Microcircuit formation following transplantation of mouse embryonic stem cell-derived neurons into peripheral nerve

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RUNNING HEAD: **Spontaneous activity in ESCMN transplants**

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NEW AND NOTEWORTHY:
This manuscript demonstrates that following peripheral transplantation of neurons derived from embryonic stem cells, the grafts are spontaneously active. The activity is produced and modulated by a number of transmitter systems, indicating that there is a degree of self-assembly of circuits.

KEYWORDS:
Peripheral nerve injury
Locomotion
Central pattern generator

AUTHOR CONTRIBUTIONS
PM, VRF, and RMB contributed to the conception and design of the study, PM acquired and analyzed the data, and PM, VRF, and RMB wrote the manuscript.

Abstract

Motoneurons derived from embryonic stem cells can be transplanted into the tibial nerve, where they extend axons to functionally innervate target muscle. Here, we studied spontaneous muscle contractions in these grafts three months following transplantation. One-half of the transplanted grafts generated rhythmic muscle contractions of variable patterns, either spontaneously or in response to brief electrical stimulation. Activity generated by transplanted embryonic stem cell-derived neurons was driven by glutamate and was modulated by muscarinic and GABAergic/glycinergic transmission. Furthermore, rhythmicity was promoted by the same transmitter combination that evokes rhythmic locomotor activity in spinal cord circuits. These results demonstrate that there is a degree of self-assembly of microcircuits in these peripheral grafts involving embryonic stem cell-derived motoneurons and interneurons. Such spontaneous activity is reminiscent of embryonic circuit development in which spontaneous activity is essential for proper connectivity and function, and may be necessary for the grafts to form functional connections with muscle.

Introduction

Spontaneous activity of neurons during embryogenesis is important for the development of neural circuits (Kirkby et al., 2013). Such activity plays a role in synapse development as well as axon path-finding (Gomez and Spitzer, 1999; Hanson and Landmesser, 2004). In early embryogenesis of the spinal cord, release of acetylcholine from developing motoneurons (MNs) has been shown to be crucial for the development of locomotor circuits (Myers et al., 2005). This is a transient requirement, as later in development eliminating cholinergic neurotransmission has little effect (Myers et al., 2005), and glutamate and glycine/GABA release from interneurons plays an increasing role in bursting behaviour (Rosato-Siri et al., 2004). Thus, various neuronal populations and various transmitter phenotypes play different roles in spontaneous bursting activity at different time points in development, and this activity is essential for the development of synapses and circuits.

Motoneurons can be derived in vitro from embryonic stem cells through exogenous application of signalling factors present in the ventral spinal cord during development (Wichterle et al., 2002). Although this results in enrichment of MNs in these cultures (Wichterle et al., 2002; Miles et al., 2004), a wide range of neuronal subtypes remains: the typical MN differentiation protocol generates about 30% MNs as well as different interneuron types: glutamatergic (10%), GABAergic (15%), and glycinergic (6%) (Deshpande et al., 2006). Some of these neurons express markers associated with specific excitatory or inhibitory ventral spinal interneuronal types (Deshpande et al., 2006). Embryonic stem cell derived motoneurons (ESCMNs) can functionally innervate muscle in culture (Miles et al., 2004; Chipman et al., 2014), or following transplantation into either developing chick embryos (Soundararajan *et al.*, 2006) or adult mouse

peripheral nerve (Yohn et al., 2008; Bryson et al., 2014; Magown et al., 2016), but we and others have had less success when transplanting purified ESCMNs. It is possible that neurons other than MNs facilitate neuromuscular innervation, possibly through inducing activity. In fact, spontaneous activity has been demonstrated in vitro in neurons derived from stem or pluripotent cells (Ban et al., 2007; Heikkilä et al., 2009; Illes et al., 2014), but whether such activity is present following transplantation or involved in innervation is not known.

We therefore asked whether there is evidence of circuit formation and spontaneous activity in ESCMNs transplanted into adult mouse peripheral nerve, isolated from the central nervous system. Our previously used model whereby neurons are implanted into the peripheral nervous system (Thomas et al., 2000; Yohn et al., 2008) avoids the growth-inhibiting environment of the central nervous system. Furthermore, this strategy ensures that all innervation following transplantation is attributable to transplanted rather than endogenous MNs. Using this peripheral nerve transplantation model, we previously reported spontaneous EMG activity in transplanted animals, but had thought this might be secondary to mechanical stimulation (Yohn et al., 2008). Here we extended these studies to characterize spontaneous circuit activity in these transplants, and found that they exhibited spontaneous and stimulation-evoked rhythmic muscle contractions. This activity was glutamate-dependent, suggesting formation of circuits with excitatory interneurons. Furthermore, GABA/glycine and acetylcholine activity modulated the circuit function. We conclude that after transplantation, a self-organized circuitry forms that is capable of driving rhythmic muscle contraction.

Methods

Embryonic Stem Cell Derived Motoneurons

Generation of mouse embryonic stem cell derived MNs has been previously described (Wichterle et al., 2002; Miles et al., 2004; Yohn et al., 2008). In summary, HBGB6 mouse stem cells expressing GFP under the motoneuronal promoter Hb9 (Magown et al., 2016) were agglomerated as embryonic bodies before differentiation with smoothen agonist (500 nM, Enzo) and retinoic acid (1 μM, Sigma) for 5 days. The presence of MNs was confirmed by the expression of GFP.

