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ORIGINAL ARTICLE

Successful implementation of cooperative handling eliminates the need for restraint in a complex non-human primate disease model

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Abstract

Background Streptozotocin-induced diabetic non-human primates are used to study efficacy and safety of innovative immunosuppression after islet transplantation. We implemented a training program for medical management of a chronic disease state.
Methods Cooperation with hand feeding and drinking, shifting, and limb presentation were trained utilizing predominately positive but also negative reinforcement in 52 animals compared with 28 macaques subjected to conventional physical and/or chemical restraint. The success and timing of behavior acquisition was evaluated in a representative subset of 14 animals.
Results Over 90% of animals were successful in behavior acquisition. Programmatically this resulted in complete elimination of chair restraint and negligible requirement for sedation. About half of the trained animals had no-to-moderate thymic involution, indicative of a substantial reduction in stress.
Conclusion Cooperative handling enhances animal well-being. This contributes to validity of scientific results and eliminates model-induced confounding that can obstruct interpretation of safety and efficacy data.

Introduction

Non-human primates (NHPs) are essential for modeling the clinical situation in transplantation among others because the NHP-immune system closely resembles that of humans. In transplantation, NHP models are used to study safety and efficacy of novel immunosuppressive compounds, immune modulation (tolerance induction), and biological products like xenografts [7, 20, 46, 49]. The translational and predictive value depends on the degree that the NHP mimics the situation of the human patient in a life-supporting transplant model. Safety monitoring relies on the evaluation of hematological, biochemical, clinico-pathological, and metabolic parameters that can be influenced by handling stress in preclinical models. Also, safety monitoring includes the assessment of adverse events in clinical inspection. Conventional methods used in handling NHPs, typically chemical or physical restraint, affect the well-being of the animal and may influence translational value by obscuring or confounding the manifestation of adverse events [6, 14, 26, 31, 37, 51]. Such assessments can be more complex in conditions where the NHP model includes an experimentally induced disease state. Type 1 diabetes (T1D) in macaques or baboons induced by the pancreatoxic compound streptozotocin (Zanosar®; Sicor Pharmaceuticals, Irvine, CA, USA) is an example of such a condition [17], which is the basis of work described in this report. In this model, animals incur additional handling in frequent metabolic monitoring [e.g., blood glucose (BG), hemoglobin A1c, C-peptide, *in vivo* glucose, or arginine stimulation tests] and subsequent administration of medications (e.g., insulin). Following islet cell transplantation, the handling burden is even higher requiring additional drug administration (e.g., oral and injectable immunosuppression, antivirals, and supportive therapeutics), blood collection (e.g., drug trough levels and immune monitoring), and physical assessments. There may be severe or rapid-onset metabolic instability requiring complex medical management and procedures to avoid serious morbidity or mortality.

The promotion of the psychological well-being of NHPs, particularly as it relates to stress from experimental procedures and opportunity to conserve species typical behavior, is a provision of US and EU standard regulation for NHPs used in biomedical research. Welfare is defined as a state of balance between positive (reward and satisfaction) and negative (stress) experiences or affective states [3, 45]. Similarly, refinement in its most progressive definition requires an approach that 'avoids or minimizes the actual or potential pain, distress, and other adverse effects experienced at any time during the life of the animals involved and which enhances their well-being' [5]. In both cases, limiting exposure to negative experiences and promoting positive experiences (through reward and stimulation) strikes a positive balance and increases the window of welfare beyond simple avoidance of distress.

In diabetic humans, compliance with management strategies that are intrinsically aversive (i.e., repeated BG measures, insulin administration) to maintain metabolic parameters close to normal often negatively affects 'quality of life'; however, a counterbalance is present in the positive health effects achieved in avoiding complications of disease [22, 39]. The NHP model of T1D is a close approximation of the human situation, and it is logical to expect that similar treatment regimens in the NHP model are equally demanding, except that in the case of the animal model, the term well-being substitutes the term quality of life. Cooperative handling is appealing in this respect having both the considerable advantage of reduced stress in interacting with handlers or burden in compliance with intrinsically aversive procedures necessary for disease management on the one hand, and improved medical care for animals through more productive interactions on the other [23, 29, 42, 50]. In addition to the perspective for refinement, the situation of an NHP trained to cooperate with research procedures opens the opportunity of less variability and confounding, which could result in lower numbers of animals required for achieving reliable outcomes, that is, a reduction can also be achieved.

Non-human primates are non-domesticated animals, suggesting a natural behavior of avoidance or flight response to humans or other species [2, 25]: this is with the remark that purpose-bred NHPs raised in captivity generally are habituated to tolerate humans in close proximity by necessitated routine husbandry interactions. Nevertheless, in our experience, these animals rarely demonstrate affiliative behaviors or bonding with humans prior to structured training: this thus indicates that the avoidance response of a non-domesticated animal is still intact, albeit to a varying extent. The way in which NHPs respond in human-NHP interactions, ranging from relatively confident to anxious, is often described in terms of temperament [9]. The animal's temperament and associated behaviors affect their perception of interactions that occur in the laboratory environment (which often are largely outside the control of the untrained animal) and their ability to effectively cope with these situations.

Historically in our program, conventional 'restraint-based' manipulations like manual chair restraint and chemical restraint were used to manage animals and perform routine research manipulations that led to 'downtime' from normal activities like exploration, feeding, grooming, and peer interactions. Recognizing an opportunity for refinement, alongside novel instrumentation techniques [15, 16, 18], we developed a training program focused on training subjects to reliably perform target behaviors that facilitate medical management in a chronic disease state and also bring certain aspects of the model closer to the clinical situation.

To accomplish this, we adapted established and expert behavioral training techniques [4, 8, 9, 27, 29, 35, 36, 41, 42] with the following goals:

- Reduce or eliminate the use of restraint for routine medical interventions associated with management of chronic disease (e.g., diabetes).
- Performance of trained behaviors in the familiar homecage environment where the animal remains in visual, auditory, and (where appropriate) tactile contact with conspecifics.
- Increase affiliative behaviors between NHPs and handlers, thereby encouraging bonding and familiarity during interactions. Medical procedures are intrinsically aversive, and fear or anxiety of these procedures can amplify the perception of pain. On the other hand, in a familiar and rewarding environment, there is relaxation or focused anticipation toward the reward, presumably diminishing the perception of pain. This is also with the aim to develop productive coping skills that allow the animal to function well even under briefly stressful circumstances: this is a relevant consideration in chronic disease models where unexpected procedures may become necessary for the health and safety of the animal.

The training program is designed to address both sides of the window of animal welfare through the reduction of negative experiences (e.g., restraint) or affective states (e.g., anxiety), and enhancement of positive experiences (e.g., cooperation, satisfaction, and reward) over the course of the life of the animal.

In this study, we analyze the effectiveness of our NHP training program that paired positive reinforcement (PRT) with typical negative reinforcers' characteristic in the captive laboratory environment, in an attempt to replace any anxiety or fear response with a positive association. We demonstrate our progression over a 10-years period from conventional NHP handling to cooperative interactions, a refinement accomplished through training NHPs in the period before enrollment in experimental protocols, using both negative reinforcement (NRT) and PRT, predominately PRT.

Materials and methods

Animals

Our studies are approved by the University of Minnesota Institutional Animal Care and Use Committee, are conducted in compliance with the Animal Welfare Act, and adhere to principles stated in the Guide for Care and Use of Laboratory Animals. Between January 2001 and July 2010, 80 NHPs were enrolled in various protocols of an islet transplantation program. The population cohort included 54 cynomolgus macaques (*Macaca fascicularis*) and 26 rhesus macaques (*Macaca mulatta*). The cynomolgus macaques comprised eight females and 46 males, aged between 2.4 and 8.2 years (median 4.1 years), and weighed between 2.5 and 5.2 kg (median 3.7 kg). The rhesus macaques comprised 11 females and 15 males, aged between 1.0 and 6.4 years (median 3.4 years), and weighed between 1.8 and 8.8 kg (median 4.6 kg).

