Functional Regeneration of the ex-vivo Reconstructed Mesocorticolimbic Dopaminergic System

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CNS reparative-medicine therapeutic strategies need answers on the putative recapitulation of the basic rules leading to mammalian CNS development. To achieve this aim, we focus on the regeneration of functional connections in the mesocorticolimbic dopaminergic system. We used organotypic slice cocultures of ventral tegmental area/substantia nigra (VTA/SN) and prefrontal cortex (PFC) on a multielectrode array (MEA) platform to record spikes and local field potentials. The spontaneously growing synaptically based bidirectional bursting activity was followed from 2 to 28 days in vitro (DIV). A statistical analysis of excitatory and inhibitory neurons properties of the physiological firing activity demonstrated a remarkable, exponentially increasing maturation with a time constant of about 5–7 DIV. Immunohistochemistry demonstrated that the ratio of excitatory/inhibitory neurons (3:1) was in line with the functional results obtained. Exemplary pharmacology suggested that GABA_A receptors were able to exert phasic and tonic inhibition typical of an adulthood network. Moreover, dopamine D2 receptor inactivation was equally inhibitory both on the spontaneous neuronal activity recorded by MEA and on patch-clamp electrophysiology in PFC pyramidal neurons. These results demonstrate that axon growth cones reach synaptic targets up to full functionality and that organotypic cocultures of the VTA/SN-PFC perfectly model their newly born dopaminergic, glutamatergic and GABAergic neuronal circuitries.

Keywords: bursts, multielectrode array recording, prefrontal cortex, substantia nigra, ventral tegmental area

Introduction

The lower brain stem structures such as the reticular formation are considered the origin of the information conveyed to the ventral tegmental area (VTA), an area that has >20 different projections to the brain. We fixed our attention on the future possibility of developing strategies of reparative medicine around these areas. For this purpose, we decided to use the VTA to prefrontal cortex (PFC) ex vivo model to study the reconstruction of the bidirectional connectivity present during in vivo adulthood. It has been suggested that DA neurons do not have specific functions, while on the contrary, they regulate and enable integrative functions in the neural systems onto which they project, but they might also play a general trophic, metabolic, or energetic role (LeMoal and Simon 1991).

Dopaminergic (DAergic) neurons in the ventral mesencephalon send projections to different forebrain structures, forming a complex neuromodulatory system crucial for many cognitive processes and motor functions (Lapish et al. 2007). These neurons are located in the substantia nigra (SN; A9 cell group) and in the VTA (A10 cell group; Oades and Halliday 1987). Prefrontal, orbitofrontal, and cingulated cortices receive the most marked innervation from the VTA; projections from the VTA to the medial prefrontal cortex (mPFC) constitute a portion of the mesocorticolimbic dopamine system (Steketee 2003). Much of this connectivity is bidirectional (Fuster 2001); the mPFC receives DAergic and GABAergic afferents from the VTA and sends glutamatergic projections to both the VTA and the nucleus accumbens. Thus, the mesocortical dopamine system is involved in a great variety of brain functions, such as working memory, attention selection, and memory retrieval, because of its interconnections with brain areas processing external information and internal information (Miller et al. 2002).

DAergic afferents from the mesencephalon seem to have a crucial role in the normal development of the PFC and in the regulation of neuronal activity in this brain area (Lewis et al. 1998). In fact, abnormal maturation and alterations of the mesencephalic projections to the PFC have been suggested to be involved in the development of sensitization induced by psychostimulants and in the pathophysiology of several disorders, such as schizophrenia (Goldstein and Deutch 1992), addiction (Steketee 2003; Kauer 2004; Van den Heuvel and Pasterkamp 2008), or attention deficit/hyperactivity disorder (Sullivan and Brake 2003).

The analysis of the formation and development of functional connections in the CNS needs suitable model systems to understand the basic, functional mechanisms (Hofmann et al. 2004). We have reconstructed parts of the mesocorticolimbic dopaminergic system using the model of organotypic cocultures of tissue slices from the VTA/SN complex and the PFC (Franke et al. 2003; Heine et al. 2007). In fact, organotypic brain slices are closer to the in vivo situation than cell cultures; unlike acute slice preparations, they are suitable for experiments over extended periods of time (Hofmann and Bading 2006) and represent the most intact culture system for studying cortex function in isolation (Gähwiler et al. 1997; Karpik and Plenz 2002; Stewart and Plenz 2008). Furthermore, by combining organotypic cultures with microelectrode array (MEA) recordings, it is possible to monitor long-term processes of neurite outgrowth and development and synapse formation at functional level (Egert et al. 1998; Beggs and Plenz 2003, 2004; Hofmann et al. 2004).

Since the final aim of a reparative medicine project is the feasibility of reconstructing the CNS region connectivity, in this first study, we wanted to focus our work on verifying the
functional aspects of the developmental connectivity. We initially characterized the developmental features of the VTA/SN-PFC cocultures maintained on MEA platforms, in order to study the functionality of the neuronal projections, which have been demonstrated by morphological methods to grow ex vivo between the 2 brain areas (Franke et al. 2003; Heine et al. 2007). Accordingly, our main aims were threefold. First, it was investigated whether a time-dependent increase in burst discharge synchronization occurs in the VTA/SN and PFC, in parallel to the reciprocal outgrowth of neuronal processes from the cocultured slices. Second, the excitatory and inhibitory components of the overall firing activity were dissected by analyzing the burst characteristics; it was studied whether the ratio of these 2 components is time-dependently changing from an early developmental (1:1) to a fully mature (3:1) pattern. Third, ɣ-aminobutyric acid (GABA) receptor (R) antagonists and D2R antagonists were applied to the cocultures in order to clarify whether their electrophysiological effects conform to the expected and developmentally defined modulation of network activity. A preliminary report of these data was presented at the 2010 Meeting of the Neuroscience Society (Dossi, Heine et al. 2010).

Materials and Methods

Materials
Primary antibodies directed against: GABA (mouse anti-γ-aminobutyric acid, 1:100; Sigma Chemical Co., St. Louis, USA), goat anti-glial filibrillary acidic protein (GFAP, 1:300; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), glutamate (mouse anti-glutamate, 1:200; Abcam PLC, Cambridge, UK); rabbit anti-glutamate 1:1000, Sigma-Aldrich, St. Louis, MO, USA), MAP2 (rabbit anti-microtubule-associated protein-2, 1:500; Millipore, Temecula, CA, USA), tyrosine hydroxylase (TH; mouse anti-tyrosine hydroxylase, 1:1000; Chemicon) and the secondary antibodies carboxyamine (Cy)2- (1:400), (Cy)3- (1:1000), (Cy)5- (1:100) conjugated to Alexa Fluor 488, Cy2- (1:400), (Cy)3- (1:1000), (Cy)5- (1:100) conjugated to Alexa Fluor 594, and Cy3 conjugated to FITC. Blocking reagents for secondary antibodies were 2% BSA, 2% goat serum, and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to stain the nuclei. Washing procedures were performed with PBS. We used secondary antibodies conjugated to dye-coupled streptavidin or biotin (1:400) (all from Jackson Immunoresearch Laboratories, West Grove, PA, USA) used. Etoilopride, SCH25390, APV, NBQX, gabazine (GZ), and muscimol (MUS) were used from Sigma-Aldrich, Italy, and Tocris Bioscience, UK, and kept as frozen stock solutions in distilled water at –20°C until diluted to their appropriate concentrations. All the experiments with the MEA technique were performed by adding the drugs in volumes that were always <1% of the total volume of the solution bathing the cocultures.

Animals
Neonatal mice (CD1® mice, Harlan Laboratories, Italy for electrophysiological experiments, or provided from the Medical-Experimental Center of the Medical Faculty of the University of Leipzig, for immunohistochemistry (IHC) of postnatal day (P) 1–3 were used for the preparation of the organotypic dissociated slice cocultures and those at the age of 14 days for IHC. The animals were housed under standard laboratory conditions, under a 12-h light/dark cycle and allowed access to lab food and water ad libitum. All the animal use procedures were approved by the respective Committees of experimental animal protection. The number of animals used was minimized as well as their sufferings.

