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Investigating brain D-serine: advocacy for good practices

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Abstract

The last two decades have witnessed remarkable advance in our understaning the role of D-amino acids in the mammalian nervous system: from the unknown, to known molecules with unknown functions, to potential central players in health and disease. D-Amino acids have emerged as an important class of signaling molecules. In particular, the exploration of the roles of D-serine in brain physiopathology is a vibrant field that is growing at an accelerating pace. However, disentangling the functions of a small chiral molecule in the complex chemical milieu of the brain requires to be familiar with distinct measurement approaches but also to currently work with delicate molecular tools which effects can be confounded. Thus, study of D-amino acids requires accurate methodologies and specific controls, and these have often been lacking. Here we outline best practices for D-amino acid research, with a special emphasis on D-serine. We hope these concepts help move the field to greater rigor and reproducibility, allowing the field to advance.

Keywords

D-Serine; NMDA receptors; analytical methods; rescue experiments; glia; neurons; serine racemase inhibitors; immunostainings

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When exploring a new signaling pathway and moving to new measurements or methodologies outside of our expertise, it is easy to follow the minimum requirements as opposed to the most rigorous ones, resulting in false findings corrupting the scientific literature. Unfortunately, this has happened several times in the field of D-amino acid (D-AA) research. Along with our quest to understand how the brain works, research on the functions of the unusual neuromodulator D-serine requires distinct methodological approaches and controls which have been lacking in many studies. Here we inspect the recent literature and outline several measures to help counter issues of irreproducibility to strengthen our scientific conclusions.

From dark to the spotlight: where do problems originate?

The discovery by Nishikawa and colleagues[1] that D-serine is present at high concentrations in the mammalian brain has not only challenged the long-cherished dogma that free D-AAs are uncommon in mammalian tissues but has also forced (neuro)biologists to reconsider the foundations of cell-to-cell signaling. The idea that D-AAs, originally considered toxic compounds, could serve important functions in mammals was quite radical and was initially dismissed since the authors had not identified pathways that produce them in the body. Considerable efforts from several groups worldwide have radically transformed this vision over the last 20 years, particularly with regard to D-serine. In the 90's, Snyder's laboratory developed specific stereoselective antibodies against D-serine to map its distribution in the brain[2] and then established D-serine's origin to the brain by purifying and cloning serine racemase (SR), the enzyme that produces D-serine[3]. At the same time, they also made a second conceptual breakthrough by demonstrating that D-serine serves as the endogenous co-agonist for the synaptic N-methyl-D-aspartate subtype of glutamate receptors (NMDARs) in the hippocampus[4], a role initially thought to be played by glycine alone[5,6]. These two discoveries have dramatically transformed our understanding of the mechanisms regulating the activity of NMDARs. According to ISI Thomson, since the first report by Nishikawa and colleagues on the presence of free D-serine in the brain there have been approximately 1500 articles published on D-serine from 1992 until 2018, and publications on this topic are appearing at an accelerating pace. As a consequence, the field has grown fast but its novelty has created an Achilles' heel.

In the brain, D-serine is synthesized from L-serine by SR, and is degraded by both SR and the peroxisome associated D-amino acid oxidase (DAAO) enzyme[7], and its levels in the synaptic cleft are controlled by the alanine-serine-cysteine-1 (ASC-1) and ASCT-1 transporters[8–10]. Glycine synaptic availability is essentially regulated by glycine transporters (GlyT)[9,11] but can also be controlled by ASC-1[8,9]. Although D-serine can also bind to the δ2 glutamate receptor (GluD2, Grid2)[12] to regulate long-term depression (LTD) of synaptic communication in the cerebellum[13], it is mostly recognized as the primary co-agonist of NMDARs at synapses of the forebrain where it regulates many functions of these receptors in synaptic plasticity, neurodevelopment and neurodegeneration[14,15]. The D-serine/NMDAR signaling pathway is an appealing route with already clear implications in the pathophysiology and treatment of human diseases as highlighted by: i) the growing literature related to its role in schizophrenia (SCZ), depression, amyotrophic lateral sclerosis, drug abuse and Alzheimer's disease (AD); ii) the expanding number of clinical trials targeting D-

serine signaling to treat patients; and iii) the increasing interest in the development of new compounds that selectively target its metabolism or transport systems[8,16].

The recognition that D-serine is the endogenous NMDAR co-agonist in forebrain, thus supporting crucial cerebral functions, has spurred its intensive study. However, because the L- and D-enantiomers share the same physical properties, its measurement is challenging. For example, quantifying D-serine using a simple migration match *via* liquid chromatography (LC) may not be sufficient, and yet is a common practice; there are too many moieties in the brain to allow simple migration matching to be robust. We note that many publications, even in high-impact journals, lack appropriate controls or use non-validated approaches for detecting D-serine and defining its function, thus leading to misinterpretation of experimental data as detailed below. Such publications propagate misinformation, biasing the field by introducing 'misconceptions'.

The D-serine issue

Although the functions of D-serine have not been questioned, its cellular origin has spawned debate[17–19]. D-serine was long considered a prototypical "gliotransmitter"[15] but recent works by several laboratories have challenged this view. They have shown that under physiological conditions, D-serine is primarily synthesized and released by neurons in the healthy brain to modulate NMDAR functions[20–23] and will only substantially derive from reactive glia in pathological conditions[24]. It appears that these past reports that D-serine can be formed in primary cultured astrocytes follows from studies under such pathological conditions.

Some recent studies published in high-profile journals continue in nourishing the D-serine gliotransmission hypothesis or argue for specific functions of the co-agonist based on experimental evidence that may be questionable. In particular, some studies fail to properly detect and quantify D-serine levels by selective immunostainings or analytical methods, in addition to the use of unspecific pharmacological or genetic interventions. Consequently, their conclusions may appear speculative due to the absence of essential controls. Therefore, as specialists in the field of D-AAs with different and complementary expertise in analytical chemistry, biochemistry, cell biology, neurophysiology and animal models, we write this essay to advocate for good practices in exploring the mechanisms and functions of D-serine in the brain, guidelines that should be used when researching additional D-AAs such as D-alanine and D-aspartate. In addition to presenting a critical review of some aspects of the D-serine literature, we hope that this perspective encourages colleagues entering the field to use good practices in their investigations and evaluations of manuscripts and grants.