All animals were purpose-bred and purchased from institutionally approved commercial vendors. They were housed in pairs or small groups of the same sex. They had free access to water and were fed biscuits (High-Protein Monkey Chow 5045; Purina Mills, St Louis, MO, USA) based on body weight (BW). Their diet was enriched liberally with fresh fruits, vegetables, grains, beans, nuts, and a multivitamin preparation. Semi-annual veterinary physical examinations were performed in all animals. Starting in January 2005, the NHPs participated in an environmental enrichment program that included social play, toys, music, and regularly scheduled access to large exercise and swimming areas.

Prior to June 2006, comprising 28 animals, there was no formal training program, and animals were instrumented using the traditional surgical method for vascular access port (VAP) placement [19]. In these animals, chemical or chair restraint was used for handling.

Starting in June 2006, involving 52 animals, we developed a novel surgical method for VAP placement [18] to facilitate venous access in the homecage, and NHPs were trained to cooperate in medical procedures including hand feeding and drinking, shifting into transport boxes for weighing, and limb presentation (blood collection, drug administration, and basic physical examination).

To assess the time investment associated with behavior acquisition and to study interanimal variability, we chose a subset of 14 animals that were demographically similar to provide direct comparison: these animals were acquired from a single vendor (Harlan, Madison, WI, USA), arrived together in our center, at the same time, and trained together over the course of 6 months starting in July 2008. This subgroup comprised 14 male cynomolgus macaques, aged between 3.8 and 4.9 years (median 4.5 years), and weighed between 3.3 and 4.8 kg (median 4.1 kg).

To assess thymus histology as an indicator of stress, we selected two studies on porcine islet xenotransplantation, one before June 2006 and one after June 2006. In both studies, diabetes was induced using a single high-dose streptozotocin injection, which was followed by transplantation of an adult porcine islet transplant into the liver: animals were maintained under chronic immunosuppression. The first study included 14 NHPs for which thymus was sampled at necropsy: islet transplantation was performed between the 1st quarter of 2002 and the 3rd quarter of 2003. In transplant recipients with a thymus in histology ($n = 10$), the median survival time after transplantation was 58 days (range 19–158 days). One animal in this series was not transplanted but was only subjected to diabetes induction and regular blood sampling as well as administration of immunosuppressants, for a period of 100 days before sacrifice. The second study included 26 NHPs from which thymus was sampled at necropsy: islet transplantation was performed between the 2nd quarter of 2007 and the 3rd quarter of 2008. In transplant recipients with a thymus in histology ($n = 20$), the median survival time after transplantation was 36 days (range 15–293 days).

In addition to the animals from those studies, one animal is presented that was not subjected to transplantation. An undetected preexisting condition (aortic stenosis) manifested in an anesthetic complication during VAP implantation requiring euthanasia. Prior to euthanasia, the animal was subjected only a few times to animal handling, that is, twice for blood sampling, since arrival at the facility 4 months earlier.

Training procedures

The diabetic condition in NHPs used in metabolic research necessitates common medical procedures like injection, blood collection, and examination on a daily basis. Animals were trained to cooperatively perform three essential tasks in the laboratory environment: hand feeding and drinking, shifting, and limb presentation (Fig. 1).

Hand feeding and drinking

Hand feeding refers to the behavior of taking food items directly from the hands of handlers, and hand drinking refers to the behavior of drinking fluids directly from a syringe held by handlers (Fig. 1). The training of these behaviors is not so much actual training (i.e., grasping food and drinking fluids is a natural behavior for the animals) as it is working with the animals to overcome the approach-avoidance conflict of doing so from the hands of staff. An approach-avoidance conflict is conceptually defined as a situation where individuals may acquire something highly desirable but in doing so they must also endure something undesirable [21]. When this concept is applied to hand feeding and drinking, the desirable end is the palatable food and drink that the animal can acquire while the undesirable outcome for the anxious or 'shy' animal is coming into close proximity with the handler offering it. Irrespective of being raised in captivity, newly introduced animals to the laboratory environment have in our experience a variable degree of ease toward staff. For instance, some animals may immediately take treats and drink from a syringe from handlers while others will shy away and maximize flight distance from handlers.

We used the following process to enable 'shy' animals to eventually overcome this approach-avoidance conflict. First, the anxiety level in relation to handler proximity of those animals was roughly assessed by handlers offering food or drink using a hierarchy of offering positions starting with the most anxiety-provoking position and working toward the least anxiety-provoking position until the animal accepts the food

Fig. 1 Illustration of trained behaviors. Top (left to right): paired non-human primates shifting from the play and swim area (one animal is moving into the shift box where the partner is waiting); hand drinking from a syringe; and hand feeding. Bottom (left to right): holding in the transport boxes, behind a plexiglass panel; routine limb presentation; and limb presentation for fluid rehydration using a vascular access port.

or drink. We assume that the most anxiety-provoking offering position to the animal is when the handler is standing directly in front of the animal's cage, facing the animal with food or syringe being offered in hand. We assumed that the least anxiety-provoking offering position is when the handler leaves the room after leaving food on the cage door or dripping fluids from the syringe onto the bars of the cage-front. An example of one of the intermediate offering positions is when the handler leaves food on the cage door then steps away from the cage and turns his/her back to the cage. After we have established the animal's comfort level on this hierarchy, a starting point is set. From this point onwards, when the animal successfully obtains the treat, the handlers progressively advance the animals by moving up to the next step in the hierarchy. This is repeated until they are readily taking treats and drinking from the syringe by hand in the most anxiety-provoking offering position. The basis for behavior change is counterconditioning: the anxiety-provoking stimulus (handler) is linked with the pleasant stimulus (food and drink). Subsequently, the stimulus of handler interaction reflects a new association of pleasure to the interaction rather than anxiety. Likewise, the term desensitization is sometimes used, as defined by Laule et al. '*Desensitization* is a highly effective training tool that can help laboratory primates tolerate and eventually accept a wide array of frightening or uncomfortable stimuli. By pairing positive rewards with any action, object, or event that causes fear, that fearful entity slowly becomes less negative, less frightening, and less stressful [29].'

Shifting

Shifting refers to the behavior of moving into a transport box, holding in the box, and exiting the box (Prima-Carrier 1HTR15; Primate Products Inc., Imomokalee, FL, USA) (Fig. 1). The most common applications for this behavior include the transport of animals from one laboratory context to another (e.g., transporting animals from their homecages to play/swimming areas and then returning to the homecages again after the animals' time in the play areas has finished) and to facilitate the conscious weighing of animals.

Training this behavior primarily employs the use of a stimulus-response reinforcement contingency. A stimulus is something that causes a behavioral response, in this case, cues or prompts are used to encourage the animal to complete the specific behavioral response of shifting from one place to another. The act of shifting is not one that is difficult or inherently physically uncomfortable to the animal: it simply requires that the handler teaches the animal where to move out of the familiar homecage environment. Initially, we used varying levels of prompting including: a simple verbal cue and physical gesture toward the box; controlled and careful use of the squeeze-back panel (SBP) to gently move the animal to the front of its cage and thus closer to the opening of the transport box; and only where necessary, the presentation of mild-anxiety 'startle' provoking stimuli to facilitate the animal's movement into the box but not items that provoke a fear response (examples of those stimuli include items like baby dolls and stuffed animals, all of which are novel items designated only for use with shifting training, and not a net or gloves that provoke a fear response). In our unit, unconventional housing or enriched cages are used, for example, cages without SBP or cages that have extensive furniture like protected hiding areas from conspecifics or added perches that negate SBP use: it is essential for animals to master the behavior such that physical prompting, like use of the SBP and mild startle stimuli which by definition are negative reinforcers, is not necessary.