Preparation of the Slice Cocultures on MEA Petri Dishes
Slice cocultures were prepared from P1–3 mice as previously described (Franke et al. 2003; Heine et al. 2007) with some modifications. Briefly, mouse pups were decapitated and the brains were removed from the skull under sterile conditions. The brains were disposed to an agar block and tissue blocks containing mesencephalic or forebrain levels were dissected, fixed onto a specimen stage of a vibratome (Leica, VT 1000S, Nussloch, Germany) with Locitite Super Attack glue (Henkel, Dusseldorf, Germany), and placed in ice-cold (4°C) solution containing the following (in mM): 87 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 7 MgCl2, 0.5 CaCl2, 2.5 KCl, 25 d-glucose, and 75 sucrose, equilibrated with 95% O2 and 5% CO2 (pH 7.4). Coronal sections of 300 µm thickness (for patch-clamp recordings, IHC) and 200 µm thickness (for MEA recordings; Shimono, Baudry and Ho et al. 2002; Shimono, Baudry and Panchenko et al. 2002) were cut. In these preparations, we did not attempt to separate the VTA and the SN; for further discussion, this area will be named VTA/SN complex. The VTA/SN and PFC slices were transferred into Petri dishes filled with the same ice-cold solution and cultured on moistened translucent membranes of tissue culture inserts (0.4 µm, Millicell-CM, Millipore, Italy) for IHC and patch-clamp recordings as well as on MEA petri dishes (30-µm diameter ITO electrodes 200 µm apart, Multichannel Systems, Germany), pretreated with Plasma Cleanner (Harrick Plasma, Ithaca, New York) and precoated with collagen 3.5 mg/mL (Collagen type I, rat tail, Millipore, Italy; Maeda et al. 2004), to cover the 8×8 microelectrode array. The sections of the VTA/SN complex and the PFC were positioned on the MEAs, in such a way that the VTA faced the mPFC (Fig. 1A) with the smallest possible distance (<300–400 µm) between them, with each slice covering almost half of the electrodes, and were kept at 37°C in 5% CO2 for 1 h before adding the culture medium (250 µL) and covered with gas-permeable covers (MEA-MEM, Ala Scientific Instruments, Inc., USA). In contrast to cultures in MEA dishes, those on tissue inserts were placed in 6-well plates, each filled with 1-mL culture medium. The medium contained 25% MEM, 25% Basal Medium Eagle without glutamine ( Gibco, Life Technologies), and 25% Horse Serum (EuroClone), supplemented with glutamine to a final concentration of 2 mM, 0.6% glucose and Pen/Strep 150 µg/mL (Sigma-Aldrich; pH 7.2). All the cultures were maintained at 37°C in 5% CO2 after 2 DIV, the incubation medium was changed with a serum-free medium consisting of neurobasal medium supplemented with B27 (Invitrogen, Italy), glutamine 1 mM, and Pen/Strep 150 µg/mL (Sigma-Aldrich, Italy). For the cultures on tissue inserts, the medium was changed 3 times a week, while for the slices on MEA dishes, the medium was changed with half volume every day. We maintained the cocultures up to 4 weeks in vitro to study the regeneration and the functional properties of the growing projections between the VTA/SN complex and the PFC. As described by Stewart and Plenz (2008), the position of the electrodes under both areas of the cocultures was established during the culture preparation and checked after 1–2 DIV, and later during development, because it has been shown that organotypic cultures adhere to the substrate, slightly expand, and flatten during postnatal maturation (Stoppani et al. 1991; Stewart and Plenz 2008).

Multielectrode Array Recordings
Data recordings were done as described (Gullo et al. 2009, 2010) with some changes. Briefly, raw analog signals sampled at 32 kHz were recorded at 36°C in CO2-controlled incubators by MEA-1060BC preamplifiers (bandwidth 0.1–8 kHz, Multichannel Systems, Germany) and detected through the MC_Rack Software (version 4.0, Multichannel Systems, Germany), using appropriate filters to separate spikes (0.25–5 kHz) and local field potentials (LFPs, 5–200 Hz, downsampled at 1 kHz; Dossi, Gullo et al. 2010; Dossi, Heine et al. 2010). For the characterization of the firing properties during the development of the cultures, MEA recordings were performed between 3 and 28 DIV; for the other experiments, we used only 12–20 DIV MEA dishes.

Spiking Activity Analysis
The spike signals were detected through the MC_Rack software using a fixed threshold (~5 standard deviations [SDs] from the background noise) and ultimately re-thresholded and cleaned of spikes having an ISI shorter than the prefixed 2.5 ms refractory period by the Offline Sorter program (Plexon Inc., USA). Durations were placed on principal component analysis (PCA)-based sorting and for multunit electrodes, we did 1 of the 2 following supplementary procedures: 1) removal of spikes by using the Mahalanobis threshold in the range 1.8–4.1, while we concurrently checked that the p of multivariate ANOVA sorting quality statistics should be <0.01 among the identified units (see...
To perform burst detection and to analyze their properties, we used the procedures described by Gullo et al. (2009), with some modifications. Briefly, for each identified unit, we computed the following characteristics: the autocorrelation function (ACF), the burst duration (BD), the number of spikes in each burst (SN), the SR, the intraburst spike rate (IBSR), the Fano Factor (FF), and the burst rate (BR) (Gullo et al. 2010). The data for all the bursts of a specific neuron were averaged in defined time segments corresponding to the control or the presence of different drugs or treatments. We were able to classify neurons on the basis of an Unsupervised Learning Approach consisting of data reducing PCA, followed by the k-means clustering procedure (Duda et al. 2000; Johnson and Wichern 2002). In the clustering processing, we also used an outlier-removing procedure, which discarded from results those units having a Mahalanobis distance from the centroid of its cluster bigger than a fixed threshold (here we used 1.4). The procedure in the PFC identified statistically different clusters, as previously described (Gullo et al. 2010), which were named with the subscript “e” (excitatory) and “i” (inhibitory). The 2 identified clusters obeyed all the following rules at the same time (in control conditions): BD_e << BD_i; SN_e << SN_i; SR_e << SR_i; IBSR_e >> IBSR_i; FF_e << FF_i. In the VTA/SN complex, we applied the procedure twice to identify different neuronal populations, with different firing properties, which could be attributed to putative DAergic, GABAergic, and glutamatergic neurons. To analyze the burst structure, we applied a scanning window of variable duration (5–30 ms) to search the start of the up-states, collect the spikes, and identify the major burst leaders (MBLs, which are those neurons capable of triggering at least 4% of the total recorded bursts; Ham et al. 2008). From the clusters defined above, it was possible to identify how the spikes belonging to each cluster are elicited during the burst time-course.

Furthermore, we applied a new procedure described in Gullo et al. (2012) that consists in a PCA-based classification of network states performed by using the following features: 1) the shape of the spike number time histograms (SNTH); 2) the number of engaged neurons (NN); and 3) the BD. The statistical significance of the classification was assessed by means of a 2-sample paired t test (P < 0.05). The states with a percentage occupation (PO) of <4% in the time segment were discarded. After the identification of the statistically different
states, the program output consisted in a series of files, associated with the 2 clusters of neurons, that described: 1) the probability density function of finding 1, 2, 3, and the 4th spikes (firing spike histograms, FSH) and its cumulative probability (cFSH) in order to investigate the mode of firing of these neurons; 2) the time histograms of the number of spikes (SNTH) and of the neurons engaged in the activity (for each time bin, NNTH), and its ratio, called excitability (EXTH) (Gullo et al. 2012).

Local Field Potential Activity
LFPs were captured simultaneously by positive and negative thresholds (± 5 SD from the background noise of each electrode) and filtered in the 5–200-Hz frequency band. Data were averaged during time segments of 600 s or more (Dossi, Gullo et al. 2010; Dossi, Heine et al. 2010; Maffezzoli and Wanke 2010).

Patch-Clamp Recordings
The cocultures were prepared as described (Franke et al. 2003). We used 8–15-day-old cocultures. To perform patch-clamp recordings, a piece of membrane containing a VTA/SN-PFC coculture was cut around the coculture, transferred to a submerged recording chamber, and continuously perfused with ACSF (saturated with 95% O₂ and 5% CO₂) at approximately 2 mL/min with temperature maintained at 30°C. The coculture was allowed to recover from the change of the medium for at least 1 h in the recording chamber before starting an experiment. A bipolar stimulating electrode (tungsten, tip separation 75 μm, Microprobes Gaithersburg, USA) was placed in the VTA region, whereas somatic whole-cell recordings were performed from pyramidal neurons of the layer 5/6 using conventional techniques (Fig. 1A). The stimulation region (VTA) and the recording site (layer 5/6) in the cocultures were visually identified with an Eclipse E600FN microscope (Nikon Instruments, Sesto Fiorentino, Italy) equipped with objective 4x, whereas cells were examined with a water immersion differential interference contrast objective 40x and an infrared CCD 100 camera (DAGE-MTI Inc., Michigan City, IN). Patch pipettes (2–3 MΩ) were pulled from borosilicate glass capillaries (WPI) with a horizontal puller (Model P97, Sutter Instrument) and filled with a solution containing (in mM): 140 K-gluconate, 10 HEPES, 1 MgCl₂, 5 NaCl, 1 EGTA, 2 Na-ATP, 0.3 Na-GTP (pH 7.3). Whole-cell recordings were performed with a computer-controlled amplifier (MultiClamp 700A; Axon Instruments), digitized (Digidata 1322 Axon Instruments) and acquired with the program P-Clamp 9.2 (Axon Instrument) at a sampling rate of 10 kHz. Electrode capacitance and offset were compensated. Liquid junction potential was not corrected. Recordings with high access resistance values (15–20 MΩ) were rejected if the access resistance changed by >15% during the experiment. Pyramidal neurons were identified, using the current clamp configuration, by evaluating the responses as action potentials to increasing depolarizing currents lasting for 1 s (Yang et al. 1996; Young and Sun 2009). In the voltage-clamp mode, holding potential was set at ~60 mV, and the currents were recorded by evoking responses after stimulation in the VTA every 30 s with bipolar symmetrical 60–100 μs pulses of 100–600 μA strength (PC controlled stimulus isolation unit ISO-STIM 01D, NPI electronic GmbH, Germany). Control and drug containing ACSF were consecutively introduced through the experiments. Glutamate sensitive currents were recorded in the PFC by stimulating newborn axons in the VTA. A single electrical stimulus applied in the VTA/SN complex produced in the voltage-clamped PFC pyramidal neuron uncontrollable currents. To avoid this problem, we had to inhibit the PFC network by continuously applying 2-μM MUS in the control solutions. As shown in Figure 10A, to study AMPA currents, we used a “normal” ACSF that contained (in mM): 125 NaCl, 2.5 KCl, 1.3 MgCl₂, 1.2 NaH₂PO₄, 2 CaCl₂, 25 NaHCO₃, 11 glucose, and 0.4 ascorbic acid; to study NMDA currents, shown in Figure 10E, we used a “Mg”-free ACSF that contained (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 2.0 CaCl₂, 26 NaHCO₃, 11 glucose, and 0.4 ascorbic acid. To obtain reasonably well space-clamped NBQX-sensitive or APV-sensitive currents, we added either NBQX 10 μM or APV 50 μM or both at the end of the experiment (Fig. 10E,F). To investigate the drug effects, we used, for each experiment, data similar to those shown in Figure 10B,F,G. We computed the percentage change between the mean (n = 10) of the areas under the curves recorded before and 10 min after the application of the D1 or D2 receptor antagonist SCH23390 and eticlopride, respectively. The areas of the NBQX-sensitive or APV-sensitive currents were calculated for 50 or 500 ms following the onset of the currents, respectively. Using Student’s t-test, the differences between pre and post drug application were considered statistically significant when P < 0.05.

