Astroglial versus neuronal D-serine: a reality check

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The activation of N-methyl D-aspartate receptors is conditioned by the binding of a co-agonist to a dedicated receptor binding site. It is now largely accepted that D-serine plays this role at many central synapses in the hippocampus, amygdala, hypothalamus, nucleus accumbens, and in the prefrontal, visual and somatosensory cortices. D-serine has been found to be synthesized, stored and released by astrocytes (Figure 1). Yet, several immuno-labeling studies and experiments in genetically modified animals have recently led to a suggestion that neurons are primarily responsible for the synthesis and release of D-serine (Wolosker et al. 2016). Here, we argue that such conclusions could have resulted from the erroneous interpretation of experimental data and that they are at odds with a substantial amount of published work.

The existence of gliotransmission is not under debate

The proponents of the neuronal-only D-serine hypothesis argue that the entire process of gliotransmission is under question, referring to a study that reported no causal link between IP3R2 receptor-dependent Ca^{2+} signals in astroglia and synaptic function (Agulhon, 2010). That study assumed that gliotransmission relies entirely on IP3R2-dependent Ca^{2+} signaling. Therefore its results suggested a flaw in such underlying assumption, rather than a disproof of gliotransmission. Consistently, improved imaging methods have since then revealed that, even under genetic deletion of IP3R2, Ca^{2+}-signaling occurs in thin astroglial processes (Srinivasan et al., 2015).

Robust evidence supports the astrocytic origin of D-serine

The astroglial origin of D-serine has been established by several independent groups using complementary techniques, including its detection using electron microscopy (Bergersen et al., 2011, Figure 1A; Takata et al. 2011; Sultan et al., 2016, Figure 1C; Henneberger et al. 2011, Figure 1D; Martineau et al., 2013, Figure 1E). Together, these studies have proposed that D-serine is synthesized in astrocytes, stored in vesicles and released via calcium-dependent exocytosis, *in vitro* and *in vivo*. Furthermore, enzymology evidence suggests that the optimal conditions exist for D-serine synthesis in astrocytes (Ehmsen et al., 2013) and D-serine degradation in neurons (Foltyn et al., 2005). Taken together, these data support the concept that D-serine originates from astrocytes, acts at synaptic NMDARs and is taken up by neurons where it is degraded (Figure 1F). To our knowledge, there has been no systematic criticism or controversy in the literature that could brand these results as “artifacts” or ignore them altogether (Wolosker et al. 2016).

The 'three pillars' of the neuronal hypothesis: fact checking

What does D-serine and serine racemase detection in neurons really mean?

Wolosker et al. argue that the presence of strong immuno-labeling for D-serine and its synthesizing enzyme serine racemase (SR) in neurons, rather than in glia (Wolosker et al. 2016), provides compelling evidence that D-serine is synthetized by neurons rather than astrocytes. However, astrocytic processes only account for 5-7% of the neuropil volume while neuronal elements make up 70-75% of it, partly because the surface-to-volume ratio of astrocytes (~25μm^{-1}) is 3-4-fold that of neurons (~7μm^{-1}) in hippocampal neuropil (Medvedev et al., 2014 and references therein). This means that, for similar cellular expression of D-serine or SR, the staining for D-serine and SR would appear 10 times stronger in neurons than in astroglia. These simple considerations illustrate the difficulties of translating tissue immuno-labelling of D-serine and SR into the predictions of their cellular abundance, or into conclusions on the cellular origin of D-serine.

Another caveat in the interpretation of immunostaining data (Wolosker et al. 2016) is to consider SR solely as a D-serine synthesizing enzyme. Indeed, SR is bidirectional and bifunctional: it can use either D-serine or L-serine as substrates, with similar affinities, and can degrade D-serine through α,β-elimination (Foltyn et al., 2005); resulting in a complex picture. In fact, enzymatic studies showed that under physiological conditions, the presence of D-serine and SR at high levels in the same cellular compartment favors the degradation activity of SR over racemization (Foltyn et al., 2005). Therefore, the suggested abundance of SR and D-serine in neurons might actually argue that neurons are a degradation compartment for D-serine.

