Degradation of extracellular chondroitin sulfate delays recovery of network activity after perturbation

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Running title

CSPGs and neuronal homeostasis

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Abstract

Chondroitin-sulfate proteoglycans (CSPGs) are widely studied in vertebrate systems and are known to play a key role in development, plasticity and regulation of cortical circuitry. The mechanistic details of this role are still elusive, but increasingly central to the investigation is the homeostatic balance between network excitation and inhibition. Studying a simpler neuronal
circuit may prove advantageous for discovering the mechanistic details of the cellular effects of CSPGs. In this study we use a well-established model of homeostatic change after injury in the crab *Cancer borealis* to show first evidence that CSPGs are necessary for network activity homeostasis. We degraded CSPGs in the pyloric circuit of the stomatogastric ganglion with the enzyme chondroitinase ABC (chABC) and found that removal of CSPGs does not influence the ongoing rhythm of the pyloric circuit, but it does limit its capacity for recovery after a network-wide perturbation. Without CSPGs, the post-perturbation rhythm is slower than controls, and rhythm recovery is delayed. In addition to providing a new model system for the study of CSPGs, this study suggests a wider role for CSPGs, and perhaps the extracellular matrix in general, beyond simply plastic reorganization (as observed in mammals) and into a foundational regulatory role of neural circuitry.

**Introduction**

Homeostatic regulation of neuronal activity is integral to neural function. Without it, neurons would be in danger of drifting toward silence or death via excitotoxicity. However, it is not known how a cell or network defines appropriate activity and regulates accordingly over the long term. Recently, the extracellular neural environment has been recognized as well placed to direct long-term cell or network-level neuronal homeostasis. One family of extracellular matrix (ECM) molecules in particular, chondroitin sulfate proteoglycans (CSPGs), have properties which are ideal for homeostatic feedback (Galtrey and Fawcett, 2007). CSPGs are produced locally by neurons and glia (Giamanco and Matthews, 2012), with dependence on cellular activity patterns (Lander et al., 1997) via Ca$^{2+}$ influx (Dityatev et al., 2007). Structural differences within the CSPG family are cell-type specific (Lander et al., 1997) and result in diverse functional roles, such as alternately preventing or encouraging neuronal structural
plasticity and pathfinding (Snow et al., 1994; Fongmoon et al., 2007; Balmer et al., 2009; Gogolla et al., 2009; Kwok et al., 2012), binding and presentation of growth factors (Galtrey and Fawcett, 2007) and modulation of ion channel properties (Snow et al., 1994; Dityatev et al., 2007; Vigetti et al., 2008; Vargas and De-Miguel, 2009; Maroto et al., 2013). The type-specificity, activity-dependence and diverse functional roles of CSPGs together suggest a way by which changes in activity output can feedback onto regulatory mechanisms – the hallmark of a homeostatic process. However, all components of this role have not been verified in one system, possibly due to technical challenges in mammalian and culture-based models. Here, we introduce the use of invertebrate systems to study CSPGs and their role in network-level neuronal activity maintenance.

The stomatogastric ganglion (STG) of decapod crustaceans, such as lobsters and crabs, is a model system for studying activity homeostasis, at cellular and circuit levels. The pyloric circuit within the STG controls the rhythmic contractions of the pylorus. The component neurons, connectivity, and activity of the pyloric circuit are known; it is comprised of ~11 neurons which generate a triphasic periodic rhythm. The STG is part of the stomatogastric nervous system (STNS) which includes connections to three other ganglia and the brain (Fig. 1A), but the STG is easily isolated from all projection neurons and neuromodulatory input via a nerve called the stomatogastric nerve (stn). If conduction along the stn is blocked or the stn is cut, known as deafferentation, the pyloric rhythm slows or stops within minutes (Thoby-Brisson and Simmers, 1998) (Fig. 1B). Over days the activity pattern is spontaneously restored in the absence of inputs through the stn, and this recovery after deafferentation has been a premier model of neuronal activity homeostasis.
The current understanding of network activity recovery after deafferentation in the STG provides a description of cellular and synaptic changes, but does not fully address what is driving these changes. After the *sin* is cut, it can no longer transport neuromodulators (NM) from anterior ganglia. This removes at least one NM-dependent current (IMt) from several cell types in the STG, altering their activity (Golowasch and Marder, 1992). Changes in intrinsic K+ and Ca2+ currents and synaptic efficacy occur after deafferentation to compensate for this loss and restore pyloric activity (Thoby-Brisson and Simmers, 2002; Haedo and Golowasch, 2006; Khorkova and Golowasch, 2007). The driving factor behind these changes is unclear; where some studies suggest activity-dependent or Ca2+-dependent changes (Golowasch et al., 1999), others suggest that the presence or absence of NM is the only signal required to induce recovery (Khorkova and Golowasch, 2007; Temporal et al., 2012). However, the time to first bout (suddenly increased frequency) after deafferentation depends on the history of network activity before deafferentation or the application of NMs which alter network activity, suggesting that recovery requires coordinated action of both activity- and NM-dependent pathways (Zhang et al., 2009).

