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RAPID REPORT | *Control of Movement*

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

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Magown P, Rafuse VF, Brownstone RM. Microcircuit formation following transplantation of mouse embryonic stem cell-derived neurons in peripheral nerve. *J Neurophysiol* 117: 1683–1689, 2017. First published February 1, 2017; doi:10.1152/jn.00943.2016.—Motoneurons derived from embryonic stem cells can be transplanted in the tibial nerve, where they extend axons to functionally innervate target muscle. Here, we studied spontaneous muscle contractions in these grafts 3 mo 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.

NEW & 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 in the grafts.

peripheral nerve injury; locomotion; central pattern generator; embryonic stem cell-derived motoneurons

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 and 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, since later in development eliminating cholinergic neurotransmission has little effect (Myers et al. 2005), and glutamate and glycine/γ-aminobutyric acid (GABA) release from interneurons plays an increasing role in bursting behavior (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 signaling factors present in the ventral spinal cord during development (Wichterle et al. 2002). Although this results in enrichment of MNs in these cultures (Miles et al. 2004; Wichterle et al. 2002), a wide range of neuronal subtypes remains: the typical MN differentiation protocol generates ~30% MNs and different interneuron types (glutamatergic, GABAergic, and glycinergic neurons) (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 in either developing chick embryos (Soundararajan et al. 2006) or adult mouse peripheral nerve (Bryson et al. 2014; Magown et al. 2016; Yohn et al. 2008), 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 in adult mouse peripheral nerve, isolated from the central nervous system. Our previously used model whereby neurons are implanted in 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 spon-

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

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 previous publications (Magown et al. 2016; Yohn et al. 2008). In summary, embryonic bodies were treated with 1 μg/ml mitomycin C (except for immediate transplants) for 2 h followed by wash, dissociation, and resuspension at 10^6 cells/10 μl of DFK10 with 10 μg/ml glial-derived neurotrophic factor (Milipore), 20 μg/ml ciliary neurotrophic factor (Chemicon), and 0.01% DNase I (Sigma-Aldrich).

Transplantation was performed in 5-wk-old mice either immediately after nerve transection or after a delay of 1, 2, or 4 wk posttransection 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 in 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 mo posttransplantation and maintained in an in vitro chamber circulating oxygenated mouse Tyrode's solution (125 mM NaCl, 24 mM $NaHCO_3$, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 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 1.5× the current needed to evoke maximum force (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

