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A loss of hippocampal perineuronal nets produces deficits in dopamine system function: relevance to the positive symptoms of schizophrenia

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Deficits in parvalbumin containing interneurons are a consistent observation in animal models and schizophrenia patients. These neurons are surrounded by chondroitin sulfate proteoglycans, forming perineuronal nets, thought to support the high firing frequencies observed in these neurons. A loss of perineuronal nets has been observed post mortem in human schizophrenia patients, however, whether this contributes to the symptoms of schizophrenia is not known. Here we directly examine the effects of chondroitinase ABC degradation of ventral hippocampal (vHipp) perineuronal nets, and demonstrate that this results in an enhanced hippocampal activity and significant increase in dopamine neuron population activity. In addition, chondroitinase-treated rats display an augmented locomotor response to amphetamine, consistent with the enhanced response to psychomotor stimulants observed in schizophrenia patients. Taken together, these data demonstrate that a loss of vHipp perineuronal nets is sufficient, in and of itself, to induce aberrant hippocampal and dopamine system function consistent with that observed in rodent models and schizophrenia patients.

Introduction

Schizophrenia is a disease affecting up to 1% of the population.[1] Whereas the exact etiology of schizophrenia is not currently known, an alteration in GABAergic function is a consistent observation in both animal models as well as post-mortem studies in schizophrenia patients.[2–8] Specifically, post-mortem studies have demonstrated a reduction in cortical glutamic acid decarboxylase;[9–11] an upregulation of post-synaptic GABA-A receptors[12,13] and a decrease in GABA uptake sites[14–16] in schizophrenia patients. There is increasing evidence that deficits in GABAergic transmission are demonstrated to occur within specific classes of GABAergic interneurons, with considerable attention being paid to those containing the calcium binding protein parvalbumin.[3] Indeed, a decrease in parvalbumin expression has been reported in a diverse variety of animal models, including the methylazoxymethanol (MAM),[5,17] neonatal ventral hippocampal lesion[6] and phencyclidine models,[7,8,18] and has been consistently demonstrated in schizophrenia patients, post mortem.[10,19,20] Although not conclusively demonstrated, this decrease in parvalbumin expression is likely attributable to, or results in, aberrant interneuron function leading to attenuated perisomatic inhibition and an augmented pyramidal neuron activity. Indeed, we have previously demonstrated that MAM-treated rats (for review see Lodge *et al.*[21]) display a decrease in hippocampal parvalbumin positive neurons, deficits in evoked gamma oscillatory activity and an increase in hippocampal neuronal activity.[5,22] This is consistent with

imaging studies in schizophrenia patients that demonstrate increases in baseline hippocampal activity that are correlated with positive symptom severity.[23–25]

An increase in hippocampal activity, particularly in ventral subfields, is able to augment dopamine system function, known to be associated with the positive symptoms of schizophrenia. Specifically, activation of the vHipp has been demonstrated to increase the number of spontaneously active dopamine neurons in the ventral tegmental area (VTA)[26–28] and this is correlated with increases in dopamine efflux in the nucleus accumbens.[27,29,30] Furthermore, we have previously demonstrated that the pathological increase in dopamine system function, observed in the MAM-rodent model of schizophrenia, is directly attributable to aberrant ventral hippocampal activity.[22] Thus, MAM-treated rats display an increase in dopamine neuron population activity and an augmented locomotor response to amphetamine administration, both of which can be normalized by tetrodotoxin inactivation of the vHipp.[22] This has led us to posit that the positive symptoms of schizophrenia, known to be associated with augmented dopamine transmission, may be secondary to a pathological increase in ventral hippocampal activity.[31] Furthermore, this increase in hippocampal activity may be attributable to a decreased function of perisomatic targeting, parvalbumin positive interneurons.