Theoretical work has suggested that activity-dependent control of ion channel distributions could be working in concert with NM-dependent intracellular Ca2+ buffering (Zhang and Golowasch, 2011), though no experimental verification of the involvement of any intracellular regulatory cascades has been published. These models assume a black-box intracellular Ca2+ sensor as the integrating component for observations of activity and feedback to the system – but this component need not be intracellular if the condition of feedback on ion channel activity is met. In fact, compartmentalization of this feedback component outside of the cell may be an advantage for network-wide regulation or prevention of interference in other intracellular
pathways. CSPGs and associated ECM molecules are candidates for this kind of long-term homeostatic regulation, as shown in recent theoretical work (Kazantsev et al., 2012).

Chondroitin sulfate (CS) structure is conserved between invertebrates and vertebrates (Medeiros et al., 2000; Sugahara and Yamada, 2000; Selleck, 2001; Fongmoon et al., 2007) but while there has been extensive study of the structural similarities, the functional roles of CS in invertebrate nervous systems have not been described. Fongmoon et al. demonstrated that invertebrate CS from cartilage functions similarly to mammalian neuronal CS, by introducing king crab CS to cultured mouse hippocampal neurons (Fongmoon et al., 2007). Invertebrate CS increased neurite outgrowth, similar to the effects of mammalian CS with comparable sulfation patterns. This is evidence that invertebrate CS has functional similarity to mammalian CS, but no studies have determined if invertebrate neurons also respond to CS in a similar way. Here we describe such an experiment and shed light on CS as a participant in homeostatic activity regulation. We show evidence of CS in crab neural tissue, then show that CS degradation in the STG alters recovery of the pyloric rhythm after deafferentation. Surprisingly, ongoing activity in an intact STNS is unaffected by removal of CS, suggesting that the influence of CS on activity regulation is specific to homeostatic pathways.

**Methods**

All experiments were performed using the STNS of the crab, *C. borealis*. The intact STNS was removed from surrounding tissue as previously described (Gutierrez and Grashow, 2009).

**Western Blots**

Crab brains were removed during STNS dissection and flash frozen in liquid nitrogen. Brains were pooled and processed as previously described (Deepa et al., 2006) using a series of
three extraction buffers. Buffer 1 (50mM TBS, pH 7.0, 2 mM EDTA, 10 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, and one cOmplete EDTA-free protease inhibitor cocktail tablet, Roche), buffer 2 (buffer 1 containing 0.5% Triton X-100) and buffer 3 (buffer 2 with 6 M urea). A subset of samples were incubated with 2.5 U/ml protease-free chondroitinase ABC (chABC, Sigma) for 2 hours at 37°C before use in a Western blotting protocol using mouse anti-chondroitin sulfate IgM CS-56 (1:10000, Sigma) as primary antibody and goat anti-mouse IgM (1:5000, KPL, MD, USA) as secondary.

**Organ Culture and Long-term Electrophysiology**

The intact STNS was removed from the crab and placed in culture using L-15 medium supplemented with 40 U/ml penicillin, 40 µg/ml streptomycin (Sigma), 1 g/L glucose, and adjusted for final salt concentrations (in mM) of: 440 NaCl, 11 KCl, 13 CaCl₂, 26 MgCl₂, 11.2 Trizma base and 5.1 maleic acid. After verification of a stable rhythm (30-60 minutes), 4 U/ml of chABC in media was added to a Vaseline well around the desheathed STG, and allowed to sit for 12-18 hours. Alternatively, media-only or denatured chABC (DNchABC) were used as controls. After this incubation, the enzyme was removed by rinsing the well three times with media. Some STGs were removed at this stage and an immunohistochemistry protocol was performed (Hudson, 2013). In other experiments, the *stn* was cut after rinsing. Sterile media was replaced every 12 to 24 hours, and the activity of the STG was monitored for at least 5 days after deafferentation. Enzyme samples were tested for relative enzymatic activity using a standard dimethylmethylene blue (DMMB) protocol (Lee et al., 2010).

