INTRODUCTION

Although it is well established that the cerebellum plays an important role in motor coordination and learning, the neuronal mechanisms underlying cerebellar function are only poorly understood. Historically, cerebellar research has focused mostly on eye movements, posture, and appendicular coordination. However, there is increasing evidence for a role of the cerebellum in the modulation of both respiratory and orofacial movements, the latter including movements such as coughing or whistling in humans, and fluid licking and rhythmic whisking in rodents (Bassal and Bianchi, 1982; Huang et al., 1993; Gozal et al., 1994; Welsh et al., 1986; Gruart and Delgado-García, 1992; Xu and Franklin, 2001; Chen et al., 2005; Dresel et al., 2005; Bosman et al., 2010; Bryant et al., 2010; Cao et al., 2012a; Farrell et al., 2012). These movements are generated largely by brainstem pattern generating circuits (e.g., Travers, 2004; Feldman and Del Negro, 2006).

The primary cerebellar output neurons are located in the deep cerebellar nucleus (DCN), which projects to a variety of areas, including thalamus, midbrain, and brainstem (Teune et al., 2000). The primary divisions of the DCN (medial or fastigial, interposed, lateral or dentate) can themselves be roughly classified with regards to motor control of body region, with medial DCN (mDCN) neurons chiefly concerned with medial body areas, including trunk, proximal limb, and head (Sugihara, 2011). Neurons in the mDCN have been shown to project bilaterally to at least two sites in the brainstem, the dorsally located vestibular nuclei, as well as to the ventromedial reticular formation (RF). Studies with transgenic mice indicate that contralateral mDCN brainstem projections are excitatory (glutamatergic), whereas ipsilaterally projecting neurons are inhibitory (glycinergic; Bagnall et al., 2009). There is strong evidence that the cerebellum modulates respiratory-related motor activity via projections of mDCN neurons to various medullary or pontine regions (Lutherer and Williams, 1988; Gruart and Delgado-García, 1992; Xu and Franklin, 1995, 1997, 2000; Zhang et al., 1999). However, the pathways by which the cerebellum engages brainstem centers controlling other orofacial movements such as licking or whisking is not known. The fact that these movements are widely represented in Purkinje cell activity in the cerebellum (Welsh et al., 1995; Bryant et al., 2010; Cao et al., 2012a) and coordinated with breathing (Welzl and Bures, 1977; Weijnen et al., 1984; Cao et al., 2012b; Deschênes et al., 2012) suggests the possibility of a common pathway with respiratory control from DCN to brainstem.

We investigated this pathway in inbred C57BL/6J (B6) mice by combining neuroanatomical and physiological approaches. Neuronal tracing studies were performed in order to carefully delineate the projection of mDCN neurons to brainstem locations associated with the generation of respiratory rhythm and mystacial whisker movements. We also recorded single unit activity of mDCN neurons in awake and behaving mice in order to determine
whether and how whisker and respiratory behaviors is represented in the activity of these neurons.

MATERIALS AND METHODS

ANIMALS

Data were collected from male and female adult C57BL/6J mice (18–30 g). Animals were maintained in standard cages in a temperature- and humidity-controlled vivarium on a 12-h light/12-h dark cycle, and were given ad libitum access to normal dry pellet food (22/5 rodent diet, Harlan Teklad, Madison, WI, USA) and water. The Animal Care and Use Committee at University of Tennessee Health Science Center approved this study, and all experiments were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996.

TRACT-TRACING EXPERIMENTS

Surgery and groups

Mice were divided into anterograde and retrograde tracing groups. Prior to surgery for tracer injections mice were anesthetized (i.p. injection) with ketamine/xylazine (100/10 ml/kg) and positioned in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The scalp was opened with a midline incision, and the skull was leveled in a stereotaxic frame (Stoelting, W ood Dale, IL, USA) or either red and green latex micro-
tive to bregma. For injection of retrograde neuronal tracers, a
= =
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DAB labeling, ranging from about 120 μm caudal to the central
and cerebellum sections were examined via light microscopy for
ing was necessary. Sections were rinsed, mounted, air-dried, and
coverslipped on silane-coated slides with DPX mountant for histology (Fluka).