Parvalbumin containing interneurons in the cortex and hippocampus are encapsulated by chondroitin sulfate proteoglycans forming an extracellular matrix known as a perineuronal net (Figure 1). Although perineuronal nets were
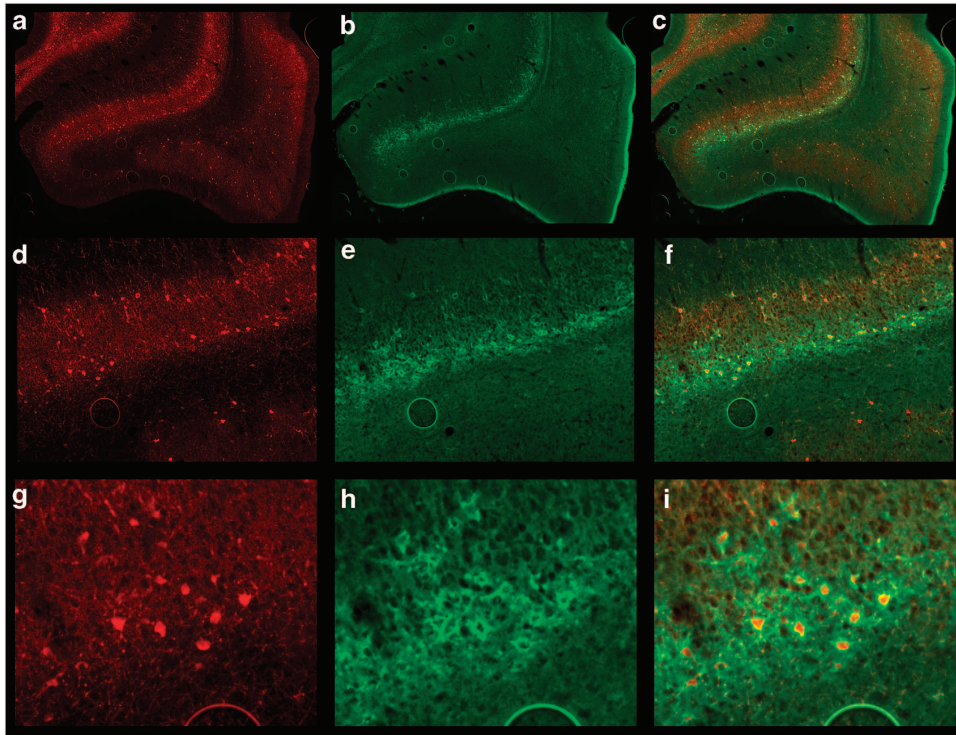
Figure 1 Perineuronal nets surround parvalbumin positive interneurons throughout the ventral hippocampus. Photomicrographs at increasing levels of magnification (from top to bottom) demonstrate the distribution of parvalbumin positive interneurons (red) and perineuronal nets (green) throughout the ventral regions of the hippocampus. The merged images (**c**, **f** and **i**) demonstrate that a significant number of parvalbumin-containing neurons are enveloped by perineuronal nets, as determined by lectin labeling.

described by Golgi over a century ago, relatively little is known about their function.[32] Recent work has suggested that perineuronal nets act to stabilize synapses,[33] support synaptic plasticity[34,35] and regulate the motility of extracellular ions.[36,37] Given that perineuronal nets appear to envelop Kv3.1b expressing neurons,[37] it has been suggested that these structures likely support the high firing frequencies of fast-spiking interneurons. Interestingly, a loss of perineuronal nets has been recently reported throughout the medial temporal lobe of postmortem schizophrenia patients.[38,39] We suggest that a loss of perineuronal nets may contribute to the aberrant hippocampal activity and subsequent dopamine hyperfunction associated with the positive symptoms of schizophrenia. Here we examine whether a loss of vHipp perineuronal nets is sufficient, in and of itself, to produce physiological and behavioral deficits associated with the positive symptoms of schizophrenia.

Materials and methods

All experiments were performed in accordance with the guidelines outlined in the USPHS Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center, San Antonio.

Animals. MAM acetate treatments were performed as described previously.[40] In brief, timed pregnant female Sprague–Dawley rats were administered MAM (25 mg kg^{-1}, i.p.) on GD17. Control rats received injections of saline (1 ml kg^{-1}, i.p.). Male pups were weaned on day 21 and housed in groups of 2–3 with littermates until adulthood (>8 weeks), at which time they were used for neurochemical studies.