Data was analyzed with custom MATLAB scripts. In most cases, the burst frequency of the PD neuron as recorded on the *pdn* was used as a proxy for pyloric frequency. If the *pdn* was not available, burst frequency of the LP neuron or PD neuron was recorded on the *lvn*. Student’s
t-tests, single-factor ANOVA, and Tukey-Kramer tests were used for statistical analysis. Standard error was used for reported confidence measures.

Results and Discussion

Chondroitin Sulfate in the STG

CS is present in invertebrates (Medeiros et al., 2000; Sugahara and Yamada, 2000; Selleck, 2001; Fongmoon et al., 2007) but studies to date have analyzed CS from connective (Gomes et al., 2011) rather than neural tissues. CS from crab cartilage has been successfully digested by chABC (Fongmoon et al., 2007), but its immunoreactivity with common anti-CS antibodies is unclear. In this study we used an antibody that binds to the chondroitin sulfate family of molecules generally (monoclonal CS-56). The CS-56 antibody displayed immunoreactivity in homogenized crab brain via Western blotting (Fig. 1C) and in the STG via immunohistochemistry (data not shown, n=6 per treatment group). The specificity of this binding, however, has not been previously verified in this system. To test this, we used a Western blot protocol used previously in the study of perineuronal nets in mammalian systems (Deepa et al., 2006) that includes a series of three extraction buffers which separate diffuse ECM (buffers 1 and 2) from more dense and cell-membrane specific types of ECM (buffer 3). We paired this protocol with chABC pretreatment of the extracts to identify specific (chABC-sensitive) vs nonspecific (chABC insensitive) binding of the antibody. We found that a portion of the binding in extracts from detergent (buffer 2) and urea (buffer 3) fractions is prevented by pretreatment with chABC, demonstrating the presence of chABC-sensitive CS in these extracts. Extract 2 shows bands around 260 kD and at high molecular weights (MW) that are reduced in the chABC-treated sample. Extract 3 has a single large band at high MW that is absent after
chABC-treatment. This is particularly interesting because perineuronal nets (PNNs) are primarily extracted by buffer 3 in mammalian systems (Deepa et al., 2006; Carulli et al., 2010), suggesting the CS found in crab brain could be PNN-like. It is important to note that the CS-56 binding in the saline-eluted fractions of brain tissue (buffer 1) was not changed after treatment with chABC, and we found diffuse staining in the STG immunohistochemistry results which was similarly unaffected by chABC pretreatment. We interpret these results as either non-specific binding of this antibody in this system or the presence of a CS variant not degraded by chABC. The mammalian CSPG decorin was also used as a positive technical control in our Western blotting protocol to ensure that the chABC enzyme was sufficiently active. The decorin control showed a high MW band before chABC treatment and no staining after treatment, indicating activity. Our results are the first demonstration of neural CS in decapod crustaceans, and show the presence of large MW molecules with chABC-susceptibility, particularly in fractions designed to isolate membrane-associated molecules and stable ternary complexes such as those found in PNNs (Deepa et al., 2006; Carulli et al., 2010). This opens up invertebrate neuroscience – with its advantages of identified cells and small, defined networks – to the study of cellular and network effects of perineuronal CSPGs.

Chondroitinase Does Not Interrupt the Ongoing Pyloric Rhythm

The pyloric circuit is comprised of four main cell types: the anterior burster neuron (AB), the two pyloric dilator neurons (PD), the lateral pyloric neuron (LP), and the three to eight pyloric neurons (PY). These neurons fire in a triphasic rhythm, in which AB and PD fire simultaneously, then LP, then PY (Fig. 1B). This rhythm can be monitored extracellularly via the
lateral ventricular nerve (lvn), through which all STG neurons send projections, or the pyloric
dilator nerve (pdn), which carries only action potentials from the PD neurons (Fig. 1A).

