobiographical memory; IP, "intermediary plexus" – the neural network between S and M; LGVCCs, L-type voltage-gated calcium channels; M, motor outputs; MEMSET, extra-navigational *Memory Set of Functions*; mPFC, medial prefrontal cortex; MTL, medial temporal lobe; MTT, Multiple Trace Theory; NAVSET, Navigational Set of Functions; NCTX, neocortex; PPC, posterior parietal cortex; PTSD, post-traumatic stress disorder; RA, retrograde amnesia; S, sensory inputs; SMSC, Standard Model of Memory (Systems) Consolidation; TTT, *Trace Transformation Theory*.

encompassing conception known as the MTT, which accepts, among other concepts, different dynamics for episodic and semantic memories.

For a number of reasons, it was not trivial to reproduce, in animal models, the clear-cut division between episodic and semantic memories observed in humans, but, similar to humans, the temporally graded RA that characterizes systems consolidation has been observed with some types of explicit memories – such as aversive memories, but not with other types – such as spatial memories, that tend to display a non-graded RA (Sutherland et al., 2010; Winocur et al., 2013), which also represents a challenge for SMSC core concepts. Actually, even in human studies there is some debate about what "episodic" really mean, with permanent HPC dependency being observed mainly in episodic memories of the autobiographical type (for a discussion, see Teyler and Rudy, 2007; Rudy, 2009).

Interestingly, both SMSC and MTT drank to some extent from the same HIT (Teyler and DiScenna, 1986; Teyler and Rudy, 2007), a very consistent early attempt to conciliate psychobiological data with neuroanatomy-of-the-day (Squire et al., 1984) plus some mathematical modeling of neural networks from the beginning of the 1970s (Marr, 1971) in order to explain the role of HPC in memory storage. HIT allowed, among other advances, the maturation of decisive concepts such as *pattern completion* and *pattern separation* (Teyler and Rudy, 2007). Another strong influence came from the seminal work of O'Keefe and Nadel (1978) that proposed the role of *Hippocampus as a Cognitive Map*, and the so called Complementary Learning Systems framework, which suggested a protective role for the HPC/NCTX interplay working to prevent catastrophic interference among similar patterns (Marr, 1971; McClelland et al., 1995; O'Reilly et al., 2014). Actually, despite invisible for many, it can be said that no modern theory of memory in cognitive psychology would exist today free from the influence at least two conceptual paradigms, the *information-processing* approach (e.g., the computer metaphor for the brain) – to this day, by far the most influential of the two (but perhaps on the negative side) – and the *connectionist* approach (e.g., parallelism, emergentism, neural networks, etc – see chapter 1 in Galotti, 2018), still scarcely explored.

## SYSTEMS CONSOLIDATION DYNAMICS: EXCEPTIONS AND ALTERNATIVE MODELS

Contextual fear conditioning and, in special, spatial learning, are among the behavioral tasks that produce more contradictory results in relation to the systems consolidation framework – i.e., they frequently produce *flat or non-graded temporal gradients* (Sutherland et al., 2008, 2010; Broadbent and Clark, 2013; Winocur et al., 2013), i.e., memories that never exhibiting independence from the HPC when retrieved. Sutherland et al. (2010) have even proposed an alternative model that would complement MTT and explain away diverging findings – the so-called DRT, according to which, instead of the "gradual and lengthy memory reorganization" of one single mnemonic entity, what happens is the rapid establishment of a dual-trace in both brain regions, with a stronger representation in the HPC, and a weaker one in the cortex. This would explain memory retrieval without an active HPC, since an extra-hippocampal trace, despite weaker, could yet be expressed in some situations. This interesting *ad hoc* hypothesis reintroduces an assumption already present – but frequently understated – in the SMSC (Squire and Alvarez, 1995), that is fully consistent with several other findings from our lab over the years (Jerusalinsky et al., 1994; Sierra et al., 2017 – see below): *cortical areas must be recruited simultaneously* with the hippocampal system during acquisition/learning in order to, later, support the temporally graded "changing of the guards" between the HPC and the NCTX, i.e., the suggested *dual trace* seems to exist at last.

One interesting conceptual suggestion originally proposed by MTT was that each time retrieval takes place, that trace would be automatically re-encoded (i.e., "re-indexed") in the HPC, meaning that the older the memory, the more "copies" of its index would be available and the easier would be to retrieve then, in thesis (Nadel and Moscovitch, 1997, 1998). This idea was devised to explain, for instance, the robustness of some old memories, or for, say, memory of items reinforced by repetition or "rehearsal." This interesting theoretical prediction, consistent with the best supporting ideas advocated by HIT (Teyler and Rudy, 2007), would be useful to account for several findings in the field of *memory reconsolidation* (Lewis, 1979; Nader et al., 2000a,b; Anokhin et al., 2002; Walker et al., 2003; Duvarci and Nader, 2004; Lee et al., 2006; Rose and Rankin, 2006; Hupbach et al., 2008; Bustos et al., 2009, 2010; Nader and Hardt, 2009; Nader and Einarsson, 2010; Hardt et al., 2010; Lee, 2010; Alberini, 2011; Haubrich and Nader, 2018) – indeed, an updated trace might even end up being expressed just as one of those index copies, slightly modified.

However, to our notice, notwithstanding the expected technical difficulties, this promising idea was never put to real test. The multiple copies scenario could, for instance, be contrasted with opposite theoretical models such as the CTT, also inspired by HIT (Yassa and Reagh, 2013), in which the HPC, through a memory reconstruction process called *recontextualization*, compensate for the deleterious effects of the competition among partially overlapping traces of aging memories, strengthening memories by semantization at the expense of contextual details.

In 2010, Winocur et al. (2010) advanced a extensively revised version of MTT – dubbed as the TTT – to incorporate the now widely accepted idea that the corticalized single episode trace is *not a mere duplicate* of the previous hippocampal version, but a *transformed* record with quite different characteristics. The transformation hypothesis differs from SMSC in that (1) it accepts the permanent HPC-dependency of detailed/autobiographical episodic, contextually bound memories, (2) the "hippocampal memory" supports the corticalization that produce a contextually poor, gist-like ("schematic") engram, and (3) that HPC-related precise memories dynamically interact/compete for dominance with

cortex-related generalized traces depending on the boundary conditions in the retrieval session (Winocur et al., 2010; Sekeres et al., 2018). The first two points were inherited from MTT, but the last one is new, and incorporates the very recent paradigm that emphasizes the parallels between HPC/precision and corticalization/generalization, i.e., the supposed connection between the neuroanatomical and the psychological/qualitative points of view.

## TWO COMPLEMENTARY APPROACHES TO SYSTEMS CONSOLIDATION

Then, coinciding with the gradual HPC disengagement in contextual fear memory expression, a number of studies have found that animals are good at discriminating between the original training context and a novel context shortly after training, whereas some weeks later they show equally robust conditioned responding to both contexts, an example of loss of contextual precision (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007; Winocur et al., 2007). The reduced HPC engagement and the increased generalization in the cognitive domain may be more than a simple coincidence, and has been suggested to reflect a specific role for the HPC in mediating detailed, discriminatory memory expression (Wiltgen et al., 2010). In this line, progressive corticalization comes at the price of having most of the details of the original experience stripped off, attaining a more generalized nature. In the limit, we may suppose this is the first step in building *schemas* – a class of fast-response cortical psychological construct which goes far beyond a mere case of generalization of information, once they act by structuring both the information gathering and their use (Ghosh and Gilboa, 2014). The transition from memory discriminative precision to generalization may be used as a measurable psychological correlate of the temporally graded neuroanatomical involvement in systems consolidation. Notwithstanding its utility as an additional tool to study the phenomenon, attention must be paid in every experimental design to avoid false positives due to the fact that there are other ways to produce the generalization of any learned information: a series of time-independent generalization protocols such as sexual hormone levels, presynaptic GABA-B inhibition or the so-called cue-induced generalization do not correlate with systems consolidation and might deserve additional control groups in some experimental designs (see Jasnow et al., 2017).

In the following sections, we will review some results from our lab that, over the years, have raised some interesting questions possibly relevant for a discussion on the nature of the engram. After replicating the phenomenon from the *neuroanatomical point of view* in two different experimental setups, finding a similar time frame between 4 and 6 weeks – despite specific differences between the protocols – we managed to *accelerate* the transition of the retrieval control from HPC to NCTX (in this case, the anterior cingular cortex) simply by increasing the amount of learning opportunities between training and remote test sessions. We then explored other, different ways to modify the time course of systems consolidation, such as reactivating the main aversive memory. In between, we investigated the need for the lately engaged neocortical area to be actively involved already during the acquisition of the behavioral task.