Positive reinforcement is given after the animal has completed the entire behavior (entry, hold, and exit). The animal is presented with a food reward or a similarly enriching reward. For example, after the animal is shifted from the homecage into the transport box, it is transported by handlers to a play area stocked with several types of desirable foods and environmental enrichments (e.g., a swimming pool, climbing apparatuses, mirrors, etc.). This was designed to quickly fade the requirement for physical prompts in favor of a simple cue (the presentation of the transport box). For regular transport to another laboratory area, juvenile animals are transported with their partner. Large adult animals (that normally are housed in pairs) may be transported individually to avoid potential injury in the confined space of the transport box.

Limb presentation

This training refers to the behavior of extending one limb out of the homecage for the handler to grasp. We trained animals to present the leg, which also is the location of the indwelling VAP, out of the cage (out means to at least the knee in larger animals and to at least mid to the upper half of the thigh in smaller animals) through a designated opening (Fig. 1). This allows the handler to take, hold, and manipulate the leg extended outside of the cage using one hand while the other hand is free to perform manipulative tasks involving the animal's foot and/or leg. There are multiple applications for this behavior pertaining to animal handling and clinical care, many of which are tasks that without animal training require manual chair restraint or chemical sedation. This behavior enables the performance of several clinical care objectives cooperatively in the familiar homecage including abbreviated physical examination (i.e., palpation of the abdomen, skin turgor assessment, oral, nasal, ocular cursory examination, auscultation, blood pressure, and oxygen saturation), heelstick for $<25\ \mu l$ blood collection, intramuscular injection, subcutaneous injection, and using a VAP (which enables central venous access) for collecting large volumes of blood and for intravenous fluid/drug administration.

Limb presentation behavior is easily the most complex of the trained behaviors for this study and, aside from its complexity, requires that handlers actually come in physical contact and grasp the animal: in the absence of training, this normally is perceived as threatening. To achieve this behavioral objective, our training process has evolved to include two primary parts, the first focusing on the action of grasping the leg (i.e., the animal allowing the handler to take, hold, and manipulate its leg outside of the cage) and the second part focusing on presentation (i.e., the animal voluntarily extending a leg outside the cage). The methods used throughout the training process are numerous and are each briefly defined below.

Exposure and response prevention (ERP) and counterconditioning are effective and well-documented techniques used in both human and animal populations to reduce and/or eliminate the anxiety elicited by specific anxiety-inducing stimuli [1, 10, 11, 32, 40, 47]. In this context, ERP is defined and utilized in the following way: the 'exposure' aspect is the direct exposure to anxiety-provoking stimuli in a gradual controlled manner, and the 'response prevention' aspect is the prevention of responses elicited by the anxiety-provoking stimuli. In order for the exposure to be effective, it is essential to preclude behaviors that are normally performed in the anxious state. For instance, in limb presentation, this is accomplished by blocking the flight response that interferes with the animal's ability to obtain the pleasurable rewards associated with the handler interaction. ERP relies on habituation and extinction of responses that result in avoidance or escape behaviors. Counterconditioning is used to pair positive rewards with anxiety-provoking stimuli in an effort to decrease elicited anxiety, effectively replacing this association with one that is positive.

Putting through, also known as passive movement training, is a method used to teach a motor skill where the student is passively moved hand-over-hand through the movement by the teacher, performed multiple times until the student is able to perform the movement without guidance by the teacher [30]. Both humans and great apes have demonstrated the utility of passive movement training, that is, placing the other's body in the desired position, in a variety of behaviors ranging in difficulty from simple hand waving in children to complex sign language in apes [30].

Prompting is the provision of cues or assistance to encourage the completion of a specific behavior. PRT is the condition in which a targeted response elicits the presentation of a pleasurable stimulus (i.e., appetitive, grooming, etc.). NRT is the condition in which a targeted response turns off or prevents the presentation of an aversive stimulus (i.e., the conventional use of the SBP to position the NHP in close proximity to the handler). Finally, omission training, also known as the differential reinforcement of other behaviors, is the condition in which a targeted response turns off the presentation of an appetitive stimulus [11].

The training plan for limb presentation is shown in Table 1, and skills trained are outlined in Table 2. Training consists of two phases, each with six subphases. Phase 1 uses passive movement training and prompting to demonstrate the limb presentation behavior to the animal. The trial duration is 3–6 minutes during which counterconditioning occurs using food treats as a positive reward. The number of trials per session is limited to avoid overexposure. Primary anxiety responses (resistance or 'escape attempt' and assaults toward the handler) needed to be absent before meeting passing criteria allowing for advancement to the next subphase. During this subsequent subphase, either the level of difficulty in the behavior increased or the behavior is consistently repeated. In phase 2, the trial duration is shorter, 45–120 s, and the number of trials that occur in a session is increased. The same passing criteria apply, but now with the added expectation of fading of prompting. During both phases, the animals continue to build on a progressive series of skills (Table 2) that are designed to prepare them for activities to which they will be exposed later, that is, after enrollment into study protocols. Each skill has a practical basis. In skill series A (Table 2):

- The handler switches hands to simulate the need to free a hand for an injection;
- lightly holding the toes is used when access to the heel is needed for heelstick blood collection;
- the animal must actively present the limb, not relying on the handler to restrain the foot in position;
- startle or quick movement toward the presented limb simulates the condition that other activities are occurring in the room by other handlers and animals that might indicate movement toward them;

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Table 1 Training plan for limb presentation

Subphase	Number of trials per session	Time between trials	Anxiety response free hold goal time	Add food reward upon limb presentation	Subtract SBP after limb presentation	Add food reward during hold	Skills training (one or more series per trial)	Subphase passing criteria
Phase 1								
1-1	1	NA	6 minutes	None	No	Yes, and jackpot after release	None/hold only	At least three consecutive sessions absent anxiety response
1-2	2	≥5 minutes	6 minutes	Yes	No	Yes	Series A	At least 1 session absent anxiety response
1-3	2	≥5 minutes	6 minutes	Yes	No	Yes	Series A and B	Same as 1-2
1-4	2	≥5 minutes	6 minutes	Yes	No	Yes	Series A and C	Same as 1-2
1-5	2	≥5 minutes	6 minutes	Yes	1/2	Yes	Series A and C	Same as 1-2
1-6	4	≥2 minutes	3 minutes	Yes	Fully	Yes	Series A and C	Same as 1-2
Phase 2								
Subphase	Number of trials per session	Time between trials	Anxiety response free hold goal time	Add food reward upon limb presentation	Subtract SBP after limb presentation	Add food reward during hold	Skills training	Subphase passing criteria
2-1	>5 ≤ 14	45–120 s	Duration of hold	Yes	Fully	Yes	Series A on 2 trials Series C on 1 trial	At least 1 session with anxiety response free holds in combination with a limb presentation score of ≥3 on all trials, of which 75% must score ≥4
2-2	>5 ≤ 14	45–120 s	Duration of hold	Yes	Fully	Yes	Opposite side of homecage Series A on 2 trials Series C on 1 trial	Same as 2-1
2-3	>5 ≤ 14	45–120 s	Duration of hold	Yes	Fully	Yes	Both sides of homecage Series A on 2 trials Series C on 1 trial	Same as 2-1
2-4	>5 ≤ 14	45–120 s	Duration of hold	Yes	Fully	Yes	Alternate handler Series A on 2 trials Series C on 1 trial	Same as 2-1 with each alternate handler
2-5	2	≥60 s	Duration of hold	No	Fully	Yes	Novel handler Series C on 1 trial	Same as 2-1 with novel handler
2-6	2	≥60 s	Duration of hold	No	Fully	Yes	Series C on 1 trial	No passing criteria (maintenance)

NA, not applicable; SBP, squeeze-back panel.

