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Dopaminergic Development of Prenatal Ventral Mesencephalon and Striatum in Organotypic Co-Cultures

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Abstract

Using organotypic co-cultures of rat embryonic day 14 (E14) ventral mesencephalon (VM and E21 striatum, we have described the developmental changes in (i) dopamine (DA) neurochemistry; (ii) numbers of DA neurons; and (iii) protein expression of tyrosine hydroxylase (TH), DA transporter (DAT), and glutamic acid decarboxylase (GAD 65/67), over 17 days in vitro (DIV). Co-cultures demonstrated changes in DA development similar to those observed in vivo. The numbers of VM DA neurons remained relatively constant, while levels of VM DA progressively increased through 10 DIV. After 3 DIV, the levels of striatal DA increased substantially, through 10 DIV. Tissue levels of DA metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) reflected changes in tissue DA concentrations, indicating that release and metabolism of DA are similar to these characteristics observed in vivo. Western blot analysis of TH protein expression revealed large increases in VM TH after only 3 DIV, followed by a decline in levels through 17 DIV; levels of striatal TH, in contrast, increased through this period. Additionally, DAT and GAD 65/67 expression increased, in both the VM and striatum, over 17 DIV. By 17 DIV, many measures of DA function had decreased from those assessed at 10 DIV, thus providing an approximate limit to the effective duration of use of this co-culture model. Our results provide a much-needed description of the neurochemical changes that occur during the maturation of VM and striatum in organotypic co-cultures. Additionally, these results provide a foundation for future studies to assess toxic challenges of the developing nigrostriatal DA system, in vitro.

Keywords

dopamine; development; innervation; substantia nigra; organotypic culture; nigrostriatal

1. INTRODUCTION

Organotypic tissue culture techniques allow neuronal tissue to be maintained for weeks in vitro, and have thus expanded the utility of in vitro preparations for neurological study. When prepared from thick (200–400 μ m) sections of neuronal tissue, organotypic cultures permit the maintenance of the three-dimensional tissue architecture observed in vivo. These cultures maintain fundamental neuronal circuitries, and neuronal and glial interactions, and they have the ability to form new neuronal synapses with other appropriate brain regions when cultured together. Two organotypic culture techniques are predominately utilized. The first is the roller-

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tube method (Gahwiler, 1988; Ostergaard et al., 1990), in which tissue is continuously rotated within a bath of medium. The second is the static membrane model developed by Stoppini and colleagues (1988); in this technique, tissue rests on a porous membrane exposed to culture medium below while the upper surface is exposed to air so that tissue oxygenation can occur. Regardless of the culture method used, the organotypic preparation allows the study of an in vivo-like anatomical system with the ease of in vitro sample manipulations, such as administration of controlled levels of growth factors (Jaumotte and Zigmond, 2005; Hoglinger et al., 1998; Meyer et al., 2001; Schatz et al., 1999) or neurotoxicants (Kotake et al., 2003; Shimizu et al., 2003; Kress and Reynolds, 2005; Testa et al., 2005).

The ability to conduct morphological assessments of ventral mesencephalic (VM) and striatum co-cultures provides support for the use of the static membrane organotypic co-culture model of the basal ganglia, given that DA neurons present in the VM will project axons to an adjacent cultured striatal tissue slice (Snyder-Keller et al., 2001; Gates et al., 2004) and form functional synapses (Tseng et al., 2006). Further analyses have shown that even the complex patch/matrix organization of the striatal tissue is maintained when donor tissue of VM and striatum is age-appropriate (Snyder-Keller et al., 2001). Findings support the use of fetal as opposed to postnatal tissue, to most faithfully model in vivo development. These studies suggest that it is important to use VM tissue in which the dopamine (DA) neurons have differentiated, yet have not begun to project DA axons/terminals to the striatum; in this way, the likely degeneration incurred with axotomy during tissue dissection can be limited.

Parkinson's disease (PD) research has utilized VM organotypic cultures, containing the DA neurons affected in PD, in the study of tissue explants for transplantation purposes, as well as to assess potential DA neurotoxicants. Researchers have used the VM cultured alone (Dickie et al., 1996; Hoglinger et al., 1998; Meyer et al., 2001; Shimizu et al., 2003), the VM in co-culture with striatum (the target of VM DA neuron terminals) (Katsuki et al., 2001; Schatz et al., 1999; Gates et al., 2004; Kotake et al., 2003; Snyder-Keller et al., 2001), and the VM, striatum, and cortex in triple-cultures, to model the basal ganglia DA system (Plenz and Kitai, 1996; Snyder-Keller et al., 2001). However, to date, studies have not adequately described the DA development, in terms of neurochemical and protein analyses, of the VM and striatum in co-culture, something that is necessary if we are to fully understand the utility of this increasingly used model system.

Here, we have used organotypic co-cultures of E14 VM and E21 rat striatum in the static membrane system (Stoppini et al., 1991; Snyder-Keller et al., 2001) to model the developing nigrostriatal DA system. We examined multiple measures of DA function including DA neurochemistry, numbers of DA neurons, and levels of DA proteins, to complement the morphological assessments made by others (Snyder-Keller et al., 2001; Gates et al., 2004). These endpoints allow us to begin to determine the relationship of the measures obtained in the VM/striatum co-culture system to similar measures obtained during in vivo DA development.