Immunohistochemistry of the Slice Cultures
After 12–14 DIV, the cultures were fixed in a solution consisting of 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in 0.1 M phosphate buffer (PBS; pH 7.4) for 2 h following 3–4 washing steps with PBS. Afterward, the cocultures were cut in 50-μm thick slices using the vibratome. After washing with Tris buffered saline (TBS, 0.05 M; pH 7.6) and blocking with 5% fetal calf serum (FCS) and 0.3% Triton X-100 in TBS, the slices were incubated in a mixture of rabbit anti-MAP2, goat anti-GFAP, and mouse anti-GABA or mouse anti-glutamate antibodies, respectively, diluted in 0.3% Triton X-100, 5% FCS in TBS for 48 h at 4°C. After incubation of the primary antibodies, slices were rinsed 3 times for 5 min in TBS. The simultaneous visualization of the different primary antisera was performed with a mixture of secondary antibodies specific to the appropriate species (IgG, rabbit, mouse, and goat). In detail, a mixture of Cy3-donkey anti-rabbit-, Cy3-donkey anti-mouse-, and Cy5-donkey anti-goat-conjugated IgGs diluted in 0.3% Triton X-100, 5% FCS in TBS was applied for 2 h at room temperature. When slices were incubated in TBS without the primary antibody, or with primary antibody, which had been preabsorbed with peptide antigen for 1 h before use, no immunofluorescence was observed.

Preparation of Tissues for the Quantification Study
CD-1® mice at the age of 14 days were transcardially perfused after CO₂-anesthesia with 1) solution 1 (2% paraformaldehyde in 0.1-M sodium acetate buffer, pH 6.5) with heparin and 2) solution 2 (4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.4 [PB]) with 0.1% glutaraldehyde. The brains were immediately removed and stored in solution 2 for 24 h followed by storage in sucrose solution (30%) for 48 h. Serial coronal sections (35 μm thick) containing the complete prefrontal cortical region (Fig. 5,4, left) were obtained using the vibratome and collected in PB. Coronal sections of 35 μm thickness containing the PFC were used for the cellular characterization/quantification. The distance between each slice on the rostro-caudal axis sampled out was 210 μm. Eight slices per staining were investigated. The slices were stained in a free-floating manner. After a blocking step in solution A (5% FCS, 0.3% Triton-X 100 in 0.05 M TBS, pH 7.6) for 30 min, the slices were incubated with antibodies (mouse anti-GABA, rabbit anti-glutamate) diluted in solution A for 48 h at 4°C. After extensive washing with TBS, the slices were incubated with the secondary antibodies (biotinylated horse anti-mouse, biotinylated goat anti-rabbit respectively; 1:65, Vector Laboratories, Burlingame, CA, USA) in solution B (1.6% normal horse/goat serum, 0.3% Triton X-100 in 0.1 M PB, pH 7.4) for 2 h at room temperature followed by extensive washing with TBS. For the detection by fluorescence microscopy, all slices were finally incubated with Cy2-conjugated streptavidin in solution A for 2 h at room temperature. Additionally, the slices were stained with the ultraviolet detectable nucleic acid dye Hoechst 33342 (17 μM, Molecular Probes, Leiden, The Netherlands), added 10 min before the end of the streptavidin incubation period. After intensive washing and mounting on slide glasses, all stained sections were dehydrated in a series of graded ethanol, processed through n-butylacetate and covered with Entellan (Merck, Darmstadt, Germany).

Confocal Microscopy
The immunofluorescence labeling was analyzed by confocal laser scanning microscopy (LSM 510 Meta, Zeiss, Oberkochen, Germany) using excitation wavelengths of 633 nm (helium/neon2), 543 nm.
(helium/neon1), and 488 nm (argon), as well as 351 nm/364 nm (ultraviolet laser, Enterprise, Hoechst staining), respectively.

**Quantification Analysis**
Pictures of the mPFC containing the anterior cingulate area and the prelimbic area were taken at the 20-fold magnification. Within the LSM pictures (600 × 600 µm), a smaller rectangle (240 × 360 µm) was arbitrarily chosen (for schematic illustration see Fig. 5A, right), and green and blue channel pictures were separated and transformed into black/white pictures (Adobe Photoshop CS3). With the help of the free access software "The Counter" (Andreas Erler; Free Software Foundation, Inc., Boston, MA, USA) clearly defined fluorescence-labeled cells and cell nuclei were counted manually (in a blinded fashion) twice by 2 persons within the selected region. Mean values of the counting data for each picture were submitted to statistical analysis (Sigma Plot® 11.0, Systat Software Inc.).

**Data and Statistical Analysis**
We used OriginPro 7.0 (OriginLab Co., Northampton, MA, USA) to analyze data and Corel Draw 15.1 (Corel Corporation, Ottawa, Canada) to prepare the figures. All the results are indicated as mean ± SEM and n indicates the number of experiments performed; drug effects were calculated as percentage of change from control values. Statistical analysis was performed with OriginPro 7.0 and SigmaStat (Systat Software Inc., Chicago, IL, USA) software package using ANOVA followed by Bonferroni test. The double and the single asterisks or hashes indicate a P of 0.01 and 0.05, respectively.

**Results**
Figure 1A shows our experimental model, namely the "static" organotypic coculture system of tissue slices from the VTA/SN complex and the PFC (Franke et al. 2003; Heine et al. 2007). We have reconstructed, as shown in Figure 1B,C, these parts of the mesocorticolimbic DAergic system to study the functional excitability properties by using a multisite MEA recording system. Through the MEA platforms, we recorded 2 different types of spontaneous activity from the VTA/SN-PFC cocultures: 1) a very fast activity shown in Figure 1D, namely bursts of action potentials and 2) LFPs shown in Figure 1E, which are characterized by frequencies ranging from 5 to 200 Hz and represent population synaptic potentials, afterpotentials of somatodendritic spikes and voltage-gated membrane oscillations, thus reflecting the input to a given brain area and its internal processing (Juergens et al. 1999; Belitski et al. 2008; Rasch et al. 2008). In Figure 1D, left, raster plots of exemplary bursts, recorded from a coculture at 5 DIV either by a number of electrodes located under the VTA/SN complex or by a number of electrodes located under the PFC are shown. Different neurons from both slices contributed to the burst, with a variable number of action potentials. The spikes recorded by one electrode under the PFC and by one electrode under the VTA/SN complex, during a 600 s recording period, are superimposed and sorted into 2 units in Figure 1D, right. Figure 1E shows some examples of LFPs, recorded from the PFC (upper row) and the VTA/SN complex (lower row). The waveforms shown in the graphs are represented as average ± SEM of 900-s recording periods. We followed the VTA/SN-PFC cocultures during their in vitro development and we characterized the developmental profile of the firing properties and network activity intra-areas and inter-areas, in order to evaluate the functionality of the newborn projections between the VTA/SN complex and the PFC.