## CLOSE ENCOUNTERS WITH SYSTEMS CONSOLIDATION

In the beginning of the 1990s, a time in which the Standard Model was still being formulated (McDonald and White, 1993; McClelland et al., 1995; Squire and Alvarez, 1995) and the phenomenon of systems consolidation wasn't even named (Dudai, 1996), when studying the role of glutamatergic and GABAergic receptors in memory formation and expression, we found that the AMPA competitive antagonist CNQX was amnestic when infused into the HPC (and amygdala – in a joint, bilateral infusion) at 1, 6, 13, 20 but not 31 days after training (Bianchin et al., 1993; Izquierdo et al., 1993a,b; Quillfeldt et al., 1996), while the same blocking effect tend to last more when injected into the ERC, effective at 1, 26, 31 but not 60 days after training (Ferreira et al., 1992a,b; Jerusalinsky et al., 1992; Quillfeldt et al., 1994, 1996). Thus, HPC and ERC appear to have naturally "switched" their roles in memory retrieval somewhere between the 20th and the 31st post-acquisition day, at least for this specific aversive task (step-down inhibitory avoidance). The fact that cortical areas were displaying sensitivity to CNQX also *before* the HPC disengagement may be due to the drug of choice and the essential nature of the glutamatergic transmission in the CA1 area (a more detailed discussion appears in the end of the section entitled "New learnings before the remote test accelerate systems consolidation").

This 2–4 weeks' interval for the disengagement of the HPC is consistent with several studies involving rodents in contextual fear learning (Kim and Fanselow, 1992; Maren et al., 1997; Shimizu et al., 2000; Wiltgen et al., 2010; Beeman et al., 2013). There are, however some important contrary findings, reporting "flat" temporal gradients (Sutherland et al., 2008; Broadbent and Clark, 2013), but besides relevant differences in experimental protocols, some of these inconsistencies may be due to the fact that most of them have employed chemical lesions, which differ from our use of pharmacological reversible blockings, both in its extent and the possible outcomes (Sutherland et al., 2010; Goshen et al., 2011; Doron and Goshen, 2017). Anyway, once even humans display different durations of RA caused by comparable hippocampal lesions (Spiers et al., 2001a,b; Cipolotti and Bird, 2006), a similar variability in experimental animals is more than expected, specially among different strains, even local substrains of experimental animals.

On the other hand, in those old studies it was remarkable to notice how cortical areas use to display a longer involvement than the HPC: thus, while the ERC was sensitive to CNQX amnestic effect at 1 (Jerusalinsky et al., 1992; Izquierdo et al., 1993a), 26 (Quillfeldt et al., 1994) and 31 days after training days (Quillfeldt et al., 1996), the PPC remained responsive after 60, and even up to 90 days post-training (unpublished results). The "stepwise" or gradual "deactivation" of the involvement of these brain structures takes place in full agreement with their neuroanatomical hodology (Marr, 1971; McNaughton and Nadel,

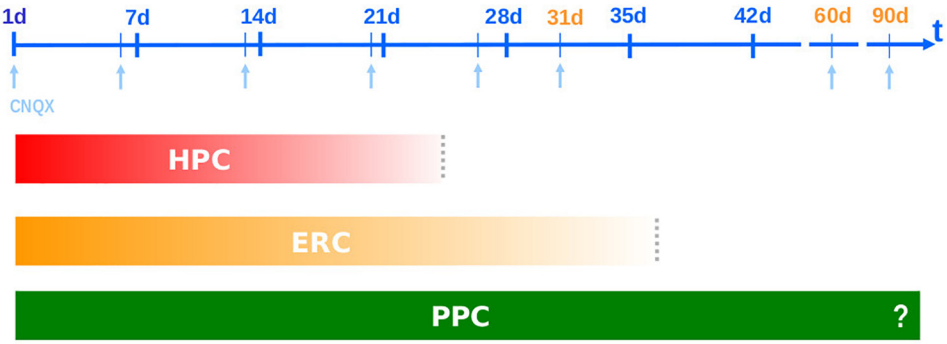


**FIGURE 1 |** Systems Consolidation: CNQX blocks the performance in the Step-Down Inhibitory Avoidance task when infused into three phylogenetically distinct brain areas – dorsal hippocampus (HPC), entorhinal cortex (ENT) and posterior parietal cortex area 2 (PPC) – resulting in different temporal frameworks: neocortical area remains in charge of memory retrieval after hippocampal and entorhinal disengagement (Quillfeldt et al., 1994, 1996; Izquierdo et al., 1997).

1990; Buzsáki, 1996; Fuster, 1997) and, consistently, with their phylogeny (Sherry and Schacter, 1987; Lavenex and Amaral, 2000; Treves, 2009; Thome et al., 2017). **Figure 1** summarizes these first findings.

There is a hierarchical organization in this time frame. Just as during learning/acquisition the sensory information flows first from multiple polymodal neocortical areas toward the paleocortex (entorhinal), and from there to the fast and iterated local circuits of the archicortex (HPC), now the processed information projects back to the associative NCTX through a paleocortical relay looking for a long-lasting storage site, closing a hierarchical loop (Teyler and DiScenna, 1986; McClelland et al., 1995; Lavenex and Amaral, 2000). This is why HPC, despite evolutionarily older, is considered the highest level of associative integration in the mammalian brain (McNaughton and Nadel, 1990) and the CNQX blockage experiments somehow unveiled the same timeline of the above hierarchical loop (Lavenex and Amaral, 2000). In this case, the representative of the associative NCTX was the posterior parietal area whose long-lasting responsiveness is in accordance with a putative role as the final residence for the engram.

## TEMPORAL FRAMEWORK FOR THE HIPPOCAMPUS INVOLVEMENT: RIGID OR FLEXIBLE?

Recently, we decided to revisit those original findings in our lab, asking *why* the HPC would need this particular time window of (then) 3–4 weeks to disengage itself from the retrieval process, originally in terms of AMPAR-mediated mechanisms, but other systems could be approached, such as the A-GABAergic one (Haubrich et al., 2016). We began by trying to replicate the above findings, but modifying three things, the drug (muscimol instead of CNQX), the aversive task (CFC) and the cortical target area: the ACC integrates the mPFC, a region that has been suggested to be of primary importance to support remote, but not recent memories (Frankland et al., 2004a,b; Teixeira et al., 2006; Ding et al., 2008; Insel and Takehara-Nishiuchi, 2013).

As consequence of these new experimental conditions, we detected a slightly longer time frame for the interplay between HPC and ACC – 4–6 weeks: HPC infusion of muscimol was amnestic in CFC-trained animals when tested at 1, 20 or 35, but not 45 days after training, while the same drug infused into the ACC produced the exact opposite scenario, being effective only at 45, but not 1, 20, or 35 days after training (Haubrich et al., 2016). This temporally graded phenomenon, despite slightly longer, still is compatible with previous findings, and represents a clear-cut instance of the systems consolidation phenomenon, despite not favoring any of the two main theories in dispute, the standard model or the MTT (Nadel et al., 2007).

## THE SYNAPTIC OCCUPANCY/RESET THEORY

Our main hypothesis was that **the duration of systems consolidation would be defined by the extent of use of the available synapses in the HPC**. Its testability, despite virtually impossible two decades ago, is becoming increasingly feasible now with the availability of high-tech tools such as opto/chemogenetics, multielectrode arrays and two-photon microscopy, despite still lacking the necessary spatial and temporal resolution (see the last section, "Testing the Theory," bellow). Of course there may be alternative explanations for our findings, but synaptic availability represents a simple, straightforward and reasonable putative model, enough to prove being valuable to explore in more depth.

Motivated, as others before, by the Hippocampal Indexing Theory (Teyler and DiScenna, 1986; Teyler and Rudy, 2007), that, as mentioned above, was a quite successful theoretical approach absorbed in different degrees by most theoretical appraisals of memory systems consolidation (SMSC, MTT, DRT, TTT, CTT, etc.), we propose that:

(1) considering that **learning a new task equals to "connecting" a set of sensory inputs (S) to a set of motor outputs (M)**, a form of higher order "pavlovian" link, that will be summoned into action in some

coordinated way during retrieval via the establishment of an *intermediate plexus* (IP) *of neural pathways* that produce the correct/learned response;

(2) considering that **those pathways would embody** (a) the spatial representation of the learned context (if learned), (b) the record of important items and subjects present, and (c) a set of efficient motor choreographies to be summoned in order to deal with what is being perceived in that moment, all these components will, at the end, assist a decision taking based on matching/non-matching between the present sensory inputs and the stored memory (Fernández et al., 2016; Agustina López et al., 2016; Krawczyk et al., 2017);

(3) considering that the HPC is such a **small brain region** in terms of number of neurons (thus, number of synapses available at each moment) – particularly in the rat (Braitenberg and Schütz, 1983; McNaughton and Nadel, 1990; Treves and Rolls, 1994; Rolls et al., 1998; Rolls and Kesner, 2006; Treves, 2009; Rolls, 2017), and

(4) considering also the **ever-growing amount of data to be continuously encoded** by any normal animal, even for experimental ones.