ESCMN Transplantation

All procedures were performed in accordance with protocols approved by the Dalhousie University Animal Care Committee, and conformed to the standards of the Canadian Council of Animal Care. Details of the ESCMN dissociation and transplantation can be found in a previous publication (Yohn et al., 2008; Magown et al., 2016). In summary, embryonic bodies were treated with 1 μg/ml mitomycin C (except for immediate transplants) for 2 hours followed by wash, dissociation and resuspension at 10^6 cells per 10 μL of DFK10 with 10 μg/ml GDNF (Milipore), 20 μg/ml CNTF (Chemicon) and 0.01% DNaseI (Sigma-Aldrich).

Transplantation was performed in 5 week-old mice either immediately after nerve transection or after a delay of 1, 2 or 4 weeks post transection as previously described (Magown et al., 2016). Briefly, the tibial nerve was transected proximal to the branching of the nerve to the medial gastrocnemius (MG). The proximal tibial nerve stump was ligated and buried into the adjacent muscle to prevent reinnervation. Ten thousand cells in 0.1 μL were transplanted in the distal tibial nerve with a glass pipette.

In Vitro Electrophysiological Recordings

The MG muscle and the transplanted tibial nerve were harvested 3 months post transplantation and maintained in an *in vitro* chamber circulating oxygenated mouse Tyrode's solution (125 mM NaCl, 24 mM NaHCO3, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5% dextrose) at room temperature (Yohn et al., 2008). Stimulation to evoke bursting activity was provided to the MG nerve with a suction electrode via a square pulse stimulator (S88, Grass Technologies) and a stimulus isolation unit (PSIU6, Grass Technologies) at 1.5x the maximal stimulus threshold (usually ~10 V, 100 μA). Three pulses of 0.2 ms at 5 Hz or 25 pulses at 50 Hz were used to elicit bursting activity. Forces were measured with a force transducer (FT03, Grass Technologies) connected to a strain gage amplifier (P122, Grass Technologies). Signals were recorded via a Digidata 1320A, using Axoscope 9.2 software (Molecular Devices). Forces were analyzed off-line. Bursts were detected using event analysis in pClamp 10 (Molecular Devices) using threshold detection set with a minimal amplitude of 0.5 mN (2 standard deviation above baseline noise recorded after nerve transection) and a minimum duration of 50 ms.

The following drugs were used: CNQX 10 μM (disodium salt hydrate, #115066-14-3, Sigma), APV 100 μM (#76326-31-3, Sigma), bicuculline 10 μM (#485-49-4, Sigma), strychnine hydrochloride 1 μM (#1421-86-9, Sigma), atropine 10 μM (51-58-8, Sigma), NMDA 5 μM (#6384-92-5, Sigma), serotonin hydrochloride (5-HT) 10 μM (#153-98-0, Sigma) and dopamine hydrochloride 50 μM (#62-31-7, Sigma). All drugs were added as a concentrated stock to the circulating Tyrode's solution to give the final concentrations indicated above.

Statistical Analysis

Statistical analysis was performed before and after drug infusion on each animal individually. Because of the high variability of responses between animals, results were not combined for analysis and the number of animals is indicating by N. For individual animals, effects of drugs (measuring multiple bursts) were compared to baseline (multiple bursts) using unpaired t-tests with Welch's correction or with a Mann-Whitney test if data groups failed a D'Agostino-Pearson normality test. When more than two groups were compared, a one-way ANOVA test was performed. A Chi-square test was performed when analyzing ratio. Results are presented as means ± standard deviations. Statistics were performed using GraphPad Prism version 6.0h for Mac (GraphPad Software, La Jolla, California USA).

Results

Motoneurons derived from embryonic stem cells were transplanted into the tibial nerve acutely after transection or after a denervation period of up to four weeks. MG forces were recorded *ex vivo* three months after transplantation (Magown et al., 2016). Out of 24 transplanted mice (the same mice as reported in Magown et al., 2016), 17 demonstrated contraction of the MG upon electrical stimulation of the transplant site, indicating functional engraftment. Of these 17 mice, 9 (53%) had rhythmic contractions, of which 6 were spontaneously rhythmic in the absence of electrical stimulation (Movie 1), and 3 had episodes of repetitive contractions evoked by either a single electrical pulse or a short train of pulses (Figure 1A). Cutting the tibial nerve distal to the transplant resulted in complete ablation of rhythmic contractions in all 9 mice. Using the nomenclature “burst” to indicate a single spontaneously terminating contraction, “bursting” to indicate repetitive bursts, and “episode” to indicate a period of repetitive bursting, transplantation of ESCMNs led to spontaneous or evocable bursting episodes in one-half (9 / 17) of the preparations.

