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Axo-axonic Synapses: Diversity in Neural Circuit Function

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Abstract

The chemical synapse is the principal form of contact between neurons of the central nervous system. These synapses are typically configured as presynaptic axon terminations onto postsynaptic dendrites or somata, giving rise to axo-dendritic and axo-somatic synapses, respectively. Beyond these common synapse configurations are less-studied, non-canonical synapse types that are prevalent throughout the brain and significantly contribute to neural circuit function. Among these are the axo-axonic synapses, which consist of an axon terminating on another axon or axon terminal. Here, we review evidence for axo-axonic synapse contributions to neural signaling in the mammalian nervous system and survey functional neural circuit motifs enabled by these synapses. We also detail how recent advances in microscopy, transgenics, and biological sensors may be used to identify and functionally assay axo-axonic synapses.

Keywords

Electron microscopy; dopamine; glutamate; GABA; serotonin; acetylcholine; norepinephrine

Introduction

In 1961, Dudel & Kuffler discovered that the major inhibitory neurotransmitter GABA acts on the presynaptic terminal of a glutamatergic neuron to decrease the release probability of this major excitatory neurotransmitter. Since then, a panoply of G-protein coupled receptors (GPCRs) and ionotropic heteroreceptors localized to axon terminals were identified to modulate neurotransmitter release (Atwood et al., 2014). Clearly most, if not all, axon terminals contain GPCR autoreceptors, which allows for autoregulatory negative feedback. Other sources of neurotransmitters exist to exert presynaptic regulatory action, including retrograde messengers sourced from postsynaptic membranes and astrocytic transmitters. Several studies now demonstrate that axon terminals synapsing on another axon terminal provide direct neural circuit routes for presynaptic modulation throughout the mammalian brain.

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Among other functions, the axo-axonic synapse allows for neurotransmitter release from the postsynaptic axon terminal that does not require action potential activity that is generated at the postsynaptic neuron's axon hillock. Such is the case for substantia nigra dopamine neurons, in which somatic action potential firing rates often fail to account for activity observed at the axon terminal (see Berke, 2018). Here, we survey structural evidence for axo-axonic synapses, discuss how this configuration may impact circuit function, and describe how recent technological advances may aid in identifying and functionally assessing these non-canonical synapses.

Structurally defining axo-axonic synapses

The gold standard in elucidating neural circuits, electron microscopy (EM), provides nanometer scale resolution critical for discerning the morphological characteristics of axon terminals and postsynaptic targets (Figure 1). Axo-dendritic and axo-somatic synapses describe interactions in which an axon synapses on the dendrite (Figure 1a) or cell body (Figure 1b) of the post-synaptic neuron. Synapses on in the axon hillock or initial segment directly regulate action potential generation of the postsynaptic neuron (Figure 1c). Axo-axonic synapses are characterized by a presynaptic element or varicosity that contains neurotransmitter-filled synaptic vesicles and forms one or more electron-dense junctions with a similarly vesicle-filled axon terminal (Figure 1d) (Peters & Palay, 1996). In some instances, axon terminals form a close parallel junction that lack the electron-dense synaptic specialization and are thus referred to as appositions (Figure 1e).

Axo-axonic synapses have been observed from the earliest use of EM, including in the retina (Kidd, 1962), olfactory bulb (Hirata, 1964), and thalamic lateral geniculate nucleus (Colonnier & Guillery, 1964). Several approaches may be used to identify the participants in axo-axonic circuits. Neuronal degenerative agents can be injected prior to tissue collection to label a projection of interest. Antibodies for neurotransmitters or receptors facilitate the identification of projections or presynaptic localization of neurotransmitter receptors. The early development of an antibody for the beta-adrenergic receptor (Strader et al., 1983), for example, enabled the detection of these receptors localized to axons within the cortex (Aoki et al., 1987).

One limitation of EM, however, is that a given sample of tissue is limited to two or three labeling products. Consequently, it is common to observe axo-axonic synapses that lack identification for either afferent, efferent, or both partners. The ability to detect synapses is influenced by sectioning protocols. This is especially true for axo-axonic synapses. One study employing serial section reconstruction of the striatum noted that the distance of the synapse of the efferent axon may be greater than ten times the size of the afferent synapse (Kornhuber & Kornhuber, 1983)! Varicosity properties such as membrane contortions may also impede detection. Therefore, the incidence of axo-axonic synapses is certainly under-reported. The following examples of axo-axonic *synapses* by presynaptic neurotransmitter type are compiled from rodent tissue, unless otherwise stated. We also describe axo-axonic *appositions* in which functional evidence supports a role for presynaptic modulation. When referring to axo-axonic synapses and appositions collectively, we use the term axo-axonic *contacts*.