**Characterization of the Development of VTA/SN-PFC Cocultures**
The first step for the characterization of VTA/SN-PFC coculture development consisted in the quantification of the following properties of spontaneous bursting activity during the time in culture (DIV): BR (bursts/min), IBSR and its peak (IBSR and pIBSR, Hz), BD (s), spike number (SN), and the percentage of spikes in bursts elicited during bursts with respect to their total number (%). We observed a gradual but significant increase of the burst frequency both in the VTA/SN complex (filled circles) and in the PFC (empty circles) from 10 different cocultures (Fig. 2A); in fact, while after the first 3 days in vitro the VTA/SN complex and the PFC showed a very low average BR (1.3 ± 0.2 and 0.5 ± 0.3 bursts/min, respectively), BR increased up to 5.1 ± 0.7 and 3.9 ± 0.6 bursts/min in the VTA/SN complex and the PFC, respectively, after 2 weeks of culture (P < 0.01). To investigate the firing activity during the functional synaptic connectivity, in panel B we show IBSR and pIBSR plots superimposed. Both parameters noticeably changed during the development in the VTA/SN complex: the former from 18.4 ± 3.2 Hz during the first days of culture to 40 ± 3.6 Hz at 13–15 DIV (P < 0.01), the latter from 86.8 ± 3.8 Hz at 1–3 DIV to 199.5 ± 9.1 Hz at 13–15 DIV (P < 0.01). At the same time, in the PFC only pIBSR significantly changed, decreasing from 290 ± 21.9 Hz at 1–3 DIV to 214 ± 10.1 Hz at 13–15 DIV, while IBSR remained constant. Furthermore, we observed that both BD and SN did not undergo a significant change during the development in vitro: in fact, BD of PFC neurons displayed a trend towards increase without reaching statistical significance, while VTA/SN complex activity showed stable BD values (Fig. 2C). On the contrary, VTA/SN complex and PFC neurons differed in their SN values only in the first days of development (10.6 ± 2.4 and 26.3 ± 3.4 spikes in burst, in the VTA/SN complex and in the PFC, respectively), with similar SN averages during the remaining in vitro maturation (18.1 ± 4.4 in the VTA/SN complex and 15.7 ± 3.7 in the PFC at 13–15 DIV; Fig. 2D).

With the help of our software, it was possible to discriminate excitatory and inhibitory neurons (Gullo et al. 2009, 2012). We analyzed in PFC the previously described parameters, distinguishing between the excitatory (empty triangles) and the inhibitory (filled triangles) clusters of neurons and these data are shown in Figure 2A–D at 1–3 and 13–15 DIV. BR increased from 1.4 ± 0.8 and 1.2 ± 0.6 bursts/min at 1–3 DIV to 5.1 ± 0.7 and 5.3 ± 0.7 bursts/min at 13–15 DIV (P < 0.01), for excitatory and inhibitory neurons, respectively (Fig. 2A), but, as expected, no difference was observed between the 2 neuronal clusters. IBSR remained stable during the time in culture (Fig. 2B), with the values of excitatory neurons being statistically different and higher than inhibitory cluster values (77.1 ± 4 and 43.3 ± 2.9 Hz at 1–3 DIV, P < 0.01; 84.8 ± 13 and 48.4 ± 6.7 Hz at 13–15 DIV, P < 0.01, for excitatory and inhibitory neurons, respectively). This result is in agreement with the well-known high SN inside the bursts of excitatory neurons, compared with the low IBSR of interneurons (Csicsvari et al. 1999; Barthó et al. 2004). On the contrary, during the first days of culturing, BD and SN of excitatory and inhibitory neurons were similar and they differed only later in the development: in fact, both BD and SN of inhibitory neurons were higher than the corresponding values of the excitatory cluster at 13–15 DIV (BD: 0.34 ± 0.14 s
and 0.46 ± 0.14 s, Fig. 2C; SN: 7.4 ± 2.4 and 16.6 ± 4.5, 
P < 0.05, Fig. 2D, for excitatory and inhibitory neurons, respectively, as expected from the known firing properties of principal cells and interneurons.

In Figure 2E, the percentage of spikes in burst was quantified with respect to the total number of recorded spikes. We observed that while in the PFC the amount of spikes being part of bursts remained constant and high throughout the development (94.9 ± 3.1% and 92.1 ± 1.9% at 1–3 and 13–15 DIV, respectively), with few spikes occurring out of the identified bursts, in the VTA/SN complex the burst firing pattern matured during the development, as indicated by the progressively increasing percentage of spikes encompassed in bursts (57.3 ± 6.2 and 89.1 ± 1.2 at 1–3 and 13–15 DIV, respectively). After 13–15 DIV, the percentage of spikes in burst remained stable for both the PFC and the VTA/SN complex.

Finally, we also computed and plotted in Figure 2F, the number of PFC excitatory and inhibitory neurons during the development of the cocultures. We observed a progressive increase of the average number of excitatory (empty triangles) and inhibitory (filled triangles) neurons, from 1–3 DIV to
9–15 DIV (excitatory cluster: 3 ± 1.2 and 22.1 ± 3 neurons at 1–3 and 13–15 DIV, respectively; inhibitory cluster: 1.1 ± 0.5 and 8.1 ± 1.3 neurons at 1–3 and 13–15 DIV, respectively). From these data, it is possible to conclude that the excitatory/inhibitory ratio in the PFC of mature VTA/SN-PFC cocultures at 13–15 DIV is ≈ 3.

On the whole, these data clearly indicate that during the in vitro development, the VTA/SN complex and the PFC tend to reach a similar mature condition, with homogeneous firing properties, in parallel with the progressive growth of new projections, which are able to link the 2 areas and allow a continuous communication between them.

To complete the picture and to illustrate the presence of remarkable LFPs, we show in Figure 2G, from the same electrode, the developmental trend observed in a representative dish. Data show a progressive maturation of the cocultures in both areas with a gradual increase in LFP amplitude (firing rate is superimposed) at 6 (a), 10 (b), 14 (c), and 17 (d) DIV, associated with a similar increase of the SR, both in the PFC (first row) and in the VTA/SN complex (second row); later in the development, the LFP amplitude remained almost stable (data not shown). On the whole, it can be noticed that the fast negative LFP deflection anticorrelates with the peak firing rate. However, the remaining waveform pattern is still under investigation since it depended on the spike units identified around the electrode.

Neuronal Populations in the VTA/SN-PFC Cocultures
To further characterize the VTA/SN-PFC cocultures, we investigated the distribution of different neuronal populations. To achieve this aim, next to incubation of the slice cocultures for 12–14 DIV, the tissues were fixed and cut in 50-µm thick slices. Afterwards labeling studies in combination with LSM using antibodies against the DAergic marker TH and against the inhibitory neuronal transmitter GABA or the excitatory transmitter glutamate in combination with the neuronal marker microtubule associated protein-2 (MAP2) as well as the glial marker GFAP were performed. The results are shown in Figures 3 and 4. Positive immunoreactivity for TH was found in the VTA/SN complex (Fig. 3A), where DAergic neurons were clearly labeled, while in the PFC only TH-positive fibers were identified (Fig. 3B). For the neurotransmitters glutamate and GABA, positive immunoreactivity was observed in the PFC as well as in the VTA/SN complex (Fig. 4). A colocalization of GABA (Fig. 4B) and glutamate (Fig. 4A) is indicated on both MAP2- (arrow) as well as GFAP- (arrowhead) positive structures, demonstrating the presence of inhibitory and excitatory neurons in this system.

As it is known that DAergic neurons represent the majority of VTA neurons (≈70%, although there is a subregional variability) and that they are intermingled with GABAergic (≈30%) and glutamatergic neurons (≈2–3%), even if the proportion can vary depending on the VTA subregions (Ungless and Grace 2012), we verified the presence of 3 different neuronal populations with distinct firing properties in the VTA/SN complex of the cocultures. We applied our software and we were able to identify 2 neuronal clusters; the least numerous cluster showed firing properties (SN; SR; FF; Fig. 3C; black histograms), which are consistent with putative GABAergic neurons (Gullo et al. 2012). The most numerous cluster was re-analyzed to see if it was possible to identify different neuronal subpopulations; we were able to identify 2 clusters, one of which was characterized by a very low firing rate and low