The retrograde tracers used in this study could be visualized using fluorescent microscopy, so no further histological process-
Analysis of histological material

Anterograde tracing: For each mouse (n = 6), brain-
stem/cerebellum sections were examined via light microscopy for

BDA injection site was found in each case to be confined to the

as 1–2% isoflurane during surgery using an Ohio Isoflurane

ELECTROPHYSIOLOGY EXPERIMENTS

Surgery

Mice (n = 8) were anesthetized initially with 3% isoflurane in oxygen in an incubation chamber. Anesthesia was maintained with 1–2% isoflurane during surgery using an Ohio Isoflurane vaporizer (Highland Medical Equipment). The depth of anes-
thesis was adjusted until the mice failed to show a withdrawal

rectal temperature was

medial RF (anteroposterior = −5.88 mm, mediolateral = 0.8 mm, and dorsoventral = −5.75 mm) or caudal ventro-
medial RF (anteroposterior = −7.08 mm, mediolateral = 1.0 mm, and dorsoventral = −6.2 mm). BDA and FG were injected via
iontophoresis (Precision Current Source, Stoelting Co., W ood Dale, IL, USA), at 2 μA (cycle 8 s ON and 8 s OFF), for a total of 10 min. Microscopes were pressure injected (60 nl) via a Picopette (Parker Hannifin Corp., Cleveland, OH, USA). The injection pipette was left in place for 10 min before and after the injection was made. Supplemental anesthetic was administered as necessary throughout the surgery to maintain the animals under
depth anesthesia.

Tissue preparation and imaging

After a 3-day survival period, mice were perfused transcardially
spheres (Lumafluor, Durham, NC, USA) was lowered into either
LLC, Denver, CO, USA) or either red and green latex micro-

Tissue preparation and imaging

After a 3-day survival period, mice were perfused transcardially
with phosphate-buffered saline and 4% paraformaldehyde. The brains were removed and placed in 4% paraformaldehyde for 1 day and then transferred to a 30% buffered sucrose solution and stored at 4°C for at least 1 week. Coronal sections (40 μm, every other section) were cut serially using a freezing microtome.

For visualizing BDA, sections were rinsed, followed by pre-
treatment with 3% H2O2 and 0.4% Triton X-100. Sections were then rinsed again and incubated in avidin-biotin complex (ABC)
solution prepared with the Vectastain ABC Elite kit (Vector Lab-

inhibitory interneurons of the dentate gyrus (DG) was assessed with a

3,3’-diaminobenzidine (DAB; Vector). The sections were then
rinsed, mounted, air-dried, and coverslipped on silane-coated slides with DAB mounting media for histology. All histological material was visualized and imaged using a Leica (DMRXA2, Leica Microsystems, Bannockburn, IL, USA) episcopic-fluorescence microscope equipped with a digital cam-
era (Hamamatsu ORCA-ER, Hamamatsu Corp., Shizuoka, Japan) and imaging software (SimplePC3, Hamamatsu Corp., Shizuoka, Japan).

Analysis of histological material

Anterograde tracing: For each mouse (n = 6), brain-
stem/cerebellum sections were examined via light microscopy for

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Lu et al. Medial cerebellar nuclear projections
Access to water in the home cages was restricted 12 h before elec-
trode post as reference. (5 stored to the same data file for off-line analysis.
Licking behavior was detected off-line to mark the timing of whisker movements using
the beam-break resulted in a brief bi-phasic potential that was
the face all but one whisker (the C4 or C3) was cut. A light-beam
sensor (Coulbourn Instruments) was placed beside the mouse’s