Glutamate

Glutamatergic projections form axo-axonic synapses or appositions in a range of brain structures. Axons arising from the prefrontal cortex (PFC) synapse on unidentified terminals in the habenula (Greatrex & Phillipson, 1982), whereas projections originating from the anterior cingulate cortex form axo-axonic synapses within the dorsal medial striatum (Wang & Pickel, 2002). Further characterization of these circuits is limited as the postsynaptic targets are presently unknown. However, a significant fraction of cortical boutons in the dorsal striatum contact putative dopaminergic terminals (Bouyer et al., 1984). Although the directionality of information flow at this synapse is unclear, physiological studies suggest that glutamate may suppress striatal dopamine release through mGluR1 receptors located on dopaminergic terminals (Paquet & Smith, 2003; Zhang & Sulzer, 2003).

An alternative approach to detecting axo-axonic synapses is through identification of heteroreceptors, or non-cognate receptors, located on axon terminals. The observation of NMDA receptors on non-glutamate releasing terminals may indicate the presence of a glutamatergic axo-axonic synapse. Indeed, presynaptically expressed NMDAR receptors are found throughout the brain to modulate a range of neurotransmitter systems (Bouvier et al., 2015; Gracy & Pickel, 1996). The bed nucleus of the stria terminalis (BNST) hosts such a configuration as NMDA receptors primarily localize to axons. Moreover, axons expressing NMDA receptors form axo-axonic appositions twice as frequently as traditional axo-dendritic or axo-somatic configurations (Gracy & Pickel, 1995). Primary sources of glutamatergic projections to the BNST arise from limbic regions such as the ventral subiculum and amygdala (Ch'ng et al., 2018). The axonal NMDA receptor expression is densest in the ventrolateral BNST, a region heavily innervated by noradrenergic projections (Freedman & Cassell, 1994), that may serve as the postsynaptic axon target. Here, adrenergic signaling induces anxiogenesis through hypothalamic-pituitary-adrenal stress axis activation and contributes to the aversive effects of drug withdrawal (Ch'ng et al., 2018). Thus, this non-canonical circuit may contribute to BNST function as an interface between stress and reward systems.

In the cortex, NMDA receptors are found on both excitatory and inhibitory axon terminals (Aoki et al., 1994; DeBiasi et al., 1996). Activation of these receptors on glutamatergic axons enhances excitatory transmission (Brasier & Feldman, 2008) and is necessary for time-dependent long-term depression (Sjöström et al., 2003). NMDA receptors located on cortical GABAergic interneurons provide a mechanism for excitatory circuits to augment local inhibitory signaling (De-May & Ali, 2013; Mathew & Hablitz, 2011). Axo-axonic glutamatergic signaling is not limited to NMDA receptors, however. Ionotropic glutamate receptors presumed to reside on GABAergic axon terminals allow cortical pyramidal neurons to inhibit neighboring pyramidal neurons through a pyramidal axon → interneuron terminal → pyramidal neuron di-synaptic circuit (Ren et al., 2007). Further investigation of glutamatergic axo-axonic synapses stands to reveal contributions of these circuits to cortical function and behavior.

GABA & Glycine

GABAergic interneurons in the dorsal horn primarily synapse on glutamatergic afferent fibers (Hughes et al., 2012). This provides modality-specific filtering of sensory information to suppress spurious activation of nociceptive circuits (Petitjean et al., 2015). A similar configuration is present in the trigeminal nuclei. Glutamatergic sensory afferents arising from the cat vibrissa (Moon et al., 2008) and tooth (Bae et al., 2005) receive GABAergic and glycinergic co-releasing terminals that gate orofacial sensory information. Glycinergic axon terminals synapsing on other axon terminals are also common in the dorsal horn (Lue et al., 1997), as well as in the trigeminal and cochlear nuclei (Clements et al., 1990).