**Figure 3.** Characterization of VTA/SN neuronal populations. (A and B) Confocal images of the DAergic marker TH in the VTA/SN complex (A) and the PFC (B). It is possible to note the presence of DAergic neurons in the VTA/SN complex, while in the PFC TH-positive fibers are labeled. (C) Properties of the 3 neuronal populations (putative DAergic, Glutamatergic and GABAergic neurons, represented as striped, white and black histograms, respectively) identified electrophysiologically on the basis of the extracellular activity in the VTA/SN complex of an exemplificative coculture at 14 DIV: spike number (SN; a), SR (in Hz; b) and Fano Factor (FF; c) (Total recorded neurons from the VTA/SN complex: 75; putative DAergic 42, glutamatergic 14, GABAergic 19). (D) Exemplificative spike waveforms from a putatively identified DAergic (a), Glutamatergic (b) and GABAergic (c) neuron of the VTA/SN complex. The thick black lines represent the average of 200 recorded spikes, while the thin black lines indicate ± SEM. (Scale bars: A, 50 μm; B, 10 μm; D, a-c, 500 ms, 20 μV).
SN (Fig. 3C, striped histograms) and insensitivity to dopamine (data not shown; Lammel et al. 2008), which are consistent with putative DAergic neurons (Steffensen et al. 1998; Ungless and Grace 2012). The other cluster showed firing properties which were intermediate between the previously identified putative GABAergic and DAergic neurons (Fig. 3C, white histograms), corresponding to putative glutamatergic neurons. We applied this procedure to the VTA/SN complex of 10 cocultures (for a total number of 391 cells) and we quantified the relative percentages of the 3 identified neuron cluster; we found that putative DAergic neurons represented 57.3 ± 3.6% of the VTA/SN cells, putative GABAergic neurons were 27.3 ± 3.8% and putative glutamatergic neurons 15.4 ± 2.4%. In Figure 3D examples of spike waveforms are shown (the thick lines represent the average waveform of 200 spikes, recorded in control condition at 14 DIV, while the thin lines indicate ± SEM): (a) putative DAergic neuron spike waveform, which shows a triphasic profile (due to high-pass filtering > 250 Hz) and a duration ≥ 1.1 ms from spike initiation to the maximal negative phase (Grace and Bunney 1983; Ungless et al. 2004; Ungless and Grace 2012); (b) putative glutamatergic spike waveform, narrower than the

Figure 4. Confocal images of multiple immunofluorescence labeling studies to characterize the expression of inhibitory and excitatory neurotransmitters in the dopaminergic VTA/SN-PFC coculture system (12–14 DIV). (A) Confocal images of the neuronal marker microtubule-associated protein-2 (MAP2), the astroglial marker GFAP and glutamate in the PFC (a–d) and the VTA/SN complex (e–h), whereas the inset in (a) and (e) represents details of the labeling exposed in (b–d) and (f–h), respectively. The immunoreactivity for MAP2 (b,f), GFAP (c,g) and glutamate (d,h) in both regions are shown. Moreover, the expression of glutamate-positive structures on neuronal fibers (arrow) and on glial fibers (arrowhead) is illustrated (Scale bars a–h: 10 µm). (B) Characterization of MAP2 and GFAP in combination with γ-aminobutyric acid (GABA) in the PFC (a–d) and the VTA/SN complex (e–h). The inset in (a) and (e) represents details of the labeling shown in (b–d) and (f–h), respectively. A positive labeling for MAP2 (b,f), GFAP (c,g) and GABA (d,h) was observed, whereas a localization of GABA-positive structures on neuronal fibers (arrow) as well as on glial fibers (arrowhead) is demonstrated (Scale bars a–h: 10 µm).
positive cells within the slices indicated small changes of the number of the labeled cells in relation to the number of Hoechst-positive cells on the rostral-caudal axis (Fig. 5D). Finally, the difference between the mean values of all counted GABAergic and glutamatergic cells in relation to the mean value of Hoechst-positive cells was calculated, and on average, we observed 6.9 ± 1 and 17.5 ± 1.4 GABAergic and glutamatergic cells, respectively (obtaining a glutamatergic/GABAergic cell ratio of 2.5). In the present quantification experiments, we found a relationship between glutamate- and GABA-positive cells of ~3:1, thus confirming the electro-physiological characterization.

**Delays and Directional Properties of Bursting During Development**

To investigate the origin of the differences in the firing properties observed during the progressive growth of the newborn projections, we compared the starting times of all of the bursts in both areas (n=10) and looked for different propagation patterns of activity. During development, 4 patterns of network bursts were identified and their percentage incidence is shown in Figure 6: (A) bursts restricted to the VTA/SN complex (VTA-only bursts), while the PFC remained silent; bursts confined to the PFC (PFC-only bursts, see inset); (B) bursts starting in the VTA/SN complex and rapidly propagating to the PFC (VTA-to-PFC bursts) and (C) bursts which arose in the PFC and back-propagated to the VTA/SN complex (PFC-to-VTA bursts). The VTA-only bursts were the predominant firing pattern during the early development, at 1–3 DIV (78.2 ± 14.5%), when we also observed PFC-only bursts (21.8 ± 14.5%), but no propagating bursts between the VTA/SN complex and the PFC (Fig. 6A–C). This can be explained by the fact that, after 1–3 DIV, the growth of new projections is still at the very beginning: thus, the connection between the 2 areas of the cocultures is limited or even absent, and thereby not sufficient to allow the activity to propagate. After 5–7 DIV, the percentage of VTA-only bursts significantly decreased to 29.8 ± 10.7% (P < 0.01), and less PFC-only bursts were observed (even if the decrease was not significant), but a remarkable increase of VTA-to-PFC bursts (61.7 ± 10.8%, P < 0.001 in comparison with the previous developmental phase) occurred. At this developmental stage, we also recorded a very limited number of PFC-to-VTA bursts (2.1 ± 1.7%). After 9–11 DIV this trend was maintained: we observed a further reduction in the incidence of VTA-only bursts (13 ± 6.1%), the disappearance of PFC-only bursts (Fig. 6A inset) and an additional increase in VTA-to-PFC bursts (86.3 ± 6%, Fig. 6B). The developmental profile of the firing patterns reached a stable condition after 2 weeks in culture: in fact, the incidence of VTA-to-PFC bursts remained almost stable (80–90%), the VTA-only bursts and the PFC-to-VTA bursts were <15% each, while no PFC-only bursts were recorded. Then, we analyzed the propagation delays in panels D,E. We observed a progressive reduction in the VTA-to-PFC burst propagation delays: the delay significantly decreased from 99.6 ± 10.8 ms at 5–7 DIV, when this type of firing patterns first appeared, to 47.4 ± 4.7 ms at 17–19 DIV (P < 0.05); then, the delay remained constant during the subsequent week of culture (Fig. 6D). These delays suggest an average velocity of ~0.02 m/s when the VTA/SN-PFC cocultures are mature; this value is far from that reported in vivo for

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**Figure 5.** Quantification of excitatory and inhibitory neurons in the PFC of CD1® mice. (A) On the left, schematic illustration of the preparation of brain slices for the quantification of GABA- and glutamate-positive cells in the PFC. Examples of the brain slices containing the PFC are shown on the right. The highlighted small squares indicate the area used for cell counting. (B–D) Quantification of GABA- and glutamate-positive cells in the PFC of CD1® mice (14 days old). Exemplary confocal microscopy images of GABA- (B) and glutamate- (C) positive cells are shown (Scale bars in B and C: 20 μm). The number of stained cells divided by the number of Hoechst stained nuclei is shown in (D) for the different slices quantified. Bar charts represent mean values for the proportion of GABA- or glutamate-positive cells respectively.

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previous one, and (c) spike waveform of a putative GABAergic neuron, which is narrower, particularly with respect to the duration from the start to the trough (Ungless et al. 2004).

Since we have previously shown that in the PFC of mature (13–15 DIV) cocultures there is an excitatory/inhibitory ratio of ≈3 (Fig. 2F), to verify that this ratio is valid under in vivo conditions, we performed a quantification study (see Methods, Fig. 5A) in the PFC of P14 mice. Immunofluorescence labeling of GABAergic and glutamatergic cells showed a number of positive cells within the studied PFC (examples are given in Fig. 5B,C). Quantification analysis of GABA- and glutamate-ergic neuron, which is narrower, particularly with respect to the duration from the start to the trough (Ungless et al. 2004).
mesolimbic (of 0.55 m/s; Deniau et al. 1980; Thierry et al. 1980) and for nigrostriatal (0.58 m/s; Guyenet and Aghajanian 1978; Lapish et al. 2007) DAergic fibers. The VTA-to-PFC burst latency is much longer compared with what is expected for monosynaptic events, but we can explain this fact considering that the projections linking the VTA/SN complex and the PFC in the cocultures have regrown in vitro after the slicing procedure and assuming that the bursts were polysynaptically generated (as indicated by the presence of multiple propagation pathways of synaptic activity within each area of the cocultures, described in the next paragraph, and as previously suggested by Overton and Clark [1997]). Differently from VTA-to-PFC bursts, we could not distinguish a clear developmental profile for the PFC-to-VTA burst delays: at 5–7 and 9–11 DIV the incidence of PFC-to-VTA bursts was too low (<2.5%) to allow the quantification of the delay; from 13–15 DIV throughout the remaining development, when this propagation pattern represented >5% of the total recorded network bursts, we calculated the delays, but the variability was too high to identify a clear developmental trend (Fig. 6E). In fact, the PFC-to-VTA propagation delay significantly decreased from 28.7 ± 9.5 ms at 13–15 DIV to 9.8 ± 1.1 ms at 17–19 DIV (P < 0.01), but then we observed a successive increment during the last week in vitro.

Taken together, these results are in agreement with in vivo experiments, which have demonstrated the functional coupling and reciprocal modulation between the VTA and PFC electrical activity (Sesack and Pickel 1992; Seamans et al. 2003; Peters et al. 2004; Tseng et al. 2006; Gao et al. 2007; Fujisawa and Buzsáki 2011).