We hypothesize **when submitted to a rich, successive series on new learning situations, the hippocampal system would easily reach maximum occupancy and might need some special maintenance**: the simplest way to do this [considering first a fixed (or restrict) set of available synapses] would be to *free* synapses previously engaged in some other representational index to become again available to hold the new memories – a kind of **synaptic reset**. In this *occupancy-reset* scenario, hippocampal synapses *might endure physical erasure* in at least two basic situations: (a) *on demand*, when the number of available, unoccupied synapses reaches a minimum, not enough to hold a new engram/trace, reset would "make space" to continue the storage process, or (b) *automatically, on a regular basis*, in the case of an "uneventful, tedious life" – typical of experimental animals that usually live for just one lifetime experience, a quite unrealistic, non-ecological situation, as Ulrich Neisser has alerted before (Neisser and Winograd, 2006) – a portion of this synaptic population would be automatically reset from time to time, a natural turnover, which could explain the timeframe of the "natural" systems consolidation observed in different experiments.

Of course this is just a first sketch, with the minimum components necessary to accommodate the experiments described in the sequence. A more detailed proposition appears in the last sections of the paper. To this point, among several assumptions, there is one that is in full accordance with HIT: the HPC will not encode the full trace of an experience inside its borders, holding just a *map* to the true location of the engram in the much more extense neocortical associative areas. The first premise above is also an epistemological commitment with the psycho-physical identity principle, a position in line with philosophical materialism, realism and systemism (Bunge, 2010), that receive different names in the scientific context, such as the "principle of functional-neural isomorphism" (Sekeres et al., 2018), when referring, for instance, to things such as the interplay between psychological phenomena and their neural representations.

## *NEW LEARNINGS* BEFORE THE REMOTE TEST *ACCELERATE* SYSTEMS CONSOLIDATION

With a well-defined systems consolidation experimental setup at hand – and if the *synaptic occupancy/reset* hypothesis is correct – we might next ask *why* does this phenomenon has this specific duration of 4–6 weeks (a period that encompasses both studies), at least for rats and in these aversive tasks. One logical possibility, derived from the finiteness of the HPC itself, would be to consider, for starts, that the number of synapses available to encode new memories is *finite and fixed/restrict*. Since these synapses should be "ready" for plasticity events, maintenance activities must be performed regularly, and we suggest that there may exist a regularly scheduled automatic "reset" of these synapses. This would naturally destroy previously used index mappings of cortical engrams, meaning that those memories would be physically deleted. Although there can be reasons to despise the omnipresent computer metaphor in the neurosciences, it is hard to resist an analogy to describe this maintenance-reset-induced-amnesia: the deletion of the FAT table in a computer's hard drive does not remove the bits of memory actually spread/intermingled all over the disk, but renders that memory virtually unrecoverable due to the loss of tracking information. We hypothesize that a similar process would be taking place in the CA1 HPC pyramidal neurons, responsible for establishing the index of each memory trace and keep track of their spread parts. So the first prediction of the *synaptic occupancy/reset theory* (SORT) is that **forgetting is just a natural consequence of this natural maintenance mechanism** (at least the passive component). The average 4–6 weeks period would represent the automatic (predetermined or scheduled) reset/erasure, once the animal is not being trained in any other task and would not be "using" those available synapses.

Next we ask: what if we interpose a series of novel learning tasks between the training and the remote test sessions? Hypothetically, this would forcefully "increment the cognitive life" of this experimental animal, and more synapses should be in demand: if the minimum limit of available synapses happens to be reached during a series of intense cognitive experiments – a reasonable supposition considering the small dimensions of the HPC – this would trigger the reset system *before* the regularly scheduled moment in order to release more, fresh synapses to build new memories. Thus the second prediction would be that **systems consolidation would endure an acceleration**, with the *switching point* that disengages the HPC and summons the neocortical areas moving to a time point *before* the completion of the regular *interval of* 35–45 days after training. The learned memory would become independent from the HPC, and dependent on a cortical areas such as the anterior cingular cortex at an earlier time point. This neuroanatomical displacement could be verified employing muscimol to check for the involvement

of each brain structure at an earlier time point, say, 20 days after training.

The result was exactly what was expected! Muscimol infusions showed us that CFC memory became independent from the HPC before the regularly scheduled time (Haubrich et al., 2016 – see Figure 3E), and was now relying upon the ACC area (*ibidem,* Figure 4E), i.e., systems consolidation was *accelerated* by multiple learning experiences, consistently with the *occupancy/reset theory*. See **Figure 2**, ahead.

This underlying hypothesis was barely sketched in the original paper (Haubrich et al., 2016): "We hypothesized that the encoding of multiple memories would result in an accelerated HPC-to-cortex information transfer in order to preserve hippocampal function of encoding new information and avoid its overload" (. . .) "It may be that such rapid reorganization occurs in order to preserve hippocampal storage capacity, allowing the HPC to continuously process new information, given that its physical storage is likely limited. This may also reduce *interference* with previously established memories." The mention to "interference" was another echo of the precursor ideas of Marr and McLelland's pioneer propositions (Marr, 1971; McClelland et al., 1995).

In support of these findings it was shown that multiple learning experiences may induce changes both in dendritic spine complexity and c-fos expression in the ACC at delays that resemble those of our remote memories (Wartman and Holahan, 2013, 2014). Most important – and a strong support for the main tenet of the indexing theory – a central role for the HPC was demonstrated in the active induction of neocortical plasticity related to memory processing, i.e., the accelerated HPC-to-ACC memory reorganization may be under control of the HPC itself (as suggested by Sutherland et al., 2010). There might exist alternative explanations for these results as, for instance, new learning inducing competition for hippocampal storage room as a side effect of the memory allocation process upon the excitatory, principal neurons of neocortical networks (Han et al., 2007; Josselyn and Frankland, 2018). For now, however, our favorite candidate mechanism for the reset mechanism might rest in processes such as neurogenesis (see, e.g., Besnard and Sahay, 2016), already shown to be induced by novel learning (Gould et al., 1999a,b; Kitamura et al., 2009). Of course, a lot more remains to be investigated.

Comparing the two sets of experiments separated by 20 years, the main difference between them was in the *duration of the observed drug effect* (compare **Figures 1** and **2**) probably due to the chosen neurochemical target. In the previously mentioned works, we have prioritized AMPAR for the pre-test blocking of retrieval, while muscimol was used only for the post-training infusions in order to evince consolidation effects (Quillfeldt et al., 1996; Izquierdo et al., 1997). Due to the existence of a similar circuitry arrangement both in the HPC and the NCTX, in which GABAergic interneurons control pyramidal glutamatergic cells through feedback and feedforward inhibition in simple, yet reliable local circuits (Pitler and Alger, 1992; Bull and Whittington, 2007; Spruston, 2008; Tremblay et al., 2016), the infusion of the GABA agonist muscimol was expected to reversibly suppress local activity (either in the CA1 area of the HPC or the NCTX), more or less the same way the AMPAR antagonist CNQX would do: the first, by stimulating GABAergic interneurons, and the last, by directly blocking glutamatergic principal neurons. However, we should consider the possibility that plasticity might have modified the level of response of these systems in different ways. Thus – and particularly in the HPC – while the responsivity of (at least some) interneurons could be reduced to near zero without drastic consequences, the same might not be possible for the principal neurons, once they happen to be the only available carrier pathway for the output of hippocampal processing.

In other words, GABAergic neurons would be freer to vary their connectivity than the principal, glutamatergic cells, and this is an decisive point since *we are studying the pharmacology of this local neural circuit, not individual, isolated neurons*. This aspect might account for the fact that CNQX *was amnestic from day* one both in the HPC and the cortical areas, ending its effect in the ERC (but not in the PPC) at the moment the structure was apparently "released from duty" (Quillfeldt et al., 1996), while in the 2016 experiment, Muscimol was effective in ACC only after the HPC ceased its engagement with memory retrieval (Haubrich et al., 2016). To some extent, the more recent experiment sounds more convincing and representative of systems consolidation, but whenever the unexpected happens, there is opportunity for a deeper peep into the processes under scrutiny: thus, these two similar, yet not identical ways to observe systems consolidation for an aversive task teaches us two additional things: (1) at least in terms of glutamatergic transmission, *cortical areas appear to be necessary from the very beginning*, right after acquisition, even if this is not apparent in every chosen experimental design, and (2) GABAergic modulation might be the locus of the central plastic events behind the transference of function observed in systems consolidation, that would be the reason why its manipulation results in a clear-cut systems consolidation time frame in both brain structures. Observation 1, for instance, have received additional support from at least two previous works of us, for instance, in Jerusalinsky et al. (1994) and, more recently, we studied a remote memory blocked by pre-training infusion of muscimol into the ACC, and managed to use reactivation/reconsolidation to rescue the supposedly lost trace and also restore the normal course of a disrupted systems consolidation – a putative case of "systems re-consolidation" (Sierra et al., 2017). The need for the presence of neocortical areas from the very beginning – despite only mobilized later in the systems consolidation process, is another exciting subject that, however, will not be further discussed here.