To allow for interanimal variability, animals remain in the subphase until passing criteria are met, for example, subphases are repeated until the animal achieves success. A trial is defined as a discrete period of time where the handler is directly working with the animal and a session as a discrete period consisting of 1 or more trials. Skills in series A–C are detailed in Table 2. Scoring for subphase passing criteria is detailed in Table 3.

Table 2 Skill building in limb presentation

Skills series A	
Preparation for routine handling	*Preparation for injection or blood collection*
Handler hand switch	Thigh pinch
Light hold toes only	Heel tap
Startle	Heel squeeze
Knee flex/extend	Foot squeeze
Leg movement on axis (backwards/forwards/up/down)	
Skills series B	
Blood glucose	*Injection*
Blunt lancet against foot	Blunt syringe against thigh
Skills series C	
Blood glucose	*Injection*
Sharp lancet, obtain sample	Sharp needle, subcutaneous

- knee flex/extend and leg movement on axis is used to encourage the animal to allow the handler to position the limb as needed;
- thigh pinch is used to simulate an injection; heel tap, heel/foot squeeze is used to simulate a heel stick with lancet for blood collection.

In skills series B and C, the use of blunt then sharp equipment is introduced. The animal's behavior acquisition is scored after each session according to criteria outlined in Table 3, with the aim to advance each animal to a score of 4 or higher on 75% of trials.

Thymus histology

At the end of studies, each individual animal is subjected to a detailed necropsy, which includes taking samples from the cardiothoracic region for histologic evaluation of the thymus. Tissue samples are fixed in phosphate-buffered formalin, embedded in paraffin, and 4-μm-thick sections are stained with hematoxylin and eosin. It is well established, both in humans and small laboratory animals, that thymus involution occurs as a consequence of aging and as a consequence of acute and chronic stress. The histologic features are different for these various conditions evoking involution [24, 43, 48]. In accord with literature, the following scoring was performed (Fig. 2):

- Normal uninvoluted or slight involution: individual lobes are located close to each other with small interlobular areas or septae. Cortex and medulla are present in normal proportions. The outer cortex shows a thin capsule extending in interlobular septae that can reach to the cortex-medulla junction. There is a clear cortex-medulla demarcation. The cortex is densely populated with small-sized lymphocytes so that the stromal compartment is not easy visualized. Blood vessels can be distinguished, and there can be a few starry-sky macrophages ('empty' small holes, resembling a starry sky) around. The medulla is less densely populated with medium-sized lymphocytes. Epithelial stroma is easily distinguished as are Hassal's corpuscles. The surrounding of Hassal's corpuscles shows individual large-sized epithelial cells indicative of activity. Blood vessels are easily visualized. Around the tightly connected lobules is a matrix of adipose tissue with well-developed vasculature.
- The first stage of involution manifests as an increase of interlobular spaces with the presence of adipose tissue or connective tissue. The thymic lobular capsule is thickened, and cortex can be slightly reduced in size. Blood vessels can be prominent at this location. There is a clear cortex-medulla demarcation, with a density of lymphocytes as in the uninvoluted tissue. Hassall's corpuscles are present in the medulla and can be surrounded by a rim of epithelial cells indicative of activity.
- In moderate involution, these changes are more pronounced, but there cortex and medulla areas can still be distinguished. Individual lobules are smaller in size and more separated from each other, either by adipose or connective tissue. Interlobular spaces are more prominent. The medulla can show Hassall's corpuscles with calcifications and necrosis indicative of loss of activity. Well-developed epithelium is less obvious. The interlobular areas contain well-developed vasculature.
- In severe involution, the individual lobes are generally small, with large interlobular spaces. Sometimes cysts are present, either in or aside the individual lobes. The cortex-medulla differentiation is lost. There are lymphocytes in the tissue, generally in variable but low density: dense lymphocyte population as present in the uninvoluted cortex is not seen. There are Hassall's corpuscles, often rather small and some with calcifications. The interlobular area shows well-developed vasculature.
- In end-stage involution, only an anlage of the original thymic lobular structure is seen, with a few

Table 3 Scoring complex behavior acquisition in limb presentation

0	No limb presentation, passive movement with handler fails
1	Limb presentation with passive movement by handler, animal relies fully on the handler
2	Limb presentation with passive movement by handler, animal relies fully on the handler with prompting incorporated
3	Animal presents limb relying on multiple prompts
4	Animal presents limb relying on single prompt
5	Animal presents limb with no prompt, cue only

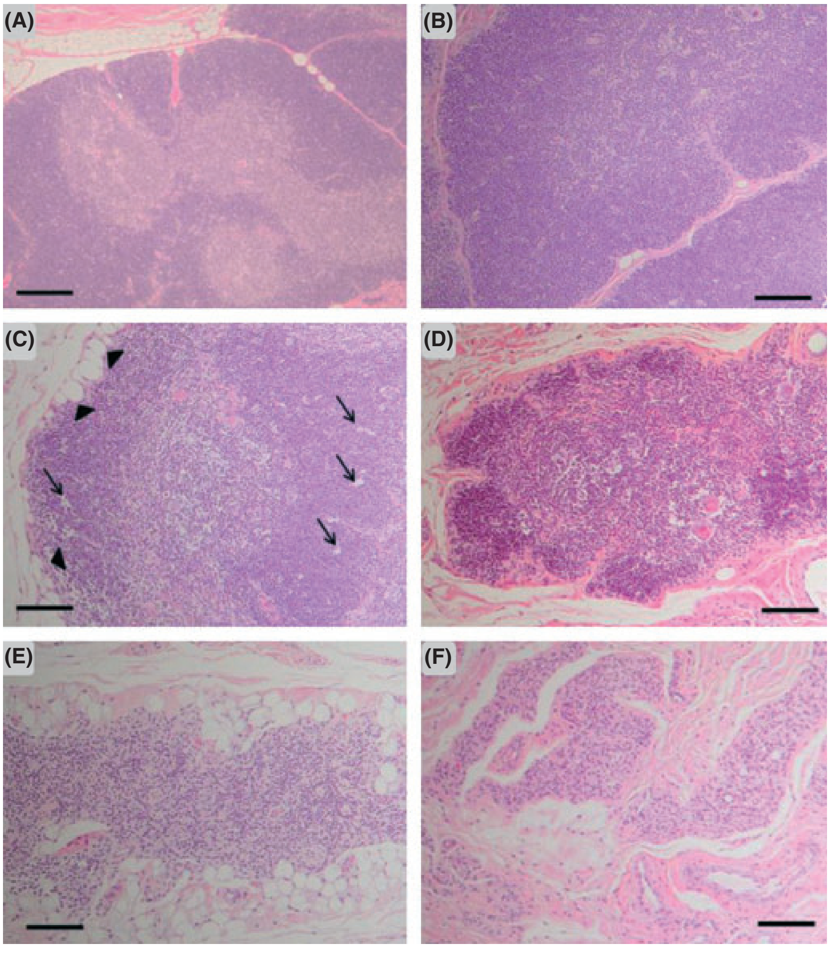

Fig. 2 Thymus histology. (A) Normal uninvoluted thymus, animal requiring euthanasia because of aortic stenosis defect and subjected only a few times to handling. (B) Uninvoluted thymus. (C) Slight involution, with some increase in starry-sky macrophages (arrows) and irregular shape of the capsule (arrowheads). (D) Moderate involution, note the presence of small-sized cortex areas. (E) Severe involution, note the absence of cortex-medulla differentiation and irregular shape of the capsule. (F) end-stage involution, with only strands of epithelial cells. Bar 100 μm (A) and 50 μm (B–F).

lymphocytes around. It is filled with epithelial strands associated with adipose tissue. Often the original capsule is no longer visible. This end-stage picture is not very common in the normal age-involuted thymus but can be seen after a period of long debilitating disease (chronic stress). The interlobular areas show well-developed vasculature.