In the dorsal raphe nucleus, a substantial proportion of GABAergic projections form synaptic triads with glutamatergic inputs. That is, for a given glutamatergic axo-dendritic synapse, inhibitory projections synapse on or appose both the glutamatergic afferent (axo-axonic) and the postsynaptic raphe dendrite (axo-dendritic) (see Figure 1f for illustration). This triadic configuration enables the presynaptic axon both pre- and post-synaptic modulatory control. In addition to directly inhibiting dorsal raphe nucleus neurons, GABA presynaptically regulates excitatory drive onto serotonergic neurons through either GABA-A receptor -mediated enhancement or GABA-B receptor -mediated inhibition of glutamatergic signaling (Soiza-Reilly et al., 2013).

Inhibitory axo-axonic regulation of glutamate signaling also occurs in the amygdala. Glutamatergic projections arising from the insular cortex primarily target GABAergic neurons of the lateral central amygdala. Local amygdalar GABAergic terminals frequently appose this excitatory projection (N. Sun & Cassell, 1993). Correspondingly, activation of presynaptic GABA-B receptors (McDonald et al., 2004) in the basolateral and lateral amygdala suppresses excitatory input (Nose et al., 1991; Yamada et al., 1999) and modulates potentiation of cortical synapses onto amygdalar pyramidal neurons (Pan et al., 2009). Inhibiting or genetically eliminating presynaptic GABA-B receptors prevents constraint of plasticity and results in generalization of conditioned fear (Pan et al., 2009; Shaban et al., 2006). Inhibitory signaling may regulate other circuits in the brain through axo-axonic synapses. Physiological studies indicate presynaptic GABA-B receptor -mediated effects in the thalamic medial geniculate nucleus (B. Luo et al., 2011) and nucleus accumbens (Manz et al., 2019).

Catecholamines

Projections containing dopamine or norepinephrine are traditionally identified in ultrastructural studies using tyrosine-hydroxylase immunostaining. Although this antibody labels all catecholamines, anatomical knowledge may be used to infer the present neurotransmitter. For brain regions innervated by both dopamine and norepinephrine projections, radiolabeled dopamine or dopamine beta-hydroxylase immunostaining can distinguish the two catecholamines. The following descriptions of catecholaminergic axo-axonic synapses follow this inferential approach.

Dopamine

In contrast to the previously described neurotransmitter systems, dopaminergic projections exhibit unique morphology that challenges traditional synaptic identification. Whereas dopamine synapses are traditionally presumed to occur *en passant* at varicosities along primarily unmyelinated axons, some studies fail to identify synapses at a given varicosity, whereas others find that these sites only account for a small proportion of dopaminergic synapses, especially in the striatum (Ducrot et al., 2020; Sesack, 2002). Ultrastructural examination throughout the brain reveals that a significant proportion of these projections fail to form synapses, suggesting that this neurotransmitter preferentially acts through volume transmission. However, dopamine terminals frequently appose other axons, often in a triad-like configuration with a shared dendritic target. Such configurations are common in the hippocampus (Sesack & Pickel, 1990) and striatum (Arluison et al., 1984; Pickel et al., 1981), where apposing terminals arise from the cortex (Bouyer et al., 1984). Triadic configurations are also present in the medial frontal cortex with dopamine terminals apposed to GABAergic axons and their targets (Verney et al., 1990). Dopaminergic axo-axonic appositions are also located in the amygdala (Asan, 1997), globus pallidus (squirrel monkey, Eid & Parent, 2015), dorsolateral BNST (Phelix et al., 1992), mediodorsal thalamus (macaque, Melchitzky et al., 2006), and cortex (Verney et al., 1990; macaque, Martin & Spühler, 2013). This pervasive parasynaptic configuration (Sesack, 2002) and the presence of presynaptic heteroreceptors (Feuerstein, 2008) suggests that dopamine may provide targeted presynaptic modulation despite lacking definitive ultrastructural evidence for synaptic contact.