2. RESULTS

2.1. Dopamine Neurochemistry

Analyses of DA and its metabolites DOPAC and HVA from the VM, striatum, and medium of developing co-cultures revealed differential changes in neurochemical measures for the two regions and media, as co-cultures were allowed to develop in vitro. E14 VM DA content increased progressively as a function of time through 10 DIV, whereas E21 striatal DA levels, which were relatively high at the time of dissection (0 DIV) fell dramatically after 3 DIV, and thereafter increased substantially, through 10 DIV (Fig. 1). Specifically, levels of DA in the VM were originally measured as 3.5 ± 0.2 ng/mg protein at the time of dissection (0 DIV),

increased to 14.7 ± 1.5 ng/mg protein after 3 DIV (not significant, ns), and increased further to 22.9 ± 3.1 and 38.5 ± 2.4 ng/mg protein after 6 or 10 DIV, respectively ($p \leq 0.001$). VM DA levels stabilized, and in fact fell slightly, to 32.4 ± 2.6 ng/mg protein (ns) after 17 DIV. Levels of striatal DA were measured at 21.6 ± 0.2 ng/mg protein at 0 DIV and fell by nearly 97%, to 0.6 ± 0.5 ng/mg protein after 3 DIV ($p \leq 0.01$). Thereafter, levels of striatal DA increased to 7.9 ± 1.6 and 23.9 ± 3.2 ng/mg protein after 6 and 10 DIV, respectively (ns, $p \leq 0.001$), before declining slightly to 19.5 ± 3.0 ng/mg protein by 17 DIV (ns). Levels of DA in the medium did not substantially differ among the 3, 6, 10, and 17 DIV time points; levels ranged from 3.0 ± 0.5 to 5.2 ± 0.7 ng/mg protein.

Striatal DOPAC levels (Fig. 2) mirrored the results of striatal DA, albeit at substantially lower values, with concentrations of 1.3 ± 0.2 ng/mg protein at the time of dissection (0 DIV), a marked loss of DOPAC by 3 DIV to 0.1 ng/mg protein ($p \leq 0.001$), and then increasing levels through 6 and 10 DIV (0.7 ± 0.1 and 1.2 ± 0.2 ng/mg protein, respectively ($p \leq 0.05$)); levels then decreased slightly to 0.9 ± 0.3 ng/mg protein by 17 DIV (ns). VM DOPAC levels (Fig. 2) did not change significantly between any pairs of sampled time points; levels fluctuated between a low of 1.0 ± 0.4 and a high of 3.2 ± 0.3 ng/mg protein over the 17 DIV. Additionally, levels of DOPAC in the medium did not significantly change over the 17 DIV; levels fluctuated from a low at 6 DIV of 4.9 ± 0.7 ng/mg protein to a high of 14.0 ± 3.4 ng/mg protein at 10 DIV. There were no significant changes in the ratio of DOPAC to DA (data not shown) in either the VM or striatum over the 17 DIV.

Measurements of striatal and VM HVA levels (Fig. 3) revealed trends similar to that for DA; levels increased through 10 DIV, before decreasing significantly by 17 DIV ($p \leq 0.001$). Specifically, striatal HVA increased from 0.2 ± 0.03 ng/mg protein at the time of dissection (0 DIV) to 1.9 ± 0.2 ng/mg protein at 10 DIV ($p \leq 0.01$), before falling to 0.9 ± 0.2 ng/mg protein at 17 DIV (ns, $p \leq 0.10$). VM HVA levels increased from 1.0 ± 0.2 ng/mg protein at 0 DIV to 3.8 ± 0.4 ng/mg protein at 10 DIV ($p \leq 0.01$), followed by a reduction to 1.1 ± 0.2 ng/mg protein by 17 DIV ($p \leq 0.001$). HVA levels in the medium were less predictable, ranging from a low of 290.9 ± 45.0 ng/mg protein at 17 DIV to a high of 417.0 ± 48.4 ng/mg protein at 10 DIV.

Additionally, since it is possible that some serotonergic neurons are present in the VM, we analyzed levels of both VM and striatal serotonin at all time points in vitro. Levels of serotonin were exceedingly low at all time points in both the VM and striatum (0.3 to 1.0 ng/mg protein) and did not change significantly over the 17 DIV (data not shown), whereas levels of DA peaked at 38.5 ± 2.4 ng/mg protein and changed dramatically over the 17 DIV.

2.2. Numbers of Tyrosine Hydroxylase Positive Dopamine Neurons

Developing VM/striatal organotypic co-cultures were immunohistochemically labeled for tyrosine hydroxylase (TH), and neuronal counts were obtained using an automated tracing/counting program at the time of dissection (0 DIV) and then after 3, 6, 10, or 17 DIV (Fig. 4). The largest numbers of TH⁺ DA neurons were seen immediately following dissection (0 DIV) (2453 ± 133). The numbers of TH⁺ neurons declined slightly, by less than 10%, after 3 DIV to 2157 ± 59 (ns); they remained constant through the next two collection points at 6 and 10 DIV (2065 ± 169 and 2034 ± 68 , respectively). It was not until the co-cultures had been maintained through the last time point (17 DIV) that a significant reduction in the number of TH⁺DA neurons was observed, with numbers declining to 53% of the levels seen at the time of dissection (0 DIV) (or 60% of the levels seen at 3 DIV) ($p \leq 0.001$).

2.3. Western Blots - Dopaminergic Protein Measures

Figure 5 describes the developmental changes in both striatal and VM TH protein expression from 0 to 17 DIV in organotypic co-cultures. At 0 DIV, striatal TH expression was at a relatively

high level (3.1 ± 0.3 standardized units, SU) before decreasing to less than 0.9 ± 0.3 SU after just 3 DIV (ns, $p \leq 0.10$). Subsequently, levels of striatal TH gradually increased to levels of 3.8 ± 1.0 SU by 17 DIV ($p \leq 0.05$). VM TH expression began at 0 DIV with levels of 2.7 ± 1.0 SU before increasing sharply to 8.7 ± 1.0 SU after only 3 DIV ($p \leq 0.05$). After 3 DIV, levels of VM TH progressively decreased through 17 DIV to levels of 3.0 ± 0.3 SU ($p \leq 0.05$), values similar to those observed at 0 DIV.