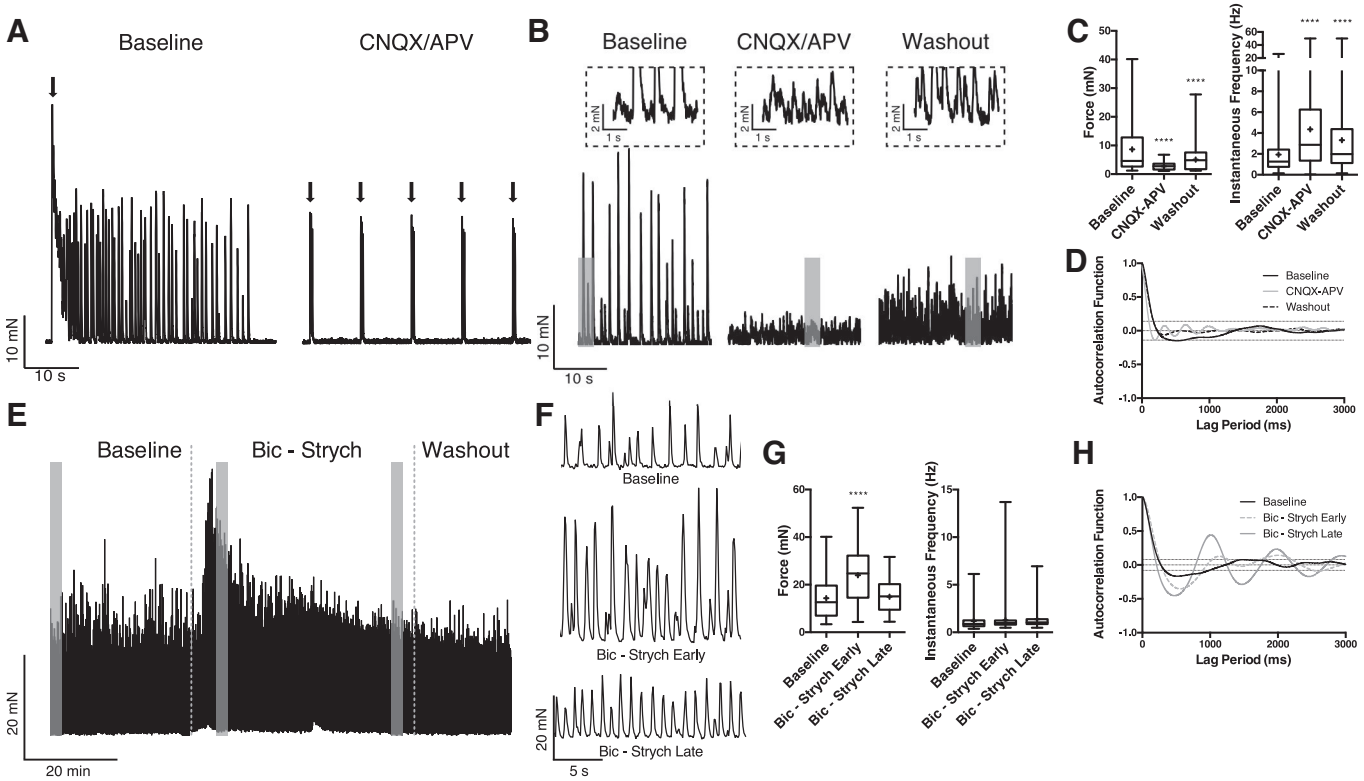

Fig. 1. Transplanted embryonic stem cell-derived motoneurons (ESCMNs) generate a neuronal circuit resulting in rhythmic muscle contractions. *A*: bursting activity evoked after three 5-Hz stimuli over 500 ms. Evoked activity was blocked after the addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and DL-2-amino-5-phosphonovaleric acid (APV) ($n = 2$ experiments). 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 *insets*. Gray 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, $P < 0.0001$. $^+$Mean. *D*: autocorrelation of baseline, CNQX and APV, and washout conditions ($n = 1$). Dotted lines represent 5% confidence interval. *E* and *F*: addition of γ-aminobutyric acid (GABA) and glycine blockers, bicuculline and strychnine, resulted in an increase in force early but not late after infusion of GABA and glycine blockers. Gray 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 gray), and late GABA/glycine blockade (solid gray). Rhythmicity can be seen after prolonged GABA/glycine blockade. Horizontal dotted lines represent 5% confidence interval.

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 SD above baseline noise recorded after nerve transection) and a minimum duration of 50 ms.

The following drugs were used: 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, disodium salt hydrate, no. 115066-14-3; Sigma), 100 μM DL-2-amino-5-phosphonovaleric acid (APV, no. 76326-31-3; Sigma), 10 μM bicuculline (no. 485-49-4; Sigma), 1 μM strychnine hydrochloride (no. 1421-86-9; Sigma), 10 μM atropine (51–58–8; Sigma), 5 μM *N*-methyl-D-aspartic acid (NMDA, no. 6384-92-5; Sigma), 10 μM serotonin hydrochloride (5-HT) (no. 153-98-0; Sigma), and 50 μM dopamine hydrochloride (no. 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 indicated by *n*. For individual animals, effects of drugs (measuring multiple bursts) were compared with 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 ± SD. Statistics were performed using GraphPad Prism version 6.0h for Mac (GraphPad Software, La Jolla, CA).