To determine the origin of the rhythmic activity, we next investigated the role of glutamatergic transmission in the contractions. Addition of the glutamate receptor blockers CNQX and APV to the preparations with evoked bursting completely prevented further prolonged stimulus-evoked bursting (N = 2; Figure 1A). That is, following glutamate receptor blockade, there was persistence of stimulation-evoked short latency contractions, consistent with our previous findings that following transplantation of these cells, NMJ transmission is cholinergic (Yohn et al., 2008; Magown et al., 2016). In transplants with spontaneous activity, the antagonists eliminated all large amplitude bursts, resulting in a significant reduction in mean amplitudes of burst forces (to 28% and

63% of baseline in the two preparations, $p < 0.05$) and a reduction in burst amplitude variance (Figure 1B, C). The remaining low amplitude bursts may reflect single motor units, as the forces recorded (< 4 mN) are similar to motor unit forces following transplantation (Magown et al., 2016). In addition to blocking the large amplitude bursts, glutamate antagonist application also led to a higher frequency of bursting (N = 2; Figure 1B, C). Autocorrelation analysis revealed no significant burst rhythmicity (Figure 1D). The loss of high amplitude bursts indicates that intrinsic glutamatergic transmission leads to synchronization of MN activity. That is, glutamatergic inputs drive coordinated ESCMN activity.

We next asked whether inhibitory inputs contribute to the rhythmicity. In one of two transplants that were spontaneously active, application of combined GABA and glycine antagonists led to a transient increase in force (Figure 1E-G). After 30 minutes without washout, forces returned to baseline (denoted “late” on Figure 1G). No further effect was seen on washout. While there was no change in frequency of the bursts, the activity became more organized over time, as demonstrated by the autocorrelogram (Figure 1H). Thus, GABA/glycine neurotransmission in the transplants limited burst amplitude, and also led to a degree of desynchronization of MN rhythmicity. Given the different time courses of these two effects, the roles of GABA and glycine in burst amplitude and burst synchrony were likely independent of one another with the former effect possibly due to MN inhibition and the latter to desynchronization of activity of the neurons involved in generating the bursting.

We next focused on the effects of cholinergic transmission, given the known role of cholinergic activity in the generation of spontaneous activity in embryonic spinal cords (Wenner and O'Donovan, 2001; Myers et al., 2005; Czarnecki et al., 2014; Gonzalez-

215 Islas et al., 2015). As nicotinic blockade would block muscle contraction, we were limited
216 to studying muscarinic responses (N = 3). In the one preparation in which bursting
217 activity was stimulus-evoked, the duration of the episode more than doubled. In
218 transplants with spontaneous activity (N = 2), application of atropine led to an apparent
219 increase in activity (Figure 2A). On closer examination of the baseline data, a
220 background activity of low amplitude bursts (2.7 ± 0.5 mN at 3.4 Hz) could be identified
221 amongst the larger amplitude bursts (17.8 ± 6.6 mN at 1.4 Hz) (Figure 2B), with each of
222 the latter was comprised of multiple contractions (Figure 2A, inset). Following atropine,
223 each large burst was a single contraction, rendering the mean instantaneous frequency
224 of the large bursts lower following atropine. The mean frequency of the low amplitude
225 bursts was also decreased (Figure 2C). Atropine also led to an increase in overall mean
226 burst force amplitudes (Figure 2D) due to the greater proportion of large amplitude
227 events (Figure 2E), but the forces of the low and high amplitude bursts were each
228 unchanged (Figure 2D). Together, these findings suggest that the large amplitude bursts
229 seen after atropine application resulted from summation of multiple small amplitude
230 bursts. In other words, muscarinic activation has several effects. It results in
231 desynchronization of MN firing, which leads to an increase in low amplitude bursts.
232 Furthermore, muscarinic receptor activation leads to high frequency, intermittent MN
233 bursting.

234 Given the above evidence of circuit formation, we asked whether transplanted ESCMNs
235 could sustain rhythmic contractions by adding the neurochemicals that induce locomotor-
236 like rhythmicity in the mouse spinal cord: NMDA, 5-HT and DA (Jiang et al., 1999). The
237 addition of these neurochemicals did not transform transplants with evoked bursting
238 activity (N = 2) into those with spontaneous activity. However, evoked bursting episodes

239 were significantly prolonged (Figure 3A, B). In the transplants that were spontaneously
240 active (N = 2), burst frequency increased (Fig 3C-D). Furthermore, the numbers of bursts
241 greater than 40 mN increased significantly, leading to an overall increase in mean
242 contraction forces (Fig 3E-F). That is, addition of NMDA, 5-HT, and DA resulted in an
243 enhancement of rhythmic motor output, raising the possibility that rhythm-generating
244 elements akin to those in spinal locomotor circuits had formed.

Discussion

We have shown that ESCMNs transplanted into the transected tibial nerve after muscle denervation can generate coordinated rhythmic bursting activity. These bursts are glutamate-dependent and are modulated by GABAergic/glycinergic and cholinergic inputs. Addition of neurochemicals that lead to locomotor activity in the spinal cord, NMDA, 5-HT and DA, promotes bursting episodes, lengthening their duration, increasing contraction forces, and increasing burst frequencies. Together, these data demonstrate that protocols to differentiate ES cells towards MN lineages generate neuronal populations capable of generating rhythmic activity.