**Is the Functional Role of Burst-Leader Neurons Changing During Development?**

Since in Figure 6 we discovered that bursts can originate from 1 of the 2 areas and propagate to the other area, we decided to investigate if, during development, some neurons maintain or loose their privilege to be the leaders. To achieve this aim, we analyzed how all the 4 previously identified firing patterns are triggered by quantifying their privilege of being major burst leaders (MBLs), namely a neuron that triggers at least 4% of the network bursts (Ham et al. 2008). We computed the percentage of both MBLs and MBL-triggered bursts in the Figure 6 experiments. We observed a similar progressive decrease of MBLs in the 2 areas during development as shown in Figure 7A (continuous lines) from ∼53% at 1–3 DIV to ∼22% at 9–11 DIV (P < 0.01 for both areas). This suggests that, during the development of the cocultures, there was a gradual increase in the potential initiation sites of activity in the networks, revealing enrichment of synaptic activity in the propagation pathways inside each area of the cocultures. This result was accompanied by a slight reduction in the percentage of network bursts triggered by MBLs (Fig. 7A, dashed lines), but this change was not statistically significant. In
role in triggering activity steadily during the development (as neuron 78a).

development (see neurons 32a and 37a in the graphs) or in the central phase of the behaviors during development: they can act as MBLs only during the early stage of the PFC at each time point. It is possible to note that neurons can have different

colonies in each panel of the graph); 3) neurons could change their behavior during the time in culture. In fact, it is possible to observe that neurons can behave as MBLs only during the early stage of development (at 3 DIV, as cells 32a and 37a) or in the central phase of the maturation of cocultures (at 9–15 DIV, like cells 32b) or they can have a predominant role in initiating network bursts steadily throughout the development (see cell 78a).

Therefore, during the time in culture we were able to observe a progressive maturation of the activity of the VTA/SN-PFC cocultures, which showed a complex plasticity of firing propagation patterns appearing concomitantly with the growth of projections from one area of the cocultures to the other.

**Disruption of the Newborn Projections Completely Abolishes the Correlated Activity of the VTA/SN-PFC Cocultures**

To test whether the correlated activity recorded in the VTA/SN complex and PFC was due to the newborn projections grown between the 2 areas of the cultures during the in vitro development, these connections were cut in the middle of the border region between the 2 slices (by using the tip of a needle, without detaching slices). Before and after the cutting procedure, the spontaneous activity was recorded for 1 h, in order to evaluate the changes caused by the disruption of the projections. Exemplary autocorrelograms of an excitatory (white) and an inhibitory (black) neuron from the PFC of a coculture, recorded from the electrodes indicated by the dashed circles, are shown in Figure 8A in the presence of intact projections and in Figure 8B after their separation along the dashed line. The disruption of the newborn projections did not change the physiological properties of the 2 clusters: in fact, as shown in Figure 8C,D, BD, SN, IBSR, FF and ACF of both excitatory (white) and inhibitory (black) neurons of the PFC did not show a significant change in response to the cut of the projections. To verify if VTA/SN-PFC synchronization was still present, we show in Figure 8E,F the raster plots of the burst activity in both areas before and after the cut. It is clear that the disruption of the axonal projections caused a complete loss of the synchronized control activity. To quantitatively verify this change, in Figure 8G,H the cross-correlograms of the spiking activity in control and after the cut of projections are shown, using a neuron of the PFC as the reference. When the axonal projections were eliminated, there was a loss of correlation in the network activity, as shown by the flat VTA cross-correlogram profile (filled circles). While in control the propagation delays of VTA-to-PFC and PFC-to-VTA bursts were in the order of tenths of milliseconds (inset to Fig. 8G), in absence of intact projections the delays appeared to be randomly distributed in the order of tenths of seconds (inset to Fig. 8H). This means that when the VTA/SN complex and the PFC were linked by the projections, there was a short

**Figure 7.** Is the functional role of burst-leader neurons changing during development? (A) Percentage of Major Burst Leaders (MBLs, continuous line) and percentage of network bursts triggered by MBLs (dashed line) during the development of the VTA/SN complex and PFC (black filled circles) and the PFC (black empty circles; n = 10). Statistical significance of MBL developmental profile (PFC: 1–3 vs. 5–7, 5–11, 13–15, 17–19, 21–23, P < 0.01; 5–7 vs. 17–19, P < 0.05, VTA/SN complex: 1–3 vs. 9–11; 13–15, 17–19, 21–23, P < 0.01; 1–3 vs. 5–7, P < 0.05). (B) Histograms of the percentage of network bursts triggered by each neuron recorded at different stages of the development (3, 9, 14 and 18 DIV) for one representative VTA/SN-PFC coculture. The neurons indicated on the x-axis include those belonging both to the VTA/SN complex and the PFC. The dotted line in each of the 4 graphs represents the threshold for a neuron to be considered a MBL (≥4% of the network bursts triggered). The insets (top right of each graph) indicate the percentage of MBLs in the VTA/SN complex and in the PFC at each time point. It is possible to note that neurons can have different behaviors during development: they can act as MBLs only during the early stage of development (see neurons 32a and 37a in the graphs) or in the central phase of the maturation of the cocultures (like neuron 32b) or they can have a very predominant role in triggering activity steadily during the development (as neuron 78a).

**Figure 7B,** data from an exemplary coculture are shown as % histograms of bursts triggered by each of the identified neuron, and we traced this development from 3 up to 18 DIV.

From the histograms (which represent 4 different stages of the maturation at 3, 9, 14 and 18 DIV) it is possible to see in detail some previously described global developmental features: 1) the number of neurons recorded from the cocultures progressively increased during the days in vitro, as evident from the x-axis; 2) the percentage of MBLs in the VTA/SN complex and in the PFC decreased during development (the values for the coculture in analysis are shown in the inset tables in each panel of the graph); 3) neurons could change their behavior during the time in culture. In fact, it is possible to observe that neurons can behave as MBLs only during the early stage of development (at 3 DIV, as cells 32a and 37a) or in the central phase of the maturation of cocultures (at 9–15 DIV, like cells 32b) or they can have a predominant role in initiating network bursts steadily throughout the development (see cell 78a).
propagation delay from the VTA/SN complex to the PFC and vice-versa; on the contrary, when the projections were not continuous but were interrupted by the cut, the propagation of activity was blocked, and the 2 areas continued to fire spontaneously according to their intrinsic frequency, each independently from the other one.

**Pharmacological Disinhibition of the Activity: Gabazine-Mediated Effects on VTA/SN-PFC Cocultures**

To demonstrate that the model of organotypic VTA/SN-PFC cocultures can be used to test the effects of different pharmacological agents (such as modulators of their activity or potentially interesting neuroregenerative substances), we applied GZ, a blocker of GABA$_\text{A}$ receptors, at 2 different concentrations (200 nM and 10 $\mu$M), known to act on phasic and both phasic and tonic inhibition, respectively (Stell and Moody 2002). In order to allow GZ to diffuse into the slice tissue properly, we recorded the spontaneous activity at each concentration for 30 min, and we have selected the second half of the recording sessions to evaluate the drug effects and to calculate the firing parameters (expressed as percentage change relative to 100% of control). In Figure 9A, the properties of the excitatory (white) and inhibitory (black) clusters of PFC cells are shown: BD of excitatory neurons increased almost 2-fold (180.1 ± 24.2%, $P<0.05$) with 200 nM GZ in comparison with control and further with 10 $\mu$M GZ (up to 337.3 ± 39.7%, $P<0.01$), while there was no change in BD of inhibitory neurons; SN and SR of the excitatory and inhibitory clusters increased with both concentrations of GZ (SN: 168.2 ± 17.7% and 145 ± 17.8% at 200 nM GZ and 284.7 ± 40.2% and 229.4 ± 40.9% at 10 $\mu$M GZ, $P<0.01$ and 0.05, for excitatory and inhibitory neurons, respectively; SR: 336.6 ± 93.9% and 279.6 ± 60.1% at 200 nM GZ, with $P<0.05$, and 732 ± 175.4% and 416.6 ± 82.8% at 10 $\mu$M GZ, $P<0.01$ and 0.05, for excitatory and inhibitory neurons, respectively). Finally, the excitability (spikes-per-neuron) of both clusters significantly increased at the high GZ concentration (260.2 ± 48% and 230.6 ± 25.6%, $P<0.05$ and 0.01, for excitatory and inhibitory neurons, respectively), while at 200 nM GZ, the effect was visible only for the excitatory cluster (174.3 ± 28.2%, $P<0.05$). Also BR increased in both areas, as shown in Figure 9B, when we blocked phasic and tonic inhibition through 10 $\mu$M GZ (224.3 ± 29.6% and 205.5 ± 47.5%, $P<0.01$ and 0.05, in the PFC and the VTA/SN complex, respectively), while the block of phasic inhibition with 200 nM GZ caused no statistically significant change.