RESULTS

Motoneurons derived from embryonic stem cells were transplanted in the tibial nerve acutely after transection or after a denervation period of up to 4 wk. MG forces were recorded ex vivo 3 mo 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 [Supplemental Movie 1 (Supplemental data for this article may be found on the *Journal of Neurophysiology* website.)], and 3 had episodes of repetitive contractions evoked by either a single electrical pulse or a short train of pulses (Fig. 1*A*). Cutting the tibial nerve distal to the transplant resulted in complete ablation of rhythmic contractions in all nine mice. With the use of 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 ($n = 2$; Fig. 1*A*), that is, following glutamate receptor blockade, there

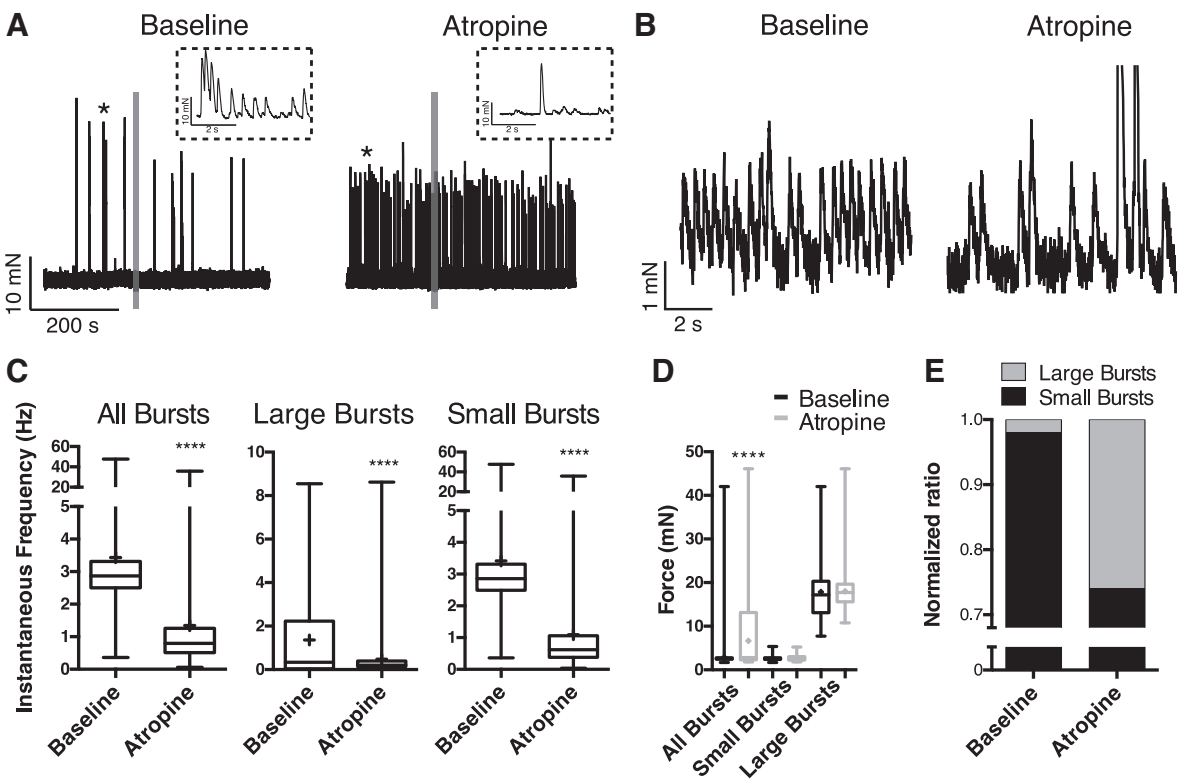

Fig. 2. Muscarinic receptor blockade alters bursting patterns produced by transplants. *A*: spontaneous activity at baseline and after addition of atropine. The addition of atropine increased the occurrence of large-amplitude bursts. Stars indicate region expanded in *inset*: note the repetitive large bursts (~2 Hz) at baseline but single burst following atropine. *B*: enlargement of 10-s regions contained within the gray bars in *A* showing a decrease in frequency of small-amplitude bursts. Postdrug forces are truncated for illustration. *C*: quantification of instantaneous frequency of bursts. Atropine decreases the overall instantaneous frequency. $^{+}$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 $P < 0.0001$.