Detecting or not detecting D-serine properly with analytical methods: that is the question

The characterization of D-serine as a cell-cell signaling molecule often requires as a first step the ability to detect and quantify the D-AA levels under the different experimental conditions. Such a measurement needs careful experimental design, appropriate analytical techniques and proper execution in order to be confident in differentiating D-serine from

other amino acids and from its L-enantiomer in biological samples. Analytical methods have matured, and we now have an array of techniques ranging from biosensors[25–27], high pressure liquid chromatography (HPLC)[28–30] and LC/LC[31] to LC/mass spectrometry[32,33] or capillary electrophoresis-laser-induced fluorescence (CE-LIF)[34–36] with well described protocols to detect and measure D-AAs (including D-serine) even at very low levels in complex matrices like biological samples. Despite the availability of multiple approaches to confirm D-serine, analysis of recent publications suggests that this D-AA was not always quantified with the controls that are outlined in Box 1 and Figure 1.

A first example is represented by a mix-up of the analytical methods used. We specifically refer a case[40] where it is stated that extracellular levels of D-serine, glycine, glutamate and GABA were analyzed by HPLC, but then the Supplementary section reports that a CE-LIF system was used following a procedure published elsewhere[41]; moreover, no electropherograms or chromatograms are shown. While confusing the descriptions of the approaches used is rare, a more common issue is the absence of experimental details on replicates, as well as not presenting electropherograms (*via* CE) or chromatograms (*via* HPLC)[42]. Most journals offer authors the opportunity to publish chromatograms/electropherograms in the Supplementary information because without them, it becomes hard to determine what was done to validate the results. Reports on D-AA characterization need to include such details.

Another fairly common issue is the poor resolution of the amino acid enantiomers (Figure 1A). As a second example, by using a combination of both HPLC and a biosensor, D-serine levels were reported to oscillate in brain samples (i.e., mouse hippocampus)[43]. We support the use of multiple measurement approaches increasing the confidence of their conclusions. However, in this case, poorly resolved D-serine and L-serine peaks render the strong conclusions difficult to support. Our goal here is to not to advocate for a specific protocol, but we do expect that the authors validate the specificity of their detection by employing a variety of controls (see Box 1).

Besides, the results obtained with biosensors should be interpreted as putative until secondary approaches validate their results. Indeed, several commercial (Sarissa LTD, UK) or home-made D-serine biosensors are based on the use of DAAO from the yeast *Rhodotorula gracilis* (*Rg*DAAO) layered on an electroconductive support. In contrast to the enzyme isolated from other sources (and commercially available), *Rg*DAAO shows a high specific activity, a stable interaction with the FAD cofactor, a high stability under experimental conditions and is not inhibited by L-amino acids[44]. *Rg*DAAO catalyzes the oxidative deamination of neutral D-AAs[44,45] and does not act either on glycine (K_m = 160 mM, i.e. two to three orders of magnitude higher than the physiological concentration), or on D-aspartate and D-glutamate ($k_{cat} \leq 1\ s^{-1}$ vs. 61 s^{-1} for D-serine), known to act also on NMDAR. The experimental evidence shows that primary and secondary amines are not substrates or inhibitors of *Rg*DAAO. However, current *Rg*DAAO-based biosensors also detect most neutral D-AAs, including D-alanine, and might non-specifically oxidize other unexpected biological substances. Although D-alanine levels in the brain and in particular the hippocampus are much lower than those of D-serine[29,31,32], the maximal activity and the catalytic efficiency of *Rg*DAAO for D-alanine is 1.7- and 30 times higher than those for D-

serine[45], so it is conceivable that DAAO-based biosensors could also detect trace amounts of D-alanine which is also known to act as a co-agonist for NMDARs. Finally, it is also important to note that the commercial *Rg*DAAO based biosensors are also sensitive to dopamine or to a series of structurally related agonists/antagonists of D1- and D2-like receptors (Dallerac & Mothet, personal communication).

D-Serine can be also measured using luminescent or fluorescent DAAO based assays[3,24,45]. But the response of these assays is nonlinear with D-serine concentration. We discourage their use which absolutely requires careful calibration for each concentration range used.

The good practice in detecting D-serine, SR and DAAO with antibodies

Another approach commonly used to visualize D-serine in tissues or cell preparations is immunocytochemistry. For D-serine, the technique was introduced first by Snyder and collaborators[2] and has the remarkable advantage of revealing the distribution of the AA in entire cells or in cellular sub-compartments. Using this method with locally produced or commercial antibodies, investigators have reported that D-serine is present in many locations throughout the central nervous system (CNS)[2,22,46,47]. Immunostainings of AAs with antibodies at first glance could sound odd since antibodies require epitopes larger than an individual amino acid. Accordingly, raising anti-D-AA antibodies requires that the immunogen of the target D-AA be conjugated to a carrier protein by glutaraldehyde, which acts as an effective linker molecule. Many companies are selling polyclonal or monoclonal anti-D-serine antibodies. Antibody specificity is often validated with an ELISA test in competition experiments.

Despite well-described procedures and methods, some studies have reported the presence of D-serine by immunostainings using less-common methods (see Box 1 and Figure 2). This is the case where immunostainings to visualize D-serine were performed in tissues perfused with a fixative containing no glutaraldehyde while using a commercial rabbit polyclonal D-serine glutaraldehyde conjugated antibody[48]. To our best knowledge, commercially available monoclonal or polyclonal D-serine antibodies require glutaraldehyde in the fixative otherwise immunodetection of D-serine is less reliable. One study reported staining of D-serine with a paraformaldehyde-based fixative but the authors used an antibody optimized for formaldehyde fixation, which is not commercially available[49].