Developmental changes in DAT expression are shown in Figure 6. There was a significant decrease in striatal DAT protein content from 0 to 3 DIV (from 6.9 ± 0.2 to 1.3 ± 0.2 SU) ($p \leq 0.001$). Levels of striatal DAT increased significantly at each time point after 3 DIV (3.1 ± 0.3 , 4.6 ± 0.2 , and 9.7 ± 0.5 SU for 6, 10, and 17 DIV, respectively), and by 17 DIV they were significantly elevated over the 0 DIV levels ($p \leq 0.001$). Levels of VM DAT did not change significantly from 0 to 6 DIV (values ranged from 3.3 ± 0.5 to 4.2 ± 0.4 SU); however, by 10 and 17 DIV, they had increased significantly over all previous determinations (9.9 ± 0.6 and 8.2 ± 0.5 SU, respectively) ($p \leq 0.001$).

Striatal GAD 65/67 protein expression (Fig. 7) remained unchanged from 0 to 3 DIV (1.7 ± 0.5 to 1.3 ± 0.4 SU, respectively) but increased significantly by 6 DIV ($p \leq 0.05$), and then again between 10 and 17 DIV ($p \leq 0.001$) (4.1 ± 0.1 , 3.6 ± 0.3 , and 8.2 ± 0.4 SU for 6, 10, and 17 DIV, respectively). VM GAD 65/67 protein levels also remained unchanged between 0 and 3 DIV (0.8 ± 0.4 and 0.5 ± 0.2 SU, respectively), before a progressive increase in expression between 6 and 17 DIV ($p \leq 0.001$) (1.8 ± 0.3 , 2.6 ± 0.2 , and 7.1 ± 0.5 SU for 6, 10, and 17 DIV, respectively).

3. DISCUSSION

During development, DA neurons follow a specific temporal pattern by which their axon project from the VM and innervate the striatum, a process that has been well documented both in vivo (Voorn et al., 1988; Gates et al., 2004) and in vitro in VM/striatal co-cultures (Snyder-Keller et al., 2001; Gates et al., 2004; Schatz et al., 1999). In vivo, approximately 80% of DA neurons of the VM are born by E12 (Gates et al., 2006) and express TH shortly thereafter (Specht et al., 1981; Gates et al., 2004). By E14, VM DA neurons have just begun to extend axons rostrally along the immature medial forebrain bundle, with subsequent striatal innervation from these axons appearing by E17 (Voorn et al., 1988; Snyder-Keller, 1991; Gates et al., 2004). Neurochemical assessments have detected DA in the striatum as early as E16 (Perrone-Capano and Di Porzio, 2000), signaling the beginning of striatal DA innervation from VM DA neurons. Striatal innervation continues through early postnatal development, as evidenced by increases in both DA terminal densities (Voorn et al., 1988) and DA neurotransmitter concentrations (Perrone-Capano and Di Porzio, 2000).

With the use of in vitro organotypic co-culture techniques, similar anatomical changes in DA innervation of the striatum have been observed. VM DA neurons project axons to the co-cultured striatum, forming functional synaptic connections (Ostergard et al., 1990; Plenz and Kitai, 1996; Becq et al., 1999; Schatz et al., 1999; Snyder-Keller et al., 2001; Gates et al., 2004). Recently, Gates and colleagues (2004) showed that the timing of DA innervation in co-cultures of E12 VM and variously aged striatum closely resembles that seen in vivo. The age of the striatal tissue is an important factor in determining the pattern and extent of DA innervation from the VM. Indeed, it has been shown that the use of postnatal striatum in co-cultures results in disparate patterns of DA innervation from that seen when late embryonic striatal tissue was used (Becq et al., 1999; Schatz et al., 1999; Snyder-Keller et al., 2001). In our co-culture system we have found that the use of postnatal striatal tissue results in less DA innervation than occurs with embryonic tissue (Snyder-Keller, unpublished observations). While studies have supplied ample anatomical evidence of the spatiotemporal similarities of

in vivo and organotypic co-culture nigrostriatal DA development, they have not adequately described the neurochemical and protein changes associated with these observations.

Changes in nigrostriatal DA neurochemistry occur as VM DA neurons begin to extend processes and innervate the striatal tissue. In vivo measurements of DA reveal relatively low concentrations of VM DA at E14, with levels in the range of approximately 1.0 ng/mg protein, increasing to 5.5 ng/mg proteins by E16-E18. Our results reveal an analogous 4-5 fold increase in VM DA from the time of dissection at E14 (0 DIV) through 3 DIV, with further increases through 10 DIV, suggesting neurochemical development in vitro that is similar to that seen in vivo. Indeed, in a similar co-culture model, Gates and colleagues (2004) showed large increases in the density of TH⁺ fibers extending from VM DA neurons throughout the VM, prior to the fibers reaching the striatum. These results are similar to what is observed in vivo; between E12 and E17, VM DA neurons begin to send projections toward their striatal targets, resulting in large increases in the density of TH⁺ fibers in the medial forebrain bundle, prior to extensive rostral projection to the striatum (Gates et al., 2004). These early increases in TH⁺ projections from the VM DA neurons provide an explanation for the observed increases in VM DA content.

The levels of striatal DA that we observed at E21 (0 DIV) are higher than levels reported at E19 by Perrone-Capano and Di Porzio (2000) and are approximately one-tenth the value of the levels observed in the adult striatum in vivo (Ling et al., 2004; Bemis and Seegal, 2004). Thus, our neurochemical values fit well with the reported trend for developmental increases in striatal DA concentrations from E16 through adult (Perrone-Capano and Di Porzio 2000). The dramatic reductions in striatal DA levels observed after 3 DIV most likely reflect axotomy-induced degeneration of DA terminals present in the striatum at the time of dissection. These findings support observations of a complete loss of striatal TH immunostaining as well as the loss of DA within a mono-cultured striatum after 5 DIV (Humpel et al., 1996; Schatz et al., 1999). Thus, observed increases in striatal DA after 3 DIV, and continuing through 10 DIV, most likely reflect the extension of new DA projections and terminals to the striatum from the VM.