was persistence of stimulation-evoked short latency contractions, consistent with our previous findings that, following transplantation of these cells, neuromuscular junction transmission is cholinergic (Magown et al. 2016; Yohn et al. 2008). 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 (Fig. 1, *B* and *C*). The remaining low-amplitude bursts may reflect single motor units, since 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 ($n = 2$; Fig. 1, *B* and *C*). Autocorrelation analysis revealed no significant burst rhythmicity (Fig. 1*D*). 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 (Fig. 1, *E–G*). After 30 min without washout, forces returned to baseline (denoted "late" in Fig. 1*G*). No further effect was seen on washout. Although there was no change in frequency of the bursts, the activity became more organized over time, as demonstrated by the autocorrelogram (Fig. 1*H*). 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 (Czarnecki et al. 2014; Gonzalez-Islas et al. 2016; Myers et al. 2005; Wenner and O'Donovan 2001). Because nicotinic blockade would block muscle contraction, we were limited to studying muscarinic responses ($n = 3$). In the one preparation in which bursting activity was stimulus evoked, the duration of the episode more than doubled. In transplants with spontaneous activity ($n = 2$), application of atropine led to an apparent increase in activity (Fig. 2*A*). On closer examination of the baseline data, a background activity of low-amplitude bursts (2.7 ± 0.5 mN at 3.4 Hz) could be identified among the larger-amplitude bursts (17.8 ± 6.6 mN at 1.4 Hz) (Fig. 2*B*), with each of the latter comprising multiple contractions (Fig. 2*A*, *inset*). Following atropine, each large burst was a single contraction, rendering the mean instantaneous frequency of the large bursts lower following atropine. The mean frequency of the low-amplitude bursts was also decreased (Fig. 2*C*). Atro-