The same precaution should be also taken for detecting SR or DAAO by Western blotting or immunostaining analysis since most commercial antibodies are not specific and have not been validated in knock-out mice for the respective proteins. Indeed, the gold standard for immune specificity is the demonstration that the epitope is absent from tissues obtained from subjects in which the expression of the antigen has been genetically silenced. Miya et al[21] were the first to apply this stringent requirement is studies of SR, establishing unequivocally that SR was expressed in neurons, primarily glutamatergic neurons but also GABAergic neurons, but not in astrocytes as was broadly accepted for a decade after their publication. The neuronal localization was confirmed by Balu et al[23] in mouse and human brain utilizing antibodies validated against SR null-mutant mice. As SR is the primary source of D-serine, Balu et al.[23] furthermore defined conditions for specific immune-staining of D-serine in

tissue sections by showing that L-serine blocking concentrations had to be increased nearly a hundred-fold above reported conditions in order to prevent cross-reactivity (false positives) with the high concentrations of astrocytic L-serine.

Detection by analytical methods or visualization by immunohistochemistry of D-serine and its metabolic enzymes (i.e., SR and DAAO) is only one set of the experimental strategies used to interrogate whether D-serine has a function in their experimental model, a second set of experimental strategies relies on integrating pharmacological and genetic interventions in order to infer or support such functions. In the next sections, we present the different approaches commonly used but consider their limitation since correlation does not mean causation (Figure 2).

The case of astrocyte poisoning and pharmacological rescue experiments

Besides analytical measurements, pharmacological experiments require careful controls. One experimental design generally used by investigators consists of blocking a particular pathway with their favorite compound A and genetic intervention, and then evaluation of the rescue by compound B, in our case D-serine. An example is given by the use of fluoroacetate (FAC) to block metabolism of glial cells and then the pharmacological rescue by adding exogenous D-serine to restore normal function. FAC is the toxic constituent of the *Dichapetalum* plant *family*[50]. When brain tissues are exposed to FAC, it is converted into fluorocitrate, an inhibitor of aconitase, and hence impairs the tricarboxylic acid (TCA) cycle in astrocytes. FAC treatment results in the accumulation of citrate and in a reduction of glutamine[51]. Although the underlying mechanism for selectively affecting glia but not neurons remains elusive, FAC has been extensively used as a selective gliotoxin to uncover the contribution of astrocytes to synaptic transmission, functional plasticity and behavior[51]. Accordingly, studies have reported that FAC application to brain tissues impairs long-term potentiation (LTP) or long-term depression (LTD) expression through decreasing NMDAR currents but not α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor function[41,52,53], and prevents allodynia[54,55] or breathing response to CO_2 levels in the brainstem[56]. These effects have been ascribed to the blockade of astrocytic D-serine release by FAC (but only based on the observation that adding exogenous D-serine rescues these NMDAR-dependent functions; see[17,57]). However, several lines of reasoning suggest that such an interpretation is speculative. First, it is also conceivable that FAC may indirectly affect the release of D-serine from neurons rather than directly block the release of the coagonist from glia[17,28]. Indeed, according to the “serine shuttle hypothesis” recently elaborated by Wolosker and colleagues[58], L-serine is initially produced exclusively in astrocytes since only astrocytes express 3-phosphoglycerate dehydrogenase (3-PGDH), the key enzyme controlling L-serine synthesis. Then, L-serine is exported to neurons where it is converted into D-serine by neuronal SR. Once released by neurons, D-serine binds to the co-agonist site of NMDARs. Interestingly, FAC-induced impairments of LTP and NMDAR-mediated synaptic currents can be also fully reversed by L-serine[28]. Yet, the transport of glucose in astrocytes from blood vessels and the glycolytic pathway are two processes highly regulated by multiple pathways[59]. It has been shown that elevation of calcium and sodium increases the uptake and utilization of glucose by astrocytes. Although speculative, we could foresee that the activity of 3-PGDH in converting 3-phosphoglycerate into L-serine

would be affected by all maneuvers (e.g., calcium clamp, FAC poisoning, …) that inhibit Ca^{2+} or Na^{+} waves in astrocytes.

Since L-serine does not affect NMDAR activity on its own, this result should support the view at first glance that FAC poisoning of astrocytes could affect the synthesis and/or the supply of L-serine to neurons. Restoring the glia-neuron shuttling by providing the D-serine precursor to neurons is thus able to fully overcome the astrocyte poisoning with FAC. However, rescue experiments with L-serine can also contribute to misleading interpretation since most commercial L-amino acids often contain D-AAs and *vice versa* as impurities at levels ranging from 0.1 to 5%. Therefore, when adding 1 mM L-serine, 1–50 μM of D-serine are added which would be sufficient to saturate the glycine site of NMDARs in most preparations. In some cases if high concentrations are used (≥1 mM) it might be necessary to purify the commercial sources of L- or D-serine with specific treatments (e.g., by enzymatic depletion) before using the amino acid for the purpose of physiological or biochemical assays. Also, the use of FAC to selectively impair astrocytes and not neurons is complicated since its glial selective action may depend on the duration of incubation and the concentration used. Indeed, FAC could also impair the metabolism of neurons themselves with prolonged exposure since neurons utilize the TCA cycle. Furthermore, FAC reduces glutamine synthesis in astrocytes and would likely impair glutamatergic synaptic transmission by reducing glutamine availability for glutamate synthesis in neurons. FAC also impairs the formation of glutamate from glucose by blocking the conversion of oxaloacetate into α-ketoglutaric acid. Finally, citrate is known to be an efficient chelator of many ions including Ca^{2+}. Therefore, its accumulation in astrocytes may likely impair several calcium-dependent physiological processes such as constitutive exocytosis. Thus, FAC by interfering with the metabolism of astrocytes may impact neuronal activity by a number of different and complementary mechanisms.