## *REACTIVATION SESSIONS* BEFORE THE REMOTE TEST *DELAY* SYSTEMS CONSOLIDATION

Under specific protocols of re-exposure to the original training context, *reactivation may* take place *during* memory retrieval and a memory that was previously acquired and already fully consolidated (in a synaptic consolidation process) would be *relabilized, becoming* again sensitive to modification or even

disruption. This allows for the integration of new information (update) and the process concludes with the *reconsolidation* of the former trace into a modified engram (Nader et al., 2000a,b; De Oliveira Alvares et al., 2008a,b; Bustos et al., 2009, 2010; Lee, 2010; Alberini, 2011). In our lab, we have been studying reconsolidation for some time, and have found, for instance, that, during a reactivation session, the concomitant presence either of a distractor (Crestani et al., 2015) or an appetitive stimulus (Haubrich et al., 2015), was able to promote a long-lasting reduction of freezing response, i.e., effectively modify the emotional valence of the originally learned tasks (CFC) to a less aversive level. In those two studies, the effects were abolished either by systemic nimodipine, or intra-hippocampal infusion of ifenprodil, which is consistent with a reconsolidation mechanism: LVGCCs, and, specifically, GluN2B-containing NMDARs appear to be common plastic components recruited in the HPC by these two different cognitive situations, once its blockage interfered with memory reconsolidation. Using reactivation/reconsolidation we have also managed to incorporate an endogenous state-dependency into previously consolidated memories (Sierra et al., 2013) and use reconsolidation to promote the consolidation of a concomitant weak learning through a synaptic tagging and capture mechanism (Cassini et al., 2013).

But memory "flexibilizing" protocols may also be employed to interfere with higher order cognitive phenomena, such as systems consolidation, in which multiple brain areas are recruited in a complex spatio-temporal choreography of engram-allocation. Two examples from our lab have managed to successfully interfere with the temporal framework of systems consolidation by inserting short *reactivation sessions* between training and the remote test (De Oliveira Alvares et al., 2012, 2013). In this experimental setup, despite checking only for one brain structure (the HPC), systems consolidation was "measured" by the psychological, qualitative modification in the ability to discriminate between original and novel contexts as advanced, e.g., by Wiltgen and Silva (2007). In other words, if memory has precision, the muscimol infused into the HPC must suppress that precise response and the animal confound the conditioning context with the novel context. At later periods, generalization (corticalization) would have take over the process and the animal would naturally not be able to discriminate between the contexts (and the HPC would become insensitive to pharmacological blockage).

And then systems consolidation was once again replicated! This time measuring the precision/generalization psychological binomium. In the training-test interval of 2 days, animals were able to discriminate well between known and novel contexts (i.e., display precision), and muscimol suppressed this capacity when infused before test into the CA1 region of the HPC (i.e., display HPC-dependency). The experimental group tested after 28 days – an interval in which the HPC was not expected to be responsible for retrieval anymore – animals did not discriminate between the contexts (i.e., they exhibit memory generalization) and muscimol did not produce any response (i.e., we detect independency from the HPC). Despite not studying any cortical target in this case, the results were a clear reproduction of half the systems consolidation viewed from the HPC perspective (De Oliveira Alvares et al., 2012 – see **Figure 1**), and consistent with previous contextual fear generalization studies (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007; Winocur et al., 2007),

Next we asked what would happen *if we interpose reactivation sessions* between the training and the remote test sessions. Subjects trained in CFC were reexposed to the original training context in the absence of the unconditioned stimulus (footshock) for three sessions of just 90 s each, once a week. After these reactivations, the control group (vehicle-injected) became again able to discriminate between the novel and the conditioning contexts, notwithstanding the long interval that normally would have lead to the corticalization/generalization of the memory trace. When muscimol was infused in the CA1, however, the ability to discriminate was gone, showing that HPC was again in charge of retrieval of this otherwise remote memory (De Oliveira Alvares et al., 2012 – see **Figure 2**).

What happened here was somehow unexpected: the HPC-dependent, precision-prone period was literally *enlarged*, i.e., the systems consolidation temporal framework was *delayed*, the exact opposite of what the new learnings have produced. The same delay was observed in another experimental setup in our lab with the insertion of *only one short reactivation session* (De Oliveira Alvares et al., 2013 – see **Figure 3**). In this second discriminative experiment, the re-exposure session was proven to consist of a real reconsolidation process of the original memory trace, since the delaying effect was suppressed by nimodipine injected i.p. before the reactivation session.

In sum, compared to new learnings, reconsolidation has produced an *opposite* effect upon the temporal framework of systems consolidation. Notwithstanding some similarities, such as protein synthesis dependency, there is an increasing list of intrinsic differences between first-time consolidation and reconsolidation, involving different membrane-bound receptors and channels, membrane insertion of ion channels, enzymatic degradation cascades, early genes, etc. (Haubrich and Nader, 2018). These differences could explain several different outcomes in different scenarios, thus observing a delay instead of an acceleration should not come as a surprise, despite not having been anticipated by the present version of the *occupancy/reset hypothesis* straightly *based on the assumption of a fixed number* of available synapses. Actually, (a) since reconsolidation should necessarily involve some degree of synaptic reorganization in order to update the original memory trace, and (b) since synaptogenesis is not an uncommon event in the hippocampal area, even out of the context of developmental critical period, we may hypothesize that the (different) kind of plasticity elicited by reconsolidation may result in an equally different outcome for instance, a direct increase of the total number of available synapses in the immediate neighborhood of the "reconsolidated" cells, at least within certain limits (there is no room for an indefinite increase of this number). In other words, there could be a second, alternative mode of operation of the set of available synapses other than that controlled by the occupancy/reset putative mechanism, now based on the complementary assumption of a variable number of available synapses.

Of course, this is highly speculative, but at least, is testable. One consequence of the local variation in the number of available synapses without the need to recruit by reset upon a fixed set would look as an expansion of the CA1 area involvement and result in a systems consolidation delay that fits what was observed in the reactivation/reconsolidation experiment (De Oliveira Alvares et al., 2012, 2013). **Figure 2** summarizes the two opposite findings that resulted either in acceleration, or delay of the systems consolidation process:

The hippocampal indexing theory suggests that this operation should tackle upon the same index of the original memory, maybe adding some extra connections here/removing others there, an operation that could or could not demand more available synapses to take place. And, as we discussed briefly above, MTT has the interesting proposition that each time a memory is retrieved, a new index would be created as a partial copy of the original trace plus some additional features integrated as an "update," a way to explain the resilience on older memories: but if reconsolidation creates a new index, it would demand more synapses and should contribute to move the ensemble of plastic, available synapses closer to the limit of occupancy, which would result in a *reset on demand* and the acceleration, not the delay of the temporal framework. Since this has not happened, something else should be going on. We can improve our model by adding another feature to it: the capacity to create new synapses, at least within certain limits (once HPC CA1 area is still such a small structure). That would be the first thought of most researchers since we use to feel comfortable with the idea that there is a "free capacity" to "produce more" (synapses, cells, etc.) and intuitively (and acritically) we comply to this comfortable position. But this may not be true.

Thus, having begun with a restricted model in which new memories must be recorded making use of a finite number of available synapses in order to survive, we have to warrant available room for the creation of *more* substrate for engram plasticity: in the above situation, if the number of synapses were *not fixed*, but variable, an increment would easily explain the delay. Maybe that is what takes place in the specific case of memory reactivation by partial mismatch of contextual cues (Fernández et al., 2016; Krawczyk et al., 2017), differing for the new learning situation, where a *total mismatch* is verified and lead to memory formation (upon a fixed set of plastic synapses). Again, be it real or not, this is a testable complementary hypothesis. We can think of it as a "toy model" designed to tie some loosen ideas and experiments with new, putative (hopefully reasonable) integrative conceptual ideas. Good theories should prioritize simplicity whenever possible, and ensure at least three things: *explainability* (have no contradictory findings), *testability* and *predictability* (Bunge, 1967, 1985). Any one of these properties is of paramount importance, and the absence of one of them will strongly limit any proposition. However, people tend to focus more on the first two properties, neglecting predictability – maybe the most important of the three. We will discuss a more complete version of the model in the next session, but before, let's bring some closing remarks on the experiments here discussed.

