UNIVERSITY OF CALIFORNIA, SAN DIEGO

Features of neurodevelopmental disorders are induced by deletion of mGluR5 or Densin in parvalbumin-positive interneurons

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by

Aaron James Kappe

Committee in charge:

Professor Terry Sejnowski, Chair
Professor Jill Leutgeb
Professor Gregory Light

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ABSTRACT OF THE THESIS

Features of neurodevelopmental disorders are induced by deletion of mGluR5 or Densin in parvalbumin-positive interneurons

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Aaron James Kappe

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Professor Terry Sejnowski, Chair

Alteration in ionotropic glutamatergic transmission onto developing inhibitory systems has been proposed as an underlying mechanism in the pathogenesis of several neurodevelopmental disorders, including schizophrenia and autism. In particular, accumulating evidence shows that alteration in the development of parvalbumin-positive (Pv+) fast-spiking interneurons can produce long-term neurophysiological and behavioral

disruptions. Here we show that separate postnatal ablations of the metabotropic glutamate receptor 5 (mGluR5) and the scaffolding protein Densin-180 (Densin) from Pv+ interneurons alter stimulus-evoked neuronal responses, oscillatory cortical dynamics and sensorimotor information processing. This implies that multiple alterations in the development of Pv+ neurons can produce both convergent and divergent neurophysiological and cognitive consequences. Together, these results suggest a fundamental role for mGluR5 and Densin in the development of Pv+ neurons, and provide a novel mechanism by which alterations in glutamatergic transmission onto this inhibitory circuitry can modify brain activity in ways that resemble specific features of neurodevelopmental disorders.

INTRODUCTION

Neurodevelopmental disorders (NDDs) involve impaired development of the brain and its circuitry and comprise several prevalent disorders including autism and schizophrenia. Autism features a variety of symptoms, often including impairments in social interactions and relationships, verbal and nonverbal communication, and stereotyped repetitive behaviors such as hand-flapping or rocking (Elsabbagh et al. 2012; American Psychiatric Association, 2013). Autism is usually apparent by age 3, and has an estimated prevalence of 0.6% in the global population according to recent reviews (Elsabbagh et al. 2012; Fombonne 2009). Schizophrenia is increasingly recognized as a neurodevelopmental disorder (Weinberger 1995; Insel 2010) with a worldwide prevalence of 1%. It is commonly recognized by the adult-onset of symptoms such as social or vocational dysfunction, cognitive deficits, and psychosis, including delusions and hallucinations (McGrath et al. 2008). Despite apparent differences between autism and schizophrenia, both these NDDs have cognitive and interpersonal deficits along with a strong genetic underpinning. Disruptions in inhibitory circuitry are thought to be a crucial bridge between the two (Carroll and Owen 2009; Dalton et al. 2005; Keefe and Fenton 2007; Lewis et al. 2012; Lisman et al. 2008).

At the cellular level, converging evidence shows that a subpopulation of GABAergic neurons, fast-spiking interneurons expressing the calcium-binding protein parvalbumin (Pv), underlies alterations in cognition and oscillatory activity observed in autism and schizophrenia (Bartos et al. 2007; Behrens and Sejnowski, 2009; Lewis

et al. 2005; Sohal et al. 2009; Marin 2012). Pv itself serves as a neurochemical marker for these neurons. Alterations in Pv expression characterize both autism and schizophrenia, as indicated by measurements in *post mortem* brains of human patients. In both these NDDs, Pv is significantly reduced in the prefrontal cortex, a key brain region implicated in regulation of executive function and social behavior (Reynolds et al. 2001; Zikopoulos and Barbas 2013). In the hippocampus, a region important for learning and memory, Pv is increased in subjects with autism and decreased in subjects with schizophrenia (Lawrence et al. 2010; Torrey et al. 2005). Pv+ interneurons are also critically involved in generation of cortical gamma (30-100 Hz) oscillations: inhibition of Pv+ interneurons suppresses gamma oscillations *in vivo*, whereas stimulation of these interneurons produces emergent gamma-frequency activity (Sohal et al. 2009). Normal gamma-oscillatory activity is thought to integrate distributed neural activity and enhance information transmission between cortical areas, with implications in functions including synaptic plasticity, local circuit synchrony, perception, attention, memory, and even consciousness (Uhlhaas and Singer 2010; Ward, 2003; Womelsdorf et al. 2007). Any deviation from normal gamma-oscillatory activity has implications for any or even all of these areas. Within NDDs, boys with autism have an excess of gamma activity that correlates positively with their developmental delay (Orekhova et al. 2007), whereas the amount and synchrony of gamma oscillations is characteristically reduced in schizophrenia during cognitive tasks and at rest (Uhlhaas and Singer 2010). Because both schizophrenia and autism appear to involve impairments distributed across multiple cortical regions, recent theories have connected the alterations in normal gamma-oscillatory activity

present in these NDDs and their pathophysiology (Uhlhaas and Singer, 2010; Marin 2012; Gandal et al. 2010; Gogolla et al. 2009). Understanding how this gamma-oscillatory activity is generated and disrupted might ultimately lead to an understanding of the pathogenesis of these prevalent NDDs.

Perturbations in the transmission of glutamate, the primary excitatory neurotransmitter in the brain, are known to disrupt Pv+ neuronal maturation and function and to produce behavioral features that resemble those of NDDs. In particular, alteration of the function of the glutamatergic receptor NMDA (NMDAR) via either downregulation, selective antagonists, or specific deletions has been strongly tied to both autism and schizophrenia (Gandal et al. 2012). The use of noncompetitive NMDA receptor (NMDAR) antagonists such as phencyclidine (PCP) and ketamine are known not only to produce schizophrenia-like symptoms in humans and exacerbate symptoms in schizophrenic patients, but also to produce several features of NDDs in rodents. In particular, auditory event-related potentials (ERPs) are similarly altered by NMDAR antagonists in humans and mice as determined by averaging the electroencephalographic (EEG) response to a stimulus over many trials (Amann et al. 2010). These drugs in humans reproduce sensory processing deficits as determined by a characteristic reduction in the negative 100 ms amplitude component (i.e. N100) seen in schizophrenia (Turetsky et al. 2008, Umbricht et al. 2000). This is matched by reductions in the corresponding N40 component in mice (Amann et al. 2010; Ehlers et al. 1992; Maxwell et al. 2006). The injection of NMDAR antagonists during the perinatal period leads to a loss of Pv expression, perturbs the maturation of

these neurons, and reduces overall power in the gamma range, results that strongly resemble those seen in schizophrenics (Powell et al. 2012).

Additional support for the role of NMDARs in Pv+ neurons in particular comes from studies in which NMDARs have been selectively deleted in Pv+ neurons, resulting in reductions in Pv+ neurons, loss of their inhibitory phenotype, and a reduction in the number of their postsynaptic contacts. This translated to alterations in gamma synchrony, an increase in repetitive behaviors, and deficits in prepulse inhibition (PPI) (Belforte et al. 2010; Carlén et al. 2012; Korotkova et al. 2010; Saunders et al. 2013; Gandal et al. 2012). PPI involves the reduction of response to a startling pulse when preceded by a weaker prepulse and is considered a measure of sensorimotor information processing that reflects the brain's ability to filter out unnecessary stimuli. PPI is characteristically reduced in schizophrenic patients (Braff et al. 2001; Amann et al. 2010), although alterations in PPI have been observed in autism as well (Perry et al. 2007; Madsen et al. 2013).

Two other proteins in the glutamatergic postsynaptic density (PSD), mGluR5 and Densin-180, are also implicated in NDDs, as their global knockouts reproduce characteristic features of NDDs. Activation of the metabotropic glutamate receptor mGluR5 positively modulates NMDAR activity at glutamatergic synapses (Awad et al. 2000; Pisani et al. 2001). Alterations in group 1 metabotropic glutamate receptors including mGluR5 are believed to account for diverse neurological and psychiatric aspects of fragile X syndrome, the most widespread cause in autism related to a single gene (Bear et al. 2004). Additionally, the global knockout of the mGluR5 in rodents is known to produce profound deficits in PPI (Mansbach et al. 1989; Kinney et al. 2003).

The global knockout of Densin-180 (Densin), a scaffolding protein localized to the glutamatergic PSD with connections to NMDARs and mGluRs, also produces deficits in PPI and selectively reduces the amount of mGluR5 and the NDD-related scaffolding protein DISC1 in the PSD fraction (Carlisle et al. 2011, Kilpinen et al. 2008, Wexler & Geschwind 2011).