Western blot. Adult MAM and saline-treated rats ($n = 5$ per group: 300–450 g) were anaesthetized with sodium pento-barbital (120 mg kg^{-1}, i.p.) and rapidly decapitated. The ventral hippocampus was dissected on ice and homogenized in disruption buffer (50 mM Tris, 150 mM NaCl, pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA: P8340). The perineuronal net fraction was isolated essentially as described previously.[41] Specifically, homogenates were centrifuged at 14 000 r.p.m. for 10 min and the pellet resuspended in disruption buffer containing 0.5% Triton X-100. The homogenate was centrifuged and the pellet resuspended in 200 μl incubation buffer (0.1 M Tris, 0.03 M sodium acetate, pH 8.0) containing 0.1 U of chon-droitinase ABC and incubated at 37 °C for 2.5 h. The homogenate was centrifuged and the supernatant analyzed as a purified perineuronal net fraction. Given that the initial washes are unlikely to release chondroitin sulfate proteogly-cans from perineuronal nets,[41] the proteins examined following chondroitinase ABC digestion should be those associated with perineuronal nets; indeed we obtained dramatically different results when analyzing chondroitin sulfate proteoglycans from the cytosolic fractions (data not shown).

Proteins were separated on a 4–20% gradient gel (Bio-Rad, Hercules, CA, USA) and transferred to polyvinyl difluoride

membranes. Membranes were blocked and incubated with either mouse antineurocan 1:100 (DSHB, Iowa City, IA, USA: 1F6-s), mouse antiphosphacan 1:100 (DSHB: 3F8-s), mouse antibrevican 1:1000 (BD Biosystems, San Jose, CA, USA: 610894) or mouse antiGAPDH 1:1000 (Abcam, Cambridge, MA, USA: ab9484), followed by HRP-antimouse 1:5000. Membranes were treated with an ECL substrate (Pierce, Rockford, IL, USA) and opposed to film prior to scanning and quantification with ImageJ.

Chondroitinase treatment. All survival surgical procedures were performed under general anesthesia in a semi-sterile environment. Briefly, male Sprague–Dawley rats (250–350 g) were pretreated with atropine (0.1 mg kg^{-1}, i.p.), anesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and placed in a stereotaxic apparatus. Chondroitinase ABC from *Proteus vulgaris* (0.05 U μl^{-1}) or penicillinase from *Bacillus cereus* (0.05 U μl^{-1}) were backfilled into a glass micropipette and pressure injected (0.75 μl) bilaterally into the ventral hippocampus (A/P − 5.3, M/L ± 5.2, D/V − 7.5 mm). The pipette was removed, the wound sutured, the rat removed from the stereotaxic frame and monitored closely until conscious. Rats were housed for at least a week before subsequent experiments.

Extracellular recordings. Extracellular recordings were performed as described previously.[22] In brief, rats (300–450 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and placed in a stereotaxic apparatus. Glass extracellular microelectrodes were lowered into the VTA ($n = 14$–16 rats per group: A/P − 5.3, M/L + 0.6 mm from bregma and from − 6.5 to − 9.0 mm ventral of brain surface) or ventral hippocampus ($n = 7$ rats per group: A/P − 5.3, M/L + 5.2 mm from bregma and from − 5.0 to − 8.5 mm ventral of brain surface). Spontaneously active dopamine neurons were identified with open filter settings (low pass: 30 Hz, high pass: 30 kHz) using previously established electrophysiological criteria,[42] whereas putative pyramidal neurons were defined as those with firing frequencies < 2 Hz as reported previously.[43,44]

Locomotor analysis. Chondroitinase- or Penicillinase-treated rats ($n = 16$–18 per group: 300–450 g) were placed in an open field arena (Med Associates, St Albans, VT, USA), where spontaneous locomotor activity in the X–Y plane was determined for 45 min by beam breaks and recorded with Open Field Activity Software (Med Associates). Rats were then injected with amphetamine (0.5 mg kg^{-1}, i.p.) and locomotor activity recorded for an additional 90 min.