While these data indicate that there is a degree of self-assembly of microcircuits, the nature and interconnectivity of these circuits is not clear. It is likely that these circuits result from connectivity between a variety of neuronal types. While neuromuscular transmission in this preparation is cholinergic, it is possible that ESCMNs release glutamate locally as they do in the spinal cord (Mentis et al., 2005; Nishimaru et al., 2005; Lamotte d'Incamps and Ascher, 2008), and this glutamate leads to bursting of ESCMNs (MacLean et al., 1997) coordinated by a high degree of MN-MN interconnectivity (chemical and/or electrical; Figure 4A). However this alone does not explain the effects of GABA/glycine, or the differential effects on force amplitudes vs rhythms when adding antagonists. For example, the results show that glutamatergic activity leads to large amplitude forces but no increase in rhythmicity, which would not be expected if the neurons producing the force-regulating output (MNs) were the same as those producing the rhythmicity. Furthermore, if the bursting resulted from MN-MN interactions alone, we would expect acetylcholine to have a synchronizing rather than

the desynchronizing effect seen. Thus, the bursting activity likely results from circuits that include interneuron types.

It is known that basic elements for the formation of rhythmic motor circuits are present in these cultures. Despite using a differentiation protocol that leads to MN enrichment (Lee et al., 2000; Westmoreland et al., 2001; Barberi et al., 2003; Peljto and Wichterle, 2011), a wide range of neuronal subtypes remains. The typical MN differentiation protocol involves the use of smoothen agonist and retinoic acid (Wichterle et al., 2002), and generates about 30% MNs as well as different interneuron types including glutamatergic, GABAergic, and glycinergic (Deshpande et al., 2006). That is, neuronal types needed for fundamental circuit formation are present. The Sutton principal leads us to suggest that the inter-preparation variability in bursting behavior is explained by differences in the proportions of the neuron types in the transplants. The present neuron types together form an “emerging” circuit capable of generating a rhythm (Figure 4B).

Embryonic Spontaneous Activity

Spontaneous activity is an essential component for the development of embryonic neural circuits (Marder and Rehm, 2005; Blankenship and Feller, 2009) and is involved in various roles, including neurite outgrowth (Metzger et al., 1998), maturation of electrical properties (Xie and Ziskind-Conhaim, 1995), synaptogenesis and axon pathfinding (Hanson and Landmesser, 2004; 2006; Hanson et al., 2008). The roles of different transmitter systems may differ at different times of development. In the early phase of embryonic circuit activity, bursting is dependent on GABAergic and cholinergic transmission, while glutamatergic effects occur at later stages (Branchereau et al., 2002; Hanson and Landmesser, 2003; Myers et al., 2005; Scain et al., 2010). Thus, multiple

transmitter systems play different roles in spontaneous activity at different times during development.

We studied rhythmic activity at a single time point when such transplants can successfully innervate host muscle (Yohn et al., 2008). The bursting activity we observed was largely glutamate-dependent, corresponding to glutamatergic predominance in late embryonic development. It is possible that earlier following transplantation, there was spontaneous activity produced by other transmitter systems similar to those in early embryogenesis, and that this activity set the stage for circuit formation.

Whether spontaneous activity is necessary for successful transplantation is not clear. We and others have observed that transplantation of purified MNs has not been successful. Furthermore, we have shown that following transplantation of non-purified ESCMNs, reinnervation is sub-optimal: force recovery plateaus at 40 to 50%, forces are not always sustained during 50 Hz tetanic stimulation, neuromuscular transmission can decrease with repeated stimulation, and motor unit sizes are smaller than expected for a reinnervated muscle (Yohn et al., 2008). Together, these anomalies point towards defects in maturation of electrical properties, synaptogenesis, axonal pathfinding, and/or neurite outgrowth and sprouting. All of these processes are dependent on MN activity. Thus, we suggest that activity of the transplants facilitates successful reinnervation and improved functional outcomes.

Functional Considerations

Investigating spontaneous activity of ES cell-derived neurons could extend our understanding of developmental neurophysiology and circuit formation (Ban et al., 2007; Heikkilä et al., 2009; Illes et al., 2014). Such knowledge could provide insight into the

314 impacts of transplanted stem cell-derived neurons on host circuits, some of which may
315 be unwanted and of clinical significance, such as uncontrollable contractions
316 (Weerakkody et al., 2013; Ilies et al., 2014). Whether the microcircuit formation that
317 resulted in spontaneous activity observed here plays an important role in the functional
318 integration of the transplants, and/or whether it produces clinically undesirable effects
319 remains to be seen.

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Figure Legends

Movie 1 – Spontaneous activity of differentiated ESCs produces rhythmic muscle contraction

Ex vivo transplanted tibial nerve under surgical microscope. Enlargement at the end of nerve represents the transplantation site. Top muscle is the medial gastrocnemius spontaneously contracting. Femur is anchored on the left with pins. Suction electrode on top of muscle for EMG recording. Stimulating electrode at the bottom is not contacting the nerve and not stimulating.

Figure 1 – Transplanted ESCMNs Generate A Neuronal Circuit Resulting In Rhythmic Muscle Contractions

(A) Bursting activity evoked after three 5 Hz stimuli over 500 ms. Evoked activity was blocked after the addition of CNQX and APV (N = 2). Arrows represent electrical stimuli. (B) Spontaneous muscle contractions at baseline, after CNQX and APV infusion, and after washout (N = 2). Spontaneous contractions were significantly reduced after CNQX and APV infusion with residual low amplitude contractions shown in the inset. Grey bars indicate region of insets showing small amplitude bursts in the background. Note the smaller scale bars and truncated events above 5 mN. (C) Quantification of force and instantaneous contraction frequency before and after CNQX and APV. **** One-way ANOVA, $p < 0.0001$. "+" represents mean. (D) Autocorrelation of baseline, CNQX and APV, and washout conditions (N = 1). Dotted lines represent 5% confidence interval. (E-F) Addition of GABA and glycine blockers, bicuculline and strychnine, resulted in an increase in force early, but not late after infusion of GABA and glycine blockers. Grey bars in (E) indicate regions depicted in (F). (G) Quantification of burst amplitude and instantaneous frequency. **** $p < 0.0001$ by one-way ANOVA (N = 1). (H)

Autocorrelation of baseline (solid black), early GABA / glycine blockade (dotted grey) and late GABA / glycine blockade (solid grey). Rhythmicity can be seen after prolonged GABA / glycine blockade. Horizontal dotted lines represent 5% confidence interval.