There is strong evidence of a role for mGluR5 and Densin in the development and function of inhibitory Pv+ neurons. In addition to the above alterations, the global knockout of Densin resulted in an altered spine morphology (Carlisle et al. 2011) and a reduction in numbers of Pv+ neurons (unpublished data from Marga Behrens), suggesting that the alterations such as the deficits in PPI might be driven by perturbations to Pv+ neurons. mGluR5 and Pv+ neurons are strongly linked, as mGluR5 is necessary for the development of synaptic plasticity in Pv+ neurons (Sahiri et al. 2008). While the global mGluR5 knockout elicited a robust PPI deficit, the selective ablation of mGluR5 from excitatory cortical glutamatergic neurons failed to produce alterations in PPI (Jew et al. 2013), indicating that proper sensorimotor function might rely on inhibitory neurons such as Pv+ neurons. To further examine the role of mGluR5 in Pv+ neurons, our group has previously shown that the selective postnatal deletion of mGluR5 from Pv+ neurons reduces the number of these neurons that reach adulthood and the number of synaptic contacts they form on the cell body of the excitatory postsynaptic neurons, suggesting decreased inhibition and altered oscillatory activity in these mice (Sejnowski et al. 2010). Despite the strong, independent linkage of NDDs to alterations in Pv+ neurons, mGluR5 and Densin, the exact relationship between the activity of mGluR5 and Densin and the function of

these Pv+ neurons is currently unknown. Here I seek to examine the role of mGluR5 and Densin in Pv+ interneurons with regard to broader electrophysiological and sensorimotor function, and how these functions might relate to the pathophysiology of NDDs. To this end, our group has selectively deleted mGluR5 and Densin separately from Pv+ neurons in mice (Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$, respectively), recorded auditory ERPs, characterized changes in oscillatory activity, and measured PPI of the startle response.

METHODS

Generation and characterization of Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice

All animal procedures were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and were approved by the Salk Institute for Biological Studies and the University of California San Diego Institutional Animal Care and Use Committees. mGluR5-floxed (Xu et al. 2009) and Densin-floxed (Carlisle et al. 2011) mice were backcrossed to C57BL/6 for at least six generations, and then separately crossed to a Pv-Cre line to generate mGluR5-deficient and Densin-deficient mice carrying the deletion only in Pv-neurons (Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$, respectively). Cre recombination in the cortex of the Pv-Cre line begins at approximately postnatal day 10 when parvalbumin expression starts, and remains stable once it reaches a plateau at approximately 4 weeks of age (Carlen et al. 2011). Effective Cre-mediated recombination in Pv^{+} neurons for Pv-mG5$^{-/-}$ was previously assessed by crossing the Pv-Cre line with mice carrying Cre-dependent tdTomato fluorescence (Ai14 line, Jackson Laboratories). mGluR5-floxed and Densin-floxed animals were used as the controls for each selective deletion.

Headstage Construction and Implantation

A 12-mm long electromyographic (EMG) wire made of stranded stainless steel and coated with nylon (diameter: 0.36 mm, PlasticsOne) was soldered into an electrode interface board (EIB-8, Neuralynx). While under isoflurane anesthesia, the EMG wire was positioned under the skin to record from the neck muscles. Epidural electrodes and the ground, all made of insulated stainless steel (diameter: 1/8 mm),

were also soldered into the electrode interface board and placed in craniotomies at a depth of 0.75 mm to collect epidural electrocorticography (ECoG) data. For Pv-mG5$^{-/-}$ and control mice, recording electrodes were placed in craniotomies in the right hemisphere at (1, 1) and (1.5, -1) in mm with regard to bregma and midline, respectively. Pv-Densin$^{-/-}$ and control mice, recording electrodes were placed bilaterally at (±1, 1), (±1.5, -1), and (±2, -3.5) and called the frontal, parietal and occipital channels, respectively. The ground electrode was positioned in the cerebellum at (0, -6). Three jewelry screws and dental acrylic fixed the implant onto the skull.

Following recovery of at least one week, implanted mice were connected to a pre-amplifier (HS-8, Neuralynx) with an interface cable to a slip-ring commutator (SL-88-10, Dragonfly) for recordings. ECoG and EMG signals were amplified and digitized (sampled at 1 kHz with 16-bit precision) by the Digital Lynx system (Neuralynx) and stored on a hard drive using Cheetah software (Neuralynx). For each recording session, animals moved freely within a 25x25 cm recording chamber. All animals were habituated to the chamber for 15 minutes before their first recording session, and no sedatives were applied before or during recordings. Recordings were obtained when the Pv-mG5$^{-/-}$ and their respective control (mGluR5-floxed) mice were 4-7 months old and when the Pv-Densin$^{-/-}$ and their respective control (Densin-floxed) mice were 5-8 months old.

Sensory Event-Related Potentials

For the recording of event-related potentials (ERPs), passive field speakers mounted on the ceiling of the recording chamber produced 10 ms Gaussian white-

noise "clicks" 25 dB above a 65 dB white-noise background every 2-3 seconds. 960 stimuli were presented in a 40 minute protocol stimulation for Pv-mG5$^{-/-}$ and control mice, whereas 1200 stimuli were presented in a 50 minute protocol stimulation for Pv-Densin$^{-/-}$ and control mice.

Recorded ECoG signals were analyzed offline in Matlab using custom-written software and the Chronux toolbox. The trials were event-locked to the stimulus, with the stimulus centered at 0 ms. All trials were baseline corrected by subtracting the median amplitude in the 100 ms before the stimulus from the post-stimulus amplitude. Individual trials with artifacts (>1 mV) and channels whose average showed no evoked response were rejected. Bilateral channels in the Pv-Densin$^{-/-}$ and control mice were tested for symmetry by comparing each time point in a 300 ms period following the stimulus with a Wilcoxon rank-sum test. Bilateral channels were combined for all animals after no differences were found between the channels. The amplitude and latency of each corresponding ERP waveform component was quantified as the minimum amplitude during a 10-30 ms window (20 ms component) and 150-250 ms window (200 ms component) along with the corresponding latency, and as the maximum amplitude during a 20-60 ms window (40 ms component) along with the corresponding latency.

Event-Related Oscillatory Activity

Spectral decomposition was performed on the amplitude data using Matlab scripts developed in-house in conjunction with the Chronux toolbox. A strong 60 Hz noise was found in the Pv-mG5$^{-/-}$ and control recordings due to the presence of a video camera. This noise was avoided by bandpassing from 2-54 Hz. The camera was

removed for the recordings of Pv-Densin$^{-/-}$ and control mice, and the corresponding 60 Hz noise disappeared. These recordings were bandpassed from 2-100 Hz. Normalized baseline power was determined by dividing the power in each frequency band by the sum of baseline power across frequencies (Carlen et al. 2011). This ratio was computed for each trial, converted to a percent, and averaged across trials for each animal. The evoked power gain from baseline, accounting for the phase-locked changes in oscillatory activity pre- and post-stimulus, was determined by dividing the power of the averaged ERPs in the 500 ms post-stimulus onset by the baseline power in a 500 ms window immediately preceding the stimulus. This ratio was converted to decibels by taking the base-ten logarithm and multiplying by ten. The total baseline power per trial was used in the evoked power gain rather than the corresponding evoked baseline power, which approaches zero as the number of trials increases. The induced oscillatory activity, referring to those oscillations not phase-locked to the stimulus, was also calculated by subtracting the evoked power from each trial of the post-stimulus power before converting again into a baseline power gain for each trial.

Principal component analysis (PCA) was performed on these two power gain measures following channel concatenation. Leave-one out cross-validation (LOOCV) was implemented on the resulting principal components by leaving out a single animal in each loop of the cross-validation and averaging the resulting success across all animals. This LOOCV used the first principal component (PC) and successively through each additional PC such that the final LOOCV included all possible PCs. The optimal number of PCs separating the genotypes was then determined along with the corresponding cross-validated success rate. LOOCV was also performed for each 2 Hz

frequency band to determine the power gain frequency bands that best separated the genotypes per channel.

Assessment of prepulse inhibition of acoustic startle response via EMG

For the recording of the startle response and prepulse inhibition (PPI) via EMG, a startle stimulus, a prepulse stimulus, and a combination of the two (prepulse + startle) were each presented 333 times in pseudo-random order during a single 50-minute recording session and overlaid a continuous 64 dB white-noise background. Startle trials consisted of a 50 ms 94 dB startle pulse of uniform white noise. Prepulse trials consisted of a 50 ms, 70 dB noise prepulse. Prepulse + startle trials consisted of the prepulse followed by the startle pulse separated by 100 ms onset to onset. Latency between trials was 2.5-3.5 s.