These last two experiments raise an important question, and even a possible objection to what we have found in the acceleration-by-new-learning experiment (Haubrich et al., 2016): couldn't it be the case that what was actually taking place was some instance of reactivation, not the mere accumulation of information that would demand more "synaptic room"? This would also make things complicate for the synaptic occupancy/reset theory, and the possibility was not directly tested in the original 2016 experiment. However, we can mention at least three reasons to reject this alternative explanation. First, both interposed tasks, despite intentionally chosen to be HPC-dependent, [a] does not involve re-exposure to the same context where CFC was learned, and [b] involve different (insufficient) exposure time, meaning that these additional tasks were unfit to reproduce the exact boundary conditions necessary to allow

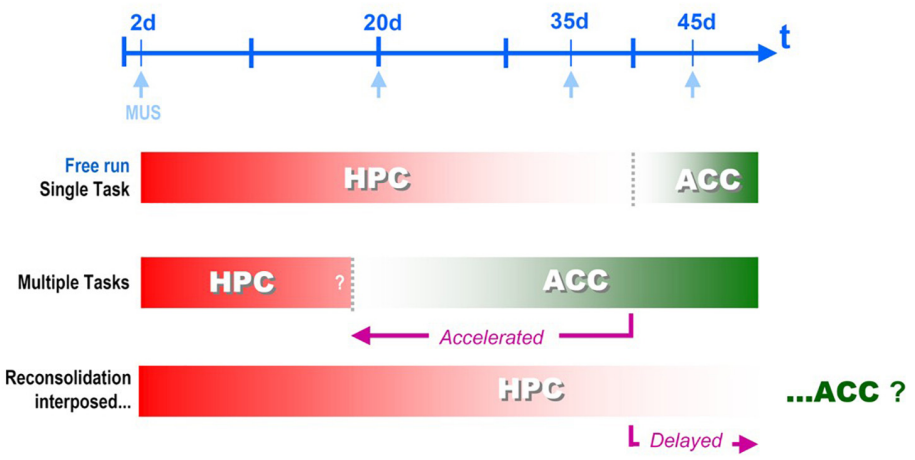


**FIGURE 2 |** Systems Consolidation temporal framework, i.e., the time for the corticalization onset after hippocampal disengagement, can be flexibilized by different behavioral interventions interposed between the training and the remote test sessions for Contextual Fear Conditioning memory retrieval: Muscimol displays different windows of effectivity when infused either into the dorsal hippocampus (HPC) or the anterior cingular cortex (ACC) depending the nature of the interposed sessions - new learning or reactivation/reconsolidation (De Oliveira Alvares et al., 2012, 2013; Haubrich et al., 2016).

for a pure reconsolidation-dependent interference that could explain the observed change in the temporal course of the systems consolidation. Second, despite being far from attaining the exact boundary conditions, considering that those interposed tasks involved actual new learnings, the induced protein synthesis could provide, among its products, diffusible plasticity-related proteins (PRPs) that could be relayed to, somehow, produce interference via a tagging-like mechanism; however, the capture of PRPs might obligatorily take place in a short period, enough to interfere with late LTP maintenance, which would be very improbable after the several days that separate the original training and the interposed tasks. Finally, we must clarify that the Haubrich et al. (2016) paper was not our first attempt to study the consequences of multiple tasks interposed during a long-lasting training-test window, but it was the only one in which the protocol worked fine. In previous attempts, we have first tried to implement an intensive training protocol, with too many different tasks along the day, and even intercalating those tasks with long exposures to enriched environments: however, most of these animals resulted more stressed than "enlightened," and the final results were inconclusive (data not published). Curiously, however, Lucas de Oliveira Alvares managed to implement an experimental protocol in which intentional stressful conditions (via aversive training intensity) was also able to accelerate systems consolidation (Pedraza et al., 2016), but the data we have did not support the idea that our multiple learning protocol caused any abnormal level of stress in order to compare both experiments. This last case of acceleration diverge from the interpretation we provided for new learning findings above, but this may be due to the more disruptive, maladaptive scenario induced, in which cells endure abnormal operation conditions (stress!) and may even suffer some degree of tissue destruction: to this point, our predictions concern mostly to healthy, non-pathological conditions, but those other conditions should receive further attention in future works.

## HIPPOCAMPUS: TWO FUNCTIONAL SETS, THREE DYNAMICAL POPULATIONS OF SYNAPSES (AT LEAST)

Indexing theory was actually an elaborate attempt to explain episodic memory with the HPC at the center of the action. This small, yet fast-processing structure would be able to automatically capture contextual information, organize it in separate single episodes, and retrieve each one of these from a partial set of cues. These abilities are consistent with its highly and recursively interconnected nature that contrasts with that of neocortical circuitry – the supposed *final destination* of the memory trace – that, despite having much more neurons (thus, synapses) to make available, is too sparsely connected to support fast encoding and efficient retrieval (Rolls and Treves, 1998; Rolls et al., 1998; Rolls and Kesner, 2006; Teyler and Rudy, 2007; Treves, 2016; Rolls, 2017). In other words, the HPC solves the two main obstacles to the feasibility of episodic memories processing: the *associative connectivity* problem, that restrains NCTX, by allowing rapid *pattern completion*, and the *interference* problem between multiple, contextually similar episodic memories, by supporting *pattern separation* capacity (Teyler and Rudy, 2007; Moser et al., 2015).

Indexing theory is still one of the best possible general proposals for a hippocampal role in memory. Due to its finite dimensions – and consequent small number of neurons – particularly in the rat, this brain area will just be able to hold a small physical record consisting of a set of cortical "coordinates" or "pointers" – the *index* – and certainly never store the whole engram itself, not even temporarily (Squire et al., 1984; Treves and Rolls, 1994; Squire and Alvarez, 1995; Teyler and Rudy, 2007). This also harmonizes with the neuroanatomical-functional fact that this phylogenetically old area represents the *highest level of information integration* in the mammal brain: it receives converging polymodal sensory data from different cortical areas, first, the parahippocampal cortex, then, the entorhinal cortex; after the completion of the trisynaptic "data crunching" and the establishment of the index for that memorized experience – information flows back to widely dispersed associative areas of the NCTX, first via the entorhinal, and then, the perirhinal cortices (Lavenex and Amaral, 2000).

**Figure 3** presents a more complete version of the *synaptic occupancy/reset theory*, integrating most of the relevant aspects it should contain, despite still sketchy and highly speculative to this point. From what we have already discussed, emerge some interesting hints and cues concerning the very nature of the engram, in the complex spatio-temporal framework of the systems consolidation process, whatever the engram may consist of.

**Figure 3** illustrates the internal organization of CA1 hippocampal area according to the *synaptic occupancy/reset hypothesis* that we describe in more detail below:

(1) **Hippocampus CA1 area is the mandatory output way for any resulting pattern** of activity previously processed by the DG-CA3 subsystem, and its projection cells might connect indirectly (via a paleocortical relay) to neocortical target areas by encompassing and tying – through the available plastic synapses – a selected ensemble of projection pathways into the NCTX. **So the *synaptic occupancy/reset hypothesis* refer basically to CA1 principal cells and their ensemble of available synapses**, although the subset of synapses might well include other cells like interneurons, due to the intrinsically "circuital nature," with local feedback/feedforward inhibition by the integration of different classes of interneurons with the principal neurons, as mentioned before;

(2) However, while the above suppositions aim to cover memory mechanisms in the HPC, that must not be the whole story for this fascinating brain region. If there is one function that is well established for the hippocampal formation and adjacent cortices is that of a ***Cognitive Map*** (O'Keefe and Nadel, 1978; Treves, 2009; Moser et al., 2015), a system that not only *organizes the representation* of the