Figure 2 – Muscarinic Receptor Blockade Alters Bursting Patterns Produced by Transplants

(A) Spontaneous activity at baseline and after addition of atropine. The addition of atropine increased the occurrence of large amplitude bursts. Stars indicate region expanded in inset: note the repetitive large bursts (~2 Hz) at baseline, but single burst following atropine. (B) Enlargement of 10s regions contained within the grey bars in (A) showing a decrease in frequency of small amplitude bursts. Post-drug forces are truncated for illustration. (C) Quantification of instantaneous frequency of bursts. Atropine decreases the overall instantaneous frequency. "+" represents mean. **** $p < 0.0001$, unpaired t-test, N = 1. (D) Quantification of force shows an overall increase in force after the addition of atropine. **** $p < 0.0001$, unpaired t-test, N = 1. (E) Ratio of large and small events at baseline and after atropine. Chi-square p-value < 0.0001.

Figure 3 – Transmitters that Evoke Spinal Locomotion Increase Activity of Transplants

(A) The addition of NMDA, 5-HT and DA resulted in an increase activity demonstrated by a prolongation of burst duration in evoked activity. (B) Quantification of episode duration. ** $p = 0.004$, Mann-Whitney test, 8 vs 4 repeats, N = 1. (C-E) In transplants with spontaneous activity, the addition of NMDA, 5-HT and DA increased the instantaneous frequency and the force of bursts. *** $p = 0.0002$ in (D) and $p = 0.0004$ in (E), unpaired t-

494 test with Welch's correction, N = 1. (F) Frequency histogram of burst amplitude at
495 baseline and after addition of NMDA, 5HT and dopamine.

496

497 **Figure 4 – Potential Schematics of Transplant Circuits**

498 (A) Bursting is produced by a subset of neurons within the transplant, possibly primarily
499 by MN-MN interactions. The circuit could be composed of an assortment of MNs and
500 interneurons or only MNs. Modulation of the circuit is provided by glutamate,
501 acetylcholine and GABA / glycine. Cholinergic release may be from MN collaterals or
502 cholinergic interneurons. Co-release of glutamate and acetylcholine from MNs is
503 depicted by the red and green boutons. MNs may be electrically coupled. Exogenous
504 NMDA, 5HT, and DA provide a modulatory effect. (B) A rhythmogenic circuit provides
505 glutamatergic inputs to MNs. Modulation of this interneuron circuit is provided by
506 extrinsic or intrinsic glutamate, acetylcholine, and GABA / glycine. Direct modulation by
507 acetylcholine and GABA / glycine onto MNs is also possible. The early effect of GABA /
508 glycine blockade producing an increase in force without a change in burst frequency is
509 shown as direct modulation of MNs. The late effect of GABA / glycine blockade is
510 depicted as acting on the rhythmogenic interneuron circuit. Exogenous NMDA, 5HT and
511 DA provide a modulatory effect. Inter-motoneuron connections (electrical or chemical)
512 could contribute to the activity seen, as could MN collaterals projecting to the
513 rhythmogenic circuit.