Recorded EMG signals were analyzed offline in Matlab. The EMG amplitude data were rectified then smoothed with a 10 ms span using a zero-phase digital filter. Individual trials were defined as a 4 s time window with the startle onset beginning at 0 ms and the prepulse at -100 ms. Responses in startle trials and prepulse + startle trials were determined by the peak value within the 10-20 ms time window following the onset of the startle pulse for each trial. Recordings were rejected if the animal's averaged startle response was 2 standard deviations from the mean of all startle responses within its genotype. Baseline activity was determined as the median of the prepulse trials for each recording, while response to the prepulse was measured by median of a 100 ms time window beginning at the presentation of the prepulse. Recordings were rejected if the prepulse response was significantly greater than baseline activity as determined by the Wilcoxon rank-sum test ($p<0.05$). The percent

PPI was calculated as follows: [100-(peak prepulse + startle response/peak startle response) x 100].

RESULTS

Alteration of sensory processing in Pv-mG5$^{-/-}$ mice

The maturation of Pv+ interneurons begins postnatally and continues through early adulthood, occurring in parallel with temporal coordination by neural synchrony (Uhlhaas et al. 2009). Pv+ interneurons are involved in the generation of gamma oscillations, with alterations in resting-state and stimulus-evoked oscillatory activity observed in several mental disorders, notably schizophrenia (Uhlhaas and Singer 2010). Our group therefore reasoned that a disruption in the maturation of this inhibitory circuitry through the deletion of mGluR5 may lead to alterations in stimulus-evoked oscillatory activity, especially due to the importance of mGluR5 for normal synaptic plasticity in Pv+ interneurons (Sahiri et al. 2008). To test this, we assessed auditory event-related potentials (ERPs) in the frontal and parietal regions via electrocorticography (ECoG). Pv-mG5$^{-/-}$ mice showed specific alterations in the grand-averaged ERPs, including increased amplitude at 20 ms and 200 ms post-stimulus, and decreased amplitude at 40 ms (Fig. 1a, b). Males displayed a lower amplitude in the frontal 150-250 ms window compared to females [Sex, $F_{(1,70)}$=5.37, p<0.05] (data not shown). There were no alterations in latency between genotypes with sexes combined, with the exception of the 150-250 ms window where Pv-mG5$^{-/-}$ mice displayed a shorter latency in the frontal channel (Fig. 1c). Additionally, male mice displayed longer latency [Sex: $F_{(1,70)}$=4.02, p<0.05] for this component in the frontal channel (data not shown). No significant differences in latency were observed for the 150-250 ms window in the parietal channel. No Genotype effect was observed

in the 20-60 ms window in the frontal channel, but a Genotype × Sex interaction was evident for the 20-60 ms latency in the parietal channel [$F_{(1,68)}$=5.15, p<0.05], with female Pv-mG5$^{-/-}$ mice exhibiting a longer latency (p<0.05) (data not shown).

Power spectral analysis revealed no differences in normalized baseline activity (Fig. 2a), while the stimulus-evoked (i.e. phase-locked) power gain was increased in the delta-theta (2-10 Hz) and gamma frequency (40-54 Hz) bands for both regions (Fig 2b). We also observed increases in stimulus-induced power gain in the gamma band in both brain regions, and increases within the delta-theta band and beta band for the parietal and frontal regions, respectively (Fig. 2c). The separation between genotypes in induced power gain could be observed quantitatively via receiver operating characteristic (ROC) curves (Fig. 2d). The frequency bands that best separated the genotypes were the 22-24 Hz band in the frontal region (65% correct classification using leave-one-out cross-validation, see Methods) and the 34-36 Hz band in the parietal region (63% correct classification; Fig 2d). We used principal component (PC) analysis to integrate information from all frequency bands and channels, finding that the first seven PCs could achieve 79% correct classification performance in our cross-validated tests.

Fewer alterations in sensory processing observed in Pv-Densin$^{-/-}$ mice

Similar to mGluR5, Densin is a protein found in glutamatergic postsynaptic density (PSD), has connections to NMDARs, and reproduces distinct aspects of NDDs in the global knockout (Awad et al. 2000; Mansbach et al. 1989; Kinney et al. 2003; Carlisle et al. 2011). The scaffolding protein Densin also reduces the amount of

mGluR5 in the PSD fraction in the global knockout (Carlisle et al. 2011). To determine whether the postnatal deletion of Densin from Pv+ interneurons (Pv-Densin$^{-/-}$) reproduces features of NDDs and whether any alterations present resemble those observed in Pv-mG5$^{-/-}$ mice, I again assessed auditory ERPs in the frontal, parietal and occipital regions by ECoG. Pv-Densin$^{-/-}$ mice showed consistent alterations in the grand-averaged ERPs around 25 and 80 ms (frontal: 79-95 ms, parietal: 23-29 and 75-95 ms, occipital: 20-26 and 78-85 ms) (Fig. 3a). Notably, these amplitude alterations were not present at the 20 and 200 minima and 40 ms maxima as observed in Pv-mG5$^{-/-}$ mice (Fig. 3b). There was a significant increase in the latency of the 20-60 ms component in Pv-Densin$^{-/-}$ mice relative to controls, but no other latency differences between genotypes were seen in other channels or components. There was increased latency in males relative to females in the frontal 20-60 ms window [Sex, $F_{(1,57)}$=4.05, $p\leq0.05$] and in the 150-250 ms window with males decreased [Sex, $F_{(1,57)}$=6.67, $p<0.05$] and increased relative to females [Sex, $F_{(1,51)}$=6.53, $p<0.05$] in the parietal and occipital channels, respectively (data not shown).

Whereas Pv-mG5$^{-/-}$ mice showed no alterations in baseline activity, the Pv-Densin$^{-/-}$ mice displayed increased baseline power within the beta frequency band (12-30 Hz) consistent across all channels as well as decreased low frequency (2-6 Hz) power in the occipital channels (Fig. 4a). This increase in baseline beta power was also present in the normalized post-stimulus power (data not shown), indicating that the increase in beta power persisted irrespective of the presence of the stimulus. Computing the stimulus-evoked power gain, which represents the gain between prestimulus and post-stimulus power, revealed increased power still present in the beta

band in the parietal and occipital channels. Increased evoked power in the alpha (8-10 Hz) band was observed in the occipital channels, while no differences were seen in the frontal channels (Fig. 4b). The separation between genotypes in evoked power gain was be observed quantitatively via receiver operating characteristic (ROC) curves (Fig. 4d). The frequency bands that best separated the genotypes were the 14-16 Hz band in the frontal and occipital region (66% and 64% correct classification, respectively) and the 4-6 Hz band in the parietal region (63% correct classification) (Fig 4d). Using PC analysis to integrate information from all frequency bands and channels, we found that the maximum classification success rate was attained using the first 49 PCs (of 150 possible), achieving 81% correct classification performance in our cross-validated tests. The comparable classification performance of Pv-Densin$^{-/-}$ to Pv-mG5$^{-/-}$ mice is likely due to the increased number of frequency bands (2-100 Hz vs. 2-54 Hz), channels (6 vs. 2), and PCs (49 vs. 7) included. Differences in stimulus-induced activity were less consistent, with decreased gamma (38-42 Hz) activity seen in the frontal channels and increased low frequency (2-10 Hz) activity in the occipital channel (Fig. 4c).

Sensorimotor information processing disrupted after postnatal mGluR5 or Densin ablation from Pv+ interneurons

Prepulse inhibition (PPI) of the startle response, used as an index of sensorimotor information processing, is impaired in the global mGluR5 KO and global Densin KO mice (Brody et al. 2004; Carlisle et al. 2011). I assessed PPI via electromyography (EMG) recordings, such that the rectified amplitude of muscle

contractions in the neck could be visualized in millisecond resolution. The result was a characteristic increase in EMG amplitude 10-20 ms following the startle pulse, with a return to baseline before another increase in amplitude around 50 ms following the cessation of the startle pulse (Fig. 5a, 6a). The ratio of the startle response following the prepulse to the startle response confirmed the inhibiting effect of the prepulse during the 10-20 ms window, and to a lesser extent in the 50-80 ms window (Fig. 5b, 6b). In both Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice, this ratio was significantly smaller (p<0.05) relative to the ratio of their respective control mice at 12 and 25 ms. The Pv-mG5$^{-/-}$ mice also have a significantly decreased ratio (p<0.01) from 67-75 ms following the onset of the startle pulse. These differences between genotypes are broader across time in Pv-mG5$^{-/-}$ mice compared to Pv-Densin$^{-/-}$ mice, indicating a greater influence of the prepulse in the Pv-mG5$^{-/-}$ mice.