While co-cultures exhibit many trends similar to those seen in in vivo neurochemical development, the levels of VM DA in co-culture were always higher than those observed in the striatum. Additionally, levels of striatal DA never surpassed levels observed at the time of tissue dissection (E21), revealing a potentially important divergence between co-culture and in vivo development. In vivo, after E16, striatal DA levels are higher than VM DA levels (Perrone-Capano and Di Porzio, 2000), and levels of striatal DA increase progressively throughout early postnatal development. We suspect that the differences in co-culture striatal and VM DA levels are due to nuances of this in vitro system, given that not all of the DA axons/terminals project immediately and directly to the striatum, as occurs in vivo (Voorn et al., 1988). These observations are in line with findings of other published reports (Gates et al., 2004; Snyder-Keller et al., 2001) and may be due to the initial spatial separation of the VM and striatal tissue in co-culture, such that growth-promoting cues from the striatum are not received until the tissue has fused (after 1-2 DIV). Another explanation for the elevated levels of VM DA may be that co-culture of an intact VM with only a small section of striatum may not provide sufficient target tissue to support the entire extent of innervation coming from the VM DA neurons. It is also important to note that, at the time of dissection (E14), the VM contains DA neurons from both the substantia nigra (SN) and ventral tegmental area (VTA). It is likely that the SN DA neurons are responsible for the majority of striatal DA innervation, while projections from the VTA neurons may remain within the VM (Lindvall and Björklund, 2004), thus providing further explanation for the elevated levels of VM DA seen in the co-culture system. Nevertheless, innervation of the striatum from VM DA neurons is occurring in a developmentally appropriate manner and the co-cultures provides an excellent model in

which to manipulate axonal guidance, or terminal formation, or to study re-innervation after neurotoxic insult of DA neurons; such processes are difficult to observe in vivo.

Levels of medium and tissue HVA and DOPAC also increase over time in vitro, supporting the functionality of the nigro-striatal system in vitro, given that these metabolites form after release and subsequent metabolism of DA. Levels of striatal DOPAC, which is formed via intraneuronal DA metabolism, closely reflect changes observed in the levels of striatal DA, specifically, a significant reduction at 3 DIV, and increases thereafter. However, striatal HVA levels are not reduced at 3 DIV. The absence of a decline in striatal HVA levels at 3 DIV may reflect an increase in extraneuronal DA metabolism, occurring because of axotomy-induced degeneration of striatal DA neuronal terminals following tissue dissection. Nevertheless, ratios of DA metabolites (specifically HVA) to DA are similar (approximately 1:10) to ratios reported in vivo in adult rat striatum (Ling et al., 2004).

To help explain the observed neurochemical changes occurring in this co-culture model, we determined the number of DA neurons present in the VM and whether changes in neuronal number occurred over time in vitro. The reported numbers of DA neurons in vivo vary greatly among studies. Reisert and associates (1990) estimated approximately 7,700 DA neurons at E14 (0 DIV in our study) and over 16,000 DA neurons in the VM by E21. Other studies have placed the total number of VM DA neurons in the adult rat at around 45,000 (Ling et al., 2004). In an organotypic culture system of solely E14 VM (our 0 DIV), Meyer and colleagues (2001) estimated approximately 4,000 VM DA neurons. Other studies, using roller-tube organotypic culture techniques, have estimated the total number of VM DA neurons to be in the low hundreds (Schatz et al., 1999; Dickie et al., 1996). Therefore, our value of approximately 2,500 DA neurons in our dissected E14 VM seems to be a reasonable figure. While DA neuron counts were constant through 10 DIV, the observed loss of DA neurons by 17 DIV may suggest an overall loss of DA neuron viability at longer time points. Although 17 DIV is a much shorter time span than the months of culture growth and viability permissible in the roller-tube method of organotypic culture (Plenz and Kitai, 1996; Jaumotte and Zigmond 2005), this static culture system provides a time frame quite adequate for observation of developmental changes in DA function, while permitting excellent maintenance of 3-D tissue architecture that is likely lost in roller-tube culture systems (Snyder-Keller et al., 2001; Schatz et al., 1999).

Determination of DA protein levels provide an additional elaboration of the parallels between this model and processes occurring in vivo. Since TH is detectable by E12 in the VM (Gates et al., 2004) and by E17 in the striatum (Voorn et al., 1988; Snyder-Keller, 1991) it is not surprising that TH was detected by Western blot in our E14 VM and E21 striatum (0 DIV). Consistent with the neurochemical measurements, an initial reduction in striatal TH levels was observed following dissection, likely reflecting axotomy-induced degeneration. From 3 DIV to 17 DIV, TH levels increased progressively, corresponding to increasingly dense striatal innervation of DA axons/terminals from the VM. Meanwhile, there was a nearly 3-fold increase in VM TH levels from 0 DIV to 3 DIV, corresponding to immunohistochemical observations by Gates and colleagues (2004) both in vivo and in vitro, that projections from the VM DA neurons increase dramatically within the VM prior to the extension of processes to the striatum. The subsequent decrease in VM TH from 3 DIV to 17 DIV likely represents the DA projections exiting the VM and finding their appropriate targets within the striatum. It is possible that these observed patterns in TH expression are additionally influenced by the initial separation of VM and striatal tissue sections, with some required time in vitro for them to adequately fuse, thereby allowing DA projections from the VM to penetrate the striatal tissue.