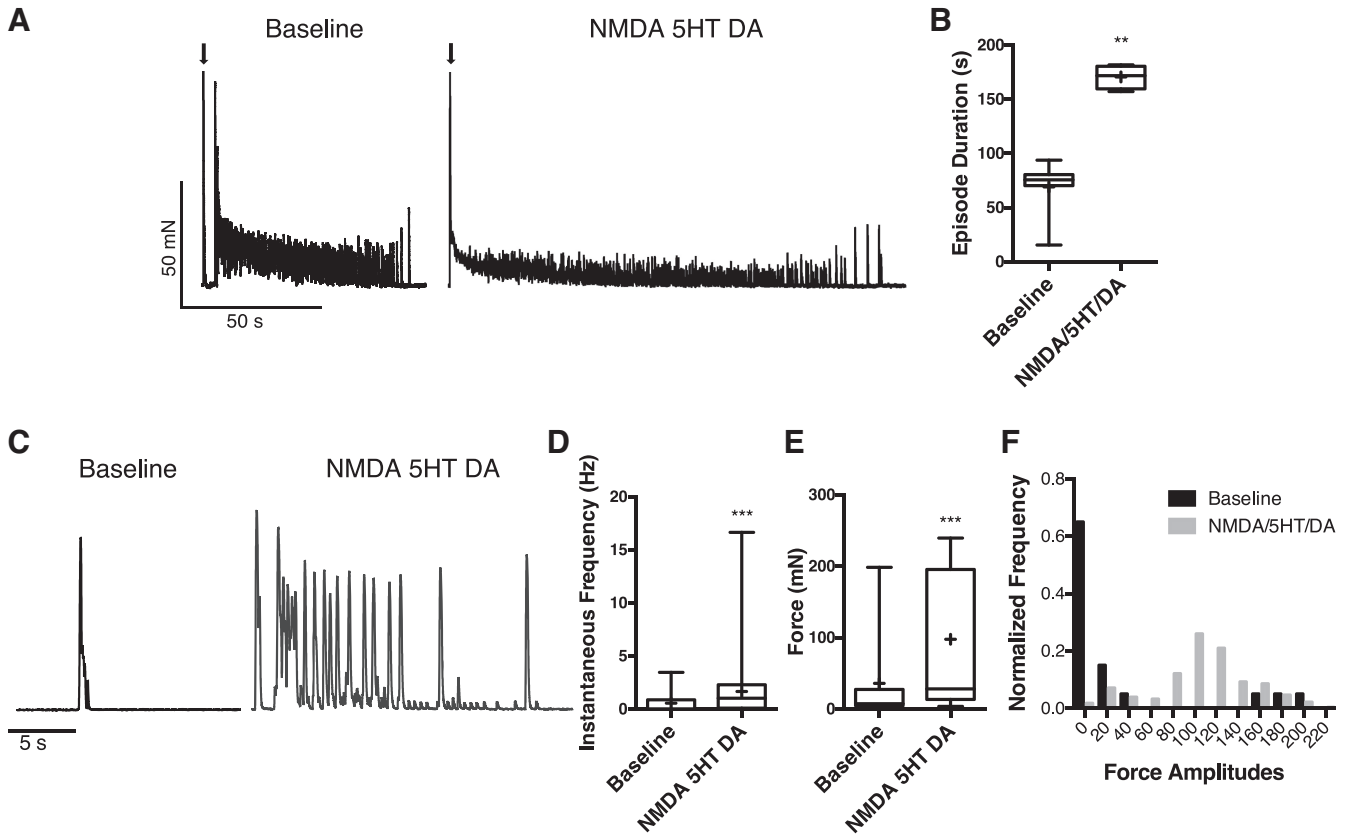

Fig. 3. Transmitters that evoke spinal locomotion increase activity of transplants. *A*: addition of *N*-methyl-D-aspartic acid (NMDA), serotonin (5-HT), and dopamine (DA) resulted in an increased activity demonstrated by a prolongation of burst duration in evoked activity. *B*: quantification of episode 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. $^{***}P = 0.0002$ (*D*) and 0.0004 (*E*), unpaired *t*-test with Welch's correction, $n = 1$. *F*: frequency histogram of burst amplitudes at baseline and after addition of NMDA, 5-HT, and dopamine.

pine also led to an increase in overall mean burst force amplitudes (Fig. 2*D*) because of the greater proportion of large-amplitude events (Fig. 2*E*), but the forces of the low- and high-amplitude bursts were each unchanged (Fig. 2*D*). Together, these findings suggest that the large-amplitude bursts seen after atropine application resulted from summation of multiple small-amplitude bursts. In other words, muscarinic activation has several effects. It results in desynchronization of MN firing, which leads to an increase in low-amplitude bursts. Furthermore, muscarinic receptor activation leads to high-frequency intermittent MN bursting.

Given the above evidence of circuit formation, we asked whether transplanted ESCMNs could sustain rhythmic contractions by adding the neurochemicals that induce locomotor-like rhythmicity in the mouse spinal cord: NMDA, 5-HT, and dopamine (Jiang et al. 1999). The addition of these neurochemicals did not transform transplants with evoked bursting activity ($n = 2$) into those with spontaneous activity. However, evoked bursting episodes were significantly prolonged (Fig. 3, *A* and *B*). In the transplants that were spontaneously active ($n = 2$), burst frequency increased (Fig. 3, *C* and *D*). Furthermore, the numbers of bursts >40 mN increased significantly, leading to an overall increase in mean contraction forces (Fig. 3, *E* and *F*). That is, addition of NMDA, 5-HT, and dopamine resulted in an enhancement of rhythmic motor output, raising the possibility that rhythm-generating elements akin to those in spinal locomotor circuits had formed.

DISCUSSION

We have shown that ESCMNs transplanted in 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 dopamine, promotes bursting episodes, lengthening their duration, increasing contraction forces, and increasing burst frequencies. Together, these data demonstrate that protocols to differentiate embryonic stem cells toward MN lineages generate neuronal populations capable of generating rhythmic activity.

Whereas 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. Although neuromuscular transmission in this preparation is cholinergic, it is possible that ESCMNs release glutamate locally as they do in the spinal cord (Lamotte d'Incamps and Ascher 2008; Mentis et al. 2005; Nishimaru et al. 2005), 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; Fig. 4*A*). 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.