Histochemistry. Anesthetized rats were perfused transcardially with saline followed by formaldehyde and their brains removed, post fixed/cryopreserved for 24 h. Coronal sections of the ventral hippocampus were used to detect expression of perineurnal nets. A subset of brains were also processed for parvalbumin immunohistochemistry. Briefly, sections were blocked (2% normal goat serum for 30 min) followed by incubation with lectin from *Wisteria floribunda* (4 μg ml^{-1}) and visualization by AlexaFluor 488 conjugated to streptavidin (1:500 for 1 h at room temperature). A subset of sections

were also incubated with rabbit antiparvalbumin 1:1000 (Abcam: AB11427)) followed by antirabbit-AlexaFluor 594 (1:1000 for 1 h at room temperature). Sections of vHipp from each rat were visualized to confirm the loss of perineuronal nets in chondroitinase ABC-treated rats.

Analysis. Electrophysiological analysis of dopamine and vHipp neuron activity was performed using commercially available computer software (LabChart v7.1). Locomotor activity was analyzed with Activity Monitor (Med Associates), whereas western blots films were scanned on an Epson Perfection V700 and optical density measured using ImageJ. Immunohistochemistry was examined on an Axio Lab.A1 Fluorescence Microscope and images taken with an attached AxioCam ICc1 digital camera. All data are represented as the mean ± standard error of the mean (s.e.m.) unless otherwise stated. All statistics were calculated using SigmaPlot (Systat Software, Chicago, IL, USA).

Materials. MAM acetate was purchased from Midwest Research Institute (Kansas City, MO, USA). The monocloncal antibodies antineurocan (1F6-s) and antiphosphacan (3F8-s), developed by RU Margolis and RK Margolis, were obtained from the Developmental Studies Hybridoma Bank. The anti-brevican antibody (610894) was purchased from BD Biosciences, whereas the antiparvalbumin (ab11427) and antiGAPDH (ab9484) antibodies were purchased from ABCAM. The HRP-anti-mouse, AlexaFluor 596-conjugated anti-rabbit, AlexaFluor 488-conjugated to streptavidin and ProLong Gold antifade mountant were obtained from Invitrogen (Grand Island, NY, USA). The Laemmli Sample Buffer, Mini-Protean gels and polyvinyl difluoride membranes were from Bio-rad. Sodium pentobarbital, atropine, protease inhibitor cocktail (P8340), amphetamine, chondroitinase ABC from *Proteus vulgaris*, Penicillinase from *Bacillus cereus* and lectin from *Wisteria floribunda* were purchased from Sigma. All other chemicals and reagents were of either analytical or laboratory grade, and purchased from standard suppliers.

Results

Western blot. A loss of perineuronal nets has been described, post mortem, in schizophrenia patients.[38,39] To examine whether this is also observed in rodent models of the disease, we examined the abundance of perineuronal net proteins throughout the vHipp of the MAM model of schizophrenia. MAM-treated rats demonstrated a significant decrease in the levels of the vHipp perineuronal net chondroitin sulfate proteoglycans, Brevican (SAL: 2.857 ± 0.662 c.f. MAM: 0.833 ± 0.104; $P < 0.05$ Two-way ANOVA, Holm–Sidak $t = 3.989$, $n = 5$ rats per group in duplicate) and Phosphacan (SAL: 2.020 ± 0.518 c.f. MAM: 0.670 ± 0.084; $P < 0.05$ Two-way ANOVA, Holm–Sidak $t = 2.661$, $n = 5$ rats per group in duplicate), but not Neurocan (SAL: 1.102 ± 0.195 c.f. MAM: 0.665 ± 0.101; $P > 0.05$), when compared to control, saline-treated rats (Figure 2).