Norepinephrine

Noradrenergic terminals forming definitive synapses of any configuration is a rarity in the cortex. Rather, a single norepinephrine neuron gives rise to over 300,000, largely asynaptic, axon terminals widely distributed across the cortex (Audet et al., 1988). These terminals frequently appose non-noradrenergic axonal processes (Séguéla et al., 1990). Whereas presynaptic-mediated noradrenergic modulation and behavioral effects are documented across a range of brain regions and synaptic mechanisms (Gilsbach & Hein, 2008), the characterization of presynaptic noradrenergic α1 receptor signaling in the PFC presents a compelling case for axo-axonic modulation. The α1 receptor is highly expressed in axon terminals that co-express vGluT1, suggesting localization to glutamatergic pyramidal axon terminals (Mitrano et al., 2012). α1 receptor activation induces a long-lasting suppression of pyramidal glutamate release probability onto GABAergic fast-spiking interneurons but not onto other pyramidal neurons (Wang et al., 2013). This circuit-specific presynaptic effect likely enhances PFC excitability (Luo et al., 2015). The behavioral consequence of such signaling is unclear; some studies report that PFC α1 receptor activation enhances spatial working memory (Hvoslef-Eide et al., 2015), whereas others demonstrate cognitive impairment (Arnsten et al., 1999). While these discrepancies may reside in the nuances of the cognitive tasks employed, they ultimately point towards a functional role for this synapse in cognition. Cortical excitability is also regulated through presynaptic α2 (Ohshima et al., 2017) and β1 (Luo et al., 2014) receptor signaling, offering additional venues for noradrenergic axo-axonic modulation.

The nucleus tractus solitarius serves as a junction for dense adrenergic and cardiovascular inputs that regulate the baroreceptor reflex. Axo-axonic synapses are readily observed in this region, with both noradrenergic (cat, Chiba & Doba, 1976) and non-catecholaminergic inputs (Kachidian & Pickel, 1993; Sumal et al., 1983) inputs synapsing on vagal terminals. Thus, this non-canonical synaptic configuration may serve to modulate blood pressure.

Serotonin

Like catecholaminergic fibers, serotonin projections exhibit a paucity of direct synaptic contacts. However, serotonergic terminals frequently appose unlabeled axons and terminals. This axo-axonic configuration accounts for approximately 30% of appositions in both the striatum (Soghomonian et al., 1989) and hippocampus (Oleskevich et al., 1991), as well as 19% in the cortex (Séguéla et al., 1989). Correspondingly, serotonin receptors are localized to presynaptic terminals throughout the brain (Feuerstein, 2008; Rodríguez et al., 1999; macaque, Jakab & Goldman-Rakic, 1998). 5-HT2A receptor activation, for example, induces glutamate release from projections impinging on pyramidal neurons in the PFC (Marek et al., 2001) and somatosensory cortex (Scruggs et al., 2000).

Beyond 5-HT2A, presynaptically -expressed 5-HT1B receptors are located on glutamatergic retinal terminals in the suprachiasmatic nucleus (SCN) and their activation suppresses optic transmission to reduce the magnitude of light-induced phase shifts (Pickard et al., 1999). Notably, these receptors are found in greater abundance on SCN GABAergic axons (presumed local interneurons) (Bosler, 1989). Here, 5-HT1B receptor activation provides a disinhibitory mechanism for SCN signaling by suppressing GABAergic transmission (Bramley et al., 2005), and in agreement, 5-HT1B receptor knock-out mice exhibit an attenuated light response (Sollars et al., 2006). Together, these two axo-axonic mechanisms provide means for serotonin to bidirectionally modulate photic regulation of circadian rhythms.

5-HT1B receptor -mediated presynaptic inhibition is also present at corticostriatal synapses, serving to persistently reduce striatal output activity (Mathur et al., 2011). Additionally, serotonergic presynaptic inhibition occurs at hippocampal CA1 local excitatory synapses (Winterer et al., 2011). This signaling bidirectionally modulates emotional memory (Eriksson et al., 2013).

Acetylcholine

Like the previously discussed modulatory neurotransmitters, the rates of cholinergic synaptic contacts are low, ranging from 14% in the parietal cortex (Umbriaco et al., 1994) to less than 10% in the hippocampus (Umbriaco et al., 1995) and striatum (Contant et al., 1996). However, there is a wealth of evidence for axo-axonically -mediated presynaptic actions of acetylcholine (Gilsbach & Hein, 2008). In the anterolateral BNST where glutamatergic and cholinergic axons form appositions, acetylcholine suppresses excitatory transmission through M2 receptor signaling on postsynaptic glutamatergic axon terminals (Guo et al., 2012). This mechanism is proposed to filter sensory input.

Conversely, activation of β2-containing nicotinic receptors located on thalamic terminals in the medial PFC induces glutamate release (Lambe et al., 2003). Arising from the midline

and intralaminar thalamus, these cortical-terminating projections are suggested to participate in arousal, attention, and affective processing (Groenewegen & Berendse, 1994). Nicotine enhances such functions, suggesting that axo-axonic circuits contribute to these effects.