In vivo DAT mRNA expression in the VM has been reported as early as E15 (Perrone-Capano and Di Porzio, 2000), while binding studies show the presence of DAT in the striatum at

relatively low levels by E18, with large increases in the early postnatal period (Jung and Bennett, 1996). Levels of striatal DAT in co-culture paralleled levels of striatal DA and TH expression; levels of DAT fell dramatically from 0 to 3 DIV, before increasing substantially thereafter. VM DAT was detected as early as E14 (0 DIV), with significant increases observed after 10 DIV and 17 DIV. Since DAT is localized to the plasma membranes of DA neurons (Nirenberg et al., 1996), and is considered to be a surrogate for DA neuronal terminal density, the reduction in striatal DAT between 0 DIV and 3 DIV strongly supports the degeneration of DA neuronal terminals following tissue dissection, while subsequent increases suggest new terminal formation within the striatal tissue. Interestingly, levels of VM DAT were also significantly increased by 10 DIV; this observation, along with the increases in VM DA and TH, suggests that some DA neuron terminals form within the VM as well as in the striatum.

Both the VM and striatum have large populations of inhibitory GABA neurons. To assess GABA function in developing co-cultures we determined the combined expression of the 65 and 67 kDa forms of GAD in both tissues. Levels of both GAD 65 and 67 *in vivo* have been shown to increase at similar rates through the late prenatal periods (Kuppers et al., 2000). Since GAD expression has been closely linked to GABA production and release (Segovia et al., 1990; Mason et al., 2001), we can assume that, in addition to increased DA activity in this VM/striatal co-culture, there is increased GABA neuronal activity (Diaz et al., 2003). GAD activity is closely regulated by DA in both the adult (Lindfors et al., 1989; Stephenson et al., 2005; Diaz et al., 2003) and developing basal ganglia (Kuppers et al., 2000). While levels of GAD increase developmentally, it has been shown that the disruption of DA neurons, via neurotoxicant administration, further increases GABA activity (Stephenson et al., 2005; Diaz et al., 2003). These observations may in part explain large increases in GAD expression at 17 DIV, a time when several measures of DA neuron integrity (numbers of VM DA neurons, and levels of VM and striatal DA and TH) show decreases, likely reflecting some DA degeneration and compensatory increases in GABA function. This finding may prove important for the use of the co-culture system in the study of neurotoxicants: our model recapitulates not only developmental changes observed *in vivo*, but also neuronal modifications, which occur after the induced degeneration of DA neurons.

Conclusions

Organotypic co-cultures of E14 VM and E21 striatum develop *in vitro* in a manner similar to that of the intact *in vivo* nigrostriatal DA system. During development, DA neurons from the VM project axons to the striatum and establish functional neuronal synapses. Formation of the nigrostriatal system results in increases in the levels of DA, TH, DAT, and GAD65/67; these increases are seen first in the VM, as DA neurons begin to sprout axons, and subsequently in the striatum, as DA terminals form and interact with GABA neurons. In organotypic co-cultures, DA neurochemical and protein measures change as expected through 10 DIV, in parallel with *in vivo* development. However, levels of DA/metabolites, as well as the numbers of DA neurons, decreased at 17 DIV, while levels of striatal DAT, TH, and GAD 65/67 increased. These results suggest either that a partial loss of DA neuron viability and function within the VM results in compensatory increases in the terminal fields of the remaining DA neurons, or else, more simply, that degeneration of DA neuronal cell bodies occurs prior to the loss of terminals within the striatum at late time points.

These results, combined with previous observations of morphological DA maturation of organotypic co-cultures, provide a much more complete and quantitative depiction of *in vitro* nigrostriatal DA development. With most measures of DA development in this system closely following those *in vivo*, this *in vitro* model provides an excellent tool with which we can assess toxicological challenges of the developing nigrostriatal DA system that are difficult to study *in vivo*.

4. EXPERIMENTAL PROCEDURES

4.1. VM and Striatum Organotypic Co-Culture Preparation

The preparation of striatal and VM organotypic co-cultures was based on methods described by Snyder-Keller et al. (2001) except that Neurobasal medium (Gibco, Carlsbad, CA) was used. Briefly, timed-pregnant Sprague-Dawley rats (Taconic Farms, Germantown, NY) (day of sperm = E0) were anesthetized using isoflurane, and fetuses were removed under aseptic conditions at E14 (for VM) and E21 (for striatum), equating to 0 DIV. Fetuses were decapitated and their brains quickly removed in ice-cold Ham's F-12 medium (Gibco, Carlsbad, CA). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

For VM dissection, the meninges were removed from E14 brains, and the VM was dissected under a stereomicroscope in ice-cooled Ham's F-12. The striatum was dissected from 300 μ m thick vibratome sections of the forebrain of E-21 fetuses. These tissue pieces were either analyzed immediately (see procedures below) or cultured together and placed approximately 1 mm apart on a porous membrane (0.4 μ m pore size), in Costar Transwell® Clear, six-well culture trays (Corning, Acton, MA), with two co-cultures per well. Culture medium consisted of Neurobasal medium (Gibco) supplemented with 2% antioxidant-free B-27 supplement (Gibco), penicillin/streptomycin/neomycin (100 μ g/ml), bicarbonate (1.2 mg/ml), HEPES (4.5 mg/ml), and glutamine (2 mM). Twenty percent horse serum was added to the medium for the first 3 days of culture; the medium was then exchanged for medium without serum and allowed to incubate for up to 17 DIV at 37°C in a 5% CO₂/95% room air humidified incubator. Tissue was used for analyses at the time of dissection (0 DIV) or cultured and subsequently removed for analyses at 3, 6, 10, or 17 DIV.