Electrophysiology and behaviors
Access to water in the home cages was restricted 12 h before elec-
trophysiological experiments. All experiments were performed
during the light cycle. During experiments, the mouse’s head
was fixed to a metal holder and the body was loosely cov-
ered with a plastic tube to limit body movements (Bryant et al.,
2009). The recording chamber was cleaned and filled with Ringer’s
solution. Up to seven recording electrodes (glass-insulated tung-
sten/platinum, 80 μm O.D., impedance, 3–7 MΩ) were inserted
into the mDCN using a computer-controlled micromanipula-
tion (System Eckhorn; Thomas Recording). Neurons in this subnucleus
were identified based on their location (bregma –6 to 6.5 mm,
0.5–1 mm lateral from midline, depth 2–2.5 mm) and firing char-
acteristics. The raw signals were bandpass filtered (280 Hz to
8 kHz) and amplified using a filter amplifier (FA32; Multi Channel
Systems). Filtered and amplified voltage signals were digitized and
stored on a computer hard disk (16 bit A/D converter; sampling rate, >20 kHz) using a CED power1401 and Spike2 software (both
Cambridge Electronic Design).

Respiratory behavior was monitored with a thermistor (Mea-
surement Specialties) placed in front of one nostril. Breathing
cycles were measured as increases and decreases in instantaneous
caused by exhal and inhale movements, respectively. Raw lacing
or respiratory signals were digitized at 1 kHz. On the left side of
the face all but one whisker (the C4 or C5) was cut. A light beam
sensor (Condor Instruments) was placed beside the mouse’s
head at the level of the nose so that the whisker would break
the light-beam during large-amplitude protraction movements.
The beam-break resulted in a brief bi-phasic potential that was
detected off-line to mark the timing of whisker movements using
a threshold procedure (Cao et al., 2012b). Licking behavior was
monitored with a piezo-resistive sensor, which created a voltage sig-

Electrophysiology data analysis
Single-unit DCN neurons were identified based on sustained spon-
taneous firing rate (8–50 Hz) and the location of lesions. Peaks and
troughs in the air temperature recordings corresponded to the
ends of expiration and inspiration cycles, respectively. The end-
of-expiration times were marked with a peak-detection algorithm
and used as temporal aligns for correlation analyses. The times
of whisker protraction movements were detected using a fixed
voltage threshold. Analyses of cross-correlations between mDCN
neuronal activity and behavior were performed using the Spike2
software (CED, Cambridge, UK). Z-scores >2 were considered to
be significant. We used the end-of-expiration times as the temporal
align for respiratory behavior.

RESULTS
Neural tracing
Injections of the anterograde tracer BDA into the mDCN of six B6
mice revealed that neurons in this subnucleus project bilaterally
primarily to two areas in the medulla: the complex of vestibular
nuclei, located dorsally (MVe and SpVe, respectively), and medial
and ventromedial portions of the RF (Figure 1). Staining appeared
both as punctate varicosities, which appear to be terminal-like
arborizations, as well as linear profiles, indicative of axonal label-
ing. Even though in each case the injection was made at roughly
the same rostral–caudal level of the DCN, at least some terminal-
like labeling was typically found in the brainstem from the most
rostral to the most caudal section examined (range about 5.8 to
7.6 mm from bregma). In the RF labeling was restricted to
medial and ventromedial regions, and was stronger on the con-
tralateral side. At more rostral levels, this was predominantly in
the gigantocellular nucleus (Gi), but also in the lateral paragigan-
tocellular nucleus (LPGi). Terminal labeling continued caudally in
the ventral medullary reticular nucleus (MdV) and medial portion
of the lateral reticular nucleus (LrV).