Neuropeptides

EM data demonstrate that neuropeptide-containing terminals serve as both afferents and efferents in axo-axonic configurations. Current knowledge of neuropeptide circuit function and directionality remains more limited compared to that of conventional neurotransmitters. Regardless, axo-axonic synapses exist between ghrelin and neuropeptide Y (NPY) - containing terminals in the arcuate nucleus (Guan et al., 2003). The SCN hosts similar configurations between somatostatin-positive and unidentified fibers (Buijs et al., 1995), and between serotonergic and NPY projections (Guy et al., 1987). NPY-releasing afferents also form axo-axonic synapses in the cat dorsal horn (Doyle & Maxwell, 1993).

Axo-axonic functional circuit motifs

Mounting evidence indicate axo-axonic contacts provide unique functional attributes to neural circuits. This configuration provides a “short circuit” by which an axon terminal synapsing on another axon terminal bypasses the somatodendritic processing (temporal and spatial summation) that occurs in traditional axo-somatic and -dendritic synapses (Figure 2a). For example, the SCN is dominated by intrinsic inhibitory signaling. GABAergic interneurons axo-axonically synapse onto other GABAergic terminals (Buijs et al., 1995; Castel & Morris, 2000), providing a direct mechanism to regulate inhibitory activity.

Axo-axonic contacts may also facilitate spatially precise control of neurotransmitter release. Whereas somatic depolarization will generally influence neurotransmitter release from all downstream axon terminals from a particular neuron, axon terminals impinging directly on other axon terminals provide fine-grained modulation of specific efferent synapses (Figure 2b). This function is especially impactful for neurons with extensive axonal arbors. Substantia nigra dopamine neurons, for example, form unmyelinated axonal arbors that sum up to 1 cubic millimeter in the dorsal striatum (Matsuda et al., 2009). Striatal dopamine signaling critically regulates action initiation (Sheridan et al., 1987), yet somatically generated action potentials may not faithfully propagate along axons to confer terminal selectivity. However, activation of striatal cholinergic interneurons, or their upstream afferents, robustly elicits local dopamine release in a behaviorally significant manner (Cachope et al., 2012; Cover et al., 2019; Threlfell et al., 2012). Although existent striatal EM data only notes a high prevalence of ChAT-labeled axons juxtaposed to (but not synapsing on) unidentified striatal axon terminals (Contant et al., 1996), the presence of nicotinic acetylcholine receptors on nigrostriatal axon terminals supports a functional axo-axonic circuit (Jones et al., 2001).

Axo-axonic contacts may also provide input regulation. Whereas postsynaptic neurons are canonically limited to either retrograde signaling or indirect multi-synaptic feedback loops to modulate their presynaptic partners, axo-axonic synapses formed by a postsynaptic axon collateral synapsing on an axon terminal that synapses on that neuron’s own dendrite, allow for direct modulation of afferent inputs (Figure 2c). Such is the case for cholinergic neurons

residing in the pedunculopontine nucleus and laterodorsal tegmentum. Cholinergic axon terminals appose projections to this area that express M2 heteroreceptors (Garzón & Pickel, 2006). Accordingly, M2 receptor activation decreases glutamatergic transmission onto cholinergic neurons (Ye et al., 2010).

Lastly, axo-axonic contacts confer an additional dimension of modulatory control over neural signaling. In canonical circuits, impinging afferents influence timing of postsynaptic action potential firing. The addition of axo-axonic configurations affords manifold control of this activity, providing granularity to neural systems. This function is magnified in more complex axo-axonic circuit configurations, such as two axons synapsing on a common postsynaptic axon (Bae et al., 2005). Serial formations of axo-axonic contacts also occur, which further amplify the modulatory resolution controlling downstream neurotransmitter release (Figure 2d). This configuration occurs in the cat trigeminal nucleus, where axo-axo-axonic di-synaptic circuits terminate on axon initial segments (Westrum, 1993).