4.2. Neurochemical Analyses

Levels of DA and its metabolites within the striatum and VM of the organotypic co-cultures were determined using high-performance liquid chromatography with electrochemical detection (HPLC-ECD), following methods developed by Bemis and Seegal (2004). Values were corrected for sample protein content using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Briefly, the VM and striatum were separated under a stereomicroscope by cutting through the co-culture where the VM and striatum had fused, and each tissue piece was sonicated in 150 μ l of 0.2M HClO₄. Medium samples of 150 μ l, were also taken and were diluted 1:1 in 0.4M HClO₄. The supernatants of these samples were used for HPLC-ECD while the pellet was dissolved in 0.1M NaOH for BCA protein analysis of the tissue samples. Neurochemical measures for both tissue and medium samples were determined as nanograms (ng) of neurotransmitter/metabolite per milligram (mg) of co-culture tissue protein.

4.3. Fixation, TH Immunohistochemistry, and Quantitative Analysis

Immediately following dissection (0 DIV), or after 3, 6, 10, or 17 DIV, co-cultures were removed, rinsed in warm phosphate-buffered saline (PBS), and fixed for 48 hr at 4°C in 4% paraformaldehyde/4% sucrose. Following fixation, the co-cultures were cryo-protected in 15% sucrose overnight before resectioning to 40 μ m using a sliding microtome (Leica, Wetzlar, Germany). Tissue sections were stored in PBS at 4°C until used for immunohistochemical labeling (no longer than 72 hr).

For immunohistochemical analysis, the sections were briefly incubated in 0.2% Triton X-100 in PBS, followed by a 2-hr incubation in 5% normal goat serum and 0.2% Triton in PBS. The tissues were then incubated for 48 hr at 4°C in a rabbit antibody to TH (1:600; Chemicon, Temecula, CA) to label DA neurons. Sections then were rinsed in PBS and incubated for another 2 hr using goat anti-rabbit Alexa Fluor® 488 secondary antibody (1:200; Molecular

Probes, Eugene, OR). Samples were imaged using a Zeiss fluorescent microscope and Zeiss Axiovision® imaging software. Neuronal cell counts of TH-immunoreactive (TH⁺) neurons were obtained using an automated counting/tracing program (Al-Kofahi et al., 2003). This counting program yielded results that varied by $\pm 10\%$ from manually obtained neuronal counts of the same images by blinded observers (data not shown). All image collection and analyses were carried out in a blinded manner.

4.4. Western Blot Protein Analysis

Samples for Western blot protein analyses were sonicated in 65 μ l of 2% Nonidet P40 with protease inhibitors. Subsequently, 10 μ l of each sample were used for BCA protein analyses (Pierce). From each homogenized sample, 10-15 μ g of protein underwent SDS-PAGE using 15-lane 4-15% acrylamide separating gels (Pierce). The gels were then blot-transferred onto PVDF membranes as described by Um et al. (2000). The membranes were blocked with 5% fish gelatin in PBS with 0.05% Tween-20, (PBST) overnight at 4°C. Primary antibody (1:1000) incubation also occurred overnight at 4°C (rabbit polyclonal raised against TH, DAT, or GAD 65/67; Chemicon). The blots were rinsed with Tris-buffered saline and incubated for 90 min with biotinylated anti-rabbit secondary antibody (1:5000; Pierce), followed by incubation with streptavidin-horseradish peroxidase (HRP) conjugate (1:10,000 in blocking buffer) for 1 hr at 4°C with rocking. Blots were then developed by incubating with Super Signal/chemiluminescent substrate (Pierce) for 5 min and assayed with a LAS-1000plus imaging system (Fuji, Stamford, CT). All blots were stripped and re-probed with anti- β -actin (1:5000; Sigma) to standardize within-blot protein loading. To compare band densities across blots, each blot included a set of rat striatal homogenate standards at 2.5, 5, 10, and 20 μ g of protein.

4.5. Statistical Procedures

All data were analyzed using one-way analysis of variance (ANOVA) and Bonferroni-corrected post hoc T-test analysis, to determine developmental changes in the numbers of DA neurons, DA neurochemistry, and DA protein expression (SPSS v.12 statistical analysis software). Results are based on sample sizes (n) of 10-15 co-cultures per measurement for HPLC-ECD; n = 6 for DA neuron counts; and n = 4 for Western blot protein measures at each time point (0, 3, 6, 10, 17 DIV). Statistically, a p-value ≤ 0.05 was considered significant. All data are expressed as the mean $\pm$ SEM.

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Abbreviations

DA, dopamine
DAT, dopamine transporter
DIV, days in vitro
DOPAC, 3,4-dihydroxyphenylacetic acid
E, embryonic day
GABA, γ -Aminobutyric Acid
GAD, glutamic acid decarboxylase
HVA, homovanillic acid
PD, Parkinson's disease
SN, substantia nigra
TH, tyrosine hydroxylase