Furthermore, we analyzed the VTA-to-PFC bursts, which represent the most frequently identified propagation pattern, and put up the question whether the treatment with GZ causes a change in the propagation delay. We observed that the propagation delay of VTA-to-PFC bursts significantly decreased to 73.7 ± 8.1% at 200 nM GZ ($P<0.05$) and to 43.8 ± 6.4% at 10 $\mu$M GZ ($P<0.01$), when compared with control (Fig. 9C). Furthermore, in one exemplary experiment, we investigated for the PFC bursts if GZ altered the properties of PFC network states. To achieve this aim, we computed the cFSH in the presence of 200 nM and 10 $\mu$M GZ and superimposed them to those of controls (represented by the thin dashed and continuous lines; Fig. 9D). Although the treatment with 200 nM GZ barely affected the control cFSH profile, 10 $\mu$M GZ caused the appearance of 2 burst states (left and right adjacent plots, with different probabilities of
occupancy, PO see Fig. 9E). These different (P < 0.05) states were characterized by an increase in the probability of having bursts with a high number of spikes (>10), both for excitatory (thick dashed lines) and inhibitory (thick continuous lines) neurons. Furthermore, the cFSHs (Fig. 9D, second row) indicated that, in addition to an increase in the spikes per burst (evident from the shift towards the right of the cFSH profile compared with control), GZ caused also an increase in the average percentage of those excitatory (thick dashed lines) but not inhibitory (thick continuous lines) neurons, which are participating in the network bursts. Finally, in order to study how the 2 neuronal clusters are progressively recruited after the burst start, we have plotted in Figure 9E the time histograms of the engaged neurons NNTHs, which describe, in the time domain, what has been previously described in Figure 9D in the spike domain. In particular, GZ at 200 nM did not change significantly the control short time-course of the burst, whereas at 10 μM it caused a pronounced BD increase of state 2 (PO, 33.9%) with respect to state 1 (PO, 59%) and an enhanced lifetime of engaged neurons with respect to control (thin dashed and continuous lines).

The disinhibition expected and observed because of the treatment with GZ on the spiking activity of VTA/SN-PFC co-cultures was also evident in the LFP activity, simultaneously recorded with the MEA platform. In Figure 9F, exemplary average traces (mean + upward error bars) of LFPs (open

Figure 9. Network disinhibition by gabazine (GZ). (A) Plots of percentage increase, with respect to control, of BD (A1), SN (A2), SR (A3) and excitability (A4) for excitatory (white) and inhibitory (black) clusters of PFC during application of 200 nM or 10 μM GZ. Excitatory and inhibitory neurons were 263 and 93 (5 experiments). Statistical significance in: A1: excitatory neurons at 200 nM vs. 10 μM GZ, P < 0.05; at 10 μM, excitatory vs. inhibitory neurons, P < 0.01; A2: excitatory neurons at 200 nM vs. 10 μM, P < 0.05. (B) Plots of percentage increase, with respect to control, of BR recorded in the PFC (grey) and in the VTA (black) during application of 200 nM or 10 μM GZ. Same experiments as in A. (C) Plots of percentage increase, with respect to control, of propagation delay of VTA-to-PFC bursts during application of 200 nM or 10 μM GZ. Same experiments as in A. Statistical significance: 200 nM vs. 10 μM, P < 0.05. All the data (mean ± SEM) shown in A–C are obtained normalizing all the values of 5 experiments to the value found in the control condition of each experiment and then averaging the normalized values. (D) For PFC, plot of cFSHs of identified network states in 200 nM and 10 μM GZ for excitatory (thick dashed lines) and inhibitory (thick continuous lines) neurons; the thin dashed and continuous lines indicate excitatory and inhibitory neuron control data, respectively. Left and right plots report data of state 1 and 2, respectively (1 exemplary experiment, 46 excitatory and 11 inhibitory neurons). (F) Same as D but for time histograms NNTHs. Upper-right legends indicate percentage occupation of states (see Methods). (F) Effect of GZ on exemplary LFPs recorded from a PFC electrode containing 2 units. The open squares indicate the average LFP waveforms (+ upward error bars) recorded in 900 s. Number of bursts for the averaged waveforms: control: 35; 200 nM GZ: 25; 10 μM GZ: 30. LFP data graphs: 4 skipped points. The superimposed upward-going thin black lines show the average spike rate increase during bursts. Notice at 10 μM an oscillatory activity. The dashed line indicates the zero level.
As shown in Figure 10, the procedure used in patch-clamp experiments and effect of D1 and D2 receptor antagonist on AMPA and NMDA currents. Data in A–D and E–H are from AMPA (NMDA, APV-sensitive) and NMDA (APV-sensitive) evoked currents (average of 10 traces), respectively. Data in B, F, and G were obtained by using SCH23390 and eticlopride, respectively.

**D2 Receptor-Mediated Modulation of the VTA/SN-PFC Coculture Activity: MEA and Patch-Clamp Experiments**

**MEA Experiments**

Since both the VTA/SN complex and the PFC contain DAergic neurons, which reciprocally innervate the 2 brain areas, we decided to test whether the spontaneous activity of the VTA/SN-PFC cocultures could be modulated by dopamine receptor blockade. We applied 10 μM eticlopride, a D2 receptor blocker, in the cocultures on MEAs and evaluated the firing parameters in the PFC. We evaluated the eticlopride effects as percentage changes (with respect to control) on BD, SR, and excitability of PFC excitatory and inhibitory neuronal clusters (average data of 4 experiments). The treatment with 10 μM eticlopride caused a significant decrease of BD and excitability of inhibitory neurons (48.5 ± 13.9% and 37 ± 13.9%, P < 0.05 and 0.01, for excitatory and inhibitory clusters, respectively). Furthermore, the BR was found to decrease for both the VTA/SN complex and the PFC to 44.7 ± 16% and 45.2 ± 18.7%, (P < 0.01 and 0.05), respectively. Taken together, these data suggest that D2 receptor blockade produces a BR decrease, that is, an increase of the down state that is obviously one of the reasons for the observed SR decrease.

**Patch-Clamp Experiments**

In order to understand the mechanism responsible for the inhibition of the spontaneous activity in presence of 10 μM eticlopride, we performed somatic whole-cell patch-clamp recordings from mPFC pyramidal neurons, stimulated through a bipolar electrode placed in the VTA region (see Methods), and studied the effect of D1 or D2 receptor blockade on AMPA- and NMDA-mediated currents. We show in Figure 10 the results obtained with 5 μM SCH23390 (a selective D1 receptor antagonist) or with 10 μM eticlopride. Exemplary recorded currents before and after drugs application in the same cell are shown for AMPA and NMDA currents in panels B, C, and F, G, respectively. We found that the effects of blockade of the 2 dopamine receptors resulted in different effects thus suggesting the functional presence of both receptor types. As shown in Figure 10 D, H for the evoked AMPA and displayed a slight but non-significant decrease in their excitability. In contrast, both PFC excitatory and inhibitory neurons exhibited a marked SR decrease (42.5 ± 17.8% and 37 ± 13.9%, P < 0.05 and 0.01, for excitatory and inhibitory clusters, respectively). Furthermore, the BR was found to decrease for both the VTA/SN complex and the PFC to 44.7 ± 16% and 45.2 ± 18.7%, (P < 0.01 and 0.05), respectively. Taken together, these data suggest that D2 receptor blockade produces a BR decrease, that is, an increase of the down state that is obviously one of the reasons for the observed SR decrease.
NMDA currents, respectively, we observed for eticlopride a reduction of both evoked AMPA and NMDA currents (67 ± 3.3% and 81.8 ± 2.9%, in 8/8 and 6/9 cells, *P* < 0.05, for AMPA and NMDA currents, respectively). This effect can thus explain the inhibitory action identified with the MEA recordings. On the contrary, SCH23390 enhanced the NMDA currents and depressed the AMPA currents, respectively (140 ± 5.3% and 75.8 ± 2.9%, in 10/13 and 12/16 cells, *P* < 0.05).

**Discussion**

In the present study we characterized the functionality of regenerating bi-directional neuronal projections connecting the VTA/SN with the PFC. These projections were reported to gradually grow under in vitro conditions and thereby close the gap between 2 adjacent slice preparations (Franke et al. 2003; Heine et al. 2007). We identified a brief, 3–7 DIV transient phase of de-synchronized spontaneous activity in either of the cocultured slices and a subsequently developing adulthood-based synaptic synchronization of the bursting pattern. We validated the newborn projections and found that they allow the neuronal activity to propagate both from the VTA/SN complex to the PFC and in the opposite direction; in the absence of these projections, the propagated activity was lost. The progressive development of the neuronal connections has been found to be accompanied by the appearance of different activity patterns. Eventually, we have proven that the VTA/SN-PFC cocultures responded to the pharmacological modulation of their GABAergic and glutamatergic innervation in a manner conforming with reactions of the adult, healthy brain.