Functional assessment of axo-axonic circuits

Despite a wealth of EM structural data, the functional differences between axo-axonic *synapses* and non-specific axonal *appositions* are unclear. Generally, it is assumed that appositions mediate “volume transmission” wherein diffusion of a neurotransmitter exerts slower, modulatory effects across many synapses, akin to that of hormones (Agnati et al., 1992). This may suggest that appositions mediate slow modulatory effects while axo-axonic synapses provide immediate control of neurotransmitter release and, therefore, behavior. However, in the dorsal striatum where cholinergic interneurons release acetylcholine to elicit local dopamine release from nigrostriatal terminals to support behavioral reinforcement (Cover et al., 2019), EM data only support an appositional relationship (Contant et al., 1996). This suggests that axo-axonal contacts may exert rapid and functionally significant events regardless of the classification as an axo-axonic synapse or apposition. Moreover, this example illustrates the need for improved methods to: 1) easily identify axo-axonic contacts and 2) assess the functional relevance of such non-canonical circuits.

Improving on EM, array tomography employs both immunofluorescence light microscopy and EM of ultrathin serial sections to allow for expanded labeling opportunities and improved z-axis resolution (Micheva & Smith, 2007). This technique facilitated the finding of GABA-glutamate terminal appositions in the dorsal raphe nucleus (Soiza-Reilly et al., 2013). Others have coupled immunofluorescence with confocal microscopy-enabled semi-automated 3D reconstruction to quantify serotonergic appositions to excitatory and inhibitory synapses throughout the limbic system (Belmer et al., 2017). Expansion microscopy, a process by which immunolabeled tissue is physically expanded, enables nanoscale resolution with light microscopy (Karagiannis & Boyden, 2018). With an expanded number of means to label presynaptic and postsynaptic-specific proteins, these approaches can be applied to identify axo-axonic circuits and provide a starting point for subsequent functional assessment.

The advent of viral and transgenic tools now allows more precise interrogation of the functional roles for axo-axonic contacts. One example arises from a mechanism by which

oxytocin release into the nucleus accumbens induces a presynaptic 5-HT1B -mediated long-term depression of excitatory transmission onto nucleus accumbens medium spiny neurons (Mathur et al., 2011) that is required for social reward (Dölen et al., 2013). Although these findings were not confirmed with EM, the experimental results support an axo-axo-axo-dendritic configuration within the nucleus accumbens by which oxytocin released from hypothalamic projections into the nucleus accumbens activates oxytocin receptors present on serotonergic dorsal raphe terminals. The subsequent serotonin release activates 5-HT1B receptors present on glutamatergic terminals synapsing on medium spiny neuron dendrites. Thus, oxytocin-induced serotonin release activates 5HT1B receptors to suppress glutamate release onto medium spiny neurons. This study illustrates how viral tools may be combined with mouse transgenic models to elucidate and functionally evaluate such non-canonical circuits (Dölen et al., 2013).

Virally-expressed biosensors may also be leveraged to probe axo-axonic circuits. These membrane-bound sensors consist of a neurotransmitter binding site that, when occupied, changes the structure's conformation to elicit photon release from a fluorophore (Leopold et al., 2019). Sensors for glutamate (GluSnFR) (Marvin et al., 2013), GABA (iGABASnFR) (Marvin et al., 2019), dopamine (dLight, $GRAB_{DA}$) (Patriarchi et al., 2018; F. Sun et al., 2018), acetylcholine ($GRAB_{ACh}$) (Jing et al., 2020), or norepinephrine ($GRAB_{NE}$) (Feng et al., 2019) may be incorporated in *ex vivo* slice physiology studies or monitored *in vivo* though implanted photometric fibers in freely moving animals. This tool may also be coupled with rodent transgenic models to examine the afferent axonal neurotransmitter release onto sensor-expressing postsynaptic terminals. Using the presumed striatal cholinergic interneuron synapse onto dopamine terminals as an example, one may express a cre-dependent acetylcholine sensor in the substantia nigra of DAT-cre transgenic mice. Recording from the striatum, one can specifically monitor cholinergic signaling onto dopamine terminals and study how this axo-axonic signaling varies during behavior or under pharmacological manipulation. This approach may also be useful for identifying novel circuits or validating preliminary findings from microscopy studies. By expressing a cre-dependent neurotransmitter sensor in the postsynaptic axon and an optogenetic activator (or silencer) in the presynaptic axon terminal, one may investigate the connectivity of a proposed synapse as a first step toward functional validation.