These stages in the process of involution are mainly based on the process of age involution and represent a chronic dynamic process. Acute or stress-induced involution follows another pattern that starts with an increase in 'starry-sky' macrophages in the cortex followed by lymphocyte depletion, first in the cortex and then in the medulla (i.e., a reverse density of lymphocyte population can be manifest during this process), and ending with a complete lymphocyte depletion. Dependent on the stressor, this process can be complete in about 1 week. If the stressor is no longer there, this is followed by recovery that takes about 2 weeks in a young–adult individual (mainly documented for rodents). In the chronic involution process described previously, there can be signs of acute involution as well: in the early phase, when there is a clear cortex visible, increased numbers of starry-sky macrophages in the cortex indicate acute stress. Also, during all stages, the capsule can be irregular and not showing the smooth lining of the cortex. Often, these irregularities are associated with a capsule of normal thickness, and irregularities areas filled with a matrix of adipose cells.

Statistics

For demographic variables, median values with ranges are presented. Data for the subset ($n = 14$) of animals studied for time to behavior acquisition are presented as mean $\pm$ SD. The number of procedures requiring chemical or chair restraint in untrained animals ($n = 28$) was compared with the number of similar procedures in trained animals ($n = 52$), in the full cohort that was subjected to islet transplantation from VAP placement to end of study or June 30, 2010,

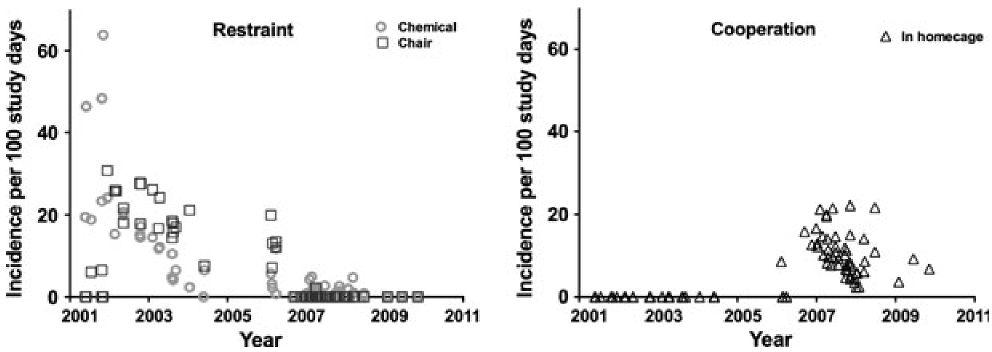

Fig. 3 Program progress. Procedures historically requiring chemical and chair restraint have been fully phased out of the program in favor of cooperative handling. Symbols represent individual animals, the X-axis indicates trial enrollment date, and the Y-axis indicates the number of handling events per 100 enrolled trial days.

whichever occurred first. Data for this series of animals are presented per animal in Fig. 3 and cohort data are presented as mean ± SD, the difference between cohorts was analyzed using a two-sided unpaired t-test. The incidence of thymic histology scores between two cohorts transplanted before (n = 14) or after (n = 26) June 2006 was analyzed using the chi-square test.

Results

General outcome of training implementation

The progress after implementation of the training in the full cohort of animals in transplant studies is demonstrated in Fig. 3, as rates of procedures per individual animal. The average rate of sedation per 100 study days was significantly reduced, from 17.5 ± 15.1 in the untrained cohort to 0.9 ± 1.2 in the trained cohort (P = 0.0001). Similarly, chair restraint was almost completely eliminated: the rate per 100 study days was 15.6 ± 9.1 in the untrained cohort and 0 ± 0.3 in the trained cohort (P = 0.0001). Instead, the trained cohort interacted cooperatively 10.4 ± 9.6 times per 100 study days for the same procedures. The overall interaction rate per 100 study days was 11.3 ± 7.0 in the trained cohort and 33.2 ± 24.2 in the untrained cohort (P = 0.0001).

Outcome of training and time investment

In the subset of 14 animals that were demographically similar and selected to study behavior acquisition, all but one successfully completed the training program, yielding a 93% success rate. This outcome is our general experience for all cohorts after the implementation of training in June 2006 (n = 52). The single animal that did not complete the training program stalled in phase 2: counterconditioning was successful in eliminating anxiety, but the animal continued to rely on the handlers to assist him in limb presentation. As a result, this animal was not allowed to enroll into a transplantation trial. Animals were trained to a level of proficiency in 19.9 ± 5.0 sessions. These sessions occurred over 88.6 ± 37.3 days that included 3.5 ± 1.1 days in between sessions (Table 4). The total time spent in phase 1 was on average 2.7 hours and in phase 2 4.3 hours (Table 4). Time spent in phase 1 was relatively similar for distinct animals, but a noticeable variability between animals was observed in phase 2 (Fig. 4).

We selected an animal that was representative for the typical pattern of behavior acquisition (Fig. 5). Anxiety generally corresponded with the introduction of novel skill series and persisted till the animal had gained sufficient experience with the behavior to pair the positive reinforcer to the behavior. At the end of the trial period, anxiety responses were no longer observed and the animal demonstrated proficiency with the behavior.

Thymus histology

One single animal requiring euthanasia because of aortic stenosis defect showed normal thymus histology compatible with a young individual without any histological sign of stress (Fig. 2A).

Almost all animals in the series of 14 animals enrolled in studies before the implementation of the training program showed severe or end-stage involution (Fig 2E,F): four cases did not reveal thymus tissue in the histologic section, which can be interpreted either reflecting a sampling error or a completely involuted thymus (Table 5). Only one case in this series

Table 4 Complex behavior acquisition: time investment

	Phase 1	Phase 2	Phase 1 and 2 in combination
Single trial time (minutes)	6.6 ± 1.0	1.5 ± 0.4	2.3 ± 0.6
Time between trials (minutes)	11.6 ± 1.2	0.8 ± 0.1	1.9 ± 0.6
Total number of trials	24.5 ± 3.1	159.2 ± 66.7	183.4 ± 66.6
Total trial time (minutes)	160.4 ± 28.3	254.9 ± 140.4	410.9 ± 154.6
Individual session time (minutes)	30 ± 2.5	24.7 ± 4.8	27.0 ± 2.6
Total session time (minutes)	323.6 ± 45.6	392.2 ± 193.7	709.5 ± 204.0
Total number of sessions	10.8 ± 1.1	15.4 ± 6.0	26.1 ± 6.2
Total number of days with training sessions	10.8 ± 1.1	14.4 ± 5.8	25.1 ± 6.0
Time between sessions (days)	3.3 ± 1.4	2.9 ± 1.3	3.5 ± 1.1
Number of days between initial session and final session	33.3 ± 13.3	40.6 ± 22.9	88.6 ± 37.3
Number of trials required to achieve proficiency level for trial enrollment[1]			130.8 ± 54.2
Number of sessions required to achieve proficiency level for trial enrollment[1]			19.9 ± 5.0

A trial is defined as a discrete period of time where the handler is directly working with the animal and a session as a discrete period consisting of 1 or more trials. Session times include both active trial time and 'downtime' between trials.

[1]When an animal scores ≥4 on at least 75% of limb presentation trials in a session the animal is considered eligible for trial enrollment. Animals complete the training program to ensure durability of behaviors.

