Published in final edited form as:

Trends Neurosci. 2017 September ; 40(9): 520–522. doi:10.1016/j.tins.2017.06.010.

Astroglial Versus Neuronal D-Serine: Check Your Controls!

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As we recently reviewed [1], studies from at least six laboratories now indicate that neurons rather than astrocytes are the major sources for D-serine and preferentially express its synthetic enzyme, serine racemase (SR), in the normal brain [1]. We described how several unforeseen artifacts misled established scientists (including ourselves) into believing that serine racemase and D-serine are localized exclusively to astrocytes and represent *prima facie* exemplars of gliotransmission. We were concerned that the purported astrocytic source of D-serine was being perpetuated to this day, despite compelling evidence to the contrary published over the past decade [1].

In their letter of rebuttal, Papouin *et al.* [2] indicate that our conclusions on the preferential expression of SR and D-serine in neurons are erroneous because they are at odds with a substantial amount of previously published research. Discarding Ockham's razor, they put forward a scattershot of possible, sometimes contradictory, explanations to justify their position. These include findings with cultured astrocytes, immunostaining that lacks the sensitivity to detect fine astrocytic processes, SR as a catabolic enzyme in neurons, etc. The problem with all the studies cited by Papouin *et al.* is the absence of adequate controls to ensure the specificity of the staining for SR and D-serine and of other detection methods such as biosensors. Antibodies are notorious sources of artifacts because it is difficult to determine if an antibody recognizes only the antigen of interest in immunohistochemistry unless negative controls include tissue that lacks the specific antigen. This is especially crucial for the anti-D-serine–glutaraldehyde conjugate antibody that has some level of crossreactivity with L-serine, which is found in extremely high concentrations in astrocytes [3,4].

The realization that the previous demonstrations of astrocytic D-serine were artifactual came to light when we and others used more-selective antibodies and negative controls consisting of SR knockout (*Srr*^{-/-}) mice. The group of Mori was the first to determine antibody specificity using *Srr*^{-/-} as a control and found only neuronal staining [5], with no detectable

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staining in astrocytes. The same result was reported by Benneyworth *et al.* [6] and Balu *et al.* [3], who observed neuronal staining for SR and D-serine under conditions where the immunoreactivity for both antibodies was abolished in tissue from *Srr*^{-/-} mice. Notably, artifactual crossreactive immunostaining in astrocytes was only eliminated by using blocking concentrations of L-serine-BSA that were 100-fold higher than those used in publications reporting apparent D-serine in astrocytes. Wolosker and Snyder also obtained similar results, showing that only ~10% of the astrocytes were positive for D-serine while 90% of D-serine-positive cells were neurons [4].

To explain the sudden disappearance of the ‘robust’ astrocytic SR and D-serine immunostaining in these better-controlled studies [1], Papouin *et al.* [2] propose a *deus ex machina*: the SR and D-serine are hidden in the tiny astrocytic processes, rendering them ‘invisible’ to the microscope. This proposal is totally inconsistent with the results of the cell-selective conditional silencing of SR expression, which is not subject to purported impaired immunohistochemical visualization. Silencing SR expression in astrocytes with inducible glial fibrillary associated protein (GFAP) promoter-Cre has negligible effects on SR expression in cortex, whereas there is a major loss of SR expression in (CAMKII α -Cre) *Srr*^{-/-} mice where *Srr* is deleted in glutamatergic neurons [6]. Moreover, to test this hypothesis of astrocyte ‘crypto-SR’, Ehmsen *et al.* [4] used a mouse line wherein enhanced green fluorescent protein (EGFP) expression is under the control of the *Srr* promoter, a sensitive surrogate marker for the cells that express SR (Figure 1). Because EGFP accumulates in the cell bodies and cannot ‘hide’ in small astrocytic processes, one would expect high levels of astrocytic EGFP expression if SR were indeed widely expressed in astrocytes. However, Ehmsen *et al.* confirmed predominant neuronal staining for EGFP (Figure 1), which was not present in non-transgenic controls [4].