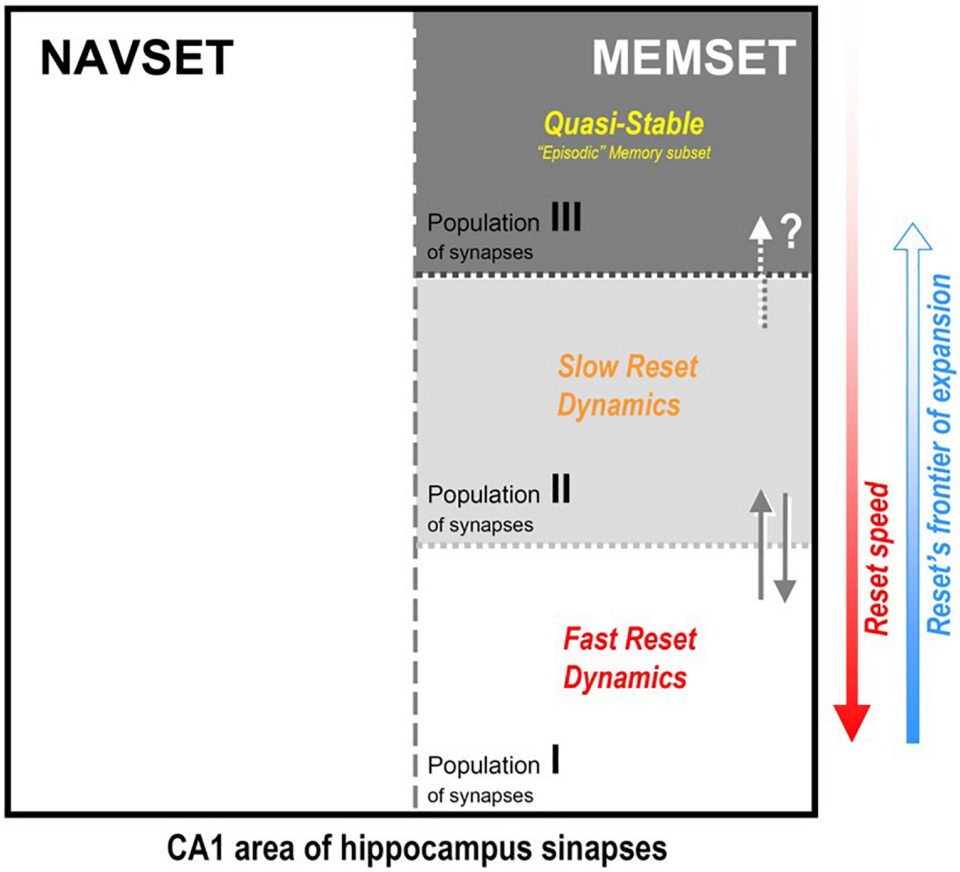


**FIGURE 3 |** Hipothetical internal organization of CA1 hippocampal area according to the synaptic occupancy/reset hypothesis (SORT, as described in the text): all active synapses would be divided in two functional (not spatial) subsets, the navigational (NAVSET) and the memory indexing (MEMSET) subsets, that might overlap to any extent (multitasking neurons). Memory-recording synapses, on their turn, might be divided in subpopulations with different degrees (speeds) of "resetability", here displaying three of them, those with faster and slower reset dynamics, and the quasi-stable one. Since MEMSET would consist of a finite, relatively constant number of synapses, the more memories exist to demand encoding/indexing, the more free, "fresh" synapses are needed, thus forcing the reset process to progressively encompass more stable synapses populations. Three population is an arbitrary division just to prove concept, once resetability might even be a property that varies continuously among the whole ensemble of MEMSET synapses.

spatial context, but actually *creates* and imposes it to the surrounding space in which the animal moves/explores. This is used to anticipate needed adaptative maneuvers to be implemented, organizing real-time navigation with simultaneous well-structured (pattern-separated) capture of environment data. **This Navigational Set of Functions (NAVSET) must always be accounted for** in any theoretical proposition of any additional hippocampal function such as memory indexing. **The extra-navigational *Memory Set of Functions* (MEMSET)**, it is reasonable to admit, **might coexist with the navigational one in the same space, sharing many (if not all) neural cells**, as has been extensively suggested elsewhere (Marr, 1971; McClelland et al., 1995; Rolls and Kesner, 2006; Knierim, 2015; Moser et al., 2015; Lisman, 2017). This is a clear *dual function system*, and although both functions seem to be *inseparable*, at least they can be *distinguished* one from the other (thus, quantified) by employing the appropriate methodology (see Bunge, 1985, p. 28). Different sets of experimental approaches have actually been studying these distinct functional outcomes of the *very same* brain structure, the HPC, and due to its intrinsic complexity, even "distinguishing" different, parallel outcomes may present sometimes a spectacular challenge to science.

(3) MEMSET contains the principal cells and local interneurons, and their available ensemble of plastic synapses, all being capable of establishing some connections between themselves, but mostly with neocortical neurons (as described in item 1, above). **We propose that all these synapses are prone to be reset by the maintenance mechanism suggested before, but with different degrees of resistance to the *erasure* process.** So, there would exist at least three intermingled populations of synapses, two "dynamical" – easier to be reset, and one robust/resilient population of quasi-stable synapses that may explain long-lasting, detailed episodic memories. Three population

types serve to illustrate the consequences of the hypothetic *differential resetability* (or erasure probability):

> **Type I – fast reset dynamical population**: for new and less relevant ("forgettable") memories; holds most new memories, but mainly those that we easily forget, which is explained by the fact that in any reset/erasure session, these are the first to go;
> **Type II – slow reset dynamical population**: for new and mostly recent necessary/useful memories of all kinds; they last longer than population I, but do not hold forever, just enough to convey their needed information;
> **Type III – quasi-stable population**: despite being the most resistant to the reset procedure, they cannot be said to be "eternal" – quasi-stable is not the same as stable – and this is consistent with the fact that even episodic memories do recede and disappear with time, even in the extreme case of HSAM patients, despite their ultra-slow forgetting;

(4) The more demand for "fresh" synapses upon the MEMSET, the more cells/synapses would be recruited and submitted to the reset procedure, starting from population I and expanding into population II (and even to III, if necessary, at least partially); in other words, those **borders between populations are movable and there is a natural direction for frontier expansion**;
(5) The **division in three populations** with "three levels of resetability" **is, of course, purely arbitrary** and practical justification: three levels is the simpler non-binary classification, a simple, but effective way to describe a system that probably is much more complex; it may be the case that MEMSET consists, say, of 4, 5, 7, 12 – whatever – different populations, or it might even be the case that those cells/synapses obey a continuous distribution (which can be consistently classified back in a finite set of discrete bins like this to facilitate understanding).

The naming of these three populations of synapses came as an analogy to the classification of stellar population types in astrophysics (Trager et al., 2008): type I (*young*, metal-rich, orbiting inside the galactic bulge), type II (metal-poor, *old*, spread farther, in the galactic halo), and type III (metal-free, ultra-massive, *very old* or of hypothetical existence). Thus, type I synapse population include the "youngest," continually recycled ones (by the reset), type II, the "older" ones, that takes more time to be reset, and type III are the "oldest," reset-resistant ones, that might account for the phenomenon of HPC-dependency displayed by human episodic memories.

## SKETCHING SOME PREDICTIONS

Besides providing a reasonable, fully HIT-compatible explanation of systems consolidation under three different frameworks – no intervention (free run), with new learnings, or with reconsolidation interposed between the training and the test sessions – SORT also imply some additional predictions:

- the model *elaborates upon one theoretical consideration discussed by the proponents of SMSC,* but not explored later by MTT or TTT: the putative connectivity changes not only can take place gradually, across weeks and months, but might also be "limited to expansion of the original axonal and dendritic fields or arborizations within these fields" (Squire and Alvarez, 1995): the functional link between HPC and NCTX is proposed as a limited, relatively fixed set of available plastic synapses that allows the selection of the correct subset of axonal projections from CA1 area to the respective cortical representations;
- the model *naturally explains both types of explicit/declarative memory dynamics – non-graded and temporally graded* – proposing the *very same underlying mechanism to explain both processes:* it explains why *episodic memories* would be prone to exhibit non-graded RA – their indexes would be supported by the so-called population III of quasi-stable, hard-to-reset synapses – and, at the same time, accommodates all the remaining temporally graded RA's in the occupancy → reset paradigm; the old controversy between HPC-dependency and HPC-independent memories, that have lead to alternative models such as MTT and TTT, can now be explained by a simpler mechanism that also can be consistent with these previous theoretical propositions;
- the model *is compatible with MTT* in the sense that it supports the idea of extra, similar indexes being created by retrieval, and do not oppose – rather complement this hypothesis proposed to explain temporally graded RA of episodic memories (Nadel and Moscovitch, 1997); it actually goes one step further by providing a putative way of directly testing it (see next section);
- the model *does not explore (as SMSC have had* – Squire and Alvarez, 1995) *the idea that frequent or constant reactivation – or "replay" – of the trace during the delay period is necessary to effectively encode long-term memory, i.e., to complete the consolidation process*; this replay process is supposed to take place during certain phases of the sleep, such as the SWS (Rasch and Born, 2013; Sara, 2017), and involves, among other aspects, the sustained activity of neighbor areas such as the parahippocampal cortex (Schon et al., 2005) as well as muscarinic cholinergic modulation (Hasselmo, 2006), just to mention two important aspects that end up integrated in an interesting theoretical model of *rule learning* that involves, as an important component, the neuromodulatory regulation of presynaptic inhibition learning (Stern and Hasselmo, 2005; Hasselmo and Stern, 2018). Notwithstanding its importance, *replay* models exceed the scope of this paper, and will not be further discussed here: suffice is to say that this phenomenon is compatible with the model, and may even help to explain how available CA1 synapses are selected to create a memory index. However, the question of neuromodulatory regulation of presynaptic inhibition over local neural circuits has been studied in lab for many years: we also have delved into the cholinergic muscarinic modulation of memory, studying M4 action (Jerusalinsky et al.,