In the same vein of artifact compromised methods is the use of genetical or pharmacological acute blockade of astrocytic inositol 1,4,5-trisphosphate (IP3) receptors with heparin, which is introduced specifically in the astrocytes, and which impairs NMDAR-dependent LTP at CA3-CA1 hippocampal synapses, a deficit rescued by exogenous D-serine[60]. Based on previous findings precisely demonstrating *in vitro* that glia may release D-serine through a Ca^{2+}-regulated process[61,62], it was claimed that astrocytic IP3 receptors contribute to the astrocyte release of D-serine thereby controlling functional plasticity at synapses. As stated above for FAC, it is also possible that blocking IP3 receptor function in astrocytes may have impacted the serine shuttle between glia and neurons, thus preventing the synthesis and release of L-serine from astrocytes and then the synthesis of D-serine by neurons. An alternative mechanism might be that IP3 receptor deletion or blockade may also impact the homeostasis of other gliotransmitters such as glutamate or ATP or most importantly glycine. Indeed, their release or uptake systems are known to be closely regulated by intracellular calcium[57,61,62,64,65]. Although highly debated for more than 20 years, astroglia have been shown to release glutamate or ATP through calcium-dependent mechanisms (putatively exocytosis) that requires intracellular calcium stores[9,57,63–65]. Therefore, impairments in synaptic plasticity (i.e., LTP) induced by genetic or pharmacological deletion of glial IP3 receptors is not specific to D-serine and would also involve these neuromodulators and potentially others (see Box 1 and Figure 2). Rescue experiments with L-serine or any other

signaling molecule whose functions depend on astrocyte integrity (i.e., glycine, glutamate, ATP) have to be considered in order to draw a definitive conclusion. Similar possibilities may be relevant for studies in which exogenous D-serine was provided directly to the slice[43] or through i.p. injection[40] to rescue the respective NMDAR-mediated dysfunctions in mice where glial exocytosis has been genetically nulled. Since invalidation of IP3 receptors or glial exocytosis are not specific interventions restricted to D-serine's function at NMDARs (validated only by the sole ability of exogenous D-serine to restore electrophysiological responses), we cannot exclude that the same inactivation would impact the release of glial glutamate, ATP, glycine and then their action on NMDARs as demonstrated by several groups at the same synapses using the same recording protocols in the same conditions, and at the same animal age[63–65].

Adding any potentiating compounds acting on the glycine modulatory site (GMS) of NMDAR like glycine, D-alanine or D-cycloserine would have the same rescuing effect as observed with exogenous D-serine in all the above studies further undermining their conclusions (Figure 2). Rescuing NMDAR function with D-serine or any compounds just indicates that the GMS of NMDAR is involved. This is a pharmacological effect, as was the case during the initial investigations considering the identity of the co-agonist of NMDARs: adding D-serine, glycine, D-alanine or D-cycloserine and observing a potentiating action of such compounds on NMDAR functions does not say much on the precise implication of a specific endogenous ligand. Our message is therefore to encourage caution in the interpretation of experiments using pharmacological agents (i.e., D-serine) to rescue deficits since these experiments would only reveal a correlation but do not demonstrate causality.

Off-target effects of some genetic and pharmacological tools to explore the functions of D-serine

Two strategies have been commonly employed to investigate the roles played by D-serine in brain activity. First, in electrophysiological experiments performed in slice preparations, the role of the endogenous agonist can be selectively removed by the perfusion of slices with a purified catabolic enzyme that selectively degrades co-agonist such as D-serine[4,66]. Using this strategy, the Snyder laboratory was the first to provide evidence that application of purified DAAO reduces synaptic NMDARs function in cultured hippocampal neurons, hippocampal slices or in juvenile cerebellar slices[4], an initial observation later confirmed by Papouin and colleagues[67]. Using this enzymatic approach, D-serine was shown to be the primary NMDAR co-agonist required for LTP at many CNS synapses[28,41,67–69], or to be required for the LTD at synapses between parallel fibers and Purkinje cells in the immature cerebellum[13]. A second more selective approach at first glance has then been introduced by the Mori laboratory[21] together with the Coyle laboratory[70] by genetically silencing SR expression. $SR^{-/-}$ mice have a 90% decrease in brain D-serine and show reduced NMDAR currents, impaired synaptic plasticity, and learning deficits[8,70,71]. This genetic approach has been made more selective through conditional methods by using Cre-recombinase expression restricted to forebrain neurons with a promoter for the α-subunit of Ca^{2+}/calmodulin-dependent kinase II or to astrocytes by a promoter glial fibrillary acidic protein to inactivated "floxed" SR gene[72].

These two approaches remain the gold standard methods to explore the functions of D-serine in brain slices or in living animals, but each have their own limitations (see Box 1). If the use of enzymatic scavengers represents an invaluable technique for neurophysiologists investigating the neuromodulation of the 'glycine' site of NMDARs in the CNS, it is important to keep in mind that the quality of the results is highly dependent on the source and purity of enzymes that the investigators are using. Important observations have been reported proposing that D-serine mediates the spread of gliogenic LTP in nociceptive pathways[73], neuropathic pain depends upon D-serine co-activation of spinal NMDARs in rats[54,55], D-serine released by astrocytes in the brainstem regulates breathing response to CO_2 levels[56], and glial D-serine controls synaptic metaplasticity in the hypothalamus[69]. One common factor in these studies was the use of commercial sources of DAAO. *Trigonopsis variabilis* DAAO from Boehringer Mannheim/Roche was active and relatively pure[4,74], but this preparation is no longer commercially available and thus could not be used in the mentioned studies. So interpretation of the results from studies based on commercial DAAO should be made cautiously since these preparations show also strong activity against D-aspartate, glycine, D-glutamate but also NMDA. We recently reviewed one effective way to manipulate these enzymes during electrophysiological recordings in acute brain slices and highlighted several experimental tricks[75] and asked investigators for prudence (Figure 2). An alternative to DAAO to eliminate D-serine is to use recombinant D-serine deaminase (DsdA) which displays a higher affinity and specificity for D-serine[66,74,76]. Enzymatic scavengers like Glycine Oxidase from *Bacillus Subtilus* (*Bs*GO) have also been successfully used to probe the relative function of glycine at NMDARs[28,41,66–69]. But, a critical analysis regarding the use of enzymes to scavenge NMDAR coagonists may be useful here. Indeed, addition of DAAO (or DsdA) together with glycine oxidase (GlyOx)[28,41,66–69], does not abolish NMDAR currents, possibly due to their insufficient activity and/or low affinity, thus preventing the complete removal of all of the co-agonists. *Bs*GO is much less active than DAAO and is normally added at concentrations (in mg/ml) up to 10 times higher than DAAO. However, only the amount of enzyme that actually adsorbs to the slice surface would be active in removing local coagonists. Therefore, the *Bs*GO local activity is likely much lower than DAAO, even when the former is added at higher concentrations (excess enzyme in the bath solution may not increase the amount of enzyme that adsorbs to the slice because of saturation of the binding sites). Perfusing tissues with medium containing an enzymatic scavenger system for extracellular D-serine or glycine does not guarantee by itself that the endogenous coagonist is fully eliminated: this must be verified by an analytical method as reported[41,69]. The considerations above suggest that quantitative estimates on the contribution of D-serine *vs* glycine achieved by the scavenging methods can be misleading knowing that in most case both enzymes when applied on slices will not fully remove the coagonists. This may explain in part the large variation and reproducibility problems regarding the relative effects of *Bs*GO in decreasing NMDAR currents.