514

Figure 1

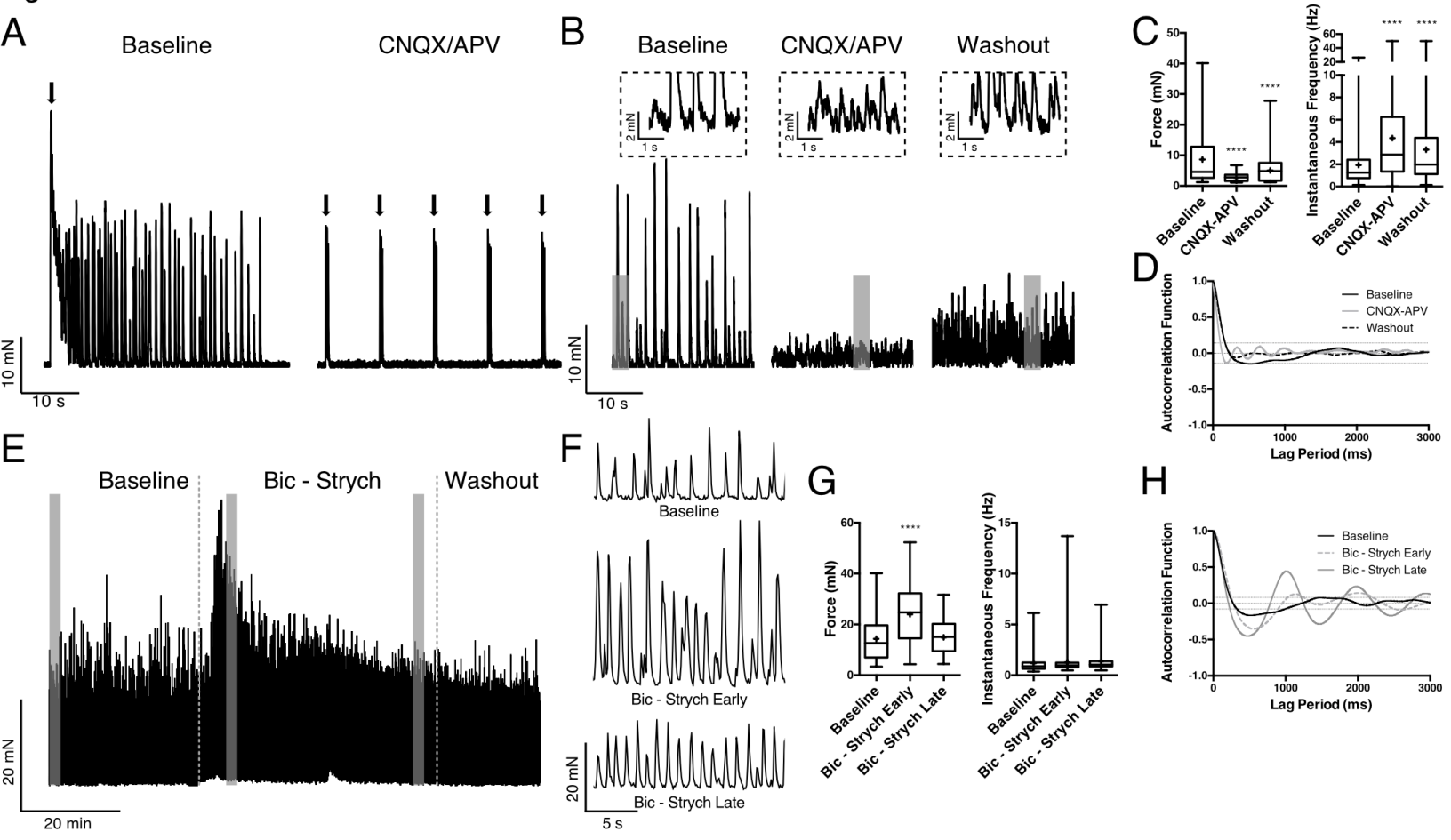

A
Baseline
CNQX/APV
10 mN
10 s
B
Baseline
CNQX/APV
Washout
2 mN
1 s
10 mN
10 s
C
Force (mN)
Baseline
CNQX-APV
Washout
****
Instantaneous Frequency (Hz)
D
Autocorrelation Function
Baseline
CNQX-APV
Washout
Lag Period (ms)
E
Baseline
Bic - Strych
Washout
20 mN
20 min
F
Baseline
Bic - Strych Early
Bic - Strych Late
20 mN
5 s
G
Force (mN)
Instantaneous Frequency (Hz)
Baseline
Bic - Strych Early
Bic - Strych Late
H
Autocorrelation Function
Lag Period (ms)