To determine if CS influences ongoing neuronal activity, we introduced chABC to
cultured intact STNSs. Dissected STNSs were placed in culture and electrical activity of the pdn
and lvn was recorded with steel extracellular electrodes for 5-7 days. In the first hour of organ
culture, treatment (chABC in medium) or sham (medium only, or denatured chABC (DNchABC)
in medium) solutions were added to a well surrounding the STG. These solutions were allowed
to sit 12-18 hours before they were rinsed and replaced with medium. No difference between
treatment groups in the nature or progression of pyloric circuit activity from intact STNSs was
seen over the duration of organ culture (Fig. 2). No significant differences were seen between the
three treatment conditions for any of the investigated activity metrics, and no qualitative
differences in activity pattern were observed. For instance, after an average of 4 days in culture
(105 +/- 9 hrs), most preparations ceased rhythmic activity, and this was common to all treatment
groups. Other metrics that were unchanged between groups included the last cell type to stop
bursting (Fig. 2A), the time and frequency of last stable bursting (defined as continuous bursting
activity with pyloric frequency >0.2 Hz, Fig. 2E), measurements of pyloric frequency at
particular time points (Fig. 2C), and the slope between the frequency at hour 20 in culture and
the frequency at the last PD bursting activity (Fig. 2D). LP-on phase, defined as previously
(Luther et al., 2003), was also calculated for the first hour of culture, 10 hours of culture, and
after 20 hours (not shown) and no differences were seen between groups (ANOVA p-values of
0.09, 0.08 and 0.11 for 1, 10, and 20 hr respectively). These results demonstrate that degradation
of CS in the STG of the intact STNS does not affect ongoing network activity for up to a week
after treatment.
This result is reminiscent of experiments in the mammalian visual system in which chABC treatment does not affect activity characteristics or ocular dominance in normal animals, but does alter ocular dominance in monocularly deprived animals (Pizzorusso et al., 2002, 2006). In this and other systems, it appears that CSPGs do not influence normal ongoing activity of mature circuits, but rather participate in cellular, synaptic, and network-wide changes only after a significant perturbation (Pizzorusso et al., 2002, 2006; Gogolla et al., 2009).

Chondroitinase Treatment Prevents or Delays Recovery

We next asked if CS in the STG participates in homeostatic feedback during recovery after deafferentation. To test this, STNSs were cultured as described above; however, in these experiments the stn was cut after replacing the treatment solutions with media (12-18 hours into culture). This removes neuromodulatory input to the pyloric circuit and causes the pyloric rhythm to slow or stop within minutes. In some cases, bursting activity goes through a period of boutning beginning 5-25 hours after the cut. Boutning is defined as alternating periods of time, on the order of minutes, in which the rhythm gets faster and slower; specifically, a bout is at least 3 pyloric cycles with an instantaneous frequency 40% above the baseline frequency, as previously described (Zhang et al., 2009). Baseline frequency was defined as the average frequency of the previous 7 minutes or a minimum of 50 pyloric cycles. Stable bursting activity returns an average of 3 days after deafferentation in control preparations (72.8 +/- 14.8 hrs) at a post-deafferentation maximum average frequency ($F_{\text{max}}$) approximately half of the original value (0.66 +/- 0.09 Hz) (Fig. 3). $F_{\text{max}}$ was defined as the maximum value of a 5-hour moving average which scanned instantaneous frequency values from 10 hours after deafferentation to the last recording.
Chondroitinase treatment before deafferentation delayed the time to maximum average burst frequency after deafferentation (Fig. 3, 4), but did not significantly alter other activity metrics (Fig. 3D, E). The $F_{\text{max}}$ of all control pyloric circuits occurred within the first 100 hours after deafferentation. During this time frame, chABC-treated preparations recovered bursting activity, however their $F_{\text{max}}$ values were lower (0.23 +/- 0.03 Hz) when compared to untreated activity and those treated with denatured chondroitinase (p<0.01) (Fig. 3C). Denatured chondroitinase and control preparations were not significantly different in any metric investigated, suggesting that it is the enzymatic activity of chABC that is mediating its effects. There was no difference between groups in the incidence of triphasic recovery patterns – 50% of pyloric circuits demonstrated triphasic activity after deafferentation regardless of treatment conditions – or the qualitative appearance of bursting activity after recovery. These results show that extracellular CS is involved in the process of recovery of pyloric activity after a perturbation, and that removal of CS via chABC prevents recovery in the first 5 days after deafferentation.