No alterations in peak startle response in the 10-20 ms window are present in either Pv-mG5$^{-/-}$ or Pv-Densin$^{-/-}$ mice with respect to their respective controls (Fig. 5c, 6c), although a trend towards increased startle is present in the Pv-Densin$^{-/-}$ mice [Genotype: $F_{(1,49)}$=3.29, p=0.076] that could become significant if 35 animals per genotype were used compared to the 24 and 29 used for the controls and Pv-Densin$^{-/-}$ mice, respectively. PPI was significantly increased in both Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice relative to their respective controls (Fig. 5d, 6d). The increased PPI in Pv-Densin$^{-/-}$ mice could result from the increased startle response, an effect more evident in the 10-20 ms window of Fig. 6a. However, this does not explain the robust increase in PPI evident in Pv-mG5$^{-/-}$ mice that show a more robust increase despite a smaller

sample size (Pv-mG5$^{-/-}$: [Genotype: $F_{(1,41)}$=5.95, p<0.05]; Pv-Densin$^{-/-}$: [Genotype: $F_{(1,49)}$=4.41, p=0.041]).

DISCUSSION

Inhibitory interneuron development plays a critical role in the postnatal maturation of brain function, with dysfunctional inhibitory circuitry hypothesized as a key mechanism in the pathogenesis of neurodevelopmental disorders (Marin 2012). Here, postnatal deletion of the glutamatergic postsynaptic density proteins mGluR5 or Densin from Pv+ interneurons each produced distinct functional alterations in neural circuitry, including deficits in stimulus-evoked neuronal responses, cortical oscillatory activity, and sensorimotor information processing. Pv-mG5$^{-/-}$ animals displayed altered ERPs in the 20, 40 and 200 ms components, with an amplitude reduction in the 40 ms component that corresponds to N100 deficits seen in autism and schizophrenia (Bruneau et al. 1999; Turetsky et al. 2008) and resembles the reduction in the 40 ms component caused by NMDAR antagonists such as ketamine (Maxwell et al. 2006). These alterations in auditory ERPs were accompanied by increased evoked and induced theta and gamma oscillatory activity. Pv-Densin$^{-/-}$ animals also displayed altered auditory ERPs, albeit with less robust alterations that were not around the extrema as observed in the Pv-mG5$^{-/-}$ animals. This translated to increased evoked beta, increased induced theta, and decreased induced gamma power as well as increased baseline beta power. Again, these cortical oscillatory changes were not as robust across channels relative to Pv-mG5$^{-/-}$ mice. Both Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice exhibited altered sensorimotor information processing in the form of increased PPI as measured by EMG. A brief summary of results of relevance to the neurodevelopmental disorders autism and schizophrenia is listed in Table 1.

The Pv+ inhibitory system is involved in the generation of neural synchrony (Sohal et al. 2009) and it is hypothesized that GABAergic deficits contribute to impairments in induced theta and gamma oscillations evident in schizophrenia (Uhlhaas and Singer 2010). Recent studies involving NMDAR ablation from Pv+ neurons resulted in increased spontaneous gamma oscillations and reductions in theta power (Carlen et al. 2012; Korotkova et al. 2010). The NMDAR antagonist ketamine yields similar findings when administered in acute doses (Lazarewicz et al. 2009), suggesting ketamine may act by blocking NMDARs on Pv+ cells (Nakazawa et al. 2012). Beyond NMDARs, I demonstrate here that the deletion of other glutamatergic postsynaptic proteins in Pv+ neurons, namely mGluR5 and Densin, also produce alterations in rhythmic activity, with altered ERPs and alterations in the theta, beta and gamma frequency bands. These findings indicate that manipulations disrupting ionotropic or metabotropic glutamate receptors or the associated scaffolding proteins on Pv+ neurons produce GABAergic impairment and altered oscillatory activity. This indicates that Pv+ inhibitory system dysfunction can lead to a variety of alterations in oscillatory activity, with gamma activity impaired with NMDAR and Densin ablation and enhanced in mGluR5 ablation.

Whereas alterations in event-related potentials and oscillatory activity were different between Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice, both groups showed increased PPI, a test that probes the brain's ability to attenuate responses to multiple stimuli. Increased PPI might reflect a hypersensitivity to incoming sensory information due to increased gamma-band activity, which is associated with heightened salience of information transfer (Sohal 2012). In support of this correlation, increased PPI and

gamma oscillations were recently observed in autism (Madsen et al. 2013; Orekhova et al. 2007). Conversely, schizophrenics display consistent deficits in PPI and gamma-oscillatory activity (Braff et al. 2001; Uhlhaas and Singer 2010). The increased auditory-evoked gamma activity observed here in Pv-mG5$^{-/-}$ mice also supports this hypothesis, although no alterations in evoked gamma were observed in Pv-Densin$^{-/-}$ mice. The novel method described here of measuring PPI in mice via subdermal EMG also allows for the simultaneous recording of cortical activity during PPI testing. The hypothesis that alterations in gamma activity are positively correlated with alterations in PPI can be empirically tested via this method.

There are two methods of assessing PPI and the startle response in rodents: the traditional method that uses a piezoelectric sensing platform and the novel method I have described using subdermal EMG recordings from the neck muscles. Increased PPI in Pv-mGluR5$^{-/-}$ mice as determined by the novel EMG method was verified by the traditional method with a very large sample size (55 control, 49 Pv-Densin$^{-/-}$) (Barnes et al., in review; for traditional method protocol, see Geyer and Swerdlow 1998). It should also be noted that the intensities of both the prepulse and startle pulse were higher than in the study presented here (74 vs. 70 dB and 120 vs. 96, respectively). Despite a larger, separate cohort and a more intense startle pulse and prepulse, Pv-mG5$^{-/-}$ mice tested via the traditional method showed increased PPI at a comparable level of significance to the cohort tested here [Genotype: $F_{(1,100)}=6.78$, $p<0.05$]. This suggests that this novel method of assessing PPI via EMG is a more sensitive measure of this phenomenon. At odds with this statement is the lack of PPI and startle differences found in Pv-Densin$^{-/-}$ mice using the traditional method (unpublished data

from Marga Behrens). However, the sample size used in this case was roughly half the size of the cohort used in this study (16 Pv-Densin$^{-/-}$ and 12 control compared to 29 Pv-Densin$^{-/-}$ and 24 control), and nearly four times smaller than the Pv-mG5$^{-/-}$ cohort that showed significantly increased PPI. Scaling up the Pv-Densin$^{-/-}$ cohort and testing via the traditional method would serve as a good test of the validity of this method, which also shows previously unobserved dynamics in the time domain such as a secondary response to the startle pulse around 50 ms (Fig. 5a, 6a) and consistent decrease in the knockout mice in the prepulse + startle/startle ratio at 25 ms following the initial EMG amplitude change (Fig. 5b, 6b).

Sensorimotor information processing as assessed by PPI is characteristically impaired in schizophrenic patients, and several reports indicate a similar impairment in autism spectrum disorders (Marin 2012). Our finding of increased PPI in both Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice was unexpected as the global mGluR5 KO and global Densin KO both had decreased PPI (Brody et al. 2004; Carlisle et al. 2011). Interestingly, mGluR5 deletion from excitatory cortical neurons showed no effect (Jew et al. 2013). This increased PPI result is not unique in the literature. Increased PPI was recently observed in autistic patients when presented with low intensity prepulse stimuli, attributed to a heightened sensitivity in detecting stimuli of low salience (Madsen et al. 2013). Increased PPI was also observed in a mouse model of Rett syndrome (Chao 2010). This suggests in tandem with the results presented here that disruptions within the inhibitory system can be associated with increased PPI. However, PPI impairments resulted from functional ablation of NMDAR from corticolimbic GABAergic interneurons during early postnatal life (Belforte et al.

2010), and NMDAR ablation from Pv+ interneurons using a Pv-Cre line left PPI unaffected (Carlen et al. 2010). This disparity of PPI effects highlights that GABAergic mediation of sensorimotor information processing is complex and that the direction of PPI alteration is highly dependent on the manipulation used, the brain region affected and the developmental time point when the alteration occurs.