As an interesting and troubling possibility, perhaps $SR^{-/-}$ mice may also have their limitations since SR in addition to produce D-serine might be also involved in the synthesis of D-aspartate[77]. Genetic deletion of SR leads to reduced levels of D-serine but also of D-aspartate[77,78]. Therefore, one can imagine a potential scenario where deletion of SR induces loss of function of both D-serine and D-aspartate at NMDAR.

Another source of possible misinterpretation comes from the use of pharmacological tools to inhibit SR or DAAO (see Box 1). Studies aiming to explore the functions of D-serine in complex system (slices or behaving animals) through the pharmacological inhibition of SR offer a compelling case. Indeed, *in vitro* screenings have identified that L -erythro hydroxyaspartate (OHAsp), phenazine-ethosulfate (PES) and phenazine-methosulfate (PMS) act as relatively selective and low-potency inhibitors for SR[79,80]. Subsequently, investigators have used the agents for exploring the roles of D-serine in different physiological processes either in brain slices or *in situ* in living animals or some animal models of disease. Thus, it has been reported that OHAsp introduced in the astrocytes impaired LTP recorded in hippocampal slices although no rundown in glutamatergic responses was observed[52] or reduced NMDA-excitatory postsynaptic currents (EPSCs)-EPSCs recorded at layer 5 pyramidal neurons[81], effects that could be fully rescued by adding D-serine back to tissues. When OHAsp was bath applied, it increased locomotor-related activity recorded from the ventral roots of spinal cord preparations from neonatal mice[82], an effect opposed to the application of DAAO (commercial source). Using bath application, PES was found to reduce light-evoked NMDAR response in the retina[83], or the EPSCs and LTP in the visual cortex[84]. Application of PES and PMS to neonatal mouse cerebellum slices impedes neuronal migration[85] and also decreases respiratory frequency when applied to brainstem slices[56]. In all these studies, the inhibitory effects of the compounds were counteracted by application of D-serine and were opposed to the enzymatic degradation of D-serine by applied DAAO, just apparently warranting the use of the SR inhibitors. In one case[83], investigators also showed that PES decreased the extracellular levels of D-serine, a parameter that may be considered as at least the basic necessary control in pharmacological studies aiming at interfering with the metabolic enzyme of D-serine. Of course, these mentioned studies proposed a significant role of the NMDAR co-agonist despite that the actual real specificity of these compounds remains totally unknown since they were identified as prototype inhibitors of SR only from *in silico* and *in vitro* screening assays. However, a simple strategy to resolve this problem would have been to concomitantly test the compounds in $SR^{-/-}$ mice, thus used as negative controls. In an ongoing and yet unpublished work (Lecouflet, Potier, Dutar, Billard and Mothet, manuscript in preparation), we observe that all these compounds have unwanted off-target effects since they display similar dose-dependent inhibitory actions on NMDAR synaptic functions and LTP in both wild-type and $SR^{-/-}$ hippocampal slices whatever the mode of administration (bath applied or injected in a single astrocyte). Notably, application of exogenous D-serine partially rescues the inhibitory effect of the compounds in both slices from WT and $SR^{-/-}$ mice. Although we cannot exclude that different modes and times of application could cause to specific *versus* non-specific effects, our observations clearly suggest that the SR inhibitor compounds currently available cannot be used to determine the functions of D-serine in complex systems (i.e. brains slices, cells) other than cell-free preparations[36] unless controls are performed with $SR^{-/-}$ mice (Figure 2). Here again, we call for a more cautious assessment of recent findings obtained using OHAsp and PES even if measurements on D-serine levels were made. Clearly, there is a need for the development of more specific SR inhibitors[86,87]. The same caution should be applied to compounds inhibiting DAAO. We encourage investigators to test their efficacity in $DAAO^{-/-}$ mice before going further in their studies. These controls seem to us absolutely necessary since

inhibitors of DAAO are already used in pre-clinical studies to treat SCZ and affective disorders[88] or for the early-phase of AD[89].