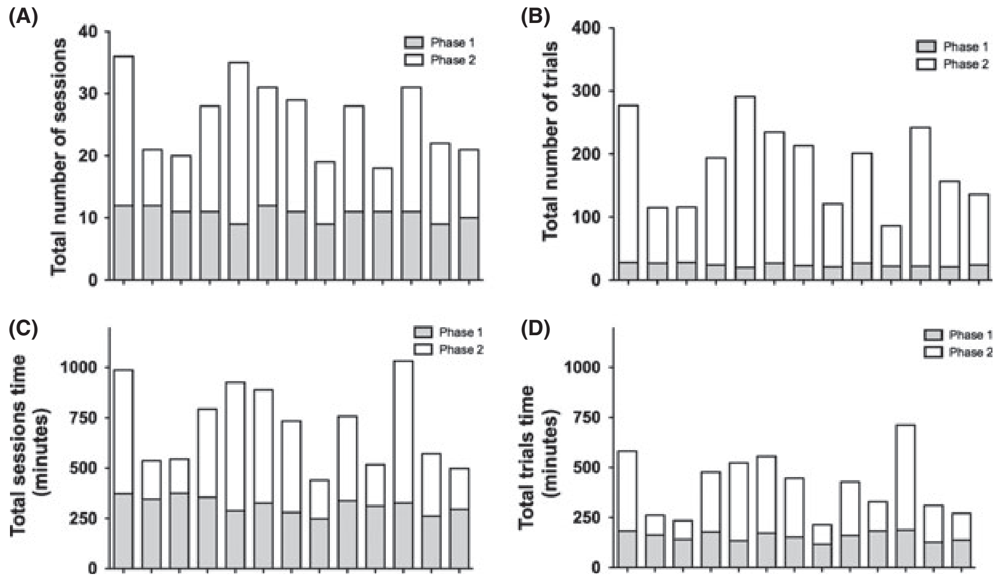

Fig. 4 Acquisition of behavior in a cohort of 14 cynomolgus macaques. Each individual bar represents an individual animal, in the same order of presentation for the four panels A–D. (A) Number of sessions to pass training criteria in phase 1 and phase 2. (B) Number of trials to pass training criteria in phase 1 and phase 2. (C) Combined session time in minutes in phase 1 and phase 2, sessions include both trial time and 'downtime' between individual trials. (D) Combined trial time in minutes in phase 1 and phase 2, starting with the cue for leg presentation to release from hold.

showed a normal or slightly involuted thymus. In the series of 26 animals enrolled in studies after the implementation of the training program, 11 showed normal thymus histology or mild-to-moderate involution (Fig. 2B,C,D), and nine showed severe or end-stage involution: six cases did not reveal thymus tissue in the histologic section. This difference between the two series reached statistical significance (Table 5, $P < 0.05$). There was no association between the extent of involution and age of the animals: regarding survival times after porcine islet transplantation, severe and end-stage involution was particularly seen in animals with prolonged survival, that is, exceeding 3 months.

Histologic signs for acute stress were seen in one of 11 animals with a normal thymus histology or mild-to-moderate involution.

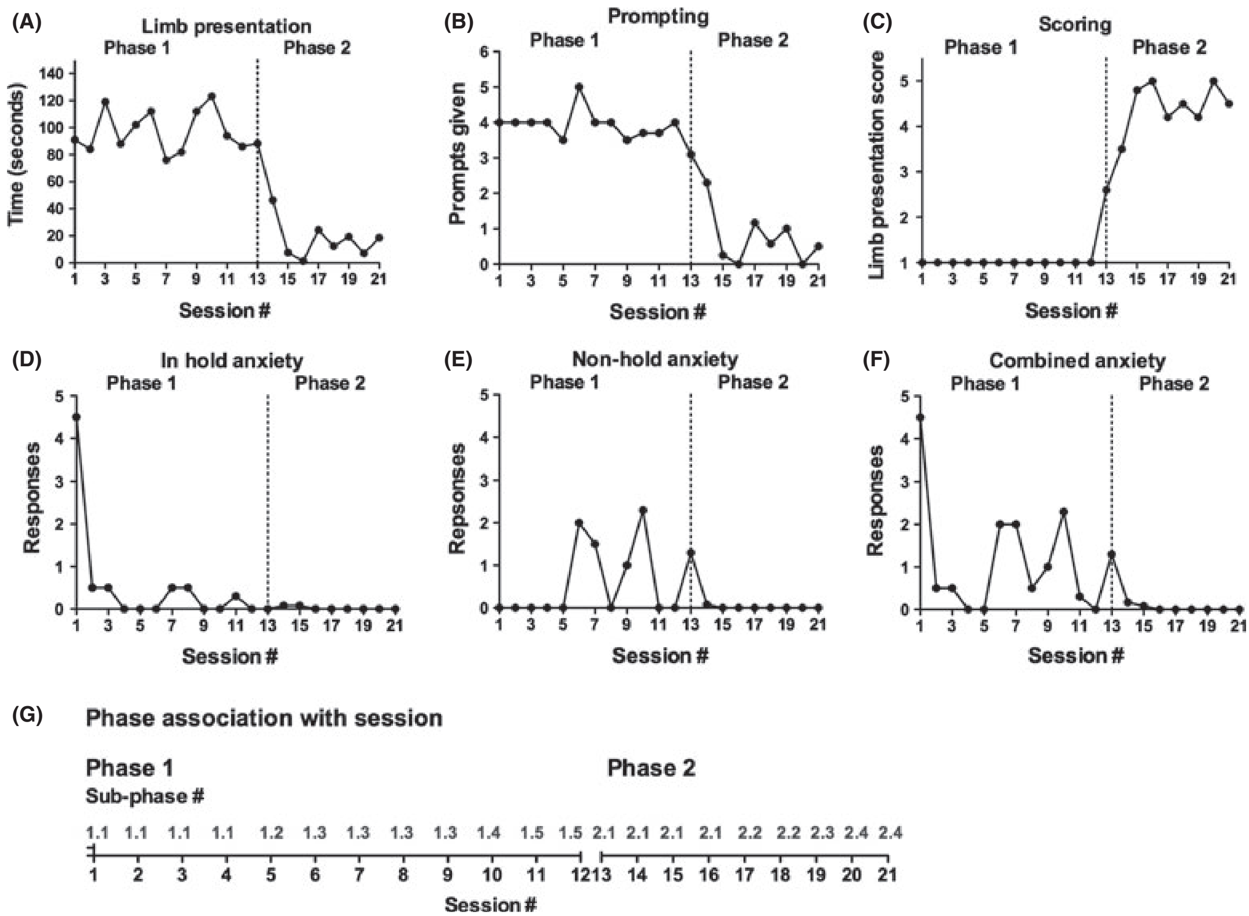

Fig. 5 Behavior acquisition in a representative animal. (A) The amount of time to limb presentation was highest during phase 1 where the animal relies on the handler to demonstrate the desired behavior. (B) Prompting was highest in phase 1, fading relatively quickly in phase 2. (C) Limb presentation scores increased in parallel with a decrease in anxiety. (D) In-hold (e.g., being grasped by the handler) anxiety was highest initially and decreased rapidly when paired with counterconditioning using paired positive reinforcement. (E) New skill introduction increased non-hold (e.g., prior to grasp) anxiety mid phase 1. (F) Combined anxiety scores were highest in phase 1. (G) The animal spent approximately 55% of time in phase 1 and 45% in phase 2. Additional sessions were required in subphases that introduced more complex skills. Note: the training plan for animal listed here differed only subtly from the current situation because of smaller number of handlers involved at that time.

These data indicate that animals after training and handling without chemical or physical restraint show a significantly lower extent of stress as concluded from histology of the thymus. It remains to be established whether the severe or end-stage involution seen in number of these animals has any relationship with the experimental protocol.