Previous work on recovery mechanisms of the pyloric circuit has shown that cessation of pyloric activity before deafferentation via PD hyperpolarization or application of low-Na$^+$ saline causes an advance in the time to first bout, suggesting that time to first bout ($t_{\text{bout}}$) is an indicator of an activity-dependent regulatory mechanism (Zhang et al., 2009). $T_{\text{bout}}$ was defined as the time from deafferentation to time of the first recorded bout that occurred after the time to half maximal activity ($t_{1/2}$), which was defined as the time from deafferentation to the time at which median-filtered (order 3) pyloric frequency first dipped below half of the pre-deafferentation pyloric frequency. $T_{\text{bout}}$ was not significantly different between the three conditions when a 95% confidence interval was used; however, the p value was notably low at p=0.075 (Fig. 3D).
suggestive (p<0.1), though not significant, delay in \( t_{\text{bout}} \) for chABC-treated STGs suggests that CS may be synthesized and secreted locally over time such that treated networks eventually go through periods of boutting and recovery. To determine if treated networks eventually recover, chABC-treated preparations were allowed to persist in culture beyond 120 hours (Fig. 4). In 3 out of 8 chABC-treated STGs, the pyloric activity did not increase in frequency past 120 hours after deafferentation. These preparations show a “flat” recovery profile, meaning that pyloric activity remains consistently very slow after deafferentation, and eventually stops. In the remaining 5 cases, activity eventually returned to control recovery values of up to half the original frequency. These preparations show a “slow ramp” recovery profile; the pyloric frequency starts slow after deafferentation, and gradually increases. The average \( F_{\text{max}} \) value after deafferentation for chABC-treated pyloric circuits (0.48 +/- 0.08 Hz) was not significantly different from values for untreated (0.66 +/- 0.09 Hz) or DNchABC-treated controls (0.62 +/- 0.09 Hz) when no time constraints were placed on the analysis period. However, the time needed for chABC-treated preparations to reach these values was significantly greater (139 +/- 23 hrs) than both untreated and DNchABC-treated circuits (p<0.05). The significant delay to reach control \( F_{\text{max}} \) values suggests that some component of the recovery mechanism is being interfered with temporarily; from this data we hypothesize that degradation of CS by chABC treatment is followed by synthesis and secretion of CS by STG neurons and glia to replace what was lost. CSPGs in mammalian systems are replenished on the order of days to weeks after chABC treatment (Lin et al., 2008), so secretion by STG neurons and glia would fit the time line of this experiment.

Across model systems, most of the neuronal effects of CSPGs fall into two categories – structural plasticity and neuronal excitability. CSPGs inhibit structural plasticity via their role in

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the glial scar after injury, axonal pathfinding and synapse formation during development (Kwok
et al., 2011). There are changes in synaptic strength during normal recovery of the pyloric
rhythm, but these changes do not appear to affect the overall wiring pattern of the circuit, and are
therefore not thought to be an important factor in recovery (Thoby-Brisson and Simmers, 2002).
For that reason, any interference in or exacerbation of these changes due to the absence of
CSPGs would likely not prevent or delay recovery, unless they also resulted in large changes in
structural plasticity or network wiring. That scenario would likely result in non-functional
network activity in both the intact and decentralized pyloric circuit, which we did not observe.
We take that as evidence that structural or synaptic changes due to chABC treatment are not a
factor in the pyloric network, but further experiments measuring the synaptic strengths of
identified synapses before and after treatment with chABC would inform this hypothesis.
We suggest that CSPG-mediated effects on neuronal excitability present a more likely
mechanism of action in the pyloric circuit. Though it is unclear how CSPGs mediate their effects
on neuronal excitability, there are three categories of evidence for potential mechanisms in other
systems: cation buffering (Vigetti et al., 2008; Hrabětová et al., 2009), interaction with cell-
surface receptors or channels (Snow et al., 1994; Maroto et al., 2013), and binding of growth
factors for later presentation to cellular receptors (Deepa et al., 2002, 2004; Kantor et al., 2004).
The lack of a clear change in the ongoing pyloric rhythm in the intact STNS after chABC
treatment suggests that ion buffering is an unlikely mechanism in our system. The other two
possibilities are consistent with the lack of change in ongoing activity and the delay in recovery
metrics in the treated pyloric circuit, if we assume that CSPGs are slowly replenished after
chABC treatment, and will be an area for future experiments.
We have demonstrated the presence of CS in the crab nervous system and its involvement in activity maintenance after deafferentation. As described, the STG is ideally suited for further study of the mechanisms by which CSPGs might be involved in activity maintenance and homeostasis.