**Formation of Propagated Excitability, Synaptic Connections and Spontaneous Activity**

The analysis of the formation of functional connections in the CNS requires an appropriate model characterized by a fast recording setup not perturbing cells for weeks. The MEA technique, known from many years, in which dissociated neuronal cells are kept in culture, is the simplest model to study the excitability neuronal activity (Keefer et al. 2001; Gramowski et al. 2004; Van Pelt et al. 2004; Tateno et al. 2005; Wagenaar et al. 2006; Gullo et al. 2009; Baltz et al. 2010). The network activity varies depending on the use of embryonic or postnatal tissues, on the application of medium with or without fetal serum and on the presence or absence of a major proliferating astrocytic layer. Indeed, after 1–2 DIV it is possible to record randomly distributed spikes (suggesting correct resting potential and density of voltage-dependent channels) with disorganized activity that rapidly reaches a synchronized and reverberating bursting pattern at about 5–7 DIV. The last characteristic suggests that: 1) neurotransmitters can be efficiently released from axon terminals, 2) synapse formation is fast, 3) excitatory and inhibitory mono- and metabo-tropic neurotransmitter receptors are homeostatically controlled. Only recently, elementary pharmacology demonstrated that the up-state bursting activity is characterized by an intrinsic connectivity mediated by anatomy-independent rules but controlled by sets of physiologically different neurons, namely excitatory and inhibitory, whose ratio is basically the same as that present in the adult mammalian cortex (Sahara et al. 2012) and their firing mode can be recognized to be completely different by computing the FF (Gullo et al. 2010, 2012). Taken together these observations suggest that, independently of the developmentally induced anatomical changes, other still unknown rules should operate, which are able to finely control the appropriate formation of all the synaptic links that govern a balanced autonomous activity.

**The Importance of Regeneration**

In recent years, many studies have been done to face the problem of CNS regeneration and many in vitro studies have analyzed the regenerative abilities of different CNS pathways, such as the serotoninergic raphe-hippocampal pathway (Papp et al. 1995), the corticostriatal (Plenz and Aertsen 1996a, 1996b) and the thalamocortical connections (Rennie et al. 1994), the perforant pathway (Hofmann et al. 2004; Hofmann and Bading 2006), the nigrostriatal system (Snyder-Keller et al. 2008) and the mesocorticolimbic DAergic pathway (Franke et al. 2003; Maeda et al. 2004; Heine et al. 2007; Kroene et al. 2009).

Although very interesting patch-clamp studies (Kroene et al. 2009) in organotypic cocultures or morphological studies in cultured VTA-PFC neurons (Gao and Wolf 2007) have been recently done, electrophysiological investigations accompanied by morphological studies were altogether scarce, and in the particularly complex mesocorticolimbic DAergic system completely nonexistent. Here we have reconstructed this system ex-vivo in the form of organotypic cocultures and demonstrated that the projections between the VTA/SN complex and the PFC are able to regenerate following an innervation pattern that is similar to that occurring in vivo (as described by Franke et al. 2003). These projections propagate spikes between the 2 areas of the reconstructed DAergic system, thus allowing a continuous and reciprocal communication. Therefore, the neuronal connections not only regenerate, but they are also functional.

**The Combination of Organotypic Cultures with Multielectrode Array Recordings**

Although many studies using organotypic cultures on MEAs concentrated on evoked activity (Egert et al. 1998; Jahnsen et al. 1999; Shimono, Baudry and Panchenko et al. 2002; Van Bergen et al. 2003; Hofmann et al. 2004; Cater et al. 2007), other groups focused on the spontaneous activity (Beggs and Plenz 2003, 2004; Hofmann et al. 2004; Hofmann and Bading 2006; Plenz and Thiagarajan 2007; Gireesh and Plenz 2008; Stewart and Plenz 2008).

We studied and characterized activity by recording spikes organized in bursts and LFPs. For each electrode and its identified sort spikes, we were able to correlate the SR with the simultaneous LFP (Figs. 1, 2 and 9). As previously described, the LFP anticorrelates with the local neuronal firing rate, with negative LFP deflections occurring at the times of increased firing rate (Pouille et al. 2009; Harris et al. 2011; Isaacson and Scanziani 2011). However, we also noticed that the fast negative deflections are followed by slow consecutive positive LFP deflections. This is in agreement with the concepts explaining feed-forward cortical circuit connectivity (Pouille and Scanziani 2001).
In the PFC, we evaluated also the excitatory and inhibitory cluster parameters, such as BD, IBSR, BR, and SN. We noticed that the differences in the firing between the 2 regions of the cocultures tended to disappear in the first 2 weeks of culture, in parallel with the growth of projections between the areas which resulted to have similar firing properties. Furthermore, in the PFC, we characterized the developmental increase in the number of excitatory and inhibitory neurons, which is in good agreement with our immunolabeling and quantification experiments.

It is important to remind that the in vitro grown projections have been demonstrated to be target-specific. Whereas in cocultures of the VTA and hippocampus, which is a minor target of DAergic fibers, a growth of fibers occurred, in cocultures of the VTA with the cerebellum, which is not a physiological target of DAergic projections, no growth of fibers into the cerebellar slices has been described (Østergaard et al. 1990; Holmes et al. 1995).

We noticed a correlated activity between the VTA/SN complex and the PFC (Fig. 6) in the form of bursts which originated in the VTA/SN complex and propagated to the PFC (VTA-to-PFC bursts) and bursts which arose in the PFC and back propagated to the VTA/SN complex (PFC-to-VTA bursts). The presence of such a bidirectional propagation of activity is in agreement with in vivo experiments, which have demonstrated the functional coupling and reciprocal modulation between the VTA and PFC electrical activity (Sesack and Pickel 1992; Seamans et al. 2003; Peters et al. 2004; Tseng et al. 2006; Gao et al. 2007; Fujisawa and Buzsáki 2011).

The VTA/SN-PFC Regeneration Toward Maturity Opens up Studies for Understanding Basic Mechanisms in Brain Networks

Activators and blockers of DAergic receptors were useful to understand the DAergic modulation of nucleus accumbens (Nac) neuronal activity in VTA-Nac-PFC triple cultures (Maeda et al. 2004). We verified how disinhibition obtained through the application of 2 different concentrations of gabazine (GABA_A receptor antagonist), known to act on both phasic and phasic and tonic inhibition (Stell and Moody 2002), and blockade of D2 receptors with eticlopride were able to modify the activity of VTA/SN-PFC cocultures. The treatment with GZ (Fig. 9) caused a strong disinhibition in both areas, as can be noted from the observed increase of BD, SN, SR, excitability, and BR, in agreement with the disinhibitory effect previously described on primary dissociated cultures (Guollo et al. 2009, 2010). By contrast, D2 receptor blockade with eticlopride caused a decrease of VTA/SN-PFC activity, as it was suggested by the BR decrease both in the VTA/SN complex and the PFC. This effect was also accompanied by a decrease of BD, SR and excitability of PFC excitatory and inhibitory neurons. These data are in agreement with the whole-cell patch-clamp experiments on VTA/SN-PFC cocultures (Fig. 10), which showed that the treatment with eticlopride causes a decrease of VTA-evoked AMPA- and NMDA-mediated currents in PFC pyramidal neurons. Thus, the patch-clamp results explain the mechanism of the inhibitory effect of D2 receptor blockade, which has been observed in the MEA experiments. However, our results with eticlopride contrast with other findings, indicating that the activation, and not the inhibition, of D2 receptors causes a decrease of neuronal excitability through the blockade of AMPA- and NMDA-mediated responses (Gulledge and Jaffe 1998; 2001; Gulledge and Stuart 2003; Tseng and O’Donnell 2004; Gao and Wolf 2007). These divergent results can be attributed to differences in the experimental conditions, as it has been reported also for other aspects of the DAergic neuromodulatory action (Seamans and Yang 2004). More recently a triple mature (16 DIV) organotypic coculture method (Kroener et al. 2009) was used to study the effects of DA modulation on synaptic activity in PFC neurons by stimulating the newborn axons by electrodes positioned in the VTA. By using patch-clamp electrophysiology and electrochemical detection of DA released, it was shown that up-states can be elicited and more important that this activity is concentration dependent.

In conclusion, extra- (Maeda et al. 2004) and intra-cellular (Plenz and Aertsen 1996a, 1996b) recording techniques, the whole-cell patch-clamp method (Snyder-Keller et al. 2008) and sometimes even MEA platforms (Hofmann et al. 2004; Hofmann and Bading 2006) proved to be useful tools to record the synchronized activity of different brain areas cocultured as slice preparations. Nevertheless, this is the first ex vivo study describing with a high temporal resolution the functional recovery of synapses after disconnection of neuronal pathways previously connecting 2 remote areas of the DAergic system in the brain. We believe that our study has a considerable significance for further characterization of neuroregeneration processes.

Authors’ Contributions

E.W., P.I. and H.F. conceived the project and aims, E.D., C.H. and H.F. developed the organotypic-to-MEA platform, E.D. did and analyzed all the MEA experiments, C.H., H.F. and K.S. did and analyzed the IHC experiments, I.S. did and analyzed the patch-clamp experiments, F.G. advised us on the MEA recordings. E.D and E.W. wrote the manuscript.

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Notes

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References