Our group has also examined the neurochemical, local circuitry, and behavioral consequences in these Pv-mG5$^{-/-}$ mice (Barnes et al., submitted for publication). We report GABAergic deficits as well as reduced inhibitory transmission as evidenced by the reduction in the frequency of mIPSCs. No alterations were found in the amplitude of the mIPSCs and in synaptic plasticity. This suggests that no reductions were present in the strength of individual Pv+ neuronal synapses despite a reduced number of Pv+ neurons and their postsynaptic contacts (Barnes et al., submitted for publication). With regard to behavior, these mice exhibited decreased recognition memory, increased compulsive-like behaviors and altered sensitivity to psychotomimetics or psychomotor stimulant agents. Interestingly, the global mGluR5 deletion impaired spatial memory (Lu et al. 1997), and the deletion of mGluR5 from pyramidal neurons decreased compulsive-like behaviors (Jew et al. 2013). This suggests that mGluR5-dependent manifestation of specific memory-domain impairments and compulsive-like behaviors rely on the neuronal population affected.

We also have preliminary results with Pv-Densin$^{-/-}$ mice for several behaviors, including nest building, novel object recognition, open field test, social interaction, and head tracking (unpublished data from Marga Behrens). However, only trends (p<0.1) were observed for time spent in the center in the open field test and for

alterations in sociability. While preliminary, these results are inconclusive as the sample size of mice used was small (5-8 mice per genotype and gender combination); a larger sample would be necessary to resolve whether behavioral alterations do in fact exist. In the global Densin deletion, Carlisle et al. found deficits in nest building and short-term memory as determined by altered object-place recognition and novel object recognition (2011). This suggests that Densin plays a role in nest building and short-term memory in non-Pv+ circuits.

Although much insight has been gained regarding the developmental and electrophysiological roles mGluR5 and Densin play within Pv+ neurons, several unanswered questions remain. There are several other oscillatory activity measures worth examining, including whether transient alterations in oscillatory activity exist in more discrete time points; these alterations might be lost by averaging across this 500 ms time window following stimulus onset. Theta-gamma coupling, thought to channel information from different sources into the hippocampus (Tort et al. 2009; Lee et al. 2012), is largely intact in schizophrenic patients, although the separate theta and gamma components are altered (Kirihara et al. 2012). Because I observe altered theta and gamma activity in these Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice, determining whether these mice also exhibit normal cross-frequency coupling would further supplement the electrophysiological characterization of these animals as a model of the NDD schizophrenia. Changes in the phase-locking factor degree (i.e. intertrial phase coherency) are also observed in NDDs such as schizophrenia (Roach and Mathalon 2008), and would be another valuable analytical technique to further characterize these mice as potential animal models of NDDs.

In summary, I have shown that postnatal ablation of mGluR5 and Densin from fast-spiking interneurons expressing Pv resulted in long-term functional alterations in neural circuitry. These findings demonstrate that disruption of glutamatergic postsynaptic density proteins in Pv+ neurons provides a novel mechanism for inducing core features relevant to several NDDs including schizophrenia, autism, and Rett syndrome. Together, these findings further support the fundamental function of this inhibitory system for balanced brain activity.

This thesis, in part, has been submitted for publication of the material as it may appear in *Nature Neuroscience*; Barnes S.A., Pinto-Duarte A., Kappe A., Metzler A., Mukamel E.A., Lucero, J., Wang X., Sejnowski T.J., Markou A., Behrens M.M., 2014. The thesis author was the third author of this material.

FIGURES

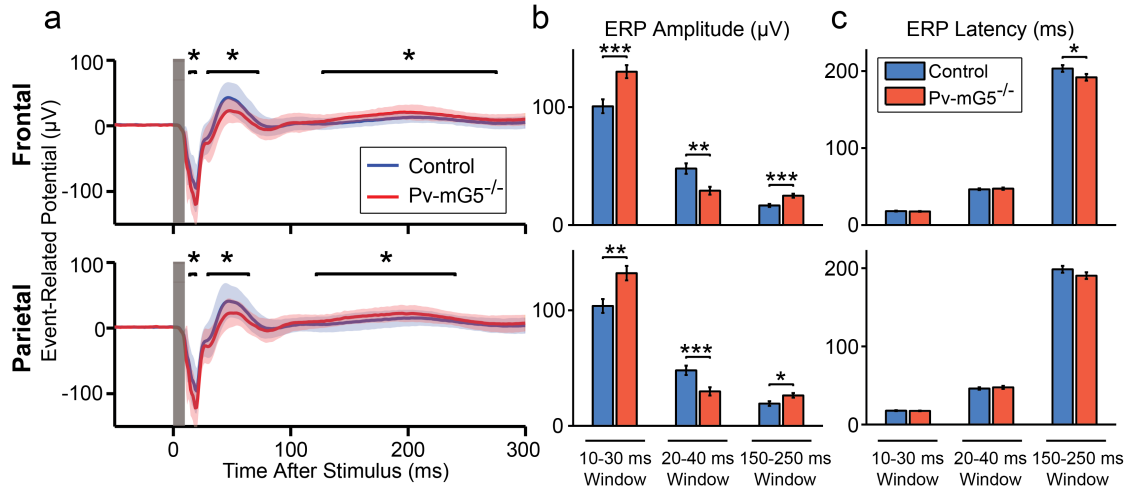

Figure 1: Pv-mG5$^{-/-}$ mice have altered auditory event-related potentials (ERPs)
(a) Auditory ERPs in Pv-mG5$^{-/-}$ mice showed alterations in amplitude surrounding the extrema in a 300 ms window following the presentation of a stimulus. Significance between genotypes was determined by the Wilcoxon rank-sum test at each time point.
(b) Pv-mG5$^{-/-}$ mice showed increased amplitude in the 10-30 ms [Genotype: Frontal, $F_{(1,70)}$=12.70, p<0.001; Parietal, $F_{(1,68)}$=10.53, p<0.01] and 150-250 ms windows [Genotype: Frontal, $F_{(1,70)}$=18.39, p<0.001; Parietal, $F_{(1,68)}$=6.57, p<0.05], and decreased amplitude in the 20-60 ms window [Genotype: Frontal, $F_{(1,70)}$=11.77, p<0.01; Parietal, $F_{(1,68)}$=12.12, p<0.001].
(c) No differences in the latency were observed in either the frontal or the parietal channel in the 10-30 ms window or the 20-60 ms window. Pv-mG5$^{-/-}$ mice show a shorter latency in the 150-250 ms window for the frontal channel only [$F_{(1,70)}$=3.84, $p\leq$0.05].
Performance was analyzed by two-way ANOVA with sex and genotype as the between group variables followed (when appropriate) by Fisher's LSD post hoc test. * denotes difference between control and Pv-mG5$^{-/-}$ mice; *p<0.05, **p<0.01, ***p<0.001. Bar graphs depict means ± SEM; data in traces depict mean ± standard deviation. Control: n = 38 (frontal), 37 (parietal); Pv-mG5$^{-/-}$: n = 36 (frontal), 35 (parietal).