1998; Ferreira et al., 2003; Sánchez et al., 2009), as well as endocannabinoid CB1 modulation (Quillfeldt and De Oliveira Alvares, 2015): both modulations act upon specific GABAergic interneurons in the CA1 area, and there are striking similarities in the way both modulations act. In the endocannabinoid study, we were able to show how the CA1 circuitry is affected in different phase of memory, operating as a "switching" mechanism between consolidation and retrieval processes, as well as between reconsolidation and extinction (Lee et al., 2006; Quillfeldt and De Oliveira Alvares, 2015);

- the model is fully compatible with the *memory reconsolidation phenomenon* (Lewis, 1979; Nader et al., 2000a,b; Anokhin et al., 2002; Walker et al., 2003; Duvarci and Nader, 2004; Lee et al., 2006; Rose and Rankin, 2006; Hupbach et al., 2008; Nader and Hardt, 2009; Bustos et al., 2009, 2010; Nader and Einarsson, 2010; Hardt et al., 2010; Lee, 2010; Alberini, 2011; Haubrich and Nader, 2018), for which there is an ever growing literature on putative mechanisms and specific "markers" that distinguish it from the consolidation of a first-learning situation; also, there are some studies on "systems reconsolidation" (Debiec et al., 2002; Wang et al., 2009; Einarsson et al., 2015; Sierra et al., 2017; Lopez et al., 2018);
- the model creates *a conceptual bridge between the two types of consolidation, synaptic and systemic*, generally treated as completely different phenomena (or phases of a wider process): by anchoring the formation of the hippocampal index simultaneously to a LTP-like process – at the synaptic level, and to the establishment of connections with efferents projecting to several sparsely distributed neocortical areas – at the systems level, the engram is formed at once, synaptically and systemically; these are not two independent phenomena anymore, but may be taken as stages of one only process;
- *forgetting* would be a straightforward consequence of this model, and if correct, might support proposals involving true erasure, specially of population I and II synapses (Rudy, 2008; Han et al., 2009; Clem and Huganir, 2010), an empirical situation that, otherwise, might be impossible to be conclusively proven, since we never could eliminate a purely negative hypothesis such as "the memory trace is not in the brain anymore";
- the model is predictive also for *dementias*, such as Alzheimer's disease, but on a first approach this would be quite trivial, because loosing neurons would automatically suppress available synapses in the HPC;
- more interesting would be to investigate its predictions in relation to pathologies involving stronger, sometimes self-reviving disruptive memories, such as those of PTSD (Ursano et al., 2010), whose mechanism might involve, for instance, the blocking/suppression of the reset mechanism – whatever it be – upon most cells/synapses, at least as part of the symptoms;
- similarly, but devoid of such devastating effects of PTSD, the very strong and detailed preservation of personal reminiscences as displayed by the HSAM individuals (LePort et al., 2012, 2017; Santangelo et al., 2018) can be consistently accommodated in this model, just by involving modifications in the reset system in population III synapses (notice that even HSAM do forget, i.e., loose details of highly details episodic memories, only extremely slowly);

## PUTTING ALL PIECES TOGETHER

**Figure 4** is a self-explainable table of possible outcomes according to the different trigger factor, and covers most of the experimental data and theoretical arguments presented have to describe the three possible general outcomes predicted for the behavior of the temporal framework of systems consolidation – acceleration, "maintenance" or delay – after being triggered by contextual degree of novelty and/or the kind of mnemonic process engaged (consolidation, retrieval, reconsolidation) in the light of the putative background of the synaptic occupancy/reset hypothesis: as shown in the second and third lines below, each trigger factor results in a different impact upon the number of available synapses, which, on its turn, recruits a different mechanism that leads to the observed temporal effects. The novelty here – and it is hopefully a *testable possibility* – is that we propose two different backgrounds, plasticity taking place upon a **fixed** versus a **variable number of available synapses in the CA1 area**.

*First/new learning plasticity* would have to produce its engrammatic embodiment working with a **fixed subset of available synapses**, with plasticity directed to select and connect to specific neocortical target areas establishing a memory trace, while other, *reactivation-induced plasticities* would be able to somehow induce an **increase in the total number of synapses in the variable subset of available synapses**, at least within certain limits. The cognitive process induced by reactivation studied above was mainly reconsolidation (De Oliveira Alvares et al., 2012, 2013; Cassini et al., 2013; Sierra et al., 2013; Crestani et al., 2015; Haubrich et al., 2015), but processes mobilizing a change in the number of available synapses may also include extinction (Bouton and Moody, 2004; Sotres-Bayon et al., 2006; Myskiw and Izquierdo, 2012; Cassini et al., 2013, 2017; Sierra et al., 2017; Haubrich et al., 2017) and possibly even the intermediary category known as subsequent learnings (Tayler et al., 2011; Crestani and Quillfeldt, 2016; Crestani et al., 2018a,b). Of course non-reactivating, *plain retrieval* is proposed as a process as inert as the consequence of a cognitively poor, uneventful life (as criticized, e.g., by Neisser, 1967): in these cases, an automatic reset would take place at regular intervals, acting as a putative maintenance mechanism.

## MEMORIES ARE SYNAPTIC PATHWAYS: TESTING THE HYPOTHESIS

In the natural sciences – as mentioned before – theories are measured not only by their *explanatory* power and *testability*, but a good *predictive* capacity is also desired (Bunge, 1967, 1985). Besides, theories are no better than the experimental data available in their support. Here, the challenge is being able

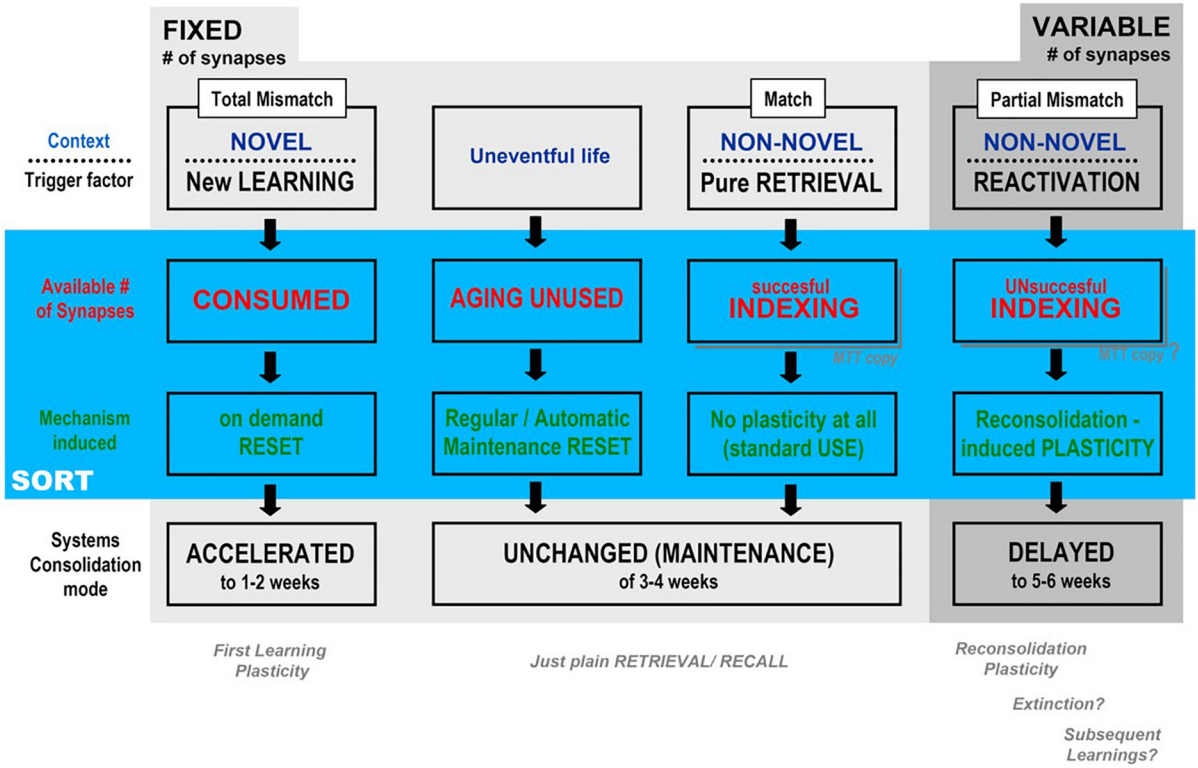


**FIGURE 4 |** Synthesis of the possible outcomes, according to the degree of contextual mismatch presented by the tasks interposed between the training and remote test sessions of the main, aversive task (e.g., CFC), according to SORT (described in the text): Departing from a fixed, finite number of CA1 synapses in MEMSET, multiple sessions of novel context presentations (new learnings) would involve more and more new information, thus demanding the reset of previously used synapses in order to "make room" for new memories, *before* the natural, maintenance reset cycle, which *accelerates* systems consolidation temporal framework. When context is recognizable (partial mismatch only), different plasticity mechanisms would be triggered that *adds* extra synapses to MEMSET in order to cover the encoding needs without engaging in "reset on demand", which would explain the observed delay in systems consolidation (see **Figure 2**).