Figure 2

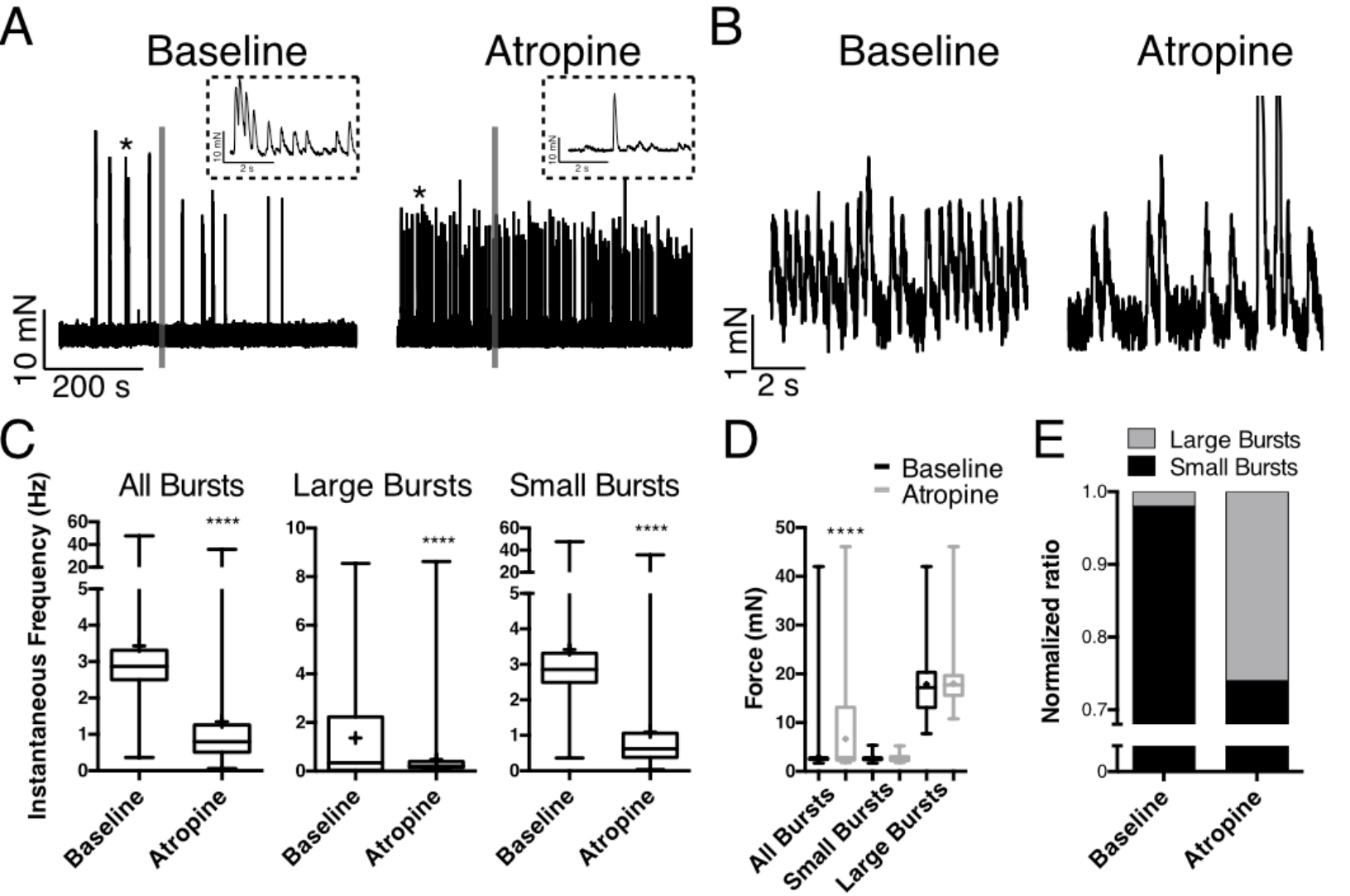

A
Baseline
Atropine
10 mN
200 s
B
Baseline
Atropine
1 mN
2 s
C
All Bursts
Large Bursts
Small Bursts
Instantaneous Frequency (Hz)
****
Baseline
Atropine
D
Baseline
Atropine
Force (mN)
****
All Bursts
Small Bursts
Large Bursts
E
Large Bursts
Small Bursts
Normalized ratio
Baseline
Atropine