VM, ventral mesencephalon
 VTA, ventral tegmental area

REFERENCES

- Al-Kofahi KA, Can A, Lasek S, Szarowski DH, Dowell-Mesfin N, Shain W, Turner JN, Roysam B. Median-based robust algorithms for tracing neurons from noisy confocal microscope images. *IEE Transactions on Info. Tech. in Biomed* 2003;7(4):302–317.
- Becq H, Bosler O, Geffard M, Enjalbert A, Herman JP. Anatomical and functional reconstruction of the nigrostriatal system in vitro: selective innervation of the striatum by dopaminergic neurons. *J. of Neurosci. Res* 1999;58:553–566. [PubMed: 10533047]
- Bemis JC, Seegal RF. PCB-induced inhibition of the vesicular monoamine transporter predicts reductions in synaptosomal dopamine content. *Toxicol. Sci* 2004;80(2):288–295. [PubMed: 15115888]
- Diaz MR, Barroso-Chinea P, Acevedo A, Gonzalez-Hernandez T. Effects of dopaminergic cell degeneration on electrophysiological characteristics and GAD65/GAD67 expression in the substantia nigra: different action on GABA cell populations. *Movement Disorders* 2003;18(3):254–266. [PubMed: 12621628]
- Dickie BGM, Holmes C, Greenfield SA. Neurotoxic and neurotrophic effects of chronic n-methyl-d-aspartate exposure upon mesencephalic dopaminergic neurons in organotypic culture. *Neurosci* 1996;72(3):731–741.
- Gahwiler BH. Organotypic cultures of neural tissue. *Trends in Neurosci* 1988;11(11):484–9.
- Gates MA, Coupe VM, Torres EM, Fricker-Gates RA, Dunnett SB. Spatially and temporally restricted chemoattractive and chemorepulsive cues direct the formation of the nigro-striatal circuit. *Eur. J. of Neurosci* 2004;19:831–844. [PubMed: 15009130]
- Gates MA, Torres EM, White A, Fricker-Gates RA, Dunnett SB. Re-examining the ontogeny of substantia nigra dopamine neurons. *Eur. J. of Neurosci* 2006;23:1384–1390. [PubMed: 16553799]
- Hoglinger GU, Sautter J, Meyer M, Spenger C, Seiler RW, Oertel WH, Widmer HR. Rat fetal ventral mesencephalon grown as solid tissue cultures: influence of culture time and BDNF treatment on dopamine neuron survival and function. *Brain Res* 1998;813:313–322. [PubMed: 9838177]
- Humpel C, Marksteiner J, Saria A. Glial-cell-line-derived neurotrophic factor enhances biosynthesis of substance P in striatal neurons in vitro. *Cell Tissue Res* 1996;286:249–255. [PubMed: 8854894]
- Jaumotte JD, Zigmond MJ. Dopaminergic innervation of forebrain by ventral mesencephalon in organotypic slice co-cultures: Effects of GDNF. *Mol. Brain Res* 2005;134:139–146. [PubMed: 15790537]
- Jung AB, Bennett JP. Development of striatal dopaminergic function. I. Pre- and postnatal development of mRNAs and binding sites for striatal D1 (D1a) and D2 (D2a) receptors. *Devel. Brain Res* 1996;94:109–120. [PubMed: 8836569]
- Katsuki H, Tomita M, Takenaka C, Shirakawa H, Shimazu S, Ibi M, Kume T, Kaneko S, Akaike A. Superoxide dismutase activity in organotypic midbrain-striatum co-cultures is associated with resistance of dopaminergic neurons to excitotoxicity. *J. Neurochem* 2001;76:1336–1345. [PubMed: 11238718]
- Kotake Y, Ohta S, Kanazawa I, Sakurai M. Neurotoxicity of an endogenous brain amine, 1-benzyl-1,2,3,4-tetrahydroisoquinoline, in organotypic slice co-culture of mesencephalon and striatum. *Neurosci* 2003;117:63–70.
- Kress GJ, Reynolds JJ. Dopaminergic neurotoxins require excitotoxic stimulation in organotypic cultures. *Neurobiology of Disease* 2005;20(3):639–645. [PubMed: 15996475]
- Kuppers E, Sabolek M, Anders U, Pilgrim C, Beyer C. Developmental regulation of glutamic acid decarboxylase mRNA expression and splicing in the rat striatum by dopamine. *Mol. Brain Res* 2000;81:19–28. [PubMed: 11000475]
- Lindfors N, Herrera-Marschitz M, Persson H. Region specific regulation of glutamic acid decarboxylase mRNA expression by dopamine neurons in the rat brain. *Exp. Brain Res* 1989;77(3):611–620. [PubMed: 2572447]
- Lindvall O, Björklund A. Cell therapy in Parkinson's Disease. *NeuroRx* 2004;1:382–393. [PubMed: 15717042]