Finally, another case of possible misinterpretation originates from the use of the iBot-Glast-CreERT2 and dnSNARE mouse lines[65], which were initially generated with the ambition to control SNARE-dependent exocytosis in astrocytes. The first model, the iBot-Glast-CreERT2 mouse line, enables the tamoxifen-inducible, stable expression of the *Clostridium botulinum* toxin serotype B-light chain (boNT/B). Once expressed, boNT/B cleaves the vesicle-associated membrane synaptobrevin-2 (Sb2), a component of the SNARE complex, which is expressed in astrocytes[43,52,57,61,62,65,90] thus impairing vesicular release. The second mouse model, the dnSNARE mouse line, expresses the dominant-negative SNARE domain of the Sb2 protein and GFP in astrocytes under the control of a tetoff tetracycline transactivator system controlled by doxycycline. These two models have been instrumental for studying gliotransmission and the role of glia in brain functions[65]. Exocytosis of gliotransmitters could be essential to normal brain function as well as in brain diseases but would require vesicles for transmitter storage, the presence of vesicular transporters for accumulating transmitters, and SNARE proteins for membrane fusion. Despite their elegancy, these tools are indirect and have limitations since they interfere with all VAMP3/Sb2-dependent fusion events including constitutive exocytosis which is important for the importation-exportation of proteins at the plasma membrane (i.e., receptors, transporters, exchangers…) and do not solely inhibit the release of gliotransmitters[57]. Inactivation of the exocytotic machinery in its whole, as achieved in the two mouse lines, most likely leads to off-target effects by rendering the astrocytes totally unable to detect or to adapt to any neuronal inputs. Therefore, astrocytes in these mice are “deaf”, i.e., unable to listen to neurons and in turn, unable to communicate with the latter, whatever the pathway being involved in this dialogue. As an example, cell surface expression and turnover of glycine transporters are regulated by calcium-dependent SNARE mechanisms and would most likely be altered in these transgenic mice[91]. Therefore, rescue seen with D-serine or any NMDAR ligands is a pure pharmacological effect with poor, if any, physiological significance on the identity of the molecule sustaining the glia-neuronal coupling. Interestingly, it has been reported that the D-serine levels measured with *Rg*DAAO-based amperometric biosensors in hippocampal slices were not affected by astrocytic dnSNARE expression regardless of time of day[43,92], an observation in conflict with a previous study[40] that reported a clear decline in the levels of D-serine in the hippocampus of the same transgenic mice. Further studies are needed to solve these discrepancies.

Conclusions

There are several important lessons to take away from the example of D-serine (and the other D-AA). This D-AA has emerged as an important signaling molecule with high hopes for more efficient therapies in multiple chronic brain disorders that affect our societies. As a consequence, any scientific publication has the potential to propagate the enthusiasm of the scientific community but also to attract the interest of the general media and the lay public by offering new hopes for treatments. This is a high level of responsibility for each scientist to assess and becomes harder when exploring a new signaling pathway outside of their expertise.

The past years have shown exciting progress in understanding D-serine biology and the coming decade is expected to continue this trend with other D-AA. Thanks to the rapid development of new animal models and the refinement of technical procedures, our knowledge about the roles of D-AAs, and particularly D-serine, in brain function has increased incredibly over the past years. Because it is crucial for science to progress as fast as possible, and since it appears likely that D-AAs are involved in a wide range of neurological and psychiatric human disorders, we encourage the use of appropriate procedures with multiple controls before delivering a message perhaps “vendeur” at first glance but that might direct the community towards dead ends.

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Abbreviations:

AD	Alzheimer’s disease
ASC-1	alanine-serine-cysteine-1
CE-LIF	capillary electrophoresis with laser-induced fluorescence
CNS	central nervous system
DAA	D-amino acid
DAAO	D-amino acid oxidase
DsdA	recombinant D-serine deaminase enzyme
EPSCs	excitatory postsynaptic currents
FAC	fluoroacetate
GlyT	glycine transporters
GMS	glycine modulatory site
HPLC	high pressure liquid chromatography
LC	liquid chromatography
LTD	long term depression
LTP	long term potentiation
NMDARs	NMethyl D-aspartate receptors
OHAsp	L-erythro hydroxyaspartate

PES	Phenazine-EthoSulfate
PM	Phenazine-Methosulfate
SCZ	schizophrenia
SR	serine racemase
TCA	tricarboxylic acid
3-PGDH	3-phosphoglycerate dehydrogenase

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Box 1 :

Best practices in exploring D-AA function

Characterization approaches

Detecting and quantifying one particular compound in brain or microdialysate samples require validated methodologies and technical training. The possibility of false positives becomes larger when measuring new categories of compounds, such as the D- from L-AAs in complex matrices. Matching the retention time of a peak in a chromatogram to a spiked standard is not sufficient to 'prove' the presence of the D-AA. Even with the much higher peak capacity of multidimensional (tandem) LC and CE, in the most convincing studies, the detection of the D-AA should be validated using secondary approaches such as enzyme degradation[37], stable or radioisotope formation experiments[38], removal of the peak *via* an antibody complexation[39], or other orthogonal approaches. This validation remains important when one considers the presence of literally thousands of metabolites within mammalian tissue and the large differences in levels of the L- and D-enantiomers, making peak confirmations important. Such orthogonal confirmations become even more important when unusual levels of D-AA are reported that do not follow expected levels[31]. Lastly, we implore authors to show chromatograms/ electropherograms for each D-AA detected or each brain region that was studied in the supplemental information; showing one or two example traces is not enough.

Accordingly, here we have outlined several best practice suggestions for D-serine: i) identify the D-serine peak *via* the retention time of standard D-serine; eliminate the corresponding peak by treating the sample with a purified recombinant-specific degrading enzyme such as DAAO or DsdA (as described in the text). These practices can be adapted to other D-AA studies. For some, such as D-alanine, the same degrading enzymes can be used, and for others, such as D-aspartate or D-glutamate, D-aspartate oxidase would be an appropriate degrading enzyme. Besides peak identification, from the change in the peak area before and after enzymatic treatment, the D-AA can be quantified even if the two peaks are not completely resolved. ii) Add greater information such as with mass spectrometry and/or additional dimensions of a separation (LC/LC or even LC3). iii) Verify the yield of D-serine recovery from the starting sample: introduce a known amount of D-serine (or even better, an isotope of D-serine, and differentiate the endogenous and labeled D-serine *via* MS) in the starting sample and verify the recovery.

Amperometric- or photonic- derived DAAO based biosensor analysis of D-serine should be used in combination with a more selective assay, such as HPLC or CE separations or measurements made in SR knock-out mice as controls. Other controls should also include the absence of signal originating from the non specific oxidation of any drug (ie agonist, antagonists, inhibitors) used in the experiments.