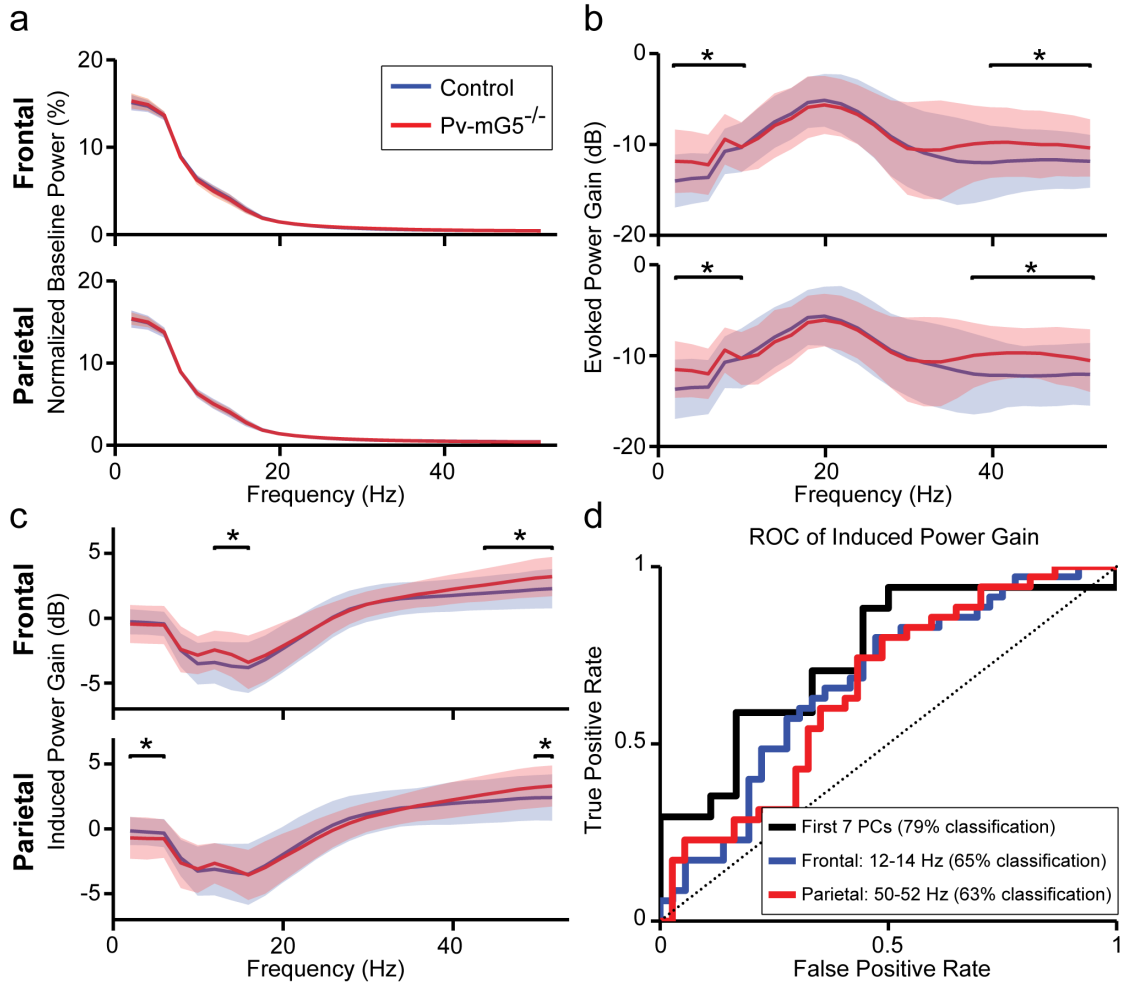

Figure 2: Pv-mG5$^{-/-}$ mice have altered evoked and induced power
(a) No differences in auditory EEG baseline power of Pv-mG5$^{-/-}$ mice were observed.
(b) Evoked power gain relative to baseline was significantly increased in the low frequency bands (2-10 Hz) and the gamma band (40-54 Hz).
(c) Induced power gain was diminished in the low frequency bands (2-6 Hz, parietal), and increased in the beta (12-16 Hz) and gamma bands.
(d) Receiver operating characteristic (ROC) analysis showed discriminability of control vs. Pv-mG5$^{-/-}$ animals using the best frontal or parietal frequency band, or using an optimized combination of frequencies (best principal component, PC). Classification rates indicate performance of a linear discriminant under leave-one-out cross validation.
Significance between genotypes in **(a, b)** is determined by the Wilcoxon rank-sum test.
* denotes difference between control and Pv-mG5$^{-/-}$ mice as determined at each 2-Hz frequency band by the Wilcoxon rank-sum test; *p<0.05. Data in traces depict mean ± standard deviation. Control: n = 38 (frontal), 37 (parietal); Pv-mG5$^{-/-}$: n = 36 (frontal), 35 (parietal).

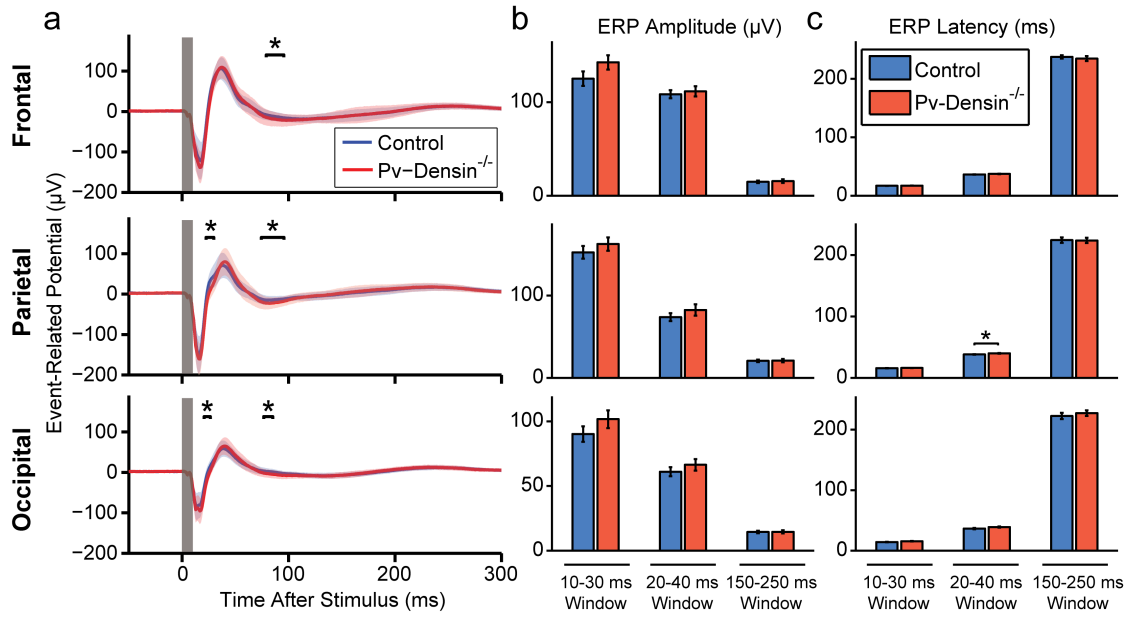

Figure 3: Pv-Densin$^{-/-}$ mice have few alterations in auditory ERPs
(a) Auditory ERPs in Pv-Densin$^{-/-}$ mice were decreased from 79-95 ms in the frontal channels, from 23-29 and 75-95 ms in the parietal channels, and from 20-26 and 78-85 ms in the occipital channels. Significance between genotypes was determined by the Wilcoxon rank-sum test at each time point.
(b) No amplitude differences were observed in any channel in either the 10-30 ms window, the 20-60 ms window, or the 150-250 ms window.
(c) No differences between the genotypes were observed in either frontal, parietal, or occipital channels in either the 10-30 ms window or the 150-250 ms window. Pv-Densin$^{-/-}$ mice showed increased latency compared to controls in the 20-60 ms window for the parietal channel [Genotype, $F_{(1,57)}=5.09$, $p<0.05$], but not in the frontal and occipital channels.
Performance was analyzed by two-way ANOVA with sex and genotype as the between group variables followed (when appropriate) by Fisher's LSD post hoc test. * denotes difference between control and Pv-Densin$^{-/-}$ mice; *$p<0.05$. Bar graphs depict means ± SEM; data in traces depict mean ± standard deviation. Control: n = 36 (frontal), 36 (parietal), 31 (occipital); Pv-Densin$^{-/-}$: n = 23 (frontal), 23 (parietal), 21 (occipital).

Figure 4: Pv-Densin$^{-/-}$ mice have altered baseline, evoked and induced power
(a) The auditory EEG baseline power of Pv-Densin$^{-/-}$ mice was increased within the beta frequency band (12-30 Hz) in all three channels, and decreased in the low frequency bands (2-6 Hz) in the occipital channel.
(b) Evoked power gain relative to baseline was significantly increased within beta in the parietal (14-20 Hz) and occipital (12-18 Hz) channels, and increased in the alpha (8-10 Hz) band in the occipital channel. No differences in evoked power gain were observed between the genotypes in the frontal channel.
(c) Induced power gain was diminished in the gamma band (38-42 Hz) in the frontal channel, and increased in the low frequency bands (2-10 Hz) in the occipital channel. No differences in induced power gain were observed between the genotypes in the parietal channel.
(d) ROC analysis showed discriminability of control vs. Pv-Densin$^{-/-}$ animals using the best frontal, parietal, or occipital frequency band, or using an optimized combination of frequencies (best principal component, PC). Classification rates indicate performance of a linear discriminant under leave-one-out cross validation.
Data in traces depict mean ± standard deviation in **(a, b, c)**, with significance between genotypes determined by the Wilcoxon rank-sum test. * denotes difference between control and Pv-Densin$^{-/-}$ mice as determined at each 2-Hz frequency band by the Wilcoxon rank-sum test; *$p<0.05$. Control: n = 36 (frontal), 36 (parietal), 31 (occipital); Pv-Densin$^{-/-}$: n = 23 (frontal), 23 (parietal), 21 (occipital).