Papouin *et al.* proposed another model whereby the astrocytic SR synthesizes D-serine, while the more abundant neuronal SR would only degrade D-serine. This argument is surprising, given their assertion that SR is expressed primarily in astrocytes. SR can indeed transform D-serine into pyruvate by α,β -elimination activity, but the efficiency of this catabolic reaction is fivefold lower than the conversion of L-serine into D-serine [7]. Thus, while the α,β -elimination activity may limit the maximal D-serine synthesis, it is difficult to envisage how neuronal SR would only catabolize D-serine as proposed by Papouin *et al.* because levels of neuronal D-serine are much higher than those in astrocytes [3,4].

Additional arguments for an astrocytic source of D-serine release come from studies using primary cultures of astrocytes [1]. Cultured astrocytes assume the characteristics of reactive astrocytes, expressing numerous proteins, including SR, that are not expressed in quiescent astrocytes. Thus, the use of cultured astrocytes to study D-serine synthesis and release provides no insight into what is normally occurring in the brain. However, traumatic brain injury (TBI) causes reactive astrocytes to proliferate in the hippocampus. It is in these reactive astrocytes that SR can be easily detected using immunohistochemistry [8]. Furthermore, the TBI-induced cognitive impairments are reversed by selectively eliminating SR in GFAP-expressing astrocytes in adulthood before injury [8]. Therefore, astrocytic SR appears to play a role in pathology where reactive astrocytes are present, but not in quiescent astrocytes that are present in normal brain.

The remaining criticisms of Papouin *et al.* deal with the lack of complete understanding of the mechanisms underlying neuronal release of D-serine. Contrary to their assertion, inhibitors of the alanine/serine/cysteine transporter Asc-1 actually reduce endogenous D-serine release from slices [9] and decrease the levels of extracellular D-serine in microdialysis *in vivo* [10], indicating that Asc-1 is a mediator of tonic neuronal D-serine release rather than its uptake. They completely misrepresent Asc-1 as a ‘pillar’ of the neuronal D-serine hypothesis. Furthermore, there is compelling evidence that neurons release D-serine under physiological conditions. Toru Nishikawa, the discoverer of D-serine in the brain, carried out *in vivo* microdialysis in the hippocampus of glutamatergic neuron-targeted *Srr*^{-/-} mice and found a 50% decrease in extracellular D-serine levels [1]. GABAergic neuronal SR and D-serine are spared in glutamatergic neuron-targeted *Srr*^{-/-} mice, likely accounting for the residual source of D-serine. Moreover, neuron-targeted *Srr*^{-/-} mice display impaired hippocampal long-term potentiation (LTP) *in vitro* [6] and *in vivo* [8], while the astrocytic *Srr*^{-/-} mouse has normal LTP.

To localize the cellular origins and effects of D-serine, *Srr*^{-/-} mouse models are more selective than several techniques invoked by Papouin *et al.*, such as calcium clamping [11], glial poisoning by fluoroacetate which blocks the L-serine to D-serine shuttle in neurons [1,11], non-specific SR inhibitors [11,12], or dnSNARE mice [1]. Most importantly, *Srr*^{-/-} mice are the only models that can validate the widely used D-serine biosensors that have unknown specificity and have been employed to demonstrate changes in D-serine without the proper controls [1]. It is possible, however, that there are species and developmental differences in D-serine and SR localizations and function that will need to be addressed case by case by carrying out parallel staining using *Srr*^{-/-} mice.

The notion of glia-derived D-serine as a neuromodulator/transmitter was first proposed more than 20 years ago by Snyder and coworkers [13]. This proposal inspired many of us to question the cherished ‘neurocentric’ dogma (chemical neurotransmission). However, ignoring the compelling evidence contradicting the astrocytic origin of D-serine and not acknowledging its obvious neuronal origin will not advance our understanding of NMDA receptor function in health and disease. This was the main message that we wished to convey in our review that was misconstrued by Papouin *et al.*

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Figure 1.

Serine Racemase Is Expressed Predominantly in Neurons. BAC-transgenic mice expressing EGFP under the control of the *Srr* promoter were stained for EGFP (green) and cell-specific markers (red). Expression is observed in principal neurons of the cortex (CTX, A–C); shown here are layer 6 polymorphic neurons identified by neuronal nuclear (NeuN) staining. Only a few cortical astrocytes identified by S100 β staining (E) also express low levels of EGFP (F, arrowheads). Scale bars, 50 μ m. Adapted from Ehmsen *et al.* [4].