Discussion

We successfully introduced a training program for cooperative interactions of NHPs, which included hand feeding and drinking, shifting, and limb presentation. Of these, limb training is the most complex one, and we used both PRT and NRT in this approach, but predominantly PRT. The advantages and disadvantages in terms of time required for routine procedures with respect to the conventional animal handling using chemical and/or chair restraint are presented in Table 6 and illustrate that the time invested in training cooperative behavior is compensated by the time required in animal handling during the conduct of experimental protocols, in particular long-term experimental periods.

Training is an intrinsic component of a multifactorial approach to meet our objective in improving the well-being of animals that also includes refinements in surgical technique and drug administration [15, 16, 18]. The goal of our NHP training program is to develop cooperative behaviors for routine planned interactions

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Table 5 Thymic histology: overview of scores and cases showing signs of acute involution

Series	Total	Normal or slight involution	Moderate involution	Severe involution	End-stage	No thymus in section
1	14	1		6	3	4
2	26	8	3	6	3	6
Signs for acute involution, in cases presented in the rows above						
1				2		
2		1	1	3		

Animals in series 1 were enrolled in experimental protocols before implementation of the training program, and animals in series 2 after implementation.

and to improve coping skills for the situation that the animals are faced with a new stimulus to which they had not been exposed before. Routine medical procedures in conscious animals may inflict momentary pain or distress in NHPs, for example, injections, blood collection, or physical examination. We opted to focus training primarily on the handler–animal relationship, using a combination of techniques orchestrated in such a way that the total sum of the interaction is positive.

In the selection of training methods, the experimental condition of the animal is very relevant to consider. In T1D, there is limited or no endogenous insulin production, so that compliance with exogenous insulin administration is ultimately not a choice for the animal. More important, in the case of severe or rapid-onset metabolic instability, reliable cooperation with medical procedures is needed to avoid serious morbidity or mortality. Using PRT only is most successful for the case where there is no intrinsic aversive consequence in the behavior performance, and the behavior is one that the animal already is accustomed to (e.g., targeting a specific cage location, hand feeding or drinking, urine collection): in other words, the behavior simply undergoes additional shaping and reinforcement for performance on cue. When more complicated behaviors are necessary that involve mildly aversive consequences (e.g., injection, capture, and blood collection), the effectiveness of using PRT alone to reliably elicit the behavior is lower [12, 42]. We chose to incorporate NRT in combination with consistent PRT counterconditioning. It is worth mentioning that techniques incorporating NRT have been suggested to be

Table 6 Comparison of handling methods for routine procedures

Method	Utility	Advantages	Disadvantages	Time approximation (minutes)
Chemical sedation (e.g., ketamine, Telazol®, midazolam)	Blood collection Examination (Comprehensive) Injection Oral drug administration via gavage Temperature Urine collection (invasive) Weights	Detailed physical examination Minimal analgesic effect Drug-induced amnesia	Drug tolerance Recovery monitoring Nausea, emesis Separation from conspecifics Injury manifesting from post sedation ataxia Metabolic	20–60 minutes
Chair restraint	Blood collection Examination (abbreviated) Injection Oral drug administration via gavage Temperature Urine collection Weights	Access to all parts of the body Conscious restraint Can accommodate lengthy procedures	Restraint associated injury Removal from conspecifics Removal from familiar homecage environment Requires special equipment Places animal in subordinate position	10–15 minutes
In homecage cooperation	Blood collection Examination (abbreviated) Injection Oral drug administration Temperature Urine collection Weights	Reduction in anxiety with routine procedures Reduced perception of pain Acquisition of research data during 'normal' conscious physiological state Positive interactions with handlers Social access to conspecifics	Access to parts of the body presented by the animal Lengthy (e.g. >1 hour) procedures are difficult to accommodate	1–5 minutes

considered only a last opportunity as these techniques have the potential to be detrimental [28, 34, 38]: the primary concern raised with NRT or ERP is the elicitation of anxiety or stress in animals, a valid concern, particularly in the absence of counterconditioning. However, transient stressors are not unequivocally detrimental. Issuing and responding to stimuli is intrinsic to behavior, both aversive and pleasurable stimuli are present and inevitable in natural environments and contrived ones [33]. Transient stress and anxiety are also normal in general behavior, and therefore, we use the term stress and not distress. 'The acute stress response (as is typical in most mammalian stressors) is adaptive and mobilizes energy to the brain. Moreover transient exposure to mildly elevated glucocorticoid levels is reinforcing. It is only with prolonged stress that the deleterious consequences of the stress response change.' Acute mild stress enhances cognition and implicit learning concerned with procedural memory and habits [13]. It can be argued that PRT–NRT combination training programs might increase training efficacy by capitalizing on enhanced and rapid learning occurring during NRT while relatively quickly transitioning associated anxiety to a rewarding state via counterconditioning. Therefore, we designed a training plan with the consideration of balance: reduction of anxiety in the situation where aversive medical procedures are indicated for medical management and increased comfort by pairing PRT, ultimately fostering a relationship of trust and predictability between the NHPs and human handlers, thereby improving safety for both.

With respect to medical management, each trained behavior contributes to improved care and better and earlier alerting of clinical symptoms of adverse events. For instance, if an animal in study that normally cooperatively takes desirable food or drink from staff very readily suddenly refuses to do so, this indicates a potential health concern such as gastrointestinal distress. Another example in our experience is diabetes induction using streptozotocin [17], which is associated with a low incidence of adverse side effects, but high severity when manifest: in animals trained to cooperative handling, adverse effects such as those associated with metabolic acidosis are much quicker recognized, so that immediate clinical care can be initiated to the benefit of the animal [17]. Clearly, hand drinking via a syringe is very useful for delivering oral medications (e.g., the animal can drink a small volume of medicated fluid mixed with a larger volume of a more palatable fluid within the syringe, and the handler can easily assess whether the correct dose was administered), measured fluid loading (e.g., giving extra oral fluids to help protect the kidneys from the effects of nephrotoxic drugs like streptozotocin), and the provision of rehydration therapy in cases like metabolic acidosis.

Shifting is a highly valuable skill that avoids the conventional approach using sedation in weight assessment. The shifting behavior is simple and rapid so that a weight measurement can be carried out in less than 5 minutes, and because of its relative ease, this encourages more frequent weight measures in the benefit of health assessment. This is especially valuable in clinically compromised animals such as those suffering from dehydration or diarrhea: weight values are very informative in such cases to monitor fluid volume deficit enabling quick fluid correction. We capitalized on this behavior in a unique situation in one animal experiencing poor oxygen saturation resulting from unexpected and sudden-onset streptozotocin-associated toxicity, in which we converted the shift box into a modified 'oxygen box' (i.e., the animal was kept in air with higher oxygen concentration) and kept the animal there for therapy periods of 20–60 minutes: this promoted rapid recovery in a life-threatening situation.

Limb presentation is by far the most useful behavior in medical management included in this study as it gives the opportunity to physically examine the animal hands-on in a conscious unrestrained state avoiding restraint-induced laboratory anomalies [6, 26, 51], collect samples (e.g., blood, urine), and administer injectable medications. However, this is also the most complicated behavior because it essentially trains NHPs for voluntary 'capture'. It is even more complicated than presentation into a blood sleeve where the animal still retains the opportunity for escape. Essentially, the animal is asked to allow the handler grasping and manipulating the leg. Prior to cooperative training, the majority of NHPs have only experienced human hands-on contact in the context of forced restraint (manual and/or chemical) and the SBP-equipped conventional homecage to immobilize the NHP into manual restraint. Subsequently, both the hands-on contact and SBP generally act as a conditioned stimulus resulting in anxiety. This is demonstrated by the anxiety-induced responses that characterize the behaviors in the initial phase of training. In this phase, animals commonly attempt assaults on the handler and attempt escapes after the leg is being held outside of the cage. Also, during this time, the animal is less likely to accept food offered from the handler's hand while being held: in this stressful position, the animal less likely is eating or drinking. The animal's posture while being held may reflect its anxiety-induced state too, for example, the animal leans as far away from the handler as possible and has its head turned completely away from the handler as well: and when the handler releases his grasp on the animal's leg, the animal may vehemently retract its leg back into the cage.