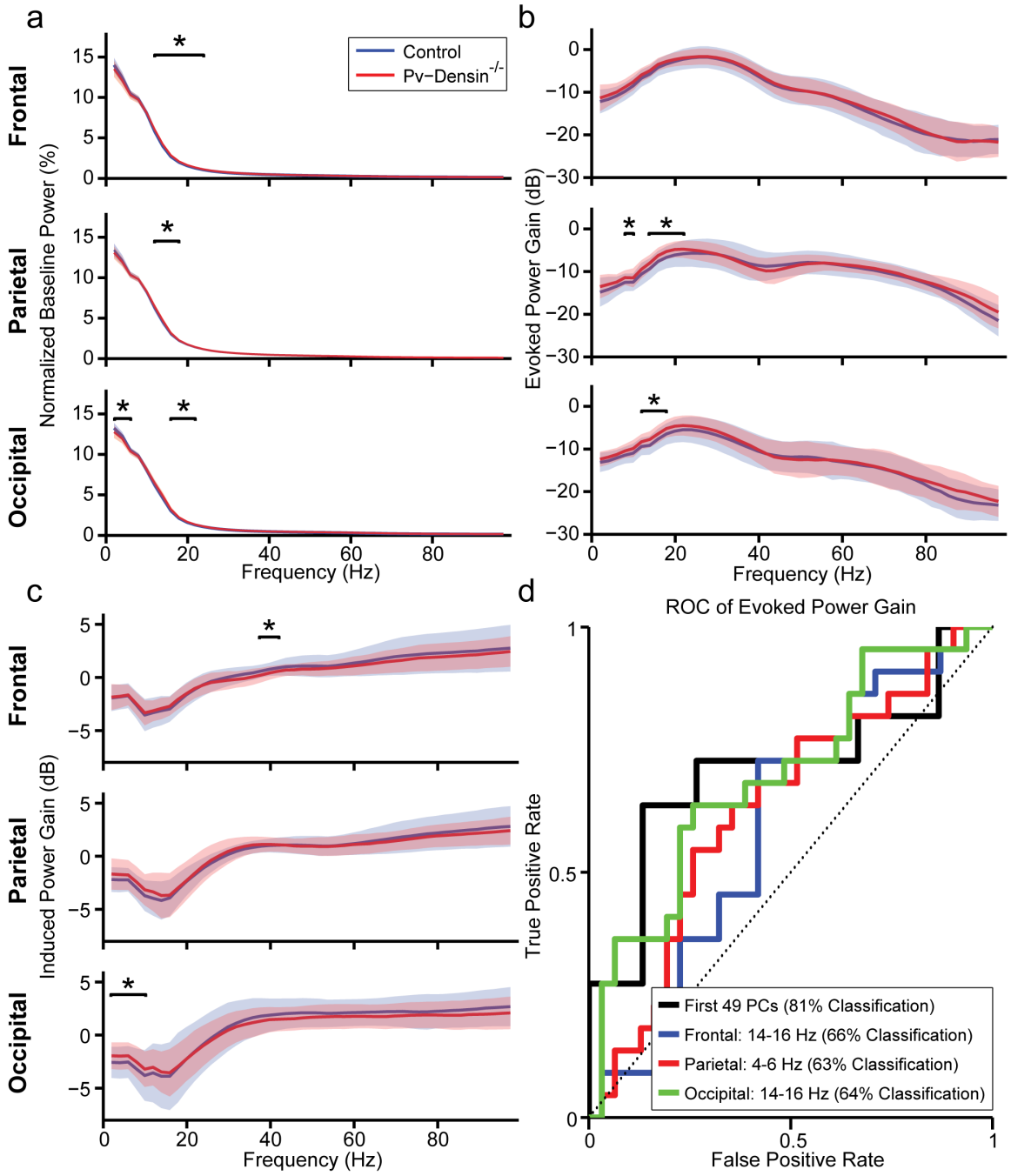
a
b
c
d
Frontal
Parietal
Occipital
Control
Pv−Densin-/-
Normalized Baseline Power (%)
Evoked Power Gain (dB)
Induced Power Gain (dB)
Frequency (Hz)
ROC of Evoked Power Gain
True Positive Rate
False Positive Rate
First 49 PCs (81% Classification)
Frontal: 14-16 Hz (66% Classification)
Parietal: 4-6 Hz (63% Classification)
Occipital: 14-16 Hz (64% Classification)

Figure 5: Prepulse inhibition of the startle response is increased in Pv-mG5$^{-/-}$ mice
(a) The averaged rectified EMG amplitude is plotted across time for each genotype and for the startle pulse alone and prepulse + startle pulse paradigms. Under their respective conditions, the startle pulse (50 ms long, 94 dB) was time-locked to 0 ms and the prepulse (50 ms long, 70 dB) to -100 ms. With or without the presentation of a prepulse, a startle response was observed between 10 and 20 ms in both genotypes.
(b) A ratio of the prepulse + startle to startle shows the effect of the prepulse on the startle response. This ratio was significantly smaller in Pv-mG5$^{-/-}$ mice from 11-17 ms, 24-28 ms, and 67-75 ms after the onset of the startle pulse. Significance between genotypes was determined by the Wilcoxon rank-sum test at each time point.
(c) No alterations were present in the startle response in the 10-20 ms window [Genotype: $F_{(1,41)}$=0.01, ns].
(d) Increased PPI was evident in Pv-mG5$^{-/-}$ mice in the 10-20 ms window [Genotype: $F_{(1,41)}$=5.95, p<0.05].
Performance was analyzed by two-way ANOVA with sex and genotype as the between group variables followed (when appropriate) by Fisher's LSD post hoc test. * denotes difference between control and Pv-mG5$^{-/-}$ mice; *p<0.05, **p<0.01. Data in traces and bar graphs depict mean ± SEM. Control: n = 20; Pv-Densin$^{-/-}$: n = 25.

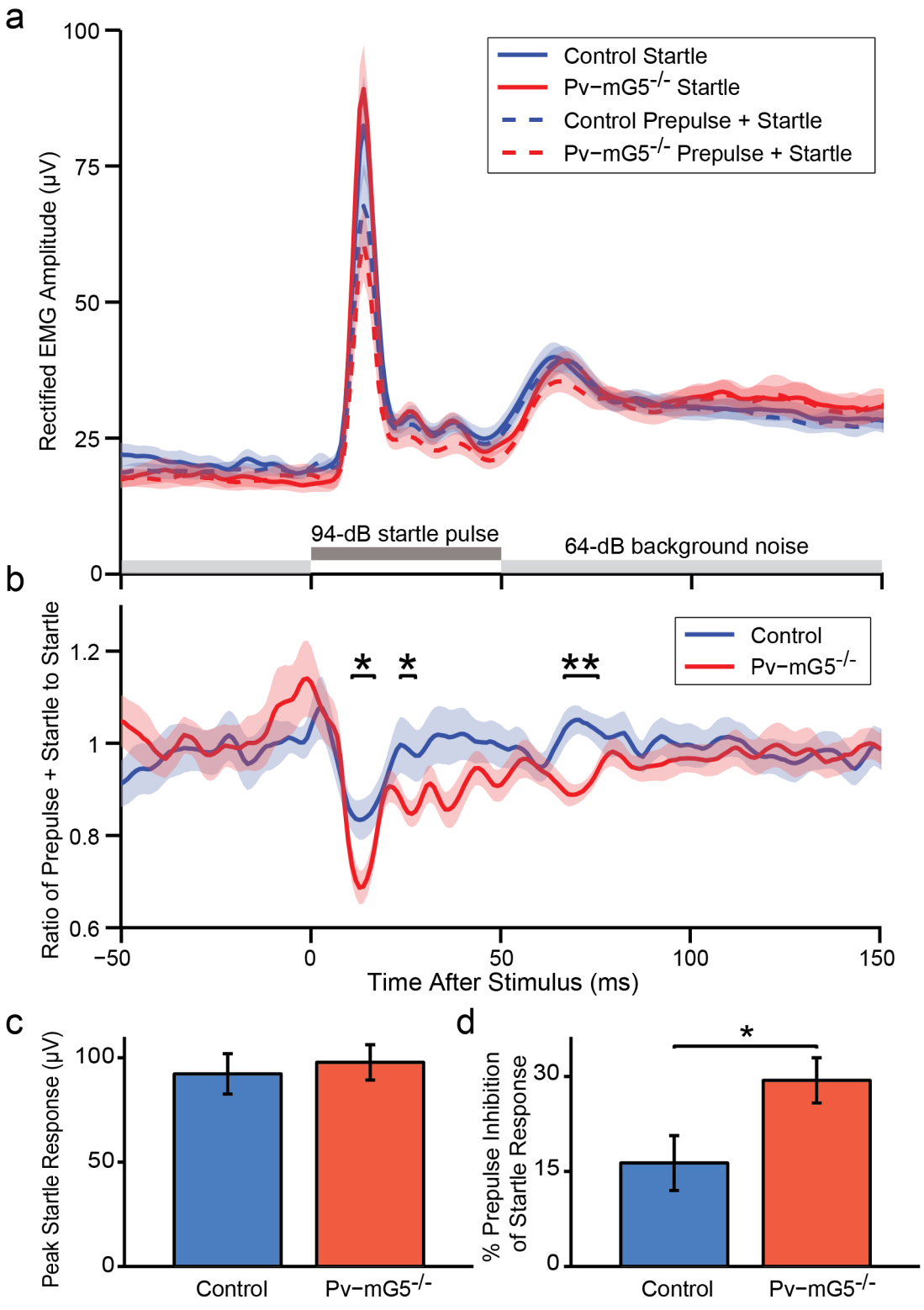
a
Control Startle
Pv−mG5-/- Startle
Control Prepulse + Startle
Pv−mG5-/- Prepulse + Startle
Rectified EMG Amplitude (μV)
100
75
50
25
0
94-dB startle pulse
64-dB background noise
b
Control
Pv−mG5-/-
Ratio of Prepulse + Startle to Startle
1.2
1
0.8
0.6
*
*
**
−50
0
50
100
150
Time After Stimulus (ms)
c
Peak Startle Response (μV)
100
50
0
Control
Pv−mG5-/-
d
% Prepulse Inhibition of Startle Response
30
15
0
*
Control
Pv−mG5-/-