- Ling ZD, Chang Q, Lipton JW, Tong CW, Landers TM, Carvey PM. Combined toxicity of prenatal bacterial endotoxin exposure and postnatal 6-hydroxydopamine in the adult rat midbrain. *Neurosci* 2004;124:619–628.
- Mason GF, Martin DL, Martin SB, Manor D, Sibson NR, Patel A, Rothman DL, Behar KL. Decrease in GABA synthesis rate in rat cortex following GABA-transaminase inhibition correlates with decrease in GAD67 protein. *Brain Res* 2001;914:81–91. [PubMed: 11578600]
- Meyer M, Matarredona ER, Seiler RW, Zimmer J, Widmer HR. Additive effect of glial cell line-derived neurotrophic factor and neurotrophin-4/5 on rat fetal nigral explant cultures. *Neurosci* 2001;108(2): 273–284.
- Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM. The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J. Neurosci* 1996;16 (2):436–447. [PubMed: 8551328]
- Plenz D, Kitai ST. Organotypic cortex-striatum-mesencephalon cultures: the nigrostriatal pathway. *Neurosci. Lett* 1996;209:177–180. [PubMed: 8736639]
- Ostergaard K, Schou JP, Zimmer J. Rat ventral mesencephalon grown as organotypic slice cultures and co-cultured with striatum, hippocampus, and cerebellum. *Exp. Brain Res* 1990;82:547–565. [PubMed: 1981356]
- Perrone-Capano C, Di Porzio U. Genetic and epigenetic control of midbrain dopaminergic neuron development. *Int. J. Dev. Biol* 2000;44:679–687. [PubMed: 11061432]
- Reisert I, Schuster R, Zienecker R, Pilgrim C. Prenatal development of mesencephalic and diencephalic dopaminergic systems in the male and female rat. *Devel. Brain Res* 1990;53:223–229.
- Schatz DS, Kaufmann WA, Saria A, Humpel C. Dopamine neurons in a simple GDNF-treated meso-striatal organotypic co-culture model. *Exp. Brain Res* 1999;127:270–278. [PubMed: 10452214]
- Segovia J, Tillakaratne NJK, Whelan K, Tobin AJ, Gale K. Parallel increases in striatal glutamic acid decarboxylase activity and mRNA levels in rats with lesions of the nigrostriatal pathway. *Brain Res* 1990;529(12):345–348. [PubMed: 2126483]
- Shimizu K, Matsubara K, Ohtaki K, Shiono H. Paraquat leads to dopaminergic neuroal vulnerability in organotypic midbrain culture. *Neurosci. Res* 2003;46:523–532. [PubMed: 12871774]
- Snyder-Keller A. Development of striatal compartmentalization following pre- or postnatal dopamine depletion. *J. Neurosci* 1991;11(3):810–821. [PubMed: 1705970]
- Snyder-Keller A, Costantini LC, Graber DJ. Development of striatal patch/matrix organization in organotypic co-cultures of perinatal striatum, cortex, and substantia nigra. *Neurosci* 2001;103(1):97–109.
- Specht LA, Pickel VM, Joh TO, Reis DJ. Light-microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. I. Early ontogeny. *J. Comp. Neurol* 1981;199(2):233–253. [PubMed: 6114114]
- Stephenson DT, Li Q, Simmons C, Connell MA, Meglasson MD, Merchant K, Emborg ME. Expression of GAD65 and GAD67 immunoreactivity in MPTP-treated monkeys with or without L-DOPA administration. *Neurobiology of Disease* 2005;20:347–359. [PubMed: 15882945]
- Stoppini L, Buchs P-A, Muller D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Meth* 1991;37:173–182.
- Testa CM, Sherer TB, Greenamyre JT. Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. *Mol. Brain Res* 2005;134:109–118. [PubMed: 15790535]
- Tseng KY, Snyder-Keller A, O'Donnell P. Dopaminergic modulation of striatal plateau depolarizations in corticostriatal organotypic cocultures. *Psychopharmacology*. 2006In Press.
- Voorn P, Kalsbeek A, Jorritsma-Byham B, Groenewegen HJ. The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neurosci* 1988;25(3):857–887.

Fig. 1.

Developmental changes in DA concentrations in the striatum and VM, of organotypic co-cultures over 17 DIV. Samples underwent HPLC-ECD for determination of DA content, and each value represents the mean $\pm$ SEM of 8-10 samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, with respect to 0 DIV, # $p \leq 0.05$, ### $p \leq 0.001$, with respect to 3 DIV %%% $p \leq 0.01$ with respect to 0, 3, and 6 DIV.

Fig. 2. Developmental changes in DOPAC concentrations in the striatum and VM of organotypic co-cultures over 17 DIV. Samples underwent HPLC-ECD for determination of DOPAC content, and each value represents the mean $\pm$ SEM of 8-10 samples. * $p \leq 0.05$, with respect to 0 DIV, # $p \leq 0.05$, with respect to 3 DIV.

Fig. 3. Developmental changes in HVA concentrations in the striatum and VM of organotypic co-cultures over 17 DIV. Samples underwent HPLC-ECD for determination of HVA content, and each value represents the mean $\pm$ SEM of 8-10 samples. [#] $p \leq 0.10$, ^{*} $p \leq 0.05$, ^{**} $p \leq 0.01$, with respect to 0 DIV; [%] $p \leq 0.10$, with respect to 10 DIV.

Fig. 4. (A) Numbers of TH-positive DA neurons in the VM of organotypic co-cultures at 0, 3, 6, 10, or 17 DIV. Each value represents the mean $\pm$ SEM of 9-12 samples. Representative images from TH-immunostained sections of VM at 0, 3, 6, 10, or 17 DIV (B-F, respectively). * $p \leq 0.05$, with respect to 0 DIV. Scale bar = 50 μ m.

Fig. 5.

Developmental changes in TH protein expression in the striatum and VM of organotypic co-cultures over 17 DIV. Samples underwent Western blotting and were probed with anti-TH antibodies (Chemicon; 1:1000). Results of each blot were standardized by standard curves of striatal homogenate, as well as β-actin protein content. Representative western blots are shown from 2 separate organotypic co-cultures at each time point. Each value represents the mean $\pm$ SEM of 4 samples. [#] $p \leq 0.10$, ^{*} $p \leq 0.05$, with respect to 0 DIV; [%] $p \leq 0.05$, with respect to 3 DIV.

Fig. 6.

Developmental changes in DAT protein expression in the striatum and VM of organotypic co-cultures over 17 DIV. Samples underwent Western blotting and were probed with anti-DAT antibodies (Chemicon; 1:1000). Results of each blot were standardized by standard curves of striatal homogenate, as well as β -actin protein content. Representative western blots are shown from 2 separate organotypic co-cultures at each time point. Each value represents the mean $\pm$ SEM of 4 samples. *** $p \leq 0.001$, with respect to 0 DIV; %% $p \leq 0.01$, %%% $p \leq 0.001$ with respect to 3 DIV; ### $p \leq 0.001$ with respect to 0, 3, or 6 DIV.

Fig. 7.

Developmental changes in GAD 65/67 protein expression in the striatum and VM of organotypic co-cultures over 17 DIV. Samples underwent Western blotting and were probed with anti-GAD 65/67 antibodies (Chemicon; 1:1000). Results of each blot were standardized by standard curves of striatal homogenate, as well as β -actin protein content. Representative western blots are shown from 2 separate organotypic co-cultures at each time point. Each value represents the mean $\pm$ SEM of 4 samples. # $p \leq 0.10$, * $p \leq 0.05$, with respect to 0 DIV; % $p \leq 0.05$, with respect to 3 DIV.