Antibody-based methods to detect D-serine and its enzymes are robust techniques that do not require expensive equipment but rely on the highly specific and sensitive reaction of the antibody with its target, and still require several precautions. Regardless of the company from which the D-serine antibody is purchased, the use of the antibody requires that: i) the paraformaldehyde-based fixative should contain glutaraldehyde to obtain

immunoreactivity in tissue/cell preparations; ii) incubation with the primary antibody should include inclusion of sufficiently high concentrations of L-serine glutaraldehyde conjugate to prevent cross-reaction of the antibody with endogenous L-serine present in the tissue/cell preparations (notably, astrocytes contain quite high concentrations of L-serine as they are the primary site of its synthesis in brain); iii) specific controls are used to pre-absorb the anti-serine antibody with D-serine glutaraldehyde conjugate in order to abolish the immunoreactivity in the biological samples[46]; and when possible, iv) use the same tissue area from SR knock-out mice, which have < 10% of wild-type levels of D-serine, treated in the same manner as the experimental tissue[17,20–23]. The specificity of the antibody for SR or DAAO should be also validated by western blot or immunostaining on tissues from $SR^{-/-}$ and $DAAO^{-/-}$ mice, respectively, since most if not all commercial antibodies are not specific for their claimed target.

Pharmacological rescue experiments

Here, we refer to experiments where a specific signaling pathway has been first pharmacologically or genetically invalidated and then D-serine is added to rescue physiological or behavioral outcomes. In many cases the pharmacological or genetical interventions are not specific to D-serine and could be rescued not only by D-serine but also by any agonist acting at the glycine site of NMDAR (i.e. glycine, D-alanine) but also by L-serine. Therefore, it is mandatory to: 1) show that the defective pathway (FAC, IP3R, and glial SNARE as examples) specifically affects the levels of D-serine over other amino acids (L-serine, glutamate, glycine) by using accurate separation and detection methods (see above), and then 2) perform rescue experiments with any agonist of the glycine site of NMDARs or L-serine.

Pharmacological inhibition of D-serine function and metabolic pathway

A first issue comes from the use of commercial preparations for DAAO (especially the enzyme from pig kidney) when probing the function of D-serine in physiological experiments. Indeed, these preparations show low activity (frequently because of inactive apoprotein production following enzyme dilution), are not pure, and thus could display off-target effects due to contamination by other enzymes like D-aspartate oxidase, which degrades glutamate, and NMDA or D-aspartic acid, which also activate and regulate NMDARs (see supplementary Fig. 1 in[74]). If commercial sources for DAAO have to be used (even when a home-made recombinant enzyme is used, i.e. *Rg*DAAO), it is necessary to carry out control experiments in parallel, measuring the impact of enzymatic treatment on the levels of amino acids other than D-serine and especially on L-serine, as done in several reports, or to use $SR^{-/-}$ mice to ascertain the specificity of the enzyme. Application of DAAO on preparations obtained from $SR^{-/-}$ mice should have no additional inhibitory effects on the responses investigators are measuring.

A second source of false positives comes from the use of pharmacological tools to inhibit SR or DAAO. The actual real specificity of these compounds remains totally unknown and could display unwanted off-target effects as illustrated for OHAsp, PES and PM, which have been largely used to inhibit SR[52,56,81–85]. Therefore, the specificity of a

putative inhibitor has to be first validated in $SR^{-/-}$ or $DAAO^{-/-}$ mice, depending on the target of the compound.

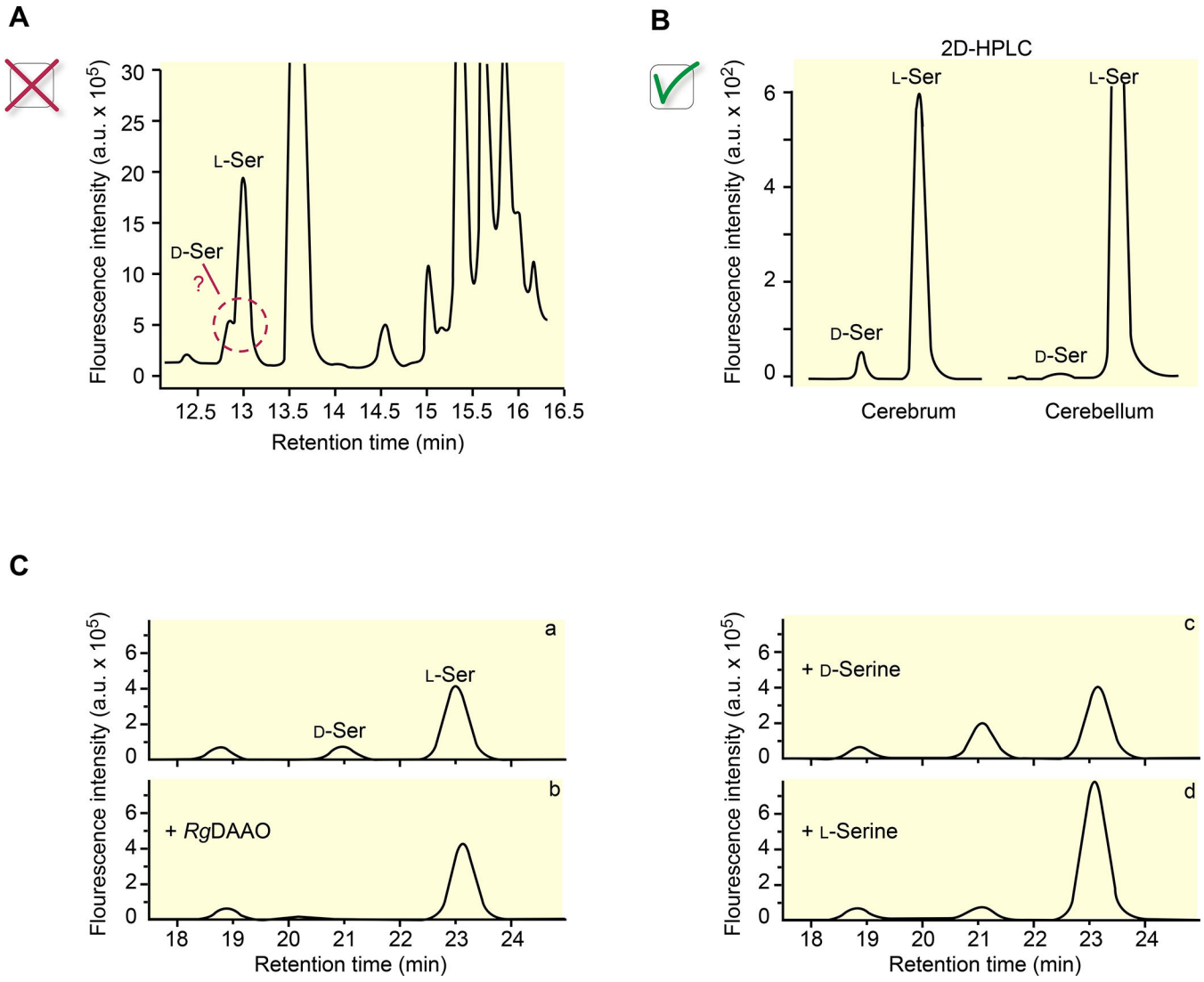

Figure 1: The bad and good practices in the detection of D-serine in biological samples.