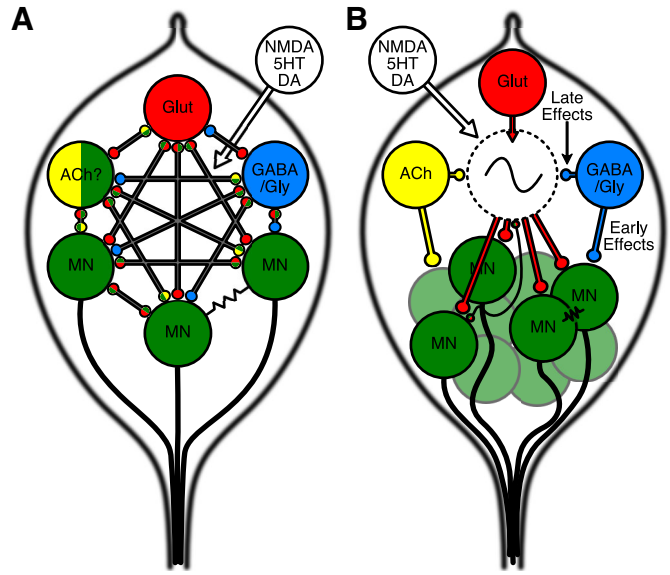

Fig. 4. Potential schematics of transplant circuits. *A*: bursting is produced by a subset of neurons within the transplant, possibly primarily by motoneuron (MN)-MN interactions. The circuit could be composed of an assortment of MNs and interneurons or only MNs. Modulation of the circuit is provided by glutamate, acetylcholine, and GABA/glycine. Cholinergic release may be from MN collaterals or cholinergic interneurons. Corelease of glutamate and acetylcholine from MNs is depicted by the red and green boutons. MNs may be electrically coupled. Exogenous NMDA, 5-HT, and DA provide a modulatory effect. *B*: a rhythmogenic circuit provides glutamatergic inputs to MNs. Modulation of this interneuron circuit is provided by extrinsic or intrinsic glutamate, acetylcholine, and GABA/glycine. Direct modulation by acetylcholine and GABA/glycine on MNs is also possible. The early effect of GABA/glycine blockade producing an increase in force without a change in burst frequency is shown as direct modulation of MNs. The late effect of GABA/glycine blockade is depicted as acting on the rhythmogenic interneuron circuit. Exogenous NMDA, 5-HT, and DA provide a modulatory effect. Intermotoneuron connections (electrical or chemical) could contribute to the activity seen, as could MN collaterals projecting to the rhythmogenic circuit.

It is known that basic elements for the formation of rhythmic motor circuits are present in these cultures. Despite the use of a differentiation protocol that leads to MN enrichment (Barberi et al. 2003; Lee et al. 2000; Peljto and Wichterle 2011; Westmoreland et al. 2001), a wide range of neuronal subtypes remains. The typical MN differentiation protocol involves the use of Sonic hedgehog and retinoic acid (Wichterle et al. 2002) and generates ~30% MNs and variable proportions of different interneuron types: glutamatergic (~5–19%), GABAergic (~12–23%), and glycinergic (~2–9%) (and ~23–34% glial cells) (Deshpande et al. 2006), that is, the neuronal types needed for fundamental circuit formation are present. We would thus suggest that the interpreparation variability in bursting behavior is explained by differences in the proportions and connectivity patters of the neuron types in the transplants, that is, the present neuron types together form an "emerging" circuit capable of generating a rhythm (Fig. 4*B*).

Embryonic spontaneous activity. Spontaneous activity is an essential component for the development of embryonic neural circuits (Blankenship and Feller 2010; Marder and Rehm, 2005) and is involved in various roles, including neurite outgrowth (Metzger et al. 1998), maturation of electrical properties (Xie and Ziskind-Conhaim 1995), and 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, whereas 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 nonpurified ESCMNs, reinnervation is suboptimal: force recovery plateaus at 40-50%, 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 toward 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 further optimizing the activity in the transplants could facilitate enhanced reinnervation and improved functional outcomes.

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

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.M., V.F.R., and R.M.B. conceived and designed research; P.M. performed experiments; P.M. analyzed data; P.M., V.F.R., and R.M.B. interpreted results of experiments; P.M. prepared figures; P.M. drafted manuscript; P.M., V.F.R., and R.M.B. edited and revised manuscript; P.M., V.F.R., and R.M.B. approved final version of manuscript.

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