Calcium sensors, such as GCaMP (Nakai et al., 2001), are commonly used to study neuronal activity and may be adapted to study non-canonical circuits in several ways. In addition to cre-dependent variants that allow for pathway-specific examination, the recent development of axon-targeting viral constructs (Broussard et al., 2018) enables activity monitoring of axon terminals arising from intrinsic neuronal populations such as GABAergic or cholinergic interneurons. Two-photon imaging also provides a means to study spatially discrete terminal activity with temporal resolution only limited by sensor kinetics. This approach was effectively employed to examine dopamine axonal activity in the context of movement (Howe & Dombeck, 2016). For appositions, coupling GCaMP with a red-shifted sensor like jrGECO (Dana et al., 2016), allows for simultaneous recording of two axon terminal populations. One may also pair a red-shifted calcium indicator with a neurotransmitter sensor to correlate neurotransmitter release from the presynaptic axon terminal with the activity of the postsynaptic axon terminal.

Whereas axo-axonic contacts have been consistently identified over the past sixty years of EM research, technological barriers hindered functional characterization of the circuits that arise from these interactions. The development of new tools to discern terminal-specific activity is finally opening doors to functionally understand the role of these non-traditional circuits in neuronal information processing and behavior expression. As these tools develop, it is apparent that a growing consideration for non-traditional synaptic configurations is warranted.

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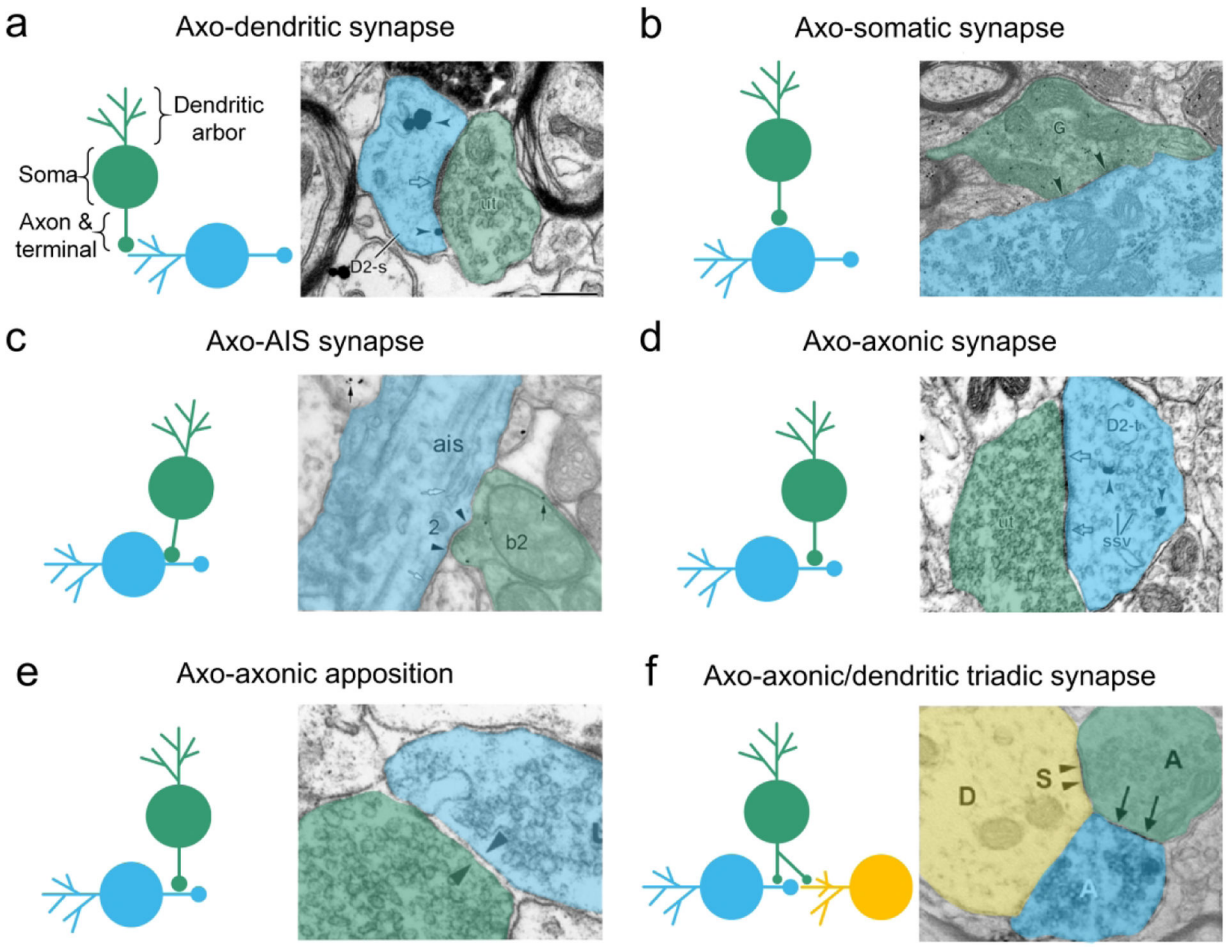

Figure 1. Canonical and non-canonical synapse types.