to directly *probe the engram*, a concept originally introduced by Richard Semon (Semon, 1904; Josselyn et al., 2017), and considered intractable until the recent emergence of new, powerful recording and labeling technological tools, such as optogenetics (Cowansage et al., 2014; Nabavi et al., 2014; Tonegawa et al., 2015a,b; Cai et al., 2016; Rashid et al., 2016) and chemogenetics (Roth, 2016; Atasoy and Sternson, 2018; Campbell and Marchant, 2018; Muir et al., 2018), usually combined with multineuronal recordings (Sakurai et al., 2018). Optogenetics, outstanding and promising as it is (Goshen, 2014; Bickle, 2016), still has to evolve as a methodology considering the technical limitations that reduce their interpretability (see Baxter and Croxson, 2013; Hardt and Nadel, 2018). Even systems consolidation has been approached with this technique (Doron and Goshen, 2017), despite some arguable preliminary findings (Kitamura et al., 2017).

In order to empirically test SORT, first we need an operational definition of *engram.* Most authors think on the engram as an *object* to be found, something that is stored *in* a particular brain structure, but, since what neural plasticity allows – in order to record learning – is the establishment of a new neural network that represents the experience, **it is much more interesting and productive to define the engram as the organizational pattern *of* that very same brain structure** – something already hinted by Pavlov (1904). Thus, an engram would be the full established neural network consisting of a collection of *multiple pathways that connects/binds,* end-to-end, S to M, i.e., the **S**ensory (or internal) input signals with the adequate **M**otor outputs – in order to allow that the right stimuli "lead" to the correct, learned response behavior. This would be the basic engram, a tripartite structure selected along animal evolution, analogous to some extent to an innate behavior circuitry (see Fuster, 1995, 1997). Additional considerations could, without much difficulty, accommodate other kinds of non-sensory inputs, such as internally generated signals, as well as non-motor outputs, including those involving imaginary or even abstract thoughts, as humans can do.

**If its true that memory traces are the neural pathways, selected and marked by plasticity at the dendritic spines level, that *connect sensory inputs to motor outputs to produce a learned response*, the direct observation of synapses in activity might be more informative than recording whole-cell activity, as many existing techniques already permit**. Observing just at the neuronal level may incur in interpretation difficulties since,

in principle, there might exist many different ways to connect the same set of neurons to obtain one same response pattern, which would lead to false positives. Another limitation derives from the fact that hippocampal neurons are known multitaskers, i.e., they simultaneously participate in different functions running in parallel, e.g., working as place cell as well as a engram-recording agent (Knierim, 2015; Lisman, 2017). Hippocampal indexes would consist of the exact relay connections necessary to store the memory/engram of one experience as a distributed network established over the large, sparse neocortical circuitry.

Thus, a subset of HPC CA1 pyramidal cells might collectively establish working connections with afferences pointing to the very same set of neocortical areas that have initially received and processed every sensory or multimodal information produced by the learning experience and conveyed to the HPC for integration and storage (Teyler and DiScenna, 1986). Of course this would *not* be a direct projection (however some might), since the efference must be relayed by at least two intermediary stations – entorhinal and perirhinal cortices – before effectively reaching neocortical targets. The difficulties of these necessarily tortuous pathways will not be elaborated here, and remains unknown. However this multilevel, stepped process resembles some classical multilayered connectionist models (Marr, 1971; Leng et al., 1994; McClelland et al., 1995; Sardesai et al., 2001), which might provide a hint to explain how a relatively small set of hippocampal cells might reach and control what is supposed to be a large number of cortical cells: studying the organization of these pathways, layer to layer, might help to understand how it works.

SORT proposes that CA1 cells establish strong synapses with previously available, yet not connected efferent axons projecting to the cortex, thus recruiting the exact set of cortical components that compose the engram. It is reasonable to consider that a direct test of this hypothesis is not technically feasible right now, at least not employing present optogenetic tools that lack resolution both in the spatial (diffusion covers only small volumes) and the temporal (fast on/off control might not reflect the longer temporal framework necessary for plastic changes to take place) domains, among other limitations (Baxter and Croxson, 2013; Kim et al., 2016; Hardt and Nadel, 2018; Sakurai et al., 2018). Thus, to visualize the whole set of CA1 (pyramidal cells') plastic synapses that might specifically be involved in connecting to those axonal efferences projecting to NCTX, it may be much more adequate to employ *fluorescent microscopy*. These techniques allow for the imaging of *dendritic spine dynamics* itself, and include confocal laser scanning, transcranial two/multi-photon microscopes, or fiber-optics endomicroscopy (Maiti et al., 2015; Sakurai et al., 2018). They have already been proven efficient to image hippocampal place cells activity in subcellular resolution during navigation (Dombeck et al., 2010), and even to study long-term memory related neural ensemble activity in the amygdala (Grewe et al., 2017). Calcium-imaging techniques such as Cal-Light or FLARE, might allow a functional readout of synaptic activity in near real time and with great spatial resolution (Sakaguchi and Hayashi, 2012). The biggest challenge would be to identify, follow and control tridimensional synaptic patterns that take place over sparse populations of neurons in a brain area, but this might be the path to meet at least the first of Mayford's experimental criteria to "definitely identify an engram for a declarative memory": "identify a learning-induced molecular and corresponding functional cellular change in a specific subset of neurons" (Mayford, 2013): the other three criteria – basically three different ways to tamper with engrams – might then follow easily. Only after this, we might consider further investigating, for instance, the nature of the putative different *levels of resetability*, that might possibly be consequence of different neurochemical and morphofunctional properties among synapse populations I, II, and III, e.g., the presence of a molecular "safeguard" system, differences hopefully detectable: clearly, population III (quasi-stable or the "episodic" memory subset) would be the most "safeguarded" of them, which doesn't mean it can't somehow be reset – erased, a fate not even the best episodic memories cannot escape.

Until a few years ago, the memory research field was subject to conform to William James's cautionary advice that "the only proof of there being retention is that recall actually takes place" (James, 1890, chapter XVI), since there was no possible way to "grasp" any material trait of "memory" to be examined: the nature of the engram has been clearly beyond the reach of most available technologies. In this aspect resided the great strength of behavioral neuroscience to this day, i.e., to be the only tool that allow "peeping" into the engram, whatever be its nature, actually "seeing the invisible." The golden standard to confirm the presence of a memory trace, however, may never cease to be basically behavioral, but whatever the real nature of the engram is, it must accommodate not only the well-described plasticity machinery underlying synaptic consolidation, but also all the essential properties behaviorally observed, such as (1) the capacity of being constantly updated by reconsolidation, (2) the slow, complex process of consolidation at the system levels, and (3) the diversity of memory systems that exist in the mammalian brain. We may still be far from a full understanding of such a complex material entity, but technological advances seem to be pushing us toward some light (Josselyn et al., 2015; Poo et al., 2016; Queenan et al., 2017).

Understanding the nature of the physical trace of memory, or process that allows experience-based behavior modification in animals, is not only important as basic knowledge, but has also practical/clinical relevance, considering how devastating pathologies of memory may be. However, as is typical in technology nowadays, any further development must be preceded by a better understanding of the basic science behind the phenomenon, which benefits immensely from some theoretical elaboration.

## ETHICS STATEMENT

The paper is a conceptual review discussing different experimental works and no new experiment was performed.

## AUTHOR CONTRIBUTIONS

JQ is the sole author of this paper and participated in all processes of writing this manuscript. In addition, he participated in many

of the manuscripts reviewed, having generated the integrative hypothesis presented in this manuscript.

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