However, the animal's hands-on interaction behavior undergoes dramatic changes as it progresses through the training process and beyond (Fig. 6), and at some point, the animal essentially displays behavior opposite to that of all the previously described anxiety-induced responses. In a relatively short period of time, the escape and assault attempts extinguish completely. The animal eats food while being held and accepts food offered from the handler's hand. The animal will face and remain in close proximity and direct sensory contact (i.e., smell, hearing, and sight) with the handler while being held and will often demonstrate affiliating behaviors (e.g., lip-smacking, grooming, etc.) with both the handler and other animals in the room. Upon release from the handler's grasp, the animal carefully and calmly retracts its leg back into the cage. Although all of these changes will take place in the majority of animals, they may not all occur at the same time. It is also during this period of behavioral change that the animal typically achieves proficiency at extending its leg out of the cage with minimal to no prompting from the handler. As the animal becomes proficient at extending its leg out of the cage, this suggests that the animal has also learned that the feared situation of having its leg held and manipulated is no longer associated with an aversive situation: this is illustrated for the case in Fig. 5 by the inverse relationship between anxiety and unassisted limb presentation. With an arguably short time investment of approximately 10–15 hours total, the animals have achieved a highly useful and durable behavior that completely nullifies the use of restraint.

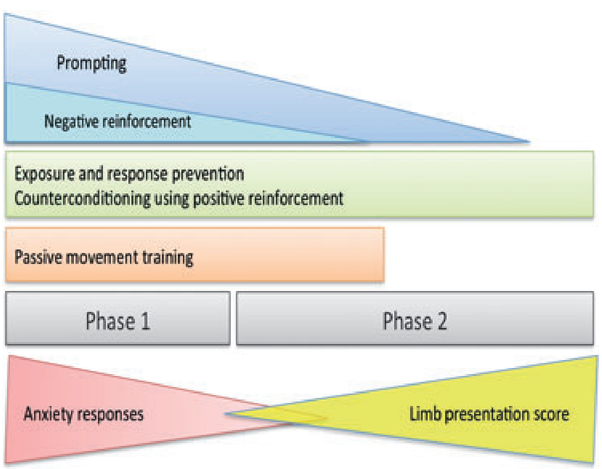

Fig. 6 Techniques associated with training phase in relation to behavior acquisition.

The scientific basis for the behavioral acquisition in animals, in particular the hands-on interaction, is given by the 'opponent process theory of acquired motivation on evoking affective changes' published by Solomon [44], and this will be presented and illustrated in more detail in the following. In brief, when a subject is repeatedly exposed to a stimulus that elicits an affective response, there are often subsequent affective phenomena observed. Upon initial exposure to the stimulus, the subject expresses an affective change in one direction away from his baseline state (i.e., affect-1); and after exposure to the stimulus, the subject expresses an affective change in the opposite direction (i.e., affect-2) before returning to the baseline state. In the case of limb presentation training, initially the animal experiences anxiety (affect-1) while grasped by the handler, and after the handler releases the leg, the animal experiences a sense of relaxation (affect-2) before returning to the baseline state.

Solomon's theory [44] describes in addition how the subject's motivation after exposure to the stimulus changes upon the diminishing affect-1 and more pronounced emergence of affect-2 and that these new motivations are likely to be very strong. Also this theory is reflected in our experiences. An animal with extended experience after leg presentation training readily presents his or her leg for any familiar handlers to achieve the positive affect accompanying the performance, and there is no intention any more to flee from the handlers. This behavior persists even under circumstances where there are no other tangible positive reinforcers. For instance, even an animal whose clinical condition (and subsequently whose appetite for food or drink) is severely compromised still readily presents its leg to the handler enabling blood sampling and the delivery of potentially life-saving therapies. Thus, the behavior is no more fully reliant on appetitive stimuli. Likewise, an animal seems confident while being held for lengthy metabolic tests in which food cannot be provided until after the test's completion. This has obvious benefits for metabolic studies where certain testing situations prohibit feeding during sampling.

As evident in Fig. 3, the behaviors are frequently employed and durable, and there is almost no fallback any more to physical/chemical restraint, including the SBP. The use of chair restraint has been completely eliminated in our unit. Besides instilling an animal with a highly practical and persevering behavior, we speculate that the combination of PRT and NRT in the training process also instills the animal with an important rudimentary coping ability [3]. Specifically, an animal trained for leg presentation has achieved a much greater sense of control over any other situation in general that might occur in the laboratory environment than an animal that is not trained for leg presentation. Even in novel situations, these animals already have basic experience with handling to the extent that contact with handlers is routinely rewarding, thus making novel interactions less anxiety-inducing. The behavior is durable and in the long-term interest of the individual. The data on thymic histology (Table 5, Fig. 2) underscore this general lower stress condition of the animal. In complicated disease models like organ, tissue or cell transplantation, combining conventional handling with physical/chemical restraint, invasive surgical procedures and chronic immunosuppression, the thymus in juvenile NHPs normally shows severe involution, and rarely an uninvoluted thymus is observed (HJ Schuurman, personal experience reviewing pathology at various NHP transplantation centers, 1993–2011). Observing an uninvoluted or moderately involuted thymus in more than 50% of cases, and signs of acute (stress-induced) involution in <25% of those cases (Table 5), clearly shows that the replacement of conventional handling by cooperative performance has abolished the stress component in causing the thymus to involute. Other factors such as chronic immunosuppression during long-term survival persist as factors influencing the involution of the thymus.

It is realized that this training method has its limitations. Behavioral antecedents like passive movement training or 'putting through' help animals learn the behavior quickly, but may induce passive behavior in progressive learning, by the adaptation to wait for 'guidance' from the trainer when cued. On the other hand, putting through may limit the frustration of the NHP when trying to satisfy the behavior. This resembles backward chaining where the teacher begins by teaching and reinforcing the performance of the final step of a desired outcome before teaching and reinforcing each of the previous steps in the process that lead to the desired outcome. As with any learning tool, this method will not work for every animal, but in our experience illustrated in a representative cohort (Fig. 4), the success rate is >93%. It is worth mentioning that considering the full cohort of trained animals, the single animal in this cohort is the only one that has failed to complete the training program, and failure was as mentioned previously in the training of limb presentation.

Finally, it should be emphasized that cooperation enables a more complete collection of experimental data through more productive interactions with animals, which is in addition realized by a substantial reduction in necessary overall interactions (Fig. 3). Also, there is no confounding by stressful events during chemical or physical restraint, which evidently yield a more reliable status of experimental results, both from *in vivo* tests and laboratory evaluations. Even in clinically compromised animals necessitating frequent handling, this is accomplished without the burden of sedation or restraint. This approach in the T1D model could be easily applied in other disease models (e.g., transplantation, rheumatoid arthritis, infectious disease, etc.) as well and also in routine toxicology/pharmacology studies. This improvement in overall well-being contributes to the validity of scientific results and eliminates model-induced confounding that can obstruct interpretation of safety and efficacy data or, in a worse case, result in non-informative animals necessitating larger numbers in experimental animal groups. Training in cooperative handling has the considerable advantages of reduced stress and improved care offering continued benefit to both the animals and handlers.

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