Four common synaptic arrangements between presynaptic (green) and postsynaptic (blue) neurons are depicted through schematic illustrations (left) and electron micrographs (right). **a**. The axo-dendritic synapse consists of the presynaptic axon terminal (green) synapsing on a dendritic element (i.e. spine or shaft) of the postsynaptic cell (blue). **b**. The axo-somatic synapse is comprised of a presynaptic axon (green) terminating on the cell body of the postsynaptic neuron (blue). **c**. An axon (green) may terminate on the axon initial segment (AIS) to modulate action potential generation of the postsynaptic neuron (blue). **d**. In an axo-axonic synapse, the presynaptic neuron (green) synapses on an axon or axon terminal of the postsynaptic cell (blue). **e**. Axo-axonic appositions describe closely apposed axon terminals with parallel membranes separated by a clear cleft and lack densities associated with synaptic elements. Note that the directionality of such synapses is often unclear, as is the case for the present example. **f**. The axo-axonic triad describes a configuration in which a neuron (green) synapses on both the presynaptic element (blue) and postsynaptic target (yellow) of an axo-dendritic or – somatic synapse. Electron micrographs are adapted from Wang & Pickel, 2002 (**a, d**), Lue, et al., 1997 (**b**), Takács et al., 2015 (**c**); Sesack & Pickel, 1990 (**e**), and Soiza-Reilly et al., 2013 (**f**).

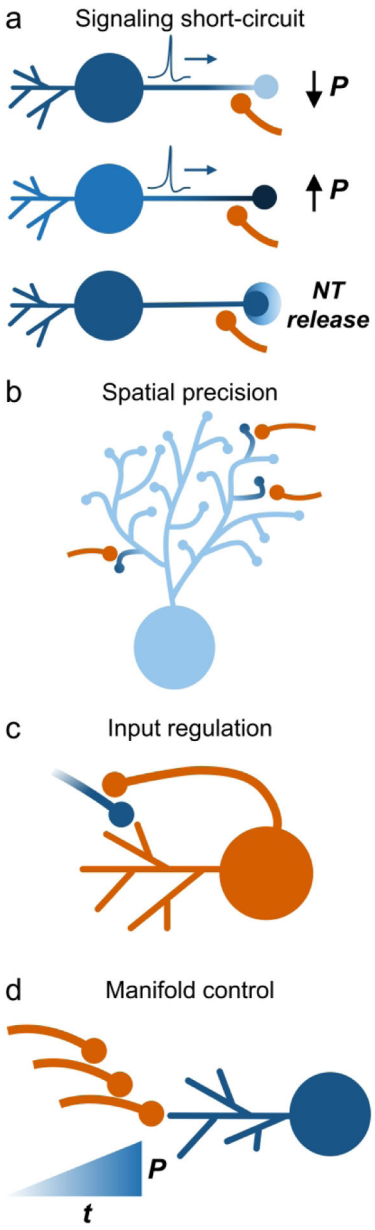

Figure 2. Axo-axonic synaptic contributions to neural circuit function

a. Axo-axonic synapses allow a presynaptic axon (orange) to negatively (top schematic) or positively (middle schematic) modulate neurotransmitter release probability (P) of the postsynaptic axon (blue). In addition, it is possible for an afferent to induce neurotransmitter (NT) release independently of the action potentials generated by the postsynaptic neuron (bottom schematic). **b**. In postsynaptic neurons with extensive axonal arborizations (light blue neuron), afferents synapsing axo-axonically (orange) provide spatially-precise neurotransmitter release or suppression. **c**. Axon collaterals from a neuron (orange) that synapse on impinging afferents (blue) provide a mechanism for that cell to regulate afferent input. **d**. Combinations of axo-axonic synapses either in convergence or serial formation (as

shown) provide granular control of neurotransmitter release probability over time (t) for canonical axo-dendritic synapses.