To date, there has been no D-serine release machinery identified in neurons

An important argument of the neuronal D-serine hypothesis is the presumed existence of a dedicated transport system. The idea that a "large fraction of D-serine release occurs by the neuronal Asc-1 transporter" (Wolosker et al. 2016) is based on studies that used various Asc-1 substrates to "artificially increase the rate of D-serine release" from D-serine-preloaded neurons or slices (see Rosenberg et al., 2013). But careful examination of these studies leads to the conclusion that, in the absence of manipulation, Asc-1 is involved in D-serine uptake by neurons, not efflux. Indeed, Asc-1 mediates a bidirectional transport and the net flux of D-serine can easily be manipulated. The cited studies used D-Ile, a substrate of Asc-1 that blocks D-serine uptake and favors its release via D-serine/D-Ile exchange. Consequently, while the authors observed a strong D-serine uptake by neuronal Asc-1 without D-Ile, they detected a rise in D-serine levels when D-Ile is applied to brain slices or neuronal cultures preloaded with D-serine (Rosenberg et al., 2013). The logical conclusion is that, under physiological conditions, D-serine is taken up by neuronal Asc-1 which, in the presence of D-Ile, can be artificially reversed into a D-serine efflux. Besides, the adult mice lacking the Asc-1 gene have unaltered D-serine levels (Safory, 2015), thus ruling out a role of this transporter in the supply of extracellular D-serine. Therefore, since ASCT-1 and ASCT-2 are unfit to transport D-serine (EC_{50} ~1.1-1.8mM), and since no D-serine vesicular transporter is known in neurons (Martineau et al., 2013), there is, to date, no conclusive evidence that neurons can release D-serine under physiological conditions.

Knock-out studies are inconclusive

The last argument in support of the neuronal view comes from a study that used cell-specific and conditional SR knock-outs (KOs) (Benneyworth et al., 2012) and concluded that the deletion of SR in neurons (nSR-KO) impairs D-serine availability while the deletion in astrocytes (aSR-KO) does not. Unfortunately, this conclusion is affected by the fact that D-serine levels were examined at very different time-points: 4-10 weeks in nSR-KOs, but 10-15 days aSR-KOs. In addition, whether SR protein is completely obliterated in astrocytes 10-15 days after the deletion of SR gene (*Srr*), and whether D-serine stores are truly depleted, is unclear. Therefore, assessing D-serine levels 10-15 days after deletion of *Srr* in astrocytes is unlikely to reveal a significant reduction of D-serine content (Benneyworth et al., 2012). Intriguingly, even though the D-serine levels were examined at a much later time-point in the neuronal KO, the study found that these levels were also intact in the hippocampus of nSR-KOs. However, because a modest reduction was detected in the cortex (exclusively) of 12-week old nSR-KOs, it was concluded that D-serine is produced in neurons. This is an unsafe conclusion at best in light of the experimental design issues mentioned above. Furthermore, the recombination induced in the nSR-KOs occurs around postnatal day 15, a time when the levels of SR protein and D-serine are still a fraction of those found in adults (Wang & Zhu, 2003). Therefore, deleting *Srr* in neurons at this critical time should have had early and strong effects on D-serine levels, unless of course, neurons are not a site of D-serine production.

Concluding remarks:

The experimental evidence for the presence of D-serine and SR in neurons is not to be dismissed, and efforts should be made to integrate it into our understanding and conceptualization of D-serine physiology (Figure 1F). Nonetheless, there is, to date, no consistent evidence indicating that neurons are a major source of extracellular D-serine, and therefore the astroglial origin of D-serine remains the best working model.