***In vivo* electrophysiology.** To confirm that a decrease in perineuronal nets results in aberrant vHipp activity, as

reported in the MAM model, we performed *in vivo* recordings from putative vHipp pyramidal neurons. Specifically, chondroitinase-treated rats displayed a significantly greater average firing rate of putative pyramidal neurons when compared to rats receiving intra-vHipp penicillinase (Penn: 0.74 ± 0.09 Hz c.f. Chase: 0.99 ± 0.07 Hz; $P < 0.05$ Rank Sum Test, $n = 41$–63 cells per group). These data suggest that a loss of perineuronal nets is sufficient to augment
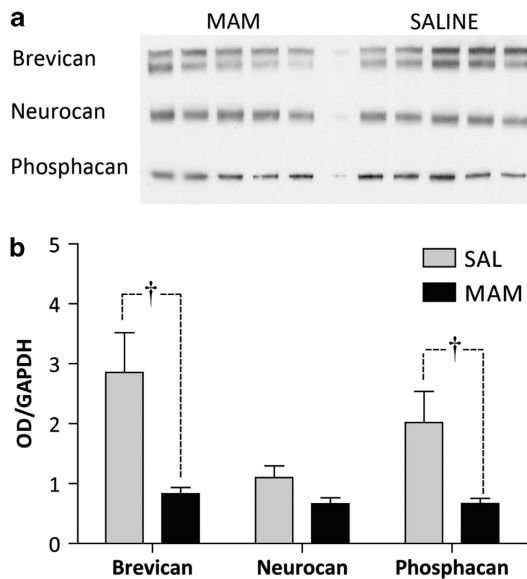

Figure 2 MAM-treated rats display decreases in the levels of the chondroitin sulfate proteoglycans associated with vHipp perineuronal nets. Representative films demonstrating the abundance of three chondroitin sulfate proteoglycans, brevican, neurocan and phosphacan are depicted in (**a**), whereas the quantitation of these data are presented in (**b**). †represents significant difference from saline-treated rats, Two-way ANOVA followed by Holm–Sidak *post-hoc*, $n = 5$ rats per group in duplicate.
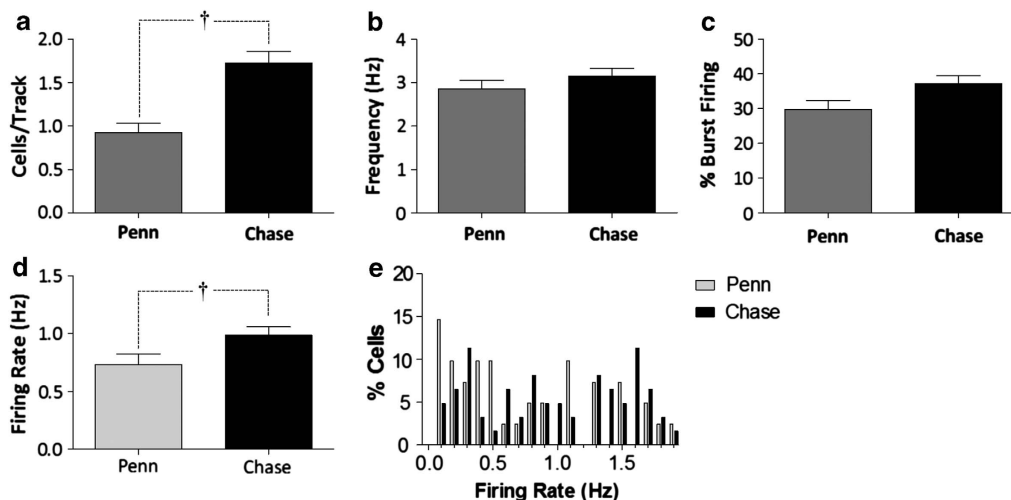
hippocampal activity, presumably due to a decrease in fast-spiking interneuron function.