We next examined labeling of neuronal cell bodies in the DCN
following injection of a retrograde tracer (FG) in the ventrome-
dial RF either at rostral (n = 6) or caudal (n = 6) levels. These
injections were targeted toward the medial RF (Gi) and varied
somewhat along the dorsal–ventral axis of the coronal plane, often
invading the LrV (caudally) and LP Gi (rostrally). In each case,
robust neuronal labeling was found in the contralateral mDCN
following brainstem injection (Figure 2). At caudal levels, the
mDCN can be divided into a medial portion (Med) and the
dorsolateral hump (MedDL); labeling was often found in both
parts. In most cases, at least some labeled neurons were also
found in the medial part of the contralateral interposed (Intp)
nucleus. Ipsilaterally, fewer labeled neurons were found in any
DCN subnucleus, including Med and MedDL. {[F(1,6) = 77.42;,
p < 0.0001]. In the mDCN, many of the FG-labeled neurons
tended to be relatively large (20–30 μm along longest axis) and
stellate in shape, often with identifiable dendritic or axonal pro-
cesses (Figure 2D). Whether injections were placed at caudal or
rostral levels, FG-positive neurons were typically found at all levels
of the DCN; moreover, the injection groups did not significantly
diff er in number of labeled neurons (Figure 2G). This equivalency
may be in part due to the spread of tracer along the rostral–caudal
axis at the injection site. However, it may also reflect potential
FIGURE 1 mDCN neurons project to the contralateral reticular formation, as revealed by anterograde tracing. (A) Tracer injection site in the mDCN (Med, approximately −6.3 from bregma). (B) Low power image of labeling in the rostral brainstem in both the ipsilateral and contralateral Gi and LPGi, approximately −6.0 from bregma. At higher power, punctate labeling indicates terminal fields in contralateral Gi (C); relatively fewer terminals are found on the ipsilateral side (D). The level for (E, F) is approximately −6.8 from bregma. (E) Low power image of labeling in the caudal brainstem, in the contralateral MdV and LRt, approximately −7.3 mm from bregma. (F) Labeling in the contralateral vestibular nucleus, approximately −7.0 from bregma. With the exception of (F), all images were prepared from a single mouse brain. Scale bars: (A, B, E, F) = 200 μm; (C, D) = 100 μm. Arrows point to examples of terminal/axonal labeling in lower power images.

uptake of FG in mDCN axons passing through the injection site (e.g., Dado et al., 1990).

It is also possible that individual mDCN neurons may collateralize to both caudal and rostral brainstem levels. To examine this possibility, we injected two different tracers (red and green fluorescent latex microspheres) at both rostral and caudal levels in the same animal (n = 3; Figure 3; Table 1). An advantage of microsphere tracers is that uptake by fibers of passage is minimal (Katz et al., 1984; Apps and Ruigrok, 2007). Injection of microspheres into rostral and caudal levels of the brainstem resulted in a number of double-labeled mDCN neurons in each case, evident at several levels of the mDCN (Figure 3E). It is germane to point out that the injections shown in Figures 3A, B also varied along the dorsal–ventral axis, with the caudal injection placed more dorsally. This result indicates that a subset of individual cells in this nucleus projects to multiple levels, and potentially different functional areas of the ventromedial RF. As with FG, labeled cells projecting to both brainstem levels co-mingled within the mDCN along its rostral–caudal axis. Overall, fewer retrogradely labeled cells were counted in the mDCN and IntP after microspheres injection relative to FG, likely due to differences in tracer efficacy and injection size. However, the general patterns were similar, and at least some double-labeled cells were found in each subnucleus (16–30% relative to total labeled cells).
We recorded spike activity from 11 mDCN single units while we next investigated whether or not DCN single-unit spike activity was related to orofacial and respiratory movements (Figure 4). We found that a subset of mDCN neurons represent more than one of these behaviors.

**DISCUSSION**

Spike activity from 11 mDCN single units was recorded while undergo-ing respiratory and orofacial movements. The activity of 11 mDCN neurons revealed strong correlations with both orofacial (licking and whisking) and respiratory behaviors, with average peak correlation Z scores of 0.88 (mean ± SD, whisking range 2.06–3.0), and for breathing of 2.53 ± 0.44 (range 2.06–3.0). Collectively, these physiological data reveal that respiratory and orofacial movements are widely represented in the spike activity of mDCN neurons and that a subset of mDCN neurons represents more than one behavior.
FIGURE 3 | A subset of mDCN neurons collateralize to rostral and caudal locations in the brainstem. (A,B) Plots of injection sites of rostral (red) and caudal (green) fluorescent microspheres in brainstem. (C) Retrogradely labeled mDCN neurons appear green or red, respectively; a subset (yellow) is double-labeled. Labeling is confined to the cell body. (D) High power picture of labeling, showing that double-labeled cells (arrows) could be reliably discriminated from single-labeled cells. Approximate level is −6.4 mm from bregma. (E) Plots of cells in the mDCN at rostral to caudal levels in a single animal (left to right); a subset of cells at each level are double-labeled (blue circles). Red and green circles correspond to cells labeled by rostral and caudal injections, respectively. Approximate rostral-caudal levels are −6.2, −6.4, and −6.6 mm from bregma (left to right). Scale bars: (C) = 100 mm; (D) = 50 mm. Atlas sections from Paxinos and Franklin (2001).

stellate-shaped neurons, similar to large projection mDCN neurons previously described (Bagnall et al., 2009). Further studies with single-cell labeling techniques may yield more precise information on the specific projection topographies of individual mDCN neurons.