References

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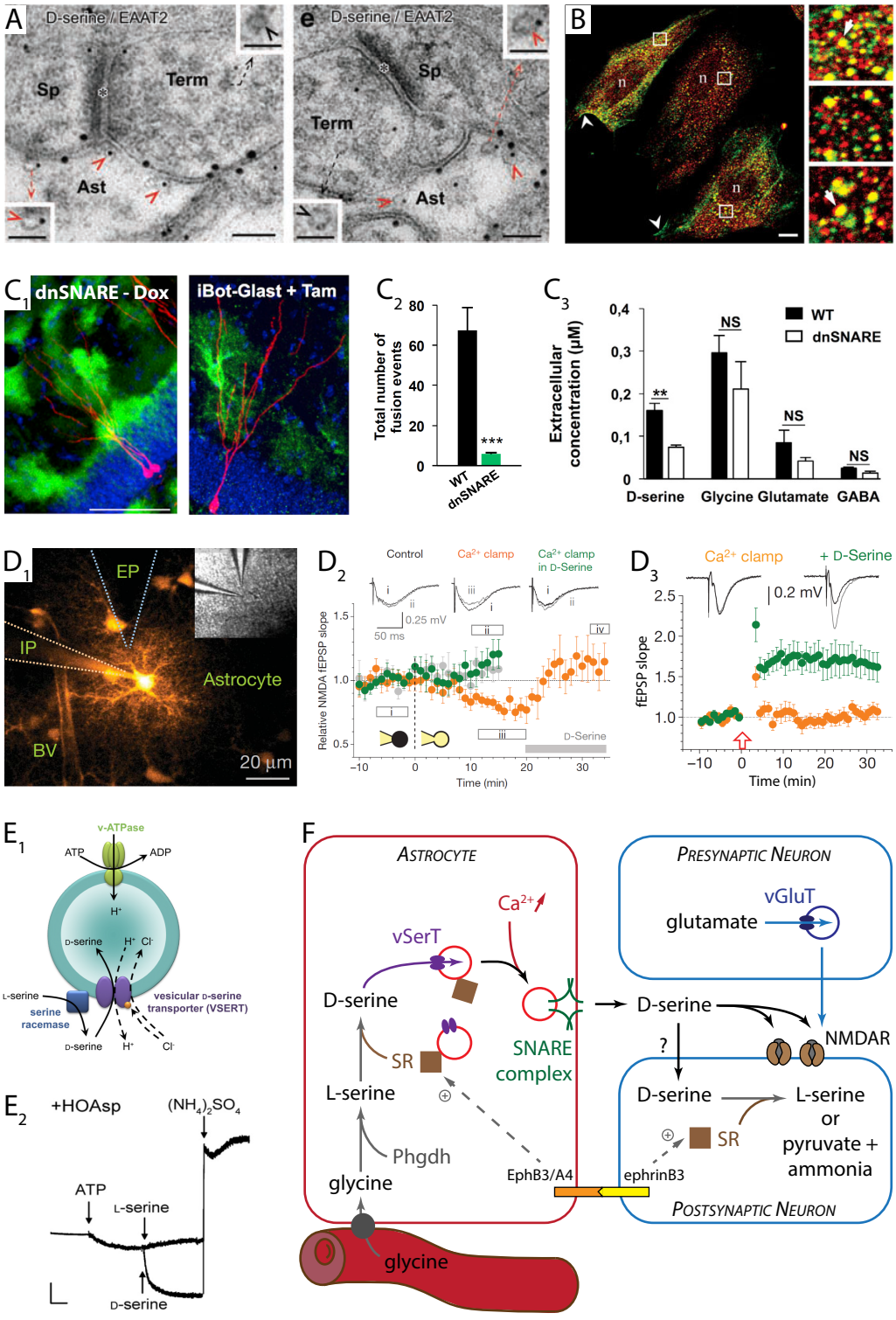
Figure Legend:

A, Using electron microscopy, D-serine is found in peri-synaptic astrocytic processes inside of, or in close association with, synaptic-like micro-vesicles (SLMVs). Note that D-serine is also found in the cytoplasm of neuronal elements, but not in vesicles (adapted from Bergersen et al., 2011. Glutamate transporter EAAT2 is labeled with large immuno-gold particles. D-serine is labeled with small immuno-gold particles. Ast: astrocytic process; Sp: dendritic spine; Term: presynaptic terminal.) **B,** Similarly, in astrocytes in vitro, D-serine is found in VAMP2 (synaptobrevin)-containing SLMVs that are recruited to the plasma membrane upon calcium stimulation (adapted from Martineau et al., 2008. D-serine is labeled in red and VAMP2 in green (eGFP-Sb2). n: nucleus). **C1-C3,** Consistently, the blockade of vesicular release with botulinum toxin or with transgenic inhibition of the SNARE-complex (dnSNARE mice) as well as the blockade of calcium activity in astrocytes, impairs extracellular D-serine availability in vitro and in vivo (Henneberger et al. 2011, Takata et al. 2011, Sultan et al., 2016). For instance, in GFAP-tTA:tetO-dnSNARE mice and iBot-Glast-CreERT2 mice, a dominant negative form of VAMP2 or the botulinum toxin (that cleaves endogenous VAMP2) are selectively expressed in astrocytes (**C1**), resulting in a 90% decrease in the total number of vesicular fusion events assessed by TIRF illumination (**C2**). Concomitantly, the levels of D-serine, assessed in slices using high performance liquid chromatography, are impaired by 50% (adapted from Sultan et al., 2016. dnSNARE – Dox: dnSNARE mouse off doxycycline, iBot-Glast + Tam: iBot-Glast-CreERT2 mouse treated with tamoxifen, green: transgene-expressing astrocytes, Red: RFP-expressing principal neuron of the dentate gyrus, Blue: Dapi staining). **D1-D3,** When astrocytes from a hippocampal slice are loaded with a "Ca^{2+} clamp" solution through a patch-pipette to maintain Ca^{2+} levels at resting concentrations (**D1**), NMDAR-mediated responses recorded in the corresponding territory are rapidly impaired (**D2**). This can be either prevented or blocked by D-serine applications. Consequently, synaptic plasticity induced with a classic protocol is prevented in the territory of a Ca^{2+}-clamped astrocyte due to the lack of D-serine (**D3**). Therefore, functionally preventing the Ca^{2+}-dependent vesicular release from one astrocyte directly impairs D-serine availability at nearby synapses (adapted from Henneberger et al., 2011. IP, intracellular patch-pipette; EP, extracellular pipette; BV: blood vessel). **E1-E2,** The exocytotic route of D-serine release by astrocytes received further confirmation through the identification of a D-serine specific vesicular transporter (vSerT) on astrocyte SLMVs, responsible for loading D-serine into vesicles (**E1**). The loading of D-serine, in the presence of ATP (required for creating a proton gradient by the vesicular ATPase), can be seen as an acidification of the vesicle in photometry experiments (**E2**). Interestingly, in astrocytes, SR was found directly bound to VAMP2-containing SLMVs (**E1**). Consistently, L-serine elicits a vesicular acidification similar to D-serine. However, when SR activity is inhibited with hydroxy-aspartate (HOAsp, **E2**) the effect of L-serine on vesicular acidification is prevented, while D-serine loading is unaltered (adapted from Martineau et al., 2013. $(NH_4)_2SO_4$ is applied at the end of the experiment to dissipate vesicular gradient; scale: 2 arbitrary units per 1 min). **F,** Current working model: in astrocytes, D-serine is readily stored in vesicles upon synthesis (protecting newly synthesized D-serine from α,β-elimination by SR). Interestingly, the D-serine precursor L-serine does not cross the blood brain barrier, and the only known L-serine production pathway in the brain parenchyma occurs via 3-Phosphoglycerate Dehydrogenase (Phgdh), an enzyme exclusively found in astrocytes (Ehmsen et al., 2013). Consequently, in astrocytes cytoplasm, SR is facing high and constant levels of L-serine but virtually no D-serine, strongly favoring the racemization activity

of L-serine into D-serine. As explained in the main text, once released in a Ca^{2+} and SNARE-dependent manner, D-serine is likely taken up by neurons (through Asc-1?) where it accumulates (hence its detection in neurons) and is degraded by SR through racemization or α,β-elimination.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C1
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Total number of fusion events
80
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WT
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C3
Extracellular concentration (µM)
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0,3
0,2
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dnSNARE
D-serine
Glycine
Glutamate
GABA
D1
EP
IP
Astrocyte
BV
20 µm
D2
Control
Ca2+ clamp
Ca2+ clamp in D-Serine
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50 ms
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1.0
0.5
D-Serine
−10
0
10
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30
Time (min)
D3
Ca2+ clamp
+ D-Serine
0.2 mV
fEPSP slope
2.5
2.0
1.5
1.0
−10
0
10
20
30
Time (min)
E1
v-ATPase
ATP
ADP
H+
D-serine
H+
Cl-
L-serine
serine racemase
vesicular D-serine transporter (VSERT)
D-serine
H+
Cl-
E2
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L-serine
D-serine
F
ASTROCYTE
Ca2+
vSerT
D-serine
SR
SNARE complex
L-serine
Phgdh
glycine
EphB3/A4
PRESYNAPTIC NEURON
vGluT
glutamate
D-serine
?
NMDAR
D-serine
SR
L-serine or pyruvate + ammonia
ephrinB3
POSTSYNAPTIC NEURON
glycine