The good checklist when detecting and measuring selected amino acids includes: a) well-resolved peaks for each amino acid (as illustrated in panels B & C, trace a). Traces displayed in B are from[30] (modified with permission). A shoulder on a peak as illustrated in panel A from[43] (modified with permission) is the sign of poorly resolved peaks, not allowing a conclusion. If such a shoulder is present, the analytical procedure showed be further optimized. b) Peak identification requires careful controls: spiking with the exogenous amino acid (panel C, traces c, d) and removing the peak with recombinant enzymes that specifically degrade the D-AA of interest (as illustrated in panel C, trace b).

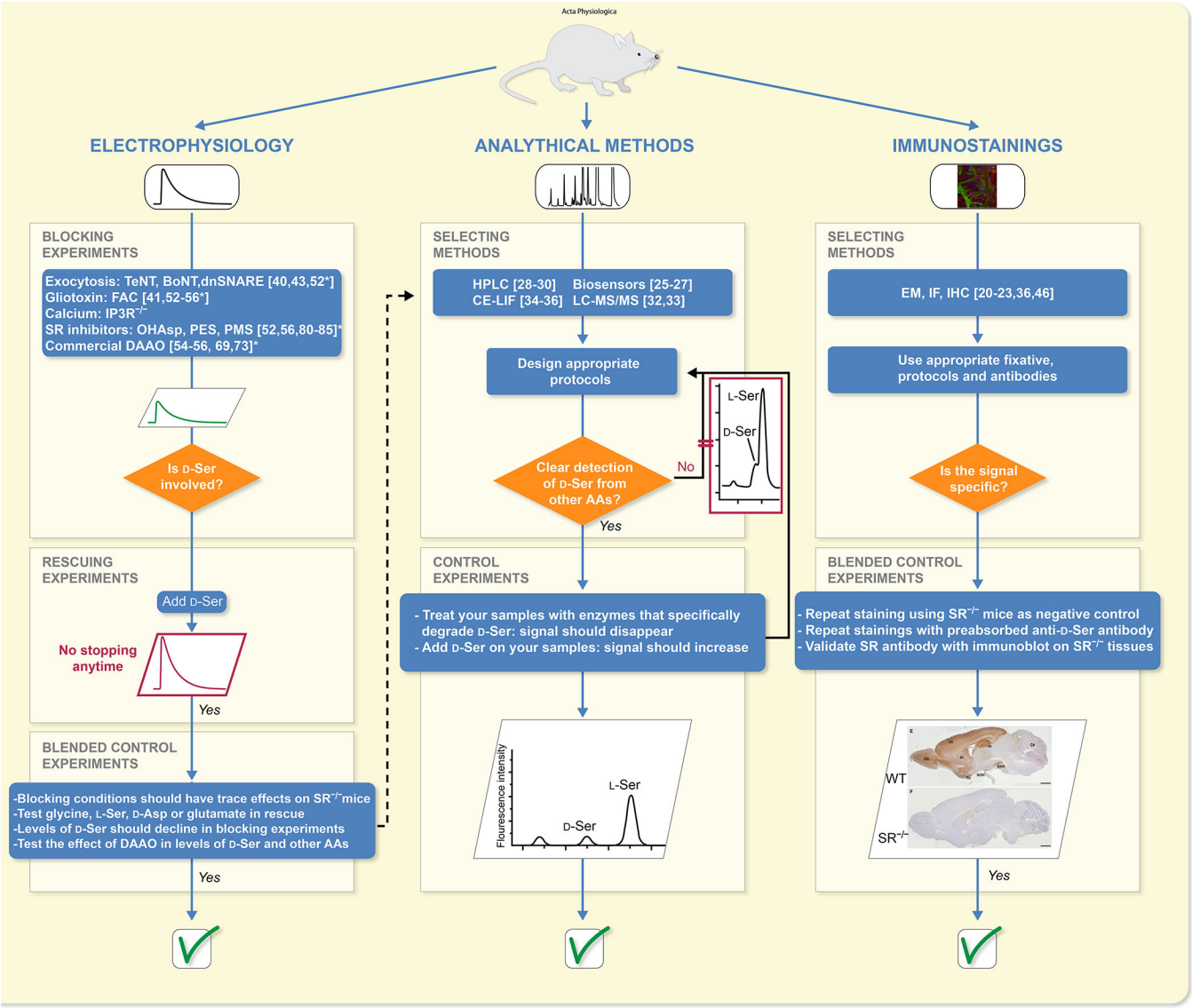

Figure 2: Chart for correct experimental strategy in exploring the function of D-serine.

Three main types of experimental methodologies are used to investigate the function of D-serine. In electrophysiological recordings, investigators use genetically or pharmacologically driven loss/gain-of functions of different pathways and perform rescue experiments. *References here are studies where problems arose in using at least one approach. Several well-described analytical methods can be used to detect and measure D-serine but all require careful management and optimization of the procedures in order to avoid confounding results, as displayed in the chromatogram extracted from[43]. Finally, immunodetection of D-serine or its metabolizing enzymes can be performed following different procedures (electron microscopy: EM; immunofluorescence: IF, and immunohistochemistry, IHC) but necessary controls should be run in order to ascertain the specificity of the signal as illustrated (modified with permission from[21]).