Figure 3

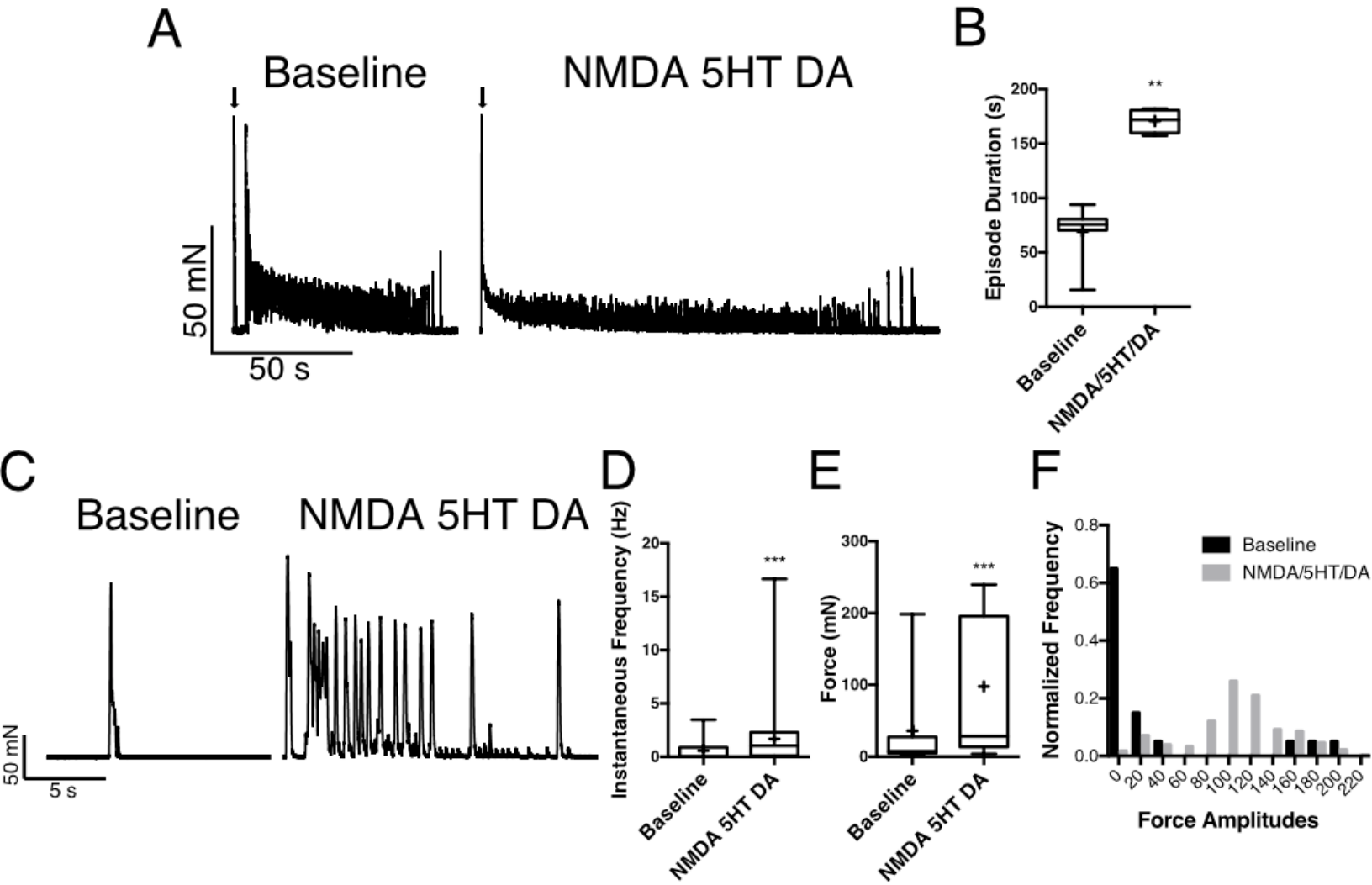
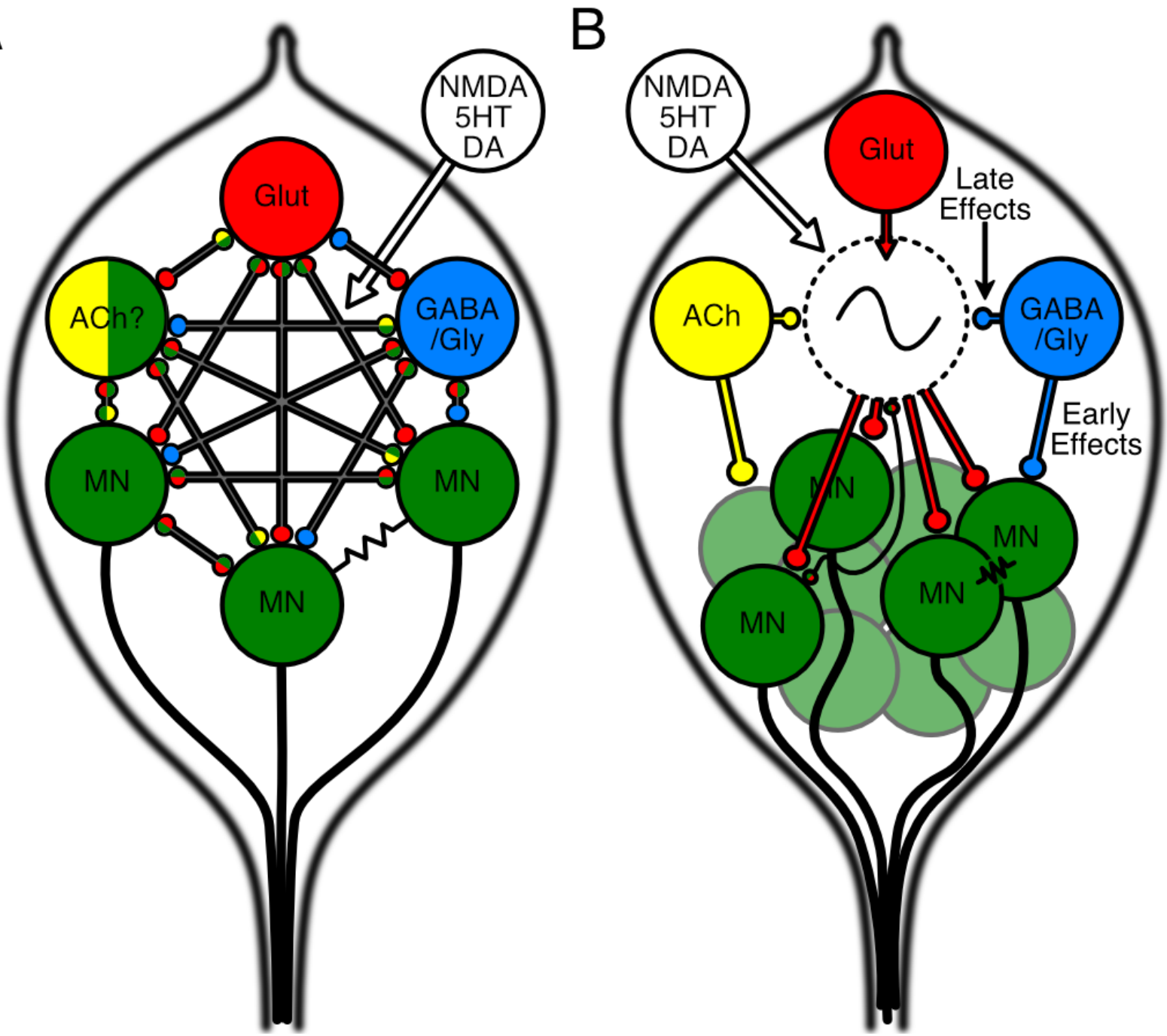

A
Baseline
NMDA 5HT DA
50 mN
50 s
B
Episode Duration (s)
**
Baseline
NMDA/5HT/DA
C
Baseline
NMDA 5HT DA
50 mN
5 s
D
Instantaneous Frequency (Hz)
***
Baseline
NMDA 5HT DA
E
Force (mN)
***
Baseline
NMDA 5HT DA
F
Normalized Frequency
Baseline
NMDA/5HT/DA
Force Amplitudes

Figure 4


A
NMDA
5HT
DA
Glut
ACh?
GABA
/Gly
MN
MN
MN
B
NMDA
5HT
DA
Glut
Late
Effects
ACh
GABA
/Gly
Early
Effects
MN
MN
MN
MN