Figure 6: Prepulse inhibition of the startle response is increased in Pv-Densin$^{-/-}$ mice
(a) The averaged rectified EMG amplitude is plotted across time for each genotype and for the startle pulse alone and prepulse + startle pulse paradigms. Under their respective conditions, the startle pulse (50 ms long, 94 dB) was time-locked to 0 ms and the prepulse (50 ms long, 70 dB) to -100 ms. With or without the presentation of a prepulse, a startle response was observed between 10 and 20 ms in both genotypes.
(b) A ratio of the prepulse + startle to startle shows the effect of the prepulse on the startle response. This ratio was significantly smaller in Pv-Densin$^{-/-}$ mice from 12-13 ms and 25-28 ms after the onset of the startle pulse. Significance between genotypes was determined by the Wilcoxon rank-sum test at each time point.
(c) No alterations were present in the peak startle response within the 10-20 ms window [Genotype: $F_{(1,49)}=3.29$, $p=0.076$].
(d) PPI was increased in Pv-Densin$^{-/-}$ mice relative to controls within the 10-20 ms window [Genotype: $F_{(1,49)}=4.41$, $p<0.05$].
Performance was analyzed by two-way ANOVA with sex and genotype as the between group variables followed (when appropriate) by Fisher's LSD post hoc test. * denotes difference between control and Pv-Densin$^{-/-}$ mice; *$p<0.05$. Data in traces and bar graphs depict mean ± SEM. Control: n = 24; Pv-Densin$^{-/-}$: n = 29.

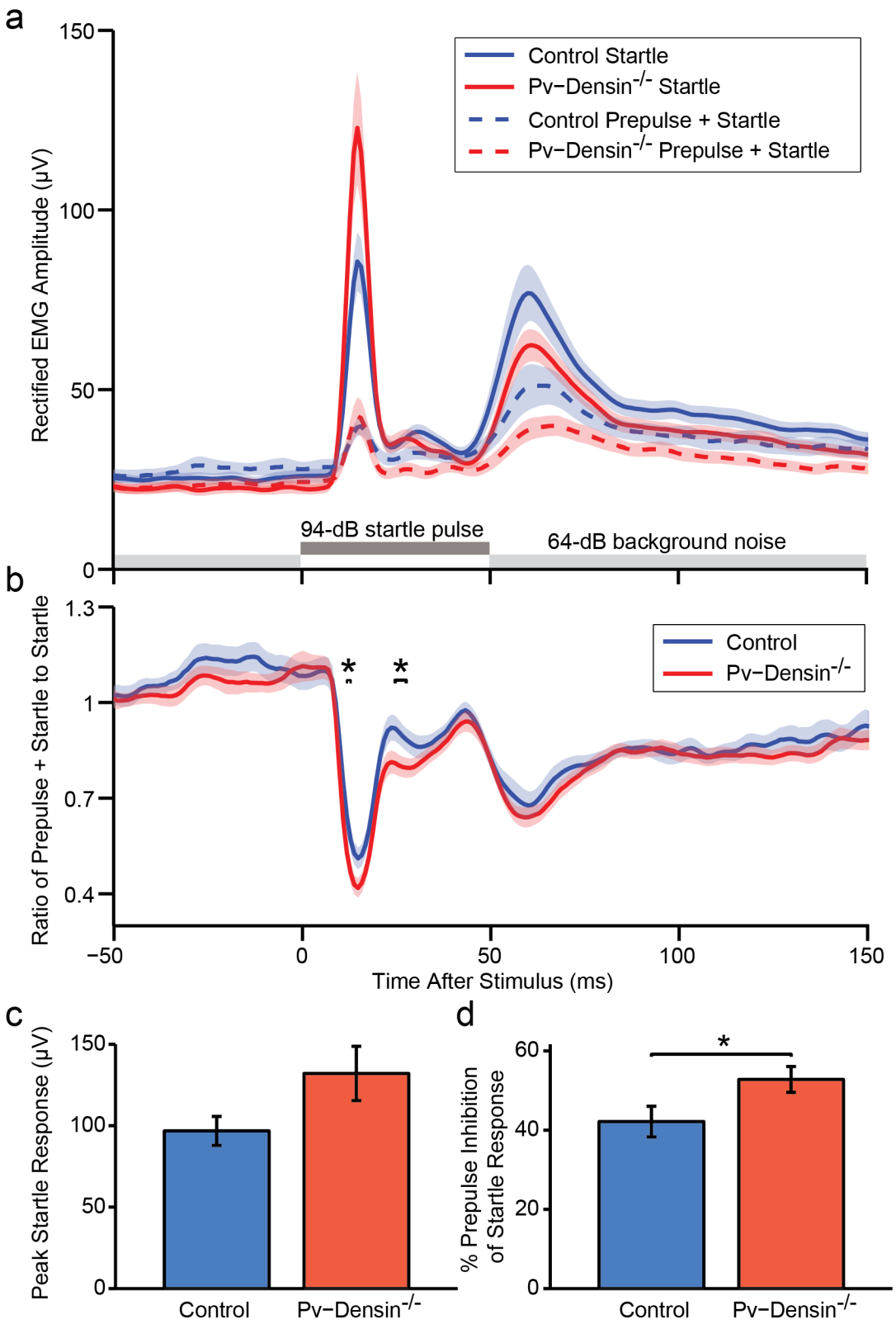
a
Rectified EMG Amplitude (μV)
150
100
50
0
Control Startle
Pv−Densin-/- Startle
Control Prepulse + Startle
Pv−Densin-/- Prepulse + Startle
94-dB startle pulse
64-dB background noise
b
Ratio of Prepulse + Startle to Startle
1.3
1
0.7
0.4
**
**
Control
Pv−Densin-/-
−50
0
50
100
150
Time After Stimulus (ms)
c
Peak Startle Response (μV)
150
100
50
0
Control
Pv−Densin-/-
d
% Prepulse Inhibition of Startle Response
60
40
20
0
*
Control
Pv−Densin-/-

Table 1: Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice have features resembling those observed in neurodevelopmental disorders

A brief list of features in the neurodevelopmental disorders autism and schizophrenia are shown alongside the relevant findings in Pv-mG5$^{-/-}$ and Pv-Densin$^{-/-}$ mice.

Citations: 1) Rogers & Ozonoff (2005); 2) Turetsky et al. (2008); 3) Rojas et al. (2008); 4) Lazarewicz et al. (2009); 5) Uhlhaas & Singer (2010); 6) Grice et al. (2001); 7) Madsen et al. (2013); 8) Braff et al. (2001); 9) Bodfish et al (2000); 10) Morrens et al. (2006)

	Pv-mG5$^{-/-}$	Pv-Densin$^{-/-}$	Autism	Schizophrenia
ERP at 40 ms (mice) & 100 ms (humans)	↓	=	=[1]	↓[2]
Baseline gamma power	=	=	=[3]	Mixed[4,5]
Evoked (phase-locked) gamma power	↑	=	↓[3]	↓[5]
Induced gamma power	↑	↑ (1/3 channels)	Mixed[3,6]	↓[5]
PPI (with low prepulse)	↑	↑	↑[7]	↓[8]
Repetitive Behaviors	↑	=	↑[9]	=/↑ in later stages[10]

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