Given that an augmented vHipp activity has been demonstrated to increase dopamine system function,[22,28,45] we recorded the activity of the population of spontaneously active dopamine neurons throughout the VTA of chondroitinase- and penicillinase-treated rats. Interestingly, the chondroitinase-induced loss of hippocampal perineuronal nets resulted in a significant increase in the number of spontaneously activity dopamine neurons observed per electrode track (Penn: 0.92 ± 0.11 cells per track c.f. Chase: 1.73 ± 0.13 cells per track; $P < 0.05$ Students *t*-test $t = -4.739$ $n = 14$–16 rats per group) without significantly altering average firing rate or burst firing (Figure 3), similar to previous observations in the MAM model.[22]

Amphetamine-induced hyperlocomotion. We have previously demonstrated that increases in dopamine neuron activity, secondary to augmented vHipp activity, result in a behavioral hyperactivity to low doses of psychomotor stimulants.[22,45] Here we demonstrate that rats treated with intra-vHipp chondroitinase ABC display a significantly increased locomotor response to amphetamine when compared to penicillinase-treated rats (Two-way ANOVA; $F_{(Treatment)} = 40.845$: $n = 16$–18 rats per group; Figure 4), demonstrating that a loss of hippocampal perineuronal nets results in behavioral alterations consistent with those observed in rodent models as well as in schizophrenia patients.[46,47]

Discussion

Here we demonstrate that a loss of ventral hippocampal perineuronal nets is sufficient to augment hippocampal activity and increase dopamine system function. Moreover, this is correlated with an enhanced behavioral response to

Figure 3 Enzymatic degradation of vHipp perineuronal nets augments dopamine and hippocampal activity. vHipp chondroitinase ABC treatment resulted in a selective increase in dopamine neuron population activity (**a**), with no significant effect on average firing rate (**b**), or burst firing (**c**) when compared to control, penicillinase-treated, rats. The degradation of perineuronal nets resulted in an increase in the average firing rate of purported pyramidal neurons throughout the vHipp (**d** and **e**). †represents significant difference from penicillinase-treated rats, Students *t*-test (Rank Sum Test if data failed normality test), $n = 14$–16 rats per group for A, $n = 88$–125 cells per group for **b** and **c** and $n = 41$–63 cells per group for (**d** and **e**).
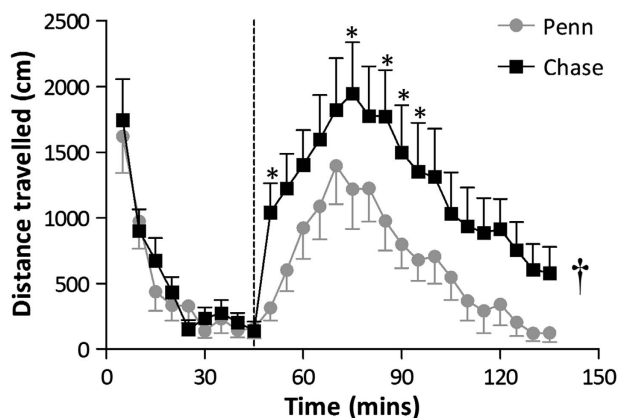
Figure 4 Enzymatic degradation of vHipp perineuronal nets results in an enhanced locomotor response to amphetamine. vHipp chondroitinase ABC treatment did not appear to alter spontaneous locomotor activity in a novel environment; however, a significant increase in the locomotor response to amphetamine (administered at the dashed line) was observed in rats with perineuronal net degradation. [†]represents a mean effect of treatment, whereas *indicates significant difference between chondroitinase ABC and penicillinase in pairwise multiple comparisons across time; Two-way ANOVA, followed by Holm–Sidak post-hoc, $n = 16$–18 rats per group).

amphetamine, a marker of positive symptoms of schizophrenia.[46,47] Given that a loss of perineuronal nets has been reported throughout the medial temporal lobe of post-mortem schizophrenia patients,[38] we suggest that alterations in extracellular matrix proteins may have a significant role in the pathophysiology of schizophrenia.