THE mDCN ENGAGES BRAINSTEM SUBSTRATES FOR RESPIRATION AND OROFACIAL MOVEMENTS

A complex grouping of respiratory-related circuits, including pacemaker and premotor neurons, can be found located along the rostral-caudal extent of the brainstem in the ventral respiratory column (vVRC) in the brainstem (Feldman and Del Negro, 2006; Smith et al., 2009). However, the mDCN may not engage
FIGURE 4 | Raw data example of DCN spike activity correlated with licking, whisking and respiratory behavior. (A) Raw data examples of single-unit DCN spike activity with licking, whisking and respiratory behavior. Under each trace are time markers marking the tongue-to-spout contact times for the licking trace, the time of whisker beam crossing for whisking trace, the end-of-expiration and inspiration times for the respiratory trace, and spike activity for the DCN spike train recording, respectively. (B) Histograms showing the inter-lick interval, inter-whisking interval, expiration-expiration interval, and inter-spike interval distribution. (C) Histograms showing the cross-correlation between DCN spikes with licking, whisking, and expiration events.

FIGURE 5 | Summary of behavior-spike cross-correlation results expressed as Z-scores of peak correlation values for breathing, whisking, and licking behavior (left, middle, and right column, respectively). Each symbol represents one mDCN unit’s Z-score for each behavior. Only Z-scores > 1 are shown. Spike activity of all 11 units was significantly correlated (Z-scores > 2, dashed horizontal line) with either breathing or whisking. Activity of five units was correlated with respiration alone, one unit’s activity was correlated with whisking alone, and the remaining five units had spike activity correlated with both breathing and whisking. One unit (red circle) had spike activity significantly correlated with all three behaviors. Box and whisker plots show median, 25th and 75th percentile (box) and 9th and 91st percentile (whiskers).

these pacemaker areas directly. The rVRC occupies a ventral and lateral position in the medulla and pons (Alheid et al., 2011), a region that did not include terminal labeling from the mDCN in the current study. Moreover, careful examination (via lesion) of certain respiratory sites in the medulla and pons by Zhang et al. (1999) indicate that neurons in a key respiratory rhythm-generating nucleus in the rVRC, the Bötzinger complex, are not necessary for mDCN modulatory effects on respiration. Instead, lesions of the Gi disrupted mDCN-dependent mediation of respiratory timing. Trace studies suggest that Gi neurons innervate respiratory premotor or motor neurons (Bystrzycka and Nail, 1983; Dobbins and Feldman, 1994), and respiratory-related neurons have been recorded from in this area (Vzaimnykh et al., 1980). Our data confirms that the projection from mDCN to this area of the brainstem is stronger on the contralateral side; Bagnall et al. (2009) posit that that contralateral projections to the ventromedial brainstem are glutamatergic and therefore excitatory, whereas the smaller (ipsilateral) projections are glycinergic and inhibitory. This reciprocal pattern suggests a mechanism for bilateral coordination.