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References


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**Figure Legends**

**Figure 1:** The STNS as a model of network homeostasis and perineuronal CS. (A) The intact STNS as maintained in culture. Pink arrows show severed connections to the brain (brain not included in culture). Grey circles indicate Vaseline wells used to isolate nerves and ganglia for administration of solutions (around STG) or electrical recordings (for lvn and pdn). Blue arrowhead indicates site of deafferentation. (B) The pyloric circuit loses, and subsequently recovers, triphasic activity after deafferentation. Top: Example extracellular recordings of the lvn nerve (d0, before; d1, within 24 hours of; d3, 72-96 hours after deafferentation) with color coded pyloric cell types in selected cycles (LP- lateral pyloric, red; PY- pyloric, green; PD- pyloric...
dilator, blue). Scale bar is 1 s. Bottom: Pyloric frequency vs. time in culture. Deafferentation occurs at t=0 (blue arrowhead). Recordings (and treatment, if applicable) began 12-18 hours earlier (data not shown in entirety). (C) Western blots using anti-CS (CS-56) primary antibody show CS immunoreactivity in homogenized *C. borealis* brain. Decorin was used as a positive control (lane d). Decorin treated with chABC (dc) was used as a negative control. Lanes marked 1-3 are crab brain samples treated sequentially with buffers 1, 2 and 3 adjacent to the same samples treated with chABC (1c, 2c, 3c, respectively), n=2.

Figure 2: chABC treatment has no effects on pyloric rhythm in long term organ culture. (A,B) Top row, untreated, n=5; Middle row, chABC-treatment, n=6; Bottom row, DNchABC-treatment, n=4. (A) Example extracellular recordings of the *lvn* at days 1 (d1) and 2 (d2), and at the times of last triphasic activity (t-Tri) and last stable bursting activity of the PD neurons (t-PD) or LP neuron (t-LP). Scale bar is 1 s. (B) Pyloric frequency vs. time in culture. Shading indicates treatment condition and duration. (C,D,E) Grey, untreated; orange, chABC-treatment; blue, DNchABC-treatment. (C) Pyloric frequency after 20 hours in culture. (D) Slope between pyloric frequency at 20 hr and frequency of last stable bursting activity. Example shown in B with purple line marked “d”. (E) Time at last stable bursting activity.

Figure 3: chABC treatment alters pyloric rhythm recovery after removal of neuromodulator via deafferentation. (A,B) Top row, chABC-treatment, n=8; Bottom row, DNchABC-treatment, n=8; Untreated (see Fig. 1B), n=6. (A) Example extracellular recordings of the *lvn* nerve (d0, before; d1, within 24 hours of; d3, 72-96 hours after deafferentation). Scale bar is 1 s. (B) Pyloric frequency vs. time in culture. Deafferentation occurs at t=0. Recordings
(and treatment, if applicable) began 12-20 hours earlier (data not shown in entirety). Shaded areas indicate treatment duration and type (orange, chABC; blue, DNchABC; white with outline in C,D,E indicates no treatment). (C) Average pyloric frequency in the 10 hours before deafferentation and maximum average pyloric frequency between 10-120 hours after deafferentation as measured by PD burst frequency. (D) Time to first bout. (E) Time to half max pyloric frequency. Error bars represent standard error. **: p<0.01; +: p=0.075

Figure 4: Recovery profiles after CSPG degradation fall into two categories. (A) Example extracellular recordings of the ivn (d1, before;d2, within 24 hours of;dX, day X after deafferentation). Scale bar is 1 s. (B) Pyloric frequency vs. time in culture. Deafferentation occurs at t=0. All culture data shown. Shaded areas indicate treatment duration with chABC. (C) Average time to Fmax. (D) Average pyloric frequency in the 10 hours before deafferentation and maximum average pyloric frequency from 10 hours after deafferentation to the last activity recorded in each experiment, as measured by PD burst frequency. *p<0.05. Error bars represent standard error. Colors as defined in Fig. 3.