As detailed above, a decreased expression of perineuronal nets has been reported throughout the temporal lobe[38] of post-mortem schizophrenia patients. Here we demonstrate that the MAM-rodent model demonstrates significantly lower levels of the perineuronal net proteins, brevican and phosphacan, throughout the ventral hippocampus. It should be noted that MAM-treated rats also display reductions in parvalbumin-containing interneurons;[5] thus, whether the decreased expression of perineuronal net proteins reflects a loss of parvalbumin neurons, or vice versa, is not currently known. Interestingly, these observed alterations in phosphacan expression are consistent with genetic association studies demonstrating that PTPRZ1, the gene encoding phosphacan, may influence the susceptibility for schizophrenia.[48] Thus, the decreased expression of phosphacan further supports the validity of the MAM model, as it appears to mimic phosphacan abnormalities in schizophrenia patients, albeit via a different mechanism.

Given that we have previously demonstrated that MAM-treated rats display augmented vHipp activity that underlies the dopamine hyperfunction and behavioral hyperresponsivity to psychomotor stimulants,[22] we investigated whether a decrease in vHipp perineuronal nets was sufficient to induce aberrant hippocampal and dopamine system function, thought to underlie the positive symptoms of the schizophrenia. Perineuronal nets throughout the vHipp were enzymatically degraded, in a region specific manner, by the administration of chondroitinase ABC from Proteus vulgaris. Chondroitinase ABC degrades glycosaminoglycans, including chondroitin sulfate and hyaluronan, disrupting the

structure of the perineuronal net.[49] Indeed, qualitative examination of the vHipp following chondroitinase ABC demonstrated a striking decrease in lectin staining to perineuronal nets (Figure 5). In contrast, the administration of penicillinase, from Bacillus cereus, did not appear to alter the distribution of perineuronal nets (Figure 5), and was therefore used as a control.

It has been suggested that perineuronal nets support the high firing frequencies of fast-spiking, parvalbumin-containing interneurons. Given that these are perisomatic targeting interneurons, a loss of function would be anticipated to increase pyramidal activity. Indeed, here we demonstrate a significantly greater firing rate of putative pyramidal neurons throughout the ventral hippocampus of chondroitinase-treated rats, when compared to penicillinase-treated controls. Interestingly, this increase in hippocampal function is qualitatively similar to that observed in the MAM model,[22] and is consistent with human imaging data demonstrating augmented hippocampal activity in schizophrenia patients.[23–25] It should be noted that hippocampal cultures treated in vitro with chondroitinase ABC do not display altered electrophysiological parameters including resistance, capacitance, resting membrane potential, nor were the amplitude or half width of action potentials of interneurons altered.[50] Thus, the results observed in the current study likely reflect alterations in interneuron function that are either a consequence of the treatment regimen (that is, one injection with animals tested >1 week later c.f. 48 h of direct exposure in vitro) or, alternatively, may require the normal patterns of afferent input observed in the intact brain. Indeed, the genetic deletion of tenascin, an integral component of the perineuronal net, results in a decrease in perisomatic inhibition and enhanced excitatory synaptic transmission of pyramidal neurons of the hippocampus,[51] consistent with the data presented here.