The location of premotor or rhythmic-generating circuits for other orofacial movements are far less defined than for respiration. Injection of retrograde tracers into the primary motor nuclei for licking and whisking (hypoglossal and facial nuclei, respectively) produce labeling in the RF including the Gi and MdV, but also the MdD, parvocellular (PCR) and intermediate (IR; Travers and Norgren, 1983; Hattox et al., 2002). For licking, evidence suggests a substrate for rhythmic licking organized among premotor neurons in the RF: neurons rhythmically active during licking can be found in both the MdD and dorsal medullary reticular nucleus (MdD)
at the level of the hypoglossal nucleus (Travers et al., 1997), as well as in the PCrIs, IRs, and GI divisions, more rostrally. For whisking, serotonergic inputs to vibrissa motor neurons (VMNs) play a key role in the pattern generation, with a necessary role for the motor neurons themselves in rhymogenosis (Hattori et al., 2003; Cranmer et al., 2007). The highest density of serotonergic premotor inputs to VMNs is found in the PGI, and we show here that the LPGi receives input from the mDCN. Although there may be overlap between substrates for licking and whisking there is also likely a degree of rostral-caudal separation, based on proximity to the primary motor nuclei, with the premotor substrate for whisking located more rostrally than that for licking. Respiration, on the other hand, appears to be widely represented in the same areas of the brainstem as licking and whisking. Overall, the specific organization of function within the GI or other RF areas is not well understood.

CEREBELLAR ROLE IN THE CONTROL OF RESPIRATORY AND OROFACIAL MOVEMENTS

There is increasing evidence that the cerebellum plays an important role in the modulation of respiration and other types of orofacial movements, such as mastication, licking, swallowing, and whisking (Williams et al., 1986; Velich et al., 1995; Xu and Frazier, 2000; Suzuki et al., 2001; Bryant et al., 2010). All of these are rhythmic movements controlled by brainstem central pattern generating circuits. It is fairly well established that the cerebellum is involved in the control of respiration, especially with regards to respiratory chemoreception and adaptation of respiratory frequency (Huang et al., 1993; Xu et al., 1994, 2001; Xu and Frazier, 1995, 1997, 2000). Other brainstem-controlled rhythmic orofacial movements with well-documented cerebellar involvement are fluid licking and whisking movements in rodents. Both complex and simple spike activity in Purkinje cells are highly correlated with licking movements in rats and mice (Velich et al., 1995; Bryant et al., 2010; Cao et al., 2012a). Bosman et al. (2010) demonstrated that Purkinje cell complex and simple spike responses differ between different aspects of mystacial whisker movements in mice. In mice whisking and respiratory movements are correlated in a complex dynamic manner, suggesting that active acquisition of orofacial (sniffing) and tactile (whisking) sensory information is well coordinated rather than independent processes (Cao et al., 2012b). Other orofacial movements such as mastication and swallowing are also coordinated with respiration (Gestreau et al., 2005). Clinical evidence suggests a role for the cerebellum in the control of swallowing movements: patients with cerebellar disease or damage often have difficulties in swallowing (dyphagia; Ramio-Torrente et al., 2006).

The neuronal mechanisms involved in most of the above cited coordination tasks have thus far received little attention and are only poorly understood. However, the anatomical and electrophysiological findings reported here support a crucial involvement of the cerebellum in the coordination of orofacial and respiratory movements. Our tracing studies suggest that mDCN neurons project to brainstem substrates of orofacial and respiratory movements, and significantly, that individual neurons may project to more than one area, commensurate with the representation of multiple movements in individual mDCN neurons. Collateralization of individual DCN neurons to multiple regions has been noted previously (Bentivoglio and Kuppers, 1982; Bentivoglio and Molinari, 1986). This projection pattern might serve to provide simultaneous cerebellar control of neuronal activity at two or more target sites.

As discussed above, there is increasing evidence for a cerebellar involvement in the coordination of brainstem generated orofacial and respiratory movements. Our electrophysiological and anatomical findings suggest a plausible neuronal substrate for a cerebellar modulation and coordination of brainstem neuronal activity. The anatomical density and complexity of the brainstem circuits poses a major challenge to more detailed investigations of the system. This challenge might best be addressed with evolving techniques of single-cell expression profiling, which have been successfully used genetically identify functionally different classes of neurons (Kodama et al., 2012).

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Fncir-07-00056 — 2013/3/29 — 11:50 — page 8 — #8
Medial cerebellar nuclear projections

Lu et al. • Medial cerebellar nuclear projections

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April 2013 Volume 7 Article 58 3

"fncir-07-00056" — 2013/3/29 — 11:50 — page 9 — #9

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential con-
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