We have previously demonstrated that the hippocampus can regulate dopamine system function via a multi-synaptic pathway including the nucleus accumbens and ventral pallidum (for review see Lodge et al.[31]). Moreover, we posit that the aberrant vHipp activity may actually be a primary pathology underlying the positive symptoms of schizophrenia. For this reason, we examined whether the chondroitinase-induced increase in hippocampal activity was sufficient to augment dopamine neuron activity in the VTA. Indeed, chondroitinase-treated rats displayed a significantly greater number of spontaneously active VTA dopamine neurons when compared to penicillinase-treated controls. The number of dopamine neurons firing spontaneously is thought to reflect the gain of the dopamine system whereby the functionally relevant, phasic signal can be amplified or dampened based on environmental context and novelty.[28] Thus, a sustained increase in the number of spontaneously active dopamine neurons would artificially ascribe high salience to events that are not typically salient, and may contribute to the positive symptoms of schizophrenia.[31] An increase in dopamine neuron population activity has been previously suggested to underlie the augmented response to psychomotor stimulants observed in animal models,[22,40,52] as well as schizophrenia patients.[46,47] This can be examined in rodent models by investigating the locomotor response to a low dose of amphetamine.[22,45,53] Indeed, we now demonstrate that
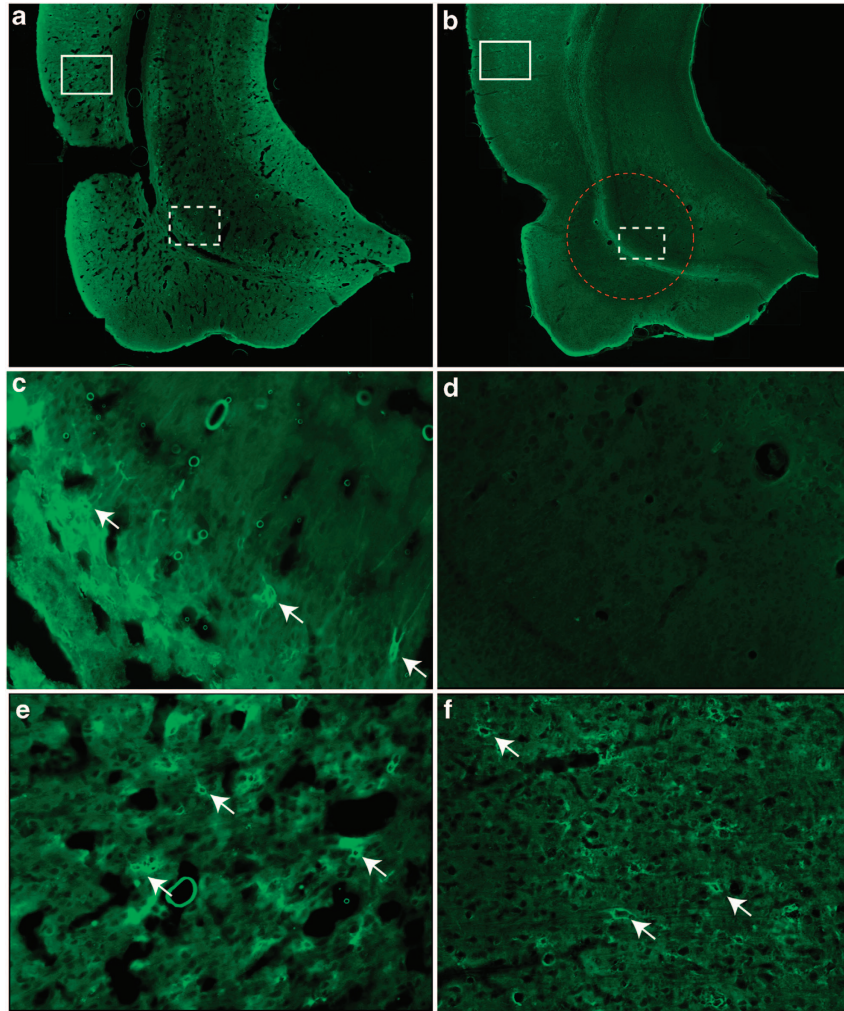
Figure 5 Intra-vHipp administration of chondroitinase ABC (**b,d** and **f**), but not penicillinase (**a,c** and **e**), results in a significant decrease in lectin staining to perineuronal nets throughout the vHipp. Photomicrographs demonstrate the extent of perineuronal net degradation, indicated by the red circle in (**b**). Chondroitinase ABC resulted in a region specific reduction of perineuronal nets observed in the vHipp (dashed box in **a** and **b**—magnified in **c** and **d**) but not in more dorsal regions of the cortex (solid box in **a** and **b**—magnified in **e** and **f**). Penicillinase-treated rats display numerous perineuronal nets (indicated by arrows) throughout both the vHipp (**c**) and cortex (**e**).

chondroitinase-treated rats display a significantly greater locomotor response to amphetamine administration when compared to penicillinase-treated rats. Taken together, these data demonstrate that a loss of hippocampal perineuronal nets is sufficient, in and of itself, to augment hippocampal activity and induce the dopamine system hyperfunction purported to underlie the positive symptoms of schizophrenia.

Conflict of Interest

The authors declare no conflict of interest.

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